# Mitochondrial DNA analysis of Bronze Age horses recovered from Chifeng region, Inner Mongolia, China*

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Accepted on September 25, 2006

**Abstract** In this study, mitochondrial DNA (mtDNA) analysis was carried out on 9 Bronze Age horses recovered from Dashanqian and Jinggouzi archaeological sites in Chifeng region, Inner Mongolia, China to explore the origin of Chinese domestic horses. Both mtDNA 16S rRNA gene and control region (D-loop) fragments of ancient horses were amplified and sequenced. The analysis of the highly conservative 16S rRNA gene sequences indicated that the burial environment of Chifeng region is suitable for the preservation of ancient DNA (aDNA). Combing 465 mtDNA D-loop sequences representing different breeds from East Asia, Central Asia, Near East and Europe, we constructed a phylogenetic network to investigate the relationship between ancient and modern horses. The phylogenetic network showed that the 9 horses were distributed into different modern horse clusters which were closely related to them representing a certain geographical distribution. Our results showed that the maternal genetic line of the ancient horses in Chifeng region was highly diversified, which contributed to the gene pool of modern domestic horses and suggested a complex origin of domestic horses in China.

**Keywords: origin of domestic horses, ancient DNA, mitochondrial DNA, 16S rRNA gene, phylogenetic network.**

The appearance of domestic horses enormously enhanced human transportation and warfare capabilities that have profoundly affected the course of civilization[1]. The origin and spread of domestic horses have become a core issue in evolutionary archaeology. Previous studies were mainly based on the analysis of morphological variability of archaeological faunal remains, which was often limited by quality and quantity[2]. During the long term preservation process, the remains lost some of their morphological identification features. In addition, the lower quantities of the remains of the early period of domestication make it difficult to get the accurate information on the origin of domestic horses. Ancient DNA (aDNA) technology provides an effective way to address above questions. As a carrier of genetic information, aDNA provides the most direct evidence to reveal the phylogenetic relationship between extinct and extant organisms through the reconstruction of past genetic structure and diversity of domestic animals, which can help to trace the evolution of domestic animals at a molecular level.

A large number of remains of domestic horses and carriages suddenly appeared at the sites of the late Shang Dynasty (3000 yr BP) in China, such as Yin Ruins in Anyang, Henan Province; Laoniupo in Xi'an, Shaanxi Province and Qianzhangda in Tengzhou, Shandong Province. However, prior to the late Shang Dynasty, there were few records of the domestic horses. Excavations from thousands of Neolithic and early Bronze Age sites in China showed that only a few sporadic fragments of tooth and bone were discovered at limited sites[3], such as Banpo in Xi'an, Shaanxi Province, Baiying in Tangyin, Henan Province, Chengziya in Zhangqiu, Shandong Province and Nansha Village in Hua County, Shaanxi Province. The lack of evidence at the early period of domestication of horses and the "sudden emergence" of domestic horses in the late Shang Dynasty makes the origin and history of Chinese domestic horses very confusing.

Chifeng region, the cradle of northern Chinese civilization, is located in the southeastern Inner Mongolia Autonomous Region. In ancient history, many nomadic tribes lived in the area. The domestication and breeding of horses played a crucial role in various aspects of their lives. To date, the remains of horses have been found at many archaeological sites of the

* Supported by National Science Fund for Fostering Talents in Basic Research (Grant No. J0530184) and National Social Science Foundation of China (Grant No. 06BKG001)

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Bronze Age in Inner Mongolia. The Bronze Age was a critical period of domestication of horses. Therefore, the reconstruction of the past genetic structure and diversity of Bronze Age horses in Chifeng region based on aDNA analysis is of great significance to the studies of the origin of Chinese domestic horses.

## 1 Material and methods

### 1.1 Sample collection

The Dashanqian site is situated in Dashanqian Village, Yongfeng Township, Harqin Banner, southwest Chifeng region (Fig. 1). The site is divided into six sections (No. KD Ⅰ—Ⅵ). The first section (KD Ⅰ) is located on the top of a small mesa, with an elevation of 765 m above sea level. Qingshui River is located to the west and south sides of this site, and the site is 12 m higher than the river bed. Excavations at KD Ⅰ revealed the deposits of Xiaoheyan Culture, Lower and Upper (2800 yr BP) Xiajiadian Culture and the Warring States period in date order, in which a large quantity of deposits belong to Lower Xiajiadian Culture Remains ( the early Bronze Age, 4000—3500$^{14}$C yr BP )[4]. A lot of stone tools for agricultural production and corns for sacrifice were found in the burials of Lower Xiajiadian Culture. In addition, pig was the main sacrificial animal in burials, and the following were cattle and goat/sheep. All of those indicated a complex economic pattern in which agriculture was dominant, and also included livestock and hunting.

Jinggouzi site in the north of Jinggouzi Village, Linxi County, north Chifeng (Fig. 1), dated to the period of the later Spring-Autumn and the early Warring States, is the remains of the late Bronze Age (2115 ± 65, $^{14}$C yr BP). The economic pattern reflected from this site suggested a developed livestock breeding, especially horses. According to the date, the place and economic pattern, Jinggouzi site possibly represents a new archaeological culture, which may be related to ancient Donghu tribes[5].

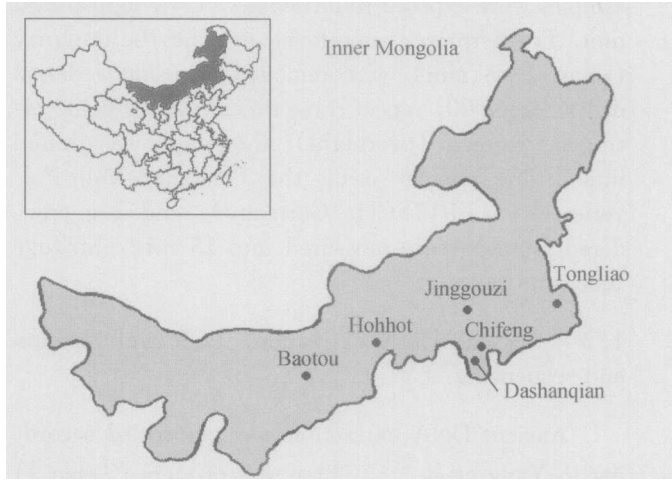


Fig. 1. Geographical distribution of Dashanqian and Jinggouzi sites in Inner Mongolia.

In this study, 9 horse remains from Dashanqian and Jinggouzi sites, dating from Lower Xiajiadian Culture to the Spring-Autumn and Warring States period (4000—2000 yr BP), were collected for aDNA analysis (Table 1). Notably, 4 of the 9 remains found at Dashanqian site, dating to the Lower Xiajiadian Culture period, were extremely similar to modern horse through morphological and morphometric analyses. Excavations from archaeological sites in Chifeng region during the period from Paleolithic Age to Qin-Han Dynasty showed that no horse bones have been found in the burial of the Lower Xiajiadian Culture period before this excavation. On the contrary, horse remains were presented only at the burial sites of the Upper Xiajiadian Culture and the Spring-Autumn and the Warring States period[6]. Undoubtedly, the 4 remains are crucial in understanding the origin of Chinese domestic horse.

Table 1. Samples data

| No. | Sites | Excavated location | Element | Expt. No. | Date |
|---|---|---|---|---|---|
| 1 | Dashanqian (KD Ⅰ) | 96KD Ⅰ T433④e | Tooth | K433 | Lower Xiajiadian Culture |
| 2 | Dashanqian (KD Ⅰ) | 96KD Ⅰ T410② | Tooth | K410 | Lower Xiajiadian Culture |
| 3 | Dashanqian (KD Ⅰ) | 97KD Ⅰ T316⑥ | Tooth | K316 | Lower Xiajiadian Culture |
| 4 | Dashanqian (KD Ⅰ) | 96KD Ⅰ T420⑥ | 3rd phalange | K420 | Lower Xiajiadian Culture |
| 5 | Dashanqian (KD Ⅰ) | 96KD Ⅰ T425② | Femurs | K425 | Later Warring States |
| 6 | Jinggouzi | 02LJM23 | 2nd phalange | LJM23 | Spring-Autumn and Warring States |
| 7 | Jinggouzi | 03LJM49 | 2nd phalange | LJM49 | Spring-Autumn and Warring States |
| 8 | Jinggouzi | 03LJM51 | Tooth | LJM51 | Spring-Autumn and Warring States |
| 9 | Jinggouzi | 03LJM52 | 2nd phalange | LJM52 | Spring-Autumn and Warring States |

### 1.2 Sample preparation

The dust and clay on the outer surface of teeth or bones were cleaned with a fur brush. The cleaned samples were exposed to ultraviolet (UV) light for 30 min. Furthermore, superficial dirt and the impurity (about 2—3 mm) were removed by using a dental drill (Strong 90, repeat three times). After liquid nitrogen treatment (overnight), the sample was ground into a fine powder using the Planetary Mill Pulverisette 6 (FRITSCH, Germany), and 2 g powdered samples were measured into 15 mL centrifuge tubes and saved at −20℃.

### 1.3 Ancient DNA extraction, PCR amplification and sequencing

Ancient DNA extraction was performed according to Yang et al.[7]. Three sets primers (Table 2) were designed from a reference sequence (GenBank X79547)[8], to amplify the 300 bp mtDNA control region (D-loop) fragment from all 9 ancient horses, and the 150 bp 16S rRNA gene fragment (Table 2) from 5 samples collected from Dashanqian site, respectively.

PCR amplifications were performed on the Mastercycler® Thermal Cycler (Eppendorf, Hamburg, Germany) in a 12.5 μL reaction volume containing 2.5 mmol/L $Mg^{2+}$, 1×buffer, 200 μmol/L dNTPs, 1.6 g/L BSA, 0.5 μmol/L of each primer, 1 U *Taq* polymerase (Promega, USA) and 2 μL DNA sample. PCR conditions were as follows: pre-denaturation at 95℃ for 5 min, 8 cycles of 92℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min; followed by 28 cycles of 92℃ for 1 min, 50—55℃ for 1 min, 72℃ for 1 min; and a final elongation step of 72℃ for 10 min. PCR products were electrophorezed on 2% agarose gel (Biowest, German), then purified using QIAEX® II GEL Extraction Kit (QIAGen, Germany). The both strand sequencing reactions were carried out on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA) using Dyeprimer Sequencing kit.

Table 2. Primers for PCR amplifications

| Amplification region | Primer[a)] | Primer sequences | Fragment length (bp) |
|---|---|---|---|
| 16S rRNA gene | L1277 | 5′-CGAATCTTCTCACTATTTTGCG-3′ | 150 |
| (Positions: 1277—1426) | H1426 | 5′-GCTTACCCCTTTTACCTTTTGC-3′ | |
| mtDNA D-loop | L15473 | 5′-CTTCCCCTAAACGACAACAA-3′ | 220 |
| (Position: 15473—15772) | H15692 | 5′-TTTGACTTGGATGGGGTATG-3 | |
| | L15571 | 5′-AATGGCCTATGTACGTCGTG-3′ | 202 |
| | H15772 | 5′- GGGAGGGTTGCTGATTTC-3′ | |

a) The numbers give the 5′-end of the primers, H and L refer to the heavy and light stands, respectively.

### 1.4 Data analysis

Ancient horse sequences were truncated to 262 bp (nt. 15494—15755) for molecular analysis. Sequences of the D-loop were aligned using the Clustal X 1.83 program to identify the position of nucleotide variation and mtDNA haplotype. We selected modern horse mtDNA sequences representing different breeds from East Asia, Central Asia, Near East and Europe for comparison[9—19]. Some Przewalski's horse (*Equus przewalskii*) sequences and ancient horse sequences from archaeological site including Berel in Kazakstan (2300 yr BP), Yakutsk in Russia (300—200 yr BP)[18], Jeju in Korea (1300—1200 yr BP)[13], Alaska in America (28000—12000 yr BP)[19] and several others in South Sweden (2000—1000 yr BP)[19] were collected from GenBank. Together, 456 sequences including 9 ancient horses were included for phylogenetic analysis. The phylogenetic network of 465 sequences was constructed using the software Network 4.1 by which the maternal genetic relationship between ancient and modern horses was established. Haplotype diversity (h) and nucleotide diversity (π) were computed using DnaSP 4.1 software. Ancient horse sequences data in this paper have been submitted to GenBank with accession numbers (DQ900922—DQ900930).

## 2 Results

### 2.1 Analysis of 16S rRNA gene sequence

Sequencing of the highly conservative 16S rRNA gene was useful in verifying the absence of post-mortem base modifications or *Taq* DNA polymerase errors, which helps to further determine the condition of the burial environment in which the aDNA pre-

served[20]. Ancient DNA sequences of 5 horses recovered from Dashanqian site were in fact identical to the reference sequence X79547, suggesting that the rate of post-mortem base modification and polymerase mispriming is very low, and the burial environment of Chifeng region is suitable for the preservation of aDNA.

### 2.2 mtDNA variations in ancient horses

Ancient mtDNA sequences were successfully retrieved from 9 samples. Comparing to the reference sequence X79547, a total 19 polymorphic sites and 8 parsimonious informative sites, representing 7.3% and 3.1% of the total DNA sequence analyzed, respectively, were found. All of these variable positions were transitions, and none of them were transversions, insertions or deletions. Seven haplotypes were determined according to the polymorphic sites. Besides, samples K410, K420 and K433 shared the same haplotype, and the other 6 sequences were unique (Table 3). Haplotype diversity and nucleotide diversity of the 9 horses were $0.917 \pm 0.092$ and $2.29\% \pm 0.25\%$, respectively. Remarkably, nucleotide diversity observed in 9 ancient horses was similar to estimates in 191 horses representing 10 breeds reported by Vila et al. ($\pi = 0.22$)[10].

Table 3. Nucleotide variation positions of 9 ancient horses

| Sample No. | Variation positions | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 7 | 7 | 7 | 7 |
| | 9 | 2 | 4 | 8 | 9 | 9 | 9 | 0 | 0 | 3 | 5 | 5 | 6 | 6 | 0 | 2 | 2 | 3 | 4 |
| | 5 | 1 | 2 | 5 | 5 | 6 | 7 | 2 | 4 | 5 | 0 | 9 | 6 | 7 | 3 | 0 | 6 | 7 | 0 |
| Ref. X79547 | T | G | C | G | A | A | A | C | G | C | A | T | G | A | T | G | G | T | A |
| K316 | C | · | · | · | · | · | G | T | A | · | · | · | · | G | C | A | · | · | · |
| K410, K420, K433 | C | · | T | A | · | · | · | T | A | · | G | · | A | · | · | A | · | · | · |
| K425 | C | · | · | · | · | · | · | · | · | · | G | · | A | · | C | A | · | · | · |
| LJM23 | C | A | · | · | · | G | · | T | · | · | · | · | · | · | · | A | · | C | · |
| LJM49 | C | · | T | · | · | · | · | T | · | T | · | · | A | · | C | A | · | · | · |
| LJM51 | C | · | · | · | · | · | · | T | A | · | · | C | · | · | C | A | A | · | G |
| LJM52 | C | · | · | · | G | · | G | T | A | · | · | · | · | · | C | A | · | · | G |

Dots denote identity with the reference sequence

### 2.3 Phylogenetic network analysis

The phylogenetic network of 456 sequences from wild, modern and ancient horses showed a star-like profile with a central node cluster A6. According to Vila et al.[10], the 9 ancient horses were distributed into lineages A, E and F, which can be further divided into several clusters[19].

Samples K316 (the early Bronze Age), LJM5 and LJM52 (the Spring-Autumn and Warring States) clustered in lineage F. Remarkably, sample K316 shared the founder haplotype of cluster F1, and was grouped into cluster F1 with horses from Europe (27.8%), Near East (22.2%) and East Asia (44.4%). East Asian horses observed in cluster F1 were Chinese Mongolian and Korean Cheju horses. Historic records suggest that Cheju horses are descendants of Mongolian horses introduced in 1276[11]. Interestingly, an ancient horse from the Yakutsk site was also grouped in cluster F1. Keyser-Tracqui et al. suggested that Yakutsk horses had Mongolian origin[18]. To sum up, the horses of Mongolian origin represented 50% of all the horses in cluster F1, suggesting a certain geographical distribution of cluster F1. LJM51 was presented in cluster F2 with horses from East Asia (13%), Near East (20%), Europe (67%). Notably, the Shetland ponies represented 50% of the horses in Europe. LJM52 and cluster F2 have a shared node 15740, which suggested they were derived from a common ancestor.

Lineage A was divided into three clusters A1, A2 and A3. Cluster A2 was restricted to the Przewalski's horses. Samples K410-K420-K433 (the early Bronze Age) and cluster A2 have a shared node 15542. LJM49 was grouped into cluster A1 with horses from East Asia (7%), Central Asia (17.8%) and Europe (78.2%). The South European ponies represented 63% of the horses in Europe, suggesting that cluster A1 may be related to South European breeds. Sample K425 was grouped into cluster A3

with horses from Near East (47.8%), Europe (39.2%), Central Asia (8.6%) and East Asia (4.4%). Notably, an ancient horse from the Berel site in Kazakstan (2300 yr BP) was also grouped into cluster A3.

Lineage E included only a small cluster E, in which the North European ponies were dominant. LJM23 and cluster E have a shared node 15521, suggesting that they were derived from a common ancestor.

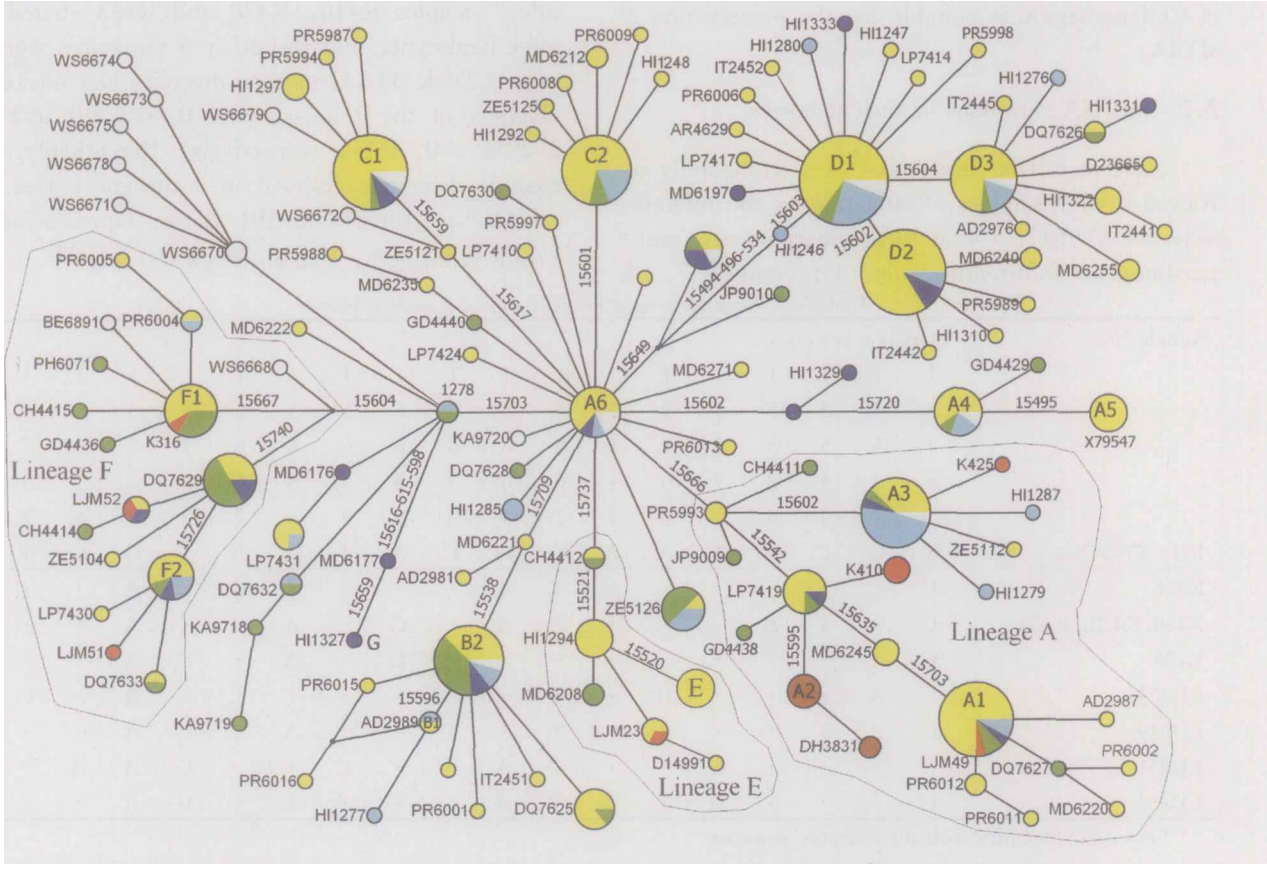


Fig. 2. Phylogenetic network of domestic horses, wild horses and ancient horses. Circle areas are proportional to mtDNA haplotype frequencies. Letters in circles represent mtDNA clusters named according to Jansen et al.[19]. The first and second letter of samples are abbreviations of GenBank accession numbers (*AD* = AF07, *AR* = AF06, *BE* = AJ87, *CH* = AF01, *DH* = AJ41, *GD* = AF35, *GZ* = AY13, *HI* = AF48, IT = AY46, JP = AF16, KA = AY04, LP = AY05、MD = AY24, PH = AF05, PR = AF46, WS = AF32, ZE = AY57). Red, sequences of ancient horses in this study; grey, ancient horse sequences from GeneBank; yellow, European horses; green, Asian horses; blue, Central Asian horses; azury, Near Eastern horses; brown, Przewalski's horses.

## 3 Discussion

### 3.1 Authenticity of aDNA results

Ancient DNA was heavily damaged and degraded into small fragments no more than several hundred base pairs after long-term underground preservation. Damage includes either base oxidation modification or base loss as well as inter-strand crosslinking, which easily leads to the exogenous DNA contamination and *Taq* polymerase mispriming in the PCR process. In this study, all the experiments were carried out in a dedicated aDNA laboratory. Samples preparation, DNA extraction and PCR amplification were performed in physically separate rooms equipped with positive pressure and air filtration. All researchers wore sterilized laboratory coats, coveralls with hood, facemasks and gloves which were frequently changed, and strict cleaning procedures were performed by regular treatment with DNA-OFF™(Q.BIO gene, Germany) and UV light. DNA-free reagents and dedicated equipment were used, and extraction and amplification blanks were used to indicate contaminants at any stage. Due to the careful research design, the authenticity of the obtained sequences was guaranteed by the following examinations: (1) all extraction and PCR reagents blanks were consistently negative throughout the study; (2) the PCR results were obtained from multiple extractions, amplifications and double-strand sequencings of the same samples; (3) the analysis of the highly conservative 16S rRNA

gene sequences indicated a favorable burial environment of Chifeng region to the preservation of aDNA; (4) throughout the study, no modern horse DNA was introduced into the laboratory, which minimized the exogenous DNA contamination; and (5) all results were repeated multiple times by two researchers, separately.

### 3.2 Ancient and modern horses

In the aDNA analysis, the nucleotide diversity was observed in 9 ancient horses which was similar to estimates in 191 horses representing 10 breeds by Vila et al ($\pi = 0.22$)[10]. Phylogenetic network showed that the 9 horses did not form a separate cluster and they were distributed into different modern horse clusters. In addition, some samples (K316, LJM49) also shared the founder haplotype of cluster. All these suggested that the maternal genetic line of ancient horses in Chifeng region were highly diversified, that contributed to the gene pool of modern domestic horses. Likewise, some ancient horses from other archaeological sites were also grouped into different clusters. Combining the early divergence time estimated by Jansen et al.[19], we suggested these maternal branches have ancient origin, not recent events. The mtDNA clusters of 9 ancient horses represented the certain geographic distribution, which reflected the multiplicity and complexity of the origin of Chinese domestic horse, and suggested that both the native origin and the external input have been probably involved in the process of the domestication of Chinese horses. The network profile also showed that breeds from different geographical regions intermingled, and some haplotypes were shared by individuals from different geographical regions, illustrating that a significant gene flow could have occurred among the breeds in different geographical range, and reflecting the widespread cultural exchange among ancient populations throughout the Euraisan range. In this study, some ancient horses with different date (the early and late Bronze Age) have the most recent common ancestor, for example, samples K425, LJM49 and K410-K420-K433 were grouped into lineage A, K316, LJM51 and LJM52 grouped into lineage F.

### 3.3 Ancient horses and Przewalski's horses

The domesticated horse is descended from the wild horse. China is the main habitat of Przewalski's horses. The plentiful fossils of Przewalski's horses have been found in the late Pleistocene faunas and human ruins in the North China ranging from the west of Xinjiang to Taiwan Strait[21]. Considering the geographic distribution of Przewalski's horses and human activity in Euraisan steppe, some scholars suggested that the early Chinese domestic horses may be derived from Przewalski's horses[22]. However, according to our results, none of 9 ancient horses were observed in cluster A2 which belongs to Przewalski's horses. It says that there is not direct maternal genetic relationship between ancient horses and Przewalski's horses. Notably, samples K425, LJM49, K410-K420-K433 and cluster A2 belonged to lineage A, indicating that they were derived from a common maternal ancestor.

In conclusion, our results revealed the maternal genetic relationships between the Bronze Age horses in Chifeng region and modern horses, which provided a valuable clue for the research on the origin of Chinese domestic horses. In the future, we will add more samples from different time and place for aDNA analysis. Simultaneously, we also hope to further investigate the patrilineal heredity of the ancient horses through the Y chromosome research. All these work will expand the phylogenetic knowledge for the origin of Chinese domestic horses.

**Acknowledgements** We are grateful to Inner Mongolia Research Institute of Cultural Reli for the help during the archaeological excavation. We also thank Dr. Tang Zhouwei and Chen Quanjia for supplying ancient horse samples, and thank Dr. Chen Yaofeng and Suratissa for the comments on and the help in writing this manuscript.

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