

Two Maternal Lineages Revealed by Mitochondrial DNA D-loop Sequences in Chinese Native Water Buffaloes (*Bubalus bubalis*)

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ABSTRACT : Little is known about the origin and genetic diversity of swamp buffaloes in China. To obtain more knowledge on genetics of the water buffalo in China, the complete mitochondrial D-loop sequences of 30 samples from 6 native types were investigated. The results revealed 12 mitochondrial haplotypes with 50 polymorphic sites. Among these polymorphic sites, there were 49 transitions and 1 transversion. The average nucleotide diversity and haplotype diversity estimated from mtDNA D-loop region in 6 Chinese water buffalo types were 0.00684 and 0.798, respectively, showing rather abundant mitochondrial genetic diversity. The Neighbor-Joining (NJ) tree of mtDNA of Chinese water buffaloes was constructed according to the 12 haplotypes. The NJ tree indicated two lineages being designated lineage A and lineage B, in which lineage A was predominant, and lineage B was at low frequency. The new lineage B was first discovered and defined in 6 Chinese water buffalo types. These results showed that two different maternal lineages were involved in the origin of domestic swamp buffaloes in China and the lineage B was probably an introgression from Southeast Asian buffaloes. (**Key Words** : Chinese Water Buffalo, Mitochondrial DNA D-loop, Diversity, Haplotype, Origin)

INTRODUCTION

China is one of the countries rich in water buffalo genetic resources in the world. The origin and evolution of Chinese water buffaloes have attracted the interests of scientists for a long time. Water buffaloes are widespread throughout 18 provinces of the Central and Southern China, primarily in Southwest and Southeast China around Yangtze valley with rice cultivation, but are also found in 2,500 meters above sea level in Shandong and Shaanxi provinces of Northwest China (Qiu, 1986). As we all know, the water buffalo of Asia (*Bubalus bubalis*) is divided into two types of the river and swamp buffalo. Because no difference in breed characteristics exists among the animals distributed in Chinese different areas, all buffaloes in China were named

Chinese water buffaloes, which belong to the swamp buffalo type according to body size, outward appearance, coat, biological characteristics and chromosome karyotype, etc (Qiu, 1986). According to the different distribution regions, the only Chinese water buffalo breed was divided into 14 local types in China (Qiu, 1986). To date, little is known about the genetics of Chinese water buffalo (Shi et al., 1995; Hu et al., 1997). This might partly be due to the lower economic importance when compared with cattle, pig, chicken, sheep and goat, limiting the interests in buffalo genetic analysis in China. However, the water buffalo holds a great economic potential in the remotely mountainous region of China such as Yunnan and Guizhou provinces etc., in which the water buffalo has the multipurpose for draught, meat and dairy.

The mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) and mtDNA D-loop sequence polymorphism have been studied in a variety of domestic animals of cattle, pigs, chickens, buffaloes, sheep and goat in China (Lan et al., 1993; Hu et al., 1997; Huang et al., 1999; Yu et al., 1999; Yang et al., 2003; Liu et al., 2004, 2006; Chen et al., 2005; Guo et al., 2005; Lai et al., 2006) and in other countries (Jeon et al., 2005; Odahara et al., 2006; Sasazaki et al., 2006). Previous studies of mtDNA D-

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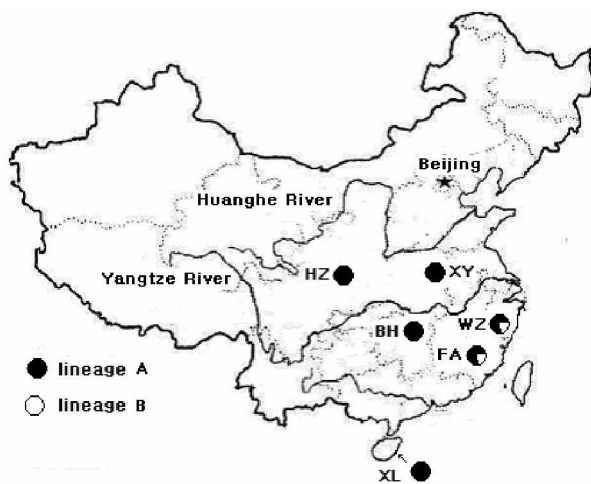


Figure 1. Geographic distribution of 6 swamp buffalo types in China. Circle area is proportional to sample sizes.

loop sequence variations have shown genetic differentiation between the swamp and river buffalo types in Southeast Asia (Lau et al., 1998), Brazil and Italy (Kierstein et al., 2004). Only 12 animals of three local water buffalo types in Yunnan province were analyzed based on mtDNA RFLP (Hu et al., 1997). No report is available, however, on mtDNA D-loop sequence variation and origins of water buffalo in China. So we examined complete mtDNA D-loop sequences of 6 native water buffalo types from 6 provinces to investigate the origin and genetic diversity of Chinese water buffaloes.

MATERIALS AND METHODS

Specimen collection

30 fresh blood samples were collected from different locations of 6 native water buffalo types in China. The geographic distribution and sources of the water buffalo

were shown in Figure 1 and Table 1.

DNA extraction and PCR amplification

DNA was extracted from these specimens using phenol/chloroform (Maniatis et al., 1982). D-loops were amplified on a T gradient DNA thermocycler (Germany) using PCR with primers constructed from the published *Cytb* genes. CB1: 5'- TAG TGC TAA TAC CAA CGG CC - 3' and *tRNA^{Phe}*. CB2: 5'- AGG CAT TTT CAG TGC CTT GC -3' (Kierstein et al., 2004). The reagent kits used for PCR were from Takara Company. The PCR reaction was carried out in a total volume of 50 μ l, and the final concentration of each component was as follows, 1 \times PCR buffer, 2.5 mM MgCl₂, 0.25 mM of each dNTP, 2.0 U *Taq* DNA polymerase, 0.3 μ M of each primer and 50 ng DNA templates. PCR was performed in a Progene thermal cycler (PE 9600). The reaction profiles included an initial denaturation at 95°C for 1 min, followed by 35 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 56°C, 1 min extension at 72°C, and then a final 10 min extension at 72°C. The amplified products were all electrophoresed by 1.0% agarose gel in 1 \times TBE buffer, with 5 v/cm voltage for 1 h. After the run, the gel was stained with ethidium bromide.

PCR products sequencing

The amplified products were purified by using the WizardTM PCR Preps DNA purification kit (Promega) according to the manufacturer's instructions. MtDNA D-loop complete sequence of the PCR product was sequenced using PCR amplification primers and four internal primers. The four internal primer sequences were as follows: CB3: 5'- CCA TCA ACA CAC CTG ACC -3'. CB4: 5'- GCG AGG ACG GAT TTG ACT -3'. CB5: 5'- CAT AAC ATT AAT GTA ATA AGG GC -3'. CB6: 5'- CCA TTC GGA GTA GTA GGG TC -3' (Kierstein et al., 2004). Sequencing

Table 1. Source of the water buffalo samples in China

Buffalo types	Abbreviations	Locality	Animal No.
Xinlong	XL	Wanning county, Hainan Province	1, 2, 3, 4, 5
Fu'an	FA	Fuzhou city, Fujian Province	1, 2, 3, 4, 5
Wenzhou	WZ	Rui'an city, Zhejiang Province	1, 2, 3, 4, 5
Hanzhong	HZ	Mian county, Shaanxi Province	1, 2, 3, 4, 5
Xinyang	XY	Guangshan county, Henan Province	1, 2, 3, 4, 5
Binhu	BH	Yueyang city, Hunan Province	1, 2, 3, 4, 5

Table 2. Haplotypes of previously published swamp buffalo breeds obtained from GenBank

Breed	Code of sequence	GenBank No.	Breed	Code of sequence	GenBank No.
Murrah	Mur-497	AF197215	Carabao	Car-352	AF197222
Murrah	Mur-481	AF197214	Carabao	Car-370	AF197223
Carabao	Car-315	AF197218	Carabao	Car-330	AY195596
Carabao	Car-320	AF197219	Carabao	Car-342	AY195597
Carabao	Car-327	AF197220	Carabao	Car-344	AY195598
Carabao	Car-336	AF197221	Carabao	Car-377	AY195599

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[      111111111 122222222 233333333 344444555 667777789 ]
[      6113686888 9044666678 9001134677 7144675569 3734477751 ]
[      8483148346 5603015708 7146791278 9936395829 5224701223 ]
WZ2  CACAAC TGGG CTTGGGCCAA AAGTGCCCGG TTGTCGCACA CGGTGTTTCC
WZ1  ..... A.....
HZ2  ..... A..... A.....
XL1  ..... A.C...
WZ4  ..... T..... A.....
FA2  ..... A..C..
HZ1  ..... T..... A..C..
FA4  ..... A..... A..C..
XL2  ..... G..... A..C..
XL3  ..... A...G..... T..... TG..... A.....
FA1  TGT. GTCA. C TC. AAATTG. GGAC. TTT. A CCACTA. . TG TAACAC. CTT
WZ5  TGTGGTCAAC TCCAAA. TG. GGACATTT. A CCA. TA... G... A..A.. CTT
    
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Figure 2. 12 swamp buffalo haplotypes and 50 polymorphic sites in 6 Chinese buffalo types.

Table 3. Haplotypes shared among Chinese native water buffalo types

Code of haplotype	Types	Code of individuals of types
WZ1	WZ, HZ, XY, BH, XL	WZ1, HZ3, HZ4, HZ5, XY2, XY3, XY4, XY5, BH3, BH4, BH5, XL4, XL5
WZ2	WZ, XY, BH	WZ2, WZ3, XY1, BH2
XL1	XL, BH	XL1, BH1
FA2	FA	FA2, FA3, FA5

was performed by using an ABI model 377 automated sequencer (PE company).

Data analysis

Sequences were edited by DNASTAR5.0 package (DNASTAR, Madison, WI). All 30 sequences of mtDNA D-loop in this study, one sequence (GenBank No. AF197215) of river buffalo as an outgroup, and 11 haplotypes belonging to swamp buffalo from Brazil (Kierstein et al., 2004) shown in Table 2 were aligned in the ClustalX package (Thompson et al., 1997). Insertions/deletions in the aligned sequences were excluded from the following analyses. The identical sequences were considered as the same haplotype. Using MEGA2.1 software (Kumar et al., 2001), Kimura 2-parameter distance matrix of all haplotypes was calculated to construct the unrooted Neighbor-Joining (NJ) phylogenetic tree (Kumar et al., 2001). Bootstrap confidence levels (BCL) of the phylogenetic tree were estimated by 1,000 replications of the data. The haplotype diversity (H) and nucleotide diversity (π) for the swamp type samples were estimated by using Dnasp software 4.0 (Rozas et al., 2003).

RESULTS

MtDNA D-loop variation and haplotypes

The mtDNA D-loop sequences reported in this paper have been deposited in the GenBank database (accession no.

DQ364160-DQ364189). Complete mitochondrial D-loop sequences, 914-916 bp in length were determined for 30 animals from 6 Chinese water buffalo types. Comparisons of these sequences revealed one animal with 914 bp mtDNA D-loop, 27 animals with 915 bp mtDNA D-loop, 2 animals with 916 bp mtDNA D-loop sequences. 12 different mitochondrial haplotypes were identified with 50 polymorphic nucleotide sites (excluding insertions/deletions), which accounted for 5.46% of a sequenced 915 bp fragment (Figure 2). Among these polymorphic sites, there were 49 transitions and 1 transversion. The mtDNA sequences comparison of the complete alignment of Chinese domestic water buffalo revealed 25 T \rightleftharpoons C and 24 A \rightleftharpoons G transitions, but only 1 C \rightleftharpoons G transversion, demonstrating the strong bias towards transitions. The strong transitional bias verified here is a characteristic of mtDNA evolution and has been observed not only in buffaloes (Lau et al., 1998; Kierstein et al., 2004), but also in other mammalian species and chickens (Liu et al., 2004, 2006; Chen et al., 2005; Guo et al., 2005; Lai et al., 2006).

Among the detected 12 haplotypes, 8 haplotypes were the unique for every buffalo type. However, three haplotypes were shared among some buffalo types, one haplotype was shared within Fu'an buffalo type (Table 3).

Phylogenetic analysis of mtDNA D-loop sequences

The 30 samples represented 12 haplotypes of the mtDNA D-loop complete sequences in Chinese native water

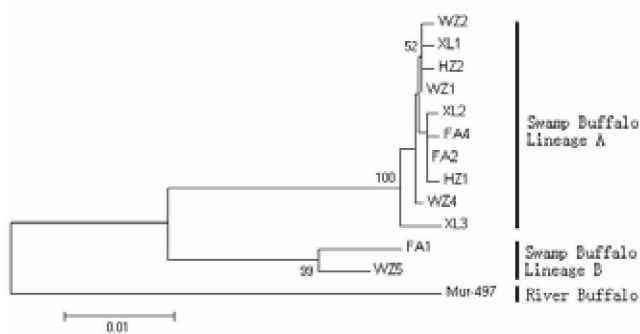


Figure 3. NJ phylogenetic tree of 12 mtDNA D-loop haplotypes in 6 Chinese buffalo types, one River buffalo sequence (Mur-497) as outgroup. Numbers at the two lineages denote the bootstrap confidence levels (BCL) of 1,000 replications (only those $\geq 50\%$ are shown).

buffalo types. The Neighbour-Joining (NJ) phylogenetic tree of haplotypes in Chinese water buffaloes was constructed (Figure 3). The NJ tree indicated two lineages being designated lineage A and lineage B. The lineage B was first discovered and defined among 6 Chinese water buffalo types in this paper. These results showed that two different maternal lineages were involved in the origin of domestic swamp buffaloes in China. The new maternal lineage B appeared in 2 types (Fu'an and Wenzhou types) of 6 Chinese buffalo types, indicating a likely origin center existed in the lower reaches (Fujian and Zhejiang provinces) of Yangtze valley (Figure 1). The lineages A and B included 10 and 2 haplotypes representing 28 and 2 samples, respectively. Lineage A was predominant (93.33%, 28/30), and was found in 6 Chinese water buffalo types. Lineage B was at very low frequency (6.67%, 2/30), and only discovered two haplotypes representing two individuals in Fu'an and Wenzhou buffalo types, respectively (Figure 2). Haplotypes, haplotype diversity (H) and nucleotide diversity (π) of 6 Chinese water buffalo types were analyzed in Table 4. The highest haplotype diversity ($H = 0.900$) in Xinlong and Wenzhou buffalo types and the lowest haplotype diversity ($H = 0.400$) in Xinyang buffalo types were observed in 6 water buffalo types. The highest nucleotide diversity ($\pi = 0.01729$) in Fu'an buffalo type and the lowest nucleotide diversity ($\pi = 0.00044$) in Xinyang buffalo type was observed,

respectively. The average haplotype diversity (H) and nucleotide diversity (π) were 0.798 and 0.00684, respectively, indicating rather abundant genetic diversity in Chinese water buffalo populations.

To investigate the relationship of swamp buffalo in Brazil (Kierstein et al., 2004) and in China, we combined all published 11 haplotypes belonging to the swamp buffalo mtDNA with 12 haplotypes of Chinese buffaloes in our data. The phylogenetic relationships among these haplotypes are shown in NJ tree (Figure 4). The NJ tree further indicated two lineages of lineage A and lineage B of swamp buffaloes in China and Brazil.

DISCUSSION

Diversity analysis of mtDNA D-loop in Chinese native swamp buffalo types

Generally, the more ancient the population was, the longer they had to mutate and accumulate the mutations. So ancient populations would be more diversified genetically and the haplotypes present in them would have more opportunities to be shared by other populations (Ward et al., 1993). The results of the present study showed that Xinyang buffalo (XY) had only two haplotypes, but other buffalo types had three or four haplotypes (Table 4). But we do not know which buffalo types were relatively ancient types (Qiu, 1986). From our results, Fu'an and Wenzhou buffalo types, which had lineage A and B, might be the relatively ancient types. Based on protein loci, Chinese scientists suggested that the genetic diversity of five Chinese water buffalo types in Yangtze valley were very slight (Shi et al., 1995). Hu et al. (1997) suggested a low level of mtDNA variation of Yunnan water buffaloes by mtDNA RFLP results. But the fairly abundant mtDNA diversity was observed in Chinese water buffalo types (Table 4). The reason is that, (i) we found a new maternal lineage, (ii) The diversity of mtDNA D-loop sequences can be detected in nucleotide level. However, protein loci and mtDNA RFLP have lower sensitivity.

Genetic differentiation of Chinese native swamp buffalo types

The origin of domestic water buffalo in China is still unclear. In our study, two mitochondrial lineages were

Table 4. Haplotype and nucleotide diversity of swamp buffalo types in China

Buffalo types	Samples	Number of haplotypes	Haplotype diversity $H \pm SD$	Nucleotide diversity $\pi \pm SD$
XL	5	4	0.900 \pm 0.161	0.00328 \pm 0.00219
FA	5	3	0.700 \pm 0.218	0.01729 \pm 0.01057
WZ	5	4	0.900 \pm 0.161	0.01705 \pm 0.01030
HZ	5	3	0.700 \pm 0.218	0.00131 \pm 0.00111
XY	5	2	0.400 \pm 0.237	0.00044 \pm 0.00053
BH	5	3	0.700 \pm 0.218	0.00088 \pm 0.00083
Total	30	12	0.798 \pm 0.068	0.00684 \pm 0.00461

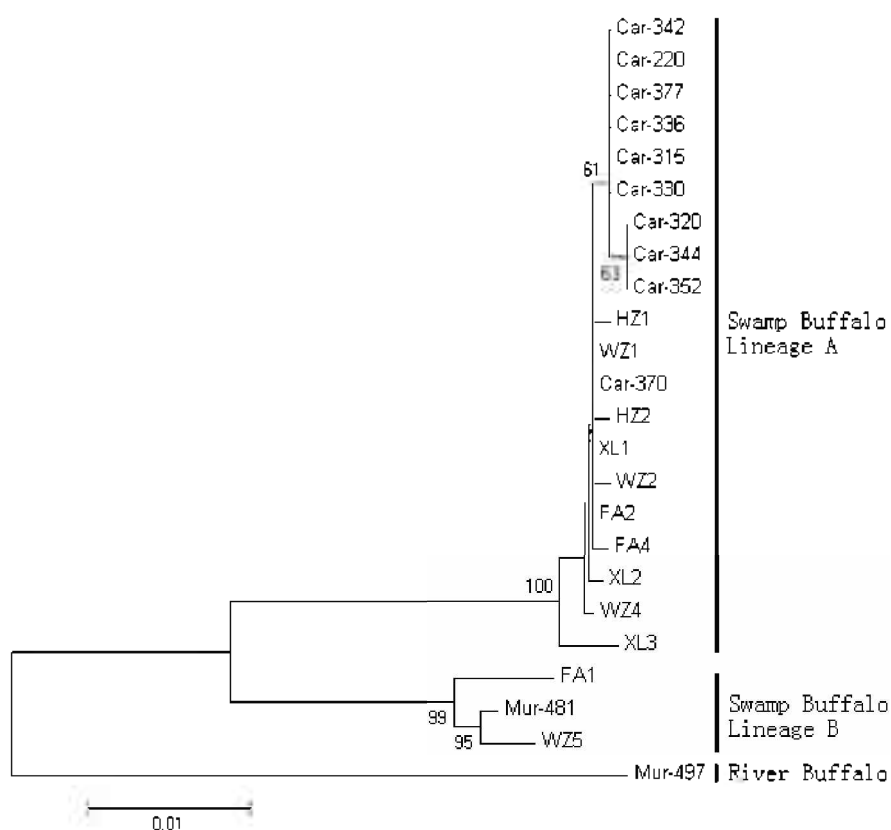


Figure 4. NJ phylogenetic tree of mtDNA D-loop of swamp buffalo in China and Brazil, one River buffalo sequence (Mur-497) as outgroup. Numbers at the two lineages denote the bootstrap confidence levels (BCL) of 1,000 replications (only those $\geq 50\%$ are shown).

found in 12 haplotypes representing 30 samples from 6 swamp buffalo types in China (Figure 3). By analyzing buffaloes from Italy and Brazil, as well as, published mtDNA data, Kierstein et al. (2004) identified an "eccentric group". The eccentric group only consists of swamp buffaloes including the Carabao and Mur-481 haplotypes from Brazil (Table 2). To further study the genetic variation of swamp buffaloes in China and the eccentric group from Brazil (Kierstein et al., 2004), we constructed the NJ tree of all haplotypes of swamp buffaloes from China and Brazil (Figure 4). Mur-481 was observed in lineage B. Therefore, we suggested that Chinese swamp buffaloes had two maternal origins.

Among 6 Chinese buffalo types, only two haplotypes representing lineage B were detected in Fu'an (FZ1) and Wenzhou buffalo (WZ5), respectively, which suggested that lineage B had influence on the origin of Fu'an and Wenzhou types. Other buffalo types in Xinyang, Xinlong, Hanzhong and Binlu type have not found lineage B partly because of small sample sizes. So, our results do not accord with the results of mtDNA RFLP (Hu et al., 1997) and mtDNA D-loop (Lau et al., 1998; Kierstein et al., 2004). The origin of Asian water buffalo is an open question. The domestication of water buffaloes most likely took place in

the civilization of the Indus, the Yangtze, and the Euphrates and Tigris in the third millennium BC (Cockrill, 1981; Kierstein et al., 2004) and/or in China during the fifth millennium BC (Chen et al., 1989). Lau et al. (1998) hypothesized that water buffalo originated in mainland Southeast Asia, and that it spread north to China and west to the Indian subcontinent, where the river type evolved and was domesticated. Following domestication in China, the domesticated swamp buffalo spread through two separate routes, through Taiwan and the Philippines to the eastern islands of Borneo and Sulawesi, and south through mainland Southeast Asia and then to the western islands of Indonesia. However, Kierstein et al. (2004) suggested the domestication of water buffaloes occurred in the Indian subcontinent 5000 years ago. Our work suggested lineage B of swamp buffalo probably an introgression from Southeast Asia. Because haplotype Mur-481 was found in Brazil, whereas Brazilian buffalo population derived from an unknown diverse ancestor population, mainly imported from India in the end of 19 century (Kierstein et al., 2004). China is an important region on origin and domestication of domestic species, in which multiple maternal origins of many domestic animals (cattle, goat, sheep and chicken) were observed (Yu et al., 1999; Liu et al., 2004, 2006; Chen et al., 2005; Guo et al., 2005; Lai et al., 2006). To clarify the

origin of water buffalo. it is necessary to conduct more samples of Chinese local swamp buffaloes.

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