HORSE TEETH PROVIDE EVIDENCE OF ANCIENT DNA PRESERVATION IN SUNGIR AND A SNAPSHOT OF MTDNA DIVERSITY

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DNA was isolated from three horse teeth found in the most recent cultural level in the Sungir burial site. One tooth was radiocarbon dated to 18,255±310 years before present (Ua-14511). Fragments of the horse mtDNA hypervariable segment I (HVS-I) were amplified from all horse teeth. The Sungir sequences cluster with the modern horse haplogroups L, K, and B as well as with the Late Pleistocene horse mtDNAs from Northeast Siberia and Central Europe. The nucleotide diversity, pairwise difference, and θ_π values demonstrated that Sungir horses had higher diversity than other Late Pleistocene and modern horses. Our analysis proved that DNA is at least survived in some remains found in Sungir. These data along with the proximity of Sungir to the Pontic-Caspian steppeland favor the model of horse domestication in this limited geographic region.

Keywords: *Sungir, horse teeth, mitochondrial DNA*

Introduction

Recent successful genome sequencing of an Upper Paleolithic human from Malta [Raghavan et al., 2014] illustrated an opportunity to carry out analogous studies from other Upper Paleolithic remains found in Eurasia. Such studies are able to shed light on ancient migrations and relationship between populations as well as on possible gene flow between hominin lineages and modern humans [Reich et al., 2010, Raghavan et al., 2014].

Genome studies of ancient people involving DNA isolation from anthropological bones and teeth often result in damage of large bones [Krings et al., 1997] or complete destruction of bone fragments and teeth [Ovchinnikov et al., 1999; Ovchinnikov et al., 2000]. To diminish the negative consequences of destructive analysis on anthropological collections it is important to evaluate the likelihood of samples recovered from specific archaeological sites containing sufficient DNA for analysis prior to initiating ancient genome sequencing projects using valuable Upper Paleolithic human remains.

Animal remains are recognized as important supporting evidence of DNA survival in human remains with which they were associated [Cooper and Poinar, 2000]. Animal DNA was also studied from such archaeological sites as Vindija and Sima de los Huesos before actual analysis of the hominin remains by high-throughput sequencing methods [Hofreiter et al., 2002; Dabney et al., 2013]. Despite the extreme sensitivity of new genome sequencing technology, it is unlikely that many Upper and Middle Paleolithic human and hominin remains represent stable sources of authentic DNA [Serre et al., 2004]. In that situation, animal bones and teeth as well as dental and bone jewelry retain their significance in estimating DNA survival in anthropological remains with which they are associated prior to submission of valuable human or hominin remains for genome analysis.

We analyzed DNA from three horse teeth found in the closest proximity to the human remains in the

Sungir burial site. Sungir is an important Mid-Upper Paleolithic settlement located not far from Moscow on the East European Plain. The remains of up to eight humans have been found in this site. The two burials are among the most famous in paleoanthropology for the unique preservation of three human remains as well as the items of the Streletskaya material culture and industry [Bader, 1984; Alexeeva et al., 2000].

The survival of DNA in the Sungir human remains is questionable [Poltoraus et al., 2000; Ovchinnikov, Goodwin, 2003]. In this paper we explore an issue on DNA preservation in the Sungir burial site and, for the first time, provide strong evidence that authentic DNA can be isolated from animal material associated with the human remains.

Material and Methods

Three horse teeth obtained from different individuals were sampled from the most recent cultural layer of the Sungir burial site dated to 19,790±80 years before present (N.O. Bader, personal communication) and were tested for the preservation of ancient DNA (fig. 1). One sample was radiocarbon dated using the accelerator mass spectrometry method in Tandem Laboratory at the University of Uppsala (Ua), Sweden.

Ancient DNA extraction and DNA contamination control techniques were used to extract mtDNA from the teeth [Ovchinnikov et al., 2000]. Fragments of teeth were cleaned up using clean abrasive paper followed by soaking in a 10% solution of NaClO for 10 min, and further rinsing in molecular-grade water. Each side was UV-irradiated for 30 min. The tooth fragments were ground to fine powder using coffee grinders. The dental powder was incubated with 0.5M EDTA (pH 8) and 5% tween-20 in an incubation oven at +37°C for 48 hours. After that, proteinase K was added to the incubation solution to a final concentration of 0.1 mg/ml and the incubation continued additional 48 hours at the same temperature. The remains of undigested dental powder were removed by centrifugation. The supernatant was used for DNA extraction with phenol and chloroform. DNA in the aqueous phase was purified using Centricon-30 microconcentrators (Amicon).

DNA amplifications were performed in 50 μl of the PCR mixture according to the manufacturer's recommendations for AmpliTaq Gold polymerase (Life Technologies). Primers specific to the hypervariable segment I (HVS-I) of the horse mitochondrial DNA (mtDNA) were used (table 1).

The PCR fragments were purified from gel slices using the QIAquick Gel Extraction kit (Qiagen) and automatically sequenced in both directions using the Big-Dye Terminator sequencing kit (Life Technologies).

All experiments were independently performed in two laboratories free from the genetic study of modern horses and other close mammal species and included both negative DNA extraction and PCR controls.

MEGA 5.2 was used to align the Sungir mtDNA sequences together with the mtDNA HVS-I sequences of 34 modern horses belonging to 18 major haplo-

Figure 1. Horse tooth found in the most recent cultural layer in the Sungir burial site

Table 1. Primers used for PCR amplification of the horse mtDNA HVS-I

Primer	3' primer coordinate	Primer sequence (5' to 3')
1F	15,443	ACCATCAACACCCAAAGCT
1R	15,561	CATAGGCCATTCATAAGATAT
2F	15,564	ACCCACCTGACATGCAATAT
2R	15,683	CTGGAAATGATTTGACTTGG
3F	15,686	TCGTGCATACCCCATCCAA
3R	15,843	CCTGAAGAAAGAACCAGATG

Table 2. Single nucleotide polymorphisms in the Sungir horse mtDNA sequences. All the sequences were compared with the horse mtDNA reference sequence (GenBank accession number X79547). A full stop indicates the nucleotide is the same as in the reference sequence X79547

	15486	15494	15495	15520	15534	15585	15602	15635	15649	15650
X79547	A	T	T	A	C	G	C	C	A	A
Sungir-1	T	.	C	G	.	A	T	T	.	G
Sungir-2	.	C	C	.	T	.	.	.	.	G
Sungir-3	.	.	C	.	T	.	T	.	G	.

groups based on the whole mitochondrial genomes and randomly selected from a group of DNA sequences having the accession numbers of JN398377–JN398457, seven modern horses representing haplogroups based on HVS-I (Batosta, Belgium, Capriola, Cheju, Kazakh, Tsushima, Yunnan), two Przewalski's horses (Przew and Prezw), the horse mtDNA reference sequence with accession number X79547, eight Late Pleistocene horses from Alaska (Pleist 1–8), 14 Late Pleistocene horses from Northeast Siberia (Pleist 9–22), and three Late Pleistocene horses from Hohlefels, Petersfels, and Vogelherd IV sites in Germany dated to 12,550–13,845 years before present (Pleist 23–25) [Xu, Arnason, 1994; Vila et al., 2001; Weinstock et al., 2005; Cieslak et al., 2010; Achilli et al., 2012]. The alignment was used to produce phylogenetic trees using the neighbor-joining method with MEGA 5.2 and the Bayesian method with MrBayes 3.1.2 [Ronquist, Huelsenbeck, 2003; Tamura et al., 2011]. To build the neighbor-joining tree, the Kimura-2 model of DNA sequence evolution and the default parameters with 1.000 bootstraps were used. For the Bayesian tree, the general time reversible (GTR) model of nucleotide evolution with gamma-distributed rate variation across sites and a proportion of invariable sites was utilized.

Internal diversity indices of the horse populations were calculated in accordance with the Kimura-2 model of DNA sequence evolution, a transition to transversion ratio of 10:1, and an alpha value of 0.12 using Arlequin 3.5 [Excoffier, Lischer, 2010].

Results

One of the Sungir horse teeth was directly dated to 18,255±310 (Ua-14511) years before present that falls within the Last Glacial Maximum that occurred in Europe 22,000–14,000 years ago (URL: http://www.esd.ornl.gov). DNA was extracted from all three horse teeth, amplified and sequenced. In total, 223 bp of HVS-I mtDNA sequences from position 15,480 to 15,702 of the horse mtDNA reference sequence were retrieved from the Singir-1 and Sungir-2 horse teeth. The shorter sequence of 214 bp between positions 15,489 and 15,702 was obtained from the Sungir-3 tooth. The single nucleotide polymorphisms are shown in table 2.

The retrieved mtDNA sequences depicted, for the first time, the Late Pleistocene horse mtDNA sequences from eastern Europe. The BLAST search revealed that all three mtDNA sequences were not found in modern, medieval or Pleistocene Alaskan, Northeast Siberian or Central European horses. The Bayesian phylogenetic analysis (fig. 2) placed the Sungir-3 sequence as a sister clade to the horse mtDNA sequences belonging to haplogroup L [Achilli et al., 2012]. Due to the within-species homoplasy in the horse mtDNA HVS-I sequences, the Bayesian approach was unable to resolve the position of many horse mtDNA HVS-I sequences on the phylogenetic tree including the Sungir-1 and Sungir-2 sequences.

The neighbor-joining phylogenetic analysis confirmed the position of Singir-3 as a sister group to the L haplogroup sequences shown by the Bayesian analysis (fig. 3). The Sungir-1 sequence was a sister group to a cluster consisting of the haplogroup K sequence (JN398420) and the Pleistocene 16 sequence of unknown haplogroup from Northeast Siberia. The Sungir-2 sequence was closely related to the haplogroup B sequence (JN398391) forming a sister group to the Pleistocene 24 sequence of unknown haplogroup from the Petersfels site in Germany. Because we were able to compare only small sections of the horse mtDNA many tree nodes were supported by low bootstrap values; this has been seen in all other works on both ancient and modern horse mtDNA [Lippold et al., 2011].

The mtDNA diversity was compared between four groups of horses including modern horses, Late Pleistocene horses from Alaska, Late Pleistocene horses from Northeast Siberia, Late Pleistocene horses from three archaeological sites in Germany, and Late Pleistocene horses from Sungir (table 3). The values of nucleotide diversity, pairwise difference, and θ_π based on the mean number of pairwise differences showed that the Sungir horses had higher diversity than all other groups including the modern horses. Only the value of θ_S based on the observed number of segregating sites was higher in the modern horses than in the Sungir sequences due to larger number of DNA sequences sampled across all 18 contemporary haplogroups. Minimal genetic diversity was found among the Late Pleistocene horses that occupied Central Europe after the Last Glacial Maximum. The Late Pleistocene horses from Alaska and Northeast Siberia demonstrated low genetic diversity as well.

Discussion

Our analysis proved that DNA can be isolated and sequenced from the Late Pleistocene animal teeth found in the Sungir burial site. The BLAST search and the positions of the three Sungir horse mtDNA sequences on the phylogenetic trees consisting of modern and Pleistocene horse mtDNA sequences provided strong evidence for the authenticity of the mtDNA sequences. This result implies that it is feasible to retrieve ancient DNA sequences from anthropological remains found in Sungir.

Using the phylogenetic analysis, we found that the Sungir mtDNA sequences are related to three haplogroups including L, K, and B. Haplogroup L is most frequent among modern horses in Europe (38.1%) followed by the Middle East (22.4%) and Asia (13.5%). The L lineages were also found in ancient horses that lived from the Late Pleistocene to the Bronze Age in Europe and Asia. Haplogroup B today is most commonly found in the Middle East (10.9%) and Europe (9.4%) and has not been reported in ancient horses. The combined haplogroup J-K was detected in modern horses of Asia, the Middle East, and Europe with the frequencies of 6.5%, 3.7%, and 0.6%, respectively as well as in ancient horses in Asia and in Europe [Achilli et al., 2012].

The mtDNA diversity in the three Sungir sequences is higher than in modern horses except the θ_S value linked to the number of segregating sites.

Table 3. The mtDNA diversity statistics in the horse populations

Horse population	Pairwise difference	Nucleotide diversity	θ_S	θ_π
Modern horses	7.24±3.46	0.033±0.017	7.902	7.238
Pleistocene horses from Alaska	5.96±3.18	0.027±0.016	5.400	5.960
Pleistocene horses from Northeast Siberia	5.72±2.92	0.026±0.015	5.660	5.723
Pleistocene horses from Germany	4.93±3.28	0.022±0.018	4.000	4.925
Pleistocene horses from Sungir	7.73±4.97	0.035±0.028	6.000	7.729

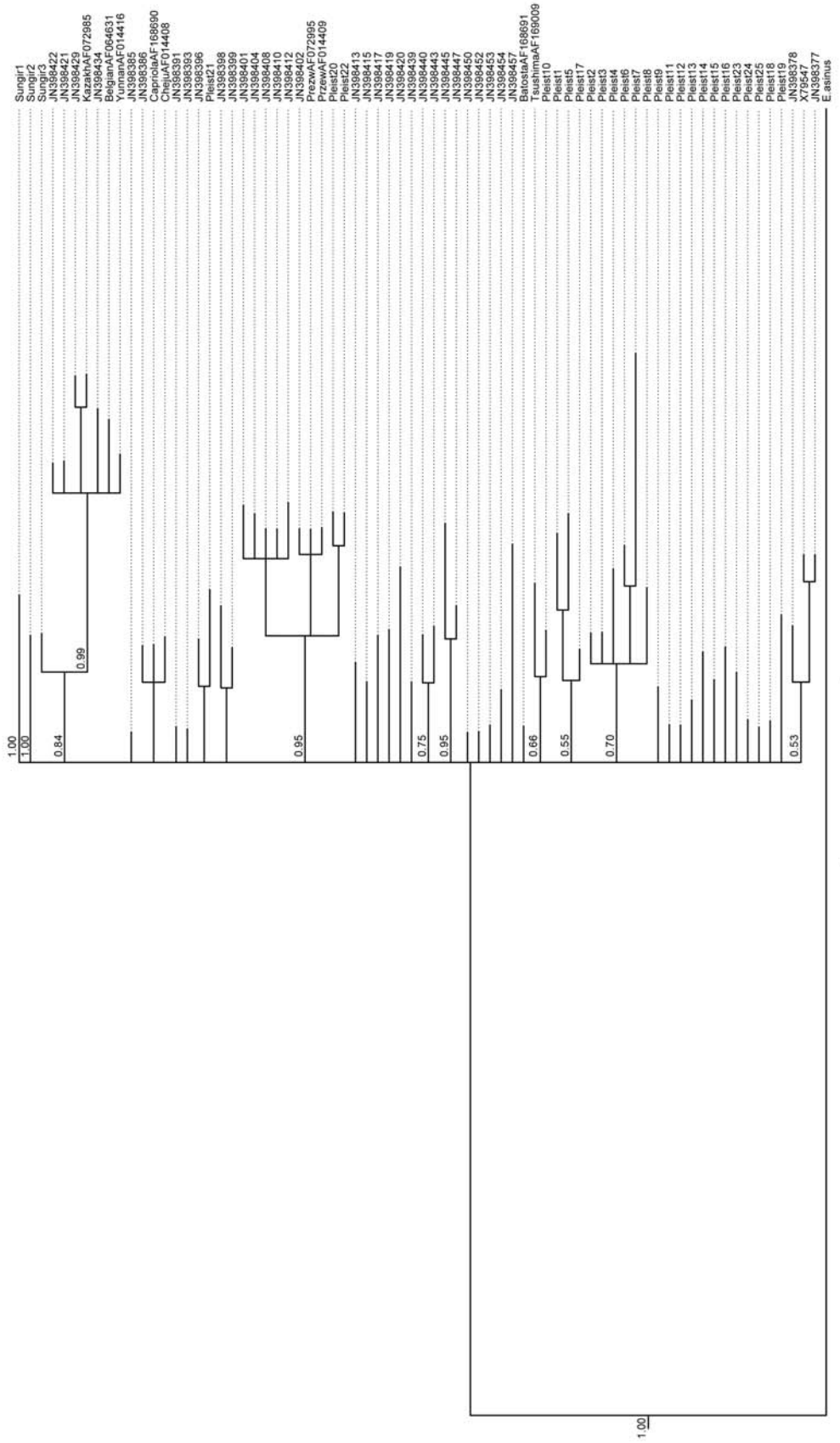

Figure 2. Bayesian phylogenetic tree shows the relationship of three Sungir mtDNA sequences with modern and Late Pleistocene horse mtDNA HVS-I. The modern mtDNA sequences are indicated by the GenBank accession numbers or the breed names. The Pleistocene mtDNA sequences are labeled as Pleist followed by a number. The root was attached to the branch leading to *Equus asinus*. The numbers refer to the posterior probability values. Branch lengths are drawn proportional to the amount of inferred changes

The genetic variation in both groups is substantially larger than in the Late Pleistocene horses from Alaska, Northeast Siberia, and Central Europe. The difference in the horse genetic diversity between Sungir and other Late Pleistocene sites and the similar genetic diversity of Sungir and modern horses taken together with the phylogenetic position of the Sungir sequences among the modern horse mtDNA sequences may have been explained by the existence of substantial number of diverse horse mtDNA lineages during the Last Glacial Maximum in eastern Europe not far away from the the Pontic-Caspian steppes where horse domestication started 12,000 years later [Kelekna , 2009].

Genetic variation in mtDNA HVS-I of domestic horses showed an unusual phylogenetic arrangement compared to other domesticated herbivores characterized by the lack of phylogeographic and breed-based patterns and insufficient statistical support with most of the nodes remaining unresolved [Vila et al., 2001; MacHugh, Bradley, 2001; Jansen et al., 2002]. Such phylogenetic reconstruction may be explained by the retention of ancestral mtDNA lineages during domestication in multiple geographic regions [Vila et al., 2001] or repeated introgression of local wild animals into spreading domestic herds from the core area of domestication [Warmuth et al., 2012]. Archaeological and zoological records suggested that horses were domesticated about 6,000 years before present in the Pontic-Caspian steppeland to the north of the Black Sea and the Caspian Sea [Bökönyi, 1974; Clutton-Brock, 1992; Anthony, Brown, 2000; Kelekna, 2009]. Around this time, wild horses had disappeared from Europe west of the steppes [Bökönyi, 1978; Drews, 2004] thus excluding western Europe from the process of initial horse domestication. Recent genetic data also supported this conclusion, finding strong evidence that horse domestication was initiated in the western Eurasian steppe corresponding to the Pontic-Caspian steppeland followed by the spread of domestic herds from the epicenter of domestication involving extensive introgression of local wild horses [Warmuth et al., 2012].

The alternative theory of multi-regional domestication originally found its support in a low mtDNA diversity in Pleistocene Alaskan horses [Vila et al., 2001]. However, it is clear today that the Alaskan horses cannot legitimize the multi-regional model due to the position of most of their mtDNA sequences in a sister group to all modern horse mtDNAs, their extinction during the Last Glacial Maximum, and the location of their range being too distant from the core area of domestication in Eurasia. In contrast, the mtDNA sequences from the Late Pleistocene horses

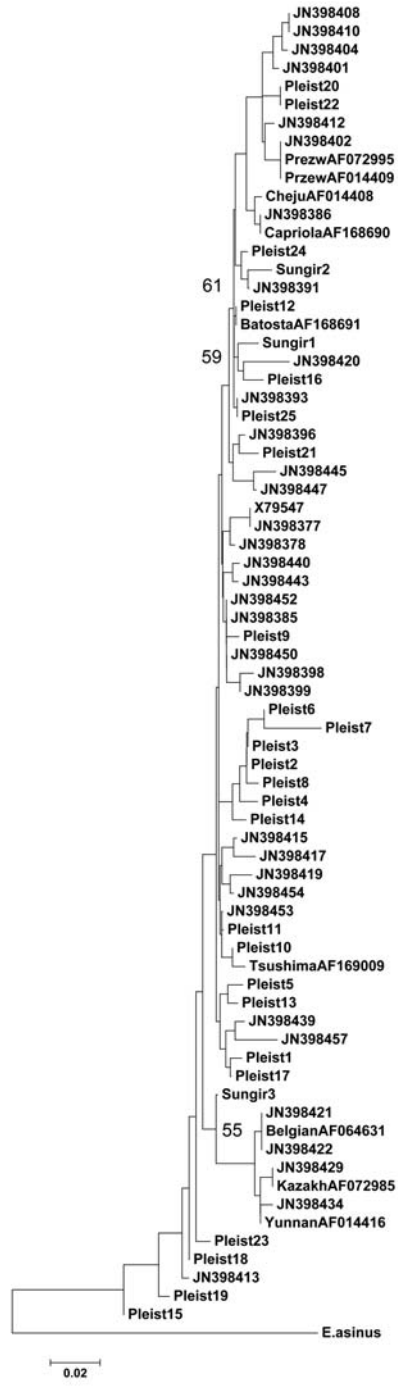

Figure 3. Neighbor-joining phylogenetic tree shows the relationship of three Sungir mtDNA sequences with modern and Late Pleistocene horse mtDNA HVS-I. The modern mtDNA sequences are indicated by the GenBank accession numbers or the breed names.The Pleistocene mtDNA sequences are labeled as Pleist followed by a number. The root was attached to the branch leading to *Equus asinus*. Only the nodes have been indicated that supported by the bootstrap values of 50% or higher. The numbers refer to the bootstrap percentage for the tree's nodes leading to the Sungir horse mtDNA sequences. Branch lengths are drawn proportional to the amount of inferred changes

of Europe and Asia may represent a tool to decipher the process of horse domestication because their mtDNA haplotypes cluster with the modern mtDNA haplogroups, demonstrating that the first domesticated horses are descendants of the Late Pleistocene horses. Based on the Sungir mtDNA sequences alone, we cannot reject the hypothesis that the horse was domesticated at several sites at several occasions as it was proposed in 2001 [Vila et al., 2001], although our data favor the hypothesis that horses were domesticated in limited geographic region [Lister, 2001]. Further studies of the mtDNA diversity in horses which had flourished in the steppe belt of Eurasia after the retreat of the ice sheets caused by the last glacial period [Mallory and Adams, 1997] as well as in horse remains found in numerous kurgans in the Pontic-Caspian region would be of crucial significance for the comprehensive understanding of the history of horse domestication. Such study has never been done.

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СОХРАННОСТЬ И ПОЛИМОРФИЗМ ДРЕВНЕЙ ДНК ИЗ ЗУБОВ ЛОШАДЕЙ, НАЙДЕННЫХ НА ВЕРХНЕПАЛЕОЛИТИЧЕСКОЙ СТОЯНКЕ СУНГИРЬ

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ДНК была выделена из трех лошадиных зубов, найденных в наиболее позднем культурном слое верхнепалеолитической стоянки Сунгирь. Возраст одного из зубов, определенный радиоуглеродным методом, – 18255 ± 310 лет (Ua-14511). Участки гипервариабельного сегмента 1 митохондриальной ДНК лошади были получены из всех трех зубов. Последовательности митохондриальной ДНК из сунгирьских лошадей близки филогенетически к митохондриальной ДНК современных лошадей, принадлежащей к гаплогруппам L, K и B, а также лошадей позднего плейстоцена из Северо-Восточной Сибири и Центральной Европы. Значения нуклеотидного разнообразия, различия между парами последовательностей ДНК и θ_π показали, что лошади из Сунгиря имели большее генетическое разнообразие, чем другие группы лошадей позднего плейстоцена и современные лошади. Наш анализ доказал, что ДНК сохранилась в некоторых останках, найденных в Сунгире. Полученные данные и относительная близость Сунгиря к причерноморско-каспийским степям подтверждают гипотезу одомашнивания лошадей в этом небольшом географическом регионе.

Ключевые слова: *Сунгирь, зубы лошадей, митохондриальная ДНК*