Characteristic of Przewalski horses population from Askania-Nova reserve based on genetic markers

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Short Report

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Abstract

Przewalski horses are considered the last living population of wild horses, however, they are secondarily feral offspring of herds domesticated about 5000 years ago by the Botai culture. After Przewalski horses were almost extinct at the beginning of the 20th century, their population is about 2500 individuals worldwide, with one of the largest breeding centers in the Askania-Nova Biosphere Reserve (Ukraine).

The research aimed to analyse the genetic structure of Przewalski horses population maintained in the Askania-Nova Reserve, as well as establish maternal ancestry based on whole hypervariable mitochondrial DNA region profiling, analysis of Y chromosome single nucleotide polymorphism unique for Przewalski horses, and coat color markers - MC1R (‘fox’), TBX3 (Dun).

The mtDNA hypervariable region analysis in 23 Przewalski horses allowed assigning them to three distinctly different haplotypes, showing the greatest similarity to the Equus caballus reference, the Equus przewalskii reference, and to extinct species - Hariontinghippus. The Y chromosome analysis using fluorescently labelled assays (FAM and VIC) differentiated horses in terms of polymorphism (MH341179.1, g731821T>C) characteristic of the species Equus caballus and Equus przewalskii. All 13 male individuals presented genotype C characteristics for Przewalski horses. The polymorphisms within genes related coat color confirmed the presence of primitive genetic variants in Przewalski horses: polymorphism of MC1R gene (ECA3g.36259552C>T) assigned all horses to the genotype with C/C alleles; the two TBX3 polymorphisms (chr8:18,227,267+1,066G>T, chr8:18,226,905A>G) assigned all horses to the genotype with G/G alleles in both loci; and in the third TBX3 1.6 kb in/del polymorphism (chr8:18,227,267) deletions were not observed.

Introduction

Przewalski's horse genome falls outside a domestic horses group, approximately 38 000–72 000 year BP (Orlando et al. 2013). Przewalski horses are considered the last living population of wild horses (Lau et al. 2009), however, the previous research based on whole genome sequencing indicated that they are feral descendants of the horses domesticated about 5000 years ago by the Botai culture in present-day Kazakhstan (Gaunitz et al. 2018). The breed's history remains unknown for nearly five millennia until Przewalski horses were rediscovered as a free-range population deemed to be wild about 150 years ago (Librado and Orlando 2021). In the 20th century, Przewalski horses almost became extinct but survived from a captive herd as a result of enormous conservation and reintroduction efforts. Currently, based on pedigree data, 11 pure species founders are distinguished, but the population cannot be genetically pure, as in the early 1900s, several zoos interbred their Przewalski horses with domestic horses to save the species and started to reproduce another two founders: a domestic horse and domestic/Przewalski hybrid (Librado and Orlando 2021; Wallner et al. 2003). Now, their population consists of no more than 2500 individuals worldwide including 900 horses in Europe and 1360 in Asia (Kerekes et al. 2021). One of the largest breeding centers for Przewalski horses is Askania-Nova Biosphere Reserve (Ukraine), where the population is maintained at about 60–70 individuals (Zvegintsova et al. 2019).
Previous studies on mitochondrial DNA (mtDNA) identified three unique haplotypes in Przewalski horses, none of which were found in modern horses - two similar ones and one substantially divergent from them (Goto et al. 2011). However, recent studies indicate evidence of four Finnhorse and one Latvian horse individuals carrying mitochondrial haplogroup previously confined only to Przewalski horse (Kvist and Niskanen 2021).

The basic coat colors genetic control in horses resides at two genetic loci, namely Extension (E) and Agouti (A) which are responsible for the three basic colors occurrence - black, bay, and chestnut (Thiruvenkadan et al. 2008). The most common color seen on Przewalski horses is bay with Dun dilution. It has been shown that Przewalski horses, as well as the Siberian horses of that time, do not have the black pigmentation recessive alleles ‘a’ in ASIP gene, which is possibly related to their living in the Asian steppes adaptation (Ludwig et al. 2009; Reissmann et al. 2016). The archaeological samples studies indicated that during domestication the selection pressure had a great impact on coat color variations (Ludwig et al. 2009). It was hypothesized that ancestral horses were characterized by black-based patterns (Thiruvenkadan et al. 2008), and all Siberian and European Pleistocene horses were bay or bay-dun (Ludwig et al. 2009). The ‘fox’ color carriers caused by MC1R recessive alleles ‘ee’ should not be present in Przewalski horses, however, the previous research showed the ‘fox’ color alleles presence in 15% of the tested Przewalski horses (Reissmann et al. 2016). The results may be the consequence of the population crossbreeding in the early days of the conservation efforts. For TBX3 gene, there is only one characteristic genotype observed in all Przewalski horses – Dun. Horses with this genotype show a diluted body color and have dark points called primitive markings including dorsal stripe and leg barring (Imsland et al. 2015).

The research purpose was to analyse the genetic structure of Przewalski horses population maintained in Askania-Nova Reserve, as well as establish maternal ancestry based on whole hypervariable mtDNA region profiling, analysis of Y chromosome SNP unique for Przewalski horses, and coat color markers - MC1R (‘fox’) and TBX3 (Dun).

**Material And Methods**

The analyses were carried out on hair follicle samples derived from 23 Przewalski horses population living in the Askania-Nova reserve (Ukraine). The 21 animals were sampled after death and the other two during immobilization by a veterinarian.

The DNA was isolated using Sherlock AX kit (A&A Biotechnology) and stored at -20°C. For the mitochondrial DNA whole hypervariable region amplification, three PCR products were designed covering a total region of 1165 bp (X79547.1; Table S1). Amplification was performed on all 23 individuals using the Phanta Ready Mix (Vazyme Biotech). PCR products were cleaned with the enzymatic method using the EPPiC Fast reagent (A&A Biotechnology) and used as a template for sequencing by the Sanger method. The PCR for the sequencing reaction was performed for each amplicon (69 samples in total) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and the products were
repurified with the BigDye XTerminator Purification Kit (Thermo Fisher Scientific). The capillary electrophoresis was performed on 3500xL Genetic Analyzer (Thermo Fisher Scientific) using POP-7 ™ Polymer for 3500/3500xL (Thermo Fisher Scientific) and the results were analysed using FinchTV (Geospiza, Inc.), BLAST and Variant Analysis (Thermo Fisher Cloud).

To analyse Y chromosome SNP, first, the amplification of X chromosome part was performed for all samples using fluorescently labeled probe unique complementary to the X chromosome (PLP1 gene, ENSECAG00000015446) and the TaqMan™ Gene Expression Master Mix kit (Thermo Fisher Scientific). Next, all of the male samples were genotyped using the allelic discrimination method with assay complementary to the Y chromosome (designed using Primer Express™) and TaqPath™ ProAmp™ Master Mix (Thermo Fisher Scientific). The SNP localized on the Y chromosome and distinguishing Equus caballus from Equus przewalskii was investigated (MH341179.1, g731821T > C). Samples with X and Y signals were recognized as male (Table S1). Real-time PCR reaction was performed on QuantStudio™ 7 Flex (Thermo Fisher Scientific).

Moreover, two coat color genes were investigated: TBX3 and MC1R. All PCRs were carried out with Phanta Ready Mix (Vazyme Biotech). Two polymorphisms at the TBX3 gene (EquCab2.0) (chr8:18,227,267 + 1,066G > T, chr8:18,226,905A > G) were detected by Sanger sequencing. The heterozygosity state of deletion (1.6 kb in/del, chr8:18,227,267) was investigated by the amplification length polymorphism method according to Stefaniuk-Szmukier et al. (2017). Melanocortin-1-receptor (MC1R) gene polymorphism responsible for ‘fox’ color (ECA3g.36259552C > T) was detected by Sanger sequencing (Rieder et al. 2001).

## Results And Discussion

The mitochondrial DNA whole hypervariable region sequencing assigned Przewalski horses to the three distinctly different haplotypes (Fig. 1a). All samples were clustered in three homogeneous groups presenting unique SNPs patterns (Table 1). The obtained sequences have been submitted to GenBank and received accession numbers: ON393914, ON393915, ON393916. The mtDNA haplotypes comparison with other Equidae mtDNA sequences available in GenBank showed an interesting association. The haplotype 3 showed the greatest similarity to the Equus caballus reference. The haplotype 2 is separated from the genus Equus, showing the highest similarity to Equus przewalskii reference (KT221845.1) and is probably low admixed with other Equidae. The haplotype 1 showed the homology to the Haringtonhippus (KT168329.2) - an extinct species, that lived in North America during the Pleistocene (Fig. 1b). The species was described by Heintzman et al. (2017) as the result of the full mitochondrial and partial nuclear genomes analysis from late Pleistocene ‘New World stilt-legged’ equids endemic to North America. They also demonstrated that Haringtonhippus falls outside of a crown group Equus however, based on our results, it can be suspected that the Haringtonhippus may have interbred with selected equines, including Przewalski horses. The geographical barrier concerning both species occurrence could be overcome through the land existing in the Pleistocene and connecting today’s Siberia in Asia with North America - called Beringia. Therefore, the gene flow between Haringtonhippus and Equus przewalskii
populations may have occurred regularly at least periodically during the Pleistocene. Moreover, the latest research confirms a small proportion of North American ancestry in Przewalski horses and observed that in present Przewalski horses some of their genomic origin is derived from relatively recent gene flow from extinct North American horses (Vershinina et al. 2021).

According to the sex chromosome analysis, 13 of 23 samples were male and all of them presented C allele in MH341179.1 g731821T>C locus, characteristic for Przewalski horses. The research previously conducted on Przewalski horses (Wallner et al. 2013) also presented C allele in this location and confirmed the g731821T>C substitution characteristics only for Przewalski horses.

The coat color genes analyses indicated only native, wild genotypes. The TBX3 gene polymorphisms were analysed. SNP1 (G in Dun, T in non-dun1) is located within the region deleted in non-dun2, 1,067 bp downstream of the deletion breakpoint at chr8:18,227,267. SNP2 (G in Dun, A in non-dun1 and non-dun2), is located 362 bp upstream of the deleted region in non-dun2 at chr8:18,226,905. The results assigned all horses to the genotype with G/G alleles in both SNP loci, and deletions were not observed. MC1R variants were also identified; none of Przewalski horses presented C to T missense mutation associated with an unfavorable ‘fox’ color variant for this species. The ability to produce phaeomelanin is attributed to the recessive alleles ‘ee’ and is not in concordance with the recommendations for the Przewalski horses (Thiruvenkadan et al. 2008).

**Statements And Declarations**

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*Ethical statement*

Non applicable.

*Conflict of interest*

The authors declare that they have no conflict of interests.

*Author Contributions*

All authors contributed to the study conception and design. Methodology: Adrianna D. Musiał, Katarzyna Ropka-Molik, Monika Stefaniuk-Szmukier; material: Nataliya Yasynetska, data collection and analysis: Adrianna D. Musiał, Katarzyna Ropka-Molik, Monika Stefaniuk-Szmukier, Grzegorz Myćka, Agnieszka Bieniek, Nataliya Yasynetska, project administration Adrianna D. Musiał, Katarzyna Ropka-Molik, funding acquisition Adrianna D. Musiał. The first draft of the manuscript was written by Adrianna D. Musiał and
all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Availability of data**

The data will be provided on request by the corresponding author.

**References**


Table

Table 1 is available in the Supplementary Files section

Figures
Figure 1

The neighborhood tree illustrates mtDNA sequence similarities between three Przewalski horses haplotypes (a) and the species which were selected as potentially most related to them (b) based on NCBI references.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- Datadepositioninformationitem.docx
- SupplementaryPrzewalskihorses.pdf