Journal of Animal and Veterinary Advances 6 (12): 1495-1499, 2007
ISSN: 1680-5593

Molecular Phylogeny and Taxonomic Status of Domestic Yak Inferred from Cytochrome B Gene Partial Sequences

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Abstract: The domestic yak (*Bos grunniens*) is the most important domesticated bovine species in the Qinghai-Tibetan Plateau. In the present study, the cytochrome b (*Cyt b*) gene partial sequences (540 bp) of 27 domestic yaks (20 sequences determined in our study and 7 sequences cited in GenBank) were analyzed. Combined with the homologous fragments of other bovine *Cyt b* sequences in GenBank, the phylogenetic trees of Bovinae were reconstructed by Neighbor-Joining (NJ) methods with *Ovis aries* as outgroup. Sequence analysis showed that, among 540 sites compared for 27 domestic yaks, 9 variable sites (1.67% of 540 sites) and 5 different haplotypes were observed, showing very low mitochondrial genetic diversity in domestic yaks. The sequence divergence and divergence time between yak (*Bos grunniens*/*Bos mutus*) and American bison were 3.33% and 1.48-1.85 million years, respectively, lower and shorter than those between yak and genus Bos (*Bos taurus*/*Bos indicus*). Phylogenetic analysis showed that yak and American bison close clustered into one embranchment, while *Bos taurus* and *Bos indicus* independently clustered into another embranchment. Our results supported that the domestic yak was domesticated from a primitive yak different from the present wild yak and the yak should be classified as an independent genus Poephagus in Bovinae.

Key words: Domestic yak (*Bos grunniens*), cytochrome b gene, molecular phylogeny, taxonomic status

INTRODUCTION

Domestic yak (*Bos grunniens*) and wild yak (*Bos mutus*) live primarily at high elevations, in the cold mountainous areas of Qinghai-Tibetan. Domestic yaks were derived from wild yaks for the production of wool, milk, meat and for manual work. Archaeological evidence suggests that the domestication of the yak was likely to have occurred about 5000 years ago by ancient Qiang people in North Tibet (Zhang, 1989; Wiener *et al*., 2003). At present, the geographical distribution of yaks extends from the southern slopes of the Himalayas to the Altai and Hangai Mountains of Mongolia and Russia (Xuebin *et al*., 2005). Approximately 14 million yaks live in China, which represents about 95% of the world population, with the residual 5% located in Mongolia, Russia, Nepal, India, Bhutan, Sikkim, and Pakistan.

Although, there are some molecular evidences about the origin and taxonomic status of the yak, these results are not consistent and there still exist noticeable divergence about yak = s taxonomic status (Ritz *et al*., 2000; Lai *et al*., 2005; Li *et al*., 2005, 2006; Gu *et al*., 2007). Cytochrome b (*Cyt b*) gene contains abundant phylogenetic information among intra- and interspecies, and 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). It is widely used in the studies on origin, taxonomy and phylogeny of the subfamily Bovinae (Kikkawa *et al*., 1997; Birungi and Arctander, 2001; Hassanin and Ropiquet, 2004). In the present study, *Cyt b* gene partial sequences of domestic yaks in China were sequenced and 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 yak in molecular level and to provide some molecular biological gists for evaluating and protecting this rare genetic resource.

MATERIALS AND METHODS

Applying simple random sampling in typical colony methods, 20 domestic yaks were selected from Hejing county Bayinbuluke district, Bayinguoleng Mongolian minority autonomy region, Xinjiang. Their blood samples were collected and taken back to the lab in an icebox, then kept at -20°C until use. The *Cyt b* gene sequences in Bovinae cited in GenBank for phylogenetic analysis were showed in Table 1.

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Table 1: Cyt b sequences cited in GenBank and accession number

Species(common name)	Accession No
Bos taurus (Domestic cattle)	V00654
Bos indicus (Zebu)	NC_005971
Bos gaurus (Gaur)	AF348593
Bos javanicus (Banteng)	DQ459558
Bos grunniens (Domestic yak)	AY684273
Bos grunniens (Domestic yak)	AY374124
Bos grunnies (Domestic yak)	AF091631
Bos grunniens (Domestic yak)	NC_006380
Bos grunniens (Domestic yak)	EF494177
Bos grunniens (Domestic yak)	EF494178
Bos grunniens (Domestic yak)	EF494179
Bos mutus (Wild yak)	AY955225
Bos mutus (Wild yak)	AY955226
Bison bison (American bison)	AF036273
Bison bonasus (European bison)	AY689186
Bubalus bubalis (Asia buffalo)	D32193
Syncerus caffer (African buffalo)	D82888
Ovis aries (Sheep)	DQ459340

DNA extraction 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 *Cyt b* gene partial sequence was amplified from total genomic DNA using polymerase chain reaction (PCR) with the two designed primers (forward: 5=- TGAAACTTCGGCTCCCTCCT-3=; reverse: 5=- CCTAAGATGTCTTTAATGGT -3= , which were situated at positions 91-110 and 673-692 in cattle *Cyt b* gene, respectively). The standard PCR conditions were as follows: 4 min at 94EC; 30 cycles of denaturation/annealing/extension with 40 s at 94EC for denaturation, 40 s at 53EC for annealing, and 50 s at 72EC for extension and 7 min at 72EC. Each PCR was performed in 25 FL 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 602 bp) were analyzed in 10% PAGE gel with a vacant comparison. Purification and sequencing procedure were carried out by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd in China.

Statistical analysis: *Cyt b* partial sequences of 20 domestic yaks were edited and aligned with reference to the *Cyt b* sequence of domestic cattle (*Bos taurus*) (Accession No. V00654) using DNASTAR package and were checked manually. 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) and nucleotide diversity (Pi) were calculated by the software DNAsp 4.1 (Rozas *et al*., 2003). Using the computer program MEGA 3.1 (Kumar *et al*., 2004), a Neighbor-Joining (NJ) phylogenetic tree based on Kimura two-parameter model was reconstructed to carry out phylogenetic analysis. Levels of resolution at internal nodes of the NJ phylogenetic tree were evaluated by bootstrap resampling with 1000 iterations (Felsenstein, 1985).

RESULTS AND DISCUSSION

Nucleotide composition of *Cyt b* partial sequences of domestic yaks: After sequenced and aligned, a 540 bp fragment of *Cyt b* gene (positions 142-681 in cattle *Cyt b* gene) was obtained in all 20 domestic yaks. Combined with 7 *Cyt b* sequences of domestic yaks in GenBank (Table 1), 27 *Cyt b* partial sequences (540 bp) of domestic yaks were analyzed in this study. No insertions/deletions were observed. The average nucleotide frequencies of T, C, A and G were 27.2, 27.3, 30.7 and 14.8%, respectively. A remarkable imbalance in base usage was observed at third positions, with infrequent use of G (3.3%) and a bias towards A+C (78.4%). The low number of Gs (3.3%) and high number of As (42.2%) at third positions indicate that the likelihood of an A to G transition is much lower than a G to A transition (Birungi and Arctander, 2001).

Nucleotide variations of *Cyt b* partial sequences of domestic yaks: Among 540 sites compared for 27 domestic yaks, a total of 9 variable sites (102, 147, 153, 183, 186, 212, 213, 234 and 318) (1.67% of all sites) were observed, of which only one site was parsimony informative polymorphic site, and only one site (the 212 th site) was amino acid substitution site. Of the 9 variable sites, the transition sites and transversion sites were 8 and 1, respectively. The transition/transversion Ratio (R) was 8.0, showing a high transition bias (Irwin *et al*., 1991). Interestingly, the transitional rate between pyrimidines (T-C) was much higher than that between purines (A-G) with a ratio of 7.0, similar to the report of Tamura and Nei (1993). 27 *Cyt b* sequences generated 5 different haplotypes (Hap01-Hap05), of which Hap01 was prevalent haplotype including 23 sequences, and other four haplotypes included only one sequence, respectively. The haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.279"0.112 and 0.00136"0.00072, respectively, showing very low mitochondrial genetic diversity in domestic yaks.

Patterns of nucleotide variations of *cyt b* partial sequences in bovinae: Among 540 sites compared for 37 *Cyt b* sequences in Bovinae, a total of 121 variable sites (22.41% of all sites) were observed, of which 79 sites were parsimony informative polymorphic sites and 42 sites were singleton polymorphic sites. Of the 121 variable sites, the transition sites and transversion sites were 16

and 2, respectively. The transition/transversion Ratio (R) was 8.0, showing a high transition bias (Irwin *et al*., 1991).

The uncorrected sequence divergences for *Cyt b* gene in Bovinae were showed in Table 2. The sequence divergence between *Bos grunniens* and *Bos mutus* (1.11%) was the lowest in Bovinae, showing very high genetic similarity between domestic yak and wild yak. The mean sequence divergence between yak (*Bos grunniens*/*Bos mutus*) and American bison (*Bison bison*) (3.33%) was much lower than that between yak and genus Bos (*Bos taurus*/*Bos indicus*) (6.83%), which indicated that the genetic similarity between yak and American bison was higher than that between yak and genus Bos. In Bovinae, the sequence divergence between genus Bos and African buffalo (*Syncerus caffer*) was the highest, suggesting very low genetic similarity between genus Bos and African buffalo.

Phylogenetic analysis: In this study, phylogenetic analysis was based on 16 *Cyt b* partial sequences (540 bp), including 5 haplotype sequences of 27 domestic yaks and other 11 homologous fragments of *Cyt b* sequences cited in GenBank (Table 1). The NJ phylogenetic tree of the Bovinae (Fig. 1) was reconstructed with *Ovis aries* (Accession No. DQ459340) as outgroup. Support for individual branch of NJ phylogenetic tree was assessed by Bootstrap Percentages (BP) computed after 1000 replicates of the closest stepwise addition option.

It can be seen from Fig. 1 that Bovinae consists of 2 clades: Clade A and Clade B. Clade A includes 4 embranchments: *Bos grunniens*/*Bos mutus*/*Bison bison* embranchment, *Bos gaurus*/*Bos javanicus* embranchment, *Bos taurus*/*Bos indicus* embranchment and *Bison bonasus* embranchment. Clade B consists of *Bubalus bubalis* and *Syncerus caffer*. Furthermore, bootstraping intensely supports that the five haplotypes of domestic yaks cluster into two branches, one branch including Hap01, Hap03, Hap04 and Hap05, the other branch only containing Hap02 and clustering with wild yak (*Bos mutus*).

Molecular phylogeny and taxonomic status of domestic yak: Phylogenetic analysis indicates that there are two

Table 2: Percentage divergence for Cyt b sequences in Bovinae (%)

	Bos grunniens	*Bos mutus*	*Bos taurus*	*Bos indicus*	*Bos gaurus*	*Bos javanicus*	*Bison bison*	*Bison bonasus*	*Bubalus bubalis*
Bos mutus	1.11								
Bos taurus	7.18	6.48							
Bos indicus	7.18	6.48	1.16						
Bos gaurus	6.31	5.93	6.85	6.48					
Bos javanicus	5.41	5.74	6.30	5.93	3.15				
Bison bison	3.70	2.96	6.30	5.93	6.67	6.30			
Bison bonasus	6.84	6.85	6.85	6.48	6.85	5.93	7.41		
Bubalus bubalis	11.10	10.74	11.30	11.30	12.04	11.67	11.11	11.85	
Syncerus caffer	11.65	11.67	13.15	12.41	12.22	12.22	12.04	12.04	8.33

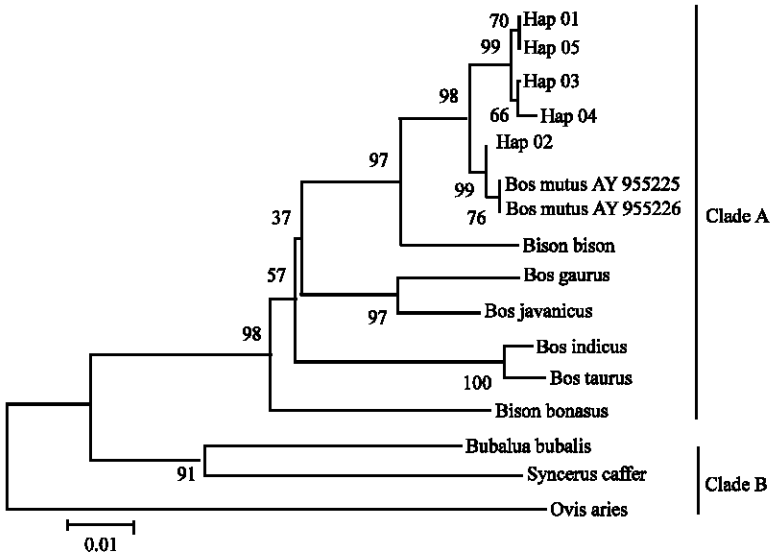

Fig. 1: NJ phylogenetic tree of the Bovinae based on *Cyt b* gene partial sequences Numbers at nodes represent bootstrap percentages (BP) (%) with 1, 000 replicates

maternal lineages (A and B) in domestic yaks (Fig. 1). The lineage A and B contains four haplotypes and one haplotype, respectively. The four haplotypes (Hap01, Hap03, Hap04 and Hap05) in lineage A contain 27 samples (96.43% of all 28 domestic yaks), while the lineage B includes only 1 sample (3.57% of all 28 domestic yaks) and it also contains all the wild yaks (*Bos mutus*). Therefore, we consider that the domestic yaks and the wild yaks originated from lineage A and lineage B, respectively. According to a molecular clock calibration of 2% sequence divergence per million years for *Cyt b* gene in Bovidae (Birungi and Arctander, 2001), the two lineages (A and B) diverged from each other 0.56 million years ago, far earlier than domestic yaks having been domesticated. So we conclude that the domestic yak was domesticated from a primitive yak different from the present wild yak.

There still exists remarkable divergence about the yak=s taxonomic status in bovinae. Based on paleontology evidence, morphologic character, microsatellite polymorphism, mtDNA or nuclear DNA sequence analysis, Linnaeus (1766), Bohlken (1961), Fan *et al.* (2000) and Ritz *et al.* (2000) regarded the yak as a subgenus Poephagus or a species of genus Bos, while Groves (1981), Olsen (1990), Geraads (1992) and Li *et al.* (2006) considered the yak as an independent genus Poephagus in Bovinae. In our study, the mean sequence divergence between yak (*Bos grunniens* /*Bos mutus*) and American bison (3.33%) was much lower than that between yak and genus Bos (*Bos taurus*/*Bos indicus*) (6.83%) (Table 2). And phylogenetic analysis showed that yak and American bison close clustered into one embranchment, while *Bos taurus* and *Bos indicus* independently clustered into another embranchment (Fig. 1). These data indicated that the genetic similarity between yak and American bison were much higher than that between yak and genus Bos. According sequence divergence, we speculated about the divergence times among different taxa in Bovinae (Birungi and Arctander, 2001). The divergence times were found to be 1.48-1.85 million years between yak and American bison, 3.24-3.59 million years between yak and genus Bos, and 0.58 million years between *Bos taurus* and *Bos indicus*, which were similar to the estimates of Gu *et al.* (2006) and Li *et al.* (2006). The evidence from paleontological data showed that domestic cattle (*Bos Taurus*) and yak started to diverge in the middle or the late Pliocene era (from 7.5-3.0 million years) (Qiu,1995), which was consistent with our divergence time estimate. From the above discussed, it can be seen that the phylogenetic relationship between yak and American bison were a lot closer than that between yak and genus Bos. So we supported the view that the yak should be classified as an independent genus Poephagus in Bovinae.

CONCLUSION

Our results indicated that there was very low mitochondrial genetic diversity in domestic yaks and the domestic yak was domesticated from a primitive yak different from the present wild yak. The data also supported the viewpoint that the yak should be classified as an independent genus Poephagus in Bovinae.

ACKNOWLEDGEMENT

We thank the anonymous reviewers for the comments on the manuscript. This research was supported by the Project of the National Natural Science Foundation of P. R. China (Grant No. 30571323)

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