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AGRONOMSKI FAKULTET

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DOKTORSKI RAD

Zagreb, 2022

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FACULTY OF AGRICULTURE

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DOCTORAL THESIS

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Mentor: izv. prof. dr. sc. Nikica Šprem

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Sažetak

Divokoza (*Rupicapra* spp.) rasprostranjena je diljem Europe gdje nastanjuje srednje i visoke planinske regije, a u iznimnim slučajevima nizinske predjele riječnih kanjona i područja na razini mora. Danas prema morfološkim i bihevioralnim svojstvima postoje dvije vrste divokoza: južna divokoza *Rupicapra pyrenaica* (s podvrstama *parva*, *pyrenaica* i *ornata*) i sjeverna divokoza *Rupicapra rupicapra* (s podvrstama *cartusiana, rupicapra, tatrica, carpatica, balcanica, asiatica* i *caucasica*). Dvije od sedam podvrsta sjevernih divokoza (*R. r. rupicapra* i *R. r. balcanica)* nastanjene su u Hrvatskoj, dok je balkanska divokoza rasprostranjena diljem Balkanskog poluotoka još u osam zemalja: Bosna i Hercegovina, Srbija, Crna Gora, Kosovo, Sjeverna Makedonija, Albanija, Bugarska i Grčka.

Trenutno znanje o genetskoj raznolikosti i strukturi populacija balkanske divokoze ograničeno je na lokalna istraživanja. U ovom istraživanju korištena je kombinacija seta mikrosatelitnih lokusa i sekvence kontrolne regije mitohondrijske DNA kako bi se istražila genetska struktura i povezanost populacija balkanske divokoze u cijelom rasponu distribucije s ciljem razvoja strategije gospodarenja i zaštite. Osim toga, korišten je isti set lokusa kako bi se utvrdila točnost postojećih povijesnih podataka o podrijetlu biokovske populacije i razjasnilo koja je podvrsta bila prisutna prije reintrodukcije balkanske i alpske divokoze na planinu Velebit s ciljem procjene i dokumentiranja genetskoga statusa izvornih i translociranih populacija.

Za postizanje ciljeva istraživanja ukupno je genotipiziran 141 uzorak balkanske divokoze s područja država Balkanskog poluotoka (osim Kosova) i 4 lubanje divojaraca (muzejski primjerci). Za izradu filogenetske mreže, prikupljeno je 56 dodatnih uzoraka koji su pripadali drugim podvrstama divokoza. Sekvenca kontrolne regije duga 376 parova baza uspješno je očitana kod 44 jedinke.

STRUCTURE model predlaže 3 genetska klastera i svrstao je jedinke iz Srbije i Bugarske u dva odvojena klastera, dok su jedinke iz ostalih zemalja pripadale istom klasteru. Analizom sekvenci kontrolne regije mitohondrijske DNA otkriveno je 30 novih haplotipova s privatnim haplotipovima u svim analiziranim populacijama i dva haplotipa dijeljena između populacija, što može biti rezultat prošlih translokacija jedinki. STRUCTURE i GENELAND analize pokazale su jasnu odvojenost reintroducirane populacije na Biokovu od divokoza nastanjenih na Prenju i značajnu genetsku sličnost između biokovske populacije i populacija uzorkovanih na Čvrsnici i Čabulji. GENELAND analiza prepoznala je balkansku divokozu s Prenja kao zaseban genetski skup, različit od populacija koje obitavaju na Čvrsnici i Čabulji. Ovi rezultati sugeriraju da su rijeka Neretva i državna cesta M17 geografske barijere za rasprostranjenost podvrste. STRUCTURE i DAPC analize muzejskih uzoraka pokazale su veću vjerojatnost da je na Velebitu prije reintrodukcije obitavala alpska podvrsta divokoze.

Rezultati ovog istraživanja su dali uvid u genetsku raznolikost populacija balkanske divokoze i pokazali uzorak arhipelaga diljem rasprostranjenosti podvrste kao posljedica fragmentacije staništa i populacija. Rezultati ovog istraživačkog rada mogu poslužiti kao smjernice za izradu akcijskog plana gospodarenja populacijama balkanske divokoze u Hrvatskoj, ali i u drugim zemljama gdje ova podvrsta obitava, te osigurati dizajn za buduću zaštitu podvrste.

Ključne riječi: Balkanski poluotok, genetska raznolikost, konzervacijska genetika, mikrosateliti, mitohondrijska DNA, populacijska genetika, reintrodukcija, *Rupicapra rupicapra balcanica*

Extended Abstract

Genetic assessment of Balkan chamois (*Rupicapra rupicapra balcanica*, Bolkay, 1925) populations status: the basis for conservation measures

Chamois (*Rupicapra* spp.) are medium-sized ungulates that inhabit alpine pastures and rocky areas on the main mountain massifs of both Europe and the Near East and, exceptionally, the low elevations of river gorges, forested and coastal areas. The currently accepted taxonomy of chamois, based on morphological, behavioral and molecular evidence, recognizes two species: the Northern chamois (*Rupicapra rupicapra*) with seven subspecies distributed on the Alps (*R. r. rupicapra* and *R. r. cartusiana*), the Balkans (*R. r. balcanica*), the Tatras (*R. r. tatrica*) and the Carpathians (*R. r. carpatica*), in western Asia (*R. r. asiatica*) and the Caucasus (*R. r. caucasica*). The Southern chamois (*Rupicapra pyrenaica*) includes three geographically isolated subspecies on the Cantabrian Massif (*R. p. parva*), the Pyrenees (*R. p. pyrenaica*) and the central Apennines (*R. p. ornata*). In the early 1900s, chamois populations in the northern Dinaric Mountains in Croatia were extirpated due to predation, natural events, and unsustainable hunting before their taxonomic classification was assessed. Several decades after local extinction, the chamois population was re-established through several reintroduction efforts between 1964 and 1978. This resulted in the current populations of Northern chamois in the northern Dinaric Mountains being descended from successfully reintroduced individuals, captured in mountainous areas in Bosnia and Herzegovina (*Rupicapra r. balcanica*) and Slovenia (*Rupicapra r. rupicapra*), except for the Dinara massif, where the only Croatian autochthonous population of Balkan chamois lives. Since different subspecies have been involved in reintroduction in the past, a contact zone has formed in the northern Velebit Mountains, where these subspecies hybridize.

The Balkan chamois is widespread on the Balkan Peninsula, along mountain massifs from Croatia in the north to Greece in the south and Bulgaria in the east. The distribution of the Balkan chamois is patchy and covers only parts of the massifs and mountain chains across the countries that form its range. The subspecies has low rates of colonization and reduced gene flow between isolated populations, which may result in genetic differentiation due to the inbreeding effect and a loss of allelic variants as a consequence of genetic drift. Reduced genetic diversity in these small and isolated populations might, in turn, cause negative impacts on fitness, resulting in decreased effective population size and, eventually, increase the probabilities of extinction. Other threats to the Balkan chamois survival are considered to be poaching, introductions of other chamois subspecies (mostly Alpine chamois), forest succession, road infrastructure, intensive livestock grazing, predation, unsustainable hunting and natural events. Due to these threats, the Balkan chamois is protected by Annexes II and IV of the European Union Habitats Directive 92/43/EEC (OJ L 206, 22.7.1992) and Appendix III of the Bern Convention (OJ L 38, 10.2.1982). The conservation and management status within the different national legislations varies between countries (members and non-members of the EU) and depends on the degree of the local communities' interest in the conservation of the subspecies. Over the past two decades, the genus *Rupicapra* has been the subject of numerous genetic studies and, although Balkan chamois has been included in several of these phylogenetic studies that used both nuclear and mitochondrial markers, it remains one of the less-studied subspecies. Current knowledge on the genetic diversity and structure of the Balkan chamois population is limited and restricted to regional-local studies. Multiplex set of microsatellite loci and a partial mitochondrial control region were used to investigate the genetic structure and connectivity of Balkan chamois throughout its distribution range to support the development of management and conservation strategies. In addition, the same microsatellite set was used to determine the accuracy of existing historical data on the origin of the Biokovo population and to clarify which subspecies was present before reintroduction of Balkan chamois to Velebit Mountain. This was done with the aim to assess and document the genetic status of both the source and translocated populations.

To test Balkan chamois genetic diversity and population structure, DNA from bone, dried skin and muscle tissue was extracted and successfully genotyped in 92 individuals of Balkan chamois and the partial control region was sequenced in 44 individuals. Additional 20 samples from Biokovo and 29 samples from three areas which might have served as source populations for reintroduction and possible recent recolonization (Prenj, Čvrsnica and Čabulja mountains) were genotyped. To clarify which subspecies was present before reintroduction to Velebit Mountain, four male chamois skulls originating from Velebit, collected around 25 years before the population local extinction were genotyped.

The Bayesian analysis suggested that Balkan chamois are divided into 3 genetic clusters, and assigned individuals from Serbia and Bulgaria to two separate clusters, while individuals from the other countries belonged to the same cluster. Thirty new haplotypes were obtained from partial mitochondrial DNA sequences, with private haplotypes in all analysed populations and only two haplotypes shared among populations, indicating the possibility of past translocations. Both STRUCTURE and GENELAND analyses showed a clear separation of the reintroduced population on Biokovo from Prenj' s chamois and considerable genetic similarity between the Biokovo population and the Čvrsnica–Čabulja population. This suggests that the current genetic composition of the Biokovo populations does not derive exclusively from Prenj, as suggested by the available literature and personal interviews, but also from Čvrsnica and Čabulja. GENELAND analysis recognized the Balkan chamois from Prenj as a separate cluster, distinct from the populations of Čvrsnica and Čabulja. This suggests that the Neretva River and the state M17 road are geographic barriers for the species dispersal, as they form a genetic boundary. Concerning the identification of the subspecies inhabiting Velebit Mountain before the reintroducitons, according to 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), Alpine subspecies showed an higher likelihood.

The subspecies' genetic composition presented in this doctoral thesis provides the necessary starting point for assessing the conservation status of Balkan chamois and allows the development of strategies necessary for its sustainable management. The use of molecular markers, in this case microsatellites and mitochondrial DNA, provided important information on the genetic diversity and evolutionary history of Balkan chamois populations.

Keywords: Balkan peninsula, genetic diversity, conservation genetics, microsatellite, mitochondrial DNA, population genetics, reintroduction, *Rupicapra rupicapra balcanica*

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Popis kratica

CR (*control region*) – kontrolna regija mitohondrijske DNA

DNA - deoksiribonukleinska kiselina

HWE – Hardy-Weinberg ekvilibrijum

IBD (*isolaton by distance*) – efekt izolacije zbog udaljenosti populacija

IUCN (*International Union for Conservation of Nature*) - Međunarodna unija za očuvanje prirode

MCMC - Markov chain Monte Carlo model

MHC (*Major Histocompatibility Complex*) - glavni sustav tkivne podudarnosti

msat - mikrosateliti

mtDNA – mitohondrijska DNA

NCBI (*National Center for Biotechnology Information - GenBank*) - Nacionalni centar za biotehnološke informacije, Banka gena

ND1 - NADH dehidrogenaza

NGS (Next Generation Sequencing) – sekvenciranje sljedeće generacije

12S - 12S ribosomski RNA gen

tRNApro – prolin transport RNA antikodon

PCR (*Polymerase Chain Reaction*) – lančana reakcija polimeraze – lančana reakcija polimeraze

POVS - područja očuvanja značajna za vrste i stanišne tipove

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Slika 2. Različiti tipovi polimorfizma kod mikrosatelita: a) polimorfizam u duljini ponavljajućeg motiva i strukture mikrosatelita; b) polimorfizam u duljini ponavljajućeg motiva analiziran kapilarnom elektroforezom, (preuzeto s: https://www.biomnigene.fr/en/our-solutions/microsatellite-analysis.html; 12.10.2022.)

Slika 3. Ispuštanje alpske divokoze u Lomsku dulibu na sjevernom Velebitu 10. rujna 1978. (izvor Frković, 2008).

Slika 4. Ispuštanje balkanske divokoze na Biokovo (izvor Šabić, 2014).

Popis priloga

Prilog 1. Znanstveni rad: Rezić Andrea, Iacolina Laura, Bužan Elena, Safner Toni, Bego Ferdinand, Gačić Dragan, Maletić Vladimir, Markov Georgi, Milošević Dragana, Papaioannou Haritakis, Šprem Nikica (2022): The Balkan chamois, an archipelago or a peninsula? Insights from nuclear and mitochondrial DNA. Conservation Genetics 23: 527–539.

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Prilog 3. Znanstveni rad: Safner Toni, Buzan Elena, Iacolina Laura, Potušek Sandra, Rezić Andrea, Sindičić Magda, Kavčić Krešimir, Šprem Nikica (2020): Microsatellite based assignment reveals history of extirpated mountain ungulate. Genetica ('s-Gravenhage) 148: 41 - 46.

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Rezić Andrea, Iacolina Laura, Bužan Elena, Safner Toni, Bego Ferdinand, Gačić Dragan, Maletić Vladimir, Markov Georgi, Milošević Dragana, Papaioannou Haritakis, Šprem Nikica (2022): The Balkan chamois, an archipelago or a peninsula? Insights from nuclear and mitochondrial DNA. Conservation Genetics 23: 527–539.	WoS	A1	Q2	2.538
Rezić Andrea, Iacolina Laura, Safner Toni, Bužan Elena, Šprem Nikica (2022): Genetic evaluation of past reintroduction programs: the case of Balkan chamois (Mammalia, Artiodactyla). Zookeys 1116: 57-70.	WoS	A1	Q2	1.546
Safner Toni, Buzan Elena, Iacolina Laura, Potušek Sandra, Rezić Andrea, Sindičić Magda, Kavčić Krešimir, Šprem Nikica (2020): Microsatellite based assignment reveals history of extirpated mountain ungulate. Genetica ('s-Gravenhage) 148: 41 - 46.	WoS	A1	Q4	1.082

Obrazloženje povezanosti istraživačkih hipoteza i objavljenih (*prihvaćenih*) znanstvenih radova	
Istraživačka hipoteza	Obrazloženje povezanosti sa znanstvenim radom
H1. Postoje bitne razlike u alelnoj i genotipskoj učestalosti između istraživanih populacija te će razlike biti izražene između geografski udaljenijih populacija	U prvom radu (Prilog 1) STRUCTURE i F_{ST} rezultati pokazuju razlike u alelnoj i genotipskoj učestalosti između istraživanih populacija, a najveća razlika je utvrđena kod udaljenijih populacija iz Srbije i Bugarske čime je potvrđena postavljena hipoteza. Hipoteza je potvrđena i u drugom radu (Prilog 2). Populacije balkanske divokoze na Biokovu su geografski udaljenije od svih ostalih analiziranih populacija i STRUCTURE je pokazao različit genetski sastav reintroducirane populacije od izvornih vjerojatno zbog efekta osnivača i nemogućnosti izmjene gena sa susjednim populacijama. Također, vidljiva je izolacija populacije na Prenju što je posljedica antropogenih utjecaja. U trećem radu (Prilog 3) vidljiva je razlika u alelnoj i genotipskoj učestalosti između referentnih populacija Balkanske divokoze i muzejskih uzoraka koji su bili stari 100 godina na osnovu čega se može pretpostaviti da su istraživane populacije u prošlosti bile suočene s naglim padom brojnosti i pojavom genetskog drifta što je vidljivo i kod sadašnjih populacija.
H2. Zbog izoliranih i fragmentiranih staništa te ograničenog protoka gena između populacija postotak parenja u srodstvu bit će visok.	Hipoteza je prihvaćena u prvom radu (Prilog 1). Indeks unutar populacijske endogamije (F_{IS}) je bio značajan kod analiziranih populacija iz Hrvatske, Bosne i Hercegovine, Crne Gore, Sjeverne Makedonije i Bugarske jer je izmjena genetskog materijala nemoguća zbog udaljenosti između populacija, fragmentacije staništa i antropogenih utjecaja. Jedino je populacija iz Srbije imala veći udio heterozigota. Sve analizirane populacije u drugom radu (Prilog 2) imale su F_{IS} vrijednost veću od 0 zbog čega je prihvaćena postavljena hipoteza. U trećem radu (Prilog 3) značajna F_{IS} vrijednost je utvrđena kod referentnih populacija podrijetlom s Biokova i Prenja.

1. UVOD

Europske populacije divokoza na temelju njihovih morfoloških i bihevioralnih karakteristika svrstane su u dvije vrste (Grubb, 2004): sjeverna divokoza (*Rupicapra rupicapra*) i južna divokoza (*Rupicapra pyrenaica*). Postoji nekoliko razlika između ovih dviju vrsta, uglavnom morfoloških, poput veličina tijela i boje dlake, te razlike u ponašanju u vezi s reproduktivnom strategijom (Cavallero i sur., 2012). Obje vrste su podijeljene na različite podvrste koje nastanjuju planinske lance Iberijskog poluotoka, Apeninskog poluotoka, Balkanskog poluotoka, Alpsku i Karpatsku regiju, Anatoliju i Kavkaz (Masseti i Lovari, 2017). Pirineji i Kantabrijsko gorje su prirodna staništa podvrsta *Rupicapra p. pyrenaica* Bonaparte, 1845. i *Rupicapra p. parva*, Cabrera, 1911. dok se ishodišna populacija podvrste *R. p. ornata* Neumann, 1899. ili apeninske divokoze nalazi na području Nacionalnog parka Abruzzo odakle su jedinke translocirane u druga zaštićena područja u središnjoj Italiji poput Nacionalnog parka Majella i Nacionalnog parka Gran Sasso i Monti della Laga (Mari i Lovari, 2006). Vrsta *Rupicapra rupicapra* se dijeli na sedam podvrsta: *rupicapra* Linnaeus, 1758., *balcanica Bolkay,* 1925., *cartusiana* Couturier, 1938., *tatrica* Blahout, 1971., *carpatica* Couturier, 1938., *asiatica* Lydekker, 1908. i *caucasica* Lydekker, 1908. (Perez i sur., 2014; Corlatti i sur., 2021). Prema Zemanová i sur. (2011), obitavanje ove vrste na velikim nadmorskim visinama različitih planinskih područja je razlog postojanja spomenutih deset podvrsta jer dovodi do fragmentirane distribucije populacija i ograničene izmjene gena. Također, divokoze predstavljaju izrazito atraktivnu lovnu vrstu te su zbog pretjeranog lova i nestanka s određenih područja vršene introdukcije i translokacije ove vrste (Zemanová i sur., 2014).

Podvrsta *balcanica* je rasprostranjena diljem devet zemalja Balkanskog poluotoka (Hrvatska, Bosna i Hercegovina, Crna Gora, Srbija, Albanija, Kosovo, Grčka, Sjeverna Makedonija i Bugarska; Slika 1) nastanjujući litice i stjenovita područja tijekom ljeta i niže nadmorske visine poput riječnih kanjona i šumskih zona tijekom zime (Papaioannou i Kati, 2007). Divokoza je autohtona vrsta u Hrvatskoj i istaknuti dio njezine prirodne baštine. Dvije od sedam podvrsta sjevernih divokoza (alpska divokoza i balkanska divokoza) prepoznate su u planinskim sustavima Dinarida u Hrvatskoj s kontaktnom zonom i hibridizacijom na području sjevernog Velebita (Šprem i Buzan, 2016). Tijekom ranih 1900.-ih, populacije divokoza Dinarskog područja su nestale iz raznih razloga, kao što su neodrživi lov, krivolov, ispaša stoke, grabežljivici i prirodne nepogode (Frković, 2009).

Štetan utjecaj čovjeka ispravljen je reintrodukcijom nakon Drugog svjetskog rata koja je zapostavila problem hibridizacije kod životinja različitog genetskog podrijetla (Apollonio i sur., 2014; Šprem i Buzan, 2016). U preglednom radu Iacolina i sur. (2019) ukazuju na problem translokacija i hibridizacije što može povećati rizik gubitka diferenciranih zaliha gena kao kod kartuzijske i tatranske divokoze. Nekoliko istraživanja provedenih na divokozama u raznim dijelovima Europe pokazala su visoku i značajnu razliku između gotovo svih populacija, čak i na mikrogeografskoj razini (Crestanello i sur., 2009) te novo izraženi efekt uskog grla, što je rezultat genetskog drifta i niske stope protoka gena (Soglia i sur., 2010; Buzan i sur., 2013). Zaključak svih istraživanja je važnost genetskih analiza kako bi se razdvojili i interpretirali uzroci raznolikosti, te definirali uvjeti koji su važni za zaštitu i gospodarenje divokozom. Za ovu svrhu, mikrosateliti i regije mitohondrijske DNA (mtDNA) su se pokazali veoma korisnim. MtDNA je pogodna za proučavanje geografske razdvojenosti populacija različitog geografskog podrijetla kao i srodnih vrsta zbog visoke stope supstitucije i majčinskog načina nasljeđivanja (Rodríguez i sur., 2009). Mikrosateliti danas predstavljaju jedne od glavnih molekularnih biljega u analizi populacijske genetike zbog njihove visoke zastupljenosti u genomu, relativno visokog polimorfizma, čak i u populacijama koje su bile izložene efektu uskog grla (Maudet i sur., 2002), te zbog lakoće umnažanja i dobivanja rezultata iz vrlo malih koncentracija DNA i niskih troškova u usporedbi s drugim molekularnim biljezima.

Balkanska divokoza zajedno s alpskom divokozom navedena je u Crvenoj knjizi sisavaca Hrvatske kao regionalno izumrla podvrsta (Tvrtković i Grubešić, 2006; Šprem i Buzan, 2016) dok se u Bosni i Hercegovini nalazi na popisu rizičnih podvrsta (Adamič i sur., 2006). Balkanska divokoza je uključena u Dodatke II i IV EU Direktive o staništima 92/43/EEZ (SL L 206, 22.7.1992.) i u Dodatak III Bernske konvencije (SL L 038/3, 01.07.2013.; Šprem i Buzan, 2016), dok je u Hrvatskoj jedino na području Dinare (identifikacijski broj područja HR5000028) zaštićena Natura 2000 ekološkom mrežom. Prema podacima Europske agencije za okoliš balkanska divokoza se u Grčkoj (8 područja) i Bugarskoj (12 područja) nalazi na popisu vrsta uvrštenih u područja očuvanja značajna za vrste i stanišne tipove (POVS). Na području Balkanskog poluotoka danas obitava oko 9000 jedinki balkanske divokoze (Corlatti i sur., 2021), od toga manje od 1000 jedinki u Bosni i Hercegovini; oko 700 jedinki u Srbiji; oko 1400 jedinki u Crnoj Gori i u Sjevernoj Makedoniji; oko 200 do 300 jedinki u Kosovu i cca. 450 do 600 jedinki u Albaniji. U Bugarskoj trenutno stanje populacija iznosi oko 2500 jedinki (Markov i sur., 2016) koje nisu ugrožene, ali još uvijek zahtijevu značajne mjere očuvanja i obnove kako bi bile dugoročno održive.

Nakon trenda opadanja koji je trajao do 2000. godine s 477 do 750 jedinki na cijelom prostoru Grčke, zbog zabrane lova i provođenja mjera zaštite dolazi do porasta u veličini populacije balkanske divokoze koja trenutno broji oko 1500 jedinki (Papaioannou, 2016; Papaioannou i sur., 2019). U Hrvatskoj, balkanska podvrsta divokoze se danas nalazi na području Dinare i Biokova. Prema Šprem i Buzan (2016) na Dinari obitava oko 60 jedinki, dok se na Biokovu nalazi oko 600 jedinki (Kavčić i sur., 2021).

1.1 Hipoteze i ciljevi istraživanja

1.1.1 Hipoteze istraživanja

1. Postoje bitne razlike u alelnoj i genotipskoj učestalosti između istraživanih populacija te će razlike biti izražene između geografski udaljenijih populacija.
2. Zbog izoliranih i fragmentiranih staništa te ograničenog protoka gena između populacija postotak parenja u srodstvu bit će visok.

1.1.2 Ciljevi istraživanja

1. Na osnovu informativnosti molekularnih biljega (mikrosatelita i mtDNA) utvrditi genetsku varijabilnost i populacijsku strukturu balkanske divokoze.
2. Odrediti genetsku povezanost odnosno udaljenost između populacija balkanske divokoze rasprostranjenih na području Hrvatske, Bosne i Hercegovine, Srbije, Crne Gore, Sjeverne Makedonije, Albanije, Bugarske i Grčke.

2. PREGLED RELEVANTNE LITERATURE

Paleontološki dokazi pokazuju da su Rupicaprini nastali tijekom miocena u Aziji i da se *Rupicapra* proširila Europom tijekom srednjeg pleistocena (Masini i Lovari, 1988). Danas je svrstana u dvije vrste: sjeverna divokoza (*Rupicapra rupicapra*) i južna divokoza (*Rupicapra pyrenaica*). Međutim, postoje kontroverze oko taksonomije vrsta i podvrsta divokoza (Crestanello i sur., 2009; Corlatti i sur., 2011) koja je podvrgnuta stalnim revizijama od početka dvadesetog stoljeća (Corlatti i sur., 2021). Prema crvenoj listi ugroženih vrsta (International Union for Conservation of Nature - IUCN), vrste *R. rupicapra* i *R. pyrenaica* su trenutno klasificirane kao najmanje zabrinjavajuće zbog čega je potrebno analizirati genetsku strukturu podvrsta divokoza da bi se utvrdio status zaštite.

2.1 Dosadašnja istraživanja podvrste *R. r. balcanica*

Balkanska divokoza je jedna od najmanje istraživanih podvrsta sjeverne divokoze, bilo da se radi o genetskim, ekološkim i drugim istraživanjima. Od ukupno 608 znanstvenih radova objavljenih o sjevernoj divokozi u periodu od 1980. do 2020. godine, samo je 14 znanstvenih radova o podvrsti *R. r. balcanica* (Corlatti i sur., 2022b). Od tih 14 objavljenih znanstvenih radova, samo su tri znanstvena rada obradila genetsku raznolikost i populacijsku strukturu balkanske divokoze ali na lokalnoj razini (Šprem i Buzan, 2016 – populacije balkanske divokoze u Hrvatskoj i jedna populacija u Bosni i Hercegovini; Markov i sur., 2016 – populacije balkanske divokoze u Bugarskoj; Papaioannou i sur., 2019 – populacije balkanske divokoze u Grčkoj). Balkanska divokoza bila je uključena i u druge znanstvene radove koji su za cilj imali proučavanje taksonomije, populacijske genetike i filogenetske analize podvrsta divokoza, ali prikupljeni uzorci za ova istraživanja nisu obuhvaćala cijeli opseg rasprostranjenosti podvrste *R. r. balcanica,* te se većinom radilo o malom broju uzoraka.

U počecima genetskog istraživanja roda *Rupicapra*, primjena različitih molekularnih biljega imala je za svrhu potvrditi geografsku klasifikaciju podvrsta predloženu u radu Couturier (1938) i Dolan (1963). Citogenetska istraživanja somatskih stanica u divokoza otkrile su kariotip s 54 akrocentrična kromosoma i s jednim parom metacentričnog kromosoma koji čine autosome (2n = 58, Gallagher and Womack 1992). Analiza genetske varijabilnosti lokusa alozima (Nascetti i sur., 1985), minisatelita (Pérez i sur., 1996) i polimorfizama dužine restrikcijskih fragmenata (RFLP) mitohondrijske DNA (Hammer i sur., 1995) pokazala je znatno veću divergenciju između populacija dviju predloženih vrsta *R. pyrenaica* i *R. rupicapra,* nego između populacija unutar iste vrste.

Razvojem novih tehnologija i metoda u području populacijske genetike doprinijelo je otkrivanju novih molekularnih biljega koji se danas primjenjuju u proučavanju genetske strukture populacija životinjskih i biljnih vrsta što pokazuju podaci Banke gena (NCBI) gdje je arhiviran veliki broj sekvenciranih cijelih genoma ili njegovih određenih regija. Pérez i sur. (2000) odredili su panel mikrosatelita goveda i koza prikladnih za analizu i kod divokoza, što su kasnije napravili i Zemanová i sur. (2015) koji su razvili različite mikrosatelitne setove za neinvazivna populacijsko-genetska istraživanja divokoza (Tablica 1). Pérez i sur. (2002) su koristili mikrosatelite da bi odredili genetsku povezanost između predloženih vrsta i podvrsta, te dobili uvid u filogeniju roda *Rupicapra*. Analiza mikrosatelita u 8 od predloženih 10 podvrsta pokazala je jasnu razliku između svakog para populacija i jasno je razdvojila dvije skupine koje odgovaraju dvjema predloženim vrstama divokoza. Pošto mitohondrijski geni evoluiraju 7 do 12 puta brže od nuklearnih, za proučavanje obrazaca evolucije analizirana je mtDNA. Rodríguez i sur. (2007) su identificirali nuklearni pseudogen citokroma b kod divokoza te su predložili da pseudogen potječe iz vrlo divergentne mitohondrijske linije koja nije opstala u mitohondriju i transponirana je u jezgru u vremenu nastanka specijacije. Usporedba ograničenog broja sekvenci mtDNA pokazuje procjenu da je trajanje razdvajanja dviju predloženih vrsta divokoza trajao oko 1,5 milijuna godina.

Rodríguez i sur. (2009) su analizirali dio sekvence citokroma b (349 parova baza) da bi istražili genetsku povezanost između predloženih vrsta i podvrsta divokoza, te da bi bolje shvatili utjecaj pleistocenskih glacijacija na nastanak podvrsta. Rezultati istraživanja Rodríguez i sur. (2009) su otkrila postojanje triju citokrom b linija: skupina „West“ kojoj su pripadale jedinke podrijetlom s Iberije i Zapadnih Alpa; skupina „Central“ kojoj su pripadale jedinke podrijetlom s Apenina i gorja Chartreuse te skupina „East“ koja je predstavljala populacije na istoku Alpa uključujući populacije balkanske divokoze. Ovo istraživanje je prošireno (Rodríguez i sur., 2010) analizom dodatnih regija DNA sekvenci koje su uključivale ND1, 12S, tRNApro, CR, mikrosatelite (20 lokusa) i obrađeno je svih 10 podvrsta divokoza. Rezultat istraživanja pokazao je neskladna filogenetska stabla dobivena analizom mikrosatelita i mtDNA gdje je potonja tvorila 3 skupine „West“, „Central“ i „East“ koje nisu odgovarale skupinama dobivenim Neighbor-Joining analizom mikrosatelita. Kontrastna filogenetska stabla za mtDNA i mikrosatelite pokazuju događaje hibridizacije među vrlo divergentnim linijama u središnjem području distribucije.

Kako bi dobili dodatne informacije o patrilinearnom filogenetskom položaju roda *Rupicapra*, Pérez i sur. (2011) su analizirali Y kromosom.

Definirali su 10 haplotipova Y-kromosoma koji su tvorili dvije haplogrupe koje se slažu s taksonomskom klasifikacijom, umjesto tri skupine prethodno formirane za mtDNA i mikrosatelite. Nakon ovog istraživanja Pérez i sur. (2013) koristili su gen za melanokortin-1 receptor (MC1R) odgovoran za boju krzna na osnovu kojeg su definirali najmanje dva odvojena klastera koji odgovaraju *R. rupicapra* i *R. pyrenaica*.

Nedavne molekularne analize kompletnih mitohondrijskih genoma (Iacolina i sur., 2021) potvrđuju prethodno objavljenu podjelu roda u tri skupine (Pérez i sur., 2017b) gdje su se dvije podudarale s klasičnim vrstama (*R. pyrenaica* i *R. rupicapra*) dok je treća skupina bila sastavljena od podvrsta *R. p. ornata* i *R. r. cartusiana*. Zbog boljeg razumijevanja filogenije divokoza, Tešija (2022) koristio je sastavljenu i anotiranu sekvencu mtDNA te dijelove jezgrinog genoma (nDNA) jer su novija istraživanja pokazala da se odnosi između pojedinih vrsta i podvrsta mogu preciznije opisati korištenjem molekularnih markera koji sadrže veću količinu varijabilnih mjesta kao što je kompletna sekvenca mtDNA. Istraživanje Tešija (2022) rezultiralo je velikom varijabilnosti u protein-kodirajućim regijama (PCG) s tim da je *R. rupicapra* imala nižu raznolikost unatoč većem broju uzoraka, dok je na razini podvrste *R. r. balcanica* (uključujući i jedan hibrid *R. r. balcanica* x *R. r. rupicapra*) bila vidljiva najveća stopa diferencijacije. Filogenetske analize kombinacije rekonstruiranih mtDNA sekvenci (maksimalna vjerodostojnost i Bayesovska analiza) s pet mtDNA sekvenci preuzetih iz Banke gena rezultirale su jednakim filogenetskim stablima kao i kod Rodríguez i sur. (2010) za rod *Rupicapra,* podijelivši ga u tri mtDNA klastera (mtDNA „West“, mtDNA „Central“, mtDNA „East“). Evolucijska pozadina roda *Rupicapra* i dalje ostaje nejasna (Corlatti i sur., 2021) jer korištenje markera s različitim načinima evolucije može rezultirati sukobljenim filogenijama, osobito kada je uključena hibridizacija između divergentnih linija.

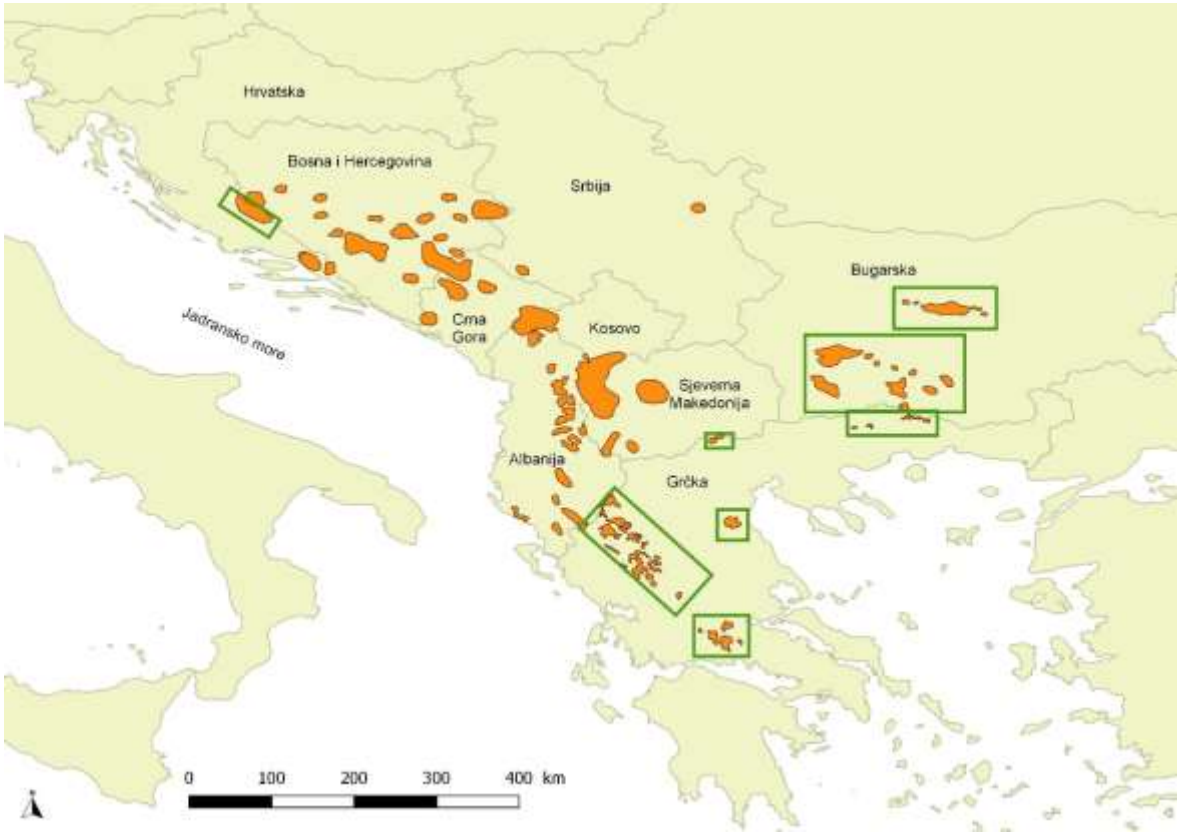
Glavni sustav tkivne podudarnosti (engl. *Major Histocompatibility Complex - MHC*) su geni koji kodiraju receptore koji prepoznaju i vežu antigene te su vrlo važni za stvaranje imunosti kod kralježnjaka. Zbog svoje varijabilnosti koja je povezana s različitim obilježljima fitnesa i održivosti populacija, smatraju se izvrsnim markerima u evolucijskoj konzervaciji. MHC DRB egzon 2 kodira aminokiseline za vezanje antigena zbog čega se može koristiti kao mjera funkcionalne raznolikosti DRB alela. Stipoljev (2022) analizirala je genetsku raznolikost egzona 2 MHC DRB lokusa skupine II u 110 jedinki iz populacija koje pokrivaju većinu područja rasprostranjenja roda *Rupicapra* korištenjem metode sekvenciranja sljedeće generacije (engl. *Next generation sequencing* – NGS). Identificirala je četrnaest novih DRB alela od koji su tri pronađena u podvrste *R. r. balcanica*.

Tablica 1. Podaci o razvijenim mikrosatelitnim lokusima za analizu populacijske genetike balkanske divokoze (prema Zemanová i sur., 2015).

Set	Lokus	Početnica	Ponavljajući motiv	Veličina raspona (pb)	Pristupni broj	C (µL)	Referenca
SET 1	OarFCB20	F: 5′-GGAAAACCCCCATATATACCTATAC-3′ R: 5′-AAATGTGTTTAAGATTCCATACATGTG-3′	$(TG)_n$	80-104	L20004	0,3	Buchanan i sur., 1994.
	OarFCB304	F: 5′-CCCTAGGAGCTTTCAATAAAGAATCGG-3′ R: 5′-CGCTGCTGTCAACTGGGTCAGGG-3′	$(TC)_n$ GC$(TC)_n$ GC$(TC)_n$ GC$(TC)_n$ $(AC)_n$	130-146	L01535	0,3	Buchanan & Crawford 1993.
	SR-CRSP-5	F: 5'-GGACTCTACCAACTGAGCTACAAG-3 R: 5'-TGAAATGAAGCTAAAGCAATGC-3	$(AT)_n$ $(GT)_n$ AT$(GT)_n$	154-174	L22197	0,2	Arevalo i sur., 1994.
	SY84	F: 5′-GAACTGAACTTGTTAGTATGTTGGG-3′ R: 5′-TTGTTATGCTTGATGTTATTTTGTTAC-3′	$(AC)_n$	170-180	AY725829	0,3	An i sur., 2005.
	CSSM66	F: 5′-ACACAAATCCTTTCTGCCAGCTGA-3′ R: 5′-AATTTAATGCACTGAGGAGCTTGG-3′	$(CA)_n$	193-255	AF232764	0,3	Barendse i sur., 1994.
	ETH10	F: 5′-GTTCAGGACTGGCCCTGCTAACA-3′ R: 5′-CCTCCAGCCCACTTTCTCTTCTC-3′	$(AC)_n$	205-213	Z22739	0,3	Toldo i sur., 1993.
SET 2	SY434	F: 5′-AAGTGTCTGGGTTCTCTTTCTCTA-3′ R: 5′-ATGTCAGTATGGGATGATGAATG-3′	$(TG)_n$	83-101	AY725834	0,3	An i sur., 2005.
	SR-CRSP-11	F: 5'-GTGCCCCATCACACATG-3' R: 5'-GTGGTTCTTTACGCTGAGCC-3'	$(CA)_n$ TG$(CA)_n$	112-132	-	0,2	Kogi i sur., 1995.
	SY259	F: 5′-GCACCACAACAAAGAGGAGC-3′ R: 5′-TGAAGACATAAGGGCGAACAG-3′	$(AC)_n$	156-166	AY725833	0,3	An i sur., 2005.
	TGLA53	F:5′-GCTTTCAGAAATAGTTTGCATTCA-3′ R:5′-ATCTTCACATGATATTACAGCAGA-3′	$(AT)_n$	132-156	GQ368903	0,3	Barendse i sur., 1994.

Set	Lokus	Početnica	Ponavljajući motiv	Veličina raspona (pb)	Pristupni broj	C (µL)	Referenca
SET 2	BOBT24	F: 5'-GAGCAAGGGAATTCAGTGGAGC-3 R: 5'-TGTATTTTACATTCAGGTCTGTGATCC-3	$(CA)_n$	148-176	-	0,3	Buitkamp i sur., 1996.
SET 3A	BM1258	F:5′-GTATGTATTTTTCCCACCCTGC-3′ R:5′-GAGTCAGACATGACTGAGCCTG-3′	$(TG)_n$	101-129	G18385	0,2	Bishop i sur., 1994.
	ILSTS030	F: 5′-CTGCAGTTCTGCATATGTGG-3′ R: 5′-GTTTCTTCTTAGACAACAGGGGTTTGG-3′	$(CA)_n$	152-180	L37212	0,3	Kemp i sur., 1995.
SET 3B	ETH225	F: 5′-GATCACCTTGCCACTATTTCCT-3′ R: 5'-ACATGACAGCCAGCTGCTACT-3′	$(CA)_n$	138-150	Z14043	0,3	Steffen i sur., 1993.
	SR-CRSP-6	F: 5'-CATAGTTCATTCACAATATGGCA-3′ R: 5'-CATGGAGTCACAAAGAGTTGAA-3′	$(GT)_n$	140-150	L22198	0,3	Bhebhe i sur., 1994.
	NRAMP1	F: 5'-GATGAGTGGGCACAGTGGCCT-3′ R: 5'-TTCAAGTGTCTTATTTACACCCATTG-3′	$(TG)_n$	196-218	AF005380	0,4	Matthews & Crawford 1998.

F – „*forward*“ početnica; R – „*reverse*“ početnica; pb – parova baza; C – koncentracija početnice (u mikrolitrima)



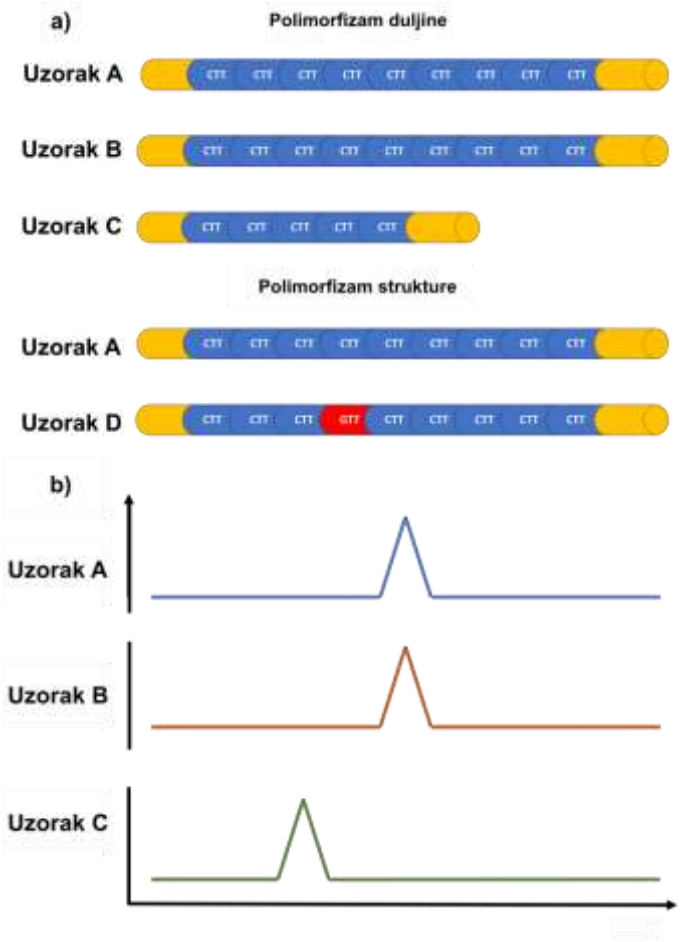
Slika 1. Geografska rasprostranjenost balkanske divokoze. Karta raspona distribucije (narančasti poligoni) izrađena je pomoću podataka preuzetih s IUCN Crvenog popisa ugroženih vrsta (preuzeto s: https://www.iucnredlist.org/resources/spatial-data-download; 02.06.2022). Zeleni kvadrati označavaju područja gdje je zaštićena Natura 2000 ekološkom mrežom.

2.2 Povijest populacija balkanske divokoze u Hrvatskoj

U posljednja dva stoljeća, prekomjerni lov, uništavanje staništa, urbanizacija, bolesti i praksa obnavljanja ili ponovnog unošenja snažno su utjecali na veličinu i geografsku distribuciju populacija divokoza (Crestanello i sur., 2009). Pretpostavlja se da su učinci ljudskih aktivnosti jači kod divokoza u usporedbi s drugim, homogenije rasprostranjenim vrstama, jer se očekuje da će život u polu-izoliranim planinskim vrhovima kod ove vrste dovesti do niske genetske raznolikosti unutar populacija i značajne razlike između njih zbog lokalne adaptacije.

Zbog fragmentacije staništa, prekomjernog lova i krivolova, introdukcije alpske podvrste, hibridizacije te mnogih drugih čimbenika neke od podvrsta su već zaštićene na nacionalnoj razini poput tatranske divokoze (Zemanová i sur., 2015). Balkanska podvrsta divokoze je zaštićena Natura 2000 mrežom jedino u Grčkoj i Bugarskoj, a u Hrvatskoj samo na području Dinare i predstavlja jedinu hrvatsku autohtonu populaciju balkanske divokoze (Slika 1).

Povijesni podaci i arheološka istraživanja potvrdili su veliku brojnost populacija divokoza u sjeverozapadnom Dinarskom gorju u Hrvatskoj, no tijekom ranih 1900.-ih, zbog neodrživog lova, krivolova, ispaše stoke, velikog broja grabežljivaca i prirodnih događaja (Frković, 2009) populacije su istrijebljene prije nego što je procijenjena njihova taksonomska klasifikacija (Miracle i Sturdy, 1991).



Slika 2. Različiti tipovi polimorfizma kod mikrosatelita: a) polimorfizam u duljini ponavljajućeg motiva i strukture mikrosatelita; b) polimorfizam u duljini ponavljajućeg motiva analiziran kapilarnom elektroforezom, (preuzeto s: https://www.biomnigene.fr/en/our-solutions/microsatellite-analysis.html; 12.10.2022.)

Posljednji zapisi o divokozi na masivu Velebita datiraju iz 1907. godine, kada je uočeno nekoliko životinja koje kasnije nisu mogle biti pronađene (Skorup, 2005). Smatra se da su posljednji primjerci odstrijeljeni u krivolovu iznad Baških Oštarija, te Jablanca u blizini Krasna (Frković, 2008). Ovaj štetan ljudski utjecaj pokušao se ispraviti ponovnim uvođenjem nakon Drugog svjetskog rata koji je zanemario genetske probleme. Translokacije divljači kao alat za obnovu ili jačanje populacija divljači za potrebe lova postala je raširena praksa u devetnaestom stoljeću (Apollonio i sur., 2014).

Pomno praćenje izvornih i translociranih populacija je često izostajalo iako je bilo ključno za razumijevanje čimbenika uspjeha i neuspjeha tih translokacija. Mnogi biotički i abiotički čimbenici mogu utjecati na uspjeh translokacije, uključujući genetsku raznolikost (Wright i sur., 2014) time što translokacije obično uključuju male izvorne populacije i ograničeni broj jedinki. Nadalje, genetski drift je jači u manjim populacijama te će narušiti genetsku raznolikost i uzrokovati međupopulacijsku divergenciju ako su populacije izolirane. Translocirane populacije najčešće imaju visok indeks unutar populacijske endogamije, te su pod utjecajem inbriding depresije (Charlesworth i Charlesworth, 1987). Dugoročno gledano, gubitak genetske raznolikosti kod translociranih populacija također smanjuje sposobnost populacije da se prilagodi budućim izazovima, odnosno gubi se njen evolucijski potencijal (Wright i sur., 2014) zbog čega je potrebno integrirati genetska ispitivanja u programe translokacije. Većina istraživanja o genetskim učincima translokacije se zasniva na raznolikosti neutralnih biljega kao što su mikrosateliti (Wright i sur., 2014). Mikrosateliti su polimorfni DNA lokusi koji sadrže ponovljene nukleotidne sekvence, obično od 2 do 7 nukleotida po jedinici (Su i sur., 2017). Duljina ponavljajućeg motiva ista je za većinu ponavljanja unutar pojedinačnog mikrosatelitnog lokusa (Slika 2a). Nasuprot tome, kada se broj ponavljanja za određeni lokus razlikuje, to rezultira alelima različite duljine (Slika 2b). Ogromna količina podataka koja proizlazi iz velikog broja mikrosatelitnih lokusa u organizmima čini ih široko prihvaćenim alatom za proučavanje genetske raznolikosti i divergencije unutar i između populacija (Su i sur., 2017).



Slika 3. Ispuštanje alpske divokoze u Lomsku dulibu na sjevernom Velebitu 10. rujna 1978. (izvor Frković, 2008).

Analiza mikrosatelita podrazumijeva umnažanje mikrosatelitnih lokusa korištenjem fluorescentno obilježenih početnica i lančane reakcije polimeraze (engl. *Polymerase Chain Reaction* - PCR). Označeni PCR proizvodi zatim se analiziraju kapilarnom elektroforezom da bi se razdvojili aleli po veličini.

Divokoze su reintroducirane u Dinarsko gorje između 1964. i 1978. godine, s jedinkama koje su potjecale iz različitih područja (Šprem i sur., 2015) pri čemu se nije razmatrala genetska struktura autohtonih i translociranih populacija. Na područje Velebita divokoze su reintroducirane u dva navrata (Frković, 2008). Prva translokacija se odvijala tijekom 1974. godine kada su ispuštene jedinke koje su uhvaćene u posebnom lovištu "Prenj“ u Bosni i Hercegovini. Divokoze su ispuštene na lokalitet Veliki lom koji se nalazi na području strogog prirodnog rezervata Hajdučki i Rožanski kukovi. Prva reintrodukcija se odvila 28. studenog 1974. kad je ispušteno 6 divokoza, a druga mjesec dana kasnije, točnije 24. prosinca kad su ispuštena 4 grla (Frković, 2008). U drugom navratu tijekom 1978. godine, ispušteno je 5 divokoza (3 ženke i 2 mužjaka; Slika 3) koje su bile podrijetlom s Kamniških Alpa u Sloveniji što je na kraju uzrokovalo hibridizaciju između alpske i balkanske divokoze (Šprem i Buzan, 2016; Kavčić i sur., 2018).

Na područje Biokova divokoza je reintroducirana prije 56 godina, točnije od 1. studenog 1964. do 23. listopada 1969. kada je ispušteno ukupno 48 jedinki (Šabić, 2014.; Tablica 2, Slika 4).



Slika 4. Ispuštanje balkanske divokoze na Biokovo (izvor Šabić, 2014).

Do 1960-ih godina nije bilo nikakvih pisanih tragova o postojanju divokoze na Biokovu. Jedini dokaz su bili arheološki ostaci divokoza u špilji Baba koja se nalazi na sjeveroistočnom dijelu Biokova. Današnje populacije balkanske divokoze na Velebitu i Biokovu su potomci jedinki koje potječu iz posebnog lovišta „Prenj“ osnovano 1961. godine koje je obuhvaćalo planinske masive Čvrsnicu i Prenj (Rapaić i Kunovac, 2020).

Prije osnivanja lovišta, Austro-Ugarska monarhija je već 1893. godine proglasila Čvrsnicu i Prenj kao zaštićena područja s ciljem zaštite staništa i rijetkih vrsta divljači (Knežević, 1893). Primorska banovina kojoj su pripadala ova lovišta je 1934. godine brojala oko 2600 jedinki (Knežević, 1893). Osim Hrvatske, s područja posebnog lovišta Prenj ukupno su translocirane 434 jedinke balkanske divokoze na područja u Bosni i Hercegovini (Gnjat, kanjon Une, Šator, kanjon Ugra, Duboka, Vranica, Sokolina, Koprivnica, Kamenica), Srbiji (Prokletije, Đerdap, Stolovi), Argentini (Ande) i Novom Zelandu (Rapaić i Kunovac, 2020).

Tablica 2. Podaci o datumu i broju reintroduciranih balkanskih divokoza na Biokovo u razdoblju od 1964. do 1969. prema Šabić (2014).

Datum	Broj reintroduciranih jedinki
31.10.1964.	7
20.11.1964.	7
26.11.1964.	6
24.09.1965.	6
03.10.1967.	5
10.10.1967.	5
10.10.1969.	6
23.10.1969.	6
Ukupno	**48**

Prema Mikuletič (1978) s Prenj planine su, u razdoblju od 1962. do 1974. translocirane 364 jedinke balkanske divokoze za ukupno 14 lovišta bivše države. Mogućnost identificiranja utjecaja povijesnih reintrodukcija na genetsku varijabilnost populacije i njezinu geografsku distribuciju od primarnog su interesa pri provedbi strategija zaštite i gospodarenja divokozama (Crestanello i sur., 2009).

3. REZULTATI I RASPRAVA

3.1. Pregled objavljenih kvalifikacijskih znanstvenih radova

3.1.1. Balkanska divokoza, arhipelag ili poluotok? Uvidi iz nuklearne i mitohondrijske DNA

Balkanska divokoza (*Rupicapra rupicapra balcanica,* Bolkay 1925) rasprostranjena je na Balkanskom poluotoku, duž planinskih masiva od Hrvatske na sjeveru do Grčke na jugu i Bugarske na istoku. Poznavanje genetske strukture populacija balkanske divokoze ograničeno je na lokalna istraživanja. U svim zemljama u kojima je prisutna smatra se ugroženom, dok akcijski plan gospodarenja postoji samo u Bugarskoj gdje je zaštićena Natura 2000 ekološkom mrežom. U Hrvatskoj je zavičajna podvrsta samo na području ekološke mreže HR5000028 Dinara, te akcijski plan gospodarenja još uvijek nije izrađen. Stoga je glavni cilj ovog istraživanja bio prikupiti informacije o genetskoj raznolikosti i populacijskoj strukturi balkanske divokoze duž njezine rasprostranjenosti kako bi se podržao razvoj strategija gospodarenja i očuvanja. S tim ciljem, koristili smo mikrosatelitne lokuse i djelomičnu regiju mtDNA (kontrolnu regiju - CR) kako bismo odgovorili na pitanja o utjecaju geografske izolacije na genetsku raznolikost populacija balkanskih divokoza.

Za potrebe ovog istraživanja ukupno je prikupljen 101 uzorak balkanske divokoze u razdoblju od 2011. do 2018. diljem lokaliteta gdje je ova podvrsta rasprostranjena (Slika 1). Uzorci su podijeljeni u osam grupa prema zemlji podrijetla: Hrvatska (HRV), Bosna i Hercegovina (BIH), Srbija (SRB), Crna Gora (MNE), Albanija (ALB), Sjeverna Makedonija (MKD), Bugarska (BGR) i Grčka (GRC). Od ukupnog broja prikupljenih uzoraka, kompletni genotipovi dobiveni su kod 92 jedinke, dok je CR mtDNA uspješno sekvencionirana u 44 jedinke.

Za analizu mtDNA prikupili smo 56 dodatnih uzoraka koji pripadaju drugim podvrstama divokoze (*R. p. pyrenaica*, *R. p. ornata*, *R. r. rupicapra*, *R. r. tatrica*, *R. r. carpatica*). Uzorci (kosti, osušena koža i mišićno tkivo) dobiveni su od jedinki odstrijeljenih u regularnim lovovima, jedinki uginulih prirodnom smrću i primjeraka iz zbirki lovačkih muzeja. Prije izolacije DNA iz kosti pripremili smo protokol demineralizacije kako bismo dobili što više izolata DNA (Buzan i sur., 2020).

Dvadeset mikrosatelita je amplificirano pomoću PCR multipleksa koji su već bili korišteni u istraživanju populacijske genetike divokoza (Zemanová i sur., 2011; Buzan i sur., 2013; Šprem i Buzan, 2016). Djelomična CR mtDNA je amplificirana korištenjem početnica i protokola opisanog u Rodríguez i sur. (2010).

U Prilogu 1. detaljno su opisane statističke metode i računalni programi koji su korišteni u prvom istraživanju za analizu populacijske strukture i utjecaja geografske izolacije na genetsku raznolikost populacija balkanske divokoze.

Nakon testiranja na prisutnost nul alela odabran je finalni set od 16 mikrosatelitnih lokusa koji su korišteni u daljnjim analizama (Tablica 1). Isključili smo četiri lokusa zbog loše amplifikacije, visokog postotka nul alela i monomorfizma. Dva isključena lokusa, SY58 i INRA121, predstavljala su iste probleme i u drugim istraživanjima (Buzan i sur., 2013; Šprem i Buzan, 2016), stoga je preporuka da se ova dva lokusa izuzmu u budućim istraživanjima vrsta *Rupicapra*. Set je dao ukupno 116 alela u osam analiziranih populacija, s prosjekom od 7,25 alela po lokusu. Samo je populacija iz Bosne i Hercegovine značajno odstupala od Hardy-Weinberg ekvilibrijuma (HWE), dok je populacija iz Srbije jedina imala višak heterozigota.

Računalni program STRUCTURE koji se zasniva na Bayesovskim metodama je korišten za procjenu broja izvornih genskih skupova (K) kod analiziranih populacija balkanske divokoze. Dodatno je primijenjena metoda opisana u Evanno i sur. (2005) kako bi se donijela odluka o najvjerojatnijem broju prisutnih genskih skupova, odnosno klastera (K) u analiziranim populacijama koja je u ovom slučaju predložila K=3 kao optimalni broj. STRUCTURE model za K=3 je pridružio jedinke iz Srbije i Bugarske u dva privatna klastera dok su ostale pripadale jednom zajedničkom klasteru (Prilog 1).

Indeks genetske diferencijacije (F_{ST} vrijednost) je pokazao slične rezultate gdje je populacija iz Bugarske imala najveću vrijednost i značajno se razlikovala od drugih populacija dok Mantelov test nije pokazao značajan utjecaj izolacije zbog udaljenosti populacija (engl. *isolation by distance - IBD*).

Kontrolna regija je uspješno amplificirana u 44 uzroka balkanske divokoze i svih 56 dodatnih uzoraka podvrsta *R. p. pyrenaica*, *R. p. ornata*, *R. r. rupicapra*, *R. r. tatrica* i *R. r. carpatica*. Sekvenca duga 376 parova baza je poravnata sa 109 sekvenci preuzetih s NCBI banke gena (pristupni brojevi AM279274-279275, EU887481-EU887488, GU951843–GU951916, KC594557-KC594572, KP730619-KP730627). Ukupno je pronađeno 30 novih haplotipova koji su uneseni u NCBI banku gena pod pristupnim brojevima MT746066 - MT746095 (HRCR17 - HRCR46).

Median-joining mreža konstruirana na osnovu sekvenci CR mtDNA pokazala je prisutnost privatnih haplotipova u svih osam analiziranih populacija balkanske divokoze dok su dva haplotipa (HRCR9 i HRCR20) bila zastupljena u više populacija ukazujući na moguće translokacije i hibridizaciju podvrsta u prošlosti (Prilog 1).

Uvid u genetsku strukturu balkanske divokoze predstavljenu u istraživačkom radu u Prilogu 1. pruža potrebnu polaznu točku za procjenu statusa zaštite, te će omogućiti razvoj strategija potrebnih za održivo gospodarenje i očuvanje populacija balkanske divokoze, ne samo u Hrvatskoj već i u drugim zemljama gdje ova podvrsta obitava.

Ključne riječi: očuvanje, genetska raznolikost, mtDNA, populacijska genetika, *Rupicapra rupicapra balcanica*

3.1.2 Tragovi povijesnih reintrodukcija u genetskoj raznolikosti: slučaj balkanske divokoze

Premještanje divljih životinjskih vrsta postala je uobičajena praksa diljem svijeta kako bi se ponovno uspostavile lokalne populacije kojima prijeti izumiranje. Sjeverna divokoza (*Rupicapra rupicapra* L.) jedna je od primjera uspješno reintroduciranih vrsta u mnogim područjima Europe, ali i na drugim kontinentima poput Južne Amerike i Novog Zelanda. Današnje populacije divokoza na Velebitu i Biokovu su potomci uspješno reintroduciranih jedinki uhvaćenih na planinskim područjima u Bosni i Hercegovini (*R. r. balcanica*) i Sloveniji (*R. r. rupicapra*). Arheološki podaci potvrđuju da su divokoze nekada živjele na planini Biokovo, ali prije reintrodukcije tijekom 1960-ih godina, nije bilo pisanih dokaza o njihovom nedavnom postojanju na tom području.

Glavni cilj ovog istraživanja je bio utvrditi točnost postojećih povijesnih podataka o podrijetlu populacije balkanske divokoze na planini Biokovo te procijeniti genetsku raznolikost i populacijsku strukturu izvorne i translocirane populacije 56 godina nakon prve reintrodukcije.

Koristili smo 16 mikrosatelitnih lokusa (Tablica 1) koji su testirani u prvom istraživanju (Prilog 1) za analizu genetske strukture tri izvorne populacije divokoza s Prenja, Čvrsnice i Čabulje te s planine Biokovo. U Prilogu 2 detaljno su opisane statističke metode i računalni programi koji su korišteni u drugom istraživanju za analizu genetske raznolikosti i populacijske strukture izvornih i translociranih populacija.

STRUCTURE model pokazao je jasnu odvojenost populacija na Biokovu od balkanskih divokoza koje nastanjuju Prenj i značajnu genetsku sličnost između biokovske populacije i populacija na Čvrsnici i Čabulji (Prilog 2). Ovi rezultati upućuju da sadašnji genetski sastav biokovskih populacija ne potječe isključivo s Prenja, što je bilo navedeno u dostupnoj literaturi (Frković, 2008; Šabić, 2014; Šprem i Buzan, 2016) i iz osobnih intervjua, već da su jedinke podrijetlom i s Čvrsnice i Čabulje. Evanno algoritam je identificirao K=3 kao optimalan broj izvornih populacija otkrivenih metodom STRUCTURE za četiri analizirane populacije i otkrio je još jedan vrh na K=5 što ukazuje na moguću daljnju podjelu genetske strukture unutar populacija.

Provedena je dodatna analiza genetske strukture populacija koristeći GENELAND računalni algoritam, koja osim genetskog dijela uzima u obzir i prostorni kontekst analiziranih jedinki. GENELAND analiza prepoznala je balkansku divokozu s Prenja kao zaseban genetski skup, različit od populacija koje obitavaju na Čvrsnici i Čabulji.

Pretpostavka je da su rijeka Neretva i državna cesta M17 geografske granice za rasprostranjenost podvrste, budući da čine barijeru za izmjenu genetskog materijala. Ova metoda je predložila četiri prostorna klastera, ali bez dodijele jedinki zadnjem tzv. „duh“ klasteru što upućuje na moguću dodatnu genetsku podjelu unutar populacija.

Naši rezultati genetske raznolikosti pokazuju da populacija balkanske divokoze s Biokova može poslužiti kao potencijalni izvor za buduće translokacije, posebno na izvorna staništa, Čvrsnicu i Čabulju, koja su trenutno ugrožena gubitkom genetske raznolikosti zbog krivolova, što za posljedicu ima visoku stopu parenja u srodstvu i pojavu genetskog drifta.

Ključne riječi: Biokovo, genetska struktura, mikrosateliti, Prenj, translokacija

3.1.3 Primjena mikrosatelita u otkrivanju povijesti istrijebljenih planinskih papkara

Tijekom devetnaestog stoljeća translociranje divljih životinja je postalo uobičajena praksa i služilo je kao način gospodarenja za jačanje ili obnavljanje populacija divljači u lovne svrhe. U to vrijeme, genetski sastav autohtonih i translociranih populacija nije razmatran. Za razliku od nekih drugih vrsta papkara, divokoza nije bila podvrgnuta opsežnim operacijama novog ili ponovnog uvođenja na određena područja kako bi se podržao potpuni oporavak povijesnog areala vrste već su se translokacije odvile uglavnom zbog lokalnih inicijativa (lovačka društva) s ciljem obnove populacija divokoza na izoliranim planinskim lancima za lovne svrhe.

Povijesni podaci i arheološka istraživanja potvrdili su veliku brojnost populacija divokoza u sjeverozapadnom Dinarskom gorju u Hrvatskoj, no zbog neodrživog lova, krivolova, predacije i drugih čimbenika tijekom ranih 1900-ih, populacije sjevernih divokoza (*Rupicapra rupicapra*) u sjevernom Dinarskom gorju su istrijebljene.

Tijekom 1970-ih izvršene su reintrodukcije u svrhu ponovnog uspostavljanja populacije na Dinarskom području (Slika 3). Translokacija se obavila iz susjednih područja u Sloveniji i Bosni i Hercegovini pri čemu su reintroducirane dvije podvrste sjevernih divokoza: alpska divokoza (*R. r. rupicapra)* i balkanska divokoza (*R. r. balcanica*). Prije reintrodukcije točna taksonomska klasifikacija autohtone istrijebljene populacije, na razini podvrsta, nije bila poznata.

Kako bismo razjasnili koja je podvrsta bila prisutna prije reintrodukcije, genotipizirali smo četiri lubanje divojaraca (muzejski primjerci) koji su bili podrijetlom s planine Velebit. Jedinke su potjecale iz vremena prije lokalnog izumiranja populacije (1886., 1893., 1895. i 1939. godina). DNA je bila uspješno ekstrahirana iz srednjeg sloja i vanjske ovojnice rogova. Kako bi se izbjegla moguća kontaminacija, laboratorijski rad s uzorcima starih kostiju (starijih od 100 godina) proveden je u namjenskom, fizički izoliranom laboratoriju za drevnu DNA. Za amplifikaciju DNA korišten je set od 20 mikrosatelitnih lokusa i protokol opisan u Zemanová i sur. (2011). Za dobivanje odgovora na postavljenu hipotezu korištene su dvije analize, Bayesovska metoda klasteriranja te diskriminantna analiza glavnih komponenti (DAPC).

Genotipovi dobiveni iz muzejskih uzoraka lubanja su uspoređeni s referentnom skupinom od 52 genotipa poznatog podrijetla prethodno analizirana u Šprem i Buzan (2016). Za referentne populacije su odabrani genotipovi koji su imali procijenjeni koeficijent pripadnosti (q) jednoj od podvrsta veći od 85%. Ovaj kriterij odabira rezultirao je s 20 genotipova koji su pripadali alpskoj i 32 genotipa dodijeljena balkanskoj podvrsti.

Tri od četiri muzejska uzorka uspješno su amplificirana za svih 20 mikrosatelitnih lokusa, dok su za četvrti uzorak dobiveni aleli na 16 mikrosatelitnih lokusa. STRUCTURE (s q vrijednostima između 0,55 i 0,73) i DAPC (s vjerojatnošću individualne pripadnosti od 0,99 i 1,00) analize pokazale su veću procijenjenu vjerojatnost da je na analiziranom području prije reintrodukcije obitavala alpska podvrsta divokoze (Prilog 3).

Rezultati ovog istraživanja daju vrijedan pokazatelj najvjerojatnijeg genetskog i taksonomskog sastava autohtone populacije divokoza koja je obitavala na sjevernom Dinarskom gorju prije provođenja reintrodukcije. Ove informacije će poslužiti kod planiranja budućih projekata reintrodukcije na ova područja koje će uzimati u obzir genetsku strukturu jedinki, da bi se ustanovile nove ali i očuvale postojeće populacije.

Ključne riječi: *Rupicapra rupicapra*, genetsko podrijetlo, povijesni uzorci, reintrodukcija

3.2. OBJEDINJENA RASPRAVA

Rezultati ovog doktorskog istraživanja su otkrili postojanje genetski i geografski različitih populacija balkanske divokoze. Rasprostranjenost ovih populacija je neujednačena i pokriva samo dijelove masiva i planinskih lanaca u zemljama u kojima se nalazi (Slika 1). Niske stope kolonizacije podvrste i smanjeni protok gena između izoliranih populacija mogu rezultirati genetskom diferencijacijom zbog parenja u srodstvu i gubitkom alelnih varijanti kao posljedica genetskog drifta (Willi i sur., 2006), kao i većom osjetljivosti na zarazne bolesti (Stipoljev 2022). Smanjena genetska raznolikost u malim i izoliranim populacijama mogla bi, zauzvrat, uzrokovati negativne utjecaje na fitnes, što rezultira smanjenjem efektivne veličine populacije i, na kraju, povećanjem vjerojatnosti izumiranja (Pelletier i sur., 2019). Ostale prijetnje opstanku divokoza na Balkanu su krivolov (Papaioannou i Kati, 2007), introdukcija drugih podvrsta divokoza, uglavnom alpskih divokoza (Iacolina i sur., 2019), sukcesija šuma (Kavčić i sur., 2019), izgradnja cestovnih infrastruktura (Kati i sur., 2020), intenzivna ispaša stoke, grabežljivci, neodrživ lov i prirodni događaji (Šprem i Buzan, 2016). Status zaštite i gospodarenja unutar različitih nacionalnih zakonodavstava razlikuje se između zemalja u kojima obitava (članice i nečlanice Europske Unije) i ovisi o stupnju interesa lokalnih zajednica za očuvanje podvrste (Anderwald i sur., 2020). Dosadašnja istraživanja genetske strukture balkanske podvrste divokoze nisu uključivala cijeli raspon distribucije i bila su ograničena na lokalna istraživanja (Bugarska - Markov i sur., 2016; Hrvatska i Bosna i Hercegovina - Šprem i Buzan, 2016; Grčka - Papaioannou i sur., 2019).

Iako se broj publikacija o *Rupicapra* rodu značajno povećao tijekom posljednja dva desetljeća, i dalje postoje velike razlike s obzirom na vrste i podvrste, a istraživanja se uglavnom fokusiraju na alpsku divokozu i, u manjoj mjeri, pirinejsku divokoza (Corlatti i sur., 2022b). Balkanska divokoza je uključena u nekoliko filogenetskih studija koje su koristile i nuklearne i mitohondrijske biljege, ali su ta istraživanja uključivala mali broj uzoraka koja su potjecala većinom s jednog lokaliteta (npr. Pérez i sur., 2002; Rodriguez i sur., 2009; Rodriguez i sur., 2010; Pérez i sur., 2011; Pérez i sur., 2013; Pérez i sur., 2017a; Pérez i sur., 2017b; Iacolina i sur., 2021; Pérez i sur., 2022).

Filogeografska istraživanja temeljena na mtDNA otkrila su tri vrlo stare skupine divokoza označene kao zapadna (mtDNA “West“), središnja (mtDNA „Central“) i istočna (mtDNA „East“) s jasnim geografskim uzorkom diljem Euroazije (Crestanello i sur., 2009; Rodríguez i sur., 2010) i njihovu prisutnost u Europi koja je procijenjena na 1,9 milijuna godina u ranom pleistocenu (Lalueza-Fox i sur., 2005; Rodríguez i sur., 2010; Pérez i sur., 2014).

To je daleko starije od starosti fosila *Rupicapra* u Europi koji su pronađeni na Balkanskom poluotoku i odgovaraju početku srednjeg pleistocena, između 780 i 750 tisuća godina (Fernandez i Crégut, 2004). Otkriveno je da je rasprostranjenost podvrsta divokoza naslijeđe kvartarne glacijalne-interglacijalne dinamike kada su mnoge svojte bile zarobljene u relativno malim refugijalnim područjima koja su uključivala južne poluotoke: Iberijski, Apeninski i Balkanski (Pérez i sur., 2022). Skupina mtDNA „West" je ograničena na Iberijski poluotok i na zapadni dio Alpa, dok je mtDNA „Central" skupina dijeljena između podvrsta *ornata* i *cartusiana*. Skupina mtDNA „East" predstavlja sve podvrste rasprostranjene od Alpa prema istoku u koju spada i balkanska divokoza. Ovu podjelu podržavaju i rezultati dobiveni kombinacijom glavnog sustava tkivne podudarnosti (MHC) i sekvenci mtDNA koji su pokazali da je posljednja glacijacija izazvala demografski pad i male podjedinice u populacijama divokoza (Mona i sur., 2008) za razliku od mnogih drugih vrsta koje su doživjele demografski pad u Sredozemnim refugijima tijekom glacijalnog maksimuma, a zatim se naknadno proširile kako su temperature rasle (Petit i sur., 2003). Rast temperature smanjio je raspoloživi teritorij za divokoze zbog čega su se formirale male podjedinice izolirane na planinskim vrhovima. Proučavanje nuklearnih biljega otkrilo je mnogo mlađu divergenciju između podvrsta od one uočene iz mtDNA i introgresiju posredovanu mužjacima u središnjem dijelu distribucije (mtDNA „Central"; Pérez i sur., 2022). Posljedično, potrebno je obuhvatiti različite molekularne biljege kako bi se proširio pogled na procese diversifikacije roda *Rupicapra* i pružili dokazi za reviziju pretpostavljene taksonomske klasifikacije (Corlatti i sur., 2021).

3.2.1. Genetska raznolikost balkanske divokoze

Genetska raznolikost istraživanih populacija balkanske divokoze (Prilog 1) je niska u usporedbi s vrijednostima objavljenim u studiji Rodríguez i sur. (2010). Ova razlika može biti posljedica većeg broja mikrosatelita koje su koristili Rodríguez i sur. (2010). Slična situacija pronađena je u istraživanju populacijske genetike alpskih divokoza (Soglia i sur., 2010), koje je pokazalo veće procjene genetske raznolikosti u usporedbi s rezultatima na istoj podvrsti u istraživanju Buzan i sur. (2013). Ove razlike u procjenama genetske raznolikosti unutar iste podvrste mogle bi se objasniti razlikama u veličini uzorka, korištenjem različitog ili većeg broja mikrosatelita, kao i zastupljenošću različitih mjesta uzorkovanja. Prosječna vrijednost opažene heterozigotnosti (0,553) bila je nešto niža od očekivane heterozigotnosti (0,581) što ukazuje na generalni višak homozigota. Uzrok tomu je posljedica prostornog strukturiranja populacija u subpopulacije (Pérez i sur., 2002; Buzan i sur., 2013).

Posebno je zanimljiva niska raznolikost Bugarske populacije, koja dolazi iz "Državnog lovnog rezervata Izvora" u zapadnim Rodopima i predstavlja glavnu izvornu populaciju za translokacije divokoza u Bugarskoj. Promatrane vrijednosti mogu biti rezultat negativnih fluktuacija u broju divokoza u nedavnoj prošlosti, dok je trenutna veličina populacije oko 250 jedinki koja je geografski izolirana (Markov i sur., 2016). Prema Hristovichu (1939), na Rodopima je procijenjeno da obitava ukupno 150 do 200 jedinki, a ukupan broj u Bugarskoj je oko 1000 divokoza. Sve do sredine 19. stoljeća balkanska divokoza imala je širi raspon rasprostranjenosti u Bugarskoj, jer je naseljavala gotovo sva prikladna staništa u Rilsko-rodopskom planinskom lancu. Krajem 19. stoljeća podvrsta je nestala s mnogih svojih područja uvođenjem dalekometnih pušaka (Avramov i Valchev, 2010). Markov i sur. (2016) sumnjaju da je populacija iz "Državnog lovnog rezervata Izvora" doživjela efekt uskog grla zbog prekomjernog lova i krivolova u svojoj novijoj povijesti, što bi moglo dovesti do kontinuiranog gubitka genetske raznolikosti. Gubitak genetske varijacije još uvijek se ne vidi u genetskom sastavu bugarske populacije iako postoje male naznake u opaženoj F_{IS} vrijednosti (0,035) koja pokazuje manje parenje u srodstvu, suprotno visokom koje su očekivali Markov i sur. (2016).

STRUCTURE i F_{ST} rezultati predstavljeni u prvom istraživanju (Prilog 1) su otkrili postojanje tri genetski i geografski različite populacije koje obitavaju na Balkanskom poluotoku. Genetska diferencijacija bila je najizraženija kod populacija iz Bugarske i Srbije. Značajne F_{ST} vrijednosti mogu se objasniti izolacijom populacija zbog geografskih obilježja planinskih lanaca u Srbiji i Bugarskoj, budući da bi mogli predstavljati prepreku protoku gena. Genetska diferencijacija uzrokovana sličnim čimbenicima pronađena je kod divokoza u drugim planinskim lancima, ne samo između (Pérez i sur., 2002; Crestanello i sur., 2009; Rodríguez i sur., 2010) nego i unutar podvrsta (Crestanello i sur., 2009; Buzan i sur., 2013; Markov i sur., 2016; Papaioannou i sur., 2019). Visoka genetska diferencijacija populacije iz Bugarske koju pokazuju i F_{ST} i STRUCTURE analize mogla bi biti rezultat male efektivne veličine populacije u Rodopima na kraju 19. stoljeća i višestrukih genetskih uskih grla s kojima se ova populacija susrela (Markov i sur., 2016). Prema Akcijskom planu za balkansku divokozu u Bugarskoj (Valchev i sur., 2006), genetska diferencijacija ove populacije rezultat je nepostojanja prirodnih koridora između populacija u Zapadnim Rodopima, Rili, Pirinu i Centralnom Balkanu, kao i fragmentacija na razini lokalnih populacija. Osim izolacije zbog fragmentacije staništa, drugi čimbenik koji je mogao dovesti do takve genetske diferencijacije mogla bi biti hibridizacija s alpskom divokozom, budući da su jedinke ove podvrste unesene 1977. godine u lovni rezervat Kormisosh (Valchev i sur., 2006).

Genetska diferencijacija balkanskih divokoza iz Srbije od susjednih populacija nije bila neočekivana, budući da je sličan obrazac uočen i kod drugih vrsta, uključujući i dinarsko-balkansku populaciju vuka (*Canis lupus*) gdje su prepoznate dvije subpopulacije: "zapadna" subpopulacija s jedinkama iz Bosne i Hercegovine i Hrvatske te "istočna" subpopulacija s jedinkama iz Srbije i Sjeverne Makedonije (Djan i sur., 2014). Čimbenici koji su uzrokovali različitu genetsku strukturu analiziranih populacija vukova uključuju efekt uskog grla, različite demografske povijesti subpopulacija, lovni pritisak, te mogućnost da rijeka Drina djeluje kao barijera izmjeni genetskoga materijala (Djan i sur., 2014.). Ova rijeka djeluje kao prirodna barijera i za druge vrste, uključujući risa (*Lynx lynx*; Melovski i sur., 2012) te divlju mačku (*Felis silvestris*; Urzi i sur., 2021), a geografske karakteristike Dinarskog gorja i kanjon rijeke Drine su možda utjecali i na balkansku divokozu, kao i lovni pritisak, što za posljedicu ima podjelu na subpopulacije. U istraživanju genetske strukture populacija divlje mačke koju su proveli Urzi i sur. (2021) identificirana je podjela na zapadne i jugoistočne populacije, ali su autori naglasili važnost ravnomjernog uzorkovanja i izbjegavanja „praznina" u uzorkovanju između analiziranih područja što bi moglo utjecati na finalne rezultate i njihovo tumačenje. Stoga bi bilo važno dodatno istražiti populacije balkanskih divokoza u Srbiji čime bi se uzrokovala sva (ili većina) područja na kojima ova podvrsta obitava. Također, bitno je kombinirati različite molekularne markere kako bi se razjasnio status i povijest populacija divokoza u ovoj zemlji i da bi se razjasnio učinak fragmentacije pogodnih staništa, povijesnih procesa ili ekoloških barijera na promatranu genetsku diferencijaciju.

S druge strane, nije pronađena značajna korelacija između genetske i geografske udaljenosti populacija. Sličan slučaj zabilježen je kod alpskih divokoza (Soglia i sur., 2010), gdje šest geografskih populacija s različitih mjesta na južnom lancu Alpa nije pokazalo značajnu korelaciju genetskih i geografskih udaljenosti. Značajna korelacija pronađena je nakon uklanjanja populacije s Lombardskih predalpa, za što su Soglia i sur. (2010) pretpostavili da je možda pretrpjela moguće posljedice utjecaja efekta osnivača (engl. *founder effect*) u kombinaciji s geografskom izolacijom i da su različite genetske karakteristike izvorne metapopulacije mogle poremetiti očekivani obrazac IBD-a.

Analiza sekvenci kontrolne regije otkrila je šesnaest privatnih i dva zajednička haplotipa (HRCR9 i HRCR20) kod balkanskih divokoza, te je pokazala da postoji izmjena gena između susjednih populacija, u ovom slučaju između Srbije i Crne Gore. Haplotipne i nukleotidne vrijednosti su pokazale relativno visoku varijabilnost među analiziranim sekvencama balkanske divokoze. Provedene filogenetske analize mtDNA sekvenci divokoza (Tešija 2022) su pokazale najveću stopu diferencijacije kod podvrste *R. r. balcanica* uključujući i jedan hibrid (*R .r. balcanica* x *R. r. rupicapra*).

Median-joining mreža odvojila je grčke haplotipove od haplotipova koji pripadaju drugim analiziranim populacijama (13 mutacija). Četiri sekvence balkanske divokoze iz Grčke analizirane u ovom istraživanju potjecale su sa sjevernog Pindskog gorja, gdje su Papaioannou i sur. (2019) otkrili veću varijabilnost među populacijama balkanskih divokoza, blizu raznolikosti većih populacija alpske podvrste u Alpama. Slični rezultati pronađeni su u istraživanju majčinski naslijeđenih biljega u drugim podvrstama divokoza (Schaschl i sur., 2003; Crestanello i sur., 2009; Rodríguez i sur., 2009; Rodríguez i sur., 2010; Buzan i sur., 2013), gdje su uočene privatne filogrupe i ograničeni protok gena između susjednih populacija. Filogeografska analiza populacija divokoza koje obitavaju na Dinaridima duž Slovenije, Hrvatske i Bosne i Hercegovine je utvrdila postojanje endemskih haplotipova balkanske divokoze na području Dinare, Biokova i Prenja kao i tragove povijesnih translokacija na području Velebita (Šprem i Buzan, 2016).

Schaschl i sur. (2003) pokazali su geografsko strukturiranje na bazi mtDNA alpskih populacija divokoza u istočnim Alpama kao posljedicu imigracije divokoza iz različitih pleistocenskih refugija oko Alpa nakon povlačenja glacijala, a ne zbog topografskih prepreka protoku gena. Za sve parove populacije proučavane u Crestanello i sur., (2009), diferencijacija je uvijek bila veća za CR nego za mikrosatelite, što je bilo i očekivano s obzirom na veću osjetljivost ovog lokusa na genetski drift.

3.2.2. Utjecaj povijesnih događaja na genetsku strukturu balkanske divokoze

Kada su se usporedile sekvence balkanske divokoze sa skupom podataka koji sadrži sve druge vrste *Rupicapra* spp. (Prilog 1), pronađen je jedan zajednički haplotip s alpskom divokozom i dva zajednička s tatranskom divokozom (HRCR9, HRCR20, HRCR38). Haplotipska mreža grupirala je balkanske haplotipove podrijetlom s Dinare u Hrvatskoj, Rodopa u Bugarskoj i Prenja u Bosni i Hercegovini s alpskim haplotipovima koji su potjecali sa Sjevernog Velebita (HRCR9). Rezultati analize mtDNA pokazuju da su se u prošlosti odvile translokacije divokoza što je dovelo do hibridizacije između podvrsta (Šprem i Buzan, 2016; Kavčić i sur., 2018; Iacolina i sur., 2019). Koristeći mtDNA Šprem i Buzan (2016.) su potvrdili postojanje hibrida između balkanske i alpske divokoze na Velebitu što je posljedica reintrodukcije podvrsta tijekom 1970-ih (Frković, 2008). Hibridi između balkanske i alpske podvrste na ovom području su imali značajno drugačiji obrazac razvoja rogova od „čistih“ podvrsta, gdje je kod hibrida zabilježena veća stopa početnog rasta rogova do 2,5 godine i znatno niže stope kompenzacije u prvih 4,5 godina života kod oba spola (Kavčić i sur., 2018). Obrasci razvoja rogova u divokoze mogu se razlikovati unutar populacija i podvrsta, a uglavnom su pod utjecajem genetske strukture, spola i dostupnosti resursa.

Veći početni rast rogova i niske stope kompenzacije u hibridnoj populaciji mogu ukazivati na učinak heterozisa, odnosno pojavu hibridne snage (Kavčić i sur., 2018).

Slični obrasci se mogu pojaviti u Rodopima u Bugarskoj, zbog izvršene introdukcije alpske divokoze na područje Kormisosh rezervata (Valchev i sur., 2006). Alternativno objašnjenje Markov i sur. (2016) je da signali introgresije koji su vidljivi iz mikrosatelita kod balkanske populacije, prije odražavaju zajednički polimorfizam predaka koji se mogao nakupiti genetskim driftom nego hibridizaciju. Pretpostavlja se da se hibridizacija događa i između balkanske i karpatske divokoze (*R. r. carpatica*) u kontaktnoj zoni u Nacionalnom parku Đerdap (Srbija; Damm i Franco, 2014) gdje su podvrste prirodno rasprostranjene. U Niskim Tatrama (Slovačka) primjenom citokroma b potvrđena je pojava hibrida između endemske podvrste divokoze (*R. r. tatrica*) i alpskih jedinki unesenih zbog potreba komercijalnog lova (Crestanello i sur. 2009).

Sekvenciranje cijelog genoma moglo bi pojasniti utjecaj hibridizacije na status zaštite, budući da bi hibridizacija između vrsta ili podvrsta, koja je rezultat legalnog i ilegalnog premještanja divokoza, mogla dovesti do gubitka lokalne prilagodbe određenim nišama (Corlatti i sur., 2022b). Buduća istraživanja o učincima hibridizacije na osobine povezane s fitnesom populacija su od iznimne važnosti za planiranje odgovarajućih mjera zaštite. Do danas, preporuke za izbjegavanje širenja hibrida općenito uključuju uklanjanje ne-endemskih populacija, definiciju jedinica zaštite (engl. *conservation units* - CU) za različite podvrste divokoza, a koje bi trebale oprezno razmotriti translokacije jedinki koje nose odgovarajuću genetsku pozadinu (Corlatti i sur., 2011) i pažljivo praćenje potencijalnih događaja hibridizacije u tijeku (Iacolina i sur., 2019).

U znanstvenim radovima u Prilogu 2 i Prilogu 3, korištenjem mikrosatelita, analiziran je genetski trag povijesnih reintrodukcija divokoza na planinska područja u Hrvatskoj i genetska raznolikost izvornih i translociranih populacija. U Prilogu 2 vidljiva je razlika u genetskom sastavu između balkanskih divokoza koje nastanjuju Prenj i ostalih analiziranih populacija u Bosni i Hercegovini (Čvrsnica i Čabulja). STRUCTURE i GENELAND rezultati, temeljeni na analizi mikrosatelitnih lokusa, otkrili su prepreke protoku gena između proučavanih populacija, ali vjerojatno i posljedicu nedavnog efekta uskog grla u populacijama, zbog istrebljenja divokoza u ratu (Frković, 2008) i lokalnu prilagodbu populacija. Strmi kanjon rijeke Neretve, jedne od najvećih rijeka Jadranskog slijeva odvaja Prenj planinu od Čvrsnice i Čabulje i čini službenu granicu između rezervata divljači „Čvrsnica“ i „Prenj“ ustanovljenih tijekom 1893. godine od strane Austro-Ugarske monarhije (Rapaić i Kunovac, 2020).

Krajobrazne značajke doline rijeke Neretve, kao i državna cesta M17, izgrađena u sklopu europske rute E73, mogu biti učinkovite prepreke prirodnom širenju podvrste, jer onemogućuju razmjenu gena između proučavanih populacija.

Ali isto tako, smanjenje populacije u posljednjem ratu je doprinijelo da male skupine divokoza ostanu izolirane u ograničenim staništima ali su geografski vrlo blizu jedna drugoj. Prirodna migracija između jedinki s Prenja i Čvrsnice trenutno je malo vjerojatna zbog prisutnosti spomenutih barijera, no to možda nije bio slučaj prije izgradnje infrastrukture. Safner i sur. (2019) su utvrdili da rijeka Kupa (duž dijela slovensko-hrvatske granice) nije djelovala kao prirodna barijera na genetsku strukturu prekograničnih populacija sjeverne divokoze (*Rupicapra rupicapra*).

Zbog migracijske krize tijekom 2015. godine, duž državne granice uz rijeku Kupu je postavljena 178 km duga ograda u obliku žilet žice, što je uzrokovalo značajni mortalitet papkara (Pokorny i sur., 2017) i što će vjerojatno uzrokovati prekid prekograničnog protoka gena. Također, divokoze karakterizira visoka fenotipska plastičnost i sposobnost migracije na veće udaljenosti zbog čega je između 2004. i 2019. zabilježeno više slučajeva gdje su jedinke stare od dvije do pet godina preplivale od 300 m do 3300 m u otvorenom moru (Kavčić i sur., 2020). U Hrvatskoj su zabilježena četiri takva slučaja dok je u Španjolskoj zabilježen jedan takav slučaj gdje je mužjak star dvije godine plivao prema otvorenom moru. Prema saznanjima Kavčić i sur. (2020) ovo je prva objavljena informacija o korištenju morskih dionica, što sugerira da čak i srednje veliki vodotoci i jezera vjerojatno neće u potpunosti blokirati kretanje divokoza. Razlozi ovog neobičnog ponašanja kod divokoza ostaju nepoznati, pretpostavka je da je jedan od uzroka neprilagođena kombinacija fizioloških potreba (npr. pojedinačni pokušaji raspršivanja).

STRUCTURE model je pokazao da je reintroducirana balkanska divokoza na Biokovu genetski sličnija populaciji s Čabulje, što sugerira da su reintroducirane jedinke u planini Biokovo možda potjecale s ovog područja kao i iz Čvrsnice (Prilog 2). Prema Jurić (1998), u lovištu Čvrsnica koje uključuje i teritorije susjedne planine Čabulje, prebrojane su 992 jedinke balkanske divokoze. Moguće je da su populacije Čvrsnica i Čabulja u prošlosti bile povezane i činile jedinstvenu populaciju, što može se zaključiti iz STRUCTURE rezultata. Zbog rata na Balkanu 1990.-ih i nastavka ilegalnog lova i krivolova, ova pojedinačna populacija se brojčano smanjila te je rascjepkana i izolirana u visokim planinskim staništima.

Kako bi se potvrdila STRUCTURE analiza, prostorni kontekst analiziranih populacija uzet je u obzir i testiran s GENELAND računalnim programom.

GENELAND analiza otkrila je sličan obrazac grupiranja jedinki kao i kod STRUCTURE modela, ali je predložila dodatni četvrti prostorni klaster duž MCMC lanca. GENELAND je među tri analizirane populacije u Bosni i Hercegovini detektirao dva prostorna klastera, dok je jedan prostorni klaster odgovarao populaciji na Biokovu.

Razliku u genetskoj strukturi između Prenja i Biokova otkrili su i Šprem i Buzan (2016.) koji su koristili BAPs algoritam za prostorno grupiranje skupina i pokazali odvajanje populacije balkanske divokoze na Prenju od populacije na planini Biokovo. Četvrti klaster koji je otkrio prostorni model GENELAND bio je takozvani "duh klaster" (Frantz i sur., 2009), budući da mu nije dodijeljena niti jedna analizirana individua. Duh klasteri nisu neuobičajena pojava, ali su još uvijek slabo shvaćeni fenomen koji može biti uzrokovan heterogenom distribucijom uzoraka (Aziz i sur., 2018). Moguće je da svi skupovi koje je identificirao GENELAND predstavljaju pravu genetsku podjelu, ali stupanj diferencijacije među njima je prenizak da bi grupiranje bilo dosljedno (Frantz i sur. 2009.). Druga mogućnost je da model GENELAND može precijeniti broj genetskih klastera kada na analizirane populacije pod IBD utjecajem (Frantz i sur., 2009). Razlika između genetske strukture divokoza koje obitavaju na Biokovu od populacija Čvrsnice i Čabulje može biti posljedica povijesnog efekta osnivača i novijeg genetskog drifta zbog izolacije i lokalne prilagodbe.

Unatoč desetljećima dugotrajnog neodrživog lova, krivolova, predacije i drugih prirodnih događaja (Šprem i Buzan, 2016), razine parenja u srodstvu (F_{IS} vrijednost) u analiziranim populacijama su i dalje umjerene. Rezultati genetskog sastava balkanske divokoze na Biokovu mogu utjecati na održivost populacije kroz vrijeme, te je vrlo važno pratiti genetske parametre reintroducirane populacije kako bi se spriječio gubitak genetske raznolikosti zbog parenja u srodstvu i genetskog drifta (DeMay i sur., 2017).

U budućim planirani projektima translokacija divokoza u svrhu očuvanja populacija ili za potrebe lova treba uzeti u obzir koje podvrste već obitavaju na tom ili širem području da bi se smanjio rizik od gubitka diferenciranih genskih fondova što može dovesti do genetskog izumiranja nekih svojti (Corlatti i sur., 2011). Translokacije su neizbježno ugrozile genetski integritet nekih populacija autohtonih europskih papkara (Linnell i Zachos, 2011), kao na primjer: jelen (*Cervus elaphus* - Senn i Pemberton, 2009; Frantz i sur., 2017), srna (*Capreolus capreolus* - Olano-Marin i sur., 2014; Biosa i sur., 2015) i divokoza (*Rupicapra* spp. - Crestanello i sur., 2009; Zemanová i sur., 2015; Šprem i Buzan, 2016). Unatoč zabilježenim osobinama povezanim s hibridizacijom (Kavčić i sur., 2018), njene posljedice na prilagodbu, životnu povijest i evolucijski potencijal ostaju uglavnom nepoznate (Iacolina i sur., 2019).

Prošle translokacije divokoza ostavile su genetski trag u novijim populacijama koji se sada može koristiti za rekonstrukciju nedokumentiranih događaja (Crestanello i sur., 2009). U slučaju reintrodukcije na Velebit kada je u dva navrata provedena reintrodukcija divokoze i to 1974. godine ispuštanjem 10 divokoza s Prenja i 1978. godine ispuštanjem 5 divokoza podrijetlom iz Kamniške Bistrice (Slovenija) zanemarena je genetska struktura autohtonih i translociranih populacija. Za određivanje taksonomskog statusa izvorne populacije na Velebitu prije reintrodukcije korištena je molekularna forenzika koja uključuje multilokusnu genotipizaciju povijesnih uzoraka i statističke alate poput testova dodjele (Prilog 3).

Na osnovu q vrijednosti STRUCTURE testa dodjele, muzejski uzorci su bili sličniji alpskoj podvrsti, ali ne u tolikom postotku da bi se dao konačni rezultat. DAPC test je sve analizirane muzejske uzorke pripisao alpskoj podvrsti, ali važno je naglasiti da DAPC ne uključuje nikakve procjene nesigurnosti za vjerojatnost pripadnosti. Nekoliko čimbenika moglo je pridonijeti nedostatku razlučivosti rezultata u obje analize.

U istraživanju evolucijske povijesti divokoza koju su objavili Rodríguez i sur. (2010), Neighbour-Joining stablo temeljeno na udaljenosti dijeljenih alela između svih podvrsta *Rupicapra* otkrilo je dvije glavne skupine koje odgovaraju južnoj i sjevernoj divokozi. Većina balkanskih i alpskih divokoza pripadala je različitim podskupinama sjeverne divokoze ali bez jasne granice razdvajanja između njih. S druge strane, njihovi rezultati STRUCTURE analize za K = 3 su pokazali da su pojedine alpske i balkanske divokoze grupirane u isti genetski klaster, vjerojatno dijeleći istu evolucijsku povijest. Ovo je dokaz da su mikrosateliti bolji markeri za nedavnu geografsku diferencijaciju populacija, ali ipak u nekim slučajevima nisu dovoljni za otkrivanje lokalnih stopa introgresije gena (niska genetska diferencijacija) koje se onda mogu podcijeniti (Oliveira i sur., 2008). Šprem i Buzan (2016) navode da je prosječna vrijednost F_{ST} između svih analiziranih populacija divokoza u Dinarskim planinama iznosila 0,103 što je vrlo niska genetska diferencijacija između referentnih podvrsta i što predstavlja dodatni problem za primijenjene testove dodjele. Nadalje, referentni i muzejski uzorci dolaze iz različitih populacija s različitim efektivnim veličinama populacije i razmakom od više od 100 godina, što bi moglo biti dovoljno da se frekvencije alela mijenjaju zbog miješanja i genetskog drifta. Treba imati na umu da STRUCTURE analiza pokušava što bolje uklopiti parametre modela kako bi objasnio uzorke uočene u podacima. Dakle, iako nije uzorkovana “prava” populacija kojoj su muzejski uzorci pripadali, STRUCTURE bi i dalje morao prikazati jednu od uzorkovanih podvrsta kao najvjerojatnije izvornu populaciju (Cornuet i sur., 1999).

Čak i uz sve opisane nesigurnosti, naši rezultati još uvijek daju vrijedan pokazatelj najvjerojatnijeg genetskog i taksonomskog sastava autohtone populacije divokoza prije reintrodukcije.

Korištenje molekularnih markera, u ovom slučaju mikrosatelita i mtDNA, dalo je važne informacije o genetskoj raznolikosti i evolucijskoj povijesti populacija balkanskih divokoza. Utvrđivanje stupnja unutarpopulacijske genetske raznolikosti važan je kriterij za određivanje budućih jedinica za zaštitu (CU) i gospodarenje (engl. *management units* - MU; Bonin i sur., 2007) da bi se osigurala dugoročna održivost podvrste diljem njene rasprostranjenosti.

4. ZAKLJUČCI

- Rezultati ovog doktorskog istraživanja pokazali su prisutnost glavnog genetskog klastera koji čini većinu analiziranih populacija balkanske divokoze i razdvajanje jedinki iz Bugarske u potpuno izoliranu populaciju, dok je populacija iz Srbije samo djelomično izolirana jer još uvijek dijeli mtDNA liniju s populacijama iz Crne Gore.
- Najveća značajna razlika u alelnoj i genotipskoj učestalost utvrđena je između populacije iz Bugarske i svih ostalih analiziranih populacija, dok se populacija iz Srbije značajno razlikovala od populacija iz Sjeverne Makedonije i Hrvatske.
- Pojava parenja u srodstvu je zabilježena u skoro svim analiziranim populacijama (F_{IS} vrijednosti), osim populacije u Srbiji koja je imala veći udio heterozigota.
- Populacije balkanske divokoze imaju uzorak arhipelaga diljem rasprostranjenosti kao posljedica fragmentacije staništa i populacija te ukupnog smanjenja veličine populacija zbog višestrukih čimbenika kao što su krivolov i neodrživ lov, povećana gustoća infrastrukture, natjecanje sa stokom za pašnjake, predacija i uvođenje drugih podvrsta divokoza, uglavnom alpske divokoze.
- Tijekom posljednjih desetljeća, balkanskoj divokozi prijeti izumiranje u mnogim dijelovima rasprostranjenosti. Treba ozbiljno razmotriti introdukciju novih jedinki u male, fragmentirane populacije zahvaćene genetskim driftom ili genetskom erozijom. Introdukcije i translokacije bi, međutim, trebale biti podržane prethodnim genetskim pregledom kako bi se spriječile neželjene negativne posljedice.
- Buduća neinvazivna uzorkovanja treba provesti tako da se pokrije većina geografske distribucije da bi se izbjegle greške uzorkovanja i kako bi se otkrila područja genetskog diskontinuiteta i identificirali (potencijalni) koridori. Dodatno, DNA analiza arheoloških i muzejskih uzoraka u slučaju Biokova pomogla bi u razjašnjavanju povijesti populacije podvrste i definiranju njezinog statusa.
- Istraživanje genetske strukture predstavljeno u ovom doktorskom radu pruža potrebnu polaznu točku za procjenu statusa zaštite balkanske divokoze. Korištenje molekularnih markera, u ovom slučaju mikrosatelita i mtDNA, dalo je važne informacije o genetskoj raznolikosti i evolucijskoj povijesti populacija balkanske divokoze.
- Dugoročna postojanost balkanske divokoze ovisi o dovoljnoj genetskoj raznolikosti za prilagodbu promjenjivom okolišu. Daljnje genetske, ekološke, imunološke, epidemiološke i demografske analize su nužne da bi se osigurao monitoring populacija balkanske divokoze te identifikacija potencijalnih rizika gubitka genetske varijabilnosti bitnih za očuvanje ove podvrste.

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Prilozi

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RESEARCH ARTICLE

The Balkan chamois, an archipelago or a peninsula? Insights from nuclear and mitochondrial DNA

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Abstract
The Balkan chamois (*Rupicapra rupicapra balcanica*) is widespread on the Balkan Peninsula, along mountain massifs from Croatia in the north to Greece in the south and Bulgaria in the east. Knowledge on the genetic structure of Balkan chamois populations is limited and restricted to local studies. Therefore, the main objective of this study was to use nuclear (16 microsatellites) and mitochondrial (partial 376 base pairs control region) markers to investigate the genetic structure of this chamois subspecies throughout its distribution range and to obtain information on the degree of connectivity of the different (sub)populations. We extracted DNA from bone, dried skin and muscle tissue and successfully genotyped 92 individuals of Balkan chamois and sequenced the partial control region in 44 individuals. The Bayesian analysis suggested 3 genetic clusters and assigned individuals from Serbia and Bulgaria to two separate clusters, while individuals from the other countries belonged to the same cluster. Thirty new haplotypes were obtained from partial mitochondrial DNA sequences, with private haplotypes in all analyzed populations and only two haplotypes shared among populations, indicating the possibility of past translocations. The subspecies genetic composition presented here provides the necessary starting point to assess the conservation status of the Balkan chamois and allows the development of conservation strategies necessary for its sustainable management and conservation.

Keywords Conservation · Genetic diversity · mtDNA · Population genetics · *Rupicapra rupicapra balcanica*

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Introduction

Chamois (*Rupicapra* spp.) are medium-sized ungulates that inhabit alpine pastures and rocky areas on the main mountain massifs of both Europe and the Near East (Corlatti et al. 2011) and, exceptionally, the low elevations of river gorges (Papaioannou and Kati 2007), forested and coastal areas (Safner et al. 2019; Kavčić et al. 2020). The current distribution of *Rupicapra* spp. has been shaped by the natural fragmentation of suitable habitats and the resulting constraints on gene flow between different populations (Buzan et al. 2013).

The currently accepted taxonomy of chamois, based on morphological, behavioral and molecular evidence, recognizes two species: the Northern chamois (*Rupicapra rupicapra*) with seven subspecies distributed in the Alps (*R. r. rupicapra* and *R. r. cartusiana*), the Balkans (*R. r. balcanica*), the Tatras (*R. r. tatrica*) and the Carpathians (*R. r. carpatica*), in western Asia (*R. r. asiatica*) and the Caucasus (*R. r. caucasica*) (Anderwald et al. 2020); and the Southern chamois (*Rupicapra pyrenaica*), which includes three geographically isolated subspecies on the Cantabrian Massif (*R. p. parva*), the Pyrenees (*R. p. pyrenaica*) and the central Apennines (*R. p. ornata*) (Herrero et al. 2020).

Over the past two decades, the genus *Rupicapra* has been the subject of numerous genetic studies in which, depending on the scope of the study, different markers were used. Microsatellites have been a useful tool in studies of population structure (Papaioannou et al. 2019; Soglia et al. 2010; Šprem and Buzan 2016), phylogeography (Pérez et al. 2002; Rodríguez et al. 2010) or conservation (Buzan et al. 2013; Crestanello et al. 2009; Markov et al. 2016). Complete mitogenome (Pérez et al. 2014; Iacolina et al. 2021) or partial regions of maternally inherited mitochondrial DNA (mtDNA) have been used to study population genetics, systematics and evolution (Hammer et al. 1995; Rodríguez et al. 2007; Rodríguez et al. 2009; Buzan et al. 2013; Šprem and Buzan 2016; Pérez et al. 2017b; Moravčíková et al. 2019). Nuclear markers such as autosomal introns (Pérez et al. 2017a) or the melanocortin-1 receptor gene (MC1R; Pérez et al. 2013), which is related to the coloration pattern of chamois fur, have been used to study the effects of historical and evolutionary events on the diversification of *Rupicapra* spp., whereas the Y chromosome use has been rare and limited to phylogenetic studies, due to the methodological difficulties associated with the marker (Pérez et al. 2011).

The effects of historical and evolutionary events on chamois diversification and taxonomy are still under discussion. Previous phylogenetic findings based on mtDNA suggest the presence of three major mitochondrial lineages within the genus *Rupicapra* which correspond to the geographic distribution of the species and are referred to as the West (*R. p. parva* and *R. p. pyrenaica*), Central (*R. p. ornata* and *R. r. cartusiana*) and East (the six remaining subspecies including *R. r. balcanica*) (Rodríguez et al. 2009; Rodríguez et al. 2010; Pérez et al. 2014). Although Balkan chamois has been included in several phylogenetic studies that used both nuclear and mitochondrial markers (Rodríguez et al. 2009; Rodríguez et al. 2010; Pérez et al. 2017a; Pérez et al. 2017b), it remains one of the less-studied Northern chamois subspecies (Kati et al. 2020). Current knowledge on the genetic diversity and structure of the Balkan chamois population is limited and restricted to regional-local studies (e.g. Bulgaria—Markov et al. 2016; Croatia and Bosnia and Herzegovina—Šprem and Buzan 2016; Greece—Papaioannou et al. 2019). The distribution of the Balkan chamois is patchy and covers only parts of the massifs and mountain chains across the countries that form its range (Fig. 1b). According to Buzan et al. (2013), the subspecies dispersals is a legacy of Quaternary glacial-interglacial dynamics when many taxa were trapped in relatively small refugial areas. This scenario supports the combined results from the major histocompatibility complex (MHC) and mtDNA sequences that the last glaciation produced a demographic decline and small subunits in chamois populations (Mona et al. 2008). The subspecies' low rates of colonization and reduced gene flow between isolated populations may result in genetic differentiation due to the inbreeding effect and a loss of allelic variants as a consequence of genetic drift (Willi et al. 2006). Reduced genetic diversity in small and isolated populations might, in turn, cause negative impacts on fitness, resulting in decreased effective population size and, eventually, increase the probabilities of extinction (Pelletier et al. 2019). Other threats to the Balkan chamois survival are considered to be poaching (Papaioannou and Kati 2007), introductions of other chamois subspecies, mostly Alpine chamois (Iacolina et al. 2019), forest succession (Kavčić et al. 2019), road infrastructure (Kati et al. 2020), intensive livestock grazing, predation, unsustainable hunting and natural events (Šprem and Buzan 2016). Due to these threats, the Balkan chamois is protected by Annexes II and IV of the European Union Habitats Directive 92/43/EEC (OJ L 206, 22.7.1992) and Appendix III of the Bern Convention (OJ L 38, 10.2.1982). The conservation and management status within the different national legislations varies between countries (members and non-members of the EU) and depends on the degree of the local communities' interest in the conservation of the subspecies (Anderwald et al. 2020). Considering the demographic independence of populations, conservation objectives should be focused on defining conservation units such as management (MUs) or evolutionarily significant units (ESUs), based on a combination of molecular, morphological, ecological, behavioral and biogeographic information that can be combined to identify populations that need to be managed together (Funk et al. 2013).

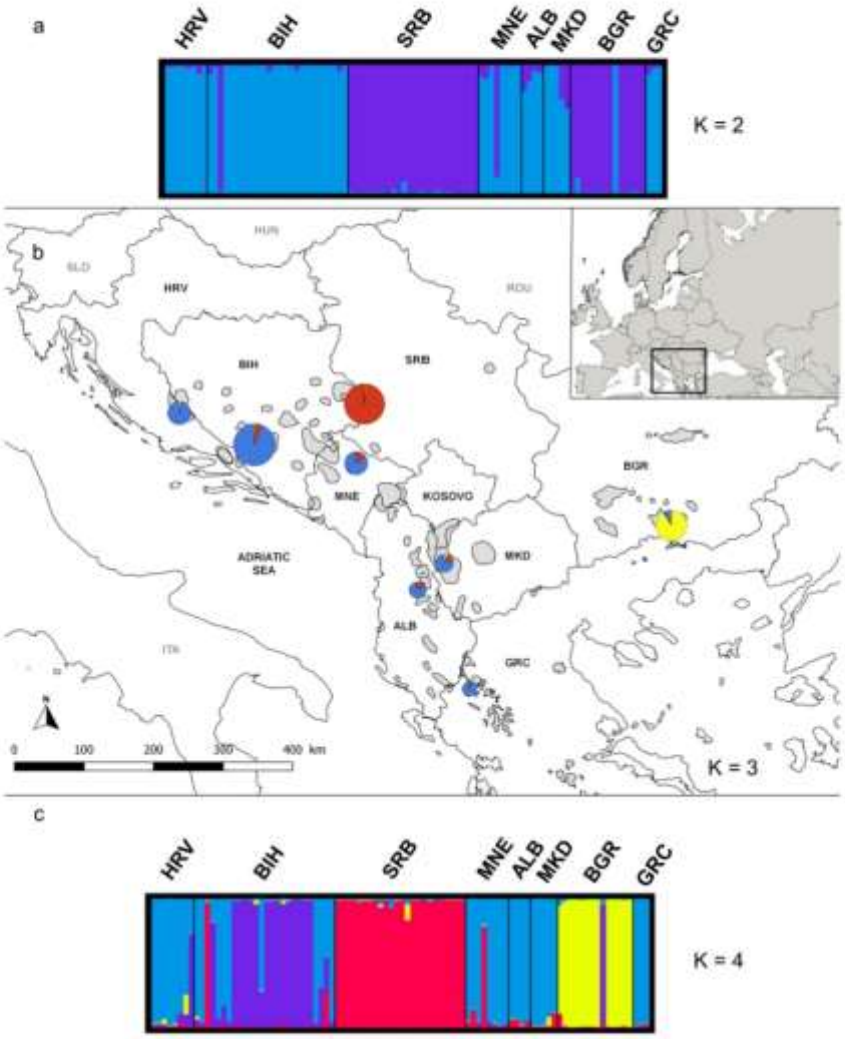


Fig. 1 Analyses of the genetic structure of Balkan chamois populations across the Balkan Peninsula. STRUCTURE admixture model results based on sixteen loci with correlated allele frequencies for (a) K = 2, (b) K = 3 and (c) K = 4. **a** Assignment of individual genotypes of Balkan chamois to clusters K = 2 as inferred by STRUCTURE. **b** Geographic distribution of the Balkan chamois genotypes. The distribution range map (in transparent grey) was constructed using shape data downloaded from the IUCN Red List of Threatened Species. Each pie chart indicates the geographic location of the sampled population. The size of the pie charts indicates the number of samples collected from a locality. Different colors of the pie chart indicate proportions of respective genetic clusters per individual Q (in %). **c** Assignment of individual genotypes of Balkan chamois to clusters K = 4. *HRV* Croatia, *BIH* Bosnia and Herzegovina, *SRB* Serbia, *MNE* Montenegro, *ALB* Albania, *MKD* North Macedonia, *BGR* Bulgaria, *GRC* Greece

The main objective of this study was to gather information on the genetic diversity and population structure of Balkan chamois along its distribution range to support the development of management and conservation strategies. With this aim, we used microsatellite genotypes and a partial region of mtDNA (Control Region—CR) to address questions regarding the influence of geographical isolation on the genetic diversity of Balkan chamois populations.

Materials and methods

Samples collection and DNA extraction

A total of 101 specimens of Balkan chamois were collected from 2011 to 2018 at sites throughout its distribution range (Fig. 1b). Of the total number of specimens collected, complete genotypes were obtained for 92 individuals, while mtDNA CR was successfully sequenced in 44 individuals (Table S.1). For population-based analyses, Balkan chamois individuals were divided into eight groups based on country of origin: Croatia (HRV), Bosnia and Herzegovina (BIH), Serbia (SRB), Montenegro (MNE), Albania (ALB), North Macedonia (MKD), Bulgaria (BGR), and Greece (GRC). For mtDNA analyses, we collected 56 additional samples belonging to other chamois subspecies (*R. p. pyrenaica*, *R. p. ornata*, *R. r. rupicapra*, *R. r. tatrica*, *R. r. carpatica*). Samples (bones, dried skin, and muscle tissue) were obtained from hunted and naturally dead animals, remains of poached individuals, and specimens from hunting museum collections. Muscle samples were preserved in 96% ethanol and stored at -80 °C until extraction.

We extracted DNA from muscle (N = 69) and skin (N = 1) samples using the commercial peqGOLD Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH) following the manufacturer´s protocol in a volume of 150 µL. DNA from bone samples (N = 22) was extracted using 400 mg bone powder following the procedure described in Buzan et al. (2020) using the QIAamp DNA Micro Kit (Qiagen) and a final volume of 100 µL.

DNA concentrations were measured with Qubit® dsDNA BR Assay Kit (Invitrogen) on a 3.0 Qubit Fluorimeter (Life Technologies).

Microsatellite amplification and genotyping

Twenty microsatellites were amplified using PCR multiplex sets already screened in studies of chamois (Zemanová et al. 2011; Buzan et al. 2013; Šprem and Buzan 2016). We used KAPA2G Fast Multiplex Mix and protocol (KAPA Biosystems Roche) to amplify the target regions. If the multiplex was unsuccessful, we repeated the analysis with single-locus PCR. Each reaction contained 6.25 µL of KAPA2G Fast Multiplex Mix, 1.7 µL primers (forward fluorescent-labeled) at various concentrations (Table S.2), 2.5 µL of DNA and ddH_2O for a final volume of 13 µL. In a few tissue and bone samples, the quality of DNA was low, so we used a volume of 5 µL of genomic DNA and reduced the amount of water for the same final volume of 13 µL. Fragment analysis was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems) using the GeneScan LIZ500 (-250) Size Standard (Applied Biosystems). Microsatellite genotypes were examined using Gene Mapper v. 4.0 software (Applied Biosystems).

Mitochondrial DNA sequencing

The partial CR of the mtDNA was amplified using the primers and protocol described in Rodríguez et al. (2010). Amplicons were visualized on a 2% agarose gel and purified with ExoSAP-IT PCR product cleanup reagent (Applied Biosystems). Forward and reverse PCR products were sequenced with the ABI BigDye terminator mix v3.1 (Applied Biosystems) followed by electrophoresis in an ABI SeqStudio Genetic Analyzer (Thermo Fischer Scientific).

Microsatellite data analysis

We used the Expectation–Maximization (EM) algorithm implemented in FreeNA (Chapuis and Estoup 2007) to estimate null allele frequencies for each microsatellite locus, since they may cause a significant heterozygote deficit and deviation of populations from the Hardy–Weinberg equilibrium (HWE). Loci that had high estimates of null allele frequencies were excluded from further analysis. The same software was used to calculate the overall genetic differentiation within the dataset, the global F_{ST} value (Chapuis and Estoup 2007).

Loci without null allele frequencies in each sampling population were tested for deviations from HWE with the Markov chain method with 10,000 dememorization steps, 500 batches and 10,000 subsequent iterations in GENEPOP ver. 4.7.2 (Rousset 2008). The exact test based on a Markov chain method implemented in GENEPOP was used to analyze pairwise linkage disequilibrium (LD) among all pairs of loci across all populations. A sequential Bonferroni procedure (Holm 1979) was applied to correct the effect of multiple comparisons tests using adjust p-values function implemented in R ver. 4.0.5 package stats (R Core Team 2020).

Genetix ver. 4.05.2 (Belkhir et al. 1996–2004) was used to calculate the mean number of alleles, observed (H_O) and expected (H_E; Nei 1978) heterozygosities for each locus in all populations as well as the inbreeding coefficient (F_{IS}) and its confidence intervals. We estimated the allelic richness in each population using the rarefaction procedure implemented in FSTAT ver. 2.9.3.2 (Goudet 2001). The number

of private alleles was estimated using GenAlEx ver. 6.502 (Peakall and Smouse 2012). The genetic differentiation between all pairs of populations (pairwise F_{ST}) was estimated using the hierfstat 0.5–7 package (Goudet 2005) in R. The respective p-values were calculated with the same package using 100 bootstraps over loci for each population pair.

STRUCTURE ver. 2.3.4. (Pritchard et al. 2000) was used to assign the Balkan chamois individuals to the most likely number of genetic clusters (K). We performed ten independent runs for each K between 1 and 10 under a model assuming admixture and correlated allele frequency with a burn-in of 10^5 steps and a run length of 10^6 Markov chain Monte Carlo (MCMC) iterations. Structure Harvester (Earl and vonHoldt 2012) was used to compare the average estimates of the likelihood of the data, $ln[Pr(X|K)]$ for each value of K and to apply the ad hoc summary statistic ΔK developed by Evanno et al. (2005) to estimate the most likely K. The same software was used to generate graphs for the mean log posterior probability of the data (mean ± SD). The results of replicated runs for each value of K were combined using the Greedy algorithm in CLUMPP ver. 1.1.2 (Jakobsson and Rosenberg 2007) and the summary outputs were displayed graphically using DISTRUCT ver. 1.1 (Rosenberg 2004). The modal cluster membership for each individual in each sampled area from the run with the highest log-likelihoods was plotted on a map using QGIS ver. 2.18.21 (QGIS Development Team 2018). The Photo Scape X software (MOOII Tech) was used for image processing.

Isolation by distance (IBD) between pairs of Balkan chamois populations was tested using the package adegenet 2.0.0 (Jombart 2008) in R. A Mantel test was applied to test the correlation between genetic distance (Edwards 1971) and geographic distances matrices.

We excluded Albania (n = 4) and Greece (n = 3) samples from all population-based analyses because of the small sample size and included them only in the individual-based analyses.

Mitochondrial data analysis

The mitochondrial sequences were manually checked and assembled in FinchTV ver. 1.5.0 (Geospiza Inc.). The 100 new sequences of CR were aligned using the ClustalW algorithm implemented in MEGA X ver. 10.0.5 (Kumar et al. 2018) together with 109 sequences retrieved from GenBank (accession numbers AM279274-279275, EU887481-EU887488, GU951843–GU951916, KC594557-KC594572, KP730619-KP730627; Table S.3). The final alignment consisted of 376 base pairs (bp) and was used to generate haplotypes in DnaSP ver. 5.0 (Librado and Rozas 2009). All newly generated haplotypes were submitted to GenBank (accessions MT746066–MT746095).

Evolutionary relationships between haplotypes were analyzed by a Median-Joining network (Bandelt et al. 1999) constructed with NETWORK ver. 5.0 (Fluxus Technology Ltd.). The weights of characters' value were set to 10, while the parameter epsilon, which specifies a weighted genetic distance to the known sequences in the dataset, was set to 0 to obtain a sparse spanning network.

Results

Within-population genetic diversity

Of the twenty microsatellites chosen for the analysis of the Balkan chamois population structure, locus MAF214 could not be amplified in 12 individuals while loci SY58 and SR-CRSP-9 showed the presence of null allele (Table S.4) and significantly deviated from HWE. These loci were therefore excluded, together with INRA121 that was monomorphic. Of the total 101 samples collected, complete genotypes were obtained for 92 individuals. We re-amplified approximately 10% of the genotypes suspected of allelic dropout and all genotypes were confirmed. The set of sixteen microsatellite loci yielded a total of 116 alleles in the eight Balkan chamois populations, ranging from 2 (ETH10, SR-CRSP-6) to 11 (BOBT24) with an average of 7.25 alleles per locus (Table S.2).

The within-population genetic diversity of the six Balkan chamois populations is shown in Table 1. The HRV and BIH populations showed significant deviation from HWE based on the exact tests in Genepop ($p < 0.05$). After applying sequential Bonferroni correction, the HRV population deviated from HWE for locus BM1258 ($p = 0.04$) while the BIH population deviated significantly ($p < 0.01$) from HWE for loci SY434, SY259 and SR-CRSP-6. The F_{IS} values varied between -0.019 (SRB) and 0.198 (MKD). The sequential Bonferroni correction applied to the linkage disequilibrium test showed a significant value only for locus CSSM66 ($p < 0.05$).

The lowest allelic richness (AR) was detected in BGR (2.522) while HRV population had the highest values (3.187). The observed number of alleles (A) across microsatellite loci ranged from 3.187 in MKD to 5.250 in BIH. All populations had 5 private alleles with exception of SRB that had none, and MNE that had only 3. The H_O varied from 0.489 in the MKD population to 0.605 in the MNE population. The H_E ranged from 0.519 (BGR) to 0.655 (HRV). SRB was the only population that showed an excess of heterozygotes.

Table 1 Genetic diversity of sixteen microsatellite markers in five Balkan chamois populations

Country /population	N	$H_O \pm SD$	$H_E \pm SD$	F_{IS} (IC 95%)	HWE	A	AR	N_{pr}
Croatia (HRV)	8	0.589 ± 0.212	0.655 ± 0.141	0.174 (− 0.077–0.236)	0.007*	4.188	3.187	5
Bosnia and Herzegovina (BIH)	26	0.546 ± 0.250	0.615 ± 0.172	0.133 (0.034–0.187)	0.000**	5.250	2.941	5
Serbia (SRB)	24	0.570 ± 0.212	0.548 ± 0.197	-0.019 (− 0.129–0.045)	0.979[ns]	3.750	2.595	0
Montenegro (MNE)	8	0.605 ± 0.235	0.619 ± 0.179	0.092 (− 0.113–0.103)	0.203[ns]	4.250	3.109	3
North Macedonia (MKD)	5	0.489 ± 0.343	0.530 ± 0.213	0.198 (− 0.213–0.198)	0.548[ns]	3.187	2.709	5
Bulgaria (BGR)	14	0.520 ± 0.213	0.519 ± 0.195	0.035 (− 0.147–0.112)	0.246[ns]	3.812	2.522	5

N—number of samples; H_O—observed heterozygosity; H_E—expected heterozygosity; SD—standard deviation; F_{IS}—inbreeding coefficient; IC 95%—95% Confidence Interval; HWE—Hardy–Weinberg equilibrium (after Bonferroni adjustment p values: ns—non—significant value; *—significant at $p < 0.05$; **—significant at $p < 0.01$); A—average number of alleles; AR—allelic richness; N_{pr}—number of private alleles

Genetic variability among populations

The global F_{ST} value for the six populations was 0.184 with a 95% confidence interval significantly different from zero (CI = 0.149 – 0.223). The lowest pairwise F_{ST} value was observed between HRV and MNE populations (0.084), while the highest value was found between SRB and BGR (0.292) populations. Pairwise F_{ST} values between BGR and all other analyzed populations were significant (Fig. S.1). Significant values were found between SRB and other populations except MNE, and between BIH and MKD.

The STRUCTURE results for K = 2 grouped the populations HRV, BIH, MNE, ALB, MKD, GRC, as well as one individual from BGR, while the other cluster contained individuals from BGR and SRB populations and one individual from BIH population (Fig. 1a). According to the Evanno method, the best model of population assignment was with three clusters (Fig. S.2). The STRUCTURE analysis for K = 3 assigned individuals from SRB and BGR to two private clusters while individuals from the other analyzed populations belonged to one common cluster (Fig. 1b). A further increase to K = 4 indicated the divergence of BIH from the other Balkan chamois populations (Fig. 1c).

Applying the Mantel test across the six populations of Balkan chamois, no significant positive relationships were found between genetic Edwards' and geographical distances ($p = 0.375$, $r = 0.082$; Fig. 2).

Mitochondrial DNA variation

MtDNA variation analysis was performed on a 376 bp sequence of CR. For the analysis of mtDNA diversity and the construction of the haplotype network, two datasets were used, one containing only individuals of Balkan chamois

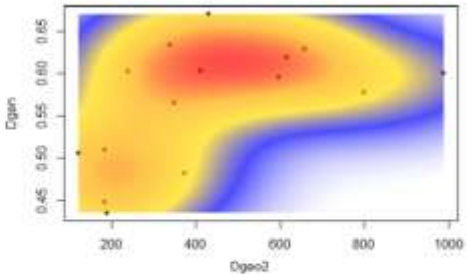


Fig. 2 Correlation analysis between pairwise Edward's genetic distances (Dgen) and the geographic distances (Dgeo; km). The colours represent the relative density of the points, with warmer colours indicating higher density, while the points represent the Edwards genetic distances between the six analysed Balkan chamois populations plotted against the Euclidean geographic distances for the same populations (HRV, BIH, SRB, MNE, MKD, BGR)

(Table S.1) and the second containing all subspecies analyzed in this study together with GenBank sequences (Table S.3). For the dataset containing only Balkan chamois individuals (44 sequences), the total haplotype and nucleotide diversity were 0.913 and 0.031, respectively (Table 2). The ALB population showed the highest values of haplotype (1.000) and nucleotide (0.030) diversity while the lowest were detected in BGR (Hd = 0.464; π = 0.009).

The second dataset consisted of 100 new CR sequences, which were aligned together with 109 sequences from GenBank. A total of 30 new haplotypes of CR were defined and deposited in GenBank under Accession numbers MT746066—MT746095 (HRCR17—HRCR46). Of these, 15 were found in Balkan chamois populations, 2 were shared

Table 2 Diversity of partial CR of Balkan chamois populations

Population	N	h	S	Hd ± SD	π ± SD
HRV	5	2	11	0.700 ± 0.218	0.015 ± 0.005
BIH	10	3	22	0.778 ± 0.091	0.026 ± 0.004
SRB	7	2	12	0.476 ± 0.171	0.015 ± 0.005
MNE	4	1	15	0.500 ± 0.265	0.020 ± 0.011
ALB	3	3	17	1.000 ± 0.272	0.030 ± 0.013
MKD	3	2	10	0.667 ± 0.314	0.017 ± 0.008
BGR	8	2	13	0.464 ± 0.200	0.009 ± 0.006
GRC	4	3	20	0.833 ± 0.222	0.028 ± 0.010
Total	44	18	48	0.913 ± 0.023	0.031 ± 0.003

N number of sequences used for analysis, *h* number of haplotypes, *S* number of segregating sites, *Hd* haplotype diversity, *π* nucleotide diversity, *SD* standard deviation, *HRV* Croatia, *BIH* Bosnia and Herzegovina, *SRB* Serbia, *MNE* Montenegro, *ALB* Albania, *MKD* North Macedonia, *BGR* Bulgaria, *GRC* Greece

between Balkan and Tatra chamois, 1 was shared between Balkan and Alpine chamois, while Alpine chamois itself had 8 and Carpathian chamois 3 haplotypes. Two haplotypes were identified in the Southern chamois (Table S.3). Alignment of 209 individual sequences revealed 136 segregating sites and the total number of mutations was 143.

In the network containing only Balkan chamois individuals (Fig. 3a), all eight sampled populations had private haplotypes, while two haplotypes (HRCR9 and HRCR20) were present in multiple populations. One individual of Balkan chamois from the HRV population was assigned the haplotype HRCR29, while others shared the haplotype HRCR9 with individuals from the BIH and BGR populations. Similarly, one individual from the HRV population shared haplotype HRCR20 with individuals from the SRB and MNE populations.

In the median-joining network obtained from 209 individuals (Fig. 3b) three haplotypes were detected where *R. r. balcanica* shared identical sequences with *R. r. rupicapra* (HRCR9) and *R. r. tatrica* (HRCR20 and HRCR38) subspecies (see Table S.3 for haplotypes information).

Discussion

Our results revealed the existence of three genetically and geographically distinct populations of Balkan chamois. Genetic differentiation was most pronounced in BGR and SRB populations while mtDNA results revealed private haplotypes in all eight sampled populations. The increased divergence between Balkan chamois populations and the existence of unique haplotypes could be a result of historical (post-glacial colonization processes) and recent events (anthropogenic impacts). The long-term persistence of subspecies depends on sufficient genetic diversity to adapt to a variable environment, understanding the genetic diversity and structure of subspecies is thus important for taking effective management and conservation action (Souza-Shibatta et al. 2018). The use of molecular markers, in this case microsatellites and mtDNA, provided important information on the genetic diversity and evolutionary history of the Balkan chamois population. Based on our results, we recommend that spatial populations should be considered MUs, in order to ensure effective management and conservation of this subspecies.

Genetic diversity

We excluded four loci from the final dataset due to either poor amplification, high percentage of null alleles or monomorphism. Two of the excluded loci, SY58 and INRA121, presented the same issues in other studies (Buzan et al. 2013; Šprem and Buzan 2016), therefore we recommend excluding these two loci in future studies of *Rupicapra* subspecies.

According to Rodríguez et al. (2010), the reason why loci (SY434, SY259, and SR-CRSP-6) in the BIH population diverged significantly from the HWE could be due to the genetic characteristics of the population (immigrants, Wahlund effect) rather than locus systematical deviation from the HWE. Since there was no deviation of these loci from HWE in other populations, we retained them in all subsequent analyses. Concordantly with our results, in the study by Šprem and Buzan (2016), the population Prenj from Bosnia and Herzegovina showed significant deviation from HWE based on exact tests and significant positive values of F_{IS}, but the deviation from HWE remained non-significant after Bonferroni correction.

The degree of intraspecific genetic diversity is an important criterion for characterizing conservation units and identifying populations to be prioritized for protection (Bonin et al. 2007). Natural selection operates with the genetic raw material of the population (Funk et al. 2013) to enable adaptation to environmental change and, eventually, evolution (Bonin et al. 2007). The interaction of genetic and demographic factors leads to a loss of intraspecific genetic diversity, especially in small and isolated populations which are threatened by inbreeding depression and thus increasing the extinction probability (Bonin et al. 2007). The Balkan chamois populations' genetic diversity analyzed in this study (Table 1) is low when compared with values reported in the study of Rodríguez et al. (2010). Here we report values of allelic richness ranging from 2.522 to 3.187, whereas Rodríguez et al. (2010) observed an allelic richness of 3.74 for the nine individuals of Balkan chamois analyzed. This difference might be due to the higher number of microsatellites used by Rodríguez et al. (2010). A similar situation was found in a study on the population genetics of Alpine chamois (Soglia et al. 2010), which showed higher

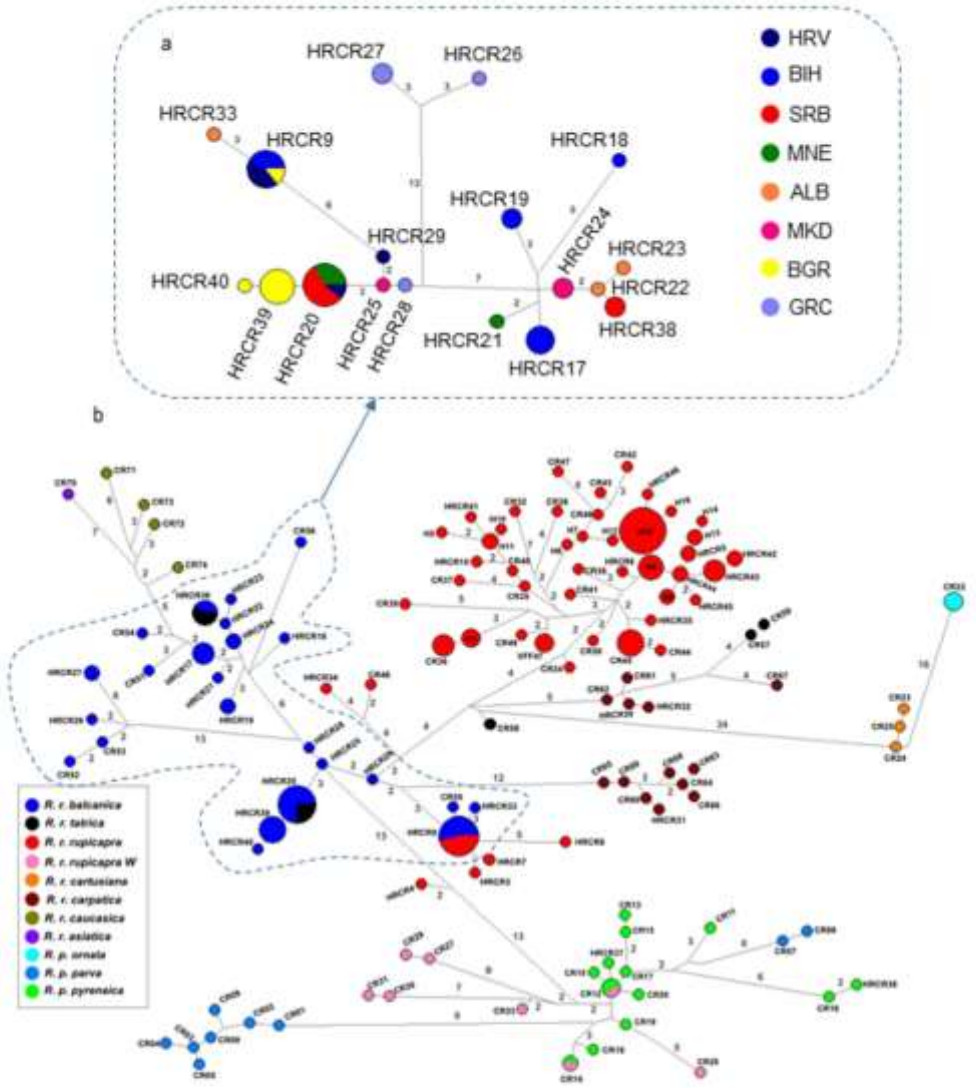


Fig. 3 Median-joining (MJ) networks of the partial CR of mtDNA. The mutations greater than 1 are indicated as grey numbers on the branches, the size of the pie chart represents the number of individuals sharing that haplotype. **a** Median-joining network of Balkan chamois, the different colors represent different countries (populations). **b** Median-joining network of newly generated and GenBank retrieved sequences (209 sequences). Pie chart colors correspond to chamois subspecies. Pie chart labels correspond to the haplotype name deposited in GenBank, haplotypes from HRCR17-HRCR46 are newly described

estimates of genetic diversity compared to the results on the same subspecies by Buzan et al. (2013). These differences in estimates of genetic diversity within the same subspecies could be explained by differences in sample size, the use of different or larger numbers of microsatellites, as well as the representation of different sampling locations. In our study, the average value of observed heterozygosities (0.553) was slightly lower than expected heterozygosity (0.581) indicating a general excess of homozygotes. This might be a consequence of the spatial structuring of populations into subpopulations (Pérez et al. 2002; Buzan et al. 2013). Of particular interest is the low diversity of the BGR population, which comes from the "Izvora State Hunting Reserve" (ISHR) in the western Rhodopes and constitutes a major source population for chamois translocations in Bulgaria. The observed values might be the result of negative fluctuations in the number of chamois in the recent past, the current census size is around 250 individuals and the population is geographically isolated (Markov et al. 2016). According to Hristovich (1939), a total of 150–200 individuals were estimated on the Rhodopes and the total number in Bulgaria was around 1000 chamois. Until the mid-nineteenth century the Balkan chamois had a wider range of distribution in Bulgaria, as it inhabited almost all suitable habitats in the Rila-Rhodopian mountain range. At the end of the nineteenth century, the subspecies disappeared from many of its ranges due to the introduction of long-range rifles (Avramov and Valchev 2010). Markov et al. (2016) suspected that the ISHR population underwent bottlenecks due to overhunting and poaching in its recent history, which might lead to a continuous loss of genetic diversity. The loss of genetic variation is not still seen in the genetic makeup of the BGR population although there are small indications in the observed value of F_{IS} (0.035) showing minor inbreeding, contrary to Markov et al. (2016) expectations of high inbreeding.

Genetic differentiation among Balkan chamois populations

Based on the pairwise F_{ST} values, the SRB population differed significantly from other populations, except MNE. A similar pattern was recorded for the BGR population which differed significantly from the other studied populations (Fig. S.1). These significant F_{ST} values could be explained by populations' isolation within the geographical features of the sampled areas in SRB and BGR (Fig. 1), as they could represent a barrier to gene flow. A genetic differentiation caused by similar factors was found in chamois in other mountain ranges, not only between (Pérez et al. 2002; Crestanello et al. 2009; Rodríguez et al. 2010) but also within subspecies (Crestanello et al. 2009; Buzan et al. 2013; Markov et al. 2016; Papaioannou et al. 2019).

The high genetic differentiation of the BGR population shown by both F_{ST} and STRUCTURE might be a result of the small effective population size in the Rhodopes at the end of the nineteenth century and the multiple genetic bottlenecks experienced by this population (Markov et al. 2016). According to the Action Plan for the Balkan chamois in Bulgaria (Valchev et al. 2006), the genetic differentiation of this population is the result of the absence of natural corridors between populations in West Rhodopes, Rila, Pirin and Tsentralen Balkan, as well as the internal fragmentation at the local population level. In addition to isolation by habitat fragmentation, another factor that could have led to such genetic differentiation could be hybridization with Alpine chamois, since individuals from this subspecies were introduced in 1977 in the Kormisosh hunting reserve (Valchev et al. 2006). The genetic differentiation of the SRB population from neighboring populations was not unexpected, since a similar pattern was observed in other species, including the Dinaric-Balkan wolf population (*Canis lupus*) where two subpopulations were recognized, the "western" subpopulation with individuals from Bosnia and Herzegovina and Croatia and the "eastern" subpopulation with individuals from Serbia and North Macedonia (Djan et al. 2014). Such a structure in the wolf population was attributed to several causes, including bottleneck, different demographic histories of subpopulations, a consequence of differences in hunting pressure, and the possibility that the river Drina acts as a barrier, separating the Peridinaric region (Djan et al. 2014). This river acts as a natural barrier between the Dinarides and the Scardo-Pindic mountain for other species, including the lynx (*Lynx lynx*; Melovski et al. 2012) and wildcat (*Felis silvestris*; Urzi et al. 2021), and its presence might have also affected the Balkan chamois, dividing it into subpopulations. The study on wildcat by Urzi et al. (2021) not only identified a division between western and south-eastern populations but also emphasized the importance of even sampling and avoiding sampling gaps between analyzed areas. It would thus be important to further investigate the Serbian chamois populations, sampling all (or most) of the areas inhabited by the species and combining different molecular markers to clarify the status and population history of chamois in this country to help disentangle the effect of discontinued suitable habitats, historical processes, life histories or environmental barriers on the observed genetic differentiation.

No significant correlation was found between microsatellite genetic distance and geographic distance (Fig. 2). A similar case was found in Alpine chamois (Soglia et al. 2010), where six geographic populations from different sites on the southern slope of the Alps showed no significant correlation of genetic and geographic distances. A significant correlation was found after the removal of the Lombard Prealps population, for which Soglia et al. (2010) hypothesized that it may have suffered from possible impacts of a founder

effect combined with geographic isolation and that different genetic characteristics of the source metapopulation may have disrupted the expected IBD pattern.

Mitochondrial DNA diversity

We observed a relatively high amount of haplotype and nucleotide diversity in the Balkan chamois population ($h = 0.913$; $\pi = 0.031$; Table 2). The Balkan chamois population from the BGR population had very low diversities compared with all other populations, which could be the result of its recent population history with multiple reductions in numbers (Markov et al. 2016). On the other hand, the ALB and GRC populations had high levels of diversity despite a very limited sample size. Here we must take into account the fact that molecular genetic studies of biodiversity that use mtDNA marker variation to characterize the existing genetic diversity of species are particularly sensitive to sample size (Phillips et al. 2018).

The analysis of 44 partial CR sequences of Balkan chamois revealed sixteen private and two common haplotypes (HRCR9 and HRCR20; Fig. 3a), indicating genetic flow among contiguous neighboring populations (SRB, MNE) and the possibility of past translocations, while geographically distant populations (BGR, GRC) were more differentiated. The median-joining network separated GRC haplotypes from haplotypes belonging to other populations (13 mutations). The four Greek Balkan chamois sequences obtained in this study were from the northern Pindus Mountains, where Papaioannou et al. (2019) detected the higher variability among the Greek populations, close to the diversity of the larger populations in the Alps. Similar results were found in the studies of maternally inherited markers in other chamois subspecies (Schaschl et al. 2003; Crestanello et al. 2009; Rodríguez et al. 2009; Rodríguez et al. 2010; Buzan 2013), where substructuring of the maternal gene pool into regional mitochondrial DNA phylogroups with limited gene flow between neighboring populations was observed. Schaschl et al. (2003) showed geographic structuring of mtDNA of Alpine chamois populations in Eastern Alps as a consequence of immigration of chamois from different Pleistocene refugia around the Alps after glacial retreat, and not due to topographic barriers to gene flow. For all population pairs studied in Crestanello et al. (2009), differentiation was always higher for the CR than for the microsatellites, which was to be expected given the higher sensitivity of this locus to genetic drift.

When comparing the Balkan chamois sequences with the dataset containing all other *Rupicapra* spp. (Fig. 3b), one shared haplotype with Alpine chamois and two shared with Tatra chamois were found (HRCR9, HRCR20, HRCR38). The haplotype network grouped Balkan haplotypes from Dinara Mt. in HRV, Rhodope Mt. in BGR, and Prenj Mt. in BIH with Alpine haplotypes from North Velebit Mt. (HRCR9). Similar results were published in Šprem and Buzan (2016), where haplotypes from the Biokovo, Dinara, Velebit and Prenj Mts. grouped with six haplotypes from the Velebit Mt. as a result of past chamois translocations (see Apollonio et al. 2014 for a review). The analysis of the complete mitogenome (Iacolina et al. 2021) revealed the presence of an *R.r. rupicapra* sequence within the *R.r. balcanica* clade. The results reported in the same study showed past reintroductions and translocations in the Northern Dinaric mountains in Croatia. Translocations and genetic introgression might as well explain the presence of the HRCR9 haplotype in the BGR population, since according to Markov et al. (2016) a few individuals of Balkan chamois from the ISHR population may have been introgressed with Alpine genes. An alternative explanation, from the same authors, suggested that the introgression signals might rather reflect shared ancestral polymorphism that might have accumulated through genetic drift.

Based on the median-joining network analysis, Balkan chamois individuals from SRB and MNE populations shared haplotypes with Tatra chamois (HRCR20 and HRCR38; Fig. 3b). The two subspecies were previously described as sister groups (Iacolina et al. 2021) and a similar result was found in the study by Rodríguez et al. (2010), in which the Neighbor-Joining tree, based on combined sequences of different regions of mtDNA, grouped an individual of Balkan chamois close to Tatra chamois. According to the supplementary files of Rodríguez et al. (2010), this one individual that grouped with the Tatra chamois originated from the Serbian Carpathians. The same results were obtained for the mtDNA cytochrome *b* region (Rodríguez et al. 2009), where the Balkan chamois shared the same haplotype with the Tatra, Carpathian, Anatolian and Caucasian chamois. The shared haplotypes between Balkan and Tatra chamois could be explained by possible historical events of introgression between subspecies or shared phylogeny, as previously suggested by Rodríguez et al. (2009).

Conclusions

Results of this study showed the presence of a main genetic cluster comprising most of the Balkan chamois populations, the separation of the BGR chamois into a completely isolated population, whereas the SRB population is only partially isolated as it still shared mtDNA lineages with the MNE chamois. Our results thus reveal that Balkan chamois populations create an archipelago rather than a peninsula as a result of habitat and population fragmentation and an overall reduction in population size due to multiple, non-exclusive factors such as poaching and unsustainable hunting, increased density of infrastructure,

competition with livestock for pastures, predation, and the introduction of other subspecies of chamois, mainly Alpine chamois.

During the last decades, the Balkan chamois has been threatened with extinction in many parts of its distribution range. The introduction of new individuals into small, fragmented populations affected by genetic drift or genetic erosion should be strongly considered. Introductions and translocations, however, should be supported by genetic screening to prevent unwanted negative consequences. We thus recommend a fine scale geographic sampling across the distribution range to detect areas of genetic discontinuity and the identification of (potential) corridors. Overall, long-term (genetic) monitoring and the establishment of MUs is warmly recommended as a basis for sustainable management of these populations and their conservation. The implementation of measures aimed at increasing genetic connectivity, while reducing the risks associated with stochastic effects are needed to improve the sustainability of Balkan chamois populations in the future. Additionally, ancient DNA analysis of archeological and museum samples would help clarifying the subspecies population history and defining its baseline historical genetic diversity.

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Author contributions Nikica Šprem and Elena Bužan conceived and designed the study. Nikica Šprem, Ferdinand Bego, Dragan Gačić, Vladimir Maletić, Georgi Markov, Dragana Milošević, Haritakis Papaioannou collected the samples. Andrea Rezić and Laura Iacolina performed the laboratory analysis. Andrea Rezić, Laura Iacolina, Toni Safner analyzed the data. Andrea Rezić wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read, revised and approved the final manuscript.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval All procedures described in the Materials and Methods section that involve animal experimentation have been approved by the Bioethical Committee for the protection and welfare of animals at the University of Zagreb Faculty of Agriculture. This committee has assessed that the use of Balkan chamois samples was in compliance with the Animal Protection Act (OG 102/17) and the Regulation on the protection of animals used for scientific purposes (OG 55/13).

Consent to participate Research did not involve human participants.

Consent for publication Research did not involve human participants.

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SHORT COMMUNICATION

ZooKeys

Traces of past reintroduction in genetic diversity: The case of the Balkan chamois (Mammalia, Artiodactyla)

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Abstract

The translocation of wild animal species became a common practice worldwide to re-establish local populations threatened with extinction. Archaeological data confirm that chamois once lived in the Biokovo Mountain but, prior to their reintroduction in the 1960s, there was no written evidence of their recent existence in the area. The population was reintroduced in the period 1964–1969, when 48 individuals of Balkan chamois from the neighbouring mountains in Bosnia and Herzegovina were released. The main objective of this study was to determine the accuracy of the existing historical data on the origin of the Balkan chamois population from the Biokovo Mountain and to assess the genetic diversity and population structure of the source and translocated populations 56 years after reintroduction. Sixteen microsatellite loci were used to analyse the genetic structure of three source chamois populations from Prenj, Čvrsnica and Čabulja Mountains and from Biokovo Mountain. Both STRUCTURE and GENELAND analyses showed a clear separation of the reintroduced population on Biokovo from Prenj's chamois and considerable genetic similarity between the Biokovo population and the Čvrsnica-Čabulja population. This suggests that the current genetic composition of the Biokovo population does not derive exclusively from

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Prenj, as suggested by the available literature and personal interviews, but also from Čvrsnica and Čabulja. GENELAND analysis recognised the Balkan chamois from Prenj as a separate cluster, distinct from the populations of Čvrsnica and Čabulja. Our results thus highlight the need to implement genetic monitoring of both reintroduced and source populations of endangered Balkan chamois to inform sustainable management and conservation strategies in order to maximise the chances of population persistence.

Keywords
Biokovo, genetic structure, microsatellite, Prenj, translocation

Introduction

The reintroduction and translocation of wild species for various purposes became a common practice worldwide and was used as a conservation tool for rescuing and re-establishing extirpated populations (Cullingham and Moehrenschlager 2013). Northern chamois (*Rupicapra rupicapra* L.) is one of the examples of successfully translocated species (Apollonio et al. 2014) in many areas of Europe (Crestanello et al. 2009; Martínková et al. 2012; Šprem and Buzan 2016), but also on other continents such as South America (Corlatti et al. 2011) and New Zealand (Christie 1964). Past translocations of chamois left a genetic signature in recent populations which can be now used for reconstructing undocumented events (Crestanello et al. 2009).

Today's populations of the Northern chamois in northern Dinaric Mountains in Croatia are descendants of successfully translocated individuals captured on mountain areas in Bosnia and Herzegovina (*Rupicapra rupicapra balcanica*) and Slovenia (*Rupicapra rupicapra rupicapra*) (Apollonio et al. 2014; Šprem and Buzan 2016). Since different subspecies were involved in past reintroduction efforts, a contact zone was formed on the northern Velebit Mountains where these subspecies hybridise (Šprem and Buzan 2016).

The Balkan chamois (*Rupicapra rupicapra balcanica*) is one of the seven recognised subspecies of the Northern chamois. It is found both in the mountainous regions of Croatia and in the mountain ranges of the eight other countries of the Balkan Peninsula, from north to south: Bosnia and Herzegovina, Serbia, Montenegro, Kosovo, North Macedonia, Albania, Bulgaria, and Greece. The lack of continuity of these habitats and overhunting in the post-Neolithic period have severely fragmented the subspecies' present distribution (Corlatti et al. 2022). In addition to low colonisation rates and reduced gene flow between isolated populations, which may lead to genetic differentiation due to the inbreeding effect and loss of allelic variants, the Balkan chamois is threatened by poaching (Papaioannou and Kati 2007), habitat change (Kavčić et al. 2019), unsustainable hunting (Šprem and Buzan 2016) and by the introduction of Alpine chamois subspecies (Iacolina et al. 2019). As a conservation measure, the Balkan chamois is listed in Annexes II and IV of the European Union Habitats Directive 92/43/EEC (OJ L 206, 22.7.1992) and in Appendix III of the Bern Convention (OJ L 38, 10.2.1982). The Balkan chamois is one of the most poorly studied subspecies of Northern chamois and

knowledge on the genetic diversity and structure of the Balkan chamois population is limited and restricted to regional-local studies (Markov et al. 2016; Šprem and Buzan 2016; Papaioannou et al. 2019; Rezić et al. 2022).

The genetic structure of the Balkan chamois population on the Biokovo has been studied only by Šprem and Buzan (2016), and few other ecological studies have included this population in a population density estimation (Kavčić et al. 2021a) and rutting behaviour (Kavčić et al. 2021b). The study of phylogenetic relationships in Šprem and Buzan (2016) revealed the existence of endemic Balkan haplotypes in the Prenj and Biokovo Mountains and a genetic richness of the historically viable Prenj population comparable to Alpine chamois studied in Buzan et al. (2013) from the south-eastern Alps. The paleontological findings in the Baba cave, which are more than ten thousand years old, confirm that chamois once lived in Biokovo (Šabić 2011) but, before the reintroduction in the 1960s, there was no written evidence of the recent existence of chamois in this area. According to historical records, the chamois on Biokovo Mt. are descendants of individuals translocated from the "Prenj" hunting district in Bosnia and Herzegovina established in 1961 (Rapaić and Kunovac 2020) which included both Prenj and Čvrsnica massifs. This hunting district had, at that time, stable and numerous chamois populations and was used for many reintroduction programmes in the Balkans (Rapaić and Kunovac 2020). The reintroduction of chamois on Biokovo Mt. was the result of the planned introduction on this area by the Union of Hunting Association, the municipality of Makarska and the Union of Hunting Association of Imotski, mainly with the aim of increasing the population size for hunting purposes (Šabić 2011). Prior to the reintroduction from the "Prenj" hunting district, assessment of the suitability of the Biokovo habitat was made and, after a positive evaluation, a first release of 7 individuals (3 males and 4 females) took place on 1 November 1964 (Šabić 2014). A total of 48 chamois was successfully reintroduced in the period between 1 November 1964 and 23 October 1969 (Šabić 2014). The success of the reintroduction of the Balkan chamois in the Biokovo Mt. is reflected by the latest population size estimate (Kavčić et al. 2021a), according to which this area is now inhabited by at least 600 individuals.

The main objective of this study was to determine the accuracy of historical data on the origin of chamois in Biokovo, and to assess and document the genetic status of both the source and translocated populations, 56 years after reintroduction, by using microsatellite markers.

Materials and methods

Ethical statement

All samples used in this study were from hunted (regular hunting activities approved by the competent Ministry of Agriculture of the Republic of Croatia within the annual game management plans) and from remains of naturally dead animals (samples from Bosnia and Herzegovina).

Population sampling

We collected 20 samples from Biokovo and 29 samples from three areas which serve as source populations for reintroduction and possible recent recolonisation (Prenj, Čvrsnica, and Čabulja Mountains). Details of sampling locations are given in Fig. 1 and Table 1. The samples were collected between 2017 and 2020. After collection, the samples were preserved in 96% ethanol, delivered and stored at -80 °C in the Laboratory of molecular ecology, University of Primorska.

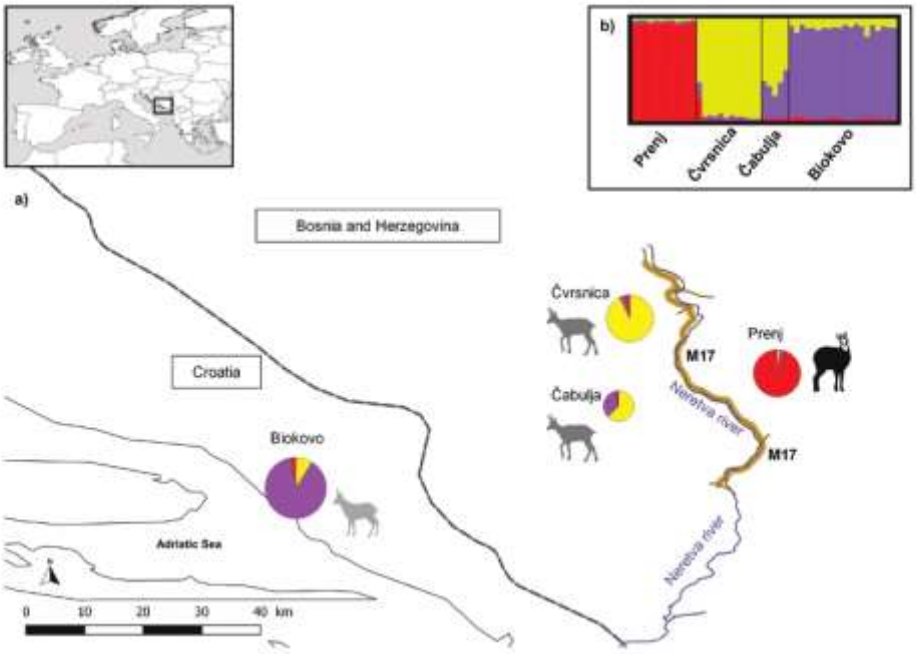


Figure 1. Results of the analysis of sixteen microsatellite loci in four Balkan chamois populations **a** geographical representation of results from STRUCTURE and GENELAND software. The pie charts show the results from STRUCTURE for $K = 3$. The different colours of the pie charts represent the proportions of each ancestral genotype per individual q (in %) in each of the four predefined Balkan chamois populations. The size of the pie charts indicates the number of samples collected at each location. The different shapes and colours of the chamois silhouettes represent the results of the spatial analysis under uncorrelated frequency model performed in GENELAND. The three spatial clusters are shown, while the assignment to the fourth ghost cluster was not shown because no individuals were assigned to it (see text for details). The dashed line indicates the national border, while the state road M17 in Bosnia and Herzegovina is marked with an orange line. The green lines represent connections with other main roads. The course of the river Neretva is marked by a blue line **b** genetic structure of the 49 Balkan chamois individuals analysed, shown as a bar plot from STRUCTURE at $K = 3$. Each vertical bar represents an individual, and the percentage of each colour corresponds to the percentage of the respective ancestral genotype. The studied populations are separated by a black line.

Table 1. Genetic diversity of four Balkan chamois populations assessed using sixteen microsatellite loci.

Population locality/ country	N	H_O (SD)	H_E (SD)	HWE	F_{IS} (*IC* 95%)	A	AR	N_P	N_e (*IC* 95%)
Prenj 43°32'03"N, 17°54'12"E/BIH	12	0.636 (0.274)	0.637 (0.150)	0.013*	0.046 (-0.147–0.116)	4.500	2.517	11	10.500 (6.500–18.600)
Čvrsnica 43°38'18"N, 17°38'30"E/BIH	12	0.552 (0.287)	0.536 (0.206)	0.011^{NS}	0.014 (-0.131–0.038)	3.937	2.223	3	7.700 (4.100–13.600)
Čabulja 43°29'11"N, 17°37'20"E/BIH	5	0.575 (0.251)	0.535 (0.187)	0.913^{NS}	0.059 (-0.415–0.089)	3.375	2.376	2	–
Biokovo 43°19'47"N, 17°63'05"E/HRV	20	0.584 (0.132)	0.597 (0.138)	0.389^{NS}	0.048 (-0.050–0.084)	4.625	2.356	6	57.200 (28.600–371.800)

BIH – Bosnia and Herzegovina, HRV – Croatia; N – number of samples; H_O – observed heterozygosity; H_E – expected heterozygosity; SD – standard deviation; F_{IS} – inbreeding coefficient; *IC* 95% – 95% Confidence Interval; HWE – Hardy-Weinberg equilibrium (after Bonferroni adjustment p values: ^{NS} – non-significant value; * – significant at $p < 0.05$); A – average number of alleles; AR – allelic richness; N_P – number of private alleles; N_e – effective population size and its confidence interval estimated with the chi-square (i.e., parametric).

DNA extraction and microsatellite amplification

We extracted DNA from tissue samples ($N = 49$) using the commercial peqGOLD Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH) following the manufacturer´s protocol in a final volume of 150 µL. DNA concentrations were measured with Qubit dsDNA BR Assay Kit (Invitrogen BR Assay Kit, Carlsbad, CA, USA) on a 3.0 Qubit Fluorimeter (Life Technologies, Carlsbad, CA, USA). Sixteen microsatellites were amplified using PCR multiplex sets previously investigated in studies with chamois (Zemanová et al. 2011; Buzan et al. 2013; Šprem and Buzan 2016; see Suppl. material 1: Table S1). The PCR protocol described in Rezić et al. (2022) was used for amplification of microsatellite regions. Genotyping errors were assessed by re-genotyping of ten randomly chosen individuals from the final data set and comparing these genotypes to the initial ones. Fragment analysis was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems) using the GeneScan LIZ500 (-250) Size Standard (Applied Biosystems). Microsatellite genotypes were analysed using Gene Mapper v. 4.0 software (Applied Biosystems).

Microsatellite data analysis

We used the Expectation-Maximization (EM) algorithm implemented in FREENA (Chapuis and Estoup 2007) to estimate null allele frequencies for each microsatellite locus, as they can cause significant heterozygote deficit and population deviation from Hardy-Weinberg equilibrium (HWE). Values of null allele frequency greater than $r \geq 0.20$ were reported (see Suppl. material 1: Table S2). FREENA software was also used to calculate global F_{ST} values and F_{ST} values for each pair of analysed populations, both with and without the use of the excluding null alleles (ENA) correction method, as described in Chapuis and Estoup (2007). The Wilcoxon Two Sample test was used to compare the corrected F_{ST} values with the original F_{ST} values and to test the signifi-

cance of null alleles in the analyses. The Wilcoxon Two Sample test was performed in R ver. 4.0.5 package stats (R Core Team 2020).

We considered each sampling location as a separate population due to limited dispersal of subspecies between mountain ranges (see Table 1). The exact probability test for each locus and population was used to test the deviation of the observed genotype frequency from HWE using the Markov chain method with 10,000 dememorisation steps, 500 batches and 10,000 subsequent iterations in GENEPOP ver. 4.7.2 (Rousset 2008). The same test, based on a Markov chain method implemented in Genepop, was used to analyse pairwise linkage disequilibrium (*LD*) between all pairs of loci in all populations. A sequential Bonferroni procedure (Holm 1979) was applied to correct for the effect of multiple comparison tests by using the adjust *p*-values function implemented in R ver. 4.0.5 package stats.

GENETIX ver. 4.05.2 (Belkhir et al. 1996–2004) was used to calculate the mean number of alleles, observed (H_O) and expected (H_E; Nei 1978) heterozygosity for each locus in all populations, and the inbreeding coefficient (F_{IS}) and its confidence intervals. The number of private alleles was estimated using the GENALEX ver. 6.502 (Peakall and Smouse 2012). We estimated allele richness in each population using the rarefaction procedure implemented in FSTAT ver. 2.9.3.2 (Goudet 2001). The same software was used to analyse the level of genetic differentiation between sampling populations (pairwise F_{ST}) and calculate their respective *p*-values using 1000 permutations.

The Bayesian clustering program STRUCTURE ver. 2.3.4. (Pritchard et al. 2000) was used to estimate the most likely number of ancestral genotypes (*K*) within the entire sample, and to estimate the proportions of each ancestral genotype in Balkan chamois individuals. We ran the analysis allowing for admixture and correlated allele frequency with ten independent runs for each *K* between 1 and 7 with a burn-in 500,000 steps followed by 10^5 Markov chain Monte Carlo (MCMC) iterations. The results of the repeated runs for each value of *K* were combined with the Greedy algorithm in CLUMPP v. 1.1.2 (Jakobsson and Rosenberg 2007), and the summary outputs were visualised with DISTRUCT v. 1.1 (Rosenberg 2004). To estimate the most likely *K*, we applied the ad hoc summary statistic ΔK developed by Evanno et al. (2005). STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used to compare the average estimates of the likelihood of the data, ln[Pr(X|K)] for each value of *K*. The same software was used to generate graphs for the mean log posterior probability of the data (mean ± SD).

The modal proportions of ancestral genotypes for each individual in each sampled area from the run with the highest log-likelihoods was plotted on a map using QGIS ver. 2.18.21 (QGIS Development Team 2018). We estimated the effective population size (N_e) using the linkage disequilibrium-based method (Hill 1981; Waples 2006; Waples and Do 2010) implemented in NeESTIMATOR V2 (Do et al. 2014). Rare alleles below an allele frequency of 0.02 were excluded (as recommended by Waples and Do 2010). The effective population size for Čabulja Mt. was not calculated due to the small sample size.

The robustness of the results of STRUCTURE was estimated by analysing the same data with the spatial Bayesian clustering model implemented in GENELAND

software (Guillot et al. 2005a). Although there are several Bayesian clustering methods that perform spatial analysis of genetic data, GENELAND was chosen because it provides the most accurate estimates of true genetic structure (Safner et al. 2011). We followed the recommendations of Guillot et al. (2005a) to set up the analysis. The algorithm was run in two steps. In the first step, the algorithm was run ten times to infer K under the uncorrelated frequency model, with the parameter indicating the degree of uncertainty of the spatial coordinates set to 10. The MCMC iterations were set to 10^6 with a thinning of 100. The number of populations was set from $K = 1$ to $K = 5$. The maximum number of nuclei in the Poisson-Voronoi tessellation was set to 300. After determining the number of population clusters in the first step, we ran the algorithm setting K to this number and leaving the other parameters as in the first step.

Results and discussion

The sixteen microsatellite loci yielded a total of 95 alleles, which varied between 2 (for locus ETH10 and SR-CRSP-6) and 10 (for locus BM1258) with an average value of 5.937 alleles per locus (see Suppl. material 1: Table S1).

The values of null allele frequencies were low for most analysed loci, except for loci ETH10, SY434, TGLA53, and SR-CRSP-6, whose frequencies were estimated to be $r \geq 0.20$ (see Suppl. material 1: Table S2). The presence of null alleles was found in all three populations from Bosnia and Herzegovina. The various factors caused by natural population mechanisms, such as disassortative mating, bottleneck, fluctuations in population size, can cause heterozygote deficit that can be interpreted as false positive presence of null alleles (Dąbrowski et al. 2014), which led to the decision to retain all analysed loci.

The Prenj population deviated from Hardy-Weinberg equilibrium (HWE) but the deviation was significant at the 0.05 level only for locus SY434 after applying sequential Bonferroni adjustment (Table 1). This may be a consequence of the recent severe bottleneck in this population, which was previously stable. Gafić and Džeko (2009) noted that the population of Balkan chamois in the Prenj hunting district, which included both the Prenj and Čvrsnica massifs, was approximately 4,000 individuals in 1966, but due to the civil war in the 1990s, the population was greatly reduced by illegal hunting, with up to 95% of the population lost. Since no deviation from HWE was observed at this locus in other populations, we retained it in all subsequent analyses. After applying the sequential Bonferroni correction to the linkage disequilibrium results, no significant value was observed.

The Prenj population had the highest values of observed (0.636) and expected (0.637) heterozygosity, and allelic richness (2.517). A similar pattern was recorded in the study of Šprem and Buzan (2016) where the Prenj population had the highest values of allelic richness, observed, and expected heterozygosity and significantly deviated from HWE. In the Šprem and Buzan (2016) study, the Biokovo population had the lowest allelic richness. The observed number of alleles (A) varied from 3.375 in the

Čabulja population to 4.625 in the Biokovo population. All populations had private alleles (N_p) and the highest number of private alleles (11) was observed in the Prenj population from Bosnia and Herzegovina (Table 1).

Effective population size was estimated for three sampled sites, excluding the Čabulja population due to small sample size (Table 1). Čvrsnica had the lowest results for N_e, although very similar to those estimated for Prenj. The higher estimates for the Biokovo population should be taken with caution, considering our results suggest the presence of multiple funding sources. Additionally, Do et al. (2014) showed that microsatellite loci could lead to a slight upward bias for the linkage disequilibrium method when the critical value is set to $p = 0.02$.

The lowest F_{ST} value was found between Čvrsnica and Čabulja ($F_{ST} = 0.024$), while the highest and significant F_{ST} value (0.084) was observed between two neighbouring populations from Bosnia and Herzegovina (Prenj and Čvrsnica; Table 2). The global F_{ST} values were 0.067 ($CI = 0.031–0.108$) without using the correction method and 0.071 ($CI = 0.038–0.112$) with the ENA correction method for null alleles. The Wilcoxon Two Sample test showed no significant differences between the corrected and original F_{ST} values ($p = 0.734$), indicating that the presence of putative null alleles did not affect the analysis (see Suppl. material 1: Fig. S1).

The algorithm developed by Evanno et al. (2005) identified $K = 3$ as the optimal number of ancestral genotypes detected by STRUCTURE analysis for four analysed populations and detected another peak at $K = 5$ suggesting a possible further genetic structure subdivision within the populations (see Suppl. material 1: Fig. S2). According to STRUCTURE results, populations from Prenj, Čvrsnica, and Biokovo had high proportions of genomes from a single ancestral genotype ($q \geq 0.88$ in all three populations), which for Prenj differ from Čvrsnica and Biokovo, while in the population from Čabulja the highest proportion of same ancestral genotype as in Čvrsnica was lower ($q = 0.63$; Fig. 1a). The difference in genetic composition between Balkan chamois from Prenj and other populations is likely due to a barrier to gene flow between the studied populations, but also probably a consequence of recent bottleneck effect, due to extirpation of chamois during the Balkan civil war (Frković 2008) and population local adaptation. Čvrsnica, Čabulja, and Prenj Mts. are restricted to habitat patches, particularly by the steep river canyon of Neretva, which might lead to their fine-scale fragmentation. The Neretva River, one of the largest rivers in the eastern part of the Adriatic Basin, separates the Prenj Mt. from the Čvrsnica and Čabulja Mts. and forms the official border between the "Čvrsnica" and "Prenj" game reserves (Jurić 1998). Landscape features of the Neretva River valley, as well as the adjacent road M17, built

Table 2. Pairwise F_{ST} values between four studied populations of Balkan chamois.

Populations	Čvrsnica	Čabulja	Biokovo
Prenj	0.084*	0.047^{NS}	0.074*
Čvrsnica		0.024^{NS}	0.072*
Čabulja			0.027^{NS}

p values: ^{NS} – non – significant value; * - significant at $p < 0.05$.

as a part of the European Route E73, can act as effective barriers for the species natural dispersal, and hinder gene exchange between the studied populations (Soglia et al. 2010; Buzan et al. 2013). Additionally, the population decline in the last Balkan civil war could have contributed by isolating small groups of chamois to restricted habitat patches, although geographically very close to each other. Individual proportions of ancestral genotypes assigned by STRUCTURE support the higher levels of admixture in Čabulja when compared to the other populations (Fig. 1b). According to the q values, only one individual from Čabulja had a q value above the threshold of 0.75 sharing similar genotype as individuals from the Čvrsnica population, while all others had admixed genotype ($q < 0.70$). One individual from Čvrsnica stood out from the others with a $q = 0.23$ proportion of ancestral genotype that was present mostly in Prenj population. As previously mentioned, natural migration between individuals from Prenj and Čvrsnica is currently unlikely, due to the presence of barriers, however this might not have been the case before the construction of the road infrastructure. STRUCTURE indicated that the reintroduced Balkan chamois population in the Biokovo Mt. is genetically more similar to the population from Čabulja, suggesting that the reintroduced individuals in the Biokovo Mt. may originate from this area, as well as from Čvrsnica, whereas animals translocated from Prenj did not leave detectable genetic signatures. According to Jurić (1998), 992 individuals of Balkan chamois were counted in the Čvrsnica hunting ground which also includes the territories of the neighbouring Čabulja Mt. It is possible that populations Čvrsnica and Čabulja were connected in the past and formed a single population, as can be inferred from the results of STRUCTURE. Due to the civil war in the Balkans in the 1990s and continued illegal hunting and poaching, this single population has dwindled in numbers and become fragmented and isolated in the high mountain habitats (statements of local people). The divergence between Prenj and Biokovo from Čvrsnica and Čabulja populations may be due to the historical founder effect and more recent genetic drift due to isolation, and local adaptation. Despite decades of long unsustainable hunting, predation, poaching and natural events (Šprem and Buzan 2016) the inbreeding levels in the analysed populations are still moderate. The results of genetic composition of Biokovo population can influence population viability through time and it is very important to monitor genetic parameters of the reintroduced population to prevent a loss of genetic diversity due to inbreeding and genetic drift (DeMay et al. 2017).

To improve the previous analyses, the spatial context of individuals was taken into consideration and tested with GENELAND. This analysis revealed a similar pattern of clustering of individuals as STRUCTURE, but suggested an additional fourth spatial cluster along the MCMC chain (see Suppl. material 1: Fig. S3). GENELAND detected two spatial clusters among the three analysed populations in Bosnia and Herzegovina, while one spatial cluster corresponded to the population in Biokovo (Fig. 1). The distinction between Prenj and Biokovo was also detected by Šprem and Buzan (2016), who used the BAPs algorithm for spatial clustering of groups and showed the separation of the Balkan chamois population of the Prenj Mt. from the Biokovo Mt. population. The fourth cluster revealed by

GENELAND spatial model was a so-called "ghost cluster" (Frantz et al. 2009) since no individual was assigned to it. Ghost clusters are not uncommon but are still a poorly understood phenomenon that can be caused by a heterogeneous distribution of samples (Aziz et al. 2018). It is possible that all the clusters identified by GENELAND represent a true genetic subdivision, but the degree of differentiation between them was too low for the clustering to be consistent (Frantz et al. 2009). Another possibility is that the GENELAND model might overestimate the number of genetic clusters when analysing populations which are affected by isolation by distance (Frantz et al. 2009).

Future studies will need to incorporate non-invasive genetic sampling, telemetry and behavioural patterns to confirm possible migration and gene flow between these populations. In the available literature there is no indication of the exact location where the animals released on Biokovo were caught. It is only known that the individuals came from the Prenj hunting district, which included two game reserves called "Čvrsnica" and "Prenj" (established in 1893 by the Austro-Hungarian Empire) and which were declared protected areas (Rapaić and Kunovac 2020). Jurić (1998) stated in his master's thesis that the translocation of Balkan chamois from the game reserve "Čvrsnica" began in 1962 and lasted until 1970. During this period, a total of 101 Balkan chamois was translocated to different areas in the Balkans, but the author did not write any additional information about the location where the animals were caught or the places where they were translocated. It is not yet known whether these data exist. Therefore, it is very important to establish standards for documenting and monitoring species translocation projects.

Conclusions

Non-invasive monitoring of genetic parameters of both reintroduced and source populations of endangered Balkan chamois, together with demographic monitoring, is crucial for sustainable management practices and improving conservation strategies to maximise the chances of population persistence. Our genetic diversity results show that the Balkan chamois population from Biokovo can serve as a potential source for future translocations, especially to the source habitats, Čvrsnica and Čabulja, that are currently threatened by loss of genetic diversity due to unsustainable hunting and poaching, leading to inbreeding and genetic drift.

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Supplementary material 1

Tables and figures

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Data type: Population genetics.

Explanation note: **Table S1**. Information about microsatellite primer sequences used for the analysis of Balkan chamois population genetics. **Table S2**. Locus/population matrix containing null alleles identified by the FreeNA software. Null allele frequencies were estimated using the EM algorithm. Frequencies with values higher than $r \geq 0.20$ were indicated. **Figure S1**. The comparison of two groups of FST values (original pairwise FST values and ENA-corrected pairwise FST values) using the Wilcoxon Two Sample test. **Figure S2**. Evanno method (Evanno et al. 2005) for selecting the representative number of clusters (K). **Figure S3**. Number of clusters along the MCMC chain for spatial analysis performed with the GENELAND software.

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SHORT COMMUNICATION

Microsatellite based assignment reveals history of extirpated mountain ungulate

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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 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

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(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_2O 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.

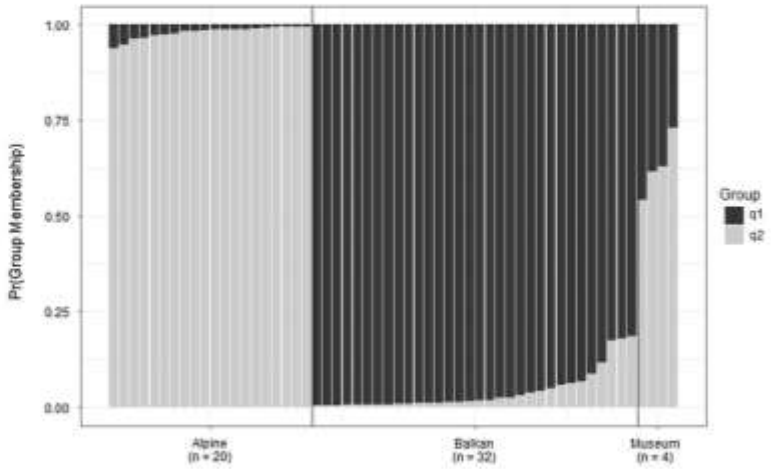


Fig. 1 Result from STRUCTURE analysis. Each bar represents one individual. Proportions of grey (*Alpine* subsp.) and black (*Balkan* subsp.) represent the contribution of each subspecies to each individual's genome.

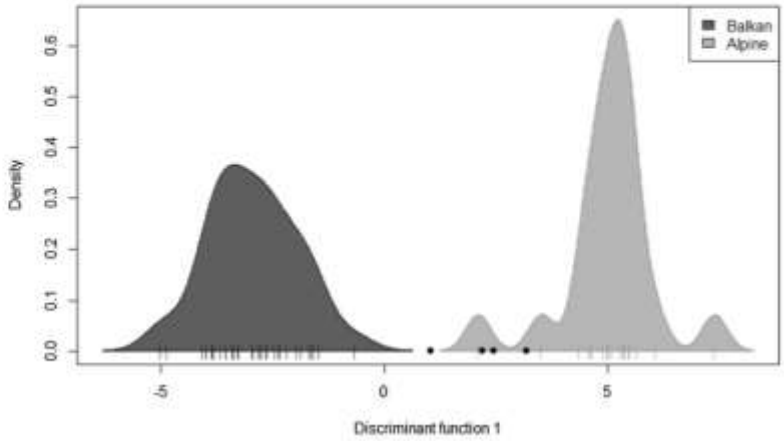


Fig. 2 Discriminant function densities for individuals from reference populations. Grey lines represent discriminant function scores for reference individuals. Circles represent discriminant function scores for the four museum samples.

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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