Theriologia Ukrainica, **20**: 58–66 (2020)
http://doi.org/10.15407/TU2008

GENETIC DIVERSITY OF THE FREE-LIVING POPULATION OF PRZEWALSKI'S HORSES IN THE CHERNOBYL EXCLUSION ZONE

Ekaterina E. Kheidorova[1], Kanstantsin V. Homel[1], Mikhail E. Nikiforov[1], Aliaksei V. Shpak[1], Valery Ch. Dombrovski[2], Marina S. Shkvyrya[3], Peter E. Schlichting[4], James C. Beasley[4], Denis A. Vishnevsky[5], Yegor B. Yakovlev[6]

[1]*Scientific and Practical Center for Bioresources of the National Academy of Sciences of Belarus (Minsk, Belarus);*
[2]*Polesie State Radioecological Reserve (Chojniki, Belarus);*
[3]*Kyiv Zoo (Kyiv, Ukraine);*
[4]*University of Georgia (Athens, USA);*
[5]*Chernobyl Radiation and Ecological Biosphere Reserve (Kyiv, Ukraine);*
[6]*I. I. Schmalhausen Institute of Zoology of National Academy of Sciences of Ukraine (Kyiv, Ukraine)*

Genetic diversity of the free-living population of Przewalski's horses in the Chernobyl Exclusion Zone. — E. E. Kheidorova, K. V. Homel, M. E. Nikiforov, A. V. Shpak, V. Ch. Dombrovski, M. S. Shkvyrya, P. E. Schlichting, J. C. Beasley, D. A. Vishnevskiy, Ye. B. Yakovlev. — The present study is aimed at evaluating the genetic diversity, genetic status and the extent of hybridization with the domestic horse for the Przhevalski's horse (*Equus ferus przewalskii* Poliakov 1881) population free-ranging in the territory of the Chernobyl Exclusion Zone (CEZ) in Belarus and Ukraine. The sample size included 12 individuals (10 sampled in the Belarusian part of the CEZ and 2 from the Ukrainian part of the CEZ). Ten microsatellites recommended by the International Society for Animal Genetics for horse genetic status and pedigree determination were used as markers in this study. The fragment analysis data obtained utilising this microsatellite panel determined that two individuals from Belarus possess no allelic variants typical for Przhevalski's horse. Most of the other individuals presented diagnostically valuable allelic variants. Demographic history analysis for the population did not indicate any drastic population shrinkage events in the population's recent history. The studied population is characterised by heterogeneous population structure with signs of inbreeding (0.21 %), intermediate level of genetic diversity (He = 0.63) and allelic richness (5.15), possesses 16 unique alleles among 2 microsatellite loci and valuable alleles for loci HMS3 and HMS7 (46.4 and 67.9 % specific alleles for Przhevalski's horse, respectively). Genetic structure evaluation for the population was performed via Bayesian population structure analysis and factorial correspondence analysis, which indicated the presence of intrapopulation genetic subdivision. Taking into account the obtained indicators of genetic diversity, we may conclude on the relatively favourable status of Przewalski's horse in the exclusion zone with good potential for the long-term existence of the species population in the wild. In order to minimise inbreeding effects and the risk of a decline in genetic diversity in the population of Przewalski's horse of the exclusion zone, as well as to increase the value of this free-living group to preserve the gene pool of the species as a whole, it is necessary to provide detailed genetic monitoring of the livestock's state, as well as develop a regional population management plan, including measures aimed to minimise the possibility of further hybridisation of wild horses with domestic ones.

Key words: Przewalski's horse, Chernobyl Exclusion Zone, microsatellites, genetic structure.

Correspondence to: Aliaksei V. Shpak; Scientific and Practical Centre for Bioresources, National Academy of Sciences of Belarus; 27 Akademičnaja St, Minsk, 220072, Belarus; e-mail: shpak.dvergr@gmail.com, orcid: 0000-0002-0563-9271

Introduction

Przewalski's horse (*Equus caballus przewalskii* Poljakov, 1881) has been permanently inhabiting the Chernobyl Exclusion Zone (CEZ) in a state of its natural freedom since 1998 when the Institute of Zoology of the National Academy of Sciences of Ukraine together with the Askania-Nova Biosphere Reserve organized the release of animals with lost pedigrees within the framework of the "Fauna" programme (Fauna: Program 1998) with a significant admixture of the blood of domestic mares. The success of their reproduction and settlement led to the formation of a self-sustaining group of free-living Przewalski's horses, which occupied a certain ecological niche in a peculiar and unique ecosystem of the exclusion zone.

The Ukrainian side of the CEZ includes up to 130 individuals (Slivinska *et al.* 2020), the maximum population estimate of the Belarusian side was about 50 individuals (Dombrovsky pers. comm.) in 2018. Thus, it may be assumed that the total number of the Przewalski's horse group in the region can reach up to 180–200 individuals. At the same time, the cases of repeated movement of individual groups of animals across the border of states are known. In order to track the real dynamics of the population size and its demography, it is necessary to conduct time-coordinated censuses of the species in the Ukrainian and Belarusian parts of the CEZ, for which it is promising to use modern high-tech equipment: webcams, trap cameras, drones, as well as methods for the individual recognition of animals.

Due to the limited number and isolation of the species population in the CEZ, the issue of studying its genetic diversity with a view of making adequate forecasts related to its long-term development and clarifying the current level of hybridity with the domestic horse becomes of particular relevance.

Earlier, we have already published the first genetic data on the polymorphism of the mitochondrial D-loop marker of Przewalski's horse in the CEZ (Kheidorova *et al.* 2020). It has been shown that this population is dominated by one haplotype, which has a pronounced genetic distance from the haplotypes of other descendants of this species from Askania-Nova. This paper demonstrates the results of the microsatellite analysis of 14 individuals of Przewalski's horses permanently living in the CEZ.

Materials and Methods

DNA analysis of tissue samples of Przewalski's horses with a view of establishing their genetic status and the degree of hybridity was carried out using standard PCR methods based on the hair samples of 12 animals collected in 2017–2018 in the territory of the Polesie State Radioecological Reserve (former inhabited localities: Daŭliady, Biarozaŭka, Viepry, Bielaja Saroka, Dubrovy, Cichin; the Republic of Belarus) and muscle tissue samples of two Przhevalski's horses from the Chernobyl Radiation and Ecological Biosphere Reserve (Ukraine). It is known that the specified sample contains a hair sample from a herd stallion (PH11_Bel). The characteristics of the samples of the investigated field material are shown in Table 1.

DNA was extracted from muscle tissue samples using a set of reagents and a standard protocol of the "ArtBioTech" manufacturer (Belarus). Hair samples were soaked for 5 min in a 2 % solution of sodium dodecyl sulfate, then rinsed 3 times in the deionized water and dried passively. DNA extraction was carried out using the commercial kit "Art DNA Mini Expert" (ArtBioTech, Belarus) according to the protocol recommended by the manufacturer, with modifications: to improve hair lysis, proteinase K (1mg/ml) and DTT (0.1M) were added to all samples. Lysis was carried out at 56 °C overnight. The concentration of obtained DNA preparations was measured spectrophotometrically and ranged from 3 to 27 ng/μL.

Table 1. Characteristics of the field material samples used in the study of the genetic status of Przewalski's horses

Таблиця 1. Характеристика зразків польових матеріалів, котрі використовувалися для вивчення генетичного статусу коней Пржевальського

No	Sample	Locality	Field material	No	Sample	Locality	Field material
1	PH1_Bel	Belarus, Daŭliady	hair	8	PH8_Bel	Belarus, Bielaja Saroka	hair
2	PH2_Bel	Belarus, Biarozaŭka	hair	9	PH9_Bel	Belarus, Daŭliady	hair
3	PH3_Bel	Belarus, Biarozaŭka	hair	10	PH10_Bel	Belarus, Dubrovy	hair
4	PH4_Bel	Belarus, Biarozaŭka	hair	11	PH11_Bel	Belarus, Cichin	hair
5	PH5_Bel	Belarus, Viepry	hair	12	PH12_Bel	Belarus, Cichin	hair
6	PH6_Bel	Belarus, Bielaja Saroka	hair	13	PH22_Ukr	Ukraine	muscle tissue
7	PH7_Bel	Belarus, Daŭliady	hair	14	PH23_Ukr	Ukraine	muscle tissue

Taking into account the specificity of the non-invasive method used for collecting hair samples and the possibility of isolating non-target DNA from them, verification of the species belonging of the studied samples of Przewalski's horses was carried out based on the analysis of the mitochondrial D-loop gene sequences obtained using primers proposed by Wilkinson and Chapman (Wilkinson & Chapman 1991).

Genetic polymorphism of the population of Przewalski's horses and the level of their hybridization with the domestic horse was studied at 10 microsatellite loci recommended by the International Society for Animal Genetics (ISAG) for investigating the genetic diversity and pedigree of horses (Table 2). These primers were previously tested for studying of Przewalski's horses (Bowling *et al.* 2003; Breen *et al.* 1994; 2009 *a–b*).

The conditions for performing STR-loci PCR are presented in Table 3.

Table 2. Characteristics of 10 microsatellite markers used to study the genetic structure and diversity of Przewalski's horse

Таблиця 2. Характеристика 10 мікросателітних маркерів, котрі використовувалися для вивчення генетичної структури та різноманітності коня Пржевальського

Locus	Chromosome	Repeat motif	Forward primer (with the label Cy5.5 on the 5´ end) Reverse primer	Fragment size, bp
HMS7	1q25	$(AC)_2(CA)_n$	5'– CAGGAAACTCATGTTGATACCATC – 3' 5' – TGTTGTTGAAACATACCTTGACTGT – 3'	167–189
HMS3	9	$(TG)_2(CA)_2TC(CA)_n$ and $(TG)_2(CA)_2TC(CA)_nGA(CA)_5$	5' – CCAACTCTTTGTCACATAACAAGA – 3' 5' – CCATCCTCACTTTTTCACTTTGTT – 3'	150–174
HTG10	21	$(TG)_n$ и $TATC(TG)_n$	5' – CAATTCCCGCCCCACCCCCGGCA – 3' TTTTTATTCTGATCTGTCACATTT – 3'	89–117
HMS6	4	$(GT)_n$	5' – GAAGCTGCCAGTATTCAACCATTG – 3' CTCCATCTTGTGAAGTGTAACTCA – 3'	153–171
VHL20	30	$(TG)_n$	5' – CAAGTCCTCTTACTTGAAGACTAG – 3' 5' – AACTCAGGGAGAATCTTCCTCAG – 3'	89–107
CA425 UCDEQ425	28q18	$(GT)_n$	5' – AGCTGCCTCGTTAATTCA – 3' 5' – CTCATGTCCGCTTGTCTC – 3'	230–250
HTG4	9	$(TG)_nAT(AG)_5AAG(GA)_5$ $ACAG(AGGG)_3$	5' – CTATCTCAGTCTTGATTGCAGGAC – 3' 5' – CTCCCTCCCTCCCTCTGTTCTC – 3'	127–141
ASB2	15q21.3 – q23	$(GT)_n$	5' – CCACTAAGTGTCGTTTCAGAAGG – 3' 5' – CACAACTGAGTTCTCTGATAGG – 3'	222–256
AHT4	24q14	$(AC)_nAT(AC)_n$	5' – AACCGCCTGAGCAAGGAAGT – 3' 5' – GCTCCCAGAGAGTTTACCCT – 3'	138–170
AHT5	8	$(GT)_n$	5' – ACGGACACATCCCTGCCTGC – 3' 5' – GCAGGCTAAGGGGGCTCAGC – 3'	128–152

Table 3. PCR temperature and time modes

Таблиця 3. Температурний та часовий режими ПЛР

Stage	Temperature (°C)	Number of cycles	Time
Pre-denaturation	95	1 cycle	10 min
Denaturation	95		45 sec
Annealing	62–60 (increment)	10 cycles	1 min
Elongation	72		1 min
Denaturation	95		45 sec
Annealing	60	30 cycles	1 min
Elongation	72		1 min
Final elongation	72	1 cycle	60 min
Hold	4		∞

Genotyping of Przewalski's horses was carried out in the GenomeLab GeXP genetic analysis system (Beckman Coulter, USA). Fragment analysis data were assessed for genotyping errors (null alleles, stuttering, large allele dropout) using the Micro-Checker software, version 2.2.3 (Brookfield 1996; Chakraborty *et al.* 1992). Additional estimation of the null allele frequency was carried out in Genepop version 4.3 using the maximum likelihood estimation of the null allele frequency (Raymond & Rousset 1995; Rousset 2008). Analysis of the genotypes matching was performed using GenAlEx v. 6.501 (Peakall & Smouse 2012, 2006). The presence of linkage disequilibrium between the investigated microsatellite loci was performed in Genepop with default settings. The deviation of the studied loci from the Hardy-Weinberg equilibrium (HWE) was assessed in Arlequin ver. 3.5.2.2 with default settings (Excoffier & Lischer 2010).

A test for a past sharp decline in the population of Przewalski's horse was performed using the Bottleneck 1.2.02 software (Cornuet & Luikart 1996). In this analysis, the two-phase mutation model was used with default settings. In addition, the data was tested relatively I.A.M. (infinite alleles model) and S.M.M. (stepwise mutational model). Presence of the statistically significant excess of observed heterozygosity for the microsatellite loci under study was assessed using the sign test, the standardized differences test, and the Wilcoxon sign-rank test. An additional assessment of the passage of the studied population of Przewalski's horse through the bottleneck was the construction of a graph of allele frequency distribution in Bottleneck and its comparison with the normal L-shaped graph corresponding to the scenario of the absence of a sharp decline in the number in the past.

Observed heterozygosity (Ho), expected heterozygosity (He) and fixation index (inbreeding index) (F) were calculated in GenAlEx. Allelic richness per locus (AR) was calculated in FSTAT 2.9.3.2 (Goudet 1995).

Bayesian analysis of population structure was performed in STRUCTURE version 2.3.4 (Falush *et al.* 2003; Pritchard *et al.* 2000). Analysis parameters in STRUCTURE were as follows: admixture model, correlated allele frequencies among populations, length of burning period = 100 000, the number of MCMC (Markov chain Monte Carlo) = 100 000, the number of tested genetic clusters (K) from 1 to 5, and the number of iterations for each cluster 20. Analysis of the STRUCTURE output to find the best K was done in Structure Harvester (Earl & vonHoldt 2012). Visualization of the summarizing graph reflecting the proportion of genetic clusters for analyzed individuals taking into account the best K value was carried out using Clumpak (Kopelman *et al.* 2015).

As an additional assessment of the presence of genetic heterogeneity for the analyzed population, the factorial correspondence analysis was performed in GENETIX v4.05.2 (Belkhir *et al.* 2004). Data visualization of factorial correspondence analysis was performed in PAST 3.0 (Hammer *et al.* 2001).

Results and Discussion

The composition of allele loci in the studied part of the Przewalski's population of the exclusion zone is shown in Tables 4–5: the HMS3 locus is represented by 7 alleles, HMS6 — by 3, HMS7 — by 7, HTG4 — by 8, HTG10 — by 4, AHT4 — by 7, AHT5 — by 3, VHL20 — by 6, CA425 — by 4, ASB2 — by 5. The most polymorphic is the HTG4 locus and the least polymorphic are the HMS6 and AHT5 loci.

In the article by Breen and coll. (Breen *et al.* 1994), the allele composition of the selected markers in *Equus caballus* and *Equus przewalskii* is given, which allowed us to determine that the HMS6 locus is fully represented by alleles characteristic of domestic horses (163, 167, 169), and the most valuable for the studied population of Przewalski's horses are the specific alleles of wild horses in loci HMS3 and HMS7 (46.4 and 67.9 %, respectively). The incidence of specific alleles in wild horses was 13.2 % overall as compared to 17.5 % of alleles characteristic of the domestic horse.

It should be noted that fragment analysis revealed two Przewalski's horses (PH1_Bel from the former populated locality Daŭliady and PH3_Bel from the former populated locality Biarozaŭka) for which no allelic variants characteristic of Przewalski's horse were observed within the studied array of microsatellite loci.

However, the overwhelming majority of the studied samples, despite the hybridity at the HMS6 locus, carried valuable alleles of Przewalski's horses at other loci. Comparison of the results obtained with the data of Bowling (Bowling *et al.* 2003) showed that the Przewalski's horse population of the exclusion zone has alleles that are both common with other descendants of horses from Askania-Nova (15 alleles at 6 loci — HMS6 — 163, 169; HMS3 — 166, 168, 178; HMS7 — 177, 181, 187; HTG10 — 93, 95, 97; VHL20 — 87, 105; ASB2 — 238, 248) and the unique alleles not known for horses from other geographic regions, for example: HTG4 — 125, 127, 129, 133, 135, 139, 141, 145; AHT4 — 124, 144, 148, 160, 162, 178, 198.

The search for matching genotypes did not reveal a complete match of those in the sample under study. However, the matching of genotypes was found for all but one locus in two individuals — PH1_Bel and PH3_Bel.

An assessment performed for the presence of genotyping errors, the frequency of null alleles, and allelic dropouts showed the signs of null alleles for three loci: HMS3, AHT5 and CA425.

Table 4. Composition of 10 microsatellite loci in the population of Przewalski's horse*

Таблиця 4. Склад 10 мікросателітних локусів у популяції коня Пржевальського

Sample	HMS7 (167–189)		HMS3 (150–174)		HTG10 (89–117)		HMS6 (153–171)		VHL20 (89–107)		CA425 (230–250)		HTG4 (127–141)		ASB2 (222–256)		AHT4 (138–170)		AHT5 (128–152)	
PH1_Bel	181	181	154	154	95	95	***163***	***163***	***87***	107	238	238	129	129	246	246	148	148	134	134
PH2_Bel	181	**187**	154	**172**	***93***	95	***167***	***169***	***87***	107	238	256	0	0	246	248	148	160	134	140
PH3_Bel	181	181	154	154	95	95	***163***	***163***	***87***	107	238	238	125	125	246	246	148	148	134	134
PH4_Bel	**179**	**187**	154	178	95	95	***163***	***163***	107	107	238	238	129	135	246	246	148	160	134	134
PH5_Bel	181	**187**	168	**172**	***93***	95	***167***	***169***	107	107	238	238	127	145	246	246	124	124	140	140
PH6_Bel	**179**	**189**	**172**	**172**	0	0	0	0	***87***	***105***	244	244	129	135	0	0	144	144	134	134
PH7_Bel	**187**	**187**	168	**172**	***93***	***93***	***169***	***169***	107	107	238	238	0	0	242	246	148	160	134	134
PH8_Bel	**179**	**187**	168	**172**	95	97	***167***	***169***	***87***	**103**	238	238	129	135	0	0	178	198	134	134
PH9_Bel	177	177	172	**172**	95	97	***169***	***169***	***87***	**103**	238	238	0	0	246	246	148	160	134	134
PH10_Bel	**179**	**185**	152	**152**	93	97	***163***	***163***	***87***	**103**	238	256	129	135	246	246	148	162	134	134
PH11_Bel	**179**	**185**	168	168	***91***	95	***163***	***167***	***87***	**103**	240	240	135	139	246	246	148	160	134	134
PH12_Bel	**179**	**185**	152	168	***93***	***93***	***163***	***169***	***87***	**103**	238	238	129	135	246	248	148	160	134	134
PH22_Ukr	***169***	**189**	166	**170**	***93***	95	***167***	***167***	107	111	238	238	133	139	236	246	148	160	146	146
PH23_Ukr	**179**	**185**	166	**170**	95	97	***163***	***163***	***87***	109	240	240	139	141	238	246	148	148	146	146

* Allele sizes found only in Przewalski's horses are **bolded**; alleles characteristic of domestic horses are highlighted in ***bold italics***; the rest of the alleles are characteristic of both wild and domestic horses, or there is no data on them.

Table 5. The number of alleles per locus in the Przewalski's horse population

Таблиця 5. Кількість алелей на локус у популяції коня Пржевальського

HMS7 (167–189)		HMS3 (150–174)		HTG10 (89–117)		HMS6 (153–171)		VHL20 (89–107)		CA425 (230–250)		HTG4 (127–141)		ASB2 (222–256)		AHT4 (138–170)		AHT5 (128–152)	
n=7	N=28	n=7	N=28	n=4	N=26	n=3	N=26	n=6	N=28	n=4	N=28	n=8	N=22	n=5	N=24	n=7	N=28	n=3	N=28
169	***1***	**152**	**3**	***91***	***1***	***163***	***12***	***87***	***10***	238	20	125	2	236	1	124	2	134	21
177	2	154	6	***93***	***8***	***167***	***6***	**103**	**5**	240	4	127	1	238	1	144	2	140	3
179	**7**	***166***	***2***	95	13	***169***	***8***	***105***	***1***	244	2	129	7	242	1	148	14	146	4
181	6	168	6	97	4			107	10	256	2	133	1	246	19	160	7		
185	**4**	**170**	**2**					109	1			135	6	248	2	162	1		
187	**6**	**172**	**8**					111	1			139	3			178	1		
189	**2**	178	1									141	1			198	1		
												145	1						

* Designations as in previous table.

An additional assessment of the frequency of null alleles in Genepop demonstrated a high probability (> 20 %) of their presence for the HMS6 locus, but the obtained value is statistically insignificant (Table 6). For other loci, the frequencies of null alleles are low.

An assessment of the presence of genotypic linkage disequilibrium did not reveal such pairs of loci in the analyzed data. An assessment of the studied loci for deviation from the Hardy-Weinberg equilibrium showed the absence of significant ($p < 0.05$) deviation only for 3 loci (HTG10, VHL20, ASB2). The presence of loci deviating from the Hardy-Weinberg equilibrium is not in most cases a basis for excluding loci from the analysis (Selkoe & Toonen 2006). Data on the level of genetic diversity and inbreeding are shown in Table 7.

It was found that average indicators of observed heterozygosity are less than those of expected heterozygosity, but in general both indicators have moderate values (0.52 and 0.63 respectively). Fixation index value indicates the presence of signs of inbreeding in the studied Przewalski's horse population, which corresponds to the obtained indicators of heterozygosity. However, it should be noted that for 3 out of 9 loci there was a complete absence of inbreeding (loci: VHL20, HTG4 and ASB2), and for 2 more loci (HTG10, AHT4) low inbreeding indices were identified. Thus, in order to conclude on the real level of inbreeding in the studied population, it is necessary to analyze a larger number of microsatellite loci.

The bottleneck analysis of the Przewalski's horse population from the CEZ did not show the presence of signs of sharp population decline in the past in the demographic history of the sample under consideration. The latter can be explained by the limited size of the studied sample and the small number of involved microsatellite loci. In addition, it is known that the test used may not be able to determine the signs of sharp population decline in the past due to the low effective size of the population itself (Höglund 2009).

Table 6. The frequency of null alleles in the Przewalski's horse population of the CEZ

Таблиця 6. Частота нульових алелей у популяції коней Пржевальського в ЧЗВ

Locus	Null allele frequency, CI	Locus	Null allele frequency, CI
HMS7	**0.1492** (0.0467…0.3093)	CA425	**0.2915** (0.1598…0.3866)
HMS3	**0.1596** (0.0420…0.3126)	HTG4	**0.0842** (0.0198…0.2616)
HTG10	0.1279 (0.0000…0.2785)	ASB2	0.0000 (No info for CI)
HMS6	0.4449 (No info for CI)	AHT4	**0.1319** (0.0425…0.2969)
VHL20	0.0000 (No info for CI)	AHT5	**0.3855** (0.1592…0.6435)

Note. CI — 95 % Confidence Interval, **bold type** — statistically significant value.

Table 7. Indicators of genetic diversity and the inbreeding level in the population of Przhevalski's horses of the CEZ

Таблиця 7. Показники генетичного різноманіття та рівня інбридингу в популяції коней Пржевальського ЧЗВ

Locus	Ho	He	AR	F
HMS7	0.714	0.814	6.706	0.122
HMS3	0.571	0.804	6.700	0.289
HTG10	0.615	0.630	3.846	0.023
HMS6	0.385	0.639	3.000	0.398
VHL20	0.786	0.709	5.357	–0.108
CA425	0.143	0.459	3.920	0.689
HTG4	0.818	0.789	8.000	–0.037
ASB2	0.417	0.361	4.746	–0.154
AHT4	0.643	0.673	6.278	0.045
AHT5	0.071	0.406	2.993	0.824
Average	0.516	0.628	5.150	0.209

Note: Ho — observed heterozygosity, He — expected heterozygosity, AR — allelic richness, F — fixation index.

Thus, further study involving a large number of individuals and microsatellite markers is required to verify the result obtained.

Bayesian analysis of the genetic structuring of the Przewalski's population showed the presence of an intrapopulation subdivision (Fig. 1). The data obtained indicate the division of the Przewalski's horse population of the CEZ into two genetic clusters (K = 2). The first cluster includes individuals 2, 5–14 (PH2_Bel, PH5_Bel — PH12_Bel, PH22_Ukr, PH23_Ukr), while the second one contains the 1-, 3- and 4th individuals (PH1_Bel, PH3_Bel, PH4_Bel). The second cluster comprises individuals with genotypes that differ from most other individuals of Przhevalski's horse from the CEZ.

One of probable explanations for this fact may be the presence of alleles that are non-specific for Przewalski's horses in them as a result of hybridization with the domestic horse. To the greatest extent, this characterizes PH1_Bel and PH3_Bel individuals.

Factorial correspondence analysis showed a different picture of the genetic structure of the studied Przewalski's horse population (Fig. 2). The presence of two genetic clusters for the considered population of Przewalski's horse was established. The first one, including all individuals, except for PH6_Bel, is from the Belarusian part of the CEZ and the second one, consisting of two individuals, is from the Ukrainian part of the exclusion zone. The presence of PH6_Bel, PH22_Ukr, PH23_Ukr individuals in the population differing in allele combinations is probably explained by the initial genetic heterogeneity of imported individuals.

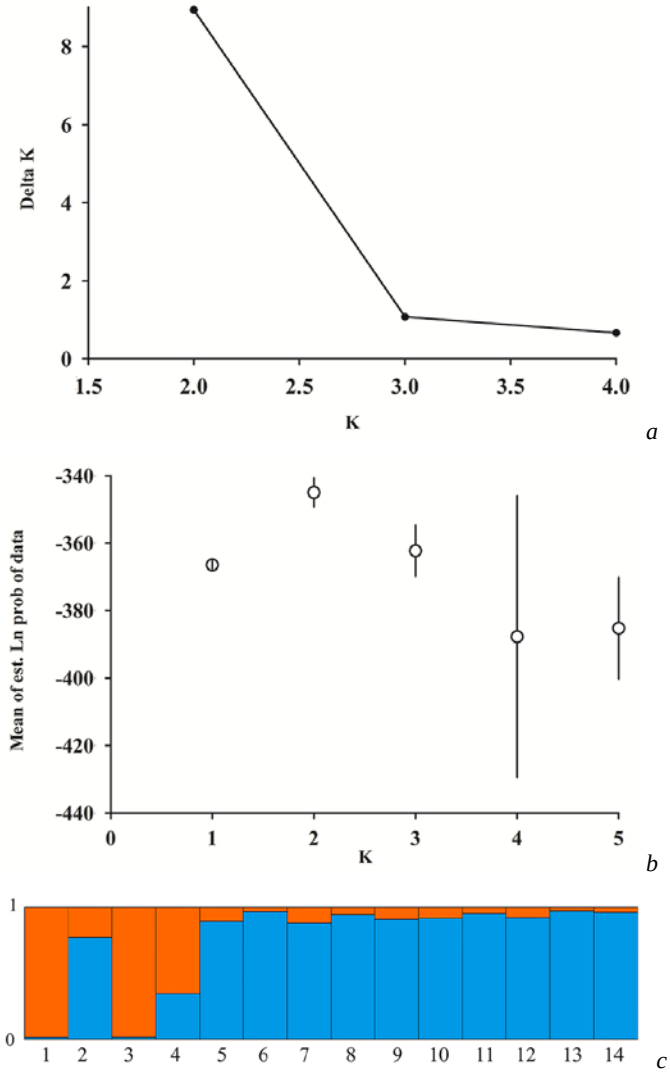

Fig. 1. Bayesian analysis of the genetic differentiation of the Przewalski's horse population in the CEZ.

Рис. 1. Байєсовський аналіз генетичної диференціації популяції Пржевальського в ЧЗВ.

Note. A — ΔK; B — estimated mean likelihoods of each number of genetic clusters (bars are standard deviation); C — the graph of genetic clusters, the column represents an individual included in the sample; column segments reflect the proportion (%) of genetic clusters for each individual; the number of identified genetic clusters (K) for the studied sample equals to two (ΔK = 8.94 at Ln Pr (2K) = –344.95); Ln Pr (2K) — the posterior estimate of the probability for a given number of clusters; the numbers on the abscissa correspond to individuals from Table 1.

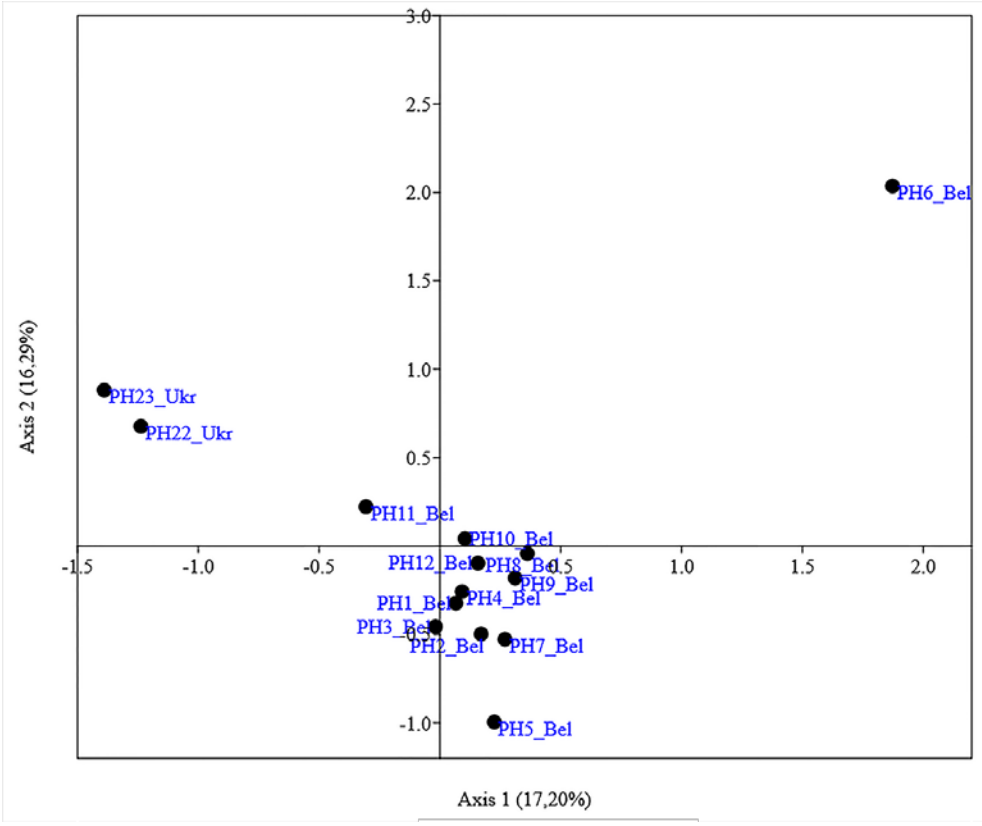

Fig. 2. Factorial correspondence analysis of the Przewalski's horse population from the CEZ.

Рис. 2. Факторіальний аналіз популяції Пржевальського із ЧЗВ.

Conclusion

The data on the genetic analysis performed shows that the Przewalski's horse population in the CEZ has a heterogeneous genetic structure with signs of inbreeding (0.21 %) and is characterized by a moderate level of genetic diversity (He = 0.63; AR = 5.15). The population possesses 16 unique alleles at 2 microsatellite loci and valuable alleles at HMS3 and HMS7 loci (46.4 and 67.9 % of Przewalski's horse-specific alleles, respectively). The low degree of hybridity with the domestic horse (the frequency of occurrence of alleles characteristic of the domestic horse is 17.5 %) is balanced by the even distribution of unique alleles of wild horses in the population (13.2 %) and by the fact that admixtures of the same alleles of the domestic horse are also characteristic of other species members in other breeding and keeping areas. Taking into account the obtained indicators of genetic diversity, we may speak about the relatively favorable status of Przewalski's horse in the exclusion zone with good potential for the long-term existence of the species population in the wild.

In order to minimize inbreeding effects and the risk of a decline in genetic diversity in the population of Przewalski's horse of the exclusion zone, as well as to increase the value of this free-living group to preserve the gene pool of the species as a whole, it is necessary to provide detailed genetic monitoring of the livestock's state and to develop a regional population management plan, including measures minimizing the possibility of further hybridization of wild horses with domestic ones.

Acknowledgements

The authors thank the editor and two anonymous reviewers for insightful comments on an earlier draft of this manuscript. The research was carried out within the framework of Government Programme on Overcoming Consequences of the Chernobyl Nuclear Power Plant Catastrophe.

References

Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste, F. Bonhomme. 2004. GENETIX4. 05, logiciel sous Windows TM pour la génétiquedes populations. *Laboratoire génome, populations, interactions, CNRS UMR*, 5000: 1996–2004.

Bowling, A. T., W. Zimmermann, O. Ryder, C. Penado, S. Peto, L. Chemnick, N. Yasinetskaya, T. Zharkikh. 2003. Genetic variation in Przewalski's horses, with special focus on the last wild caught mare, 231 Orlitza III. *Cytogenetic and Genome Research*, **102** (1–4): 226–234. CrossRef

Breen, M., P. Downs, Z. Irvin, K. Bell. 1994. An equine tetranucleotide repeat: microsatellite MPZOOB. *Animal Genetics*, **25**: 124.

Breen, M, P. Downs, Z. Irvin, K. Bell. 2009*a*. An equine tetranucleotide repeat: microsatellite MPZ001. *Animal Genetics*, **25** (2): 123–123. CrossRef

Breen, M., P. Downs, Z. Irvin, K. Bell. 2009*b*. Intrageneric amplification of horse microsatellite markers with emphasis on the Przewalski's horse (E. przewalskii). *Animal Genetics*, **25** (6): 401–405. CrossRef

Brookfield, J. F. Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology*, **5** (3): 453–455. CrossRef

Chakraborty, R., M. D. Andrade, S. P. Daiger, B. Budowle. 1992. Apparent heterozygote deficiencies observed in DNA typing data and their implications in forensic applications. *Annals of Human Genetics*, **56** (1): 45–57. CrossRef

Cornuet, J. M., G. Luikart. 1996. Description and Power Analysis of Two Tests for Detecting Recent Population Bottlenecks From Allele Frequency Data. *Genetics*, **144** (4): 2001–2014. CrossRef

Earl, D. A., B. M. vonHoldt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, **4** (2): 359–361. CrossRef

Excoffier, L., H. E. L. Lischer. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10** (3): 564–567. CrossRef

Falush, D., M. Stephens, J. K. Pritchard. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, **164** (4): 1567–1587. CrossRef

Fauna: Program. 1998. *«Fauna» of the Chornobyl Nuclear Power-Plant Exclusion and Absolute Resettlement Zones*. Ministry of Extraordinary Situations of the Ukraine Pres, Kyiv.

Goudet, J. 1995. FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *Journal of Heredity*, **86** (6): 485–486. CrossRef

Hammer, Ø., D. A. T. Harper, P. D. Ryan. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica*, **4** (1): 9.

Höglund, J. 2009. Evolutionary Conservation Genetics. Oxford University Press. CrossRef

Kheidorova, E. E., M. E. Nikiforov, K. V. Homel, A. V. Shpak, V. V. Shakun, V. Ch. Dombrovski, M. G. Shkvyrya. 2020. Analysis of D-loop mitochondrial marker polymorphism of Przewalsky horses *Equus caballus przewalskii* Poljakov, 1881 and proposals for the species conservation in Belarus. *Proceedings of the National Academy of Sciences of Belarus. Biological series*, **65** (3): 275–282. CrossRef

Kopelman, N. M., J. Mayzel, M. Jakobsson, N. A. Rosenberg, I. Mayrose. 2015. Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, **15** (5): 1179–1191. CrossRef

Peakall, R., P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research — an update. *Bioinformatics*, **28** (19): 2537–2539. CrossRef

Peakall, R., P. E. Smouse. 2006. genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6** (1): 288–295. CrossRef

Pritchard, J. K., M. Stephens, P. Donnelly. 2000. Inference of population structure using multilocus genotype data. *Genetics*, **155**: 945–959. CrossRef

Raymond, M., F. Rousset. 1995. GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity*, **86** (3): 248–249. CrossRef

Rousset, F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources*, **8** (1): 103–106. CrossRef

Selkoe, K. A., R. J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, **9** (5): 615–629. CrossRef

Slivinska, K., N. Yasynetska, D. Klich. 2020. Przewalski`s Wild Horses and Their 18th Years Management in the Chornobyl Exclusion Zone, Ukraine. *Proc. International Symposium Ecology 2017*, Ercyes University, 90–98.

Wilkinson, G. S., A. M. Chapman. 1991. Length and sequence variation in evening bat D-loop mtDNA. *Genetics*, **128** (3): 607–617. CrossRef