Six complete mitochondrial genomes from Early Bronze Age humans in the North Caucasus

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ABSTRACT

The North Caucasus region is rich in early Bronze Age sites, with burials yielding many artifacts, including those from the Chekon, Natukhaevskaya, Katusvina-Krivitsa kurgan groups (at Krasnodar Krai, Russia) and Klady kurgan (near Novosvobodnaya Village, Republic of Adygea, Russia). According to the mainstream archaeological hypothesis, these sites belong to the Maikop culture (3,700-3,000 years BC), with Novosvobodnaya communities representing an offshoot of Maikop ancestry. However, due to specific differences in Novosvobodnaya artifacts, the Maikop and Novosvobodnaya assemblages could represent two synchronous archaeological cultures living in almost sympatry but showing independent ancestry, from the Near East and Europe respectively. Here, we used target-enrichment together with high-throughput sequencing to characterize the complete mitochondrial sequence of three Maikop and three Novosvobodnaya individuals. We identified T2b, N1b1 and V7 haplogroups, all widely spread in Neolithic Europe. In addition, we identified the Paleolithic Eurasian U8b1a2 and M52 haplogroups, which are frequent in modern South Asia, particularly in modern India. Our data provide a deeper understanding of the diversity of Early Bronze Age North Caucasus communities and hypotheses of its origin. Analyzing non-human sequencing reads for microbial content, we found that one individual from the Klady kurgan was infected by the pathogen *Brucella abortus* that is responsible for zoonotic infections from cattle to humans. This finding is in agreement with Maikop/Novosvobodnaya livestock groups, mostly consisting of domestic pigs and cattle. This paper represents a first mitochondrial genome analysis of Maikop/Novosvobodnaya culture as well as the earliest brucellosis case in archaeological humans.

Keywords Novosvobodnaya site, the Maikop culture, ancient DNA, mitochondrial haplogroup, brucellosis

Introduction The Neolithic period and the beginning of Bronze Age represent an essential transitional period in the history of Europe colonization. Based on the archaeological data, there are several controversial hypotheses that describe Europe colonization. However, there are only few genetic/genomics studies of ethnical characteristics of Neolithic and Bronze Age human populations that can provide an additional source of information on the migration routes of European ancestors (Bramanti et al. 2009; Brandt et al. 2013; Chikhi et al. 1998; Haak et al. 2005; Skoglund et al. 2012). Moreover, recent study attributed most of present-day Europeans to at least three highly differentiated populations: west European hunter-gatherers, ancient north

Eurasians related to Upper Palaeolithic Siberians and early Middle East farmers who contributed to agriculture origin in Europe (Lazaridis et al. 2014).

To date, several studies about Europeans from Bronze Age were published but data about genetic diversity and mitochondrial haplotype presence of Caucasus Bronze Age populations are unknown (Allentoft et al., 2015; Haak et al. 2015; Gamba et al. 2014).

The Maikop culture was a main archaeological culture in the North Caucasus in the Early Bronze Age. It had several development stages and spanned the period of 3,700-3,000 years BC. First Maikop culture kurgans were excavated by Nikolay Veselovsky in 1897 near Maikop City (Republic of Adygea, Russia) (Rezepkin 2012). Since then, abundant archaeological material was found near Maikop City and the Caucasus and Black Sea Region (Fig. 1)

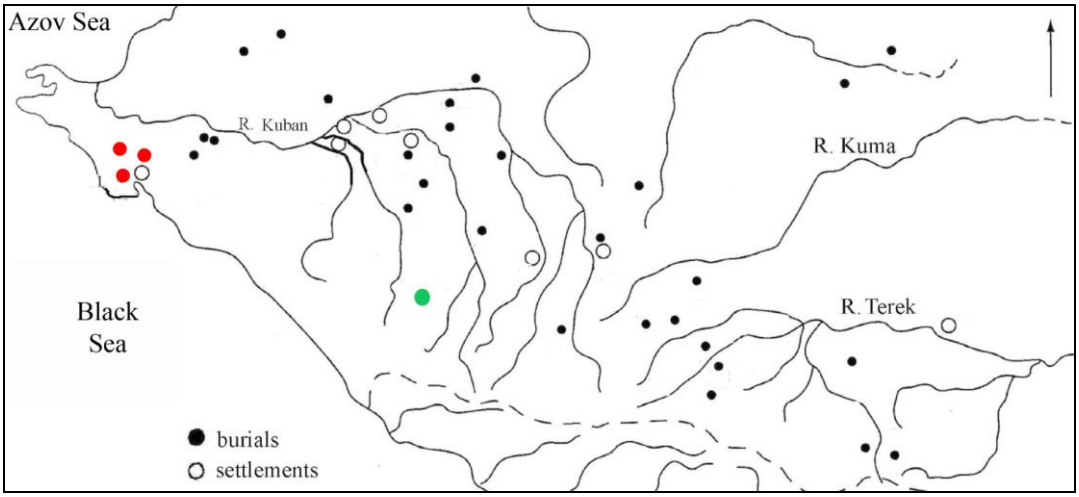

Fig 1. The North Caucasus Early Bronze Age significant archeological sites (modified after Rezepkin 2012). Red circles – Maikop culture burials (Natukhayevskaya Village, Katusvina Krivitsa-2 and Chekon), specimens from those were used in this study, black circles – other Maikop culture burials, white circles – Maikop culture settlements, green circle – Klady burials (Novosvobodnaya settlement), specimens from those were used in this study. (https://www.google.com/maps/d/viewer?mid=zGhBH1rfe2eE.kfpObKz6NZr0)

The mainstream archaeological view suggests the presence of only one Maikop culture/community in this area, which had Near Eastern cultural ancestry (Iessen, 1950; Munchaev 1975). At the same time, there is another hypothesis about the Western-European origin of the part of the Early Bronze Age Caucasus community based on the stratigraphic disposition of tombs in kurgans and artifacts found near Novosvobodnaya site (Republic of Adygea, Russia). Unlike the mainstream one, this hypothesis suggests the presence of the European cultural and potentially genetic flow to the North Caucasus at the turn of the Neolithic

and Bronze Age. Moreover, the Novosvobodnaya tombs could be the eastern wing in the development of the north central European gallery graves, which include the Funnel Beaker (TRB) culture (Rezepkin 2012).

In the past few years, the methods for ancient DNA extraction and analysis from archaeological material have been developed to allow retrieval of genomic information from a variety of sample types: hairs (Miller et al. 2008; Rasmussen et al. 2011), mummified tissue (Keller et al. 2012), calcified bones and teeth (Allentoft et al. 2012; Skoglund et al. 2012), and plant remains (Martin et al. 2013; Yoshida et al. 2013).

Moreover, millions or even billions of DNA sequences may be derived from ancient biological samples due to massive capacity of modern platforms for Next-Generation Sequencing (NGS) (Skryabin et al. 2009). Methods of modern genomics can be successfully applied to archaeological problems. The riddle of human migration in Europe at the turn of the Neolithic and Bronze Ages has become particularly attractive for archaeological genetics (Deguilloux et al. 2011; Haak et al. 2005; Izagirre and de la Rua 1999; Skoglund et al. 2012; Sykes 1999).

In the previous investigation, we applied the NGS approach to study ~5,000-year-old human remains from the Klady kurgan grave (Novosvobodnaya site). Sequencing of the mitochondrial (mt) DNA with 13.4x coverage enabled us to determine the mtDNA haplogroup for that individual as V7 (Nedoluzhko et al. 2014). In the current study, we aimed at investigating the Early Bronze Age ancient communities from the North Caucasus and establish their origins and development by combining archaeological and genetic data. Here, we present the pilot ancient mitochondrial DNA analysis of 6 individuals, who lived in the piedmont area of the North Caucasus at approximately 3,700 to 3,000 years BC. Further investigation may require a power of hundreds ancient mt genomes as well as nuclear genomic data to support the archaeological hypotheses of Europe colonization.

Ancient remains of our ancestors provide information about the *Homo sapiens* origin, migration history and even diseases. For instance, deep sequencing of ancient strains of tuberculosis and plaque revealed important pieces of information about the origin of European epidemics throughout the centuries (Dabernat et al. 2014; Mutolo et al. 2012; Nguyen-Hieu et al. 2010; Wagner et al. 2014). However, molecular identification of historic pathogens can be complicated by limited information in public genetic databases and diversity of soil nonpathogenic contaminants that leads to false positive results in genetic analysis (Campana et al. 2014).

Brucellosis is an important livestock and human disease in many parts of the world. According to the WHO reports, brucellosis keeps patients from normal activity, thus, making it one of the major economic and medical problems in developing countries.

Based on several studies, *Bruccella*-induced diseases were common in Antiquity (Bendrey et al. 2008; Capasso 1999; Kousoulis et al. 2012; Papagrigorakis et al. 2006; Shapiro, Rambaut and Gilbert 2006) and the Middle Ages (Isidro 2009; Mutolo et al. 2012). For the first time, we report a case of *Brucella abortus* in ancient human remains from the early Bronze Age North Caucasus using the ancient DNA analysis. This finding suggests that brucellosis affected early farmers in Europe, at least in the Caucasus.

Material and methods

Samples

Ten ancient human bones and teeth were collected for analysis. Human bones from the burials of the Maikop and Novosvobodnaya sites were used for ancient DNA (aDNA) analysis (which were only successful for five specimens, see below) (Supplementary 1). Excavations were conducted by A. Rezepkin's group (the burial beneath Klady near Novosvobodnaya Village, Republic of Adygea, Russia, during the expedition of the Institute of History of Material Culture of Russian Academy of Sciences), and by A. Shishlov's group of the Novorossiysk Historical Museum (in the kurgan group Natukhaevskaya-3 (Fig. 2) and the sites Katusvina Krivitsa-2 near Novorossiysk, Krasnodar Krai, Russia). The remains were dated from 3,700 to 3,000 years BC using radiocarbon analysis. Different biological samples excavated from burial beneath Klady, Katusvina Krivitsa-2 and Chekon from the same stratigraphic horizon as well as cultural artifacts in burials were used to support age determination (Gei and Zazovskaya 2013; Rezepkin 2012; Shishlov et al. 2009; Shishlov et al. 2015; Trifonov 2004) (Table 1).

Fig. 2. Skeletal remains in the kurgan burial in Natukhaevskaya-3 (Krasnodar Krai, Russia). Maikop culture

DNA extraction and sequencing

Ancient DNA was extracted from bone powder in the aDNA facilities from the Centre for Geogenetics (University of Copenhagen, Natural History Museum of Denmark), following the methodology described in Orlando et al., 2013. DNA-libraries were prepared using a NEB Next Quick DNA Library Prep Master Mix set for 454 (New England Biolabs, UK) with adapter primers based on Illumina Sequencing Platform following Der Sarkissian et al. 2015. Amplified DNA libraries were quantified using a high-sensitivity chip on a 2100 Bioanalyser instrument (Agilent, USA). Amplified DNA libraries were enriched for their mitochondrial content using the FleXelect Mitochondrial DNA enrichment kit (Flexgen, Netherlands), using probes overlapping across 10 to 40% of their sequence length (a detailed list of the oligonucleotide probes for mtDNA enrichment is available upon request). DNA-libraries were sequenced using single-end and paired-end reads with different length on the Illumina platform (Supplementary 2, 3)

Ancient DNA analysis and microbial profiling

Sequencing reads were processed through PALEOMIX (Schubert et al. 2014), mapping was done against the mitochondrial reference sequence (Genbank Accession Nb. NC _012920.1) using Bowtie 2 under the "*very*-sensitive" and "*rescale*" options. We used mapDamage2 (Jonsson et al. 2013), as implemented in PALEOMIX, to model post-mortem DNA damage from

nucleotide mis-incorporation patterns for each individual library. We then used such models to downscale base quality scores according to their probability of being DNA damage by-products in order to reduce the impact of nucleotide mis-incorporations in downstream analyses. Positions showing sequence variants were called using the VarScan software (v 2.3.5) and a *p-value* of 0.01 (Koboldt et al. 2012). The mitochondrial haplogroups were determined based on the SNPs with the HaploGrep web tool (Kloss-Brandstatter et al. 2011).

To exclude modern reads from ancient DNA samples, we used Pmdtools (Skoglund et al. 2014). All samples were passed through pmd tools with threshold that equals at least to 0. In situation when we faced with conflicting SNPs (SNPs that are belong to different haplotypes), we used SNPs with lower *p-value* or used pmdtools with more stringent threshold (up to three).

We used contamMix-1.0.10 kindly provided by Dr. Philip Johnson in order to estimate of contamination levels in mitochondrial data. This software implements the procedures described in Fu et al. 2013, estimating contamination from the fraction of target mitochondrial DNA sequences that match any genome from a comparative panel (here, a worldwide set of 311 mitochondrial genomes) better than the consensus NC _012920.1 Table 1). In order to conduct principal component analysis (PCA) of our samples with ancient and modern samples, we used 101 Bronze Age samples (Allentoft et al., 2015) and a set of 311 worldwide mitochondrial genomes (Fu et al. 2013). For PCA plot construction, we used R package "bios2mds" version 1.2.2.

To profile microbial communities, we used MetaPhlAn tool (Segata et al. 2013) with bowtie2 parameter *very-sensitive.* To confirm that reads are relevant to Brucella pathogen, DNA reads, which were filtered using PALEOMIX, also were mapped on *Brucella abortus* genome (NC_016795.1) following the methodology described in Skoglund et al., 2014.

1

2

3 Table 1. Material description and summary of mtDNA haplogroup analysis from Early Bronze Age North Caucasus samples

Sample ID	Sample description	Sample origin, also see Fig. 1	Archaeological description	GPS coordinates	Laboratory mark	^{14}C interval based on available data	Mitochondrial haplogroup (rCSR)	Contamination (contamMix), %	Coverage of mtDNA, X
1	tooth	Klady, Kurgan 11, Grave 4, near Novosvobodnaya settlement, Republic of Adygea, Russia	Burial without archaeological artifacts	$44^0$229 N, $40^0$249 O	Dates of burials from the same stratigraphic horizon were used	3700 – 3300 BC (Rezepkin 2012; Trifonov 2004)	T2b	3.1	7,7664
2	tooth and bone	Klady, Kurgan 25, Grave 1, near Novosvobodnaya settlement, Republic of Adygea, Russia	Burial with Maikop culture artifacts	$44^0$229 N, $40^0$249 O	Dates of burials from the same stratigraphic horizon were used	3700 – 3300 BC (Rezepkin 2012; Trifonov 2004)	M52	2.6	32,1150
3	tooth	Klady Kurgan 23, Grave 1, near Novosvobodnaya settlement,	Novosvobodnaya site, with specific Novosvobodnay	$44^0$229 N, $40^0$249 O	Dates of burials from the same stratigraphic	3700 – 3300 BC (Rezepkin 2012; Trifonov 2004)	V7	2.8	13,4358

		Republic of Adygea, Russia	a artefacts		horizon were used				
4	lower tooth	Kurgan 2, Burial 1, Natukhaevskaya-3, near Novorossiysk city, Krasnodar Krai, Russia	Burial with Maikop culture artifacts	$44^{0}53$ N, $37^{0}33$ O	ЛЕ-9715	4000-3000 BC	U8b1a2	3.9	10,9832
5	tooth with fragment of jaw	Katusvina Krivitsa-2, near Novorossiysk city Krasnodar Krai, Russia	Burial without archaeological artifacts	44°54 N, 37°32 O	Dates of burials from the same stratigraphic horizon were used	3700 – 3300 BC (Rezepkin 2012; Trifonov 2004)	N1b1	4.2	53,8832
6	tooth with a jaw fragment	Chekon, near Anapa city, Krasnodar Krai, Russia	Burial with Maikop culture artifacts	Data not yet	Dates of burials from the same stratigraphic horizon were used	3700 – 3300 BC (Gei and Zazovskaya 2013)	U8b1a2	5.4	32,5017

Results

We used target-enrichment coupled with high-throughput Illumina DNA sequencing to retrieve mitochondrial DNA sequences for 5 out of 10 Early Bronze Age individuals from the North Caucasus (two from Novosvobodnaya burials and three from Maikop culture archaeological sites). We also included the complete mitochondrial genome from a Novosvobodnaya sample (Klady Kurgan 23, Grave 1) individual, that was characterized in the previous study (Nedoluzhko et al. 2014). A total of 518,556,514 sequencing reads from 5 enriched libraries were generated. Despite enrichment, most sequencing reads consisted of environmental (bacterial) DNA sequences (Supplementary 2), a recurrent problem in ancient DNA analyses (Green et al. 2010). Following read alignment against the reference mitochondrial genome sequence and stringent quality filters, we analyzed the complete mitochondrial genome sequence from five ancient individuals at an average depth-of-coverage of 7.7X to 53.8X (Supplementary Table 2).

Ancient DNA is known to degrade into short fragments over time; cytosine residues (C) located at the ends deaminate to uracil (U) and turn into thymine (T) during sample preparation (PCR). The frequency of terminal C → T substitutions in samples dated older than 300 thousand years could be up to 60% and higher (Orlando et al. 2013).The substitution frequency was calculated using MapDamage 2.0. The frequency of C → T substitutions at the 3'- and 5'-ends of the DNA libraries exceeded 20 - 30% in the samples that were in the pipeline (Supplementary 4).

Here we report the results of five mtDNA genomes sequencing of ancient humans that lived during the Early Bronze Age in the North Caucasus (archaeological sites are dated about 3,700 - 3,000 BC). The SNPs revealed during the analysis (Supplementary 8) indicate that mtDNA of three samples from Novosvobodnaya belongs to haplogroup V7 (Nedoluzhko et al. 2014), T2 and M52, and that one of Maikop samples belongs to haplogroups U8 (in two specimens) and N1 (Table 1). Our samples were analyzed using Principal Component Analysis (PCA) with previously sequenced Bronze Age (Allentoft et al., 2015) and modern mtDNAs demonstrating that five out of six North Caucasus individuals clustered with individuals of the Bronze Age European cultures (Supplementary 5).

In addition, the bacterial profile of the Early Bronze Age North Caucasus samples was analyzed using MetaPhlAn (Segata et al. 2012). Most bacteria were typical for soil, with exception of *Brucella abortus* that was identified in the ancient remains from Novosvobodnaya (Table 2; Figure 3; Supplementary 6). MapDamage 2.0 and phylogenetic analysis based on of

our sequencing data is shown in Fig. 4 and Supplementary 7. The phylogenetic analysis confirmed that ancient human from Novosvobodnaya suffered from brucellosis.

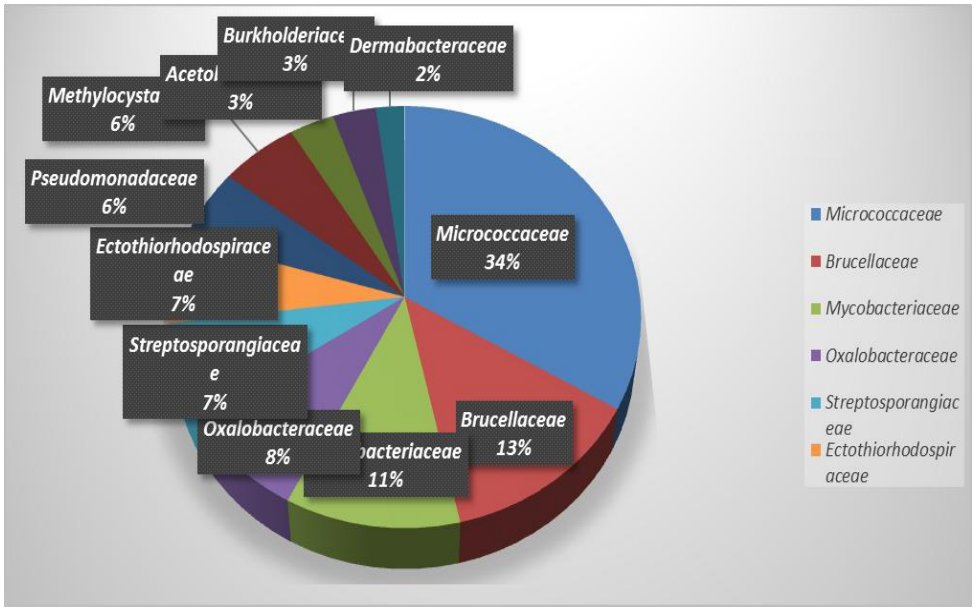

Fig 3. Microbial profiling of Novosvobodnaya (Kurgan 25, Grave 1) bones using MetaPhlAn

DNA reads, which were filtered using PALEOMIX, were also mapped on *B. abortus* genome to confirm that the reads are indeed relevant to Brucella pathogen. From 797 to 1757 reads were mapped on *B. abortus* genome depending on threshold value (0-3). Consistent with its ancient origin, *B. abortus* reads were highly fragmented, with average read lengths of 51–75 bp, and displayed clear signs of C→T deamination damage at the 50 termini, typical to ancient DNA (Figure 4).

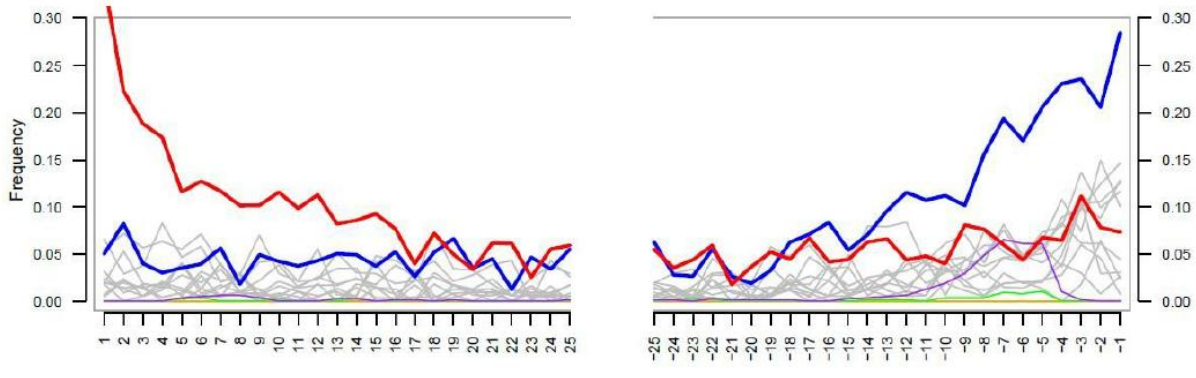

Fig. 4. Postmortem DNA damage patterns in the *Brucella* reads

This brucellosis case is the earliest one found among *Homo sapiens* using ancient DNA analysis. Our findings suggest that brucellosis affected early farmers in Europe, at least in the

Early Bronze Age of the North Caucasus. It is possible that brucellosis could be a common disease in ancient human populations.

Table 2. Microbial profiling of Novosvobodnaya (Kurgan 25, Grave 1) bones using MetaPhlAn with very sensitive local (VSL) parameters and with/or without duplicates (D/ND). MetaPhlAn score is percent of the abundances in each clade

Sample origin	VSL-ND	MetaPhlAn score	VSL-D	MetaPhlAn score
Kurgan 25, Grave 1. Novosvobodnaya, Republic of Adygea, Russia	*Brucella_abortus*	1.13782	*Brucella_abortus*	1.09073
	Conexibacter_woesei	1.0632	*Conexibacter_woesei*	1.05678

Discussion

The Early Bronze Age cultures in the North Caucasus got involved in the orbit of Near Eastern civilizations and were directly related to the events occurring in Mesopotamia and the Eastern Anatolia regions (Munchaev 1975; Rezepkin 2012).

The Chalcolithic Eastern Anatolia and Uruk were among the most important cultural areas in the fourth millennium in the Near Eastern region. The mainstream archaeological opinion proposes that the origin of the Maikop culture sites is directly connected to the expansion of the Late Chalcolithic societies of Eastern Anatolia to the North Caucasus. According to the archaeological evidences, this was the first expansion in the North Caucasus (Munchaev 1975).

The second cultural impulse probably came at the time when Uruk was in the middle stage of its development in the Near East spreading far to the north and coming into the contact with the Late Chalcolithic that had an effect on the sites and artifacts in the North Caucasus. At the same time, according to some authors, there were also several penetration events to the North Caucasus from Western Europe (Funnel Beaker culture) that introduced megalithic features to the burial ceremony and a number of ceramic artifacts (Rezepkin 2012). The proposed Western European influence on the Early Bronze Age in the North Caucasus is still under discussion.

The third stage of North Caucasus culture formation was directly associated with Uruk expansion to the North Caucasus steppes (Rezepkin 2012).

By the middle of 2016, several mitochondrial haplogroups of Paleolithic and Mesolithic Europeans were identified using ancient DNA analysis. Most of them are members of mtDNA haplogroup U (U2, U4, U5a, U5b, and U8) and M (Posth et al. 2016). Neolithic revolution and expansion of Neolithic farmers to Europe in 7,000 – 6,000 BC carried new mtDNA lineages with a generally higher diversity (e.g., H, HV, V, K, J, T2, X, W, N1a). As a result, mtDNA haplogroup U eventually became very rare in Central Europe (Brandt et al. 2014) and haplogroup M totally disappeared in Europe during Last Glacial Maximum (Posth et al. 2016). Interestingly, known mtDNA haplogroups described for Pontic steppe Yamnaya culture (Early Bronze Age) are U, T2, H, W (Allentoft et al. 2015; Haak et al. 2015)

In our study, we for the first time investigated genomic diversity of Early Bronze Age ancient cultures of the North Caucasus to better understand mtDNA ancestry of peoples who lived in the region. We identified several mitochondrial haplogroups distributed in Paleolithic and Neolithic Europe. We suggest that haplogroup U, found in the samples from the Maikop burials near Chekon and Novorossiysk, is a legacy of Upper Paleolithic hunter-gatherers that lived in Europe and adjacent regions 40–10 ka.

In addition, Neolithic mtDNA haplogroups were identified in Novosvobodnaya and Natukhaevskaya burials. There are several archaeological hypotheses about the origin of North Caucasus Early Bronze Age cultures, and one of them considers the European cultural gene flow in this region, particularly, of the Funnel Beaker Culture (TRB) (Rezepkin 2012). Our results support the influence of Neolithic cultures on North Caucasus societies but prove neither European nor Middle Eastern origin of these cultures.

Humans that originally migrated from Africa to Eurasia had L3 mtDNA haplogroup, which gave rise to the two basal non-African clades, haplogroups M and N approximately 63,000 ka ago (Wallace, Brown and Lott 1999). Surprisingly, one sample discovered near Novosvobodnaya had mitochondrial haplogroup M52. Recent investigations, which were conducted on European Paleolithic ancestry, unexpectedly identified mtDNA lineage M in individuals prior to the Last Glacial Maximum (LGM). Today, this lineage is absent in Europeans, although it is found at high frequency in modern Asians, particularly in modern India, Australasians, and Native Americans. (Eaaswarkhanth et al. 2010; Macaulay et al. 2005; Posth et al. 2016).

Our mtDNA analysis suggests that North Caucasus Early Bronze Age cultures could spread to the North Caucasus during the Early to Middle Neolithic, when Near East societies began their expansion to Europe. Another presumption is that progeny of Near East Neolithic farmers colonized North Caucasus during the Chalcolithic and Uruk periods. Some archaeological artifacts and burial ceremony propose European "footprints" in Caucasian cultures (Rezepkin 2012) but the mtDNA analysis didn't clearly support this assumption. The Early Bronze Age Caucasus was a huge "population shaker" with archaeological data available to study North Caucasus Early Bronze Age cultures origin. However, a whole-genome DNA sequencing or analysis of the hundreds ancient mt genomes of Novosvobodnaya and Maikop remains may be required to resolve this archaeological puzzle.

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