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ORIGINAL PAPER

Genetic variation of European mouflon depends on admixture of introduced individuals

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Abstract

In the early twentieth century, European mouflon was introduced in Croatia, while all introductions in Slovenia occurred in the 1950s and 1960s. Although majority of the introductions were historically documented, occasional cases involving individuals of unknown origin have likely contributed to a mixed genetic pool in established colonies. To understand the impact of past management and the potential founder effects on contemporary mouflon populations, we performed the first genetic study of the species in these two countries. Utilising next-generation sequencing of both mitochondrial control region (mtDNA CR) and major histocompatibility complex (MHC DRB exon 2), our study scrutinises the genetic diversity and structure of these populations. Additionally, the origins and genetic variability of mouflon in Croatia and Slovenia were compared with reference samples from Czech Republic, Sardinia (Italy), and Corsica (France). The mtDNA haplotype network showed that the majority of mouflon from Slovenia are closely related to mouflon from Sardinia, and only few shared the same haplotypes with mouflon from Croatia. Some mouflon from mainland Croatia share identical or closely related haplotypes with individuals from the initially established population in this country (on the Brijuni Archipelago), while others belong to a distinctly different cluster. We found five MHC alleles previously reported for mouflon in Europe, and genetic diversity was similar in both studied countries. We observed an excess of the Ovar-DRB1*07012/*07012 genotype, and only a few individuals exhibited the advantageous genotypes for parasite infection (Ovar-DRB1*0114 allele and Ovar-DRB1*0324/*0114 genotype). Genetic data showed that the population origins are generally in agreement with the written historical records, although we found signals of release of extra individuals into certain colonies.

Keywords *Ovis gmelini musimon* · Mitochondrial DNA · MHC DRB exon 2 · Introduction · Haplotype network

Introduction

After their extinction in mainland Europe, European mouflon (*Ovis gmelini musimon)* have been reintroduced since the eighteenth century in several European regions (Türcke

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and Tomiczek 1982; Apollonio et al. 2014; Guerrini et al. 2015). The species is now distributed in 21 European countries and lives both in enclosures, game reserves and in the wild, with population numbers varying greatly by country and region. The most numerous populations live in the Czech Republic, Germany, Hungary, Austria, France, and Slovakia (Nasiadka et al. 2021). The remaining 15 countries are inhabited by local populations comprising, all together, no more than a few thousand individuals that live in separate, isolated groups (Tomiczek and Türcke 2003). While historic records are available for some of the introduced populations, others have multiple/mysterious origins (Türcke and Schmincke 1965; Uloth 1972; Cugnasse 1994; Piegert and Ultoh 2000; Andreotti et al. 2001; Boitani et al. 2003). The introduction of mouflon in Europe was primarily for hunting purposes but also with the intent of increasing genetic diversity within small populations negatively affected by inbreeding (Heroldová et al. 2007). Further motivation for new introductions was transfer of small groups of mouflons from the Sardinian-Corsican "reservoir" (e.g. on the island of Giglio, Italy, where founders from Sardinia were released), aiming to create a genetic backup, i.e. to minimise the risk of extinction of the oldest European island populations (Barbato et al. 2022). Nowadays, mouflon are an integral part of the fauna of many ecosystems, inhabiting mainly mountainous environments (Ruiu 1989; Le Pendu et al. 1996; Cransac and Hewison 1997; Garel et al. 2005; Özdirek 2009; Bourgoin et al. 2011), although some (sub) populations also occur along the seacoast or in lowland areas (Garel et al. 2005; Krapinec et al. 2013; Kavčić et al. 2020; Nasiadka et al. 2021).

In Croatia, mouflon were first introduced in 1911 at the Brijuni Archipelago (on the largest island of Veliki Brijun, in total surface 555 ha; northern Adriatic Sea) by a German wildlife trader who supplied many European zoos, mostly from his private zoo in Germany (Rotondi 1937). This action was followed by multiple introductions of the species on the Croatian mainland (Krapinec et al. 2013; Kavčić et al. 2020). Overall, 2000 individuals were introduced at eighteen sites, most of them originating from the Brijuni Islands, but also from the Czech Republic, Slovakia, and Hungary (Krapinec et al. 2013). In Slovenia, the first successful introduction was recorded in 1953, followed by about ten introductions in different hunting grounds in the 1960s; in this country, mouflons were mainly introduced from the Brijuni Islands, but also from Austria and Germany (Fabjan 1965; Krže 1975; Kryštufek 1991). Today, there are 10–12 established colonies in eight hunting management districts in Slovenia (Fabjan 1965; Krže 1975; Galjot and De Brea-Šubic 1998; Hafner and Černe 2018; Gozdarski inštitut Slovenije 2023).

The mouflon has found favourable conditions in both countries (Krapinec et al. 2013; Hafner and Černe 2018; Kavčić et al. 2020), and several colonies have been established in various types of environment. The species inhabits mostly steeper and rocky areas at altitudes up to 2000 m (Krapinec et al. 2013; Hafner and Černe 2018). The mouflon habitat in both countries is dominated by forests in some places, and scrubland or non-forested areas (grasslands, other lightly vegetated areas) in others. While the colonies in the Alps have recently experienced a significant reduction due to wolf predation (Hafner and Černe 2018), the remaining colonies located outside of wolf territories have maintained stability, with occasional declines and increases in some areas (Galjot and De Brea-Šubic 1998; Hafner 2004; Krapinec et al. 2013). In many areas, mouflon lives sympatrically with other wild ungulates, especially with red deer (*Cervus elaphus*), axis deer (*Axis axis*), European roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*), or livestock, especially domestic sheep (*Ovis aries*), with which mouflon can sometimes hybridise (Šprem et al. 2023).

A better knowledge on the genetic characteristics (including origin) of wildlife populations is an essential tool for effective, science-based management. Mitochondrial DNA (mtDNA) is a commonly used marker to evaluate the historical origin of populations and to identify the source populations used for translocations. Using mtDNA, researchers revealed that the mouflon from Sardinia and Corsica belong to the most prevalent haplogroup (HPG-B) found in domestic sheep breeds (Hiendleder et al. 1998; Meadows et al. 2011; Satta et al. 2021). However, mtDNA genetic distance between the Corsican-Sardinian mouflon clade and other clades, including domestic sheep breeds, in this haplogroup is notably high. The phylogenetic tree further indicates an early separation of the Corsican-Sardinian clade from the lineage that evolved into domestic sheep (Sanna et al. 2015). Sardinian mouflon gene pool harbours the oldest clade of the HPG-B haplotype, which is preserved in the introduced populations in mainland Europe.

Founder-created populations tend to have small effective population size and low genetic diversity, and they may suffer from inbreeding depression, i.e. loss of individual fitness due to inbreeding (Coltman et al. 1999; Keller and Waller 2002; Taylor et al. 2017), which, in turn, might negatively affect their dynamics and persistence (Bozzuto et al. 2019). This can also cause a low major histocompatibility complex (MHC) variability, known as MHC depletion. Pathogen-driven balancing selection has been proposed as one of the most important evolutionary forces for the maintenance of MHC polymorphism (Piertney and Oliver 2006). Mechanisms resulting from the "rare-allele advantage" hypothesis are thought to maintain the high diversity of MHC genes at the population level. The hypothesis assumes that rare MHC alleles that have a higher efficiency in pathogen recognition provide the host with an advantage (Bernatchez and Landry 2003). For

instance, in domestic sheep, MHC variants significantly contribute to protection against nematodes, which are the most common gastrointestinal parasite in sheep (Paterson et al. 1998). Portanier et al. (2019) discovered that certain alleles and heterozygosity of MHC exon 2 alleles impact the abundance of gastrointestinal nematodes in female mouflon. This suggests the occurrence of heterozygote advantage, effects of rare alleles, and/or fluctuating selection in mouflon population. High diversity of MHC alleles allows wider range of pathogen recognition and it could also hinder the efficiency of the immune response. Low MHC diversity within a population can make population more susceptible to parasite infection (Kurtz et al. 2004). Although pathogen-mediated selection is important for preserving MHC functional variation, other mechanisms, such as disassortative mating preferences, maternal-foetal interactions, recombination, and gene duplication, have been suggested as alternative or complementary mechanisms maintaining MHC diversity (Miller and Lambert 2004; Spurgin and Richardson 2010; Juola and Dearborn 2011).

The dynamics observed in introduced mouflon populations across Europe (e.g. high reproductive success and rapid population growth; Garel et al. 2005; Kaeuffer et al. 2008) suggest that recovery may be very rapid in this taxon. This could be a consequence of balancing selection maintaining advantageous genetic variation within populations and playing crucial role in adaptation, thereby causing genetic differentiation across populations (Williams 1966; Portanier et al. 2018; 2019). Therefore, it is crucial to understand MHC genetic diversity and the history of populations in order to understand how genetic variation in functional genes is maintained as a consequence of genetic drift and the rate of allele fixation. This knowledge can help prevent the onset of inbreeding depression (Edmands 2006) and enable the future potential increase of functional alleles (Portanier et al. 2018; 2019).

Several scattered and isolated mouflon populations in Slovenia and Croatia with different histories provide an opportunity to address questions regarding the effects of past management and introductions on genetic diversity and structure in this species. In our study, we used neutral loci (partial fragment of mitochondrial control region, mtDNA CR) and adaptive major histocompatibility complex (MHC DRB exon 2) to analyse genetic variation and structure of selected mouflon populations from both countries. We hypothesised that established populations show depleted genetic diversity, due to the small number of funders. In an effort to determine the origin of introduced mouflon in Slovenia and Croatia, we analysed population genetic composition of populations in these two countries, supplemented by additional samples from the Czech Republic, Corsica (France), and Sardinia (Italy), as well as already published data from GenBank (from Corsica, Sardinia, and Germany).

Material and methods

Study areas and sampling

Animals used in the study were either legally harvested during the hunting season or collected as roadkill or natural death. No animal was shot or otherwise killed for the purposes of this study. Samples of harvested animals were collected by hunters or wildlife researchers immediately after harvest between 2020 and 2021. Tissue samples were preserved in 70% ethanol and blood samples were stored at − 20 °C until analysis.

Study areas were in the following five countries (Fig. 1): Slovenia (four areas), Croatia (eight areas), the Czech Republic (one area), Italy (Sardinia; two locations), and France (Corsica; one location) (Supplementary material, Table S1).

In line with our objectives, most of samples included in the analysis (102 in total) were collected in Slovenia ($n = 49$) and Croatia ($n = 40$). In addition, we analysed 9 samples from the Czech Republic, 2 from Corsica, and 2 from Sardinia.

Mitochondrial DNA CR haplotypes

In the mtDNA CR analysis, we incorporated the identified haplotypes from Slovenia, Croatia, and the Czech Republic (Supplementary material, Table S1). Additionally, we included five haplotypes from Sardinia and two from Corsica belonging to mitochondrial haplogroup HPG-B, which had already been published in GenBank (accession numbers KR011772, KR011774, KR011775, KR011776, MG489885, KR011781, and KR011782) by Sanna et al. (2015) (Supplementary material, Table S2).

In the mtDNA haplotype network analysis, we further incorporated additional sequences of *Ovis* sp. downloaded from GenBank (Supplementary material, Table S3).

Major histocompatibility complex DRB exon 2 alleles

For the MHC analysis, we included all 102 samples from five countries. Due to amplification failure, we were unable to include MHC data from three samples collected in Croatia and two samples from the Czech Republic; therefore, 97 of our samples were analysed in this part of the study. Available sequences data on GenBank ($n = 5$) were also included in the analysis (accession numbers: MK593470-MK593471-MK593472 (*Ovis gmelini musimon*; Corsica), MG000544

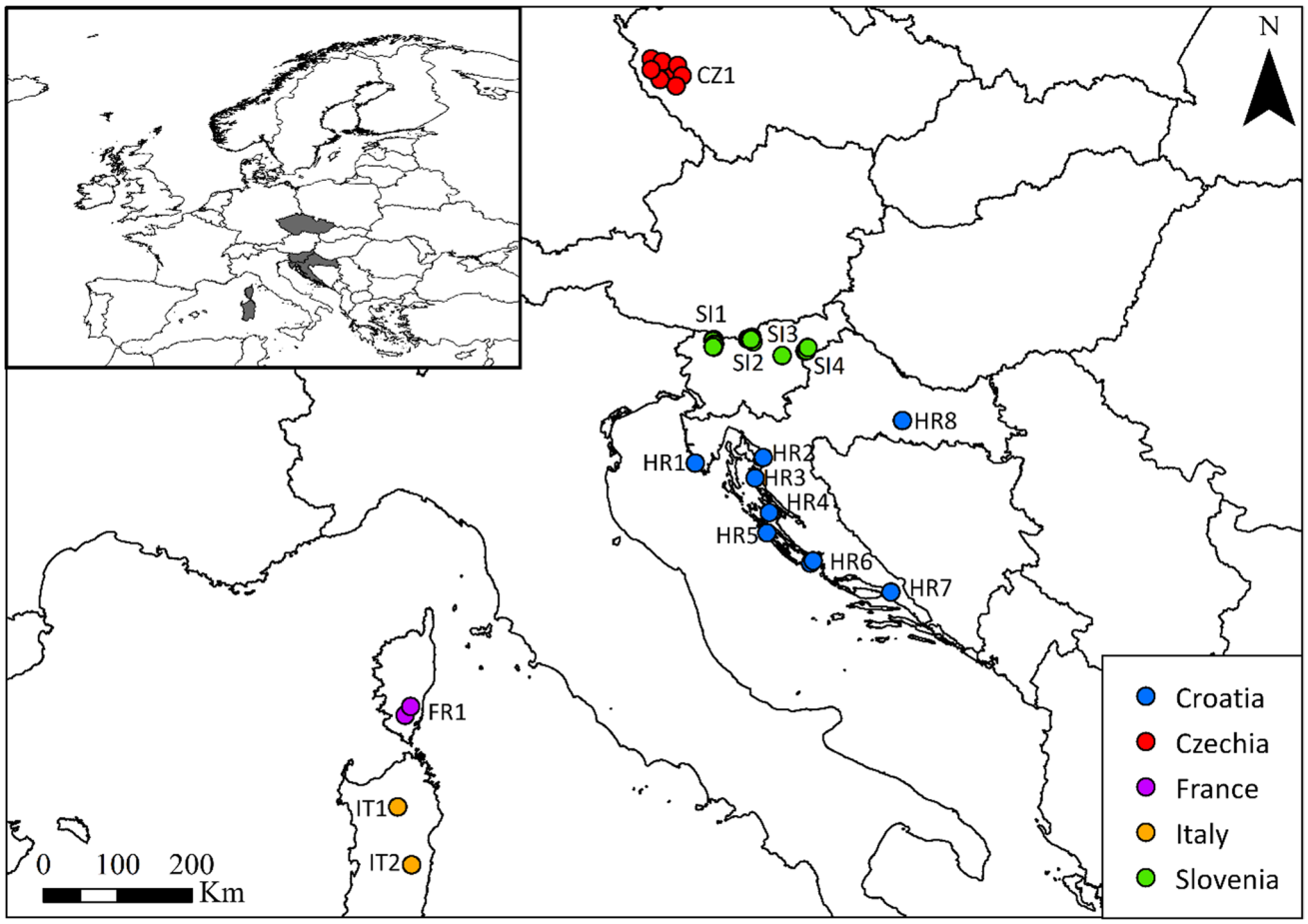

Fig. 1 Study areas with abbreviations; see Supplementary material, Table S1 for details on the studied individuals and names of the localities

(*Ovis aries*; Ghana), AY884007 (*Ovis aries*; USA); Supplementary material, Table S4).

DNA extraction and quality control

Blood and tissue samples were extracted with the Qiagen QIAamp® DNA kit, following the manufacturer's instructions (Qiagen, Germany). DNA concentration was measured with Qubit 3.0, using Qubit dsDNA BR Assay Kit (Thermofisher Scientific, USA).

Mitochondrial control region

We amplified fragments of the mitochondrial control region with primers CR1F (CCCACTATCAACACCCAAAGCT) and CR2R (TCATCTAGGCATTTTCAGTGCCTT), previously published in Sanna et al. (2015). All polymerase chain reactions (PCR) were performed in a total volume of 20 µl (details of PCR protocol are given in Supplementary material, Table S5). Sanger nucleotide sequencing was performed on SeqStudio Genetic Analyzer (Thermofisher Scientific, USA) using BigDye Terminators v3.1 (Applied Biosystems, Foster City, CA, USA). CodonCode Aligner 4.27 (CodonCode Corporation, USA) was used to align the forward and reverse sequences. The resulting consensus sequences were aligned using ClustalW 4.0, implemented in MEGA 11 (Tamura et al. 2021).

Genetic diversity was estimated with the following parameters: (i) haplotype diversity with standard deviation ($Hd \pm SD$), (ii) nucleotide diversity ($\pi \pm SD$), (iii) number of haplotypes summarised for each sampling location along with explicit number of unique haplotypes (h), and (iv) number of polymorphic sites (P). All parameters were assessed with the program DnaSP v.6.12 (Rozas et al. 2017).

The relationship among haplotypes was evaluated by constructing median-joining haplotype network (MJN; Bandelt et al. 1999) using PopART (Leigh and Bryant 2015) and ArcGIS 10.4 (ESRI, Redlands, CA, USA) (Fig. 2 and Fig S1).

Genetic differentiation among mouflon populations was tested by analysis of molecular variance (AMOVA), aiming to assess the extent of the genetic structure between populations. Molecular variance was analysed at two hierarchical levels: within and between countries (defined as groups,

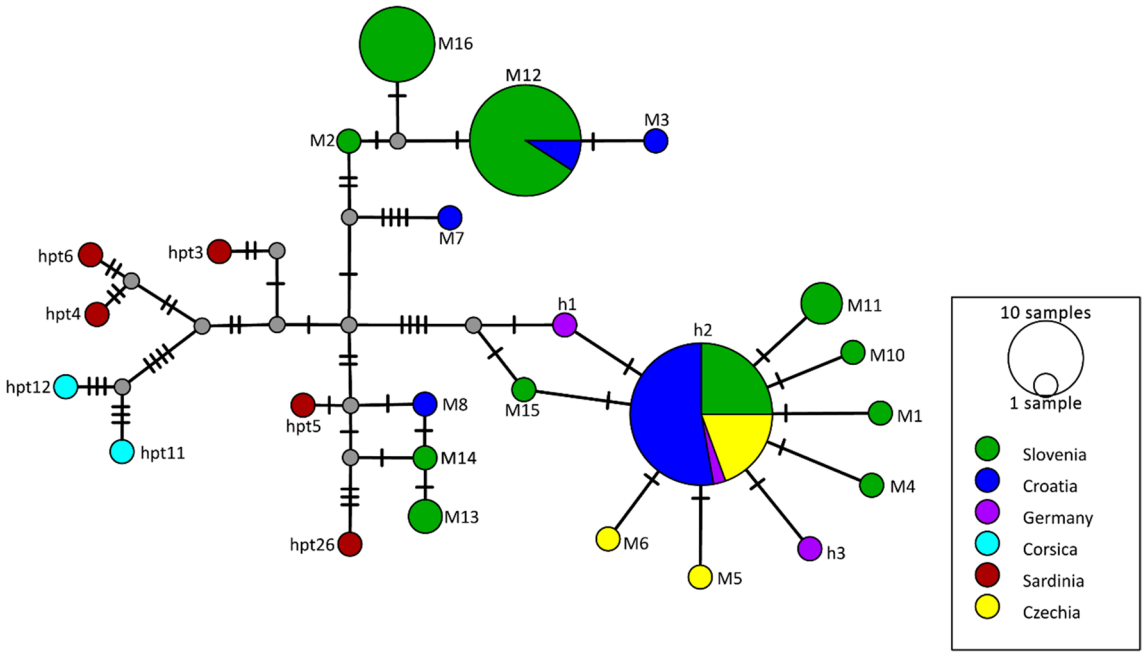

Fig. 2 Median-joining network of mtDNA CR haplotypes of studied European mouflon populations (Supplementary material, Table S1 and S2). The number of mutations between haplotypes is indicated by perpendicular lines. The size of the circles is proportional to the frequency of the haplotype, while colours identify the geographical origin of the sample

where Italy is restricted to Sardinia and France to Corsica). We categorised groups by country, i.e. under the assumption that population management, which contributes to population genetic differentiation, is tailored to each country specific conservation strategy. Non-parametric permutation procedures (Excoffier et al.1992) were performed to test the significance of genetic structuring at each covariance component. The comparison between groups was carried out by calculating the pairwise fixation index (pairwise F_{ST} index) with 1000 permutations by measuring genetic differentiation (Reynolds et al. 1983; Slatkin 1995). AMOVA, global F_{ST}, and pairwise F_{ST} assessments were performed with Arlequin version 3.5.2.2 (Excoffier and Lischer 2010).

MHC diversity

We amplified a 249-bp fragment of the exon 2 of the MHC DRB gene using the primers LA31 (5′-GATCCTCTCTCT GCAGCACATTTCCT-3′) and LA32 (5′-TTCGCGTCACCT CGCCGCTG-3′), which were initially designed for cattle (Sigurdardóttir et al. 1991). Primers were uniquely extended to identify individuals. We performed PCR amplification in triplicate in 20-µl reaction mixtures (for details, see Buzan et al. 2022).

The amplicons from the triplicates were pooled and purified with magnetic particles Agencourt® AmPure® (Agencourt Bioscience Corporation, A Beckman Coulter Company, Beverly, MA, USA), following the manufacturer's instructions. Concentrations of pooled and cleaned amplicons were quantified by Qubit 3.0 fluorometry using Qubit dsDNA BR (Broad range) Assay Kit reagents (Thermofisher Scientific, USA). Samples were normalised to 3 ng and combined into a final library, which was again purified with Agencourt® AmPure® magnetic particles. For the separation, sizing, and quantification of dsDNA final library amplicons, we used Agilent DNA High Sensitivity Kit on a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), according to the manufacturer's recommendations. We normalised the library to 100 pM, which was then multiplicated and bound with Ion Sphere particles (ISPs) using the Ion 520 and 530 Kit-OT2 reagent kit. Ion 530 chip on Ion Torrent S5 (Thermofisher Scientific, Waltham, MA, USA) was used for sequencing.

For allele calling, we used the pipeline in the Amplicon Sequence Assignment (AmpliSAS) web tool developed for high-throughput genotyping of duplicated polymorphic genes, such as MHC (Sebastian et al. 2018). Filtering of raw data was performed with AmpliCLEAN by removing reads with a Phred quality score < 20 and filtering of all reads < 250 bp and > 300 bp. AmpliSAS clusters true variants with their potential artefacts based on the platform-specific error rates. We used AmpliSAS's default parameters for Ion Torrent sequencing technology: a substitution error rate of 0.5% and an indel error rate of 1%. An accurate length was required to identify the dominant sequence within a cluster. We did not expect more than two DRB

variants per individual, so we kept the "minimum dominant frequency" clustering threshold at 25%, based on previously published work on mouflon (Portanier et al. 2019). We discarded variants with a frequency < 1% within an amplicon. True variants of the DRB exon 2 fragments were aligned and translated into protein sequences to check for evidence of pseudogenes, such as the presence of premature stop codons or indels. A maximum of 200,000 reads per amplicon was used for allele calling, and we repeated the analysis three times for each sample.

The unique sequences were aligned, edited, and confirmed to be *Ovis gmelini musimon* MHC DRB exon 2 alleles by comparing them with alleles downloaded from GenBank (Supplementary material, Table S4) using MEGA 11 (Tamura et al. 2021). DnaSP was used to calculate the average number of nucleotide differences (k) and number of segregating (variable) sites (S). The average pairwise nucleotide distances (Kimura 2-parameter model; K2P) and Poisson-corrected amino acid distances were calculated in MEGA for the following: overall, antigen binding sites (ABS), and non-ABS. The locations of the putative ABS and non-ABS were inferred from the human MHC II molecule structure (Brown 1993). Values for nucleotide diversity (π), 4 Nμθ for autosomal genes of diploid organism theta (θ), Tajima's D based on the site frequency spectrum from DNA sequences, and allele number (A) were calculated using DnaSP. Observed and expected heterozygosity values (Ho and He) and pairwise F_{ST} between groups were calculated and tested with Arlequin. Due to the small number of samples, populations from Sardinia and Corsica were excluded from the estimation of diversity parameters. We tested conformance of the allelic frequencies with Hardy–Weinberg expectations within groups, and overall, by using the complete enumeration algorithm of Louis and Dempster (1987) as implemented in Genepop 4.75 (Raymond 1995). The same software was used to test specific hypotheses of heterozygote excess and deficit, and differences in allele and genotype frequencies between clusters/groups were assessed with Markov chain Monte Carlo approximations of Fisher exact tests (Raymond and Rousset 1995). MHC-based population structure in groups was assessed by analysis of molecular variance (AMOVA) implemented in Arlequin with 10,000 permutations.

Results

Mitochondrial sequence analysis

We successfully amplified 1145 bp long mtDNA CR fragments from 81 out of 98 samples from Slovenia, Croatia, and the Czech Republic (Supplementary material, Table S1). The sequenced mtDNA CR fragments were deposited in GenBank (accession numbers (OR843181 (M1)-OR843196 (M10))).

Mitochondrial genetic diversity

A total of 17 (M1–M16, h2) haplotypes were detected in our samples from Slovenia, Croatia, and the Czech Republic. Combined with haplotypes from Sardinia, Corsica, and Germany downloaded from GenBank, 26 haplotypes were included in the median-joining network of mtDNA CR haplotypes (Supplementary material, Table S1, S2). Haplotypes that have been previously uploaded in GenBank were named according to Meadows et al. (2011), Sanna et al. (2015), and Mereu et al. (2019).

The most common haplotype in the newly analysed populations was h2 (42%) followed by haplotypes M12 (25%) and M16 (12%) (Supplementary material, Table S1). We found 14 new mtDNA CR haplotypes in Slovenia and Croatia. The haplotypes M1, M2, M4, M10, M11, M13, M14, M15, and M16 were present only in Slovenia, haplotypes M3, M7, M8, and M9 only in Croatia, while M12 was shared between Croatia and Slovenia. Two new haplotypes, M5 and M6, were additionally detected in the Czech Republic. The total number of variable sites was 45 and was higher in the Slovenian group ($n = 20$; 44%), than in the Croatian one ($n = 15$; 33%) (Table 1). Interestingly, haplotype M12 and its associated close by haplotypes form a distinct cluster separate from the larger cluster dominated by the most common haplotype, h2. Furthermore, haplotypes M8, M13, and M14 reveal a unique genetic makeup distinct from both h2 and M12 haplotypes, possibly indicative of a secondary introduction from Sardinia. Additionally, the independent cluster centred around haplotype M12 may be linked to an additional introduction from mainland Europe.

The overall haplotype diversity was 0.991 ± 0.013 and nucleotide diversity was 0.008 ± 0.001. The Slovenian group had higher nucleotide ($\pi = 0.007 \pm 0.001$) and haplotype diversity ($Hd = 0.776 \pm 0.039$) compared to the Croatian

Table 1 Genetic variation in the analysed European mouflon populations

	P	h	Hd (SD)	π (SD)	Tajima's D
Slovenia	20	11	0.776 (0.039)	0.007 (0.001)	1.282
Croatia	15	6	0.333 (0.124)	0.002 (0.001)	− 1.234
Czech Republic	6	3	0.417 (0.191)	0.001 (0.000)	− 1.728
Sardinia	18	5	1.000 (0.126)	0.008 (0.001)	*
Corsica	6	2	1.000 (0.500)	0.005 (0.001)	*
Overall	45	21	0.991 (0.013)	0.008 (0.001)	− 0.762

P, number of polymorphic sites; h, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; SD, standard deviation

*Populations from Sardinia and Corsica were excluded due to small sample sizes

group ($\pi = 0.002 \pm 0.0001$ and $Hd = 0.333 \pm 0.124$, respectively) (Table 1).

Population genetic differentiation among studied groups

The median-joining network of mtDNA CR haplotypes (Fig. 2) shows a star-shaped topology. The composition of the clusters separates the original populations from Sardinia and Corsica from the introduced populations (Slovenia, Croatia, Czech Republic, and Germany). The most common haplotype (h2) is shared by mouflon from all introduced populations and is separated by nine and twelve mutation steps from two haplotypes from Sardinia (hpt5 and hpt26, respectively). The M8 haplotype from introduced populations in Slovenia and Croatia is separated only by two mutation steps from the Sardinian hpt5 haplotype, which represents the closest link in the network between the source and introduced haplotypes. The remaining haplotypes from Corsica and Sardinia form a branched, separate network of divergent haplotypes with more than five mutation steps between individual haplotypes.

Most haplotypes from Slovenia, Germany, and the Czech Republic are separated from the central haplotype (h2) by a single mutation step, forming a star-shaped cluster indicative of recent expansion. Exceptions are haplotypes M2, M8, M13, and M14 present in Slovenian and Croatian samples that are closer to Sardinian haplotypes hpt5 and hpt26, as well as haplotypes M3, M7, M12, and M16 present in Slovenian and Croatian samples that are closer to the Sardinian haplotype hpt3.

The extended median-joining network (Supplementary material, Fig. S1) including mouflon from Asia, Anatolia, Cyprus, and the Urals (Demirci et al. 2013; Hiendleder et al. 1998; Meadows et al. 2011; Lv et al. 2015) showed a similar network topology as previously described by Satta et al. (2021), supporting the existing topology between source populations and introduced populations with the star-shaped cluster of haplotypes revealed in our study, with the haplogroup h2 being the most widespread and present both in Asia and Europe.

The global F_{ST} value (0.348) was significant, indicating high genetic differentiation among the groups for the mitochondrial marker studied (Frankham et al. 2002). The pairwise F_{ST} comparisons also showed genetic differentiation between groups, with high and significant values. Genetic differentiation was the highest between the groups from Corsica and the Czech Republic. The only low and not significant F_{ST} value was between the Czech Republic and Croatia (Table 2).

Hierarchical AMOVA showed that the genetic variation among the five considered groups (Croatia, Slovenia, the Czech Republic, Corsica, and Sardinia) can be explained by both differences within (58.2%) and between groups (41.7%) (Table 3).

Table 2 Pairwise F_{ST} values between studied populations of European mouflon

	Slovenia	Croatia	Czech Republic	Corsica
Croatia	**0.349**	/	/	/
Czech Republic	**0.440**	0.022	/	/
Corsica	**0.561**	**0.797**	**0.930**	/
Sardinia	**0.294**	**0.594**	**0.686**	**0.363**

Values in bold are significant ($p < 0.05$)

Table 3 AMOVA parameters of mtDNA CR in the studied European mouflon groups

Source of variation	d.f	SS	Variance components	Percentage of variation	Fixation index
Among groups	4	106.07	1.75	41.76	**0.42**
Within groups	85	207.54	2.44	58.24	

Values in bold are significant ($p < 0.05$)

MHC genetic diversity

We successfully amplified MHC DRB exon 2 from 95 samples from Slovenia, Croatia, and the Czech Republic as well as from two samples from Sardinia and two from Corsica. We also included in the analysis MHC DRB exon 2 alleles obtained from five sequences in GenBank. We found 5 functional alleles for MHC DRB exon 2, encoding different amino acid sequences, all previously described by Herrman et al. (2005) and Portanier et al. (2019). No evidence of multiple locus amplification was found, confirming previous reports for European mouflon and domestic sheep (Herrmann et al. 2005; Portanier et al. 2019). A summary of the allelic variants found in each individual is provided in the Supplementary material (Table S1).

Of the 95 mouflon genotyped, 84 (88%) were homozygous, with 57 (60%) of them homozygous for the most common allele Ovar-DRB1*07012. Interestingly, no homozygous individual was found with the Ovar-DRB1-B allele (Table S1). The two most frequent alleles across all countries were Ovar-DRB1*07012 (with the highest frequency: 58%) and Ovar-DRB1*SA023F3 (23%). The other three alleles had a frequency < 10%. The number of MHC alleles was one in the populations from the Czech Republic and Sardinia, two in Corsica, four in Slovenia, and five in Croatia (Fig. 3). The MHC DRB exon 2 alleles that were present in Sardinia and Corsica were also found in Slovenia, Croatia, and the

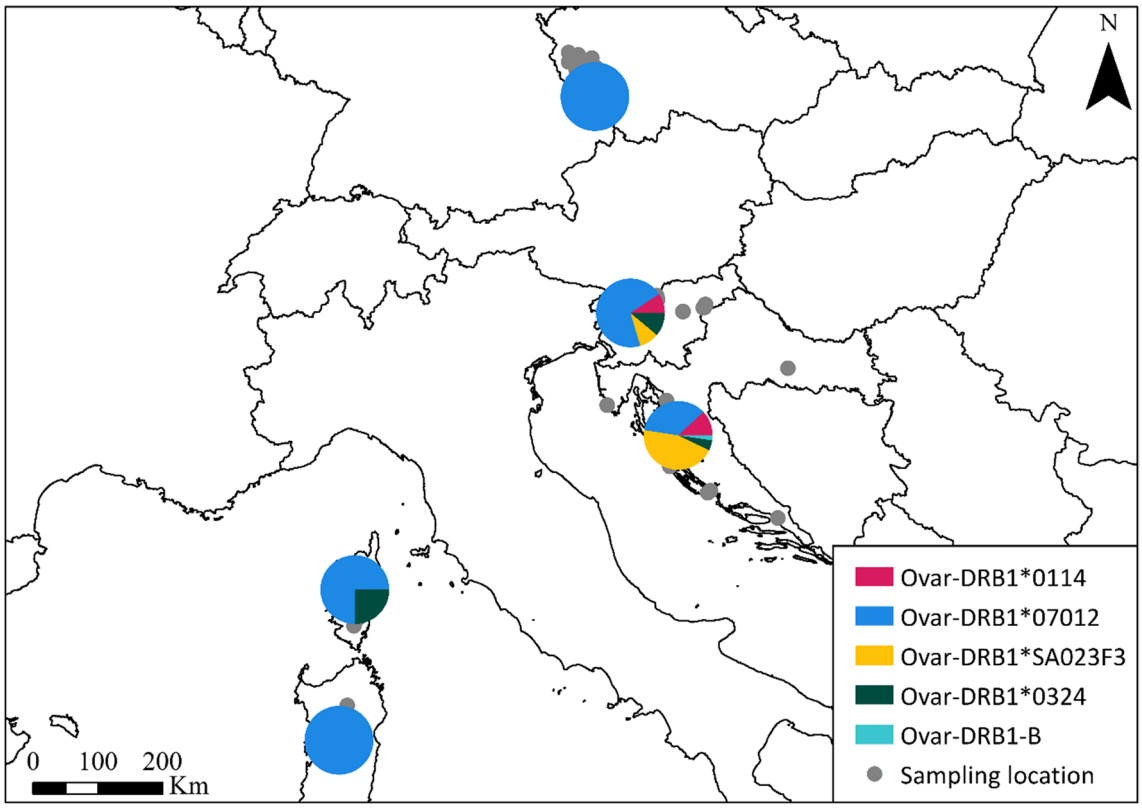

Fig. 3 Sampling locations (grey dots) and DRB1 exon 2 allele frequencies in European mouflon from Slovenia, Croatia, Czech Republic, Sardinia, and Corsica. Pie charts show DRB allele frequencies in each group/country (see Supplementary material, Table S1 for details on the studied individuals)

Czech Republic. We had the opportunity to analyse only two samples from Sardinia and two from Corsica, and we found one allele in Sardinia (Ovar-DRB1*07012) and two alleles in Corsica (Ovar-DRB1*0324 and Ovar-DRB1*07012). These alleles were previously identified in mouflon from National Hunting and Wildlife Reserve in the Caroux-Espinouse Massif, France (Portanieret al. 2019). In introduced populations from continental Europe, we additionally found a third allele (Ovar-DRB*0114) described previously for the mouflon from France (Portanier et al. 2019), the allele Ovar-DRB1-B previously described by Herrmann et al. (2005), and the allele Ovar-DRB1*SA023F3 described by Yaro et al. (2019).

Nucleotide alignment analysis revealed 33 segregating (variable) sites distributed across 83 codons. The overall nucleotide evolutionary distance and amino acid evolutionary distance were 8% and 16%, respectively. The average number of nucleotide differences between alleles was $k = 18.6$ (Table 4).

Table 5 presents values of diversity parameters of MHC DRB exon 2 for mouflon from Slovenia and Croatia (results for the Czech Republic, Sardinia, and Corsica were excluded due to the small sample size). Allelic richness was similar for populations (geographical groups) from Slovenia and Croatia ($Ar = 2.559$ and 2.948, respectively). In both countries, expected heterozygosity ($He = 0.413$ and 0.631) was higher

Table 4 Nucleotide and amino acid diversity of all MHC DRB alleles found in European mouflon

Parameters related to nucleotide differences		Overall nucleotide evolutionary distance			Amino acid evolutionary distance		
k	*S*	All sites	ABS	Non-ABS	All sites	ABS	Non-ABS
18.6	33 (23*)	0.08 (0.01)	0.19 (0.06)	0.06 (0.01)	0.16 (0.03)	0.49 (0.15)	0.10 (0.03)

k, average number of nucleotide differences; *S*, the number of segregating (variable) sites; *ABS*, antigen binding sites; *Non-ABS*, non-antigen binding sites

*SD is given in parenthesis, except for *S*, for which the number of non-synonymous sites is provided

Table 5 MHC DRB exon 2 genetic diversity of European mouflon from Slovenia and Croatia

Geographical group	*n*	*A*	*Ar*	*Ho*	*He*	π (SD)	θ	Tajima's *D*	F_{ST}
Overall	86	5	2.880	0.116	0.572	0.035 (0.005)	0.029	0.636	
Slovenia	49	4	2.559	0.102	0.413	0.035 (0.005)	0.025	1.100	
Croatia	37	5	2.948	0.135	0.631	0.034 (0.005)	0.027	−0.011	**0.210**

Deviation from Hardy–Weinberg equilibrium (not shown) was significant for overall and group data. The bold value is significant

n, number of individuals; *A*, number of alleles; *Ar*, allelic richness; *Ho*, observed heterozygosity; *He*, expected heterozygosity; π, nucleotide diversity; θ, 4 Nμ for autosomal genes of diploid organisms

Table 6 Analysis of molecular variance of MHC alleles in European mouflon from Slovenia, Croatia, the Czech Republic, Sardinia, and Corsica

Source of variation	d.f	SS	Variance components	Percentage of variation	Fixation index
Within groups	177	43.953	0.063	79.8	**0.139**
Among groups	2	6.536	0.248	20.2	**0.020**

Values in bold are significant ($p < 0.05$)

Table 7 Relative rates of non-synonymous (*dN*) and synonymous (*dS*) substitutions (with standard errors), calculated for DRB exon 2 alleles for antigen binding sites (ABS) and non-ABS. Statistical significance was tested using the one-tailed *Z*-test with standard errors resulting from 10,000 bootstrap replicates

Main parameters	Overall	ABS	Non-ABS
N	83	16	67
dS (SE)	0.06 (0.03)	0.00 (0.00)	0.07 (0.03)
dN (SE)	0.09 (0.02)	0.25 (0.08)	0.05 (0.02)
ω (*dN*/*dS*)	1.61	**3.21**	0.05
p value	0.11	0.01	0.96

Significant value is bolded

N, number of codons

than observed (*Ho* = 0.102 and 0.135). Tajima's *D* values were positive for Slovenia and negative for Croatia, but neither was significant. The pairwise F_{ST} values between populations from Slovenia and Croatia were significant and equal to 0.210, indicating genetic differentiation. The average nucleotide diversity was $\pi = 0.035$ and was very similar between the two countries.

Hierarchical AMOVA revealed that most of the MHC DRB exon 2 genetic diversity of mouflon was due to within-group variation (79.8%), i.e. due to variability among different populations/colonies in each country (see Fig. 3), while 20.2% of the MHC variability was due to differences among groups/countries (Table 6).

Non-ABS and ABS sites in MHC DRB alleles

Of the estimated 83 codons, 67 (81%) were for non-antigen binding sites and 16 (19%) were antigen binding sites. The global estimates of ω, averaged across all codon sites, and the codon-based *Z*-test of selection showed the presence of positive selection at the MHC DRB exon 2 locus. The non-synonymous mutation rate (*dN* = 0.09) exceeded the synonymous one (*dS* = 0.06) (Table 7).

Discussion

According to historical records, the mouflon from Sardinia and Corsica were first introduced to Austria in 1731 (Uloth 1972). The next translocation to Northern Italy was in 1780 (Türcke and Tomiczek 1982). The first introduction of significant number of mouflon into the wild in mainland Europe took place in 1868 in the area that is now part of Slovakia (Uloth 1972). Our analysis revealed that mouflon from Croatia, Slovenia, Germany, and the Czech Republic share the most common mtDNA CR haplotype, or have closely related country specific ones. Overall, mouflon from studied populations from these four countries are genetically closely related to haplotypes from Sardinia and Corsica.

A star-like pattern of haplotype network (Fig. 2), in which a very common haplotype, presumably ancestral, is located at the centre of a network and connected to rarer haplotypes by independent mutation steps, is usually regarded as an indication of a population that has recently expanded in size from one or a small number of founders (Avise 2000). Most of the haplotypes from Slovenia and the Czech Republic were closely related and separated by one mutation step from the central haplotype h2 (Fig. 2). The central position of h2 in the network indicates that this haplotype, likely originated from Germany, functions as one of the ancestors of the populations introduced in Slovenia and Croatia, as this haplotype is present in all studied populations in both countries, consistently with the historical record that the mouflon was introduced from Sardinia to Germany and from there to other European areas, including Slovenia and Croatia. Therefore, the results of the mtDNA CR are largely consistent with written historical data indicating Germany

as the source population of mouflon in Slovenia and Croatia (Rotondi 1937; Fabjan 1965). However, our results also suggest the presence of additional sources, since both countries have haplotypes (M8, M14, M13) more closely connected to Sardinia than to Germany, which is probably a consequence of the additional translocation of the mouflon from Sardinia to the Brijuni Islands as a gift from Italian government to the former Yugoslav president Tito (Pers. comm. E. Kolić). Likewise, haplotype M12 and its associated haplotypes discovered in Croatia and Slovenia constitute an independent, distinct cluster. This finding probably suggests the occurrence of possible unrecorded translocations of mouflon in both countries.

In light of the limited number of Sardinian samples included in our study, we assume that all haplotypes found in the Croatian and Slovenian populations during the translocation events also exist within the Sardinian population. However, in order to definitively confirm this assumption, it is imperative to obtain a larger sample set from Sardinia. By acquiring a more extensive sample size, we can ensure a comprehensive representation of the Sardinian population and ascertain the presence of these haplotypes during the translocation events.

To position the mtDNA CR haplotypes within a broader evolutionary context, we performed a network analysis including additional sequences of *Ovis* sp. from Europe and Asia, which shows a separation between Asian and European haplotypes (Supplementary material, Fig. S1). This haplotype network also revealed a "star-like" genealogy for the most common haplotype h2. Haplotype h2 was shown to belong to the most common haplogroup HPG-B which includes some sheep breeds (Satta et al. 2021) and is recurrent in Middle East, Asia, and Europe (Sanna et al. 2015). It is thus not surprising to find downloaded samples from Asia sharing haplotype h2 with our samples. Within the European cluster, populations showed mostly private haplotypes. Nevertheless, our results show the genetic differentiation between Corsico-Sardinian and introduced mouflon, as previously already described (Mereu et al. 2019; Satta et al. 2021).

A high number of unique haplotypes (i.e. singletons) and low nucleotide diversity in populations from Slovenia and Croatia indicate that these populations likely underwent population expansion, probably as a consequence of the post-reintroduction management measures (which include selective/trophy hunting with higher hunting pressure towards males, but also subsequent introductions control) implemented in both countries (Fabjan 1965; Krže 1975; Galjot and De Brea-Šubic 1998; Krapinec et al. 2013; Hafner and Černe 2018; Kavčić et al. 2020).

Overall, haplotype diversity for Slovenian and Croatian populations was high (0.776 and 0.333, respectively) while nucleotide diversity was low (0.007 and 0.002). Such combination of high haplotype and low nucleotide diversity can result from a rapid demographic expansion after a period of low effective population size (Grant and Bowen 1998), such in the case of rapid demographic expansion in novel distribution ranges after founding (Abduriyim et al. 2018; Stipoljev et al. 2021; Šprem et al. 2021). Additionally, higher nucleotide diversity, as also shown in the haplotype network pattern of mouflon in Slovenia, is probably the result of substantial introduction and translocation from different populations compared to Croatia, where most populations were established by individuals from the Brijuni Islands. Interestingly, our recent study (Šprem et al. 2023) also documented a hybridisation between European mouflon and feral sheep on the Croatian island of Dugi otok, where a group of individuals, phenotypically indicative of possible introgression, was photographed and subsequently confirmed as hybrids through SNP array analysis.

Pairwise F_{ST} comparisons were significant and showed a strong degree of differentiation for all groups/countries except between the Czech Republic and Croatia. This lack of differentiation is likely the consequence of the translocation history of mouflon in Croatia, where the population on the Brijuni Islands was founded by a German wildlife trader Carl Hagenbeck from his private zoo (Rotondi 1937). A positive relationship between genetic variation and number of sources is not only known from reintroduced native species (Biebach and Keller 2012) but also from introduced populations of alien species (Ellstrand and Schierenbeck 2000; Kolbe et al. 2004; 2008; Dlugosch and Parker 2008).

Our study confirmed that mouflon populations (both introduced on the continent and historically introduced to Sardinia and Corsica) have low MHC diversity (only five alleles were found in 97 samples), possibly due to a combination of historical founder effects in the original population of Sardinia and Corsica (Chessa et al. 2009; Sanna et al. 2015; Barbato et al. 2017; Mereu et al. 2019) and low effective population sizes of established colonies on Brijuni Islands as well as in Croatian and Slovenian mainland (Fabjan 1965; Krže 1975; Türcke and Tomiczek 1982; Galjot and De Brea-Šubic 1998; Krapinec et al. 2013; Guerrini et al. 2015; Kavčić et al. 2020).

Mutations in the ABS domain are usually associated with differential disease resistance (Langefors et al. 2001; Portanier et al. 2019). In our dataset, we observed much higher evolutionary distances between nucleotides and amino acids in ABS than in non-ABS (19% vs. 6%; 49% vs. 10%), indicating a direct effect on binding properties for antigenic peptides (Jones et al. 2006) as it has been previously already described (Gutierrez-Espeleta et al. 2001; Dicks et al. 2019).

Portanier et al. (2019) found that specific alleles and heterozygosity of MHC exon 2 alleles have effects on faecal egg counts and faecal oocyte counts of gastrointestinal nematodes in female mouflon. This suggests the occurrence

of heterozygote advantage, effects of rare alleles, and/or fluctuating selection in the Caroux-Espinouse mouflon population. On the contrary, no association was detected between genetic diversity and resistance to coccidia parasites, indicating that different parasite classes are impacted by different genetic drivers (Cox 2001). Portanier et al. (2019) also found a marked gradient between the most parasitised MHC DRB genotype (Ovar-DRB1*07012/*07012) and the least parasitised genotype (Ovar-DRB1*0324/*0114), with a statistically significant difference also between Ovar-DRB1*0324/*0324 and Ovar-DRB1*0324/*0114 genotypes, leading to a 57.2% decrease in faecal egg abundance. In their study, the presence of the allele Ovar-DRB*0114 led to a 56% decrease in faecal oocyte of nematodes between individuals carrying or not carrying this allele.

In our sample set, we found Ovar-DRB*0114 allele in only 4 individuals. Overall, we also found only 4 individuals with the advantageous genotype (Ovar-DRB1*0324/*0114). This genotype was present in three mouflon from Slovenia and in just one from Croatia. On the contrary, genotype Ovar-DRB1*07012/*07012 was present in 56 individuals: 36 in Slovenia, 12 in Croatia, 5 in the Czech Republic, two in Sardinia, and one in Corsica. The genotype Ovar-DRB1*0324/*0324 was present only in one individual from Slovenia. In the present study, DRB1*0114 allele was the rarest allele, which could indicate that mouflon populations in Slovenia and Croatia are not specifically adapted to the resistance against gastrointestinal nematodes.

Herrmann-Hoesing et al. (2008) studied the impacts of genetic diversity of MHC DRB alleles on ovine progressive pneumonia virus resistance in domestic sheep. They evidenced that alleles DRB1*0324 and DRB1*0114 were associated with a higher provirus level, while DRB1*07012 allele was associated with a lower susceptibility for this virus. The authors explained that these differences were linked with specific amino acids encoded by the diverse alleles and determining the immune response. Provirus and macro-parasitic strongyles are very different pathogen types, and probably the functional links between genetics and resistance could indeed be expected when considering different parasite classes (Ortego et al. 2007; Ezenwa et al. 2010; Ruiz-López 2020).

There is currently limited monitoring of parasite infections of mouflon in both countries. In Slovenia in the last 10 years, only eight individuals have been examined, revealing that two individuals had lung parasites (*Protostrongylus* and *Dicrocoelium dendriticum*), three individuals had intestinal parasites, two individuals had bacterial infections (*Mannheimia haemolytica*), and one individual had a skin parasite (Pers. comm. G. Vengušt). Although mouflon in Croatia have not been examined for any diseases, information provided by wildlife managers suggests an increase in mortality due to bacterial infections (*M. haemolytica*) transmitted from sheep in the mouflon colony near the town of Senj, in the northern Adriatic (Pers. comm. R. Beck and J. Tomljanović).

Given the established positive relationship between MHC genetic diversity and parasite resistance in mouflon, it is of utmost importance to prioritise research on this aspect. Disease and infections have a substantial impact on the dynamics of mouflon populations and present a crucial challenge in their management.

The DRB locus showed signs of positive selection as relative rates of non-synonymous mutations were higher compared to synonymous (the overall ratio $\varpi = 3.21$), which is similar to that found in other wild ungulates in Europe, for example northern chamois (*Rupicapra rupicapra*) and southern chamois (*Rupicapra pyrenaica*) (Cavallero et al. 2012; Buczek et al. 2016; Pérez-Espona et al. 2019). Positive selection most likely affects only a few codons at a few time points but, due to the small number of alleles, we lack statistical power to test this hypothesis.

Genetic differentiation of the studied mouflon populations at MHC DRB gene revealed past demographic history of populations established by founder effect and expanded in isolation. The pairwise F_{ST} between populations from Slovenia and Croatia was high and significant. Additionally, the hierarchical AMOVA strongly supports differentiation within populations in the same geographic group/country, and also differentiation between Slovenian and Croatian mouflon.

The observed heterozygosity of mouflon in both countries (Slovenia: 0.102; Croatia: 0.135) was much lower compared to expected heterozygosity (Slovenia: 0.413; Croatia: 0.631). Additionally, nucleotide diversity was low (Slovenia: 0.035; Croatia: 0.034) compared to mouflon population in France (0.91) (Portanier et al. 2019). Allelic richness is slightly lower in Slovenia than in Croatia, which may also be related to the past translocation and management, due to probably smaller effective size of mouflon populations in Slovenia.

In conclusion, our study provides evidence for the substantial impact of mouflon translocations on genetic diversity within populations. As a result of founder effects, genetic drift, inbreeding, and subsequent isolation, mouflon populations in Slovenia and Croatia exhibit low levels of both neutral and functional genetic diversity. In light of the connection between functional genetic diversity and parasite resistance, it is imperative for future studies to underscore the pivotal role of population management strategies (including possible reintroductions and translocations of individuals of different genetic origin) in augmenting genetic diversity. Furthermore, there is a need to contribute additional data to support science-based management of mouflon populations. Specifically, when introducing or translocating mouflon in the context of conservation and genetic reinforcement strategies, it may be beneficial to maximise the admixture

of founder or translocated individuals in order to increase parasite resistance through increased genetic diversity (Cahn et al. 2011). To maintain genetic diversity in introduced populations, it appears to be more beneficial to release animals from various sources rather than many animals from a single source. Both mitochondrial DNA and MHC polymorphism can serve in combination as valuable markers for studying translocation pathways and evaluating population susceptibility to various infections and diseases.

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Declarations

Competing interests The authors declare no competing interests.

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