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Taxonomy and Phylogenesis of Chinese Yak Based on the Complete Sequence of Mitochondrial Cytochrome b Gene in Tianzhu White Yak, *Poephagus grunniens*

[1,2]J.L. Bai, [2]J.Y. Jiang and [2]J. Yang

[1]Gansu Engineering Research Center for Animal Cell, No. 1, New Village of Northwest, Lanzhou, 730030, China

[2]College of Life Science and Engineering, Northwest University for Nationalities, No. 1, New Village of Northwest, Lanzhou, 730030, China

Corresponding Author: J.L. Bai, Gansu Engineering Research Center for Animal Cell, No. 1, New Village of Northwest, Lanzhou, 730030, China

ABSTRACT

It is well known that yak is originated from China but fact regarding its taxonomy and evolutionary relationship with other species of Bovini, is largely disputed. Here, we cloned 1140 bp complete mitochondrial cytochrome b (cytb) gene in Tianzhu with yak (JF946750). The origin, taxonomy of the Chinese yak and its phylogenetic relationship with other 8 species of Bovini were discussed based on the cytb. Results showed that Tianzhu white yak had high identity to Qinghai black breed with the minimum sequence divergence of 0.5%. The sequence divergence between yak and cattle/zebu (8.0-8.5%) was higher than that between yak and American bison (3.4-4.1%). Phylogeny analysis also found that domestic yak and wild yak clustered first of all, then gathered with American bison and other Bovini species, indicating yak and American bison were higher genetic comparability than that of other species. The findings sustained the idea that in the choice of nomenclature both the domestic yak and the wild yak belong to the subgenus of *Poephagus*. The approximate divergence time between domestic yak and wild yak was 0.50 Million Years Ago (MYA), while the yak and cattle/zebu, American bison, European bison, Asian buffalo/African buffalo was 4.00-4.25, 1.7-2.05, 3.80-3.85 and 6.70-6.95 MYA, respectively. We speculated that the ancient yak lived in the northeastern part of Eurasia during the Quaternary had been shifted south to the cold area of the Qinghai Tibetan Plateau (QTP) during the metaphase of the Pleistocene era. Some of them acclimated to today's wild yak, the others were domesticated by the ancient Qiang people at least 4500 years ago. The sequential evolution could be predicted that buffalo was first to divided into Asian buffalo and African buffalo among the species of Bovini during the end of Miocene and the early of Pliocene. In the end of Pliocene, the Bovini genera were evolved to *Bos*, *Bison* and *Poephagus*. *Poephagus* which branched off from the middle of Pleiocene, was the latest evolved genera among the Bovini species.

Key words: Yak, Bovini, cytochrome b gene, phylogenetic relationship, divergence time

INTRODUCTION

Yaks (*Poephagus grunniens*) are distributed across the QTP and adjacent highlands (Gerald *et al*., 2003). QTP is the largest continuous high-elevation ecosystem in the world, occupying nearly 2.5 million km^2 of the Asian continent and reaching an average elevation of more

than 3000 m as sea level. More than 14 million yaks are currently herded there and they provide many of life's necessities (food, hids, dung fuel and transport power) for the nomadic Tibetan pastoralists living in this extremely harsh region (Gerald *et al.*, 2003).

The yak (domestic and wild yak) is originated from China. Based on the petrifactions found in the northern China, Siberia and Alaska, researchers suggest that the forefather of the today's domestic yak and wild yak (*Poephagus mutus*) was a primitive wild yak that lived about 2.50 MYA (Cai, 1992). Investigation of 20 microsatellite loci indicates that the divergence time between yak and cattle (*Bos taurus*) was 0.57-1.53 MYA, between yak and the American bison (*Bison bison*) was 0.39-1.04 MYA (Ritz *et al.*, 2000), showing the genetic relationship between yak and American bison is more close than yak and cattle. More molecular evidence, either from mitochondrial DNA (mtDNA) or from nuclear genome, have been obtained that yak and American bison is first to cluster to the same clade among other species of Bovini (Tu *et al.*,1998, 2002; Hassanin and Ropiquet, 2004; Li *et al.*, 2006b; Xie *et al.*, 2010; Zhao *et al.*, 2011; Qiu *et al.*, 2012; Ma *et al.*, 2013). However, so far some envidence derived from archeology and historical records still haven't been reached a consensus about the orign of yak and its taxonomy. The ancient Tibetans in the northern Tibet had captured and domesticated wild yak for agricultural use during the Paleolithic era 5000-10000 years ago (Gerald *et al.*, 2003; Li *et al.*, 2006b). Chinese archeologists discovered fossils in Tibet, Sichan and Qinghai province in the 1950s indicating that the domestic yak appeared 4500 years ago (Cai, 1992). These findings were consistent with the report by Li (2004) who used microsatellite markers to determine the period of yak origin.

The yak has similar morphological characteristics to American bison and cattle, so researchers focused on the phylogentic relationship between yak and other species of Bovini (Li *et al.*, 2006a; Gu *et al.*, 2007; Xie *et al.*, 2010). The earliest report by Gray in 1843 suggest that yak was identified as *Poephagus* according to the morphological differences between genus *Bos* and *Bison bison*. Olsen (1991) found that the yak has the same arrangement of premaxillaries, maxillaries and nasals as in *Bison* and are different from the structure in *Bos*. Geraads (1992), using a matrix of 57 cranial characters and 32 taxa of fossil of differentt Bovini species, concluded that the yak is close to the *Bison*. These results were consistent with the studies from Li *et al.* (2005) and Hassanin and Ropiquet (2004). In contrast, according to the different morphological characteristics of species among Bovini, Bohlken (1961) first grouped the yak with cattle, followed by gaur (*Bos gaurus*) and banteng (*Bos javanicus*) in one clade which then clustered with the American bison and European bison (*Bison bonasus*). Same conclusion was also made by Hartl *et al.* (1988) and Ritz *et al.* (2000) from biochemical genetics and microsatellite loci, respectively.

Tianzhu white yak, with 0.8 million population, is one of the unique species of Chinese yaks that centralized distribution in Tianzhu Tibetan Autonomous County, Gansu Province in China which is located in the eastern end of the Qilian mountain and the northern edge of the QTP. The aim of the present study was to research the taxonomy of the Chinese yak and the phylogenetic relationship between the yak and other Bovini species using the complete sequence of mitochondrial cytochrome b gene.

MATERIALS AND METHODS

Animals: Tianzhu white yak was selected from Tianzhu White Yak Breeding Farm in Xidatan Village, Xidatan Town, Tianzhu Tibetan Autonomous County, Wuwei Prefecture, Gansu Province which is located in the eastern end of the Qilian mountain and the northern edge of the QTP (102°02'-103°29'E; 36°29'-37°41'N). Animals were followed the guidelines stated in the Guide

for the Care and Use of Agricultural Animals in Research and Teaching in Gansu Province, China. The 10 mL of anticogulated carotid arterial blood was sampled from each adult female yak (n = 4).

Genomic DNA isolation: Genomic DNA extraction was performed using animal blood genomic DNA extraction kit (Tiangen, China) according to the manufacturer's instruction.

Primer design: Primers were designed according to the nucleotide sequence of cytb of *Poephaguss grunniens* mitochondrion complete genome (NC_006380) by Primer Premier 5.0 software and confirmed by primer-BLAST in http://www.ncbi.nlm.nih.gov/tools/primer-blast/. The sequence of forward primer is 5'-CATCGTTTGTCATTCAACTACA-3'and the reverse primer is 5'-TGTTCTCCTTTTCTGGTTTAC-3'.

Gene amplification: Gene amplification of cytb was performed with 5 min at 94°C for one cycle, followed by 35 cycles of denaturation for 45 sec at 94°C, 45 sec of annealing at 55°C and 1 min of extension at 72°C; with the final extension step for 10 min at 72°C for one cycle. Analyze 5 μL of each sample on a 0.9% agarose/EtBr gel. Each 20 μL reaction system was comprised of 1 μL genomic DNA (200 ng μL^{-1}), 1 μL forward primer (10 μM μL^{-1}) and 1 μL reverse primer (10 μM μL^{-1}), 1 μL dNTP mix (10 μM μL^{-1}), 2 μL 10×buffer, 0.5 μL Taq DNA polymerase (5 U μL^{-1}), 13.5 μL ddH_2O. Expected target gene fragment was then subcloned to T-easy vector. Positive plasmid was sequenced in Sangon Biotech (Shanghai, China) Co., Ltd.

Data analysis: Using cytb of sheep, *Ovis aries* (AF010406) as outgroup taxa, phylogenetic analysis of Tianzhu white yak was performed based on the cytb (JF946750) comparison with representative species of Bovini, such as *Poephagus grunniens* (Qinghai black breed, AY684273), *Poephagus mutus* (wild yak, AY955226), *Bos taurus* (cattle, AY952966), *Bos indicus* (zebu, AF492350), *Bison bison* (American bison, AF036273), *Bison bonasus* (European bison, AY689186), *Bubalus bubalis* (Asian buffalo, D88631) and *Synerus caffer* (African buffalo, D82888). The analysis of sequence alignment, arrangement and the sequence divergence were carried out using DNAStar 5.02 software (DNASTAR Inc., 1996). The overall transition/transversion bias was calculated as following equation:

$$R = [A*G*K_1+T*C*K_2]/[(A+G)*(T+C)]$$

where A, T, C, G was represented nucleotide substitution frequence, K_1 and K_2 were indicated the transition/transversion rate ratio of purines and pyrimidines using the maximum parsimony method of MEGA 5.0 software (Tamura *et al*., 2011) by selecting the Kimura 2-parameter model. Phylogenetic tree was constructed by Neighbor-joining method with bootstrap replication test (1000 replications). The divergence time of different species of Bovini was estimated by the 2% molecular clock of the cytb gene sequence per million years (Birungi and Arctander, 2001).

RESULTS

Cloning of cytb in Tianzhu white yak: As shown in Fig. 1, about 1225 bp segment containing cytb in Tianzhu white yak was amplified by PCR using genomic DNA as template. Sequencing result indicated that the full length of cytb in Tianzhu white yak was 1140 bp which has been and conferred GenBank Accession Number with JF946750, encoding 380 amino acids. Content of base A and T, base G and C is 57.89 and 42.11% in cytb, showing obviously higher contents of base A and T than that of base G and C.

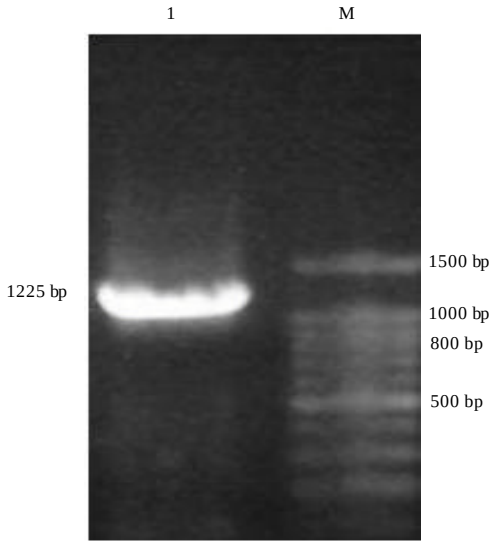

Fig. 1: Electrophoresis of mitochondrial cytochrome b gene amplified by PCR M: 100 bp DNA marker; 1: Cytochrome b (cytb) gene, about 1225 bp segment containing 1140 bp cytb in Tianzhu white yak was amplified by PCR using genomic DNA as template

Table 1: Percentage divergence for mitochondrial cytochrome b gene sequence in Bovinae and outgroup (%)

Parameters	*Poephagus grunniens* (Tianzhu white)	*Poephagus grunniens* (Qinghai black)	*Bos mutus*	*Bos taurus*	*Bos indicus*	*Bison bison*	*Bison bonasus*	*Bubalus bubalis*	*Syncerus caffer*
Poephagus grunniens (Tianzhu white)									
Poephagus grunniens (Qinghai black)	0.5								
Poephagus mutus	1.0	1.0							
Bos taurus	8.5	8.3	8.0						
Bos indicus	3.9	8.3	8.0	1.6					
Bison bison	7.7	4.1	3.4	7.1	7.1				
Bison bonasus	13.9	7.5	7.6	6.0	6.0	7.3			
Bubalus bubalis	13.7	13.6	13.7	14.5	14.5	14.1	13.2		
Syncerus caffer	17.8	13.4	13.5	14.4	14.4	14.1	13.3	9.9	
Ovis aries	17.8	18.0	17.8	19.0	19.0	18.0	16.8	16.1	16.4

Sequence divergence of Bovinae species based on cytb: The percentage divergence for cytb gene sequence in *Bovinae* and its outgroup, sheep, *Ovis arise* are list in Table 1. In *Poephagus*, Tianzhu white yak has high identity to Qinghai black breed with the minimum sequence divergence of 0.5%, while the sequence divergence between *Poephagus grunniens* and *Poephagus mutus* is 1.0%, between cattle and zebu is 1.6%. Sequence divergence between *Poephagus* and *Bos* (8.0-8.5%) is higher than that between *Poeghagus* and *Bison bison* (3.4-4.1%), indicating genetic identity between *Poephagus* and *Bison bison* is more closer than that between *Poephagus* and *Bos*. However, sequence divergence between *Peophagus* and *Bos* (7.5-7.7%) is similar to that between *Poephagus* and *Bison bonasus* (8.0-8.5%). The average

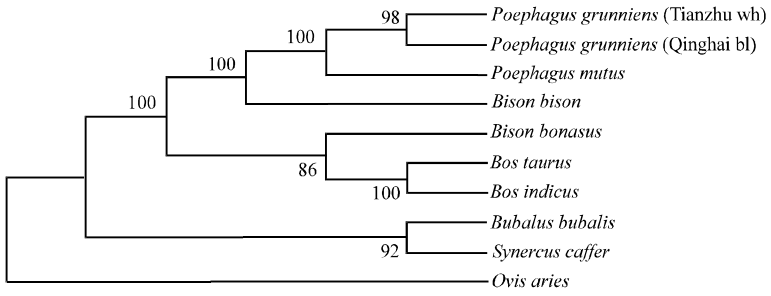

Fig. 2: Phylogenetic tree of Bovini based on cytb gene by Neighor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 5.1

divergence of *Ovis aries* and species of *Poephagus*, *Bos*, *Bison*, buffalo is 17.9, 19.0, 17.7 and 16.6%, respectively. The maximum divergence between *Poephagus* and buffalo, *Bos* and buffalo was also identified (Table 1), representing minimum genetic identity between them.

The maximum composite likelihood estimation of the pattern of nucleotide transition rate of cytb gene among different Bovini species was the major source of substitution, accounting for 82.67%, in which predominant T/C transition was 55.04%, whereas transversion substitution rate was 17.33%, in which C/A and T/A was the predominant replacement, with 5.28 and 4.99% variation rate, respectively. The Ts/Tv of cytb among different species was 4.737, showing higher transition bias.

Phylogenetic analysis of Bovinae species based on cytb: Based on the 1140 bp mitochondrial cytb gene sequences, a phylogenetic tree of Bovini was constructed using the Neighbor Joining Tree method (Fig. 2). The result shows that Bovini species were clustered in one group, while the outgroup (sheep, *Ovis aries*) was clustered in another group. Bovini which had eight species, could be divided into two groups. Buffalos, including *Bubalus bubalis* and *Syncerus caffer*, were clustered in one group (BP = 90%), with the other six species clustered in another group (BP = 100%). Among these six species of Bovini, Tianzhu white yak and Qinghai black yak were firstly clustered in one group (BP = 99%), then clustered with *Poephagus mutus* (BP = 100%), followed with *Bison bison* in one group (BP = 100%). Meanwhile, *Bos Taurus* was initially clustered with *Bos indicus* (BP = 100%), then clustered with *Bison bonasus* (BP = 89%).

Estimation of divergence time of Bovini species based on cytb: The Ts/Tv rate of cytb among the different species of Bovini was 4.737, higher than the critical value 2.0 (Knight and Mindell, 1993) and showed higher transition bias. Therefore, the molecular clock of the cytb gene sequence was 2% per million years in Bovinae which was consistent with Birungi and Arctander (2001) and chosen to estimate the divergence time between different species of Bovini.

The sequence divergence of Tianzhu white yak and Qinghai black yak was 0.5% and the divergence time was 0.25 MYA. Both the sequence divergence of cytb between Tianzhu white yak and wild yak, Qinghai black yak and wild yak was 1.0 and the divergence time between them was 0.50 MYA. The sequence divergence of cytb between yak (Tianzhu white, Qinghai black and wild breed) and cattle/zebu, yak and American bison were 8.0-8.5 and 3.4-4.1%, the divergence time was 4.00-4.25 MYA and 1.7-2.05 MYA, respectively. The results indicated that the divergence time between yak and American bison was much later than other species of Bovini. The divergence time (sequence divergence) between the yak and European bison, Asian buffalo/African buffalo was 3.80-3.85 MYA (7.6-7.7%), 6.70-6.95 MYA (13.4-13.9%), respectively.

DISCUSSION

Origin of the yak: It is generally accepted that close ancestor of today's domestic yak and wild yak has been lived in the northeast Eurasia in the new tertiary period 2.5 MYA. During the Quaternary Ice Age, the original yak were pressured to move southward to the central Tibetan plateau because of the climate changes which became the distributing area of today's wild yak, whereas those yaks in northeast Eurasia were driven to extinction (Cai, 1992). The domestic yak was acclimated from wild yak by acient Qiang people in Qiangtang area of northern Tibet about 5000-10000 years ago in the Paleolithic era (Gerald *et al*., 2003; Li *et al*., 2006b). Evidence has been found that the yak raising industry appeared during the terminal Chinese Neolithic period (about 2800 BC-2 300 BC) (Cai, 1992). Chinese archaeologists have found domestic yak fossils and related relics in some places of in Tibet, Qinghai, Sichuan dated from 4500 years ago, showing that the yak was domesticated before 4500 years (Liu *et al*., 1989). Li (2004) reported that the divergence time of Chinese yak was existed before 8000 years according to the investigation of 20 microsatellite loci which is consistent with historical data and archeological studies.

Based on the complete sequences of cytb, we suggest that the divergence time between Tianzhu white yak and Qinghai black yak was 0.25 MYA, both Tianzhu white yak and wild yak, Qinghai black yak and wild yak was 0.50 MYA, earlier than archeology findings (about 4500 years ago) (Liu *et al*., 1989), the historical recorded time of yak domestication (5000-10000 years ago) (Gerald *et al*., 2003; Li *et al*., 2006b), microsatellite loci investigation of Chinese yak origination (8000 years ago) (Li, 2004) but a little bit later than Chinese yak divergence time from phylogenesis study based on complete sequence of cytb (280000 years ago) (Xie *et al*., 2010). Therefore, the ancestor wild yak lived 500000 years ago was probably a close evolutionary ancestor of the domestic yak and today's wild yak which is in agreement with the cytb study (0.55 MYA) (Li *et al*., 2006a). The divergence time of yak and American bison was 1.70-2.05 MYA which is consistent to the cytb study (1.70-2.10 MYA) (Li *et al*., 2006a), similar with that presumed by mtDNA-RFLP (1.10-2.20 MYA) (Tu *et al*., 2002) and by microsatellite loci (0.57-1.53 MYA) (Ritz *et al*., 2000). The divergence time between yak and cattle/zebu (4.00-4.25 MYA) which is in agreement with the results of Li *et al*. (2006b) (4.0-4.2 MYA) and Qiu *et al*. (2012) (4.9 MYA) and different from Tu *et al*. (1998) (1.01-1.02 MYA), was far earlier than that of yak and American bison. Therefore, we thought it was not possible that the ancestor of the yak was divergent from the close ancestor of *Bos taurus* and *Bos indicus* but probably from an ancestor of American bison. That is to say, the yak and American bison probably have a common direct ancestor.

Based on the present study and fossil records (Cai, 1992), historical data (Cai, 1992), archaeological data (Liu *et al*., 1989) and molecular biology data (Hassanin and Ropiquet, 2004; Guo *et al*., 2006; Li *et al*., 2008; Xie *et al*., 2010; Qiu *et al*., 2012), we have estimated the

approximate evolutionary clues of the yak. The ancestors of American bison and primitive wild yak which lived in the northeastern part of Eurasia during the Quaternary, had been differentiated due to the climate changes. The ancient yaks shifted south to the cold area of the QTP and evolved during the metaphase of the Pleistocene era. Some of them acclimated to today's wild yak, the others were domesticated by the ancient Qiang people at least 4500 years ago.

Taxonomy of the yak: The yak belongs to Artiodactyla, Ruminatia, Bovidae and Bovinae in animal taxonomy. But there is still big controversy of the order classification for yak. Numerous attempts have been made to clarify phylogenetic relationships among the genera of the Bovini, including the yak but the results have been ambiguous. The earliest report of Linnaeus in 1766 listed yak as *Bos grunniens* of *Bos* which was consistent with Bohlken's results from morphological characteristics that yak and catle first grouped, followed by gaur and banteng in one clade, then clustered with the American bison and European bison (Bohlken, 1961). Gray in 1843 identified yak as *Poephagus* according to the morphological differences between genus *Bos* and *Bison bison* which was consistent with the opinions of Groves (1981), Olsen (1991) and Geraads (1992) from the head bone features and archaeological study.

Since the late 1980s, the sequences of mtDNA of yak have been used for the phylogenetic studies of *Bovidae*. Miyamoto *et al.* (1989) sequenced the 12S rRNA and three tRNA genes and a 247 bp partial hypervariant D-loop fragment of four taxa in the Bovini and the results showed a similar topology of phylogeny with Groves (1981), the yak grouped with the *Bison bison* first, with an average divergence of 2.6% for the conservative rRNA/tRNA genes and 9.1% for the D-loop fragment and then followed by *Bos taurus* (Kraus *et al.*, 1992). Ward *et al.* (1999) using a partial mtDNA control region of 667 base pairs, found that the percentage nucleotide divergence of *Bos indicus* and *Bos taurus* from the yak was 24.23 and 29.53%, respectively but that of American bison and European bison from the yak was only 12.28 and 16.59%, respectively. Similar results have been obtained by Hassanin and Douzery (1999); Li *et al.* (2006b); Xie *et al.* (2010) from complete sequence of cytb, Zhao *et al.* (2011) from cytochrome c oxidase subunit 3 (cox3), Zhang *et al.* (2012) from mtDNA D-loop, Hassanin and Douzery (1999) from mtDNA 12S rRNA, aromatase cytochrome P-45.

Within the nuclear genome alone, Wall *et al.* (1992) confirmed the position of yak as more closely related to the bison and wisent than to *Bos taurus* and *Bos indicus* as earlier suggested by Groves (1981). Hassanin and Douzery (1999) sequenced the promoter segment of the lactoferrin-encoding genes of nuclear genomic DNA in the yak alongside with other *Bovidae* species, showing the yak clustered with the American bison first and then grouped with *Bos taurus* in the phylogenetic tree. More similar molecular evidence has been made by Ritz *et al.* (2000) from 20 bovine microsatellite markers, Buntjer *et al.* (2002) from 361 fingerprinting Bovini markers of AFLP, Fan *et al.* (2000) from exon 4 of kappa casein, Li *et al.* (2005) from exon 2 of MHC-DRB3. At present study, the average percent nucleotide divergence between yak (Tianzhu white, Qinghai black and wild yak) and cattle/zebu was 8.37% which was considerably higher than that between yak and *Bison bison* (3.8%), indicating that genetic identity between yak and American bison is higher than that between yak and cattle/zebu. Phylogenetic analysis showed that yak was clustered with American bison but not with cattle/zebu. According to the 2% per million years mutation rate of cytb, we speculated the divergence time between yak and American bison was 1.90 MYA which is later than that between yak and cattle/zebu (4.18 MYA), elucidating that the yak has the closest genetic relationship to American bison. It is clear that for the domestic yak and

the wild yak, the subgenus, *Poephagus,* seems more appropriate than *Bos*. This finding is in agreement with the results made by Gerald *et al*. (2003), Li *et al*. (2005) and Li *et al*. (2006a).

The divergence time of buffalo from other species of Bovini was 6.60-7.26 MYA, whereas the divergence of yak from cattle/zabu, Bison bison from cattle/zebu was 4.15-4.25 and 3.55 MYA, respectively, which is in agreement with the report of Li *et al*. (2006b). The divergence time of cattle from zebu was 0.8 MYA, consenting with the result of Ritz *et al*. (2000). Similar to the result of Li *et al*. (2006a), the divergence time between yak and American bison was 1.7-2.05 MYA. Combination with paleontological evidence (Osborn, 1990) and morphological characteristics, we estimated that buffalo was first to divided into Asian buffalo and African buffalo among the species of Bovini during the end of Miocene (23.00-5.3 MYA) and the early of Pliocene (5.2-1.8 MYA). In the end of Pliocene, the genera of the Bovini were evolved to *Bos*, *Bison* and *Poephagus*. *Poephagus* which branched off from the middle of Pleiocene, was the latest evolved genera among the species of Bovini.

CONCLUSION

We cloned the complete sequence of cytb in Tianzhu with yak (JF946750). The origin and taxonomy of the yak and its phylogenetic relationship with other 8 species of Bovini are discussed based on the 1140 bp cytb gene. In the choice of nomenclature both the domestic yak and the wild yak belong to the subgenus of *Poephagus*. The ancient yak lived in the northeastern part of Eurasia during the Quaternary had been shifted south to the cold area of the QTP during the metaphase of the Pleistocene era. Some of them acclimated to today's wild yak, the others were domesticated by the ancient Qiang people at least 4500 years ago. We also speculated that buffalo was first to divided into Asian buffalo and African buffalo among the species of Bovini during the end of Miocene and the early of Pliocene. In the end of Pliocene, the Bovini genera were evolved to *Bos*, *Bison* and *Poephagus*. *Poephagus* which branched off from the middle of Pleiocene, was the latest evolved genera among the Bovini species.

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