

## Mitochondrial DNA analysis of ancient sheep from Altai

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### Summary

A comparative analysis of the genetic diversity of ancient and modern sheep can shed light on the origin of these animals and their distribution as well as help to evaluate the role of humans at each formation stage of different sheep breeds. Here we isolated ancient DNA and performed sequencing of the mitochondrial DNA D-loop from 17 sheep bone remains (~4000–1000 years old) found in the archaeological complexes in the south of Altai (Western Siberia). The length of the sequences obtained ranged between 318 and 586 bp. The haplotype diversity and nucleotide diversity were  $0.801 \pm 0.081$  and  $0.0096 \pm 0.0014$  respectively. The average number of nucleotide differences was ~3.1. Nucleotide sequence analysis revealed that 15 specimens were nested within previously described A,B,C,D and E lineages and that two specimens had a basal position relative to the rest of the analyzed samples. A relatively high diversity of sheep haplotypes, including the presence of two basal haplotypes, indicates that the Altai region may have been a transport route of human migration. Further ancient DNA analysis of other specimens and deeper genome sequencing of samples with novel haplotypes is needed to better understand the demographic history of sheep in Southern Siberia.

**Keywords** aDNA, archaeological remains, domestication, haplotyping, lineage, *Ovis aries*, sequencing, Western Siberia

The sheep (*Ovis aries*) is one of the early domesticated animals that played an important role in the life-support system of ancient societies. Different sheep breeds spread throughout Eurasia and other continents due to migration and multifaceted interactions among human groups. It is assumed that sheep were domesticated around 9–11 thousand years ago in the area of the Fertile Crescent (Demirci *et al.* 2013). The subsequent husbandry of these animals was accompanied by natural and intense artificial selection and led to over 1400 breeds (Scherf 2000). During that time, human activity played a remarkable role in the transfer of certain genes associated with the formation of necessary economically useful traits. A comparative analysis of the genetic diversity between ancient and modern sheep can shed light on the origin of these animals and their distribution and also help in evaluating the role of human

beings at each formation stage of different sheep breeds (Kijas *et al.* 2012).

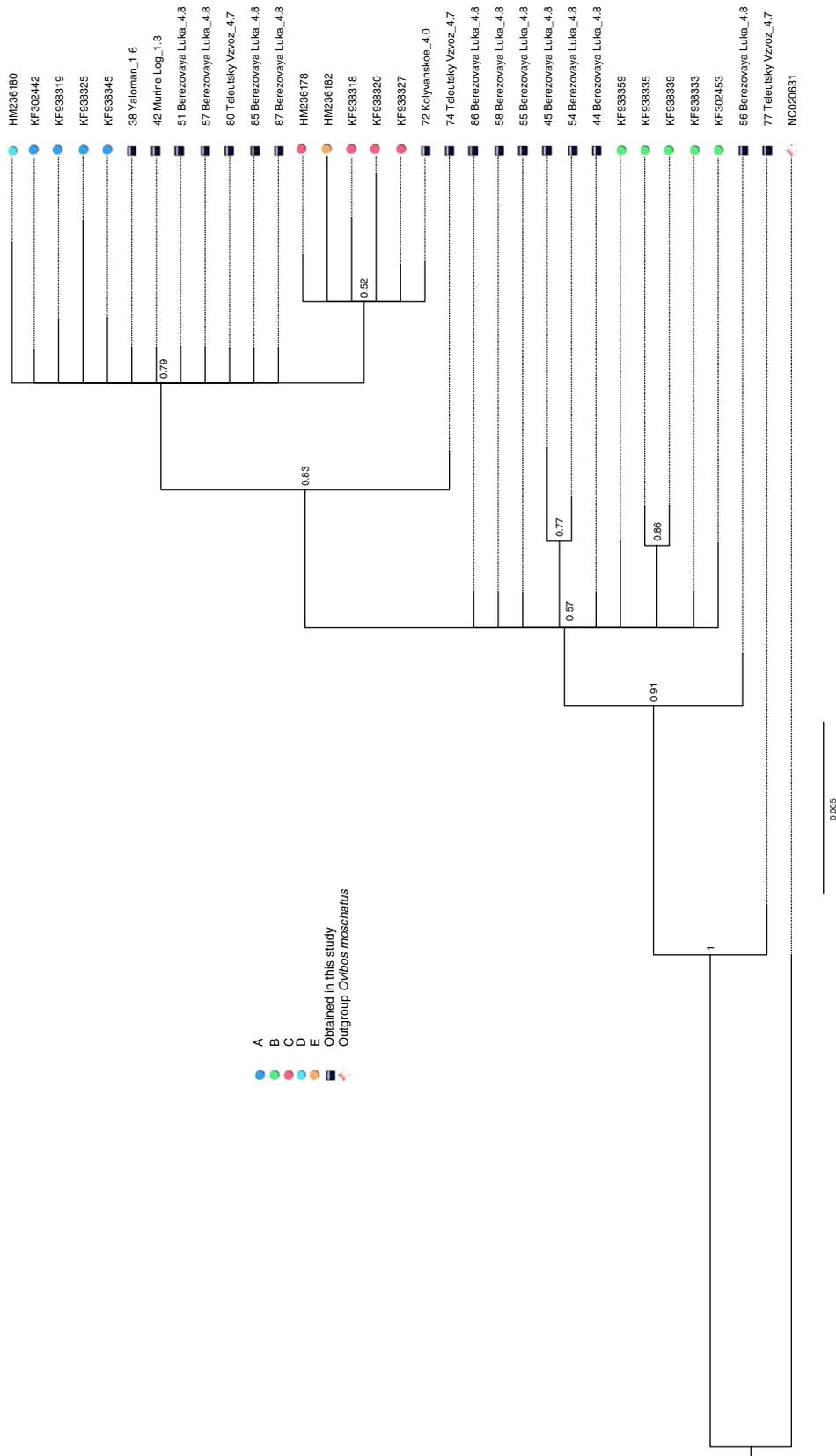
The Mongolian Plateau Region played a significant role in the 'transport interchange' of migratory flows five to seven thousand years ago (Lv *et al.* 2015). Therefore, archaeological sites located in this and adjacent areas may contain important information for understanding the origin and demographic histories of different populations of domestic sheep. They also allow for observing the possible pathways of animal spread to determine if the ancient herdsmen used routine selection methods to create the most productive meat, dairy and wool sheep breeds (Chessa *et al.* 2009; Rannamäe *et al.* 2016).

One of the key issues of modern archeology is to determine the period of the establishment of a productive economy in Siberia (north of the Mongolian Plateau) (Kirjushkin *et al.* 2005). Obvious evidence of such an important historical stage is the presence of sheep bone remains in the archaeological sites of ancient herdsmen from Western and Southern Siberia (Fig. SA1 in Appendix S1). The 'Afanasiev culture' discovered in Altai is considered to be the most ancient (the first half of the

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**Figure 1** A rooted phylogenetic tree of a 316-bp fragment of sheep mitochondrial control region with *Ovibos moschatus* as an outgroup constructed with MRBAYES v3.2.6. Node labels, Bayesian posterior probabilities; A, B, C, D and E, major haplogroups identified in previous work. Phylogenetic positions after the ABCDE split cannot be solved, but the basal position of samples 56 and 77 are supported by reliable posterior probabilities.

third millennium BC). Among the archeological sites studied thus far, the ancient settlement Berezovaya Luka is of great importance, as it has been characterized as a cultural and economic center referring to Eluninskaya archaeological culture. The excavation site yielded an enormous amount of material, of which domestic animal bones dominated (about 99% of all artifacts). It has been proven that the bones of sheep and goats prevailed (over 55%) (Kosinzev 2005). The studied bones of sheep were relatively well preserved, even in comparison with more recent samples from other sites. These and other findings from the ancient and medieval monuments of Western Siberia, Eastern Kazakhstan and Mongolia provide an opportunity to start an extensive analysis of population demography using ancient DNA. The aim of this study was to investigate the mtDNA D-loop variability of Bronze Age ancient sheep from Altai and the relationship between ancient and modern sheep, thus to obtain valuable genetic information for understanding the origin of Altai domestic sheep.

In this work we studied 15 skeletal remains of sheep from the several Early Bronze Age archaeological sites and two skeletal remains from the Early Middle Age archaeological sites (Table S1). The aDNA isolations were carried out according to the already-described method in a specialized laboratory dedicated to ancient DNA analysis, with some modifications (Pääbo *et al.* 1988). Negative control was used as one of the stringent quality assurance criteria. The mtDNA D-loop fragments were amplified by PCR using specific overlapping primers—Dov1–Dov10 (Table S1)—which flank the region from 15 956 bp to 16 555 bp. We analyzed sequences obtained in this work as well as modern sheep haplotypes from all earlier described haplogroups (A, B, C, D and E) and the mtDNA sequence of *Ovibos moschatus* (NC\_020631.1) as an outgroup. The MRBAYES v3.2.6 package was used to construct phylogenetic trees (Huelsenbeck & Ronquist 2001), which were visualized and manipulated in FIGTREE v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Divergence times were estimated by BEAST 1.8.3 (Drummond *et al.* 2012). As a calibration node, we used previously estimated divergence time of major haplogroups A, B, C, D and E ( $0.8 \pm 0.2$  Ma) (Lv *et al.* 2015).

Ancient DNA was isolated from 17 samples of sheep remains found in the archaeological complexes in Altai for the first time, and the sequences of mtDNA control regions were determined. The GenBank accession numbers of the sequences are shown in Table S1. The haplotype diversity and nucleotide diversity were  $0.801 \pm 0.08$  and  $0.0096 \pm 0.001$  respectively. The average number of nucleotide differences was 3.1. Previous work on genotyping modern Chinese, Mongolian and Tibetan sheep have shown greater haplotype and nucleotide diversity, which may be explained by the longer lengths of the analyzed sequences (Luo *et al.* 2005; Liu *et al.* 2016). Analysis of ancient samples from the Early Bronze Age in China has shown lower nucleotide diversity ( $0.0076 \pm 0.0053$ ) (Cai *et al.* 2011).

The aligned sequences here had a total length of 316 bp, which slightly reduced the number of polymorphic sites. The phylogenetic position of sequences was inferred from a phylogenetic tree (Fig. 1). An initial haplotyping revealed that samples 44, 45, 54, 55, 58 and 86 perhaps belonged to lineage B (6/17, 35.3%) whereas samples 38, 42, 51, 57, 80, 85 and 87 belonged to lineage A (7/17, 41%). Samples 72 and 74 could possibly be placed in an uncertain position within the A, C, D and E lineages. Samples 56 and 77 had a basal position relative to the rest of the analyzed samples. We hypothesize that ancient sheep may have been more diverse, that the level of haplotype polymorphism was higher than in modern times and that it is necessary to consider extinct lineages G (which includes haplotype 56) and F (which includes haplotype 77). A sequence similarity search using BLAST revealed that the haplotype 77 was most similar to sequence DQ791112.1, obtained from an Iberian domestic sheep (*O. aries* LA08) and that haplotype 56 was most similar to JN574151.1, isolated from *O. aries* voucher TKDAG21; both sequences presumably belong to haplogroup B. The approximate time of haplotype divergence within haplogroups was estimated using BEAST 1.8.3 as 1.7 Ma ago for sample 77 and as 0.96 Ma ago for haplotype 56 (see Fig. SA4 in Appendix S1). As a calibration node we used previously estimated divergence time of the major haplogroups A, B, C, D and E ( $0.8 \pm 0.2$  Ma ago).

Thus, at least two sheep lineages (A and B) occurred in Altai since the Early Bronze Age, which correlates with the literature data describing the route of the major colonization process of Middle Eastern sheep to eastern Eurasia (including Mongolia, China and India) through the Caucasus and Central Asia at approximately 4.5–6.8 ka (Lv *et al.* 2015). Earlier, it was demonstrated that haplogroup A was characteristic of the Early Bronze Age in China (Cai *et al.* 2011). At the present time, haplogroup A remains dominant (58.73%) followed by haplogroup B with an average frequency of 24.68% and haplogroup C with an average frequency of 16.59%. (Luo *et al.* 2005). Here we showed first that in Early Bronze Age Altai both haplogroups (A and B) occurred along with some novel previously undescribed haplogroups. The existence of two basal haplotypes, greater haplotype diversity and nucleotide diversity indicate that there was a great variety of sheep breeds in the past. This indicates that a highly heterogeneous pre-domesticated sheep population was recruited and that a genetic bottleneck took place, but the bottleneck was not as severe as for some other domestic animal (Kijas *et al.* 2012). This also can be explained by the hypothesis that Altai could have been a migratory corridor for sheep dispersal or that the region has benefited from an active exchange between different cultures.

Future projects will concentrate on whole genome sequencing of certain samples, which will help to further expand the phylogenetic and evolutionary analysis. In parallel, we plan to conduct targeted next-generation sequencing, implemented through the recent availability

of meaningfully large collections of SNPs (Kijas *et al.* 2012; Lühken 2012). Further genetic studies of ancient samples of sheep, particularly a whole-genome sequencing of novel undescribed haplogroups, and targeted next-generation sequencing will expand the understanding of both the early stages of the domestication process and the evolutionary history of domestic sheep of Altai in the Early Bronze Age.

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## Supporting information

Additional supporting information may be found online in the supporting information tab for this article:

**Table S1** General information about gathering places, ancient monuments and samples, reference and practical sequences from Genbank.

**Appendix S1** Mitochondrial DNA analysis of ancient sheep from Altai.