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REINTRODUCTION SHAPES THE GENETIC STRUCTURE OF THE RED DEER (*CERVUS ELAPHUS*) POPULATION IN BELARUS

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Key words

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Abstract

The red deer (*Cervus elaphus*) is considered a valuable and important ungulate species with significant ecological role and high importance as a game species in Europe. Its local population in Belarus had undergone extended periods of decline in the past, followed by multiple reintroduction campaigns and management policy adjustments during the Soviet and post-Soviet periods, which eventually led to a recent spike in estimated population numbers. Along with increasing the numbers, those reintroductions have made the understanding of the structure and origins of the populations for the purpose of proper management and sustainable long-term growth much more complicated. Information on the origin of the reintroduction stock has often been lacking, while control of the red deer population dynamics in Belarus is currently limited to indirect survey of putative population numbers, with no utilization of contemporary genetic analysis. Here we report an estimate and interpretation of the red deer population structure in Belarus based on the analysis of microsatellite genotype data from 118 individuals of the red deer from the most well-known groups across Belarus. These specimens were genotyped using a novel multiplex panel of 14 microsatellite loci with various levels of polymorphism. We describe two red deer subpopulations with overlapping ranges that form the Belarussian metapopulation. We also report estimates of their genetic diversity, gained from the analysis of molecular variance, Bayesian analysis of genetic structure, differentiation indices, genetic bottleneck event analysis, and standard genetic diversity metrics. Based on the geographical distribution of subpopulations, their genetic differentiation and known history of red deer reintroductions in Belarus, we consider that both these subpopulations emerged mostly out of the patterns of animal release during two separate periods of reintroduction. We also suggest appropriate population management adjustments arising from the issue of anthropogenic reintroductions that determine the population structure in this managed species.

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Реінтродукція формує генетичну структуру популяції оленя благородного (*Cervus elaphus*) у Білорусі

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Резюме. На території Європи олень шляхетний відноситься до цінних мисливських видів копитних, який має високе екологічне значення і статус одного з ключових мисливських видів на континенті. При цьому конкретно білоруська популяція оленя шляхетного характеризується складною історією, включаючи декілька випадків критичного зменшення чисельності, скорочення ареалу і подальше відновлення завдяки заходам з реінтродукції виду, що проводилися у радянський і пострадянський періоди. Кампанії щодо відновлення чисельності, боротьби з браконьєрством і модернізація біотехнічних методів підтримування популяції зрештою призвели до швидкого росту чисельності білоруської популяції оленя шляхетного за останні роки. Однак заходи з реінтродукції також значно ускладнили характеристику походження і генетичної структури білоруської популяції оленя шляхетного через нестачу інформації про походження особин і локалізації розселень, тоді як сучасний моніторинг популяційної динаміки оленя шляхетного у Білорусі зводиться до непрямого обліку чисельності передбачуваних популяцій у межах лісництв і мисливських угідь, хоча сучасні методи генетичного аналізу, що здатні надати більш точні відомості про походження особин і популяційну структуру для коректного виділення одиниць управління, дотепер не застосовувалися. У цій роботі представлено результати визначення генетичної структури білоруської популяції оленя шляхетного на основі даних мікросателітного генотипування 188 особин, взятих із більшості відомих популяційних груп на території країни, за допомогою панелі з 14 мікросателітних локусів з різним ступенем поліморфізму. Наведено характеристику двох субпопуляцій, ареали яких перекриваються і яких виявлено в межах білоруської метапопуляції, а також оцінку їхньої взаємної генетичної диференційованості й внутрішнього генетичного різноманіття, одержані в результаті аналізу мікросателітних даних. Запропоновано гіпотезу походження субпопуляцій у результаті двох розділених у часі кампаній випуску тварин, а також заходи господарського управління популяціями відповідно до виявленої генетичної структури.

Ключові слова: олень шляхетний, копитні, мисливські види, генетична структура, реінтродукція, збільшення популяції, мікросателітний аналіз, Білорусь.

Introduction

The Belorussian population of the red deer has a rather complicated history, including multiple declines, artificial reintroductions from various sources, and eventual rapid growth. The species is known to be practically non-existent in Belarus by the 18th century [Romanov 2000], with a series of efforts to restore the local population following soon, aiming to establish suitable deer hunting grounds. This was accomplished through several reintroduction campaigns using various stock [Kozlo 1972; Shakun *et al.* 2021]. The latest campaign was undertaken from 2006 until 2020, and included releases of deer taken from local or foreign stock in hundreds of natural habitat localities across Belarus, with a total of about 3200 released animals, according to the National Plan on the Red deer population management by Shakun and Veligurov. By 2022, most of the regions utilized for reintroduction have shown significant growth of local red deer populations, suggesting a successful reintroduction. However, the reintroduction effort did not include any measures for genetic control and monitoring of the reinforced and new populations, while the data on the stock material used for some of the reintroductions is scarce. The only measure of control over reintroduction effects was limited to indirect census across hunting grounds and preserves [Shakun *et al.* 2021].

Research into ungulate populations across Europe has shown that aside from supporting population growth, reintroductions can have unforeseen consequences and leave populations vulnerable and harder to manage further on [Nussey *et al.* 2006; Dellicour *et al.* 2011; Niedziałkowska *et al.* 2012; Queiros *et al.* 2019]. Founder effect and heterozygocity loss due to the Wahlund effect during relocations pose risk for a loss of genetic diversity and fitness, and, eventually, along with hunting pres-

sure and habitat loss due to anthropogenic effects, to population decline. Another potential issue is the creation of isolated populations incapable of maintaining stable population growth due to limited adaptability in a new habitat [Reed & Frankham 2003; Spielman *et al.* 2004; Frankham 2005]. Additionally, we note that the genetic structure and diversity of the red deer in Belarus has never been studied before.

In this context, a study of genetic structure and diversity in the Belarusian red deer is of significant interest, as we can finally make a thorough genetic description of one of the important and least studied populations of this valuable species on the continent, while also observing the genetic effects of a wide-scale reintroduction campaign right after its conclusion. Problematic population groups requiring additional attention for conservation can be revealed in this process, as we observed the effects of a massive reintroduction campaign on the structure of a model nation-wide ungulate population. All of this would serve the development of a more effective and scientifically rigid approach to population management [Apollonio *et al.* 2017; Ralls *et al.* 2018; Rodger & Clulow 2021].

Our goals in the present study are to reveal and explain the genetic structure in the Belarusian population of red deer, to estimate the genetic diversity within the population, and to provide recommendations to account for the genetic factor in population management of the species. Therefore, we have set out to genotype and analyse the Belorussian deer population using microsatellite markers.

Materials and methods

The studied population

The Belarusian red deer population was estimated to have a size of about 36 000 animals as of late 2021, which is a steady increase from 31 000 in 2020[1]. The population has shown continuous growth since 2006 (Fig. 1), which is due to the reintroduction campaigns of that period, as well as due to the adoption of scientifically based contemporary practices of population conservation and management according to the data on the Environmental protection in the Republic of Belarus in 2021 (by the National Statistical Committe of the Republic of Belarus).

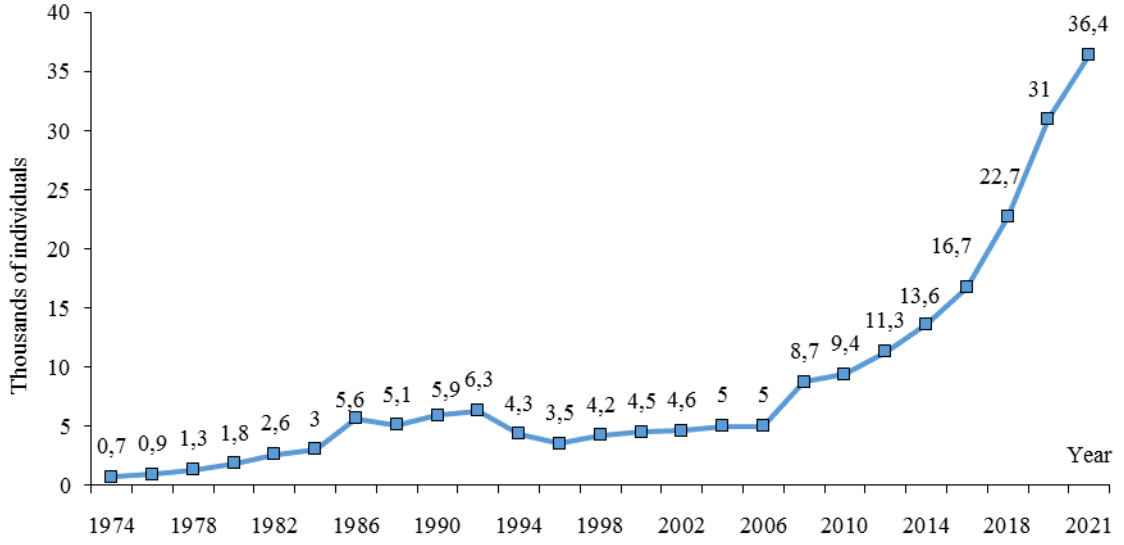

Fig. 1. Dynamics of the red deer population in Belarus in the last decades, according to data from the Ministry of Forestry of the Republic of Belarus.

Рис. 1. Динаміка чисельності оленя шляхетного на території Білорусі за останні десятиліття, відповідно до даних Міністерства лісового господарства Республіки Білорусь.

[1] Reports on the management of the hunting economy for 2020–2022. Ministry of Forestry of the Republic of Belarus, Minsk.

The species in the study area is characterised by emergence of the original population out of haplotype groups A and C, originating from the Iberian and Balkan refugia, respectively [Ludt *et al.* 2004; Niedziałkowska *et al.* 2021, 2011; Skog *et al.* 2009]. The population suffered a drastic decline due to anthropogenic pressure in the 18th and 19th centuries, and then another decline in the first half of the 20th century. Throughout the entire 20th century, the Belarusian population had relatively low abundance (<3000 individuals), distributed across only a part of potential range [Kozlo 1972; Romanov & Kozlo 2002; Shostak 1974; Shostak *et al.* 1974].

Starting from 1955, a reintroduction campaign accomplished release of stock deer from Belovezhskaya Pushcha National Park (further referred to as Belovezha Forest) and Voronezh Preserve into new habitats [Kozlo 1972; Shostak *et al.* 1974]. The campaign was cut short in the 1990s after resettling about 1600 animals due to socioeconomic issues, but reinstigated in 2006. This campaign managed to release about 3200 deer of Belovezha Forest stock, as well as animals from Lithuania, Hungary, and Austria across multiple Belarussian habitats, but without documenting the data on the animal stock thoroughly enough for many specific releases. Cases of Caucasian sika deer (*Cervus nippon*) from the Caucasus and Altai wapiti (*Cervus elaphus maral*) releases are known too [Shakun 2011; Shakun & Veligurov 2018].

The Belarusian part of the red deer range is characterised by minimal presence of major geographical obstacles for the animal's migration, aside from major rivers and national borders, while the range itself is significantly fragmented due to human activity, mainly of dispersed deforestation [Pirozhnik & Martsinkevich 2006]. This makes deer migration between various groups in the range possible, but slow and unlikely on a short timescale, which is the condition that necessitated reintroductions in the first place.

Analysis outline

We based the present study on statistical analysis of genotypes obtained from microsatellite fragment sets, amplified from genomes of a wide sample of red deer, made up from individuals harvested along most of the Belarusian part of its range. Genetic structure was determined from genotypes through Bayesian analysis and AMOVA (Analysis of Molecular Variance). We then incorporated the obtained genetic structure into the same set of genotype data for further analysis of genetic diversity and differentiation of the discovered subpopulations.

Sampling

We have sampled 118 red deer specimens from every administrative region of Belarus, including 22 individuals in the Vitebsk region, 29 in Brest, 16 in Grodno, 25 in Minsk, 20 in Gomel, and 6 in the Mogilev region, which we estimate to be sufficient to reveal the most possible genetic groups using microsatellite analysis [Hale *et al.* 2012]. Figure 2 shows the geographic distribution of genetic samples used in the present study.

Samples were harvested from free-ranging animals killed in legal hunts or culls, and provided for research by cooperating forestry and preserve authorities, at which point they were entered into the Genetic Bank of Wildlife of SSPA 'SPC NAS of Belarus for Bioresources' and stored in cryogenic conditions until further use. For sampling material, we used soft tissues or antlers. The total sample includes both males and females of various estimated ages (from several months old to 8-year-old) collected in 2010 to 2021. The full list of samples is presented in Supplement 1.

The map illustrations were created using QGIS 3.24.2 (by QGIS Development Team) and OpenStreetView map files (based on OpenStreetMap contributors).

DNA extraction

The two types of available tissue material led us to utilizing two methods for DNA extraction: for soft tissue samples we used the Jena Bioscience 'Animal DNA Preparation' commercial kit, following the manufacturer's protocol with slight alterations; and for antler samples, we used a method based on the work by Hoffmann and Griebeler [2013].

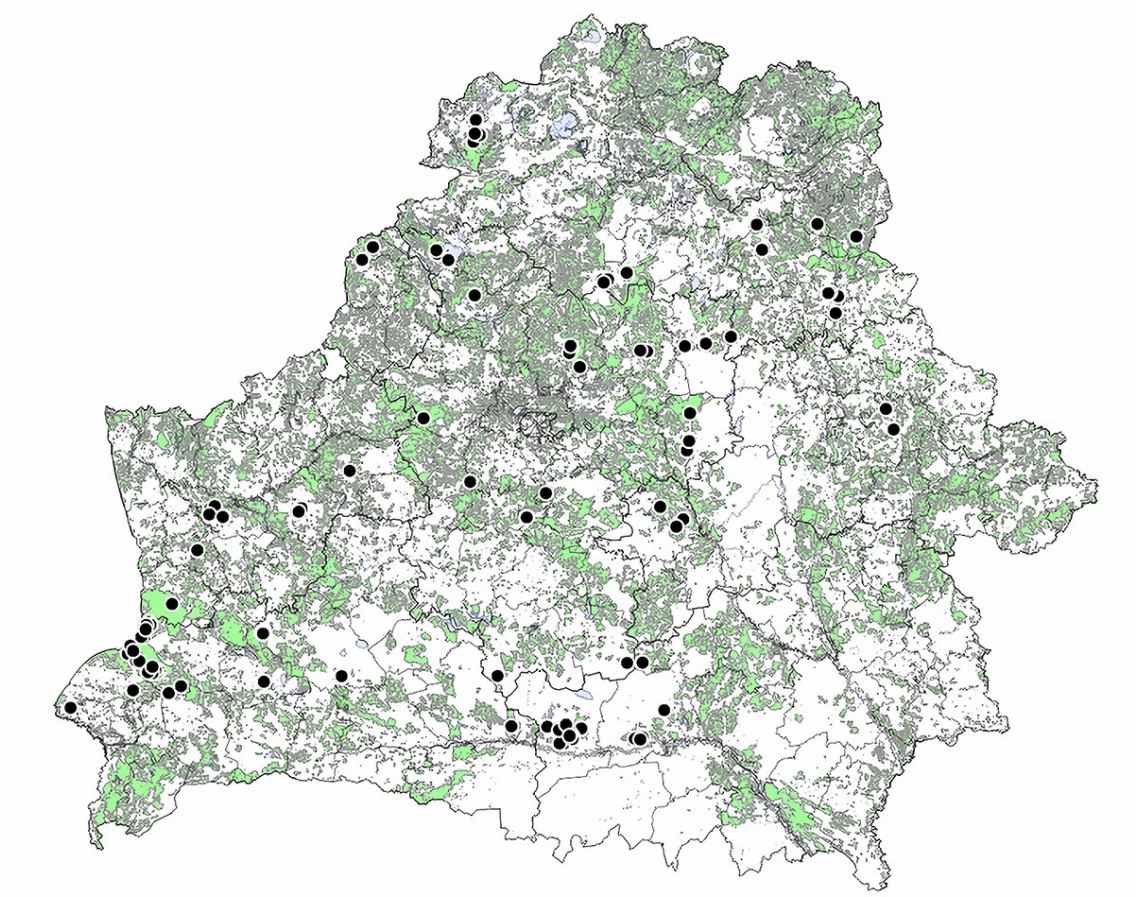

Fig. 2. The map of spatial distribution of genetic samples across the studied area. Black dots represent harvesting locations for individual samples.

Рис. 2. Карта просторового розподілу генетичних зразків оленя шляхетного, використаних у цьому дослідженні. Чорні точки позначають місця збору зразків.

DNA extraction with the Jena Bioscience 'Animal DNA Preparation' commercial kit is based in lysing minced tissue samples (5–10 mgs) with proteinase K at 56°C, with subsequent alkaline protein precipitation, isopropanol nuclear acid extraction, ethanol washing, rehydration and purification from co-purified RNA using RNAse A. We altered the protocol by increasing proteinase K (ArtBio-Tech, Belarus) concentration to 0.5 mg/ml, and increasing the lysis duration to 12 hours.

To extract DNA from antler material, we drilled pieces of antlers into powder, soaked the antler powder in EDTA, lysed the cells with proteinase K, NaCl, and SDS, and extracted the nucleic acids by centrifuging the lysate with ammonium acetate and chloroform, with further ethanol washing and rehydration. We based this method on the work by Hoffman and Griebeler [2013].

For individual extractions, we took 10 mg of powdered antlers, soaked it in 750 µl of 0.5M EDTA solution for 1 hour at 56°C, lysed the soaked mass for 24 hours in 400 µl of lysis solution (50mM Tris-HCl, 25 mM EDTA, 0.1M NaCl, 0.5 mg/ml proteinase K, 2% w/w SDS). We then precipitated DNA with 3.75M ammonium acetate at -20°C, extracted the nucleic acids pellet by centrifuging with 300 µl of pre-chilled chloroform, washed the pellet in 98% and 70% ethanol, and rehydrated the pellet in 200 µl of ddH_2O.

Extractions with either method included negative and positive extraction controls, with further verification via PCR amplification. The obtained DNA solutions were measured for concentration and purity using a 330P Implen nanospectrophotometer and were stored at -20°C until further use.

Table 1. A full list of *Cervus elaphus* microsatellites used in the analysis, the utilized multiplex reactions, STR size ranges, total number of alleles per locus, literature sources for microsatellites, and primer sequences

Таблиця 1. Повний перелік мікросателітних локусів для *Cervus elaphus*, використаних в аналізі, діапазони розмірів алелів, кількість алелів на локус послідовності олігонуклеотидів для ампліфікації та організація по мультиплекс-сумішам

Multiplex mix # and it's T_a	Locus	Size range, b.p.	N all'.	Source	Primers sequences
MP I 57 ^{0} C	Haut14	108–154	19	[Thieven *et al.* 1997]	F CCAGGGAAGATGAAGTGACC R TGACCTTCACTCATGTTATTAA
	T193	159–231	19	[Jones *et al.* 2002]	F AGTCCAAGCCTGCTAAATAA R CTGCTGTTGTCATCATTACC
	BM1818	227–267	16	[Bishop *et al.* 1994]	F AGTGCTTTCAAGGTCCATGC R AGCTGGGAATATAACCAAAGG
MP II 59 ^{0} C	MM12	83–105	11	[Mommens *et al.* 2009]	F CAAGACAGGTGTTTCCAATCT R ATCGACTCTGGGGATGATGT
	T156	130–202	16	[Jones *et al.* 2002]	F TCTTCCTGACCTGTGTCTTG R GATGAATACCCAGTCTTGTCTG
MP III 57 ^{0} C	T268	143–267	11	[Jones et al 2002]	F ATTCCCTTCTCCAGTGTATG R GATGATAACAGCTCAACAGATC
	BM4208	209–255	22	[Bishop *et al.* 1994]	F TCAGTACACTGGCCACCATG R CACTGCATGCTTTTCCAAAC
MP IV 49 ^{0} C	TGLA57	82–128	10	[Bishop *et al.* 1994]	F GCTTTTTAATCAGCTTGCTG R GCTTCCAAAACTTAACAATATGTAT
	TGLA126	105–205	12	[Bishop *et al.* 1994]	F TTGGTCTCTATTCTCTGAATATTCC R CTAATTTAGAATGAGAGAGGCTTCT
	T530	243–361	14	[Jones *et al.* 2002]	F GTCCTCACAGCAGCTCTATG R GCATTCTTTAGAACTCCAACTG
MP V 59 ^{0} C	ETH152	174–239	23	[Steffen *et al.* 2009]	F TACTCGTAGGGCAGGCTGCCTG R GAGACCTCAGGGTTGGTGATCAG
	IOBT965	86–118	8	[Kühn *et al.* 2009]	F GGGGTTGTGGGTAAGCGGAGTT R GATCTAGCGCCAGACAGACGTGTCA
MP VI 55 ^{0} C	INRA35	107–122	-	[Vaiman *et al.* 1994]	F TTGTGCTTTATGACACTATCCG R ATCCTTTGAAGCCTCCACATTC
	T26	312–392	15	[Jones *et al.* 2002]	F GTTCCAATAGACACGCTCAT R TGCCATAGTTTTTCCTACCTT
No mix 65 ^{0} C	Cer14	198–278	18	[DeWoody *et al.* 1995]	F TCTCTTGCGTCTCCTGCATTGAC R GAGACCTCAGGGTTGGTGATCAG

Microsatellite panel

For genotyping, we selected a novel panel of microsatellite (STR) markers [Valnisty 2019] out of all known markers used in earlier research of wild red deer across Europe and North America. In order to avoid the known limitations of pitfalls of microsatellite markers, such as excessive polymorphism bias and uninformative results [Moss *et al.* 2003, Abdul-Muneer 2014; Galinskaya *et al.* 2019; Reiner *et al.* 2019], we included markers characterised by various levels of polymorphism into the panel. The selected markers were bundled into sets for multiplex reaction using Multiplex Manager v1.2 software [Holleley & Geerts 2009] according to their annealing temperature, lack of undesirable mutual primer interactions such as dimerization, and minimal size range overlap. To do so, we obtained these characteristics from original published sources and research utilizing those markers, and refined it with additional empirical testing and necessary adjustments.

For ensuing automated fragment analysis, the forward-facing primers used for every locus were marked with a By5 (646 nm absorption maximum, 662 nm emission maximum) at the 5' end. The panel and characteristics of included STRs are given in Table 1.

STR amplification and fragment size measurement

To amplify the chosen microsatellite loci, we bundled them into multiplex reaction mixes [Henegariu *et al.* 1997] according to Table 1. Each multiplex reaction was carried out in a 30 µl volume. Reactions contained 10X ammonia sulphate PCR buffer (Primetech, Belarus) diluted to a 0.9X final concentration, 0.2 mM of unmodified dNTPs, 2–2.5 mM of $MgCl_2$, 1.5 mg/ml of BSA, between 0.15 and 0.5 pM of each primer, 1 unit of Taq DNA polymerase (ArtBioTech, Belarus), 5-20 ng of sample DNA and PCR-grade water to adjust volume [Lorenz, 2012].

Table 2. Protocol of PCR amplification for microsatellite multiplexes. Protocol stages follow from top to bottom. T—stage target sample temperature; N—the number of cyclic repeats for a stage. T_a corresponds to MP annealing temperature according to Table 1

T, °C	Time, sec.	N
95	180	1X
95	45	
$T_a - (0{,}5 \times N_i)$	45	10X
72	90	
95	45	
T_a	45	30X
72	90	
72	900	1X

Таблиця 2. Протокол ПЦР-ампліфікації мікросателітних локусів. Етапи слідують згори донизу. T — температура зразка протягом етапу; N — число циклічних повторів етапу. Величина T_a відповідає таблиці 1 за локусами.

$MgCl_2$ and primer concentrations varied between specific primers and reactions in order to achieve balanced product concentrations. Amplification itself followed a touchdown-type PCR protocol [Korbie & Mattick, 2008], specified in Table 2. In order to ensure minimal contamination risk, all PCR-related work was done using sterile DNA-free plastic consumables and reagents, in a laminar flow cabinet, in a dedicated PCR-prep room, spatially separated from sample processing and post-PCR workflows.

To control the PCR quality, we utilized positive and negative controls when setting up reactions, as well as PCR product separation via 1% agarose gel electrophoresis, with subsequent visualization through UV gel-documentation. We used a 20 cm phoresis chamber with sodium borate buffer, the 100+ bp DNA Ladder size reference marker (Evrogen, Russian Federation) and a Gel Doc™ XR+ system (Bio-rad, US).

Verified amplicons were used to measure fragment sizes through automated linear polyacrylamide electrophoretic separation and laser detection using a Beckman Coulter GeXP genotyping system. We used a cubic fragment migration model without dye mobility correction and DNA size standard 400-reference marker for modelling fluorescence data. We then interpreted fluorescence data manually, with two researchers working independently for mutual cross-validation.

Ten percent of randomly selected samples were re-analysed starting from DNA extraction to fragment size data in order to confirm solid reproducibility of fragment sizes.

Genotype data analysis

We subjected the collected fragment size genotype data to binning with TANDEM v1.09 software [Matschiner & Salzburger 2009]. Binned data was checked for genotyping errors using MicroChecker v2.2.3 software [van Oosterhout *et al.* 2004].

We conducted an analysis of molecular variance (AMOVA) in Arlequin v3.5 [Excoffier & Lischer 2010] using 5000 Markov Chain Monte-Carlo (MCMC) iteration and four grouping models for individuals: administrative regions (oblasts), putative populations, cardinal directions (North / South / East / West), and groups shown by Bayesian analysis clustering. The resulting values of percentage variations among populations were used to test hypotheses concerning the corresponding structuring model. We also used this set of models for an exact test of population differentiation, conducted in Arlequin v3.5 as well.

Bayesian analysis of population structure and individual assignment was conducted in STRUCTURE v2.4.3 [Pritchard *et al.* 2000; Falush *et al.* 2003]. We ran simulations for prior cluster values (K) from 1 to 10, with 10 simulations for every K value, each simulation utilizing 50 000 MCMC iterations after a 10 000 iterations burn-in period. Simulation followed the settings for admixture model with inferred Dirichlet parameter (prior value = 1.0) and variable α across subpopulations, as well as correlated allele frequencies, without using harvesting locations as prior (no LOCPRIOR). We chose the admixture model and no LOCPRIOR due to certain knowledge of massive reintroduction events in the recent past of the studied red deer population, as well as the species'

rare but still substantial migratory mobility. After obtaining the simulation data, we determined the most probable number of clusters using the Evanno method [Evanno *et al.* 2005] as implemented in STRUCTURE Harvester v0.6.94 [Earl & vonHoldt 2012], using 10 simulations for every K value.

The obtained K value probabilities were analysed, and individual assignment probability was drawn from simulation summary for the most probable K, using assignment probability value Q, summed from the same 10 simulations for the given K using CLUMPACK [Kopelman *et al.* 2015]. Individuals with Q>0.6 according to the sum of simulations were assigned to the corresponding cluster. Additionally, the same genotype dataset was later re-analysed using the same model settings save for 10-times increase in MCMC iterations, and also used in another re-analysis following a no admixture model and uncorrelated allele frequencies. The additional analyses have shown results for K values and individual assignment effectively identical to the original analysis, and therefore will not be discussed in more detail.

Then we used the STR genotype data supplemented with population assignment information for individuals to determine the compliance of outlined subpopulations with the Hardy–Weinberg equilibrium (HWE), as well as measure their genetic diversity values and differentiation. We measured the expected (H_e) and observed (H_o) heterozigosity, allelic richness (Ar) and Wright's inbreeding coefficient (F_{is}) for diversity values. For population differentiation, we measured F_{st} [Holsinger & Weir 2009], D_{st}, G_{st} [Nei, 1978], G_{st}' [Hedrick 2005], and D_{jost} [Jost 2008]. Both diversity coefficients and differentiation index values were calculated in diversity ver. 1.9.90 [Keenan *et al.* 2013] for R Statistical Software ver. 4.1.1 (by R Core Team).

For differentiation indices, we used estimated values with 1000 iteration bootstraps to obtain confidence intervals. We jointly used multiple differentiation indices due to known limitations and pitfalls specific to every singular metric [Ryman & Leimar 2009; Meirmans & Hedrick 2011], as simultaneous evaluation of several indices in the context of other data can give us more accurate conclusions. Evaluation of the indices was carried out using relative range classification based on earlier research of the same species, establishing further classes: no differentiation ($X< 0.1$); weak differentiation ($0.1<X<0.5$); and strongly pronounced differentiation ($X\geq0.5$). For F_{st}, we considered any $X>0.15$ to indicate strong differentiation.

To determine the presence of population declines in the past by measuring genetic bottleneck effects, we used BOTTLENECK v1.2.02 [Piry *et al.* 1999] with settings for a two-phase model (TPM) with a 95% stepwise-mutation share in the TPM and 12% variance, and two-tailed Wilcoxon test. To visualise the hypothesised genetic structure we conducted a factor correspondence analysis (FCA) in Genetix v4.05.2 [Belkhir *et al.* 2004] using the same genotype and population assignment dataset, with graphic representation of FCA analysis in PAST v4.03 [Hammer *et al.* 2001].

Results

Locus INRA 35 was excluded from the analysis due to excessive genotyping errors. An analysis re-run with 10% of samples has shown full reproducibility of data with an error margin of 0.4 bp.

Genotyping quality test in Micro-Checker v2.2.3 did not indicate a high probability of genotyping errors or null alleles for any of the remaining 14 loci. Fragment size binning TANDEM v1.09 has shown a rounding error of < 0.5 b.p. for 10 out of 14 utilized STR loci. All the microsatellite loci in the analysis displayed polymorphism within the analysis sample, with a range of 8 to 23 alleles per locus.

AMOVA results have shown the highest variation percentage (11.4%) for grouping based on Bayesian analysis outcomes, against administrative regions (4.62%), putative populations (7.24%), and cardinal directions (9.85%). At the same time, AMOVA indicated the absence of significant differentiation between subpopulations for every model of genetic structure. Exact test of population differentiation presented the same outcome. The outcomes of Bayesian analysis of genetic clustering in STRUCTURE (Fig. 3) have indicated the presence of two strongly pronounced genetic clusters ($K = 2$) within the studied sample as the most probable model of genetic structure, according to the Evanno method, with $\Delta K = 33.186$ (Fig. 4).

Fig. 3. Individual assignment probability graph for the studied red deer sample according to a CLUMPACK summary of 10 Bayesian simulations in STRUCTURE for K = 2. Each vertical column (n = 118) represents an individual specimen. Colouration represents median probability of an individual's assignment to Group A (blue) or B (orange).

Рис. 3. Графік ймовірностей індивідуальної приналежності особин оленя шляхетного до генетичних кластерів за об'єднаними даними 10-ти Баєсових симуляцій STRUCTURE при K=2 у CLUMPACK. Кожен вертикальний стовпчик позначає окрему особину (n = 118). Кольорова заливка позначає медіанну вірогідність приналежності особини до групи A (синій) або B (оранжевий).

We also considered the K = 3 and K = 4 hypotheses, but they were rejected due to the lack of sensible correlations with spatial or biological factors, as well as the Evanno method outcomes. We presume that additional clusters represent differentiation within various genetic lines rather than population structure.

Considering both Bayesian analysis of genetic structure and AMOVA results, we conclude that the studied sample includes two main genetic clusters, corresponding to two subpopulations in the Belarusian red deer population. We labelled these clusters as Group A (n = 89) and Group B (n = 30). The genotype data was organised accordingly for further analysis. Visualisation of the two genetic clusters by plotted factorial correspondence analysis (Fig. 5) shows tighter genetic clustering for Group A. Group B appears to show greater genetic distances between its members.

Estimates of genetic diversity in the outlined subpopulations indicate a deficit of heterozygocity for most loci (Table 4). Both groups do not seem to be close to a Hardy–Weinberg equilibrium (p<0.01) and show an increased level of inbreeding. Group A and Group B show a very similar level of genetic diversity, despite the differences in sample sizes (89 and 30, respectively).

Estimates of genetic differentiation (Tables 5–6) suggest a weak, but sufficiently pronounced (0.1 < X < 0.5) differentiation between the two outlined groups according to most of the utilized indices. Testing for population decline in the past using BOTTLENECK has shown a likely bottleneck event for Group A according to the two-wailed Wilcoxon test (p = 0.00085), but did not provide any solid confirmation for Group B (p = 0.19373).

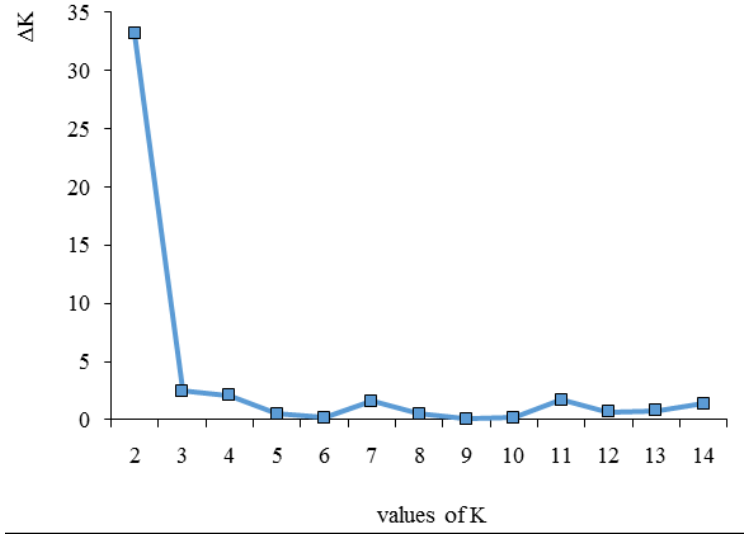

Fig. 4. A graph of ΔK value for increasing values of K for a set of 10 Bayesian simulations of genetic structure in the sampled red deer population.

Рис. 4. Графік зміни величини ΔK залежно від показника K для набору з 10-ти Баєсових симуляцій генетичної структури дослідженої популяції оленя шляхетного.

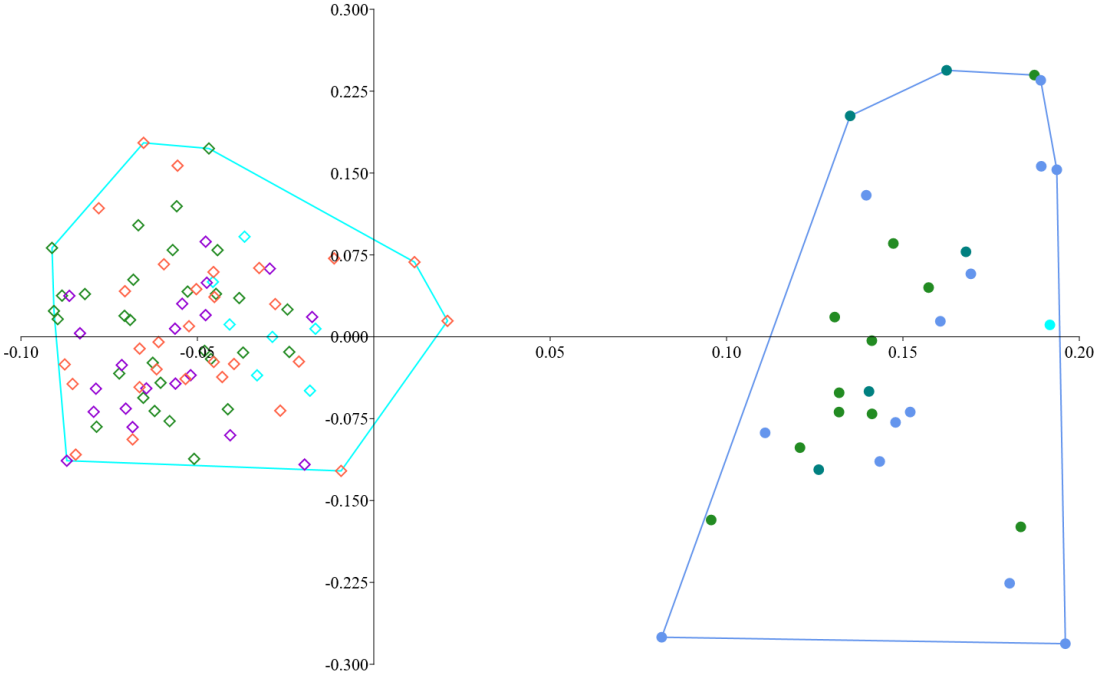

Fig. 5. FCA plot for microsatellite genotypes of the Belarusian red deer sample. Data points represent individual specimens. Point shape represents assignment to Group A (diamond) and Group B (dot). Point colour corresponds to the geographical origin of the sample: green for Minsk, Grodno, and Mogilev oblasts (central regions), red for Brest and Gomel oblasts (southern region), blue for Vitebsk oblast (northern region), purple for Belovezha Forest (south-western region), turquoise for Berezinsky Bioshpere Reserve (north-central region), and teal for Braslaw Lakes National Park.

Рис. 5. Графік з результатами факторного аналізу мікросателітних даних для генетичних кластерів. Окремі зразки позначені фігурами. Форма фігури позначає приналежність особини до Групи А (ромб) або Групи Б (точка). Колір позначає походження зразка: зелений — Мінська, Гродненська та Могильовська області (центральний регіон); червоний — Брестська та Гомельська області (південний регіон); синій — Вітебська область (північний регіон); фіолетовий — НП «Біловезька Пуща (південно-західний регіон); бірюзовий — Березинський біосферний заповідник (північно-центральний регіон); синьо-зелений — НП «Браславські озера».

Discussion

Genetic structure

The genetic clustering data we acquired shows that the red deer population structure has a weak correlation with putative populations or management units in Belarus. The significant correlation we discovered is the one with the geography of the recent reintroduction campaigns. When we tested the harvest locations for individuals belonging to Group A against Belovezha Forest area or areas that saw releases of red deer from Belovezha Forest stock after 1989, we saw a strong correlation ($p < 0.0001$ according to Fisher's exact test). A similar test could not be conducted for Group B and earlier releases due to the limited information on the exact locations of those releases.

We interpret these results as an indication of how reintroductions shaped the population structure of the red deer in Belarus stronger than any other factor. Animal releases did not augment the existing population groups of red deer, but instead formed new population groups that reflect reintroduction patterns [Wilson *et al.* 2006]. Similar situations were already reported for several red deer populations that have been recovered through reintroduction efforts [Niedziałkowska *et al.* 2021; Nussey *et al.* 2006; Pérez-González *et al.* 2010; Queiros *et al.* 2014].

Table 4. Locus genetic diversity characteristics for Group A and Group B: A—the number of detected alleles; Ar—the allelic richness coefficient; H_o—observed heterozygocity; H_e—expected heterozygocity; HWE—the *p*-value for a χ^2-test of the group's fitness for Hardy–Weinberg equilibrium; F_{is}—Wright's inbreeding coefficient. *P*-values >0.05 are given in bold

Таблиця 4. Параметри генетичного різноманіття у виділених популяційних групах за даними мікросателітного аналізу для генетичних кластерів, представлені за локусами: A — число встановлених алелів на локус; Ar — показник алельного багатства; H_o — спостережена гетерозиготність; H_e — очікувана гетерозиготність; HWE — величина *p* для χ^2-тесту на відповідність груп рівновазі за Харді–Вайнбергом; F_{is} — коефіцієнт інбридингу за Райтом. Жирним виділено величини p < 0,05

Locus	Group A						Group B					
	A	Ar	Ho	He	HWE	F_{is}	A	Ar	Ho	He	HWE	F_{is}
Haut14	16	11.52	0.74	0.87	0.00	0.14	19	15.30	0.80	0.90	0.00	0.11
T193	18	14.16	0.71	0.91	0.00	0.21	13	11.77	0.87	0.89	**0.11**	0.03
BM1818	12	9.61	0.65	0.83	0.00	0.22	11	9.38	0.67	0.84	0.00	0.21
MM12	8	5.34	0.69	0.57	0.00	-0.21	7	6.23	0.57	0.68	**0.74**	0.17
T156	13	7.70	0.76	0.76	0.00	-0.01	12	10.44	0.80	0.86	0.03	0.07
T268	10	8.22	0.70	0.83	**0.10**	0.15	7	6.90	0.55	0.81	0.05	0.32
BM4208	18	13.61	0.74	0.89	0.00	0.17	17	14.75	0.83	0.92	0.04	0.10
IOBT965	5	4.26	0.65	0.59	0.01	-0.09	8	7.03	0.70	0.63	0.00	-0.10
T26	14	10.92	0.75	0.86	0.00	0.13	16	13.52	0.77	0.90	0.01	0.15
Cer14	14	10.49	0.70	0.85	0.00	0.18	12	10.49	0.70	0.85	0.04	0.18
TGLA57	6	4.32	0.08	0.40	0.00	0.80	5	3.79	0.14	0.16	0.00	0.15
TGLA126	9	4.96	0.58	0.61	0.00	0.05	9	7.49	0.82	0.77	0.01	-0.07
T530	13	11.63	0.75	0.87	0.00	0.13	12	10.44	0.61	0.86	0.00	0.30
ETH152	19	8.95	0.22	0.60	0.00	0.64	8	6.02	0.13	0.44	0.00	0.70
Global	175	8.98	0.62	0.74	0.00	0.16	156	9.54	0.64	0.75	0.00	0.15

Table 5. Averages of estimated indices for pairwise differentiation between Group A and Group B. The 'BS average' row contains averages of bootstrap (1000 iterations) values. '-95% CI' and '+95% CI' rows contain upper and lower 95% confidence intervals for bootstraps

Measure	G_{st}	G_{st}'	D_{Jost}	F_{st}
Average	0.057	0.457	0.177	0.106
BS average	0.070	0.482	0.227	0.118
-95% CI	0.061	0.439	0.181	0.099
+95% CI	0.080	0.525	0.276	0.138

Таблиця 5. Усереднені показники попарної генетичної диференціації між групами A і B: одержані середні значення нижніх і верхніх меж 95% довірчих інтервалів і середні величини за результатами бутсрепа (1000 ітерацій)

Table 6. Locus-specific values of estimated pairwise differentiation between Group A and Group B

Locus	D_{st}	G_{st}	G_{st}'	D_{Jost}	F_{st}
Haut14	0.03	0.03	0.49	0.47	0.05
T193	0.00	0.00	0.08	0.08	0.01
BM1818	0.00	0.00	0.03	0.02	0.00
MM12	0.16	0.20	0.88	0.85	0.34
T156	0.06	0.07	0.68	0.65	0.13
T268	0.01	0.01	0.10	0.09	0.02
BM4208	0.02	0.02	0.53	0.51	0.04
IOBT965	0.00	0.00	0.00	0.00	0.00
T26	0.01	0.02	0.27	0.26	0.03
Cer14	0.03	0.03	0.38	0.36	0.05
TGLA57	0.35	0.55	0.99	0.97	0.67
TGLA126	0.03	0.05	0.26	0.22	0.09
T530	0.01	0.01	0.18	0.17	0.02
ETH152	0.00	0.01	0.02	0.02	0.01

Таблиця 6. Показники попарної генетичної диференціації між групами A і B, вказані за локусами

We propose considering that the contemporary population of red deer in Belarus as consisting of at least two subpopulations, corresponding to the abovementioned Group A and Group B genetic clusters, have spread, respectively, across the central-southern and northern parts of the country with partial overlap (Fig. 6). Out of these two subpopulations, Group A can be described as a product of the reintroduction efforts that took place in 2006–2020.

The origin of the second subpopulation can be surmised from the population differentiation data: all differentiation indices save for G_{st} without the Hedrik correction indicate weak differentiation, appropriate for populations exhibiting significant levels of admixture, or for populations that diverged recently. The G_{st} estimate is likely to be underestimating the actual differentiation due to the sample including a sufficiently high level of diversity, regardless of the actual differentiation level [Ryman & Leimar 2009]. Bayesian analysis and FCA have revealed limited admixture, which seems to indicate recent divergence as a more likely explanation. Neither AMOVA nor exact test of differentiation report any significant differentiation, which is expected in a highly structured population with recent divergence and consequent low pairwise F_{st} values [Balloux & Lugon-Moulin 2002].

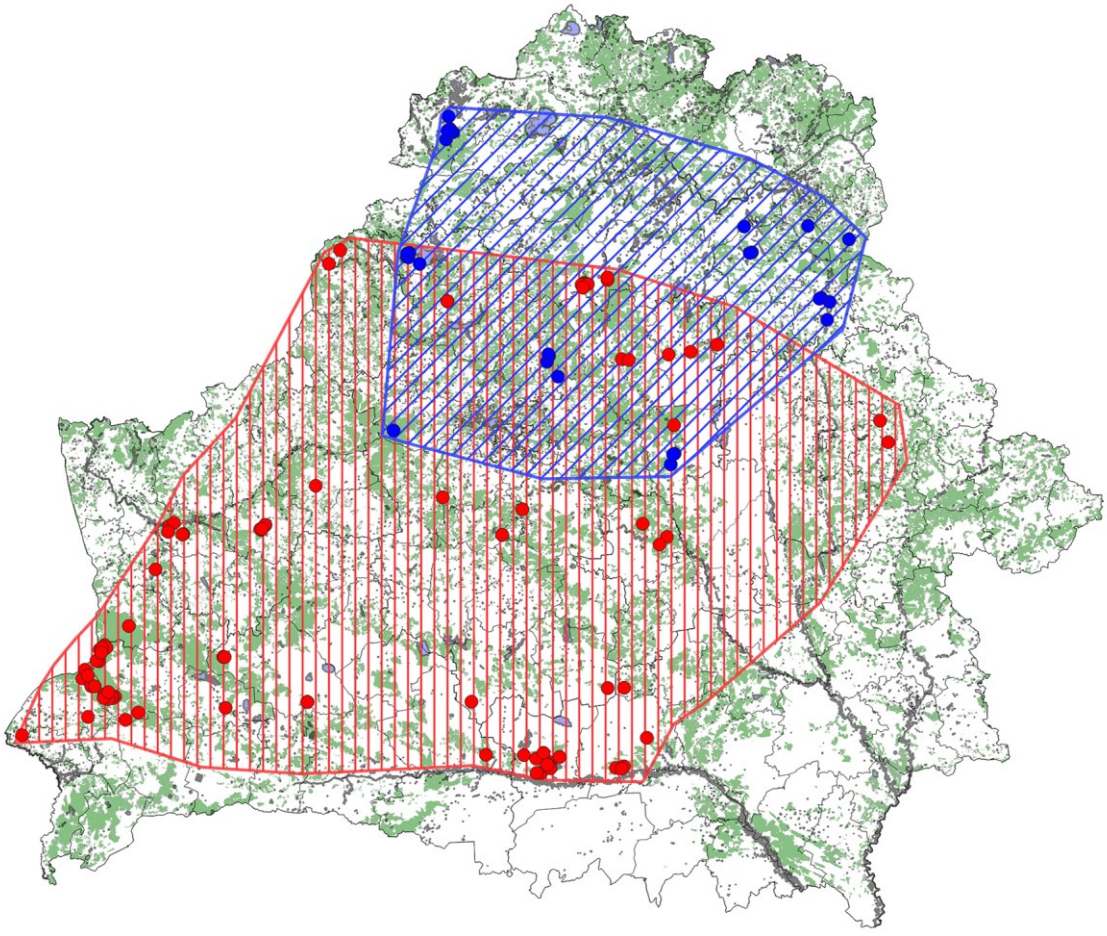

Fig. 6. Geographic distribution of genetic clusters in the Belarusian red deer population across the country. Red dots represent harvesting locations for individuals assigned to Group A; blue dots represent individuals assigned to Group B. Areas filled with respective colour and hatching (vertical for Group A, diagonal for Group B) represent approximate ranges of the respective clusters.

Рис. 6. Карта географічного поширення генетичних кластерів у білоруській популяції оленя шляхетного. Червоні точки позначають місця збору зразків, віднесених до групи А; сині точки — місця збору зразків, віднесених до групи В. Заштриховані відповідним кольором (вертикальними штрихами для групи А та діагональними для групи В) площі позначають приблизну географічну локалізацію кластера.

We interpret these results as indicators of recent divergence between Group A and Group B subpopulations, with earlier resettlement from Belovezha Forest being the point of divergence. Essentially, we argue that Group B is not a regional autochthonous population of any recentness, but rather a similar product of an earlier reintroduction effort using the same Belovezha Forest stock, which took place during the period of 1955–1989 [Kozlo 1972; Shostak *et al.* 1974] and included release of about 2400 animals in various habitats across Belarus.

This conclusion complements the deviation from HWE for both populations, as the latter is explained by the consequences of founder effect caused by reintroduction. BOTTLENECK analysis supports this hypothesis as well, as possible bottleneck event in the past of Group A reflects the population's decline during the period of 1989–1998, while the groups forming the divergent Group B reportedly maintained stable low numbers [Shakun 2011].

An alternative explanation for the revealed population structure would be the effect of cross-border gene flow. This is highly improbable for Group A, as the range of that group is limited by national borders reinforced with strong physical barriers for animal migration. Similar explanation for Group B is made more complicated by the use of Voronezh and Altai stock animals for the 1958–1989 reintroduction efforts, in addition to the Belovezha Forest stock. However, neither the published literature sources nor the known practice of north-eastern forestlands in Belarus do not support the idea of a consistent, significant influx of animals from Russia. The hypothesis is also challenged by the known population dynamics in the region, as well as the red deer's known patterns of cross-border migration, with the animals' strong tendency to phylopatry creating the need for reintroductions in the first place.

There are facts of animals from Lithuania and Austria being released in areas affiliated with both Group A and Group B, although the number of those animals was small [Shakun *et al.* 2021]; the locations of their release are known, and the distinct genetic stock of those animals does not show for those areas. The specified animals either failed to adapt to the new environment after release, were deeply assimilated into the larger population, or were completely missed in the sampling. The latter is a possible explanation for one specific group in the far north of Belarus, with deer population of Verkhnyadzvinsk administrative district having a number of Western European stock animals in its reintroduction stock, while being absent from our current sample, inviting further research. An attempt to distinguish the admixture of dubious stock in the wider population is possible, but would require a significantly wider and more detailed analysis [Frantz *et al.* 2017].

Genetic diversity

Estimates of genetic diversity for the Belarusian deer population do not indicate a dangerous level of inbreeding in either of the outlined subpopulations: the diversity metrics are comparable with many stable and successful populations of Europe [Feulner *et al.* 2004; Dellicour *et al.* 2011; Niedziałkowska *et al.* 2012; Zachos *et al.* 2016]. We suggest that a degree of variety in the stock material could play a positive role for the populations' genetic diversity, as aside from Polish, western Russian, Lithuanian, and Austrian stock, reintroductions have utilized a limited number of Altai wapiti (*Cervus elaphus maral*). Specific conclusions on the extent and effect of this hybridization would require more in-depth research. In any case, reintroduction campaigns played a decisive role in the formation of the observed level of genetic diversity [Krojerová-Prokešová *et al.* 2015].

However, it is hard to predict the long-term effects of significant genetic relatedness for animals sharing one cluster across significant distances as anthropogenic pressure increases along with the population numbers. The continuous stability and growth of the population would depend on adequate management strategy, better structuring of animal removal during hunts, timely measures for habitat preservation and quality veterinarian control [Andersone-Lilley *et al.* 2010; Apollonio *et al.* 2017; d'Aprile *et al.* 2020].

Population management

The described population structure is central to the issue of defining management units within the Belarusian red deer population, as it illustrates that factual subpopulations extend far beyond the

reach and jurisdiction of specific organisations conducting population management at the moment. Continuous growth and stability of the population requires considering the factual population groups in the management strategy. It could be argued that the observed population structure is likely to rapidly fragment into smaller groups due to fragmented environment and the species' philopatry, however, the existing structure itself suggests otherwise—Group B has remained as a singular subpopulation for more than 30 years since the reintroduction campaign that created it. FCA shows a degree of genetic radiation for this group, but significant isolation is likely to take multiple generations and decades of time.

Hybridization and fragmentation of two genetic clusters into several smaller subpopulations is possible, but the process would take too long to build current population management policies around its eventual outcomes.

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