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NEW DATA ON THE GENETIC DIVERSITY OF EUROPEAN BISON (*BISON BONASUS*) IN BELARUS

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New data on the genetic diversity of European bison (*Bison bonasus*) in Belarus. — K. V. Homel, K. Śliwińska, A. A. Valnisty, M. E. Nikiforov. — The paper presents data on the assessment of the genetic diversity of five subpopulations of the European bison (*Bison bonasus*) in Belarus — from the National Park "Belovezhskaya Pushcha", the National Park "Pripyatsky", the Osipovichi district (Mogilev area), SEI "Berezinsky Biosphere Reserve" and the Grodno region. In general, the work includes 30 samples of muscle tissue from the collection of Gene bank of wild fauna in SSPA "SPC NAS of Belarus on Bioresources" (Minsk, Belarus). Microsatellites were used as markers to assess genetic diversity, structure, and search for signs of a sharp decline in the size of bison subpopulations in the past. A total of 11 microsatellite markers were used, recommended by the Food and Agriculture Organization of the United Nations for cattle research. The analysis of *B. bonasus* subpopulation from the NP "Pripyatsky" showed signs of passing through the genetic bottleneck. All studied subpopulations are characterized by a similarly low genetic diversity level in all analyzed indicators (mean number of alleles, allelic diversity, observed and expected heterozygosity). The expected heterozygosity (He) for the three subpopulations from the NP "Belovezhskaya Pushcha", the NP "Pripyatsky" and from the Osipovichi district ranged from 0.37 to 0.39. For the studied subpopulations, the values of the fixation index were negative. The assessment of the presence of genetic structuring between the subpopulations of bison from the NP "Belovezhskaya Pushcha", the NP "Pripyatsky" and from the Osipovichi district based on the values of such indexes as Fst and D_{Jost} which showed no signs of genetic differentiation, which is also confirmed by principal coordinates analysis (PCoA). The European bison conservation in Belarus has required tremendous efforts in the past. So far, even though the impressively large population size reached in Belarus, *B. bonasus* status still should not be considered as stable, which is closely linked to aspects of its overall low genetic diversity. Our research confirmed the low genetic variability of Belarusian subpopulations. Therefore, the more extensive research concentrated on identifying genetic diversity is necessary to ensure the beneficial control of gene flow and register a potential correlation of unfavorable gene variants with possible inbreeding depression. These attempts are required to lay the groundwork for the management and protection of the European bison in Belarus.

Keywords: European bison, *Bison bonasus*, subpopulations, genetic diversity, genetic structuring, Belarus.

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Introduction

The European bison history symbolizes successful efforts to revive an endangered species. In the past, significant efforts have been made to restore this species, which has completely disappeared in its natural habitat. Individuals from zoos in Europe were used for breeding and released into the wild. Despite the success of reintroduction in various regions of Europe, the species remain to be endangered, retaining the status of "vulnerable" in the red list of the International Union for Conservation of Nature (IUCN) (Olech 2007).

According to the pedigree book, by late 2018, there were 7,532 European bison in the world, of which 5,368 are free-living (Raczyński & Bołbot 2019). The current demographic situation with this species seems to be satisfactory, but given the factors associated with genetic diversity, the existence of the European bison in the long term is considered to be under threat. Recently, potential and real health threats for populations associated with genetic variation have been mentioned (Luenser *et al.* 2005; Oleński *et al.* 2020).

In the last few decades, a significant contribution was made to the revival, increase in the number and research of the European bison in Belarus. Currently (2019 data), there are 1,937 individuals

of *B. bonasus* in the country (26 % of the world population), 1,870 of which are forming a free-living population (35 % of the world population) concentrated in 9 subpopulations (Shakun 2019). For comparison, in Poland, there were 1,820 individuals (2018 data), including 1,613 individuals in 6 free-living subpopulations (Shakun 2019).

The main threat to the European bison is low genetic variability. Due to the small number of founding specimens, the species suffers from a limited gene pool with a high degree of relatedness. Due to the founder's individuals geographical origins, two lines were restored: a Lowland and a Lowland-Caucasian. The Lowland-Caucasian line level of inbreeding is 26 %, the Lowland line — 44 % (Pucek *et al.* 2004). For comparison, inbreeding above 12% affects the inbreeding depression in cattle, resulting in a decrease in fertility and negatively affects young animals' proper development (Sewalem *et al.* 2006).

Despite the noticeably lower level of inbreeding of the Lowland-Caucasian line than the Lowland line, there is evidence of inbreeding depression, which has been especially noticed in females (Kobryńczuk 1985). The level of inbreeding does not affect the survival of young individuals of the Lowland line, while it affects individuals of the Lowland-Caucasian line (Olech 2003).

Low genetic variability caused by the genetic bottleneck effect and subsequent continuous closely related interbreeding has been confirmed in bison by a number of studies. The control region of the D-loop (mtDNA) analysis showed that the Lowland line is represented by three haplotypes, which indicates the origin of all *B. bonasus* from 3 females. Most of the examined individuals represent a single haplotype (Wójcik *et al.* 2009).

Due to the close relationship between bison and cattle, identifying the genetic polymorphism of cattle was a base in several *B. bonasus* research. Gralak et al. (2004) found that the expected average heterozygosity (He) is 0.40 (in the range from 0.13 to 0.53) with an average number of alleles per polymorphic locus of 2.5. Other authors (Luenser *et al.* 2005) showed that the average heterozygosity (He) is 0.5, with an average value of 2.3 alleles per polymorphic locus. The expected heterozygosity of Lowland line ranged from 0.23 to 0.39, depending on the author and method (Gralak *et al.* 2004; Luenser *et al.* 2005; Tokarska *et al.* 2009; Tokarska *et al.* 2015).

Various studies highlight the low level of genetic diversity of the European bison (Hartl & Pucek 1994; Gralak *et al.* 2004; Luenser *et al.* 2005; Radwan *et al.* 2007; Wójcik *et al.* 2009) not only based on the results of the analysis of nuclear DNA (microsatellites) and mitochondrial genes, but also protein variability (Gębczyński & Tomaszewska-Guszkiewicz 1987; Hartl & Pucek 1994), blood types (Sipko *et al.* 1995), genes of the main histocompatibility complex (Udina *et al.* 1994; Radwan *et al.* 2007; Łopieńska *et al.* 2003; Babik *et al.* 2012; Mikailova & Voytyukhovskaya 2014), kappa-casein gene (Sipko *et al.* 1994; Burzynska & Topczewski 2009), panels of single nucleotide polymorphism (Pertoldi *et al.* 2009).

Comparative studies of the Polish and Belarusian populations have revealed significant differences between them. The effective size of the Belarusian population is 23 % higher than the Polish one, which is due to the detection of allelic variants in Belarusian *B. bonasus*, which were never found in the Polish population, but it is present in the Lowland-Caucasian line (Tokarska *et al.* 2015).

It is currently impossible to enrich the general *B. bonasus* gene pool, as there are no unrelated animals. It mostly concerns populations of Lowland origin, where the loss of genetic diversity is expected.

Therefore, the European bison populations require constant genetic monitoring, including the developing management actions to reduce the consequences of inbreeding.

The study aims to assess the genetic variability of previously unstudied subpopulations of bison in Belarus and compare the obtained features with available data for both Belarusian and Polish populations.

Materials and Methods

To assess the genetic diversity of the Belarusian subpopulations, a panel of 11 microsatellite markers were used, which is recommended by FAO (Food and Agriculture Organization of the United Nations) for genetically assessing cattle (Commission ... 2011) (Table 1).

The study included samples of muscle tissue from the collection of Gene bank of wild fauna deposed in SSPA "SPC NAS of Belarus on Bioresources". The European bison tissue samples were collected from animals that died for various reasons (diseases, poaching, culling) between 2006 and 2019. A total of 30 individual samples from 5 regions were used to analyze the genetic diversity of the European bison in Belarus (Fig. 1).

DNA extraction from biological samples was conducted using commercial reagents, Blood, Animal, Plant DNA Preparation Kit (Jena Bioscience, Germany), which included purification on the binding silica membrane.

PCR was performed in a 25 µl reaction mixtures containing 2.5 µl 10X Taq buffer ($(NH_4)_2SO_4$), 2.5 µl 10X dNTPs mix (2 mM of each dNTP), 3 µl $MgCl_2$ (25 mM), 2 µl forward and reverse primers (5 pmol/µl), 1 unit of Taq-polymerase, 2 µl of DNA sample and 10.9 µl ddH_2O on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc.). Amplification of target DNA sites was carried out under the following conditions: initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at the specified temperature for each specific microsatellite locus (Table 1), elongation at 72 °C for 45 seconds, and a final elongation at 72 °C for 5 minutes.

Fragment analysis of amplicons was performed using commercial protocols, reagents, and software for the genetic analysis system GenomeLab GeXP (Beckman Coulter, USA). The raw fragment size data was checked by hand independently twice.

Table 1. Microsatellite markers used for Belarusian European bison's subpopulations genotyping

Таблиця 1. Панель мікросателітних маркерів, використаних для оцінки генетичної різноманітності субпопуляцій зубра в Білорусі

№	Locus	Primer sequence (5′–3′)	Annealing temperature, ◦C
1	ETH3 F ETH3 R	GAACCTGCCTCTCCTGCATTGG ACTCTGCCTGTGGCCAAGTAGG	55–65
2	SPS115 F SPS115 R	AAAGTGACACAACAGCTTCTCCAG AACGAGTGTCCTAGTTTGGCTGTG	55–60
3	TGLA227 F TGLA227 R	CGAATTCCAAATCTGTTAATTTGCT ACAGACAGAAACTCAATGAAAGCA	55–56
4	TGLA122 F TGLA122 R	CCCTCCTCCAGGTAAATCAGC AATCACATGGCAAATAAGTACATAC	55–58
5	BM2113 F BM2113 R	GCTGCCTTCTACCAAATACCC CTTCCTGAGAGAAGCAACACC	55–60
6	INRA23 F INRA23 R	GAGTAGAGCTACAAGATAAACTTC TAACTACAGGGTGTTAGATGAACTC	55
7	BM1824 F BM1824 R	GAGCAAGGTGTTTTTCCAATC CATTCTCCAACTGCTTCCTTG	55–60
8	ETH10 F ETH10 R	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCTC	55–65
9	ETH225 F ETH225 R	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	55–65
10	BM1818 F BM1818 R	AGCTGGGAATATAACCAAAGG AGTGCTTTCAAGGTCCATGC	56–60
11	TGLA126 F TGLA126 R	CTAATTTAGAATGAGAGAGGCTTCT TTGGTCTCTATTCTCTGAATATTCC	55–58

Note. F — forward primer, R — reverse primer.

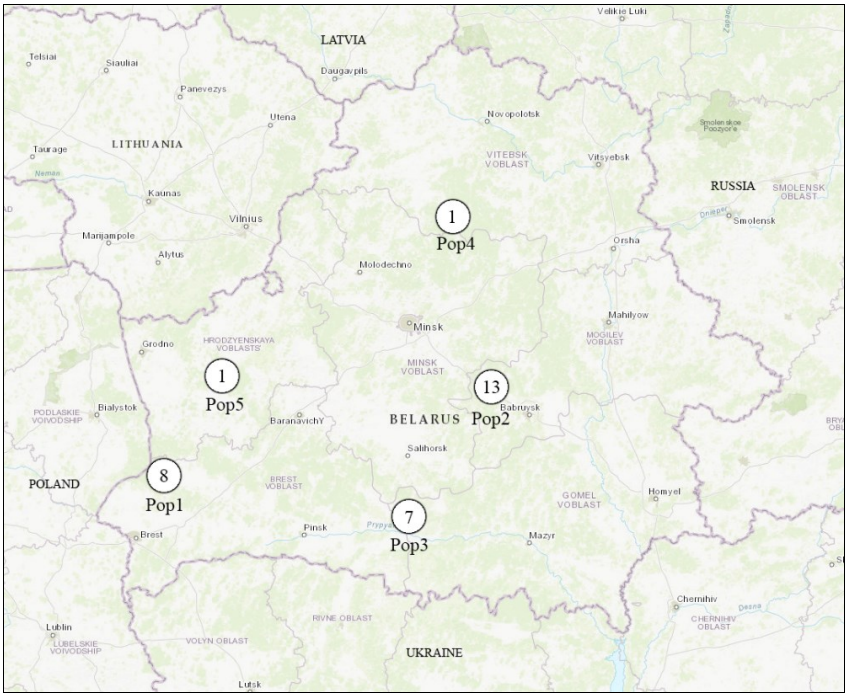

Fig. 1. European bison's subpopulations distribution: Pop1 — NP "Belovezhskaya pushcha", Pop2 — Osipovichi district, Pop3 — NP "Pripyatsky", Pop4 — SEI "Berezinsky Biosphere Reserve", Pop5 — Grodno region; numbers in circle — sample size.

Рис. 1. Розподіл проб зубра, використаних в роботі.

Pop1 — НП «Біловезька пуща», Pop2 — Осиповицький район, Pop3 — НП «Прип'ятський», Pop4 — ДПЗ «Березинський біосферний заповідник», Pop5 — Гродненська обл.; цифри в колі — обсяг вибірки.

The frequency of null alleles for the microsatellite loci under consideration was analyzed in the Genepop version 4.3 (Raymond & Rousset 1995; Rousset 2008). Analysis of the presence of linkage disequilibrium between microsatellite loci was carried out in the Arlequin version 3.5.2.2 (Excoffier *et al.* 2007). Parameters used: 1000 permutations, statistical significance level at $p < 0.05$. Evaluation of the presence of deviations in the frequency of genotypes from the expected ones in accordance with the Hardy-Weinberg equilibrium was also calculated in Arlequin with the following settings: number of steps in Markov chain — 1000 000, number of dememorization steps — 100 000.

To detect a sharp decline in the number of European bison subpopulations in the past, Bottleneck 1.2.02 software was used (Cornuet & Luikart 1996). For the TPM (two phase model) model, the default settings were used, as well as those recommended by the authors — proportion of SMM (stepwise mutational model) in the TPM (%) = 0.000, variance for TPM = 0.36. In addition, the analysis used the I. A. M. (infinite allele model) and S. M. M. models. The reliability of the presence of an excess of heterozygotes in the studied loci was evaluated using three tests — sign test, standardized differences test and Wilcoxon's sign-rank test.

The calculation of genetic diversity indicators was carried out with R software using the package diveRsity v. 1.9.90 (allelic richness (AR)) (Keenan *et al.* 2013) and GenAlEx v. 6.501 (mean number of alleles (Na), observed (Ho) and expected (He) heterozygosity, fixation index (F)) (Peakall & Smouse 2006; Peakall & Smouse 2012). Allelic richness was calculated using 1000 re-samples. Pop4 and Pop5 (SEI "Berezinsky Biosphere Reserve" and Grodno region) populations did not provide any allelic richness and fixation index data due to extremely small sample sizes.

To analyze the genetic differentiation between subpopulations from the NP "Belovezhskaya pushcha", the NP "Pripyatsky" and from the Osipovichi district the D_{Jost} и Fst indices in R were calculated using the diveRsity package. To calculate the 95 % confidence interval (CI) for the specified indices, the bootstrap repeats parameter value was 1000. In addition, to search for signs of genetic structuring of subpopulations, principal coordinates analysis (PCoA) was conducted in GenAlEx. The PCoA calculation was based on a distance matrix with data standardization. Visualization of the result for PCoA was performed in PAST version 4.0 (Hammer *et al.* 2001).

Results of Studies and Discussion

Estimation of the frequency of null alleles for the microsatellite loci used shows their presence for the SPS115 locus in the subpopulations from the Osipovichi district (Pop2) and the NP "Pripyatsky" (Pop3) (Table 2). Singular signs of null alleles were noted for the loci INRA23 (NP "Belovezhskaya Pushcha", Pop1) and BM1818 (Osipovichi district, Pop2). The presence of null alleles for these loci is most likely due to the uneven representation of alleles in specific analyzed subpopulations.

Analysis of the presence of linkage disequilibrium did not show a stable relationship between the microsatellite loci studied, that is for some subpopulations such a relationship was not found at all (Pop1, Pop4, Pop5), and for the other subpopulations (Pop2 and Pop3), the relationship was present, but it changed depending on the subpopulation under consideration. The presence of signs of linkage disequilibrium can be explained by the characteristics of the studied samples — a small size of the studied subpopulations and their low genetic variability. Accordingly, the presence of a true linkage disequilibrium between loci is unlikely, especially given their physical separation (located on different chromosomes) (Commission... 2011). Analysis of the correspondence of the genotype frequency to the Hardy-Weinberg equilibrium showed a deviation for the TGLA227 and ETH225 loci. The presence of loci that deviate from the Hardy-Weinberg equilibrium is not, in most cases, a reason for excluding loci from the analysis (Selkoe & Toonen 2006).

The search for signs of sharp population decline in the past revealed them for the subpopulation from the NP "Pripyatsky" (Pop3) based on the shift of the allele frequency distribution profile (shifted mode) from the L-shaped one. All other tests showed no signs of populations passing through the genetic bottleneck. The found genetic bottleneck features for Pop3 can be explained, among other things, both by features of specific individuals included in the sample, and by the history of the formation of this subpopulation from an initially small number of individuals with low genetic diversity.

All studied subpopulations are characterized by a similarly low level of genetic diversity in all analyzed indices (Table 3). Negative values of the fixation index were identified for *B. bonasus* subpopulations (see: Table 3). Such values are commonly due to an excess of heterozygotes due to negative assortative mating or the heterotic selection of individuals (Peakall & Smouse 2006; Peakall & Smouse 2012). In our case, the presence of negative values of the fixation index can be associated with a low number of alleles for a number of loci, which leads to the appearance of heterozygous genotypes, more often than expected in accordance with the frequency of genotype distribution according to the Hardy-Weinberg equilibrium.

Table 2. Estimated null allele frequencies for microsatellite loci in Belarus European bison's subpopulations

Таблиця 2. Частота нульових алелей для мікросателітних локусів в білоруських субпопуляція зубра

Locus / Population	Pop1 (CI)	Pop2 (CI)	Pop3 (CI)	Pop4 (CI)	Pop5 (CI)
ETH3	0.079 (0.000…0.295)	0.000	0.000	No inf.	0.000
SPS115	No inf. inf.	**0.609** (0.384…0.812)	**0.535** (0.155…0.855)	0.000	No inf.
TGLA227	0.000	0.000	0.000	0.000	0.000
TGLA122	0.000	No inf.	0.000	No inf.	No inf.
BM2113	No inf.	No inf.	No inf.	No inf.	No inf.
INRA23	**0.723** (0.468…0.923)	0.563	0.612	0.000	0.000
BM1824	0.000	0.024 (0.000…0.212)	0.000	0.000	0.000
ETH10	0.000	0.000	0.000	0.000	No inf.
ETH225	0.276	0.213	0.215	No inf.	No inf.
BM1818	0.354	**0.480** (0.226…0.731)	0.378	No inf.	No inf.
TGLA126	0.000	0.000	0.000	No inf.	No inf.

CI — 95 % confidence intervals, **bold** — statistically significant, No inf. (no information) — not enough alleles in the sample. Pop1 — NP "Belovezhskaya pushcha", Pop2 — Osipovichi district, Pop3 — NP "Pripyatsky", Pop4 — SEI "Berezinsky Biosphere Reserve", Pop5 — Grodno region.

Table 3. Summary of the genetic diversity indices at 11 microsatellite loci among the European bison's subpopulations

Таблиця 3. Показники генетичної різноманітності досліджених субпопуляцій зубра за даними поліморфізму 11 мікросателітних локусів

Population	N	Na	AR (95 % CI)	Ho	He	F
Pop1	8	2.364	2.16 (1.8182...2.3636)	0.409	0.374	-0.118
Pop2	13	2.727	2.20 (1.8182...2.5455)	0.413	0.369	-0.096
Pop3	7	2.273	2.12 (1.9091...2.2727)	0.468	0.393	-0.173
Pop4	1	1.455	–	0.455	0.227	–
Pop5	1	1.364	–	0.364	0.182	–

N — sample size, Na — mean number of alleles, AR — allelic richness, Ho — observed heterozygosity, He — expected heterozygosity.

Analysis of the presence of genetic differentiation between subpopulations of the European bison from the NP "Belovezhskaya pushcha", from the Osipovichi district and the NP "Pripyatsky" did not reveal statistically significant differences in D_{Jost} and Fst (Table 4). Similarly, no isolated clusters were observed according to the principal coordinates analysis data (PCoA) for 5 analyzed samples (Fig. 2). The first two axes explained 53.2 % of the total data variability.

According to the literature, three microsatellite loci from the NP "Belovezhskaya Pushcha" were monomorphic (TGLA227, BM2113, INRA023; Gralak *et al.* 2004; Kostyunina *et al.* 2020), two of which (TGLA227, INRA023) were polymorphic for the European bison subpopulations from our work. Since the total sample size in the present study was larger (n = 30) than from the population mentioned above (n = 22), we detected a larger number of alleles. Contrariwise, some studies show that the Belarusian and Polish populations of *B. bonasus* from the NP "Belovezhskaya Pushcha" differ significantly (Mikhailova & Medvedeva 2013; Tokarska *et al.* 2015).

Table 4. Genetic differentiation among the European bison's subpopulations

Таблиця 4. Показники генетичної диференціації між дослідженими субпопуляціями зубра

Populations	D_{Jost} (CI)	Fst (CI)
Pop1 vs Pop2	0.0056 (-0.0092… 0.0308)	0.0685 (-0.0143… 0.1872)
Pop1 vs Pop3	0.0000 (-0.0051… 0.0145)	0.0113 (-0.0431…0.0945)
Pop2 vs Pop3	0.0000 (-0.0053… 0.0203)	0.0207 (-0.0423… 0.1211)

CI — 95 % confidence intervals.

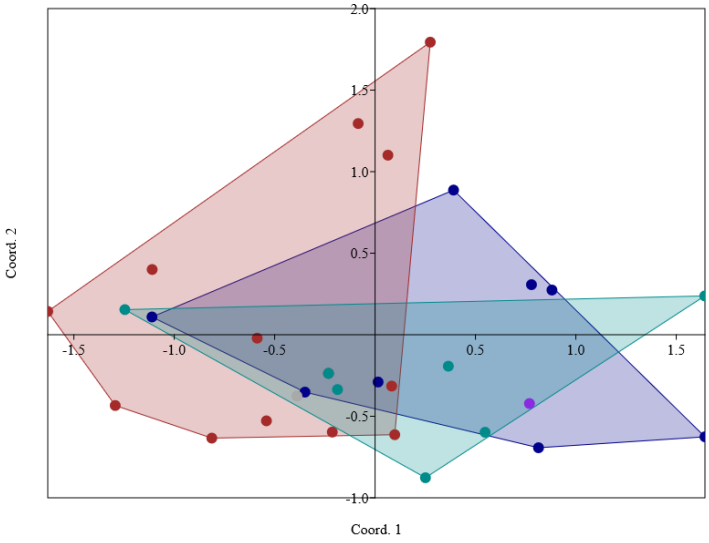

Fig. 2. Principal coordinates analysis of the European bison's subpopulations: Pop1 — blue color, Pop2 — brown color, Pop3 — turquoise color, Pop4 — gray color, Pop5 — purple color.

Рис. 2. Аналіз головних координат для субпопуляцій зубра: Pop1 — синій колір, Pop2 — коричневий колір, Pop3 — бірюзовий колір, Pop4 — сірий колір, Pop5 — фіолетовий колір.

Some alleles are not present in the Polish but exist in Belarusian one, which is explained by the possible presence of alleles of the Lowland-Caucasian line in the Belarusian population of the European bison (Tokarska *et al.* 2015). These alleles can exist in the Belarusian population, as they could come directly from 18 ancestors from the Prioksko-Terrasny breeding center (Bunevich *et al.* 2006), where the introgressive hybridization of two lines probably occurred (Tokarska *et al.* 2015). As a result, in this case, the potential presence of additional alleles in the Belarusian population could explain the increase in the value of observed and expected heterozygosity compared to the purebred line of the Lowland European bison.

Our study results show that the average number of alleles in *B. bonasus* from Belarus is relatively low (Na = 2.18). In total, the European bison have from 2 to 4 microsatellite alleles for different loci and usually, their number does not exceed three (Luenser *et al.* 2005; Tokarska *et al.* 2011; Tokarska *et al.* 2015). As soon as the number of alleles drops to two, the expected heterozygosity does not exceed 0.5. According to microsatellite data, the overall average heterozygosity for the population of the European bison is relatively low (He = 0.31; Tokarska *et al.* 2009). The Belarusian population heterozygosity is slightly higher than the average (He = 0.317), but this still indicates its relatively low genetic variability. The expected heterozygosity for the Belarusian subpopulation from NP "Belovezhskaya Pushcha" for 11 microsatellite loci (10 of which were the same as in the present study) was reported as noticeably lower (He = 0.277; Kostyunina *et al.* 2020) compared to the value of this indicator obtained for the same population in our study (He = 0.374). It may be due to the higher sample size in the previous study than in the current one (n = 42 and n = 8, respectively).

Given the history of the formation of the entire free-living Belarusian population of European bison, it is expected that the indicators of genetic diversity will be low and will not vary significantly in different subpopulations. The overall low genetic differentiation of the European bison populations has been observed before (Burzyńska *et al.* 1999; Luenser *et al.* 2005; Radwan *et al.* 2007) and it is related to the history of the species that passed through a few genetic bottlenecks and, subsequently, been returned to nature from a limited number of founder individuals (Pucek *et al.* 2004; Wójcik *et al.* 2009). In this regard, it is assumed that the Belarusian population was subjected to inbreeding, which is confirmed by the analysis of the pedigrees of the founding individuals (Kobryńczuk 1985; Pucek *et al.* 2004).

Other studies of the European bison showed that the inbreeding coefficient (Fis) based on microsatellite data indicates a deviation from panmixia during the study period (Tokarska *et al.* 2009). The indicators of this coefficient for subpopulations in this study were negative, which indicates that mating does not occur with the closest relatives within the subpopulations.

Genetic analysis of the European bison from Belarus did not reveal significant differences between subpopulations from the NP "Belovezhskaya Pushcha", from the Osipovichi district and the NP "Pripyatsky". Our results indicate a low genetic differentiation of the whole Belarusian population, which is comparable to previous studies of this species.

Overall, it should be noted that the genetic certification of Belarusian subpopulations of the European bison requires more extensive and detailed research concentrated on the identification of genetic diversity to ensure the beneficial control of gene flow and to register a potential correlation of unfavorable gene variants with possible inbreeding depression. These attempts are necessary to lay the groundwork for the management and protection of the European bison in Belarus.

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