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Molecular Phylogeny of the Gayal in Yunnan China Inferred from the Analysis of *Cytochrome b* Gene Entire Sequences

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ABSTRACT : The gayal (*Bos frontalis*) in China is a very rare semi-wild and semi-domestic bovine species. There still exist remarkable divergences on the gayal's origin and taxonomic status. In the present study, the *cytochrome b* (*Cyt b*) gene entire sequences (1,140 bp) of 11 gayals in Yunnan China were analyzed. Combined with other bovine *Cyt b* sequences cited in GenBank, the phylogenetic trees of genus Bos were reconstructed by neighbor-joining (NJ) and maximum parsimony (MP) methods with *Bubalus bubalis* as outgroup. Sequence analysis showed that, among 1,140 sites compared for 11 gayals, 95 variable sites (8.33% of all sites) and 6 different haplotypes were observed, showing abundant mitochondrial genetic diversity in gayals. Both NJ and MP trees demonstrated that gayals in this study were markedly divided into three embranchments: one embranchment clustering with *Bos gaurus*, another clustering with *Bos taurus*, and the third clustering with *Bos indicus*. The result of phylogenetic analysis suggested that the gayal might be the domesticated form of the gaur, and a great proportion of the gayal bloodline in China was invaded by other bovine species. (**Key Words :** Gayal (*Bos frontalis*), *Cytochrome b* Gene, Molecular Phylogeny)

INTRODUCTION

The gayal (*Bos frontalis*), also called mithan or mithun, is found in China only in the Dulong River and Nujiang River Basin in Yunnan Province, and in Menyu and Luoyu regions of the Tibet Autonomous Region where the altitude ranges between 1,500 M-4,100 M. It is also found in Assam in India, East Bengal and Kachin state in the northern part of Burma (Simoons, 1984; Giasuddin and Islam, 2003b; Nyunt and Win, 2004; Namikawa, 2005; Mao et al., 2005; Deng et al., 2007; Xi et al., 2007). The gayal in China is a very rare semi-wild and semi-domestic livestock. The gayal in Yunnan Province was named as "dulong or drung ox", because it was firstly domesticated by the Dulong Tribe. The dulong is large-framed and adult males can reach 120 to 150 cm tall with a body mass ranging from 400 to 500 kg, compared with 350 to 400 kg for the cows. Adult dulongs are dark brown to black with white stockinged legs. The horn bases are rugged, tapering gradually upwards, protruding out from both sides of the head and bending relatively upwards. The males have fleshy necks with evident dewlaps and relatively low dorsal humps.

The gayal was classified as a separate subgenus, together with Bali cattle (*Bos banteng*), the kouprey (*Bos sauveli*) and the gaur (*Bos gaurus*), and distinct from European cattle (*Bos taurus*) and zebu cattle (*Bos indicus*) (Williamson and Payne, 1977). There are three major hypotheses about the origin of the gayal (Walker et al., 1968; San et al., 1980; Winter et al., 1984; Payne, 1991; Ritz et al., 2000; Tanaka et al., 2004; Verkaar et al., 2004; Ma et al., 2007): (1) that it was a domesticated gaur; (2) that it was a hybrid descendant, from crossing of gaur (*Bos gaurus*) and ordinary domestic cattle (*Bos indicus* or *Bos taurus*); and (3) that it was an independent species descended from a wild Indian bovine which is now extinct. Of these, the first is most favored.

As one of the important protein-coding genes in mitochondrial DNA (mtDNA), the *cytochrome b* (*Cyt b*) gene contains abundant phylogenetic information among intra- and interspecies, and it is considered to be a good marker to study on genetic differentiation and phylogenetic relationships among species within the same genus or the same family (Browers et al., 1994; Zardoya and Meyer, 1996). *Cyt b* gene is widely used in studies on origin, taxonomy and phylogeny of the subfamily Bovinae

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Table 1. The list of species, accession number, source and code

Species	Accession number	Source	Code
Bos taurus	V00654	GenBank	Bos taurus
Bos indicus	NC_005971	GenBank	Bos indicus
Bos gaurus	AF348593	GenBank	Bos gaurus
Bos javanicus	D82889	GenBank	Bos javanicus
Bos grunniens	AY955225	GenBank	Bos grunniens
Bos sauveli	AY689189	GenBank	Bos sauveli
Bubalus bubalis	D88635	GenBank	Bubalus bubalis
Bos frontalis	EF061227~EF061237	This study	BF01~BF11

(Kikkawa et al., 1997; Birungi and Arctander, 2001; Hassanin and Ropiquet, 2004). In the present study, *Cyt b* gene entire sequences of 11 gayals in China were analyzed. These data, combined with *Cyt b* sequences of other bovine species in GenBank, were used to perform phylogenetic analysis in order to explore the molecular phylogeny and taxonomic status of the gayal and to provide some molecular biological gist for evaluating and protecting this rare genetic resource.

MATERIALS AND METHODS

Animals

Applying simple random sampling in typical colony methods in the central area of habitat (in Nu River City of Yunnan Province), 11 gayals were selected. Blood samples were collected and taken back to the laboratory in an icebox, then kept at -20°C until use. The *Cyt b* gene sequences of other cattle cited in GenBank for phylogenetic analysis are shown in Table 1.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from blood using standard procedures, involving treatment with SDS and proteinase K and subsequent phenol/chloroform extraction (Wall et al., 1992). The entire mitochondrial *Cyt b* gene (1,140 bp) was amplified from total genomic DNA by the polymerase chain reaction (PCR) with the two primers: L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and H15915R (5'-GGAATTCATCTCTCCGGTTTACA AGAC-3') (Irwin et al., 1991). The standard PCR conditions were as follows: 4 min at 94°C; 30 cycles of denaturation/annealing/extension with 40 s at 94°C for denaturation, 40 s at 52°C for annealing, and 90 s at 72°C for extension; and 8 min at 72°C. Each PCR was performed in 25 μl reaction volume with 2.0 units *Taq* DNA polymerase (TaKaRa biotechnology (Dalian) Co. Ltd in China) and about 100 ng DNA as template. The PCR products (each about 1,246 bp) were analyzed on 1.0% agarose gel with a vacant comparison. Purification and sequencing procedures were carried out by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd in China.

Data analyses

Cyt b gene entire sequences (1,140 bp) of 11 gayals were edited and aligned with reference to the *Cyt b* sequence of the domestic cow (*Bos taurus*) (Accession No. V00654) using DNASTAR package and were checked manually. The sequences of 11 gayals were deposited in GenBank under Accession Nos. EF061227~EF061237 (Table 1). Pairwise comparisons of observed sequence differences, number of transitions and transversions, and nucleotide composition by codon position were analyzed using the computer program MEGA 3.1 (Kumar et al., 2004). The haplotype diversity (Hd) (using Equation 8.4 in Nei (1987), except that n was used instead of 2n) and nucleotide diversity (Pi) (using Equations 10.5 or 10.6 in Nei (1987)) were calculated by the software DNAsp 4.1 (Rozas et al., 2003). Neighbor-joining (NJ) and maximum parsimony (MP) methods were used to reconstruct the phylogenetic trees. The NJ phylogenetic tree was reconstructed on the basis of a Kimura two-parameter model using the computer program MEGA 3.1 (Kumar et al., 2004). The MP phylogenetic tree was generated by heuristic search routines with 1,000 random-addition sequences and TBR branch swapping using the software PAUP* 4.0 (Swofford, 2000). Levels of resolution at internal nodes of two phylogenetic trees were evaluated by bootstrap resampling with 1000 iterations (Felsenstein, 1985).

RESULTS AND DISCUSSION

Nucleotide composition of *Cyt b* entire sequences of gayals

After being sequenced and aligned, a 1,140 bp fragment including the entire mitochondrial *Cyt b* gene was obtained in all 11 gayals. No insertions/deletions were observed. The average nucleotide frequencies of T, C, A, and G were 25.8%, 29.6%, 31.4% and 13.3%, respectively. A remarkable imbalance in base usage was observed at the third positions, with infrequent use of G (3.5%) and a bias towards A+C (82.0%). The low number of Gs and high number of As at the third positions indicates that the likelihood of an A to G transition is much lower than a G to A transition (Birungi and Arctander, 2001).

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                                                                                               111111111 11111
             111 1111222333 3333333333 4444445555 5555566666 6666677777 7778888888 9999999999 9000000000 01111
       156889011 2579137012 2446678999 0124691133 4466923457 8999911256 7790134789 0000026778 9045666678 90112
      9879143257 1645946424 7250354036 8408280347 0619213442 1347947998 4757340052 0345940591 9540568940 24466
BF01  TATTCCACGA TGTCATTCCC CCTATTCGTA GGTCTTATTT CCCCTTCCTC AAATGCTGCT AACGATTATG CAATTTTTAT ATTTCATACT AAGCC
BF02  CGCCTA.TAC CACTGCCT.T TT.GCCTACC AACTAC.CCC TTTTCCTTCT GGGCCTAAAC .C.AGCC.CA TTGCACCCGC GCCCTGCCTC GGA.T
BF03  .......... .A........ .......... ......G... .......... .......... .......G.A .C........ .......... .....
BF04  CGCCTAGTAC CACTGCCTTT TTCGCC.ACC AACTAC.CCC TTTT...TCT GGGCCTAAAC GCTA.CC.CA TTGCACCCGC GCC.T.CCT. ...TT
BF05  CGCCTA.TAC CACTGCCT.T TT.GCCTACC AACTAC.CCC TTTTCCTTCT GGGCCTAAAC .C.AGCC.CA TTGCACCCGC GCCCTGCCTC GGA.T
BF06  .......... .A........ .......... ......G... .......... .......... .......G.A .C........ .......... .....
BF07  .......... .......... .......... .......... .......... ..G.A..... ........A. .......... .......... .....
BF08  CGCCTA.TAC CACTGCCT.T TT.GCCTACC AACTAC.CCC TTTTCCTTCT GGGCCTAAAC .C.AGCC.CA TTGCACCCGC GCCCTGCCTC GGA.T
BF09  CGCCTAGTAC CACTGCCTTT TTCGCC.ACC AACTAC.CCC TTTT...TCT GGGCCTAAAC GCTA.CC.CA TTGCACCCGC GCC.T.CCT. ...TG
BF10  CGCCTA.TAC CACTGCCT.T TT.GCCTACC AACTAC.CCC TTTTCCTTCT GGGCCTAAAC .C.AGCC.CA TTGCACCCGC GCCCTGCCTC GGA.T
BF11  CGCCTA.TAC CACTGCCT.T TT.GCCTACC AACTAC.CCC TTTTCCTTCT GGGCCTAAAC .C.AGCC.CA TTGCACCCGC GCCCTGCCTC GGA.T
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Figure 1. Polymorphic sites in *Cyt b* gene entire sequences of 11 gayals. The dot means the same base as the first sequence.

Nucleotide variations of *Cyt b* entire sequences of gayals

Among 1,140 sites compared for 11 gayals, a total of 95 variable sites (8.33% of all sites) (Figure 1) was observed, of which 94 sites were phylogenetically informative sites and 11 sites were amino acid substitution sites. Of the 95 variable sites, the transition and transversion sites comprised 84 and 11, respectively. The transition/ transversion ratio (R) was 7.64, showing a high transition bias (Irwin et al., 1991). Interestingly, the transitional rate between pyrimidines (T-C) was higher than between purines (A-G) with a ratio of 2.23, similar to the report of Tamura and Nei (1993). Eleven *Cyt b* sequences generated 6 different haplotypes (hap01-hap06): hap01, hap04, hap05 and hap06 including only one sequence (BF01, BF04, BF07 and BF09, respectively); hap02 including five sequences (BF02, BF05, BF08, BF10 and BF11); and hap03 including two sequences (BF03 and BF06). The haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.744±0.091 and 0.0336±0.00697, respectively, showing abundant mitochondrial genetic diversity in gayals.

Phylogenetic analysis

In this article, phylogenetic analysis was based on 13 *Cyt b* sequences, including 6 haplotype sequences of 11 gayals and 7 *Cyt b* sequences of other bovine species cited in GenBank (Table 1). The NJ and MP phylogenetic trees of genus Bos (Figures 2 and 3) were reconstructed with *Bubalus bubalis* (Accession No. D88635) as outgroup. Support for individual branches of two phylogenetic trees was assessed by Bootstrap Percentages (BP) computed after 1,000 replicates of the closest stepwise addition option.

It can be seen from Figure 2 and 3 that both the NJ and MP phylogenetic trees support almost the same topology. There were three embranchments for gayals in both phylogenetic trees. The first embranchment, consisting of hap01, hap03 and hap05, clustered together with *Bos gaurus* at the BP value 100% in both NJ and MP trees. The second embranchment, including only hap02, clustered together with *Bos taurus* at the BP value 100% in the NJ tree and 99% in the MP tree. The third embranchment, including hap04 and hap06, clustered together with *Bos indicus* at the BP value 99% in the NJ tree and 95% in the MP tree. The results suggest that the gayal might have close relationships with *Bos taurus*, *Bos indicus* and *Bos gaurus*.

Molecular phylogeny and taxonomic status of the gayal

Phylogenetic analysis showed that gayals in our study were divided into three embranchments (Figures 2 and 3). The second and third embranchments close clustered with *Bos taurus* and *Bos indicus*, respectively, which suggested that the gayal might contain a maternal origin of *Bos taurus* or *Bos indicus*. However, the researches on descriptive characteristics, karyotype, blood protein polymorphism and microsatellite analysis (Walker et al., 1968; San et al., 1980; Simoons, 1984; Nie et al., 1999; Tu et al., 2000; Ritz et al., 2000; Tanaka et al., 2004), have shown that *Bos frontalis* is distinctly different from *Bos taurus* and *Bos indicus*. Therefore, the gayal could not have originated from *Bos taurus* or *Bos indicus*, and its maternal bloodline of *Bos taurus* or *Bos indicus* might be the mtDNA introgression of *Bos taurus* or *Bos indicus* into the gayal's ancestor through interbreeding during historic times. This scenario is apparently reasonable, as the gayal can interbreed with domestic cattle (*Bos taurus* and *Bos indicus*) and the female offspring may be fertile, but the male offspring may not always be fertile (Simoons, 1984; Huque et al., 2001; Giasuddin et al., 2003a; Nyunt and Win, 2004; Tanaka et al., 2004). In China, Fan et al. (2005) first reported the meat

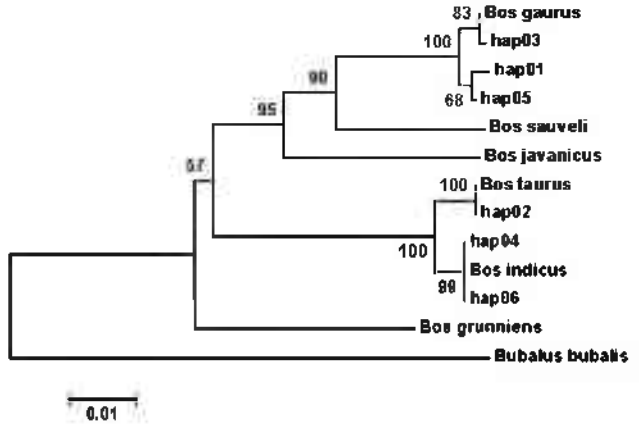

Figure 2. Molecular phylogenetic tree of genus Bos reconstructed by NJ method based on *Cyt b* sequences. Numbers at nodes represent bootstrap values (%) with 1,000 replicates.

characteristic of descendants of crossbreds between gayal bulls and Yunnan yellow cattle cows. Mao et al. (2005), based on their field investigation, reported that there are crossbreed descendants of gayal and domestic cattle from the Dulong River Basin to the Nu River Basin, which was verified again by our investigation. In Yunnan, gayals are kept in a semi-feral stage and mainly used as a ready source for ceremonial occasions. They are reared under a range system and the only bondage with the owner is a periodical visit for salt licks. This half-domestic and half-wild breeding pattern increased the chance of the gayal contacting domestic cattle. In our study, 7 *Cyt b* sequences of gayals (63.64% of 11 sequences) belonged to the second and third embranchments, which indicated that a large proportion of the gayal bloodline in China was invaded by other bovine species.

Winter et al. (1984) proposed that the gaur was the wild ancestor of the gayal according to karyotype, red blood cells and haemoglobin type. This view was subsequently supported by Ritz et al. (2000), Tanaka et al. (2004) and Verkaar et al. (2004). Our data of phylogenetic analysis indicated that the first embranchment of gayals was closely allied with the gaur (*Bos gaurus*) (Figures 2 and 3). The nucleotide divergence between *Bos frontalis* (the first embranchment of gayals, including hap01, hap03 and hap05) and *Bos gaurus* was only 0.53%, far less than the divergences between *Bos frontalis* and *Bos sauveli*/*Bos javanicus* (4.27%/5.64%). These results indicated that there was very close kinship between the gayal and the gaur, and the gayal might be the domesticated form of the gaur. The hypothesis that the gayal was an independent bovine species (Walker et al., 1968; San et al., 1980; Ma et al., 2007) was not supported by the results presented in this study.

Based on the sequence analyses and phylogenetic analyses, we think that the gayal might be the domesticated

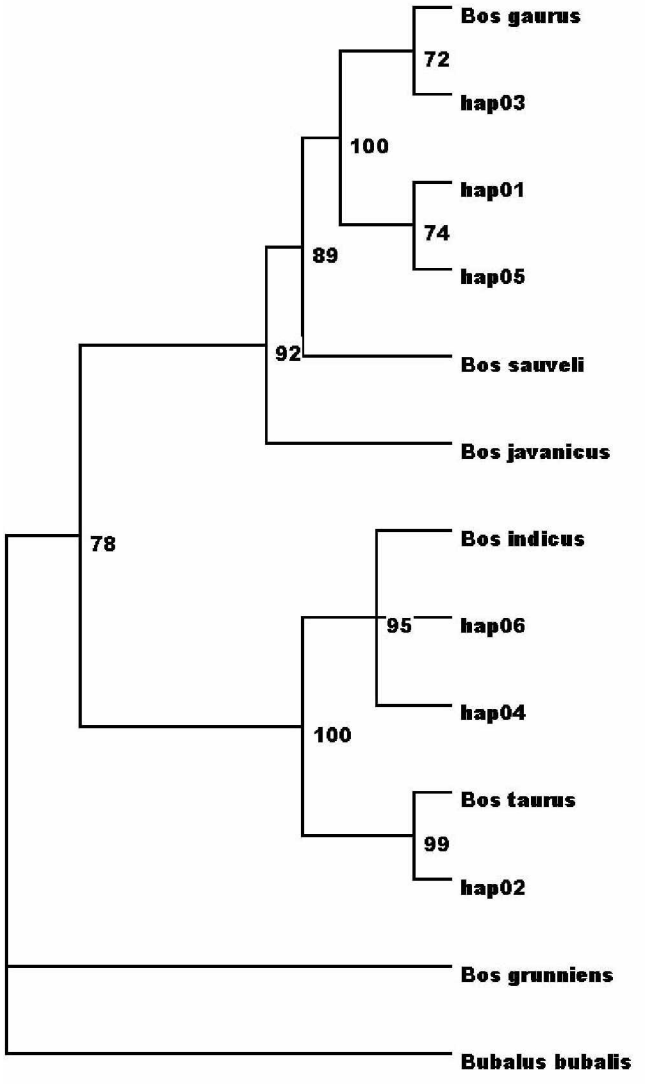

Figure 3. Molecular phylogenetic tree of genus Bos reconstructed by MP method based on *Cyt b* sequences. Numbers at nodes represent bootstrap values (%) with 1,000 replicates.

form of the gaur, and a great proportion of the gayal bloodline in China was invaded by other bovine species. Further nuclear data are needed to confirm this taxonomic classification of the gayal.

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