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# Ancient DNA study of *Cervus canadensis* unearthed from the Royal Sacrificial Site of the Northern Wei Dynasty in Inner Mongolia Autonomous Region, China

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ABSTRACT

The practice of animal sacrifice was widespread in ancient societies, and the study of sacrificial animals holds significant importance. In this study, three samples excavated from the Royal Sacrificial Site of the Northern Wei Dynasty in Inner Mongolia Autonomous Region, China were initially identified as Cervinae subfamily animal based on their morphology. Through alignment analysis and principal component analysis, these samples were identified as belonging to the species *Cervus canadensis*. Meanwhile, through phylogenetic analyses and genetic distance calculations, it was determined that all three samples were of the *Cervus canadensis alashanicus* subspecies, proving the utility of ancient DNA technology in accurate subspecies identification. The analysis of the mitochondrial D-loop region revealed that the ancient *Cervus canadensis alashanicus* dated to 3800 BP and 5100 BP differed significantly from the modern ones in opposition to 1500 BP samples which were similar to them. The low genetic diversity of modern *Cervus canadensis alashanicus* was formed at least 1500 years ago and have lasted till today. In addition, as suggested by historical documents and archaeological studies, it is evident that the reverence for deer within the Tuoba Xianbei population had become a significant element of their culture and belief system. The identification of *Cervus canadensis* species in this study serves as evidence of the Northern Wei Emperor’s utilization of totem animals as sacrificial offerings to heaven.

## 1. Introduction

Animal resources are essential for human societies, which especially applies well to ancient societies where animals fulfill various needs such as food, clothing, transportation, labor, and religious practices (Yuan, 2010). Among these uses, animal sacrifice held a prominent position as one of the primary methods of animal resources utilization (Lv and Gong, 2016). Zooarchaeological studies have revealed that the utilization of animals as sacrificial offerings became a traditional practice during the Neolithic period. During the Shang and Zhou periods, animal sacrifice was more prevalent (Yuan, 2007). From the Qin and Han Dynasties onwards, emperors and other members of the noble class would regularly organize grand sacrificial ceremonies, and these ceremonies involved the sacrifice of a significant number of animals (Yan, 2011). As written in historical records, not only the Han Chinese ethnic group but also ancient Chinese ethnic minorities practiced animal sacrifice in their rituals (Xu and Sun, 2022). In ancient societies, animals used for sacrifices encompassed not only domesticated mammals such as cattle, dogs, pigs, and sheep but also wild mammals like deer and tigers. Birds, fish, and shellfish were used in sacrificial rituals as well (Wang, 2014).

The Northern Wei Dynasty (386–534 CE), founded by the Xianbei ethnic group, had a rich array of ritual activities, ranging from the worship of heaven and earth to the reverence of ancestors and other deities (Zhao, 2020). As a regime established by ethnic minorities, the sacrificial rituals of the Northern Wei Dynasty reflect their own distinctive ethnic characteristics. What accompanied their geological fusion with the Han Chinese group was cultural assimilation. This borrowing and adaptation of Han culture contributed to their even more distinct and characteristic sacrificial practices (Liu and Zhao, 2018). As a nomadic tribe, the Tuoba Xianbei loved and worshiped wild animals. For example, they named official positions after animals such as dragons and birds, and set up royal gardens to raise wild animals (such as tigers,

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deer, etc.) for entertainment (Yang, 2007). In addition, Xianbei ethnic group made use of animal resources, a typical example of which is animal sacrifices. Throughout the various developmental stages of the Northern Wei Dynasty, whether it was the primitive stage and the early Shengle era (258–398 CE) or the Pingcheng era (398–494 CE) and the subsequent Hanization reforms under Emperor Xiaowen's rein, sacrificial animals were always a crucial part of these rituals (Xu, 2007; Liu, 2018). Sacrifices to Heaven was a significant ceremonial practice for emperors of the Northern Wei Dynasty, and the offering of animals as sacrifices was involved as well (Yang, 2002). It is evident that the practice of using animals for sacrificial offerings was widespread during the Northern Wei Dynasty, therefore studying the practice of animal sacrifice during that period is of great significance.

The ancient Chinese nomadic groups had a long-standing tradition of deer worship. Deer held significant cultural and symbolic importance among these communities, many ethnic groups attached political and religious significance to deer, and deer-totem legends, visual arts, and rituals had long existed (Wang, 2008). Rock paintings dating back over 10,000 years depict an abundance of deer images, indicating that deer held a prominent place among the animals revered by the northern nomads during that era (Li, 2012). The deer stones, discovered across the Eurasian steppe, stand as enduring symbols of the nomadic people's deer culture worship, with thousands of these ancient artifacts scattered across the region (Seng, 2014). The Xiongnu people held a strong reverence for deer as well, as evidenced by the discovery of deer-patterned decorative plates at numerous archaeological sites, underscoring the people's profound worship and emotional attachment to deer (Zhang, 2022). Furthermore, the ancient Mongolian people passed down a legend about "Cang Lang Bai Lu", which further confirmed their totemic reverence for deer (Zha, 1990).

In this paper, ancient DNA analysis was conducted on three samples of remains that were morphologically identified as Cervinae subfamily animal unearthed from the Royal Sacrificial Site of the Northern Wei Dynasty in Inner Mongolia Autonomous Region, China. The samples underwent alignment analysis, phylogenetic analysis, and genetic distance calculation to identify the species and investigate the molecular genetics of the species. In addition, as inspired by historical documents and archaeological findings, this study attached great importance to analyzing the totem worship culture of the Xianbei ethnic group. The present study is expected to provide fresh perspectives and valuable insights into the history and culture of the Northern Wei Dynasty.

## 2. Materials and methods

### 2.1. Archaeological site introduction and sample collection

The Royal Sacrificial Site of the Northern Wei Dynasty, also known as the "Dam-Peak Site", is situated on the peak of the Centipede Dam in the Daqingshan District of Wuchuan County, Hohhot City, Inner Mongolia, with geographic coordinates of 111.08°E and 40.91°N (Qu and Li, 2023). In 2019, the Institute of Cultural Relics and Archaeology of the Inner Mongolia Autonomous Region conducted archaeological excavations at the Royal Sacrificial Site of the Northern Wei Dynasty. The site is 1660 m in altitude. The area is mountainous. The climate here is dry all year round and cold in winter with a large temperature difference (Yang, 2019). The site locates in the interlaced area of agriculture and animal husbandry (Jing, 2023). The site has a diameter of 98.5 m and is divided into five parts, namely the altar, the inner moat, the internal wall, the outer wall, and the outer moat arranged from the inside to the outside. The archaeological excavation revealed ritual pottery and iron artifacts within the altar area. Additionally, a small number of skulls and limb bones from horses and sheep, which were used for rituals, were unearthed at the bottom of the inner moat. (Chinese Archaeological Society, 2021). According to the radiocarbon dating of the excavated remains as well as historical records and archaeological research, the site was determined exist from 430 to 490 CE (Zhao et al., 2021). According to historical documents, it is speculated that the Royal Sacrificial Site of the Northern Wei Dynasty was used by Emperor Xiaowen for offerings to Heaven (Zhang and Da, 2021). The discovery of this site offers valuable evidence pertaining the ritual system and history of the Northern Wei Dynasty.

The three samples analyzed in this study were excavated from the bottom of the altar at the Royal Sacrificial Site of the Northern Wei Dynasty, and were identified morphologically as Cervinae subfamily animal, but their specific species need to be further determined. The site overview and the information concerning the ancient samples analyzed in this study are shown in Fig. 1 and Table 1.

### 2.2. DNA extraction, library construction and high-throughput

Firstly, 1–2 mm of the sample surface was removed using an electric sanding tool and sterilized disposable drill bits. This was done to minimize contamination from external sources. After the surface was removed, the teeth were soaked in a 10 % sodium hypochlorite solution for 15 min. They were then rinsed with diethylpyrocarbonate (DEPC) water and soaked in anhydrous ethanol for 5 min. Subsequently, the samples were exposed to a UV lamp until completely dry. On the following day, the teeth were further sanded using an electric sanding tool and sterilized disposable drills to obtain a powder sample weighing between 50 and 100 mg. The ancient DNA extraction method used in this study was based on the approach described by Dabney (Dabney et al., 2013). The extraction solution was obtained using the MinElute® PCR Purification Kit. For library construction, we adapted and optimized the experimental method developed by the Max Planck Institute in Germany (https://www.protocols.io/view/a-z-of-ancient-dna-protocols-for-shotgun-illumina-36wgq529xgk5/v2/guidelines). Mitochondrial capture was performed on iGeneTech Bioscience. Finally, double-end sequencing was performed using the Illumina Hiseq X Ten platform, which is a high-throughput sequencing technology.

### 2.3. Authenticity of the sequencing results

In accordance with the ancient DNA contamination prevention protocol, all pre-PCR procedures were conducted in a dedicated ancient DNA laboratory at Jilin University, while the post-PCR steps were carried out in a separate and geographically distant laboratory. Prior to each experiment, the workspace was exposed to ultraviolet light for 30 min. Throughout the experiment, pipettes and the super-clean table were regularly wiped with bleach. All personnel in the laboratory wore protective clothing, including sterile disposable caps, masks, and gloves. Additionally, all disposable consumables used during the experiments were DNA-free grade. To detect any potential contamination, blank controls were included at each stage of DNA extraction and amplification, all of which yielded negative results.

Ancient DNA often has C to T or G to A mutations during experimental amplification due to damage caused by hydrolysis and oxidation (Seguin-Orlando et al., 2015). The sequencing data underwent analysis using MapDamage v2.2.1 (Jónsson et al., 2013) to assess terminal damage, which exhibited a pattern consistent with ancient DNA damage demonstrating substitutions at the 5′ end (left) and 3′ end (right). The red line shows C to T and the blue line shows G to A substitutions. This ensures the authenticity and reliability of the ancient DNA data (Supplementary Fig. 1).

### 2.4. Data processing

Raw data were processed using the PALEOMIX v1.3.7 pipeline (Schubert et al., 2014). Firstly, adapter sequences were identified and removed using AdapterRemoval v2.2.0 (Schubert et al., 2016). During the processing, reads below 35 base pairs (bp) in length were filtered out, and bases with a quality below 20 were discarded. The double-end data was then merged. A total of 154 mitochondrial genomes including

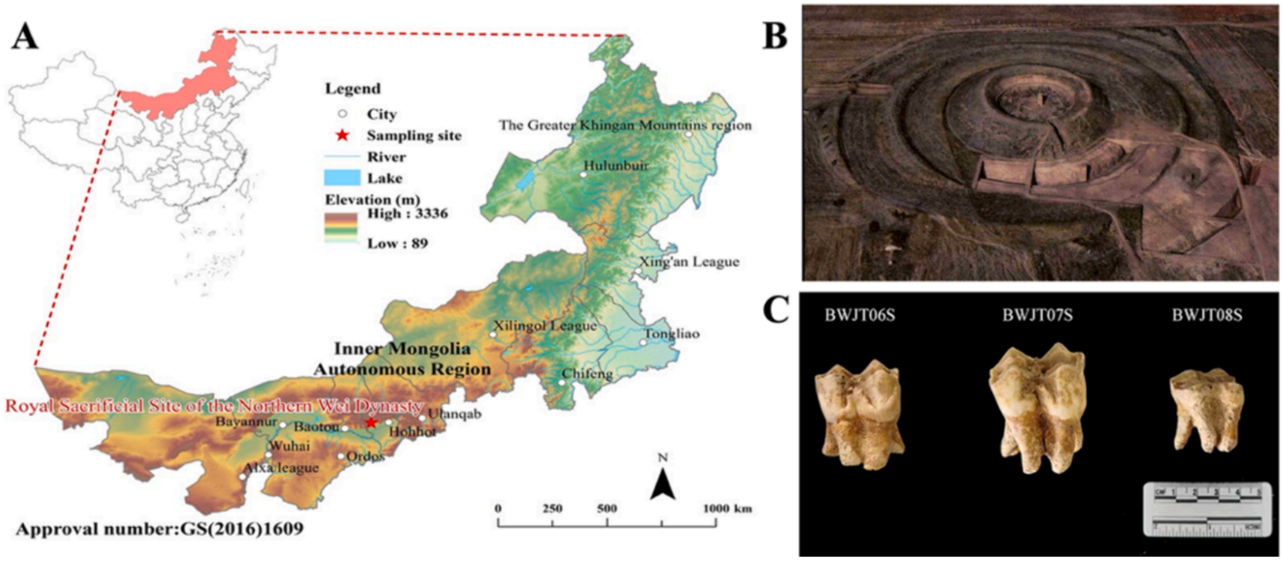

**Fig. 1.** (A) Location of the Royal Sacrificial Site of the Northern Wei Dynasty; (B) Excavation area of the site; (C) Sample of remains analyzed in this study.

**Table 1**
The information concerning the ancient samples analyzed in this study.

| Lab code | Archaeological code | Element | Dynasty | Age | Morphology | Experimental result |
|---|---|---|---|---|---|---|
| BWJT06S | JT1 | Tooth | Northern Wei Dynasty | 430–490 CE | Cervinae | success |
| BWJT07S | JT2 | Tooth | Northern Wei Dynasty | 430–490 CE | Cervinae | success |
| BWJT08S | JT3 | Tooth | Northern Wei Dynasty | 430–490 CE | Cervinae | success |

three different families of animals – Bovidae, Cervidae and Equidae (Supplementary Table 1) – were selected as reference sequences and aligned with them simultaneously using the BWA v0.717 aln algorithm (Li, 2013) to identify the specific species. When selecting reference sequences, given that some parts of Cervidae family animals bear resemblance to bovine animals in terms of skeletal morphology (Taylor et al., 2021), and that the samples excavated from the Royal Sacrificial Site of the Northern Wei Dynasty are mainly horses and sheep, which may lead to inaccurate identification or misidentification, we selected 154 mitochondrial genome sequences nearly covering all the species within the Cervidae, Bovine, Equine families as the reference sequences. After that, the mitochondrial genomes of the identified species were used as the reference sequences for alignment, and then MinQuality was set to 25. PCR duplicates were discarded using the MarkDuplicates command in Picard v2.20.0 (Sacco et al., 2017). All local realignments around indels were performed using GATK v3.7.0 for base quality rescaling and end trimming (McKenna et al., 2010). Sequencing quality and mitochondrial coverage were assessed using Qualimap v2.2.1 (Okonechnikov et al., 2016). Mitochondrial consensus sequences were extracted using ANGSD v0.931 (Korneliussen et al., 2014). Finally, the resulting mitochondrial consensus sequences were searched online using BLAST (https://blast.ncbi.nlm.nih.gov) to obtain the species information of sequences and to further identify the species type.

### 2.5. Data analysis

When doing alignment, we first carried out separate alignment, that is, the raw reads were aligned with the 154 reference sequences one by one. Considering that the mitochondrial genomes of related species are similar, which may lead to very close results between the number of reads and coverage aligned to reference of the separate alignment, thus affecting the identification, we adopted combined alignment strategy. We combined 154 mitochondrial genome reference sequences of Bovidae, Cervidae and Equidae species into one reference sequence. This combined reference sequence, containing 154 mitochondrial genomes which are equivalent to 154 chromosomes of this reference sequence, was then used to analyze the three samples against the aligned reference sequence following the practice in 2.3, that is, raw reads were aligned only once with the combined reference sequence, and due to the "competition", raw reads were preferentially aligned to the most consistent reference genome (chromosome), and would not be repeatedly aligned to other reference genomes (chromosomes). Based on the total number of base pairs and coverage of each chromosome, the species of the samples were determined.

To further explore the species relationships of the excavated samples and to identify the subspecies of three samples, we downloaded 46 mitochondrial genome data of Cervinae including 2 ancient data (Table 2) from NCBI GenBank (https://www.ncbi.nlm.nih.gov/) to construct a dataset called database1 (Supplementary Table 2). Multiple sequence alignment of data in database1 and three samples in this study was performed using MUSCLE v3.8.1 (Edgar, 2004). The R package "adegenet" (Jombart, 2008) in conjunction with R v4.1.2 (R Core Team, 2022) was utilized for performing the principal component analysis (PCA). The results were visualized by plotting using "ggplot2" (Wickham, 2016). We used the species *Hydropotes inermis*, which belongs to the genus *Hydropotes* within the family Cervinae mitochondrial complete genome sequence EU315254.1 as the outgroup. The best alternative models were determined using ModelTest-NG v0.1.6 (Darriba et al., 2020) based on the Bayesian Information Criterion (BIC). The model was selected HKY+I+G4. The phylogenetic tree was constructed using RAxML-NG v0.9.0 (Kozlov et al., 2019), and the final tree visualization was done using iTOL (Letunic and Bork, 2021). In addition, we calculated genetic distances between individuals using MEGA 11 (Tamura et al., 2021) and used the R package "pheatmap" (Kolde, 2019) in conjunction with R v4.1.2 to draw heatmaps.

To analyze the ancient samples from the Royal Sacrificial Site of the Northern Wei Dynasty within a broader spatial and temporal context, we downloaded mitochondrial D-loop region (931 bp) data of the *Cervus*

**Table 2**
Information of ancient mitochondrial genome data in database 1.

| Experimental Code | NCBI No. | Species | Data (YBP) | Unearthed place | Reference |
|---|---|---|---|---|---|
| CADG522 | MT784751 | *Cervus canadensis alashanicus* | 3800 ± 30 cal | Zhaodong, Heilongjiang, China | Xiao et al., 2020 |
| CADG527 | MT784752 | *Cervus canadensis alashanicus* | 5100 ± 30 cal | Binxian, Heilongjiang, China | Xiao et al., 2020 |

*canadensis alashanicus* from NCBI GenBank (https://www.ncbi.nlm.nih.gov/) to construct a dataset called database2 (Supplementary Table 3). Meanwhile, we extracted the D-loop region (931 bp) of the mitochondrial genome sequences for three ancient samples in this study and five samples of the *Cervus canadensis alashanicus* in database1. Multiple sequence alignment was performed using MUSCLE v3.8.1 (Edgar, 2004). The R package “adegenet” in conjunction with R v4.1.2 was utilized for performing the principal component analysis. The results were visualized by plotting using ggplot2. We used DnaSP v6.0 (Rozas et al., 2017) to determine haplotypes of mitochondrial sequences, and then Arlequin v3.5.2.2 (Excoffier and Lischer, 2010) to calculate different haplotype frequencies. PopART v1.7 (Leigh and Bryant, 2015) was used to construct median-joining network. Finally, DnaSP v6.0 was used to calculate Nucleotide diversity ($\pi$).

## 3. Results and analysis

### 3.1. Sequencing and identification results of ancient samples

DNA was successfully extracted from the three ancient samples in this study. In order to determine the specific species of the samples, we aligned the raw data of the three samples to the mitochondrial genome reference sequences of 33 Cervidae family, 116 Bovidae family and 5 Equidae family. We initially chose the five species that had the highest coverage of the reference sequences of the alignment (Supplementary Table 4), and then extracted the mitochondrial consensus sequences of the five corresponding species and performed the BLAST online search to obtain the species information of the sample sequences. We found that all three samples were aligned to the mitochondrial genome reference sequences with the highest coverage and number of reads aligned to reference, which were Cervidae family Cervinae subfamily *Cervus* genus *Cervus canadensis*. In addition, we further combined the 154 mitochondrial genomes into one reference sequence for alignment, and the three samples were compared to the species with the highest total number of base pairs aligned to reference and coverage, all of which were *Cervus canadensis* (Supplementary Table 5). The results obtained by two alignment methods were consistent, where the species with the largest total number of reads/base pairs and the highest coverage aligned to reference belonged to the *Cervus canadensis*.

The mitochondrial genome coverage obtained after alignment and analysis of the three samples separately using the whole mitochondrial genome of *Cervus canadensis* (NC_039923.1) as the reference sequence were all greater than 1 X and the number of sites covered by the mitochondrial genome was greater than 10,000 (Table 3).

### 3.2. Results of mitochondrial genome analysis

#### 3.2.1. Principal component analysis

Principal component analysis (PCA) on the mitochondrial genome data of the Cervinae subfamily including the three ancient samples in this study and those in database1 was conducted (Fig. 2). The Cervinae subfamily consists of four genera: *Cervus*, *Elaphurus*, *Dama*, and *Axis* (Sheng, 1992). As depicted Fig. 2, the first principal component (PC1) effectively distinguished the Cervinae subfamily based on their respective genera, while the second principal component (PC2) successfully differentiated the species within each of the four genera. Notably, the three samples analyzed in this study clustered with *Cervus canadensis*, corroborating the findings from the alignment analysis mentioned earlier.

#### 3.2.2. Phylogenetic analysis

To determine the phylogenetic position of the ancient samples, we performed phylogenetic analysis on the data in Section 3.2.1. A maximum likelihood tree was developed using the mitochondrial genome sequences, with *Hydropotes inermis* serving as the outgroup (Fig. 3). It was observed from maximum likelihood tree that the Cervinae subfamily were grouped into four clusters corresponding to the four genera and that each species was denoted by a branch within its respective genus. Notably, within the *Cervus canadensis* species, different subspecies, such as *Cervus canadensis alashanicus*, *Cervus canadensis songaricus*, *Cervus canadensis kansuensis*, *Cervus canadensis xanthopygus*, and *Cervus canadensis nannodes* were found to be sister branches to *Cervus nippon*. This finding was consistent with previous study (Xiao et al., 2020). Besides, three ancient samples analyzed in this study were clustered together with the five *Cervus canadensis alashanicus* samples. Therefore, it can be concluded that all three samples belong to the *Cervus canadensis alashanicus* subspecies, validating the utility of ancient DNA analysis in species identification and revealing the evolutionary process of species.

#### 3.2.3. Genetic distance calculation

To validate the accuracy of the phylogenetic analysis results, we calculated the genetic distances between individuals using the same data in phylogenetic analysis. Fig. 4 shows that the genetic distance between the three ancient samples analyzed in this study and *Cervus canadensis alashanicus*, *Cervus canadensis songaricus*, *Cervus canadensis kansuensis*, *Cervus elaphus canadensis*, and *Cervus canadensis nannodes* are short. Moreover, they are genetically closest to the five *Cervus canadensis alashanicus* samples analyzed, and the genetic distances between each of the three ancient samples are also closest as well. These results further support the conclusion that all the three ancient samples belong to the *Cervus canadensis alashanicus* subspecies in terms of genetic distance.

### 3.3. Results of mitochondrial D-loop region analysis

#### 3.3.1. Principal component analysis

To explore the relationship between ancient and modern samples, we extracted the mitochondrial D-loop region (931 bp) sequences of the

**Table 3**
Sequencing information of ancient samples analyzed in this study.

| Experimental Code | Total Number of Raw Reads | Species with the Highest Coverage of Aligned to Reference | Coverage of Aligned to Reference (X) | Number of Reads Aligned to Reference | Number of Sites Aligned to Reference | Number of Sites in the Reference | Average Fragment Length (bp) |
|---|---|---|---|---|---|---|---|
| BWJT06S | 8,233,136 | *Cervus canadensis* | 206.5613 | 537,992 | 15,663 | 16,430 | 83.2613 |
| BWJT07S | 3,195,442 | *Cervus canadensis* | 115.4971 | 150,069 | 14,479 | 16,430 | 80.6883 |
| BWJT08S | 4,362,456 | *Cervus canadensis* | 179.3194 | 269,670 | 14,828 | 16,430 | 78.8178 |

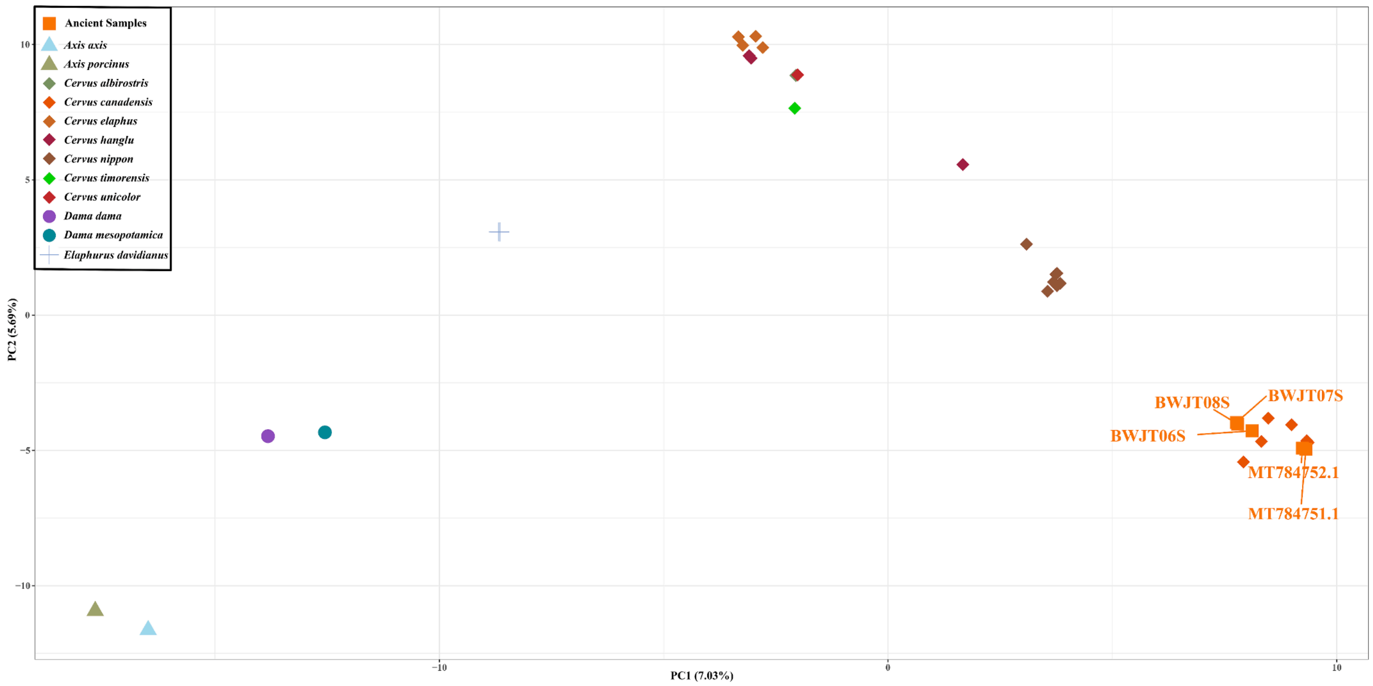

**Fig. 2.** PCA based on mitochondrial genome of Cervinae subfamily.

three ancient samples in this study and five *Cervus canadensis alashanicus* samples from Database1. mitochondrial D-loop region sequences with those of modern *Cervus canadensis alashanicus* samples from Database2 to perform a principal component analysis (Fig. 5). Of the five samples from Database1, two were ancient samples from Heilongjiang, China (CADG), dated to 3800 BP (MT784751.1) and 5100 BP (MT784752.1), respectively. As shown in Fig. 5, the two samples from Heilongjiang are distantly located from the other samples. In contrast, the three samples excavated from the Royal Sacrificial Site of the Northern Wei Dynasty (BWJT) cluster well with different modern samples, indicating that they are genetically closer to modern samples.

### *3.3.2. Median-joining network*

A median-joining network (Fig. 6) was constructed based on the data obtained in Section 3.3.1, and the network revealed that two samples from the Royal Sacrificial Site of the Northern Wei Dynasty had D-loop sequences identical to the central, most common haplotype (Hap 2) of the network and the third (differing in one mutation step) was identical to another mtDNA sequence of modern deer individual (Hap 1). These findings suggest that the three samples from the Royal Sacrificial Site of the Northern Wei Dynasty are genetically related to modern *Cervus canadensis alashanicus* populations.

### *3.3.3. Nucleotide diversity calculations*

All the samples mentioned in Section 3.3.1 were categorized into three groups: Ancient Heilongjiang samples (CADG), Royal Sacrificial Site of the Northern Wei Dynasty samples (BWJT), and modern samples. Subsequently, we calculated the nucleotide diversity ($\pi$) for each group. Our calculations revealed that CADG exhibited the highest nucleotide diversity ($\pi = 0.00236$), whereas BWJT and the modern samples displayed relatively lower nucleotide diversity ($\pi = 0.00112$ and 0.00102, respectively). Despite the nucleotide diversity of BWJT being slightly higher than that of the modern samples, they both exhibit low nucleotide diversity in general.

## 4. Discussion

### *4.1. Molecular genetic studies of the Cervus canadensis alashanicus*

*Cervus canadensis*, a species within the Cervidae family, the Cervinae subfamily and *Cervus* genus to be specific, is closely related to *Cervus elaphus* and *Cervus hangul*, as well as the *Cervus nippon* (Doan et al., 2022; Hu et al., 2019). *Cervus canadensis* mainly distributes in China, Russia, Mongolia, Canada and the United States, etc. (Lorenzini and Garofalo, 2015). The *Cervus canadensis alashanicus*, classified as a Class II nationally protected wildlife species in China, is currently found only in the middle part of the Helan Mountain, an important geographical boundary between Ningxia and Inner Mongolia. Among all the subspecies of *Cervus canadensis* in China, the *Cervus canadensis alashanicus* is the smallest in size, its population has consistently remained at a relatively low level, and it is geographically isolated, accounting for at least partially the low genetic diversity of the *Cervus canadensis alashanicus* as pointed out by related studies (Gao et al., 2020). The *Cervus canadensis alashanicus* is vulnerable to environmental changes and disease (Gao, 2020). Moreover, anthropogenic interference, such as habitat destruction, hunting, and other disturbances, further deteriorates the situation of this species (Gao, 2020). Although many efforts have been made to protect the *Cervus canadensis alashanicus* population, including the establishment of protected areas and increased conservation measures, the population remains small. Therefore, the continuous conservation of this population is critical to ensure its survival and genetic diversity (Gao et al., 2017).

In this study, we analyzed the D-loop region of three samples from the Royal Sacrificial Site of the Northern Wei Dynasty (1500 BP), two ancient samples from Heilongjiang, China (3800 and 5100 BP, respectively), and modern *Cervus canadensis alashanicus* samples. The identification of the two Heilongjiang samples as *Cervus canadensis alashanicus* suggests that this subspecies had a wider distribution and more genetic exchanges in history and that population decline, migration, and introduction might have occurred subsequently (Xiao et al., 2020). As suggested by the PCA (Fig. 5) and median-joining network (Fig. 6) results, *Cervus canadensis alashanicus* living 3800 BP and 5100 BP was genetically far to the population 1500 years ago, while the population

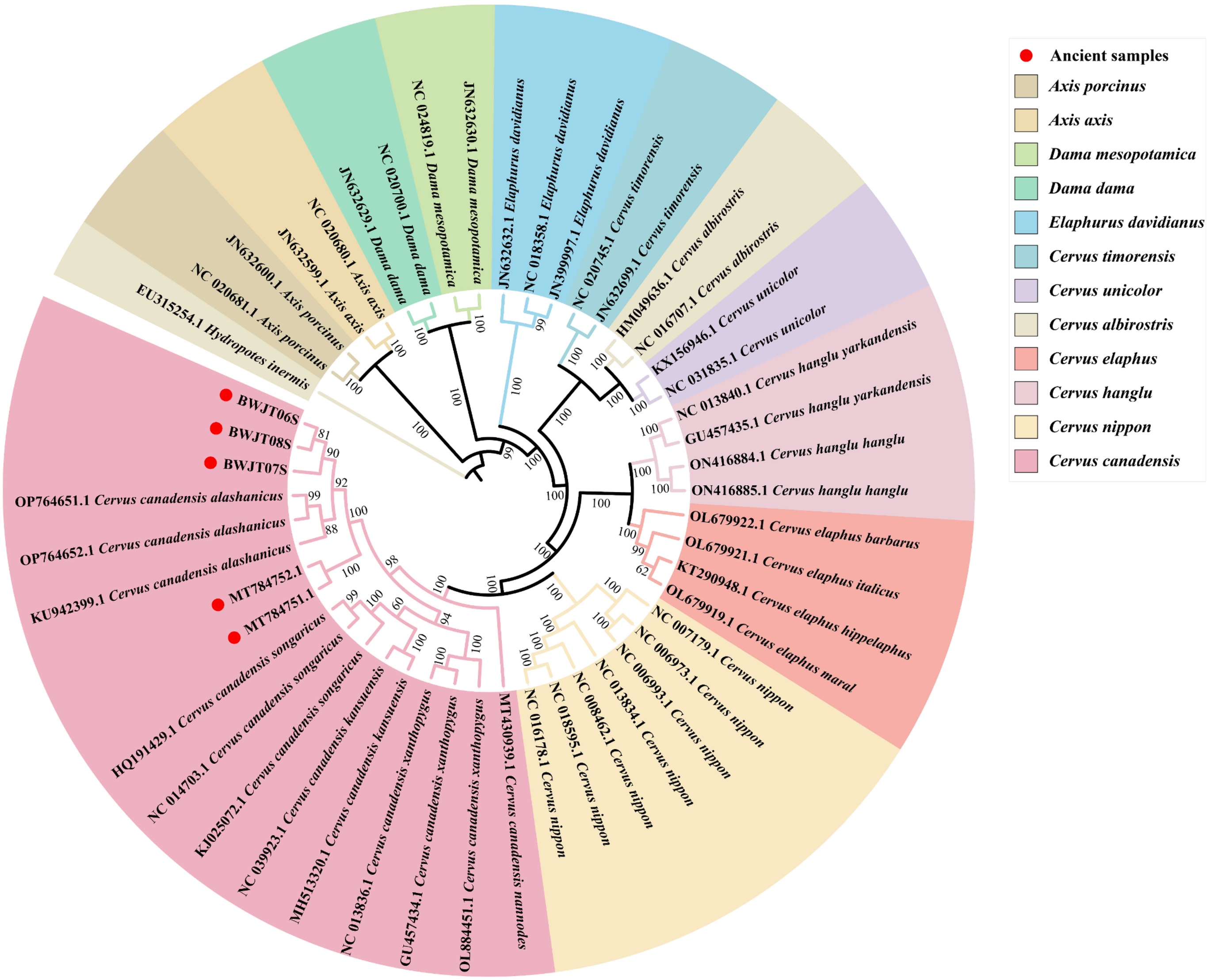

**Fig. 3.** ML phylogenetic tree based on mitochondrial genome of Cervinae subfamily.

1500 years ago was genetically close to the modern population. The ancient samples dated to 3800 BP and 5100 BP differed significantly from the modern ones in opposition to 1500 BP samples which were similar to them. The results of the nucleotide diversity calculation also support these findings, as the nucleotide diversity of the Royal Sacrificial Site of the Northern Wei Dynasty samples and the modern samples are close to each other, and both of them are lower than that of the ancient samples from Heilongjiang. These results indicate that the low nucleotide diversity of modern *Cervus canadensis alashanicus* was formed at least 1500 years ago and remained up to now. As mitochondrial genome data of *Cervus canadensis alashanicus* is scarce, especially that of ancient samples, the above nucleotide diversity analysis results were obtained based on a small sample size of ancient samples, and we will enlarge the mitochondrial genome data size to perform more in-depth analyses in the future. However, it remains unclear whether the three samples in this study were local or transported from other regions for Sacrifice to Heaven, thus we cannot draw conclusion regarding whether the distribution of *Cervus canadensis alashanicus* during the Northern Wei Dynasty was larger than that of the modern population. This question can be answered by further research which examines and compares ancient samples from different periods and regions to gain a better understanding of this species' distribution changes in history.

### *4.2. Deer cultural worship of the Northern Wei Xianbei*

The deer totem was an indispensable crucial element for Xianbei ethnic group, and it had cultural and religious significance especially during the rule of the Tuoba Xianbei in the Northern Wei Dynasty (Zhang, 2011; Zhao and Yang, 2016). *The Book of Wei* says that with the guidance and assistance of the "divine beast", the Tuoba Xianbei people overcame challenges during their migration (Wei, 1999). Research suggests that the "divine beast" revered by the Tuoba Xianbei people is actually the deer (Wang, 2008). Referring to it as a "divine beast" signifies the reverence and worship of the deer within the Tuoba Xianbei culture. Opinions diverge regarding whether the species of the "divine beast" is deer. A majority of scholars argue that the *Rangifer tarandus*, commonly known as reindeer, aligns better with the characteristics of "resembling a horse and emitting sounds akin to cattle", and that the *Rangifer tarandus* is large in size, good at traversing forests and swamps and capable of carrying heavy stuff (Gan and Sun, 1982; Zhuang, 2012). On the other hand, some researchers propose that the *Cervus canadensis* was the totem animal of the Tuoba Xianbei (Gao, 2023). Due to the fact that the Northern Wei emperors attached great significance to the Sacrifice to Heaven and that they held reverence to *Cervus canadensis*, thus it is plausible that they would use their totem animal as sacrificial animals, which provides evidence that *Cervus canadensis* is the totem animal of

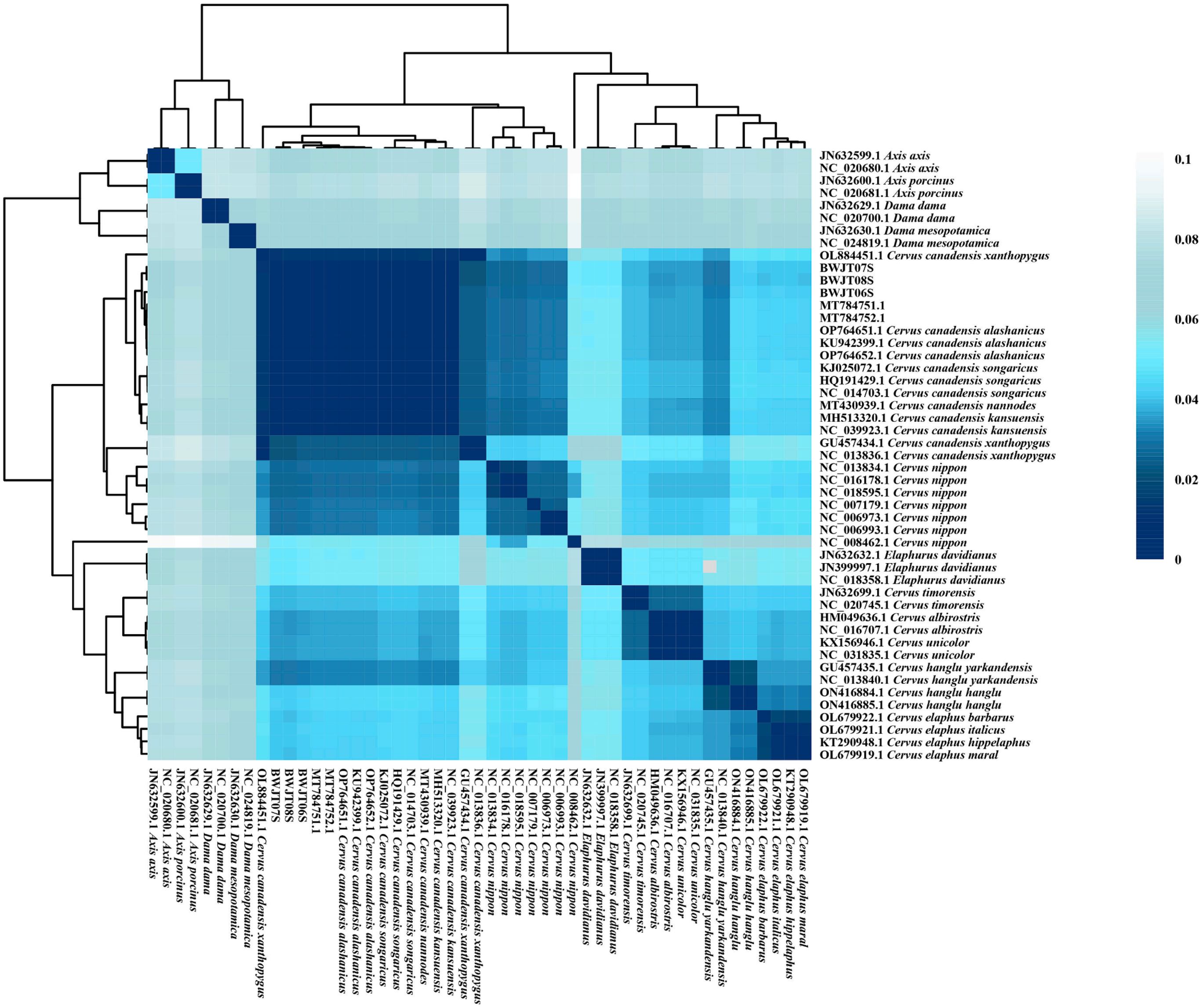

Fig. 4. Pairwise Distances heatmap based on mitochondrial genome of Cervinae subfamily.

Tuoba Xianbei people.

## 5. Conclusion

It was quite common to use animals for sacrificial rituals in the Northern Wei Dynasty. We carried out ancient DNA analysis on three samples morphologically identified as Cervinae subfamily animal excavated from the Royal Sacrificial Site of the Northern Wei Dynasty. Through alignment analysis and BLAST search, the samples were identified as *Cervus canadensis* within the Cervinae subfamily of the *Cervus* genus, which is in line with the results of principal component analysis. Phylogenetic analysis and genetic distance calculation further identified that all three samples were *Cervus canadensis alashanicus*, proving that ancient DNA technology is capable of accurate species/subspecies identification. Analysis of the mitochondrial D-loop region revealed the ancient *Cervus canadensis alashanicus* dated to 3800 BP and 5100 BP differed significantly from the modern ones in opposition to 1500 BP samples which were similar to them. Apart from that, it was found that the low genetic diversity of modern *Cervus canadensis alashanicus* was formed at least 1500 years ago and remained up to now. In addition, as shown by historical documents and archaeological findings, the deer worship by the Tuoba Xianbei people had become a crucial element of their culture and beliefs. The species identification of *Cervus canadensis* provides evidence that the Northern Wei emperors sacrificed the totem-worshipped animal to honor heaven.

## Authorship contribution statement

Guangjie Song and Dawei Cai conceived and designed the experiments. The specimen was provided by Wenping Zhang. The experiments were performed by Guangjie Song. Guangjie Song and Dawei Cai conducted data analysis. The manuscript was written by Guangjie Song. Dawei Cai and Wenping Zhang revised the manuscript. All authors read and approved the final manuscript.

## CRediT authorship contribution statement

**Guangjie Song:** Writing – original draft, Data curation. **Wenping Zhang:** Writing – review & editing. **Qingchuan Bao:** Writing – review & editing. **Dawei Cai:** Writing – review & editing, Data curation.

## Declaration of competing interest

The authors declare that they have no known competing financial

**Fig. 5.** PCA based on mitochondrial D-loop region (931 bp) of *Cervus canadensis alashanicus*.BWJT: ancient samples from the Royal Sacrificial Site of the Northern Wei Dynasty, CADG: ancient samples from Heilongjiang, modern: modern samples.

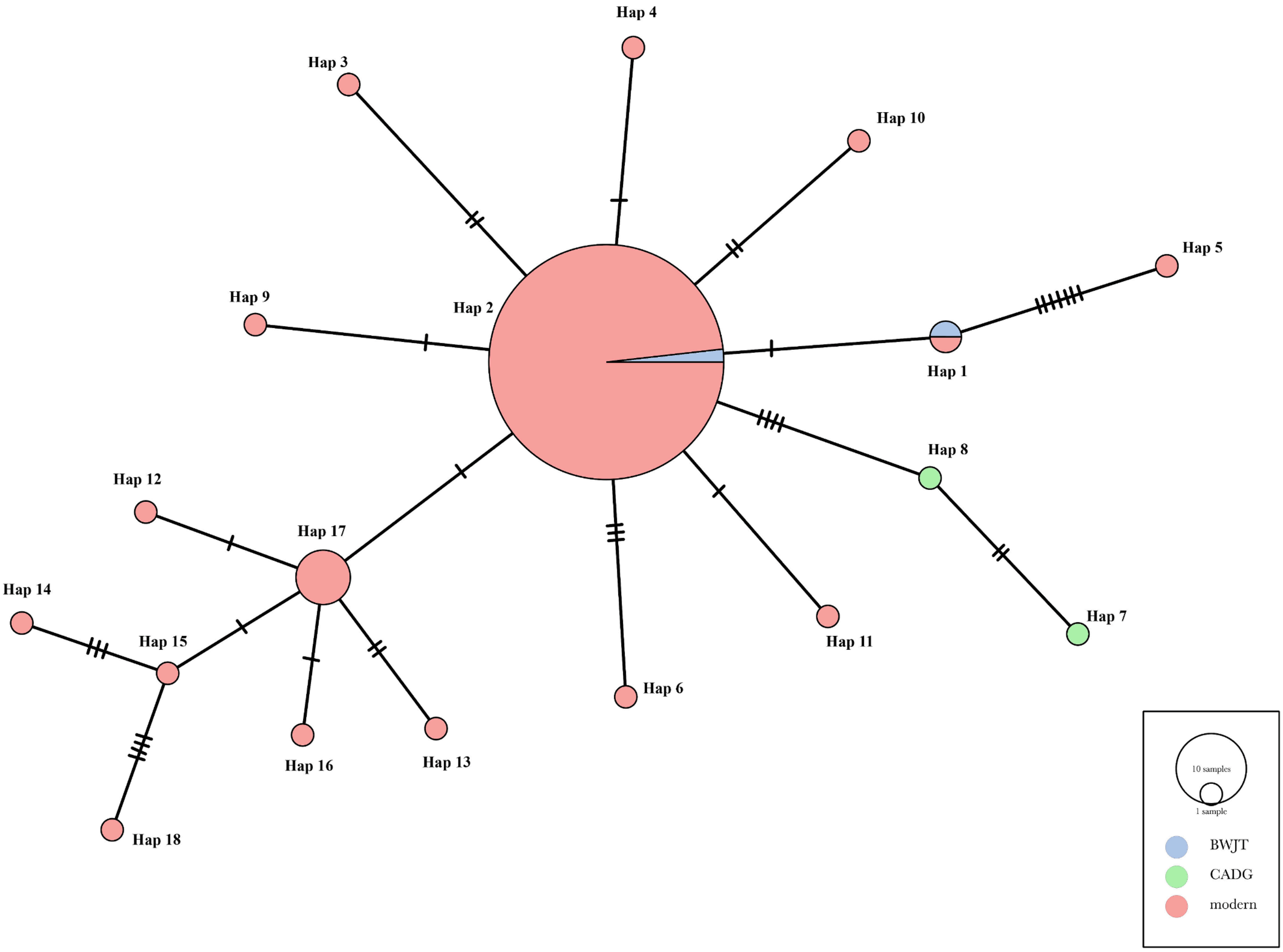

**Fig. 6.** Median-joining network based on mitochondrial D-loop region (931 bp) of *Cervus canadensis alashanicus*.BWJT: ancient samples from the Royal Sacrificial Site of the Northern Wei Dynasty, CADG: ancient samples from Heilongjiang, modern: modern samples.

interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

All novel sequences were deposited in GenBank of NCBI (https://www.ncbi.nlm.nih.gov/)under the accession numbers PP191140-PP191142.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jasrep.2024.104633.

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