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Preliminary results of determining the mitochondrial genetic diversity of Ovodov horses in Southern Siberia

Kusliy M.A.[1]*, Malikov D.G.[2], Klementiev A.M.[3], Graphodatsky A.S.[1], Vorobieva N.V.[1], Molodtseva A.S.[1]

[1] *Institute of Molecular and Cellular Biology, SB RAS, Department of Diversity and Evolution of Genomes, Laboratory of Animal Cytogenetics, Novosibirsk, Russia*

[2] *V.S. Sobolev Institute of Geology and Mineralogy, SB RAS, Laboratory of Cenozoic Geology, Paleoclimatology and Mineralogical Indicators of Climate, Novosibirsk, Russia*

[3] *Institute of the Earth's Crust, SB RAS, Irkutsk, Russia*

** kusliy.maria@mcb.nsc.ru*

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Motivation and Aim: Published genetic data [1–4] on the Ovodov horse made it possible to expand the range of this species, identified by morphometric analysis results, to the northern regions of China and rejuvenate the time of extinction of *Equus ovodovi* to the middle Holocene, while the low degree of genetic diversity of the Ovodov horse Holocene population was identified as the main reason for the extinction. Since the sample of Ovodov horses from Siberia studied at the DNA level (2 samples) was small compared to Ovodov horses from China (21 samples), we expanded it and obtained almost complete mitogenomes (mitochondrial genomes) for 10 Late Pleistocene Ovodov horses from Southern Siberia. The main objectives of the study were to confirm the northern boundary of the range of the Ovodov horse (*Equus ovodovi*), identified on the basis of morphological data (56° north latitude), and to conduct a comparative analysis of the mitochondrial genetic diversity of the Ovodov horse populations of Siberia, China and other populations of wild and domestic species of the genus *Equus*.

Methods and Algorithms: All experiments were conducted in a special laboratory for ancient DNA research, in accordance with the basic authenticity criteria [5]. Ancient DNA isolation from bone samples was carried out according to the protocol described in the article by Vorobiova and co-authors [6]. Ancient DNA fragment libraries were obtained using TruSeq Nano DNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol with a few modifications: sample purification using MinElute PCR Purification Kit (Qiagen), 12 cycles of library amplification. Two rounds of library enrichment were performed based on a previously published method [7]. Paired-end sequencing of the enriched libraries was performed on the MiSeq platform (Illumina) using MiSeq v2 Reagent Kit (300-cycles, 2x150bp). The PALEOMIX BAM Pipeline v1.3.2 [8] was used to trim and collapse reads, align reads against the Ovodov horse mitogenome reference sequences, filter out PCR duplicates, and reassess base quality scores in accordance with the probability of post-mortem damage. Consensus mitogenome sequences were obtained using the bioinformatics software platform Geneious Prime v2024.0.4 with a set threshold of the 60 % highest quality. The phylogenetic tree was constructed using the program MrBayes v.3.2.6 [9] (8 million generations, sampling frequency – 1000, the first 25 % of trees were discarded) based on multiple mitochondrial genome sequence alignment performed in the MAFFT v1.4.0

program [10] and the best-fit partitioning schemes and models of molecular evolution defined in PartitionFinder v2.1.1 [11]. The final phylogenetic tree was visualized using the program FigTree v1.4.4. F_{ST} and nucleotide diversity values were obtained using an integrated software package Arlequin v3.5.2.2 [12].

Results: The graphs of nucleotide misincorporations and the average library fragment sizes for the studied Siberia Ovodov horse samples confirm the ancient origin of the investigated samples. Because the ND1 gene was incompletely assembled in many samples, we excluded it from the multiple alignment, resulting in 98–100 % coverage of the remaining mitogenome sequence for all samples, with an average coverage depth of 47,22 times. The belonging of the samples from Krasnoyarsk to *Equus ovodovi* species confirms the extreme northern border of this species range at 56° north latitude. Based on obtained by us mitogenome sequences of 10 Ovodov horses from Siberia and mitogenome sequences of 123 non-caballine and 122 caballine horses from nucleotide databases (GenBank, the European Nucleotide Archive), we carried out phylogenetic reconstructions. They showed that all the studied Ovodov horses of Siberia are located in clade B of the studied species in the phylogenetic tree, together with all Late Pleistocene and most of the Holocene Ovodov horses from China. The phylogeographic structure is noticeable, since the Ovodov horses of China and Siberia are located in different subclades of clade B, which indicates a certain degree of differentiation of the Ovodov horse populations of these regions. However, the alternation of these clades indicates the interpenetration of the mitochondrial gene pools of these populations during the Late Pleistocene. The F_{ST} analysis confirmed a moderate degree of differentiation between the populations under consideration, which may be due to the fact that the studied horses from China and Southern Siberia lived at opposite extremes of this species range, determined by genetic data. To obtain a more detailed phylogeographic structure of *Equus ovodovi* species, it is necessary to study samples from the central part of the range: the south of Eastern Siberia, Mongolia. We also determined that the value of nucleotide diversity of the studied populations of Ovodov horses in Southern Siberia and China is extremely low and close to that of the populations of wild Iberian horses of the Copper Age, historical quaggas and Ragusano donkeys of the island of Sicily [13, 14], geographically isolated or on the verge of extinction. This most likely indicates that the *Equus ovodovi* species has been endangered on the territory of Siberia and China since at least 40 kya. However, in China the species survived until the mid-Holocene. When, as for the territory of Siberia, we determined only the preservation of representatives of the species a little after the Last Glacial Maximum (end of the Pleistocene). Whether the species survived in Southern Siberia until the middle of the Holocene remains open to question. Perhaps the bone remains of Holocene Ovodov horses from Siberia have not yet been found. Also, such a difference may be due to features in environmental conditions, competition for resources with other species of horses with which their habitats overlapped (the wild horse). In order to find out the genetic diversity of the species under study during its heyday, it is necessary to study the Ovodov horse samples dating back to the period 84–74 kya, the time of its maximum population. However, these samples have not yet been discovered.

Conclusion: Our research showed that the range of the Ovodov horse (*Equus ovodovi*) in Siberia extended north to at least 56° north latitude. Conducted phylogenetic reconstructions and population genetic analysis showed a moderate degree of differentiation between the Ovodov horse populations of Siberia and China, which indicates a certain degree of geographic isolation of the extreme northwestern and

southeastern parts of the range. The mitochondrial genetic diversity we determined in the horse populations of Siberia and China shows that the species was on the verge of extinction since at least 40 kya. Whether the Ovodov horse species survived in Siberia until the mid-Holocene requires new expeditions and genetic research.

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