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Ancient DNA reveals evidence of abundant aurochs (*Bos primigenius*) in Neolithic Northeast China

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ABSTRACT

Ancient DNA analysis of 24 archaeological bovid remains recovered from large Neolithic (6300 BP to 5000 calBP) pit and ditch features at Houtaomuga, Northeast China, identified 23 of these samples as aurochs (*Bos primigenius*). These DNA-based identifications contrast with the morphological analysis of the remains, which identified them as *Bison exiguous*. The abundance of auroch remains at this site contradicts the general assumption that this species was not present in large numbers in Neolithic China. It also suggests archaeologists need to revise the notion that wild aurochs played an insignificant role in the lifeways of Neolithic peoples in China. Furthermore, phylogenetic analyses of a 294 bp fragment of the mitochondrial DNA (mtDNA) D-loop indicate the identified aurochs belong to a unique haplogroup (Haplogroup C) that is indigenous to East Asia and made no direct contribution to modern domesticated cattle *Bos taurus.* Moreover, temporal changes in haplotype frequencies were observed among the identified aurochs, suggesting population fluctuations potentially caused by human hunting activities occurred among Chinese aurochs during the Neolithic. This study also identified one sample (HT31) radiocarbon dated to ca. 5500–5300 calBP as *Bos taurus*, making it one of the earliest known taurine cattle specimens in China. HT31's location in Northeast China and early date points to the existence of another entrance for domesticated cattle into China, the Northeast China Route via the Mongolian Steppe.

1. Introduction

Paleontological evidence indicates indigenous aurochs (*Bos primigenius*) were widespread in North China during the late Pleistocene (Chow, 1953; Hu, 1959; Qiu, 2006), but their fate in Neolithic China is less well documented. To date, only a few Neolithic bovid remains from China have been morphologically identified as *Bos primigenius* (Li et al., 1980; Huang, 2003). In addition to these morphologically identified remains, two recent studies have also identified remains from Central and Northeast China as *Bos primigenius* through ancient DNA analysis (Brunson et al., 2016; Zhang et al., 2013). Phylogenetic analysis of the complete mitogenome of an isolated cattle mandible (ca. 10,660 calBP) recovered from a riverbed in Northeast China indicates it belongs to an auroch lineage indigenous to China (Zhang et al., 2013). In addition, Brunson et al. (2016) genetically identified three oracle bones (ca. 3900 BP) from Central China as *Bos primigenius*. These genetic data confirm *Bos primigenius* was present in China during the Neolithic. However, due to its relative scarceness in the archaeological record the taxon is assumed to have played an unsubstantial role in the lifeways of Neolithic peoples in China.

In this study, we report on the DNA-based species identification of a large number of *Bos primigenius* remains recovered from Neolithic (6300 BP to 5000 calBP) pit and ditch features at Houtaomuga in Northeast China. Morphologically, most of these bovid skeletal remains were initially identified as *Bison exiguous,* consistent with the notion that this cold-adapted species was widespread in Northeast China (Wei, 1989).

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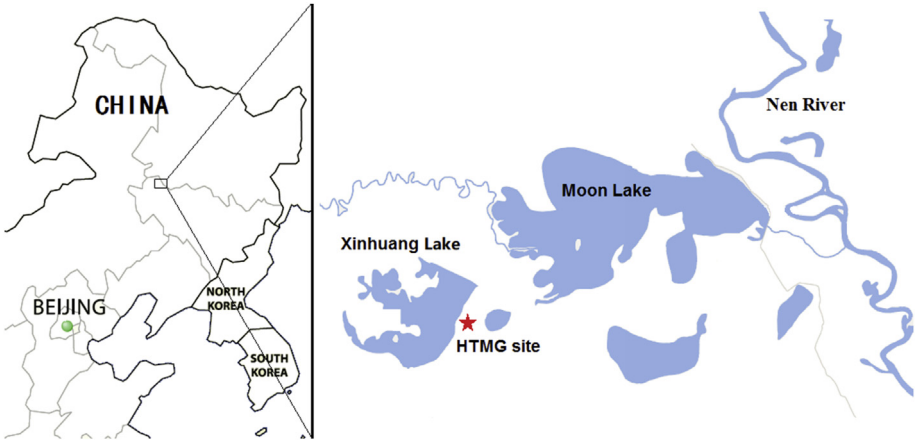

Fig. 1. Maps showing Houtaomuga's (HTMG) landscape and its geographical location in China.

The DNA analysis has not only corrected the species identification assigned to these bovid remains, but also allows for the reassessment of the interactions between people and wild aurochs in ancient China.

2. Materials and methods

2.1. Houtaomuga

Houtaomuga (45.39° N, 123.47° E) is an archaeological site located on the southeastern shore of Xinhuang Lake in Da'an County, Jilin Province, Northeast China (Fig. 1). As part of the Songnen Plain, this region is surrounded by mountain ranges to the west, north, and south, as well as numerous rivers and lakes to the east. Currently, the region's climate is characterized by warm wet summers with a mean temperature of 26–28 °C, and very cold winters with mean temperatures of −22 to −28 °C (Li et al., 1982; Wu and Zhang, 2006), making it one of the coldest regions in China. Ecologically and historically, the region is considered to be a transitional zone between nomadic pastoralism and agriculture (Ren and Zhang, 1998).

The site was first identified in 1957 and was subsequently systematically excavated from 2011 to 2014 by a joint team from Jilin University and the Jilin Provincial Institute of Archaeology. Preliminary studies of the site have constructed a chronology consisting of seven phases with the earliest phase being Phase 1 (ca. 13,000–11,000 calBP) and the latest being Phase 7 (AD 907–1234) (Wang et al., 2012; Kunikita et al., 2017). Phase 3 (ca. 6300–5500 calBP) and Phase 4 (ca. 5500–5000 calBP) at Houtaomuga represent two Neolithic stages (Wang et al., 2012; Kunikita et al., 2017). Excavations of the site have revealed several large Neolithic pit and ditch features filled with faunal remains (Fig. 2). Bovid remains, morphologically identified as *Bison exiguous*, dominate the faunal assemblages from these features, although other taxa, including fish, reptiles, birds, and other mammals, are also represented. A detailed analysis of the faunal assemblage from one of these features (ditch G2) conducted by Zhang (2015) found that 2546 of the 2757 identified specimens consisted of bovid elements. Based on their analysis of the material from ditch G2, Zhang (2015) hypothesized the faunal assemblages recovered from these features might be refuse from ritual feasting activities or the manufacturing of bone artifacts.

2.2. Preparation of bone samples

In total, 34 bovid samples (33 teeth and 1 bone) recovered from Neolithic pit and ditch features at Houtaomuga were selected for ancient DNA analysis (Table 1). In general, the gross morphology of the teeth and bone selected for analysis is well preserved (Fig. 3). Samples HT1-HT15 are from Phase 3 (ca. 6300–5500 calBP), whereas samples HT16-HT34 are from Phase 4 (ca. 5500–5000 calBP) (Table 1). Although the majority of the analyzed bovid samples were not directly dated, their assignment to Phase 3 or 4 is supported by the stratigraphic placement of their respective cultural contexts, as well as these contexts association with dated materials. Moreover, one sample (HT05) from Phase 3 and two samples (HT28 and HT31) from Phase 4 were then sent to Beta Analytic Inc. for radiocarbon dating. HT05 (No. Beta-445,990) was radiocarbon dated to ca. 5900–5700 calBP, while HT28 (No. Beta-445,988) and HT31 (Beta-445,989) were radiocarbon dated to ca. 5500–5300 calBP (2 sigma calibration).

Prior to DNA extraction, the surfaces of the samples were first cleaned with a brush, and then removed with a Dremel tool. A small piece (1–2 cm) of tooth or bone was subsequently removed and then submerged in a 5% sodium hypochlorite solution for 5–10 min, rinsed with clean water and 100% ethanol, and UV irradiated for 30 min on each side. The decontaminated samples were then ground into a fine power with a liquid nitrogen grinding mill (Mill 6750, SPEXP CetriPrep, USA).

2.3. DNA extraction and PCR amplification

DNA was extracted from approximately 200 mg of bone or tooth powder using a modified silica-spin column method (Yang et al., 1998). Approximately, 100 μl of DNA solution was collected from each sample and used in subsequent PCR amplifications. A 294 bp fragment of the mtDNA D-loop was obtained by amplifying two overlapping fragments with two sets of primers designed using a *Bos taurus* reference sequence (Genbank accession number V00654) (Anderson et al., 1982): for fragment 1, forward primer L16022 (5′-GCCCCATGCATATAAGC

Fig. 2. A large ditch feature (G1) at Houtaomuga filled with faunal remains. All of the Phase 3 samples analyzed in the study (HT01-HT15) were collected from this ditch feature.

Table 1
Bovid remains for ancient DNA analysis in this study.

	Lab Code	Element	Arch Code	JLU Lab			Beijing Lab			Repeated
				Extract.	Frag.1	Frag.2	Extract.	Frag.1	Frag.2	Species ID
Houtaomuga Phase 3 (ca. 6300-5500 CalBP)	HT01	Tooth	11DHAIIIG1①:143	1	2/3	2/3				Yes
				2	1/2	1/2				*B.p.*
	HT02	Tooth	11DHAIIIG1④:93	1	2/3	1/3				Yes
				2	1/2	2/2				*B.p.*
	HT03	Tooth	11DHAIIIG1①:149	1	2/2	2/3				Yes
				2	2/2	1/2				*B.p.*
	HT04	Tooth	11DHAIIIG1②:35	1	2/2	2/3	1	3/4	3/5	Yes
				2	1/1	1/2				*B.p.*
	HT05	Tooth	11DHAIIIG1:1025	1	2/2	2/3				Yes
				2	1/1	1/2				*B.p.*
	HT06	Tooth	11DHAIIIG1④:96	1	2/3	2/3	1	3/3	3/4	Yes
				2	1/2	1/2				*B.p.*
	HT07	Tooth	11DHAIIIG1①:142	1	1/2	2/3				Yes
				2	2/2	1/2				*B.p.*
	HT08	Tooth	11DHAIIIG1①:145	1	2/3	1/3				Yes
				2	1/2	2/3				*B.p.*
	HT09	Tooth	11DHAIIIG1:669	1	2/2	2/2				Yes
				2	1/1	2/2				*B.p.*
	HT10	Tooth	11DHAIIIG1④:97	1	2/2	2/2				Yes
				2	1/1	1/2				*B.p.*
	HT11	Tooth	11DHAIIIG1④:99	1	2/2	2/2				Yes
				2	1/1	1/1				*B.p.*
	HT12	Tooth	11DHAIIIG1:553	1	2/2	2/2				Yes
				2	1/1	1/1				*B.p.*
	HT13	Tooth	11DHAIIIG1:941	1	2/2	2/3				Yes
				2	1/1	1/1				*B.p.*
	HT14	Tooth	11DHAIIIG1①:40	1	2/2	2/2				Yes
				2	1/1	1/1				*B.p.*
	HT15	Tooth	11DHAIIIG1:148	1	2/2	2/3				Yes
				2	1/1	1/2				*B.p.*
Houtaomuga Phase 4 (ca. 5500-5000 CalBP)	HT16	Tooth	13AHAIGJ01	1	2/3	2/3				Yes
				2	1/1	1/1				*B.p.*
	HT17	Tooth	13DHAIG2 T0102	1	2/2	2/3				Yes
				2	1/1	1/2				*B.p.*
	HT18	Tooth	13DHAIG002	1	0/3	0/3				
				2	0/3	0/3				
	HT19	Tooth	13DHAIH036④	1	0/3	0/3				
				2	0/3	0/3				
	HT20	Tooth	13DHAIH004①	1	2/2	2/3	1	2/3	3/5	Yes
				2	2/2	1/1				*B.p.*
	HT21	Tooth	13DHAIH004③	1	0/3	0/3				
				2	0/3	0/3				
	HT22	Tooth	13DHAIH006	1	0/3	0/3				
				2	0/3	0/3				
	HT23	Tooth	13DHAIH036①	1	2/2	2/3	1	3/3	2/3	Yes
				2	1/1	1/2				*B.p.*
	HT24	Tooth	13DHAIG001	1	2/2	2/3	1	3/4	3/5	Yes
				2	1/2	1/3				*B.p.*
	HT25	Tooth	13DHAIG003	1	0/3	0/3				
				2	0/3	0/3				
	HT26	Tooth	14DHAIG001:1	1	0/3	0/3				
				2	0/3	0/3				
	HT27	Tooth	4DHAIG001:2	1	0/3	0/3				
				2	0/3	0/3				
	HT28	Tooth	4DHAIG001:3	1	1/2	2/3				Yes
				2	2/2	1/2				*B.p.*
	HT29	Tooth	4DHAIG001:4	1	0/3	0/3				
				2	0/3	0/3				
	HT30	Tooth	14DHAIT1206:5	1	2/2	2/2				Yes
				2	1/1	2/2				*B.p.*
	HT31	Tooth	14DHAIG001:6	1	3/4	2/3				Yes
				2	2/2	2/2				*Bos taurus*
	HT32	Tooth	14DHAIG001:7	1	0/3	0/3				
				2	0/3	0/3				
	HT33	Bone	14DHAIG001:8	1	0/3	0/3				
				2	0/3	0/3				
	HT34	Tooth	14DHAIT1306:9	1	0/3	0/3				
				2	0/3	0/3				

Note: Extract. refers to DNA extraction repeat; Frag.1 and Frag. 2 refer to the 157 bp (Fragment 1) and 175 bp (Fragment 2) fragments amplified respectively using two different sets of primers; "#/#" indicates the number of times a fragment was successfully amplified and the number of attempted PCR amplifications; B.p. stands for Bos primigenius.

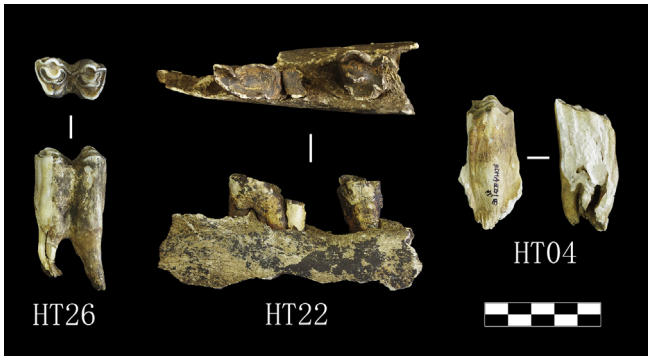

Fig. 3. Images showing three of the tooth samples used for ancient DNA analysis in this study. All three of the samples exhibit good morphological preservation, but only one (HT04) yielded PCR products. All of the other tooth and bone samples used are in similar conditions.

AAG-3′) and reverse primer H16178 (5′-CACGCGGCATGGTAATTAAG-3); and for fragment 2, forward primer L16137 (5′-TTCCTTACCATTA GATCACGAGC-3′) and reverse primer H16315 (5′-GGAAAGAATGGAC CGTTTTAGAT-3′). These two sets of primers target amplicons that are 157 bp and 175 bp long, respectively.

PCR amplifications were performed in a Mastercycler Personal thermal cycler (Eppendorf, Hamburg, Germany) in a 25 µl reaction volume containing 2 µl of DNA solution, 1 U *TransStart™ TopTaq* DNA polymerase (TransGen Biotech, Beijing, China), 1x PCR Buffer, 0.2 mM of dNTP, and 0.2 µM of each primer. The PCR conditions consisted of an initial denaturation period of 4 min at 94 °C followed by 36 cycles of 55s at 94 °C (denaturation), 55s at 52 °C-55 °C (annealing), and 55s at 72 °C (extension), with a final extension step of 10 min at 72 °C.

PCR products were isolated via electrophoresis using 2% agarose gels (Biowest, Germany) and then purified with a QIAquick® Gel Extraction Kit (Qiagen, Germany). PCR products were bidirectionally sequenced with the amplification primers at Jilin University (JLU) using a BigDye Terminator v3.1 sequencing kit on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA).

2.4. Data analysis

The obtained DNA sequences were edited and aligned using MAFFT 7 (mafft.cbrc.jp/alignment/software/) (Katoh and Standley, 2013). Haplotype diversity (Hd ± SD), nucleotide diversity (Pi ± SD), and mean number of pairwise differences (MNPD ± SD) were calculated with Arlequin 3.5 (cmpg.unibe.ch/software/arlequin35/) (Excoffier and Lischer, 2010). Variable positions were identified in MEGA 6.0 (www.megasoftware.net/) (Tamura et al., 2013) by comparing the HT sequences with a *Bos taurus* reference sequence (Genbank accession number V00654). To examine temporal trends in the frequency of the identified haplotypes, a three-dimensional statistical parsimony network was constructed using the R script TempNet (web.stanford.edu/group/hadlylab/tempnet/) (Prost and Anderson, 2011).

Bayesian (BI) and Maximum likelihood (ML) phylogenetic trees were constructed in order to evaluate the relationship between the HT samples and other modern and ancient bovids. In addition to the sequences obtained from the HT samples, publicly available sequences from several *Bison* and *Bos* species were also included in the phylogenetic analyses (Table S1). A buffalo (*Bubalus bubalis*) reference sequence (Genbank accession number NC_006295) was used as the outgroup in both the ML and BI phylogenetic analyses. The best-fit nucleotide substitution model (HKY85 + G + I) was determined with jModelTest2 (https://github.com/ddarriba/jmodeltest2) (Darriba et al., 2012) based on the BioNJ topology and using the Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC). The BI phylogenetic tree was constructed using MrBayes 3.25 (http://mrbayes.sourceforge.net/) (Ronquist et al., 2012). To construct the BI tree, twenty million generations of the Markov chain Monte Carlo (MCMC) chains were run, with sampling occurring every 1000 generations, and the first 25% trees were discarded as burn-in. The ML phylogenetic tree was constructed with PhyML3.0 (www.atgc-montpellier.fr/phyml/) (Guindon et al., 2010) and branch support was evaluated through the approximate likelihood ratio test based on the Shimodaira-Hasegawa-like procedure (aLRT SH-like). The BI and ML trees were visualized with TreeGraph 2 (http://treegraph.bioinfweb.info/) (Stöver and Müller, 2010) and iTOL v3. (http://itol.embl.de) (Letunic and Bork, 2016), respectively.

2.5. Contamination controls and prevention

All pre-PCR procedures were conducted in a dedicated ancient DNA laboratory at JLU that is physically separated from the post-PCR laboratory. A series of strict criteria for authenticating ancient DNA sequences were applied as described by Cooper and Poinar (2000). To remove potential contaminant DNA, working areas and benches were frequently cleaned with bleach and exposed to UV radiation. All workers wore protective clothing consisting of coveralls with hoods, facemasks, and gloves. In order to ensure the reproducibility of the results, at least two independent DNA extractions and multiple PCR amplifications of each DNA extract were carried out for each sample in the JLU lab. To detect any possible contamination, blank controls were included in each DNA extraction procedure and PCR setup.

2.6. Independent replication

To further verify the authenticity of the DNA data, five samples (HT04, HT06, HT20, HT23, and HT24) underwent repeat independent DNA extraction in the dedicated Ancient DNA Laboratory at the Institute of Archaeology, Chinese Academy of Social Sciences in Beijing. DNA was extracted in the Beijing Lab using a silica-spin column method (Yang et al., 1998) similar to the one employed in the JLU lab. PCR amplifications were conducted in a Mastercycler Personal in a 30 µl reaction volume containing 50 mM KCl, 10 mM Tris–HCl, 2.5 mM MgCl2, 0.2 mM dNTP, 0.3 µM each primer, 5 µL of DNA extract, and 1.5–3.0 U AmpliTaq Gold ™ (Applied Biosystems). PCR amplifications were carried out in the Beijing Lab using the same primers used in the JLU lab. The PCR conditions consisted of 60 cycles at 94 °C for 30s (denaturing), 52–55 °C for 30s (annealing), and 72 °C extension for 40s, with an initial 12 min denaturing period at 95 °C. PCR products were visualized through electrophoresis on a 2% agarose gel and were sequenced by Invitrogen™ (Shanghai, China) in both directions using the amplification primers.

3. Results

3.1. Diversity of the ancient DNA sequences

Reproducible sequences were successfully obtained from 24 of the 34 analyzed bovid remains. The DNA sequences obtained from each of the HT samples matched those obtained from internally repeated DNA extractions and PCR amplifications conducted in the JLU Lab (Table 1). In addition, the independent repeat extraction of DNA from five samples in the Beijing Lab yielded DNA sequences concordant with the sequences the JLU Lab obtained from these five samples. All 24 DNA sequences obtained from the HT samples have been deposited in GenBank with accession numbers MG279460-MG279483.

Based on comparisons with a *Bos taurus* reference sequence V00654, a total of 30 variable positions were identified and 13 haplotypes (Bp1-Bp13) were defined (Table 2). The most common haplotype (Bp4) was identified in 25% of the samples and is shared by samples from both Phase 3 and 4. The next most frequent haplotypes, Bp5 and Bp12 (21% and 13% of samples, respectively), are shared by multiple samples from

Table 2
Variable nucleotide positions of the 294 bp mitochondrial D-loop fragment amplified in this study.

Haplotype	Variable nucleotide positions																														Sequence Name	
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Phase 3	Phase 4
	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6		
	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2		
	5	6	6	8	8	8	8	9	1	1	2	2	2	3	3	5	5	0	1	1	3	3	3	4	5	6	6	7	8	9		
	7	7	8	3	4	5	6	5	0	9	1	5	7	7	9	4	8	0	2	9	1	2	3	8	0	0	4	4	1	2		
V00654	G	A	T	A	C	T	G	A	C	T	G	A	C	T	C	A	G	–	C	C	C	C	G	C	A	C	G	C	C	C		
Bp1	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	–	.	.	.	.	.	T	.	.	.	.	.	.		HT31
Bp2	A	.	C	G	T	C	.	.	T	C	.	T	.	.	.	.	.	A	T	T	T	.	.	.	G	T	A	.	.	.	HT12	
Bp3	A	.	C	G	T	C	.	.	T	C	.	T	.	.	.	.	.	A	.	.	T	.	.	.	G	T	A	T	.	.		HT30
Bp4	A	.	C	G	T	C	.	.	T	C	.	T	.	.	.	.	.	A	.	.	T	.	.	.	G	T	A	.	.	.	HT07 HT09 HT13	HT17 HT20 HT23
Bp5	.	G	C	G	T	C	.	G	.	C	.	T	T	C	.	G	.	A	.	.	T	.	.	T	G	T	A	.	.	.	HT03 HT04 HT08 HT11 HT15	
Bp6	.	G	C	G	T	C	.	G	.	C	.	T	T	C	.	G	.	A	.	.	T	.	.	T	G	T	A	.	T	T	HT10	
Bp7	.	G	C	G	T	C	.	G	.	C	.	T	T	C	.	.	.	A	.	.	T	.	A	T	G	T	A	.	.	.	HT01	
Bp8	.	G	C	G	T	C	.	G	.	C	.	T	.	C	.	.	A	A	.	.	T	.	.	T	G	T	A	.	.	.		HT22
Bp9	.	G	C	G	T	C	.	G	.	C	.	T	.	C	.	.	.	A	.	.	T	.	.	T	G	T	A	.	.	.		HT27
Bp10	.	.	C	G	T	C	.	.	.	C	.	T	.	C	.	.	.	A	.	.	T	T	.	.	G	T	A	.	.	.	HT16	
Bp11	.	.	C	G	T	C	.	.	.	C	.	T	.	C	.	.	.	A	.	.	T	.	.	.	G	T	A	.	.	.		HT24
Bp12	.	.	C	G	T	C	.	G	.	C	.	T	.	.	.	.	.	A	.	.	T	.	.	.	G	T	A	.	.	.	HT02 HT06 HT14	
Bp13	.	.	C	G	T	C	.	G	.	C	.	T	.	.	T	.	.	A	.	.	T	.	.	.	G	T	A	.	.	.		HT28

Note: Nucleotide positions are numbered according to GenBank accession no. V00654.

Phase 3, but are both absent among the Phase 4 samples. The remaining ten haplotypes were only represented by a single sample. All of the HT samples, except for HT31, share a distinctive set of sequence variations (Table 2): 16,068C- 16,083G- 16,084T- 16,085C- 16,119C- 16125T- 16,231T- 16,250G- 16,260T- 16,264A, and the insertion of A at 16,200.

An examination of Table 2 also reveals the distinctiveness of HT31 as it is significantly different from the other HT samples, but very similar to the *Bos taurus* reference sequence V00654. Further comparisons made through BLAST searches of GenBank and phylogenetic analyses supports the identification of HT31 as domesticated *Bos taurus*. Furthermore, the BLAST search of HT31 revealed a 100% sequence similarity with a sample from Korean Jeju yellow cattle (Genbank accession number AY337540). As it is not an auroch, HT31 was removed from all subsequent population sequence analyses of the HT samples.

The overall genetic diversity estimates for the HT samples were as follows: Hd = 0.8893 ± 0.0441, Pi = 0.0156 ± 0.0088, MNPD = 4.61 ± 2.34. It is interesting to note that the genetic diversity of the HT samples is higher than that of early domesticated cattle remains (approximately 4000 BP) (Table 3) from China despite the latter being from multiple locations and time periods (Cai et al., 2014). One potential explanation for this pattern is that the HT samples are from undomesticated wild aurochs. Generally, wild populations are expected to be more diverse than domesticated populations.

3.2. Phylogenetic trees

Sequences from several *Bison* and *Bos* species were included in the construction of phylogenetic trees of the HT samples, which used *Bubalus bubalis* as an outgroup (Table S1). In total, 148 DNA sequences were used to generate both the BI (Fig. 4) and ML (Fig. S1) trees. In both trees, all of the HT samples except HT31 clustered together to form a strongly supported distinctive group (96% posterior probability support and 96% aLRT SH-like). This group is relatively close to both *Bos taurus* and *Bos primigenius*, but very different from comparative *Bison* species, such as *Bison priscus* and *Bison bonasus*. As expected, the HT group also includes a sequence (Genbank accession number KF525852) from a 10,660 year-old *Bos primigenius* mandible recovered from the same geographic region (Zhang et al., 2013), which the original paper codes as haplogroup C. A recent study (Brunson et al., 2016) of oracle bones from China has also identified samples (n = 3) with two haplogroup C haplotypes similar to the KF525852 sequence.

It is interesting to note that haplogroup C clusters with haplogroup R, seemingly indicating a close relationship (Fig. S1). However, this cannot be easily explained as haplogroup R is confined to Italy, whereas haplogroup C has only been identified in China. However, when the whole mitogenome is considered, haplogroups C and R are found to be separate from each other (Zhang et al., 2013). Consequently, it is reasonable to believe that the close haplogroup C-R relationship evident in our phylogenetic trees is due to the short length of the DNA fragment that we examined.

Table 3
DNA sequence diversity of HT samples and early domesticated cattle remains from China.

Diversity	HT Samples	Ancient Cattle Remains (Cai et al., 2014)
Hd	0.8893 ± 0.0441	0.8128 ± 0.0411
Pi	0.0156 ± 0.0088	0.0094 ± 0.0059
MNPD	4.61 ± 2.34	2.27 ± 1.27

3.3. TempNet analysis

A TempNet analysis was conducted in order to examine whether any changes in the frequency of the identified haplotypes occurred among the Houtaomuga aurochs between Phase 3 and Phase 3 (Fig. 5). The results of the analysis indicate the number of haplotypes seems to have increased from Phase 3 (6/14) to 4 (7/9), but only one haplotype (Bp4) identified in Phase 3 persists into Phase 4 while the others disappear. Although new haplotypes show up in Phase 4, the topographic change in the network shows the loss of a major branch in Phase 4, suggesting a dramatic population fluctuation occurred between Phase 3 and 4.

4. Discussion

4.1. Authentication of the ancient DNA data

The authenticity of the ancient DNA sequences obtained from the HT samples is supported by multiple lines of evidence: 1) all pre-PCR procedures in the JLU and Beijing labs were conducted in dedicated ancient DNA labs physically separated from the post-PCR laboratories and housed in different buildings; 2) blank extraction and negative PCR controls processed alongside the HT samples failed to yield PCR products, indicating a lack of systematic contamination; 3) the genetic data indicate all but one of the samples are *Bos primigenius*, which excludes

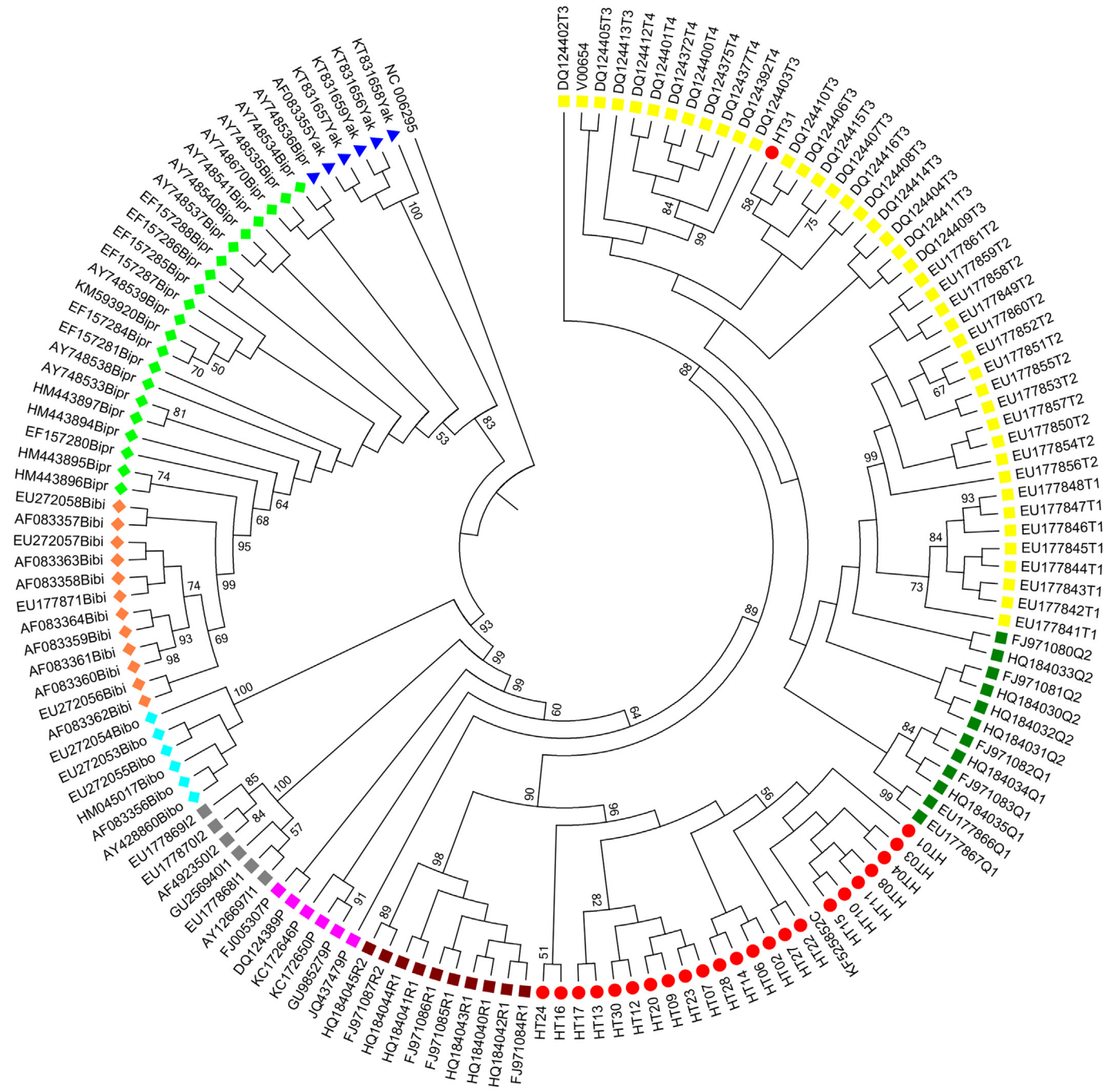

Fig. 4. Bayesian phylogenetic tree displaying the relationship between the D-loop sequences obtained from the Houtaomuga bovid samples and sequences from *Bison* and *Bos* species accessioned in GenBank. A total of 148 D-loop sequences were used to construct the tree. Bayesian posterior probabilities are provided for nodes supported by posterior probabilities ≥50%.

the possibility of contamination from modern sources as this taxon is long-extinct in China; 4) multiple haplotypes were identified, suggesting a lack of systematic cross-sample contamination; 5) the results make phylogenetic sense as the BI and ML trees indicate the HT *Bos primigenius* samples belong to an East Asian *Bos primigenius* haplogroup; 6) the sequences obtained from each of the HT samples were internally replicated in the JLU Lab through repeat DNA extractions as well as repeat PCR amplifications and DNA sequencing; 7) the DNA sequences obtained from five of the HT samples were independently replicated in the Beijing Lab; 8) the relatively high success rate (70.59%) is corroborated by Houtaomuga's location in one of the coldest regions in China, which makes the site's environmental conditions conducive to DNA preservation.

4.2. Species identification of Bos primigenius and its paleontological significance

The DNA sequence analyses indicated all 24 of the samples with amplifiable DNA clustered within *Bos* haplogroups (Fig. 4). This indicates they should be confidently identified as a *Bos* species rather than any *Bison* species as originally suggested by the morphology-based identifications. Furthermore, DNA sequence analyses showed significant sequence differences between all but one of the HT samples and reference sequences from domesticated *Bos taurus*, indicating they

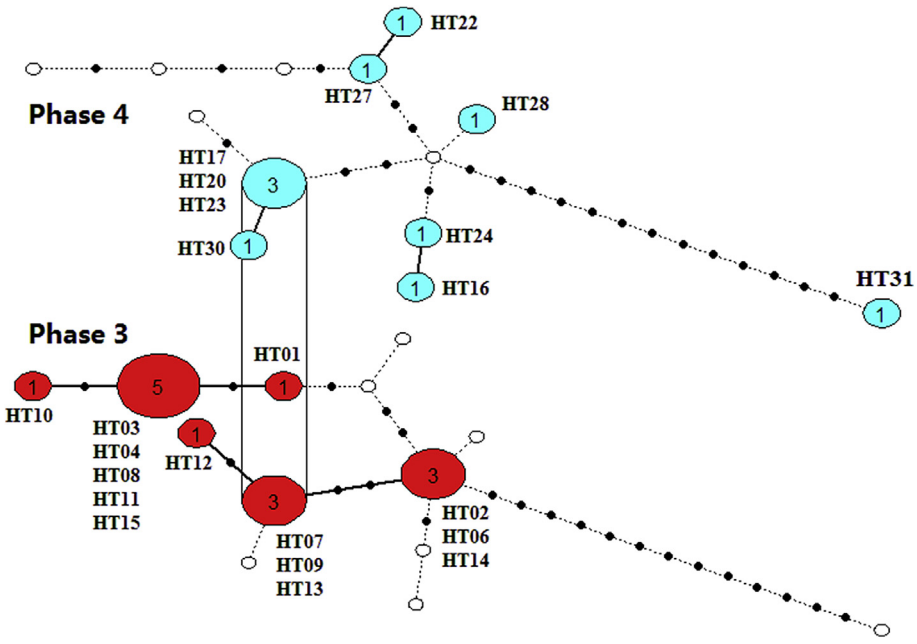

Fig. 5. Temporal network documenting the relationship between the haplotypes observed among the Houtaomuga bovid samples from Phase 3 and Phase 4. Haplotypes are represented by ellipses, with the area of each ellipse being proportional to the haplotype's frequency. A haplotype that is not found in a particular phase but is present in the other phase appears as a white ellipse. Haplotypes present in both layers are connected by vertical lines.

should be identified as *Bos primigenius* rather than domesticated *Bos taurus*.

Previous paleontological and zooarchaeological studies indicate three bovid species (*Bison exiguous*, *Bos primigenius*, and *Bubalus wangsjoki*) were abundant in Northeast China until the Late Palaeolithic (Tong et al., 2013). *Bos primigenius* and *Bubalus wangsjoki* are generally assumed to have gradually become extinct during the Neolithic period as few faunal remains of these two species have been reported from the region. Conversely, *Bison exiguous* was believed to have thrived until the late Neolithic as numerous bovid remains from multiple archaeological sites (eg., Honghe in Heilongjiang; Shuangta in Baicheng), including the samples in this study, have been identified as *Bison exiguous* (Zhang, 2015).

Our identification of numerous Phase 3 and 4 bovid remains from Houtaomuga as *Bos primigenius* contradicts this notion and suggests the taxon was abundant in Northeast China until at least 5000 calBP. Moreover, our identification of purported *Bison exiguous* remains as *Bos primigenius* also raises paleontological questions about the fate and status of *Bison exiguous* in the region. Due to the potential misidentification of *Bos primigenius* remains as *Bison exiguous*, the abundance of *Bison exiguous* in Northeast China, if this species indeed existed in region, has likely been overestimated. Moreover, even if *Bison exiguous* was present in the region the taxon, on account of being cold-adapted, may not have persisted in abundant numbers during the warm Holocene Megathermal (8500 to 3000 BP) (Shi et al., 1992). Some scholars even question the validity of *Bison exiguous's* status as separate species, suspecting *Bison exiguous* may just be steppe bison *Bison priscus.* which raises further questions about the taxon's abundance (Tong et al., 2013).

However, it should be noted that the absence of DNA-identified *Bison exiguous* in our sample may not be sufficient to refute the existence of *Bison exiguous* in the region. Our study's small sample size (34 samples in total) and the fact that all of the samples are from a single archaeological site limits our dataset's ability to confirm the absence of *Bison exiguous* in the region. In addition, 10 of the analyzed samples failed to generate any DNA sequences, raising the possibility the some of these samples may be *Bison exiguous* that failed due to primer mismatches.

4.3. Archaeological significance of Chinese aurochs

The phylogenetic analyses of the HT samples identified as *Bos primigenius* clearly indicate they cluster within haplogroup C, which has been previously identified in other *Bos primigenius* remains from China (Brunson et al., 2016; Zhang et al., 2013). Consequently, haplogroup C likely represents wild aurochs indigenous to China and East Asia. As it is only present in modern East Asian cattle, Haplogroup T4 was also once hypothesized to have originated from *Bos primigenius* indigenous to East Asia (Mannen et al., 2004). However, subsequent analyses have shown that T4 is derived from T3, which originated in the Near East, and that a founder effect can adequately explain T4's distribution being restricted to East Asia (Achilli et al., 2008). Like *Bos primigenius* in Europe, Chinese *Bos primigenius* were not likely domesticated. The genetic diversity of the HT samples is higher than early (ca. 4000 BP) domesticated *Bos taurus* in China, which may serve as a good indication of their wild-type status.

The abundance of *Bos primigenius* remains in the Houtaomuga faunal assemblage suggests wild aurochs played an important role in the lifeways of Neolithic peoples in Northeast China. Due to their importance, aurochs could have potentially influenced the subsistence practices of the inhabitants of Houtaomuga, as well as their social and cultural developments. Securing the large number of aurochs needed to generate the large bovid assemblages found in the pit and ditch features at Houtaomuga would have required planned hunting activities. Based on the results of age-at-death and cut mark analyses, Zhang (2015) has proposed the faunal assemblages from the pit and ditch features may be the accumulated refuse of ritual feasting activities or leftover materials from the preparation of bone artifacts. As such, the large number of aurochs taken by the inhabitants of Houtaomuga may have been used for these purposes.

DNA data alone may not be able to reveal much information about the hunting practices or strategies Houtaomuga's inhabitants used to hunt aurochs. However, such data can potentially reveal information about some of the population changes caused by human hunting pressures. The TempNet analysis indicated the genetic diversity of Houtaomuga *Bos primigenius* changed between Phase 3 and 4, indicating population fluctuations may have occurred during this period (Fig. 5). Due to our small sample size and the inclusion of samples from only one archaeological site, it is difficult to decipher the nature of this suspected population change, and whether it was the result of natural processes or

human activities, or both. By analyzing additional samples from other nearby sites in the future, we may be able to test the following hypothesized scenario. In Phase 3, wild aurochs were abundant, and people could easily hunt multiple individuals from the same large population with many shared and related maternal lineages. The multiple shared haplotypes represented among the Phase 3 samples might support this scenario. However, in Phase 4, large populations might have been reduced in size and/or some local populations could have become extinct as a result of human hunting pressures, forcing people to go farther afield in order to obtain sufficient numbers of aurochs. Consequently, as reflected in the temporal network, the hunted aurochs would have more haplotypes and less shared haplotypes. Through the analysis of additional samples, in the future, we may be able to test the hypothesis that hunting, without sustainable strategies, may have contributed to reduction and eventually disappearance of *Bos primigenius* in the region.

4.4. History of domesticated Bos taurus in China

The apparent abundance of *Bos primigenius* in Late Neolithic China (East Asia) raises the possibility that indigenous aurochs could have been domesticated in China or interbred with domesticated *Bos taurus*. If such a domestication or interbreeding event did occur, some modern or ancient domesticated Chinese cattle would be expected to belong to these indigenous aurochs' haplogroup (haplogroup C). However, haplogroup C has not been reported among the mtDNA sequences that have been obtained from the thousands of modern and ancient (Lai et al., 2006; Lei et al., 2006; Cai et al., 2014) domesticated cattle from China that have been sampled to date. This suggests indigenous Chinese aurochs did not make any lasting genetic contribution to ancient or modern domesticated cattle in China. This may be due to biological or behavioral factors that prevented the domestication of indigenous Chinese aurochs and/or their interbreeding with domesticated *Bos taurus*. Estimation of the time to the most common recent ancestor of haplogroup C suggests this haplogroup might have existed in East Asia for 38,378 years (Troy et al., 2001 calibration) or 45,574 years (Shapiro et al., 2004 calibration). Such a timescale along with East Asia's geographic isolation might have been sufficient for the development of reproductive barriers that limited East Asian aurcoh's ability to interbreed with domesticated *Bos taurus*. This scenario can be supported by the observation that even within the less isolated Europe only low frequencies of local auroch haplogroups (P and R) have been reported among modern cattle, suggesting limited interbreeding occurred between domesticated *Bos taurus* and European aurochs (Achilli et al., 2008, 2009; Bonfiglio et al., 2010).

The significant genetic differences that exist between indigenous Chinese *Bos primigenius* and modern and ancient domesticated Chinese cattle suggests domesticated cattle in China originated from outside of the country. This hypothesis has been previously proposed by many zooarchaeological studies (Flad et al., 2009; Yuan et al., 2007). All of the earliest Chinese bovid remains morphologically identified as domesticated *Bos taurus* (no DNA confirmation yet) are from archaeological sites in the Gansu Province of Northwest China (e.g., Fujiamen ca. 5600 BP, Shizhaocun and Xishanping ca. 5400–4700 BP, and Xishan ca.5500–4900 BP), making Northwest China a logical entrance route for domesticated *Bos taurus* into China (Zhao, 1995; Zhou, 1999; Yu et al., 2011). The establishment of the Silk Road approximately 3000 years later in the same region provides further support for this hypothesis.

An unexpected result of this study was that sample HT31 could be confidently DNA-identified as domesticated *Bos taurus*. Phylogenetic analyses indicate this individual belonged to haplogroup T3, which originated in the Fertile Crescent, the center of the cattle domestication (Edwards et al., 2007). HT31 was radiocarbon dated to ca. 5500–5300 calBP (Beta Analytic Inc. Beta-445,989), making it one of the earliest known domesticated *Bos taurus* specimens in China. Its location in Northeast China contradicts the aforementioned notion that Northwest China was the entrance route for domesticated cattle into China (Flad et al., 2009; Yuan et al., 2007), but supports the hypothesis (Zhao, 2015) that Northeast China was also an important entrance route for domesticates into China. Based on archaeobotanical studies, Zhao (2015) has proposed the Mongolian Steppe could have also served as an effective west-east trading and exchange route that entered China through Northeast China. Following their entrance into Northeast China, domesticated *Bos taurus* were likely exchanged into other regions of China and East Asia. BLAST searches revealed HT31 shares a haplotype with an indigenous Korean cattle breed (Jeju yellow cattle), suggesting domesticated cattle were subsequently transported into Korea from Northeast China (Yuk et al., 1979). Although only one sample among our analyzed skeletal samples was identified as *Bos taurus*, its appearance in the Houtaomuga faunal assemblage might mark the beginning of the end of abundant wild aurochs in the region.

5. Conclusions

Ancient DNA analysis was carried out on 34 Neolithic bovid remains from Houtaomuga, an archaeological site in Northeast China. Through vigorous contamination controls and reproducibility tests, authentic and reproducible ancient DNA data was obtained from 24 samples.

1. In total, 23 of the 24 bovid remains from Houtaomuga with amplifiable DNA were genetically identified as *Bos primigenius* rather than the species to which they were morphologically assigned, *Bison exiguous*. The correction of these misidentifications provides an opportunity to revise the notion that aurochs played an insignificant role in the lifeways of Neolithic peoples in China.
2. Phylogenetic analyses of a 294 bp fragment of the mitochondrial D-loop indicated all of the identified aurochs belong to a unique haplogroup (haplogroup C) indigenous to China (East Asia) and made no direct contribution to ancient and modern domesticated *Bos taurus* in China.
3. The abundance of auroch remains in large pit and ditch features at Houtaomuga suggests this species may have been abundant in Northeast China during the Neolithic. However, temporal changes in the frequency of the haplotypes observed among the identified aurochs suggests some dramatic population fluctuations may have occurred among the taxon during this period.
4. As one of the earliest radiocarbon dated (ca. 5500–5300 calBP) and DNA confirmed domesticated *Bos taurus* specimens in China, sample HT31 points to the existence of another entrance route for domesticated cattle into China, the Northeast China Route via the Mongolian Steppe.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jas.2018.08.003.

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