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RESEARCH ARTICLE

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Study on the origin and taxonomic status of yak (*Poephagus*) using cytochrome *b* gene of mitochondrial DNA

Abstract There still exists a remarkable difference on the yak's taxonomic status in *Bovinae*. Primers designed according to the mitochondrial gene sequences of *Bos taurus* reported were used to amplify and sequence the yak's cytochrome *b* gene, and the whole sequence of cytochrome *b* gene was finally obtained. Using *Ovis aries* as outgroup taxa, the phylogeny about the representative species of *Bovinae* was analyzed. Results showed that among the different species, the ratio of transition/transversion (*Ts*/*Tv*) of Cytochrome *b* gene was 4.9, suggesting that the mutation was not saturation. The percentage nucleotide sequence divergence between yak and *Bovinae* was 8.0%–8.6%, which was higher than that of yak and *Bison bison*. Phylogeny analysis found that *Poephagus grunniens* and *Poephagus mutus* clustered first before gathering with *Bison bison*, indicating higher genetic comparability than that of *Bos*. The results sustained the idea that *Poephagus grunniens* and *Poephagus mutus* shared one ancestor—the primitive yak. The approximate divergence time between these two species was 0.55 million years. The data also supported the viewpoint that the yak is classified into *Poephagus* of *Bovinae*, including two species of *Poephagus grunniens* and *Poephagus mutus*.

Keywords *Bovinae*, yak, mitochondrial DNA, cytochrome *b* gene, origin, taxonomic status

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1 Introduction

The yak, known as the "Boat on the Plateau" and "almighty livestock", originating from China, is the prominent livestock breed and distributes only in a restricted region of the Qinghai-Tibet Plateau and nearby areas at an altitude of more than 3 500 m (Wiener et al., 2003). In animal taxonomy, the yak belongs to the *Bovinae* and *Bovidae ruminatia*, *Artiodactyla*. However, there still exists a dispute on the yak's taxonomic status in *Bovinae*. Linnaeus (1766) treated yak, *Bos taurus* and *Bos indicus* as *Bos grunniens*, which was named as *Bos*. In 1843, Gray cited yak as an independent *Poephagus* according to the morphological differences among yaks of *Bison bison* and *Bos* (Gray, 1843). Similar results came from other studies by different standards: the characteristics of the head skull from Groves (1981), the bone of the forehead, maxilla and nose from Olsen (1990, 1991), and the characteristics of 57 bones and 32 fossils from Geraads (1992). However, Bohlken discovered that the phylogenetic relationship between *Bison bison* and *Bison bonasus* was close according to their morphological characteristics, and the same relationship was observed between yak and *Bos taurus* (Bohlken, 1958; 1961).

With the development of molecular biology, scholars from different countries are trying to use different kinds of molecular markers to study the phylogenetic relationship in different genus of *Bovinae*, but there still exists a remarkable difference on the yak's taxonomic status in *Bovinae*. One viewpoint is that yak can be classified as an independent genus of *Bovinae*, namely *Poephagus* (Miyamoto et al., 1989; Hassanin and Douzery, 1999; Li et al., 2005). The other viewpoint is that yak can be a category of one subgenus or genus of *Bos* (Fan et al., 2000; Ritz et al., 2000). Given that there is still no definite conclusion, mtDNA study becomes an increasingly more important tool for understanding the origin of a species, molecular evolution and phylogene because of such characteristics as rapid evolutionary rate, big population genetic variation, simple molecular structure, complete understanding of the DNA sequence and rare recombination (Gray et al., 1999; Saccone et al., 2000; Wu et al., 2003; Zhao et al., 2005). The cytochrome *b* gene is becoming the

most commonly used protein-coding gene for phylogenetic analysis of the mitochondrial genome and of the intergenus and genus of *Bovidae* (Miyamoto et al., 1989; Lau et al., 1998; Hassanin and Douzery, 1999). To confirm the phylogenetic relationship between genus and species of *Bovinae* and taxonomic status of yak, our study sequenced the complete cytochrome *b* gene and blast with Cytochrome *b* gene sequences of the representative species in *Bovinae* phylogenetic analysis was applied at the molecular level to discuss the origin of yak and its taxonomic status in *Bovinae*.

2 Materials and methods

2.1 Sample collection and DNA extraction

10 mL blood samples were obtained respectively through venipuncture from four mature *Jiunong* yaks (2♀, 2♂) in Sichuan Province. Samples were collected with tubes containing anticoagulant citrates dextrose (ACD) and then stored at −30°C prior to use. Genomic DNA was extracted following the methods of phenol / chloroform as described by Li (2004).

2.2 Polymerase chain reaction (PCR) amplification, cloning and sequencing

Polymerase chain reaction primers were designed to amplify the yak's cytochrome *b gene* according to the mitochondrial gene sequences of *Bos taurus* (GenBank access No. AY526085). The primer sequences were as follows:

Forward: 5′-CCATAAATAGGTGAAGGTTTCG-3′;
Reverse: 5′-TTGATGGTGAGACTGCAGTT-3′.

Each 25 μL PCR reaction contained 30 ng of genomic DNA, 1.5 mmol/L $MgCl_2$, 1 × reaction buffer, 0.2 mmol/L of dNTP, 0.01% glutin, 1.0 U of *Taq* DNA polymerase and 0.2 μmol/L of each primer. Amplification consisted of initial denaturalization at 94°C for 5 min and 35 amplification cycles including denaturalization at 94°C for 30 s, annealing at 64°C for 45 s, extension at 72°C for 60 s and a final extension of 72°C for 10 min.

The purified PCR products obtained using a Geneclean III kit (Q. Biogene Co.) were ligated to the pMD 18-T Cloning vector (TaKaRa Co.) according to the manufacturer's instructions, and then transformed into competent DH5α cells. The plasmid DNA was extracted using the BioDev Plasmid Rapid Isolation Kit (BioDev Co.) and sequenced on the Model ABI377 Fluorescent Sequencer (Perkin-Elmer).

2.3 Computer and statistical methods

The representative species in *Bovinae* such as wild yak (*Poephagus mutus*), *Bos taurus*, *Bos indicus*, *Bison bison*, *Bubalus bubalis* and *Sycenrus caffer* were chosen. Their cytochrome *b* gene sequence was downloaded from the GenBank (Table 1) using *Ovis aries* (GenBank access number AF010406) as outgroup taxa of the phylogenetic tree, whose phylogenetic relationship is close to *Bovinae*.

Table 1 Source of sequences for cytochrome *b* gene in *Bovinae*

No.	Species	Common name	GenBank access No.	Sequence length/bp
1	*Poephagus*	Yak	AY374124*	1 140
2	*Poephagus mutus*	Wild yak	AY955226	1 140
3	*Bos taurus*	Cattle	AY952966	1 140
4	*Bos indicus*	Zebu	AF492350	1 140
5	*Bison bison*	American bison	AF036273	1 140
6	*Sycenrus caffer*	African buffalo	D82888	1 140
7	*Bubalus bubalis*	Asian buffalo	D88631	1 140

Note: * stands for sequences produced during this work.

The analyses of sequence alignment, arrangement and the percentage of nucleotide sequence difference were carried out between the determined sequence and GenBank by DNAStars5.02 software (DNASTAR Inc.). The base composition, variable sites and the transition/trans-version (*Ts*/*Tv*) were accounted for by MEGA3.1 software (Kumar et al., 2004). The phylogenetic tree was constructed by the NJ method of MEGA31 software (Kumar et al., 2004), while the bootstrap value was deduced by a bootstrap test (1 000 replicates).

3 Results

3.1 Sequence of cytochrome *b* gene

By means of clone sequencing, we obtained the complete cytochrome *b* gene sequence. Its total length was 1 140 bp, coding 380 amino acid and including *Bos taurus*' cytochrome *b* sequence. In the sequence, the rates of four nucleotides A, T, G and C were 26.5%, 28.8%, 31.6% and 13.1%, respectively, and the rate of AT (58.1%) was significantly more than GC (41.9%). The sequence has been submitted to GenBank, and the access number is AY374124.

3.2 Variation of cytochrome *b* gene in *Bovinae*

In *Bovinae,* the length of the cytochrome *b* gene was 1 140 bp, and the average contents of T, C, A and G were different, which were 25.8%, 29.4%, 31.6% and 13.2%, respectively. The content of G C (42.6%) was less than that of A T (57.4%). There were 233 polymorphic sites in the sequence, with 20.4% of polymorphic sites and 92 single polymorphic sites accounting for 39.5% of the whole site, while 141 parsimony informative polymorphic sites accounted for 60.5% of the whole site.

The percentage of divergence for cytochrome *b* gene sequences in *Bovinae* and outgroup shown in Table 2 demonstrated that genetic comparability between *Poephagus grunniens* and *Poephagus mutus* was the highest, and the percentage of nucleotide sequence divergence was 1.1%, which was lower than that between *Bos taurus* and *Bos*

Table 2 Percentage of divergence for cytochrome *b* gene sequences in *Bovinae* and outgroup

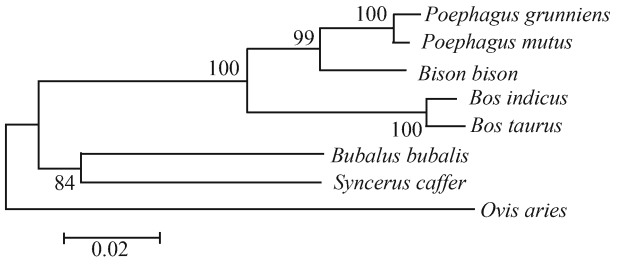
Species	*Poephagus grunniens*	*Poephagus mutus*	*Bos taurus*	*Bos indicus*	*Bison bison*	*Bubalus bubalis*	*Sycenrus caffer*
Poephagus mutus	1.1						
Bos taurus	8.6	8.6					
Bos indicus	8.0	8.0	1.6				
Bison bison	4.2	3.4	7.7	7.1			
Bubalus bubalis	13.5	13.7	14.3	14.5	14.1		
Sycenrus caffer	13.0	13.5	14.9	14.4	14.1	9.9	
Ovis aries	18.3	18.1	19.4	19.5	18.3	16.0	16.6

indicus (1.6%). Meanwhile, genetic comparability between *Poephagus grunniens*, *Poephagus mutus* and *Bison bison* was higher than that between the group as a whole and *Bovinae*, while comparability between the yak and *Sycenrus caffer* was the lowest. The percentage of nucleotide sequence divergence between *Ovis aries* and *Bovinae* was 16.0%–19.5% (with an average of 18.0%) and the average percentage of nucleotide sequence divergence between sheep and yak was 18.2%.

Only one base pair deletion was found, with the others covered by base substitution in cytochrome *b* gene sequence variations of *Bovinae*. In the base substitution, the transition (84 times) was significantly higher than the transversion (17 times), and the transition/transversion (*Ts*/*Tv*) was 4.94/1, indicating that the transition was the main polymorphism variation of cytochrome *b* gene in *Bovinae*. In transition, the variation of T/C found dominated (72.61%), and C/A variation dominated transversion (52.94%).

3.3 Phylogenetic analysis of *Bovinae*

The phylogenetic tree of *Bovinae* (Fig. 1) was constructed based on cytochrome *b* gene sequences by the Neighbor-joining method. It showed that *Poephagus grunniens* and *Poephagus mutus* clustered at first, with the bootstrap percentage (BP) of node at 100%, and then gathered with *Bison bison* with the BP rate of node over 99%. Finally, *Bos indicus* and *Bos Taurus* clustered together, with the BP rate of node over 100%. *Bubalus bubalis* and *Syncerus caffer* were in one group.



Notes: The length of the branch means divergence; the nodal parameters support that the 1 000 times repetition analysis on sampling examination.

Fig. 1 Phylogenetic tree of *Bovinae* based on cytochrome *b* gene sequences by neighbor-joining method

4 Discussion

4.1 Origin of yak

As far as the origin of the yak is concerned, scholars agree with a monodoctrine that the animal has a common original source. Namely, the original yak is the ancestor of *Poephagus grunniens* and *Poephagus mutus*. "The Breed of Cattle in China" also suggests that there were no other theories than monogenesis—a viewpoint held with academic support (Zheng, 1986). According to fossils of yak found in North China, Mongolia, Siberia, Alaska and the northern part of central Asia, it can be proved that yak lived in the northeast of Eurasia in the new tertiary, about 2 500 thousand years ago. Thus, the original yak in the tertiary may be considered the close ancestor of *Poephagus grunniens* and *Poephagus mutus*. This area became warmer due to quaternary glaciations, forcing the original yak to migrate southward and live in the Qinghai-Tibet Plateau, which became the habitat of today's wild yak, while yak in the northeast of Eurasia died out (Hou, 1991; Cai, 1992; Wang, 2004). Today's yak was domesticated from wild yak (Zheng, 1986; Olsen, 1990). In the Paleolithic Age, about 10 thousand years ago, the ancestors of Tibetans living in the Qiangtang of northern Tibet began to domesticate wild yak chronically, and domestic yak ultimately came into being. The breeding industry emerged during the Longshan cultural period in the terminal Neolithic Age (about 2 800 BC–2 300 BC) (Qian, 1979; Cai, 1992).

In this study, the transition/transversion (*Ts*/*Tv*) of cytochrome *b* gene among different species of *Bovinae* was 4.9, higher than the critical value of 2.0 (Knight and Mindell, 1993), which showed higher transition bias. According to the results of mitochondrial gene sequences of *Bovidae* (Bettine and Terence, 2001), *Bovinae* (Laura et al., 1996) and *Poephagus* (Lai et al, 2005), the mutation of cytochrome *b* gene sequences among different species was not saturated. According to the molecular evolutionary clock 2% proofread per million years from the analysis of mitochondrial cytochrome *b* gene sequences in *Bovidae* (Birungi and Arctander, 2001), the approximate divergence time between *Poephagus grunniens* and *Poephagus mutus* was 0.55 million years, earlier than the time of *Poephagus mutus*'s initial domestication estimated by Cai (1992). This showed that *Poephagus mutus* is not the ancestor of *Poephagus grunniens*, which confirms that *Poephagus grunniens* and *Poephagus mutus* came from original yak about 0.55 million years ago. A conclusion can be drawn that *Poephaus grunniens* originated from the northeast of Eurasia, with *Poephaus grunniens* and *Poephagus mutus* from the same ancestor-original yak. The approximate divergence time is about 0.55 million years, and *Poephagus mutus* is not the ancestor of *Poephaus grunniens*.

4.2 Taxonomic status of yak

The phylogenetic relationship of each species, especially the taxonomic status of yak in *Bovinae,* has become a major topic of studies in molecular evolution and phylogenetics. There exists an argument on whether yak is an independent genus or a subgenus in *Bovinae*, and no confirmed conclusion has been reached.

By appearance, the yak is much like individuals in the genus *Bovinae* with a strong and small body, making the exact taxonomic status of yak in *Bovinae* a puzzle to zoologists for a long time. Linnaeus (1766) put yak into the *Bovinae* genus based on its morphological features, which was consistent with the result clustered by Bohlken (1958, 1961). However, there was a remarkable difference between *Bos* and *Bison bison* in morphological characteristics (Li et al., 2006). Based on that difference, Gray classified yak as *Poephagus* of *Bovinae* (Gray, 1843), which was in accordance with the results concluded by Groves (1981), Olsen (1990, 1991) and Geraads (1992) from the characteristics of skull bones and the study of yak fossil. Ritz et al. (2000) analyzed the phylogeny of the representative species in *Bovinae* using 20 microsatellite markers. The results showed that *Bos taurus*, yak and *Bibos* belong to a subgenus of *Bos* respectively, while *Bison bison*, *Bison bonasus* and *Bubalus bubalis* are classified as independent genus respectively. Fan et al. (2000) put yak, *Bos taurus*, *Bison bonasus*, *Bos gaurus* and *Bos indicus* into a subgenus of *Bos* according to the 363 bp sequence of κ-casein fourth exon. Wang (2004) found that the phylogenetic relationship between yak and *Bison* was closer than that between yak and *Bos* using the D-loop sequence, which was consistent with the results of Miyamoto et al. (1989), Hassanin and Douzery (1999), Kraus et al. (1992) and Ward et al. (1999) using a mitochondrial sequence. Based on the phylogenetic tree constructed by polymorphism of exon2 of *MHC DRB3*, Li et al. (2005) found that *Bovinae* were clustered in five genera: *Bubalus*, *Syncerus*, *bos*, *Bison* and *Poephagus*, which supported the idea that *Poephagus* is a separate genus.

Our study showed that in *Bovinae,* the average percentage of nucleotide sequence divergence between yak (including *Poephagus grunniens* and *Poephagus mutus*) and *Bos* (including *Bos taurus and Bos indicus)* was 8.3%, which was higher than that of yak and *Bison bison* (3.8%). Phylogenetic analysis revealed that *Poephagus grunniens* and *Poephagus mutus* clustered first, indicating a higher genetic comparability between the two species and a closer phylogenetic relationship than those between *Poephagus grunniens* and *Bos*. According to the molecular evolutionary clock 2% each million years inferred from the analysis of mitochondrial cytochrome *b* gene sequences in *Bovidae* (Birungi and Arctander, 2001), the approximate divergence time between the buffalo and the other species of *Bovinae* was 7.0 million years, 4.0–4.2 million years between yak and the two species *Bos taurus* and *Bos indicus* in *Bovinae*, and 3.6–3.9 million years between *Bison bison* and *Bovinae*—conforming with the time period speculated by Xie (1985). The approximate divergence time between *Bos taurus and Bos indicus* was 0.8 million years, which was consistent with work by Ritz et al. (2000) using microsatellite markers, and 117–211 million years between yak and *Poephagus mutus*. On the basis of the estimated divergence time, the evidence of paleontology (Osborn, 1990) and the morphological characteristics (Xie, 1985), there is an argument about the evolution in *Bovinae* that the species began to diverge at the end of middle Miocene. The buffalo first began to differentiate during the late Miocene-early Pliocene into *Bubalus bubalis* and *Sycenrus caffer.* The *Bovinae* then differentiated in late Pliocene into *Bos taurus and Bos indicus*, and the yak and urus finally differentiated during the middle Pleistocene. Therefore, in the discussion on whether the yak belongs to a genus or a subgenus, it is more reasonable that the yak be classified as an independent genus in *Bovinae—Poephagus* (Corbet, 1978; Feng et al., 1986; Wiener et al., 2003; Li et al., 2005; 2006). In conclusion, the yak should be classified as *Poephagus* of *Bovinae*, and *Poephagus* includes two species of *Poephagus grunniens* and *Poephagus mutus*.

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