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Wild or domesticated: DNA analysis of ancient water buffalo remains from north China

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ABSTRACT

Recent zooarchaeological studies on water buffalo (*Bubalus* sp.) remains from China and south Asia question the traditional view that water buffalo were first domesticated in Neolithic China over 7000 years ago. The results from several recent population genetic studies of modern domesticated buffalo (*Bubalus bubalis*) are not consistent with each other, placing the original center of buffalo's domestication in south Asia, southeast Asia, or China. This paper reports a study using an ancient DNA approach to analyze water buffalo remains from Neolithic sites in north China to investigate their affinities with modern domesticated water buffalo, and to shed light on the origin of modern domesticated water buffalo in China.

A 169 bp fragment of D-loop mitochondrial DNA was successfully amplified and verified for 13 of 24 bone samples obtained from seven archaeological sites along the Wei River valley in Shaanxi Province, China. The bone samples which yielded positive DNA can be dated to 8000–3600 cal. BP. The phylogenetic analysis of the obtained DNA sequences along with modern water buffalo sequences indicated that the ancient water buffalos were not the direct ancestor of modern domesticated water buffalo. However, the phylogenetic analysis, along with BLAST searches of these ancient DNA sequences, did demonstrate their relatedness to water buffalo more so than to any other bovid species, confirming the existence of indigenous wild (but now extinct) water buffalo species (*B. mephistopheles*) in ancient China.

The DNA analysis of these ancient remains failed to establish direct links between modern domesticated water buffalo (*B. bubalis*) and indigenous water buffalo (*B. mephistopheles*) from ancient China. If further DNA studies of more ancient remains from other regions of China confirm the observation of solely indigenous water buffalo species in ancient China, it would suggest modern water buffalo might not have been first domesticated in China.

1. Introduction

Modern water buffalo (*Bubalus bubalis*) in China are traditionally believed to have been first domesticated in the Yangtzi River region during the Neolithic period around 7000 years ago (Bellwood, 2005: 125; Chang, 1986: 211; Chen and Li, 1989; Han, 1988). Many archaeological sites from the Neolithic and Bronze Age in China have uncovered buffalo remains, morphologically identified as *Bubalus mephistopheles* (e.g., Teilhard de Chardin and Young, 1936; Wei et al., 1990), thus leading to the assumption that modern water buffalo *B. bubalis* was first domesticated from wild *B. mephistopheles* in China. Based on morphological studies of ancient buffalo remains from south Asia, Meadow and Patel challenge this premise and argue that water buffalo was first domesticated in south Asia (Patel, 1997; Patel and Meadow, 1998). Recent studies on Neolithic buffalo remains from China have also cast doubt on the domesticated status of *B. mephistopheles* (Liu et al., 2004, 2006). However, morphological analysis of ancient bones alone has not been able to reveal exactly how these different buffalo forms were related to one another, in part due to the unavailability of modern wild *B. mephistopheles* reference collections, since all wild water buffalo in China have become extinct.

Genetic studies of modern water buffalo from various regions in Asia have also produced divisive conclusions, placing the original domestication event in south Asia (Kierstein et al., 2004; Kumar et al., 2007a,b), southeast Asia (Barker et al., 1997; Lau et al., 1998), and China (Lei et al., 2007). The controversy within these genetic studies is caused in part by the lack of reference DNA sequences from ancient wild and domesticated water buffalo, which are critical to root the phylogenetic tree of modern domestic water

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buffalo to their original geographic regions. Another shortcoming is a lack of DNA sequences from modern wild water buffalo populations (*B. arnee*) which are considered to be the progenitor of modern domesticated water buffalo. A small number of wild water buffalo that have survived in south Asia and southeast Asia could provide the much needed wild water buffalo reference DNA sequences, but few DNA sequences are available from these populations (Flamand et al., 2003). Therefore, extinct water buffalo, such as those recovered from archaeological sites in China, represent an invaluable source of genetic information for tracing the temporal and regional history of water buffalo in Asia, and for studying the domestication history of modern water buffalo.

In this study we focused on archaeological water buffalo remains uncovered from the Wei River valley in Central Shaanxi Province, north China, in order to bridge the gap between genetic and archaeological data. We have managed to successfully analyze 13 samples from four sites in Shaanxi Province, China (Fig. 1), dating to the Neolithic and Bronze Age periods. By obtaining DNA sequences from ancient indigenous buffalo and comparing them to modern reference data, we could examine the relationships on the molecular level between Neolithic–Bronze Age water buffalo and modern domesticated water buffalo in China. A direct link, if established, would provide strong evidence supporting the traditional view of Chinese water buffalo domestication.

2. Materials and methods

2.1. Archaeological water buffalo remains

Abundant ancient buffalo remains have been unearthed in the Wei River valley, morphologically identifiable to three species, *B. telhardi*, *B. youngi*, and *B. mephistopheles*. While the first two species appear to have become extinct during Pleistocene, only the latter one (*B. mephistopheles*) survived to the Neolithic and Bronze Age in the Holocene. All three indigenous Chinese water buffalo species became extinct in antiquity, thus ancient DNA analysis of the remains seems to be only approach to effectively test the link between ancient water buffalo and their modern counterparts in China. A total of 24 buffalo bone samples were obtained from sites in Shaanxi Province (Fig. 1) and subjected to DNA tests in the Ancient DNA Laboratory of Simon Fraser University, Canada. Among these samples, 20 were recovered from three excavated Neolithic sites of Guantaoyuan, Baijia and Kangjia (c. 8000–4000 cal. BP). The Guantaoyuan buffalo remains were identified as *B. mephistopheles* based on morphological characteristics of the horncore (Hu et al., 2007), while the Baijia and Kangjia remains were only identifiable to *Bubalus* sp. (Liu et al., 2001; Zhou, 1994). The other four bone samples were from incomplete skulls recovered from sand deposits of the Wei River bank by local farmers and subsequently stored at the Banpo Museum and the Shaanxi Institute of Archaeology in Xi'an. Two skulls from Tianmashachang and Gengbei were morphologically identified as *B. mephistopheles*, and dated to the early Bronze Age (Table 1). The other two skulls from Dongkou and Mao'erliu respectively, were identified as *B. youngi* and dated to late Pleistocene or even earlier (Fig. 1 and Table 1).

From each bone specimen two pieces (1–2 cm in length each) were cut and prepared for the initial DNA extraction and the repeat analysis. We first conducted a small pilot project with six samples reflecting good morphological preservation from Kangjia (Fig. 2). Based on the high success rate for DNA extraction (5/6 samples), the additional 18 samples were then processed. Altogether 13 samples from Baijia, Kangjia, Tianmashachang and Gengbei produced positive results. These successful bone samples appeared to be in good morphological and physical condition, judging from their solid bone matrix, and were from more recent archaeological contexts (c. 8000–3600 cal. BP) relative to the other specimens in this study.

2.2. Bone decontamination and DNA extraction

The surface of the bone samples was physically abraded with sandpaper to remove possible surface contamination resulting from the handling of the ancient bones and comparative reference specimens. A vigorous chemical decontamination protocol (Yang et al., 2004, 2005) was then applied: a small piece of each bone sample was placed into a 15 mL tube and soaked with 10% or 100% commercial bleach solution for 5–10 min (Table 1), the sample was then immersed in 1 N HCl for 1–3 min and then in 1 N NaOH for 1–3 min before being rinsed with ample amounts of ultra-pure water. Wet bone samples were UV irradiated in a Crosslinker for 30 min on each side. The decontaminated bone samples were then ground into coarse powder using a mortar and pestle, or ground into fine powder using a liquid nitrogen grinding mill (Table 1).

A modified silica-spin column method was used for DNA extraction (Yang et al., 1998, 2004, 2005). The powdered samples were incubated at 50 °C overnight with 3–5 mL of lysis buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 0.5 mg/mL proteinase K) in a rotating hybridization oven. After centrifugation for 20–30 min, 1.5–3.0 mL of supernatant was transferred to an Amicon centrifugal

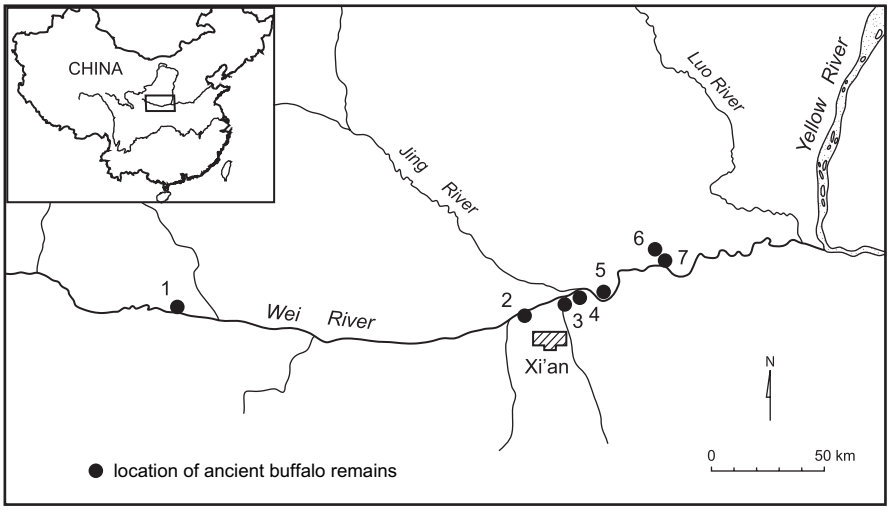

Fig. 1. Location of ancient buffalo remains: 1, Guantaoyuan; 2, Mao'erliu; 3, Tianmashachang; 4, Gengbei; 5, Dongkou; 6, Kangjia; 7, Baijia.

Table 1
The antiquity and morphology of the analyzed faunal remains and the DNA results

Lab code	Site	Morph-ID	cal. BP	Element	Arch. context	Bleach %	Grinding[a]	DNA	Age at death
BF1	Kangjia, Lintong	*Bubalus* sp.	4600–4000	Radius	F264:7-12	10, 100	MP, LNM	KJ1	Adult
BF2	Kangjia, Lintong	*Bubalus* sp.	4600–4000	mc	F264:7-15	10, 100	MP, LNM	Failed	Old adult
BF3	Kangjia, Lintong	*Bubalus* sp.	4600–4000	mt	H71:B6	10, 100	MP, LNM	KJ2	Adult
BF4	Kangjia, Lintong	*Bubalus* sp.	4600–4000	Radius	H71:B16	10, 100	MP, LNM	KJ2	Juvenile
BF5	Kangjia, Lintong	*Bubalus* sp.	4600–4000	Tibia	H77:B17	10, 100	MP, LNM	KJ1	Adult
BF6	Kangjia, Lintong	*Bubalus* sp.	4600–4000	Skull	H77:B54	10, 100	MP, LNM	KJ2	Young?
BF14	Kangjia, Lintong	*Bubalus* sp.	4600–4000	mc px	F264:7-10	10, 100	MP, LNM	KJ1	Adult
BF15	Kangjia, Lintong	*Bubalus* sp.	4600–4000	ph 1	H77:B26	10, 100	MP, LNM	KJ1	Young
BF16	Kangjia, Lintong	*Bubalus* sp.	4600–4000	mt ds	H71:B80	10, 100	MP, LNM	Failed	Adult
BF17	Kangjia, Lintong	*Bubalus* sp.	4600–4000	ph 2	H71:B26	10, 100	MP, LNM	Failed	Juvenile?
BF18	Kangjia, Lintong	*Bubalus* sp.	4600–4000	Tooth	F263:14-2	10, 100	MP, LNM	KJ2	Juvenile
BF19	Kangjia, Lintong	*Bubalus* sp.	4600–4000	Mandible	F263:7	10, 100	MP, LNM	KJ2	Young/older
BF20	Kangjia, Lintong	*Bubalus* sp.	4600–4000	Radius	F264:7-11	10, 100	MP, LNM	KJ2	Adult
BF53	Baijia, Lintong	*Bubalus* sp.	8000–7000	ph1	82SLBT5	100	LNM	Failed	Adult?
BF54	Baijia, Lintong	*Bubalus* sp.	8000–7000	ph1	82SLBT	100	LNM	Failed	Adult?
BF55	Baijia, Lintong	*Bubalus* sp.	8000–7000	Humerus	83SLBT312(2a)	100	LNM	KJ2	Adult?
BF56	Baijia, Lintong	*Bubalus* sp.	8000–7000	Tooth M3	82SLBT5:2	100	LNM	Failed	Adult?
BF57	Guantaoyuan, Baoji	*B. mephistopheles*	7300–7000	Horncore	H240:3	100	LNM	Failed	Adult?
BF58	Guantaoyuan, Baoji	*B. mephistopheles*	7300–7000	Calcaneus	H187:3	100	LNM	Failed	Adult?
BF59	Guantaoyuan, Baoji	*B. mephistopheles*	7300–7000	Tooth M3	H244:1	100	LNM	Failed	Adult?
BF60	Tianmashachang, Baqiao	*B. mephistopheles*	3700–3600	Skull		100	LNM	KJ2	Adult
BF65	Dongkou, Lintong	*B. youngi*	28,000	Skull		100	LNM	Failed	Adult
BF66	Maoerliu, Sanqiao	*B. youngi*	>43000	Skull		100	LNM	Failed	Adult
BF77	Gengbei, Gaoling	*B. mephistopheles*	3700–3600	Skull		100	LNM	KJ1	Adult

[a] MP, mortar and pestal; LNM, liquid nitrogen grinding mill.

filter, Ultra-4 (Millipore, Billerica, MA). The extract was concentrated to less than 100 μL and then purified using QIAquick columns (QIAGEN, Hilden, Germany), from which approximately 100 μL of DNA solution was collected for each sample.

All of the bone samples underwent DNA extraction twice; most of reproduced extractions were from two separate pieces of the original bone sample, although a small number of samples were reproduced using bone power remaining from the first extraction.

2.3. PCR primer design and PCR amplification

Since the majority of water buffalo mitochondrial DNA sequences in GenBank are from the control region (D-loop), this study focused on PCR amplification of a short fragment from the same region. Based on the available reference sequences, forward primer F213 (5′-TAG TAC ATT AAA TTA TAT GCC CCA T-3′) and reverse primer R381 (5′-GCA TGG TAA YTA AGC TCG TGA TCT A-3′) were designed to amplify a 169 bp fragment of D-loop mtDNA from the degraded ancient DNA samples. Since the bone samples could only be confidently identified morphologically as bovids, the PCR primers were designed not to exclude the amplification of cattle DNA.

PCR amplifications were conducted in a Mastercycler Personal (Eppendorf, Hamburg, Germany) in a 30 μL reaction volume containing 50 mM KCl, 10 mM Tris–HCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 mg/mL pig-gelatin (used in place of BSA to avoid possible contamination from bovine DNA), 0.3 μM each primer, 3.0 μL DNA sample and 1.5–3.0 U AmpliTaq Gold™ (Applied Biosystems). PCR was run for 50–60 cycles at 94 °C for 30 s (denaturing), 55 °C for 30 s (annealing), and 72 °C extension for 40 s, with an initial 12 min denaturing period at 95 °C. Five microliters of PCR product were visualized via electrophoresis on a 2% agarose gel using SYBR Green™ staining. PCR products were purified using QIAGEN's MinElute™ purification kits and were subjected to direct sequencing.

The sequencing was carried out using both primers, F213 and R381 respectively to obtain sequences from both directions. The sequencing was performed on an ABI 3100 (Applied Biosystem) at the Mobix of McMaster University of Canada or on ABI 3730XL (Applied Biosystem) at Macrogen, Seoul, Korea (http://www.macrogen.com). The obtained electropherograms were edited and compared using ChromasPro (http://www.technelysium.com.au). Sequences of both the forward and reverse primers were removed from the 169 bp amplified sequence, generating a 113 bp sequence for subsequent sequence analysis.

2.4. DNA sequence analysis

The edited DNA sequences were initially subjected to GenBank BLAST searches with subsequent phylogenetic analysis conducted using MEGA3 software (Kumar et al., 2004). Since only a short fragment of mtDNA was amplified from the ancient samples, the reference DNA sequences obtained from GenBank for phylogenetic comparison were truncated to obtain equivalent DNA sequence lengths. As a result, multiple haplotypes in GenBank were found to share the same truncated DNA sequence. In total, 283 water buffalo sequences were retrieved from GenBank (mainly from several major population genetic studies, including Kierstein et al., (2004), Lei et al., (2007), Kumar et al., (2007b), Lai et al., (unpublished), and Zhang et al., (unpublished), and once truncated, formed only 75 unique haplotypes (Fig. 4). These redefined shortened haplotypes sometimes represent as many as 83 and 89 original haplotypes in GenBank. In order to maximize the representativeness for modern water buffalo genetic diversity for the shorten haplotype, unpublished GenBank DNA sequences were also included in the reference dataset for this study. Using Kimura 2-parameter model, MEGA3 (Kumar et al., 2004) was employed to calculate average pair-wise nucleotide substitution within and between swamp water buffalo, river water buffalo and ancient water buffalo.

2.5. Contamination controls

DNA analysis was conducted in the dedicated ancient DNA laboratory which is specifically designed for and dedicated to ancient DNA work. Strict contamination control protocols were followed: (1) the pre-PCR lab and the post-PCR labs are situated in two buildings with separate ventilation systems; (2) blank DNA extractions and negative PCR controls were all undertaken to monitor contamination; (3) no modern water buffalo DNA samples were

analyzed in the lab, preventing the occurrence of contamination from modern sources. One modern water buffalo bone sample from Zhejiang Province of China was processed (albeit outside of the ancient DNA laboratory) once the DNA analysis of most of the ancient remains was completed, with the modern bone DNA revealing a perfect match to multiple sequences of swamp water buffalo from GenBank (haplotype XY1 in Fig. 4).

3. Results

3.1. PCR amplifications

In spite of the PCR amplification failure for several ancient water buffalo samples from an archaeological site in south China (data not shown), most of the ancient DNA samples from Kangjia yielded strong PCR amplifications and produced reproducible DNA sequences (Table 1). The Tianmashachang sample, the Gengbei sample, and one of the four Baijia samples also produced DNA sequence, accounting for a total of 13 successful samples (Table 1).

3.2. Multiple sequence alignment and phylogenetic analysis

BLAST searches indicated that none of the ancient DNA sequences matched sequences from GenBank without significant sequence differences, though the closest matches were with modern domesticated water buffalo sequences. The multiple alignments of the ancient DNA sequences and two modern DNA sequences (the most common haplotype of swamp, and river water buffalo in Fig. 4) are designed to display the variation among the ancient sequences and their significant difference from their modern counterparts (Fig. 3).

As the modern reference sequences from GenBank were significantly truncated to make them comparable to the ancient sequences, these truncated DNA sequences were first tested to evaluate their phylogenetic informativeness. When compared with their original complete sequences, the truncated sequences produced similar phylogenetic trees, indicating that they were still representative of the original, longer reference DNA sequences. Fig. 4 is a phylogenetic tree (NJ with 2000 bootstrap tests using Kimura 2-parameter model) constructed using MEGA 3 when with cattle (*Bos*) as the outgroup. All redefined haplotypes of modern domesticated water buffalo were clustered into either swamp water buffalo or river water buffalo groups, showing two reciprocally monophyletic clades of swamp and river water buffalo.

All 13 ancient DNA sequences were found to belong to four unique haplotypes within two haplogroups (KJ1 and KJ2) (Figs. 3 and 4). All four ancient DNA haplotypes were distant from the clades of river and swamp water buffalo with haplogroup KJ2 closer to the root of the common ancestor of both swamp water buffalo and river water buffalo.

3.3. Diversity of ancient DNA sequences

It might not be appropriate to calculate the genetic diversity of past water buffalo populations based on the ancient DNA sequences obtained from this limited study, as we cannot ensure that each bone sample represents a single water buffalo individual in the assemblages. Effort was therefore made to estimate DNA sequence divergence of ancient DNA haplotypes and compare it with that of swamp water buffalo and river water buffalo: the group average of pair-wise nucleotide substitutions is 0.048 (Kimura 2-parameter) with standard error (SE) 0.016 (2000 bootstrap replicates) for the ancient DNA haplotypes, which is higher than that of river water buffalo (0.028 with SE 0.008) but lower than that of swamp water buffalo (0.066 with SE 0.016). The inter-group divergence of the ancient remains is 0.085 (SE 0.023) with swamp water buffalo and 0.156 (SE 0.035) with river water buffalo, indicating marked differences between the ancient water buffalo and modern swamp and river water buffalo.

4. Discussion

4.1. Authenticity of the ancient water buffalo DNA

The water buffalo sequences obtained from the ancient remains can be considered authentic due to the following observations: (1) contamination from modern water buffalo DNA can be generally excluded since no modern water buffalo DNA samples were extracted and analyzed before the ancient samples were processed; (2) contamination by reference bone specimens during the morphological studies can be minimized since the analyzed bone samples have gone through a vigorous decontamination process; (3) contamination from previously amplified PCR products is minimized since the established protocols separate the pre-PCR activities and the post-PCR work; (4) the obtained ancient DNA sequences are significantly different from any modern domesticated water buffalo DNA sequences, which is consistent with the fact that these ancient water buffalo are now extinct; (5) most DNA sequences have been successfully repeated (see the exception below); (6) good DNA preservation can be supported by the bones' good morphological preservation (see Fig. 2) and their excavation from sites located in temperate north China rather than in hot and humid subtropical south China (a parallel project to extract DNA from over 10 water buffalo remains from south China failed; data not shown).

Another line of evidence to demonstrate the effectiveness of our contamination controls could be drawn from the general lack of contamination with cattle DNA throughout this study. Although the primers we used in this study are also perfectly suitable for amplification of cattle DNA, we only encountered one such contamination event in over 200 PCR setups in this study. One water buffalo DNA sample failed to generate the same sequence through repeated PCR amplification, and rather surprisingly, the second DNA sequence was from cattle. Fortunately, the second DNA extract from a separate piece of the same bone confirmed its identity as water buffalo. The source of contamination is unknown at this stage since BSA was replaced by pig-gelatin within the PCR reaction, but the incident raises legitimate concerns over the ancient DNA analysis of common domesticated species as DNA from their modern counterparts can be easily introduced into work areas (Leonard et al., 2007).

In addition, the extinct nature of the indigenous Chinese water buffalo has provided the best criterion for quickly detecting any contamination from modern buffalo. The uniqueness of the obtained DNA sequences in fact increases our confidence in accepting the authenticity of the DNA data in this study.

4.2. DNA species identity of B. mephistopheles

The placement of the ancient DNA sequences in the phylogenetic tree (Fig. 4) indicates their relatively close affiliation to, but significant difference from both swamp water buffalo and river water buffalo. For the majority of the samples, the initial BLAST search in GenBank corresponded only to modern water buffalo, while a few matched with some other different species. Therefore, it is reasonable at this stage to conclude that the ancient samples are from water buffalo-like animals (unless closer DNA sequences are found from GenBank in the future). Since *B. mephistopheles* has traditionally been used as species name to classify the now extinct ancient indigenous Chinese water buffalo remains of the Holocene, and two of our DNA samples were from two identifiable *B. mephistopheles* skulls (Tianmashachang and Gengbei), we consider *B. mephistopheles* as a valid separate species identity for these ancient

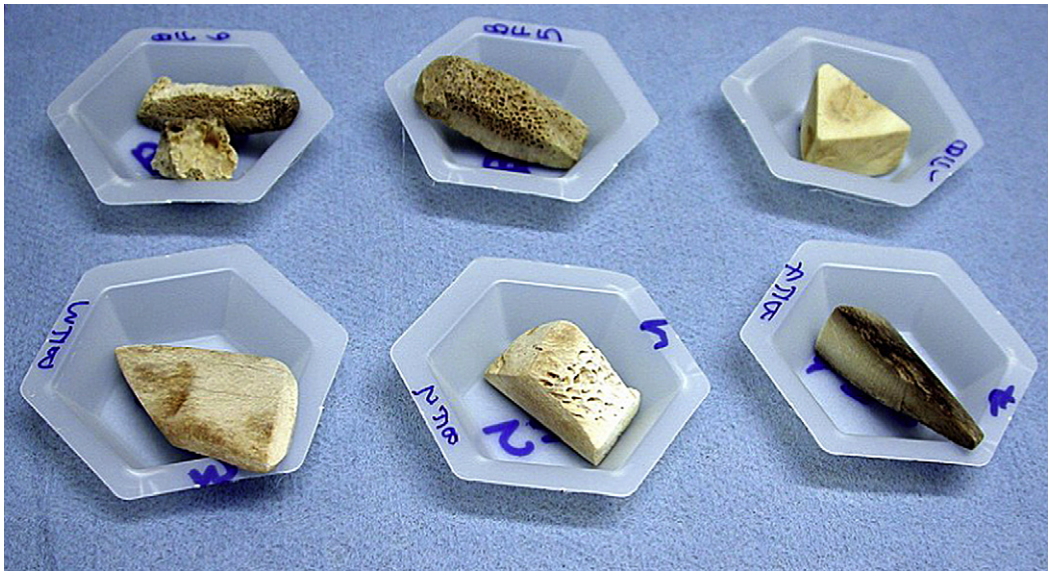

Fig. 2. Water buffalo bone samples BF1 to BF6 before DNA extraction in the lab, showing good morphological preservation. (for scale, the length of each weight-boat side is approximately 25 mm).

remains and as a result, assign the ancient DNA sequences to *B. mephistopheles*.

When examining Fig. 4, it is challenging to interpret the significance of the two clearly separated maternal lineages KJ1 and KJ2 from our ancient DNA sequences. The separate lineages may reflect the huge genetic diversity of wild *B. mephistopheles* populations or perhaps indicate two subspecies of *B. mephistopheles*. The latter possibility can be effectively rejected by the provenances of the remains: the bones from two lineages are sometimes derived from the same archaeological features (Table 1). By definition, two subspecies could not naturally inhabit the same area, and therefore, it can be speculated that KJ1 and KJ2 unlikely represent two subspecies. Based on the DNA divergence, there might be a slight possibility that the KJ1 and KJ2 may represent two separate species. But the skeletal remains that yielded KJ1 (Gengbei) and KJ2 (Tianmashachang) were both identified as *B. mephistopheles*. More research is required to examine skeletal morphological variations of *B. mephistopheles*.

4.3. Wild or domesticated water buffalo

Although only a short PCR fragment was analyzed in this study, the phylogenetic analysis is still very informative for inferring the genetic history of the ancient remains and in shedding new light on their relationships with the origin of modern domesticated water buffalo. Judging from the phylogenetic pattern (Fig. 4), the indigenous water buffalo represented by those ancient remains are unlikely to have represented any earlier members of modern domesticated water buffalo and at best, it may only represent a close evolutionary relationship with the ancestor of modern domesticated water buffalo. We fail to observe any ancient sequences which are clustered more closely with modern domesticated water buffalo clades. No ancient DNA haplotypes were found in the domesticated clades (Fig. 4), showing that those ancient water buffalo did not contribute maternally to modern water buffalo.

In this study, although only the Tianmashachang and Gengbei samples can be confidently identified as *B. mephistopheles* morphologically, the other successfully amplified buffalo samples previously identified only to the genus level (*Bubalus* sp.) can now be confidently classified as *B. mephistopheles* because they all share the same haplogroups. Thus, we can conclude that these wild *B. mephistopheles* from Shannxi Province made little genetic contribution to the domesticated water buffalo. We have multiple bone samples representing *B. mephistopheles*, which allow us to more confidently draw the conclusion that *B. mephistopheles* was not the direct ancestor of modern water buffalo.

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                    10        20        30        40        50        60        70        80        90        100       110       120
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| .
Swamp Type          GCATATAAGC GGGTACACAA ACATGCATGA TAGTACATAG TACATTCAAT TATTGATCGT ACATAGTGCA TTCAAGTCAA ATCCGTCCTC GCCAACATGC -----ATAT- -CCCCT-CCA C 113
River Type          ..G.....T. AA.....T.. .......... .......... .....AT... .......... ......CA.. ..T..... ....A.T... AT..... -----G...- -...T.-... T 113

    <KJ1>
BF1                 .......... AA.....TG. .....T..A. .......... .....A..G. .......... ....G.CA.. ..T..... ....A.T... .......... ------...C- -...T.-... T 113
BF5                 .......... AA.....TG. .....T..A. .......... .....A..G. .......... ....G.CA.. ..T..... ....A.T... .......... ------...C- -...T.-... T 113
BF14                .......... AA.....TG. .....T..A. .......... .....A..G. .......... ....G.CA.. ..T..... ....A.T... .......... ------...C- -...T.-... T 113
BF15                .......... AA.....TG. .....T..A. .......... .....A..G. .......... ....G.CA.. ..T..... ....A.T... .......... ------...C- -...T.-... T 113
BF77                .......... AA.....TG. .....T..A. .......... .....A..G. .......... ....G.CA.. ..T..... ....A.T... .......... ------...C- -...T.-... T 113

    <KJ2>
BF3                 .......... AA.....T.. .....TG... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------...C- -...T.-... T 113
BF4                 .......... AA.....T.. .....TG... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------...C- -...T.-... T 113
BF6                 .......... AA.....T.. .....TG... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------...C- -...T.-... T 113
BF18                .......... AA.....T.. .....TG... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------...C- -...T.-... T 113
BF19                .......... AA.....T.. .....TG... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------...C- -...T.-... T 113
BF20                .......... .A.....T.. ......G... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------...C- -...T.-... T 113
BF55                .......... AA.....T.. .....TG... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------....- -...T.-... T 113
BF60                .......... AA.....T.. .....TG... .......... .....A.... .......... ......CA.. ..T..... ....A.T... AT..... ------...C- -...T.-... T 113

Bos-taurus          .......... AA.C...TG. C.-.CT..AG C........A .....AT... ......CT.. .....A.A.. ..-.T..... ..T.A.T..T .AT.GT..AT CTATT....A TT..T.A... T 119
Bos-indicus         .......... AA.....TG. T..-.CT..A. .........A .....A.... ....A..T.. .......A.. ..-.TA.... ....A..... AA......AT CTACT....A C...T.-... . 118
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Fig. 3. The amplified D-loop sequence with the primer sequences removed. The swamp type water buffalo reference sequence was retrieved from GenBank's whole mitochondrial genome sequence NC_006295 (it is also the most common haplotype in GenBank, coded as XY1-(83) in Fig. 4) (Lei et al., 2007). The river type water buffalo reference sequence has been coded as Jaf-01-(89) (Kierstein et al., 2004) in Fig. 4, and represents the most common sequence for river water buffalo in GenBank. The GenBank accession number for the *Bos taurus* sequences is NC_006853 and NC_005971 for the *Bos indicus* sequence. The dot indicates identical sequences and the dash represents a deletion/insertion.

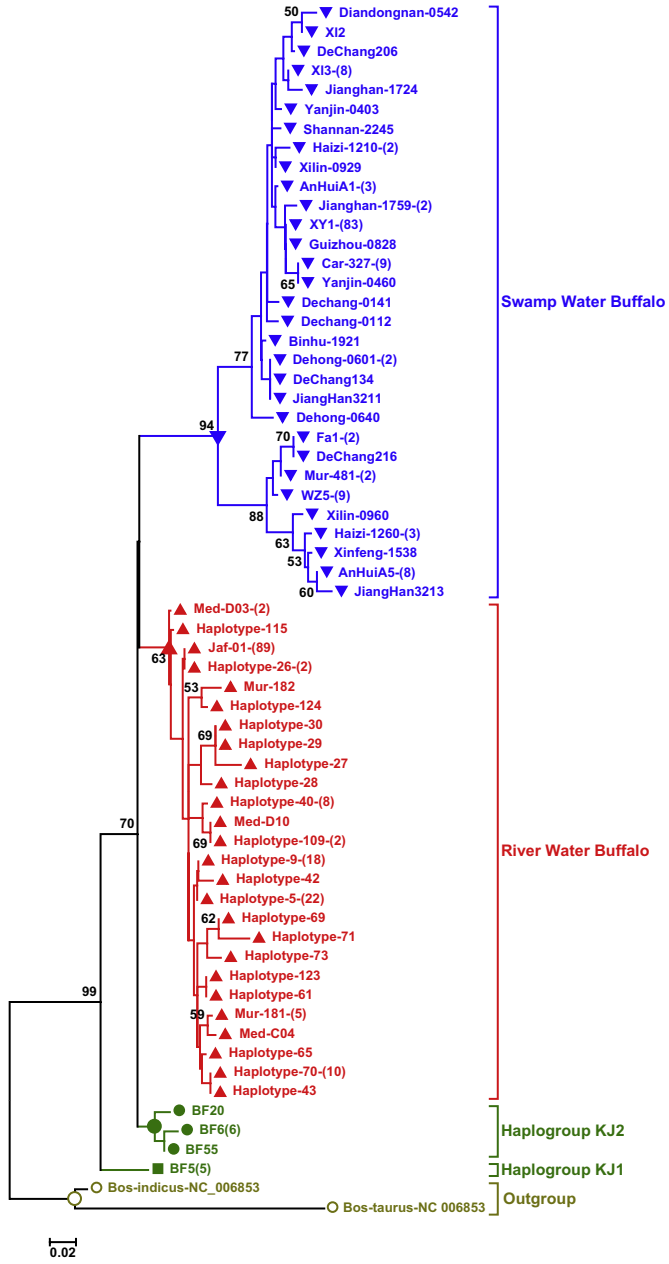

Fig. 4. Phylogenetic tree displaying the haplotype variation of ancient water buffalo and modern water buffalo. The tree was composed using the short mtDNA D-loop fragment (see Fig. 3) using Mega3 software (NJ with Kimura 2-parameter) with *Bos indicus* and *Bos taurus* as the outgroups. The numbers at the nodes indicate those bootstrap values above 50% after 2000 replications. The 57 reference sequences were those redefined unique haplotypes from 283 sequences currently stored in GenBank (mainly from Lei et al., 2007; Kierstein et al., 2004; Kumar et al. 2007b; Lai et al., unpublished; Zhang et al., unpublished). The haplotype names were chosen from those originally presented in GenBank. The number inside the brackets indicates frequency of a particular haplotype in GenBank, and for these shorter haplotypes, their names were chosen randomly from original GenBank haplotypes.

Zooarchaeological studies on *B. mephistopheles* remains from several Neolithic sites in south China have indicated that *B. mephistopheles* from the sites were not domesticated animals (Liu et al., 2004, 2006). It could be expected that DNA analysis of ancient *B. mephistopheles* remains in this study should also reveal the signal of their "domesticated" or "wild" status. Generally, domesticated animals should show low DNA diversity due to the bottle-neck effect caused by human selections in association with domestication process and cultivation practices. The calculated DNA sequence diversities of unique haplotypes in this study show that the ancient water buffalo diversity (0.048) lies between swamp water buffalo (0.066) and river water buffalo (0.028). By no means, however, should this comparison be used to support a "domesticated" status of the ancient water buffalo assemblage due to severe sampling bias. Both domesticated swamp and river water buffalo samples were collected from vast geographic regions, including India, China, Italy and even Brazil (for recently transferred breeds) (Kierstein et al., 2004; Kumar et al., 2007a,b; Lau et al., 1998; Lei et al., 2007), while the ancient water buffalo DNA samples are all from sites situated within an area only 30 km in radius. For modern water buffalo, we could expect a much lower DNA diversity if the study samples were all from a small area even within a 100 km radius. Due to this sampling bias, we can argue that genetic diversity of these ancient DNA samples should be considered to be high, and may be high enough to be indicative of "wild" status.

4.4. Implications for domestication history of modern water buffalo in Asia

Three population genetic studies of modern water buffalo (Kierstein et al., 2004, Kumar et al., 2007a,b, Lau et al., 1998) point to different regions in south Asia or SE Asia as the origin of domestication of water buffalo. The exclusion of China as a domestication center could potentially be due to a sampling bias, as none of the studies incorporated DNA samples from modern Chinese water buffalo. Moreover, the three studies all failed to incorporate wild-type water buffalo (*B. arnee*) DNA samples into their genetic analyses.

A very recent genetic study of modern Chinese domesticated water buffalo (swamp water buffalo) (Lei et al., 2007), has revealed that the Chinese water buffalo are not significantly different from previously published data from Brazilian/Italian swamp water buffalo, which originated in Asia (Kierstein et al., 2004). However, this study identified two lineages (A and B) in the Chinese swamp buffalo assemblages, and calculated the divergence time for the two lineages as 18,000 BP. The authors then concluded that the swamp water buffalo originated independently in China while the river water buffalo originated from India.

Our ancient DNA data are not supportive of the conclusion that domesticated swamp buffalo originated in China, since no direct linkage between the indigenous *B. mephistopheles* and modern Chinese domesticated water buffalo can be established based on the ancient DNA data. In addition, the suggested occasional introgression of wild water buffalo into domesticated swamp buffalo as suggested by others (Lei et al., 2007) cannot be validated by ancient DNA data, since no *B. mephistophele* DNA sequences have ever been reported from any modern water buffalo samples. The absence may serve as additional evidence to show that *B. mephistophele* was not the progenitor of modern water buffalo.

Recent zooarchaeological studies have noticed that the morphological characteristics of *B. mephistopheles* from north and south China dating to the Neolithic and Bronze Age are very similar (Liu et al., 2004, 2006), suggesting only one buffalo species existed in China during the Holocene. As demonstrated in this study, all the analyzed water buffalo remains (even those that were previously tentatively identified as domesticated water buffalo) were found to likely belong to one species. If there was indeed only one wild water buffalo species during early and middle Holocene in China, modern water buffalo were unlikely domesticated from those wild water buffalo. Nevertheless, there is a slight possibility that ancient water buffalo DNA sequences from north China as represented by data in this study could be somewhat different from those of south China. If so, DNA data from south China may potentially show closer linkages to modern water buffalo. Clearly, retrieval of DNA from south China water buffalo remains will hold the key to these outstanding

questions, and we are currently continuing our efforts to extract DNA from south China samples.

At the moment, our understanding of the water buffalo domestication issue is also constrained by the lack of DNA data from modern populations of south Asian wild water buffalo, *B. arnee*. This animal is considered to be the progenitor for modern domesticated water buffalo, but it is unclear about genetic variations within this species, and how they are related to domesticated river and swamp forms. Until these modern wild-type sequences are collected and analyzed, rooting phylogenetic trees for available water buffalo data with *Bos* as the outgroup will only continue to produce ambiguous trees.

Our data fail to establish any direct linkages between ancient water buffalo remains from north China to modern water buffalo in China. As a result, we cannot falsify the hypothesis based on recent studies from zooarchaeology, biology and genetics that modern water buffalo were first domesticated in regions outside China: south Asia or southeast Asia as suggested by others.

Liu et al. (2006) have also examined other lines of evidence to investigate the origin of domesticated water buffalo in China from zooarchaeology, historic documentation and artifacts. The preliminary data from these analyses also fail to support the hypothesis of Chinese origin of water buffalo domestication.

4.5. Future DNA research

The origin of domesticated water buffalo is still an open question. Given that river and swamp types may have been domesticated separately from different wild ancestors in different regions (Kumar et al., 2007a,b), our research should focus on the relationships between modern and ancient buffalo species in particular regions by continuously employing multidisciplinary approaches, including archaeology, zooarchaeology, and modern and ancient DNA analysis. Our current study points to several directions for future research.

First, ancient DNA analysis represents an important approach in tracing the progenitors of domesticated water buffalo, since it can provide both regional and temporal evidence of genetic variation. We need to evaluate the classification of *B. mephistopheles* with further DNA tests on more ancient buffalo remains from different regions, in order to determine if more than one indigenous buffalo species existed in China during the Holocene. Furthermore, DNA sequences from ancient water buffalo remains will provide a more appropriate outgroup than *Bos* in the phylogenetic study of these animals.

Second, genetic data from modern 'wild-type' (*B. arnee*) should provide crucial information for interpreting the relationship between ancient DNA sequences and modern water buffalo. A comparative DNA study of the ancient Chinese indigenous buffalo and modern wild buffalo from south Asia should help us to clarify the relationship between these wild species and their affiliations to the modern domesticate.

Third, since the swamp buffalo is distributed mainly in northeastern India, southeast Asia and southern China, DNA from modern domesticated buffalo from southwest China (particularly Yuannan and Guangxi provinces in China) and its adjacent regions in southeast Asia (Burma, Laos, Vietnam and Thailand) and northeast India should be the focus of further study, in order to trace the origins of domesticated swamp water buffalo in this region.

5. Conclusions

Ancient DNA sequences have been successfully retrieved and analyzed from 13 of 24 water buffalo bone samples. The DNA sequences, when compared with other modern water buffalo DNA data, shed new light on ancient indigenous populations of water buffalo in China and their relationships to modern domesticated water buffalo in Asia.

Based on the DNA sequences recovered in this project, the indigenous water buffalo *B. mephistophele* from Shaanxi Province displays no direct connection with modern domesticated water buffalo, failing to support the assumption that water buffalo were first domesticated in China. Although DNA samples in this study were obtained from a limited geographic region, the data show that these remains are from a wild buffalo species which was not closely related to the ancestral population of modern domesticated water buffalo in China.

The results from our analysis have clearly demonstrated that ancient DNA provides a new window into the study of ancient wild buffalo and the processes of buffalo domestication. It can be expected that DNA analysis of more ancient water buffalo remains from other regions will provide a clearer picture of water buffalo domestication, and eventually resolve ongoing debates on the origins of domesticated water buffalo in Asia.

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