Mitochondrion 12 (2012) 500–505

Contents lists available at SciVerse ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

# Mitogenomic analysis of the genus *Pseudois*: Evidence of adaptive evolution of morphological variation in the ATP synthase genes

Quekun Peng [a], Lei Tang [a], Shuai Tan [a], Zhigang Li [b], Jifei Wang [b], Fangdong Zou [a,*]

[a] Sichuan Key Laboratory of Conservation Biology on Endangered Wildlife, Key Laboratory of Bio-resources and Eco-environment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu, Sichuan 610064, PR China
[b] Ningxia Helan Mountain National Nature Reserve Administration, Ningxia 750021, PR China

ARTICLE INFO

*Article history:*
Received 6 December 2011
received in revised form 21 June 2012
accepted 10 July 2012
Available online 20 July 2012

*Keywords:*
Adaptive evolution
Mitochondrial genome
*Pseudois*
Morphological variation
ATP synthase

ABSTRACT

The genus *Pseudois* includes two variable taxa, blue sheep (*Pseudois nayaur*) and dwarf blue sheep (*Pseudois schaeferi*), that exhibit notable geographic variation in morphology and ecological niche, suggesting the potential for significant adaptive differentiation between these two goats. Blue sheep are broadly distributed in the Tibetan Plateau and peripheral mountains through Central Asia, while dwarf blue sheep are only found in the gorges of the upper Yangtze River (Jinsha River) near Batang county, Sichuan province and adjacent mountains. Although they are all adapted to high altitude environments, endangered dwarf blue sheep show unique morphological variation and niche shifts compared to blue sheep. Mitochondria play important roles in oxygen usage and energy metabolism. The energetically demanding lifestyles of these high altitude species may have altered the selective regimes on mitochondrial genes encoding proteins related to cellular respiration. Here, we compared the sequences of 13 protein-coding genes in the mitochondrial genome of dwarf blue sheep with those of blue sheep to understand the genetic basis of morphological variation. Using neighbor-joining, maximum-likelihood and Bayesian approaches, we estimated rates of synonymous ($d_S$) and nonsynonymous ($d_N$) substitutions. Independent analyses showed that no $\omega$ ratio was larger than 1, suggesting that all mitochondrial 13 genes were under the purifying selection. Surprisingly, we found that the $\omega$ ratio ($d_N/d_S$) of the ATP synthase complex (ATP6 and ATP8) in blue sheep is sixteen times that of dwarf blue sheep (0.340 compared to 0.021). This result was confirmed by a separate analysis of ATP synthase genes from two additional *P. schaeferi* individuals and two *P. nayaur* individuals. We hypothesize that the large body size and diverse feeding styles are factors influencing the nonsynonymous substitutions in the ATP synthase complex of blue sheep.



## 1. Introduction

The species of genus *Pseudois* include two morphologically variable taxa, blue sheep (*P. nayaur*) and dwarf blue sheep (*P. schaeferi*), whose habitats in the Tibetan Plateau and peripheral mountains can induce severe environmental stress associated with decreased oxygen pressure, cold temperatures, increased levels of UV radiation, steep slopes, and scarce food supplies. Since dwarf blue sheep were discovered in the gorges of the upper Yangtze River (Jinsha River) near Batang county, Sichuan province (Schafer, 1937), their morphological identification and taxon status in the genus *Pseudois* has generated many controversies. Based on the comparison of morphological data and early molecular data, dwarf blue sheep were designated as either a new subspecies *P. nayaur schaeferi* (Cao et al., 2003; Feng et al., 2001; Haltenorth, 1963) or a new species *P. schaeferi* (Groves, 1978). However, based on additional molecular analyses, Zhou et al. (2003) and Zeng et al. (2008) found them to be a paraphyletic group and morphologically distinct population of *P. nayaur szechuanensis*, and suggested that the rank of a full species or subspecies was not appropriate.

In recent years, an increasing number of investigations into the habitats of these two blue sheep populations in China (including Yunnan, Sichuan, and Gansu provinces) provides more detail regarding their behavior (Liu et al., 2010; Long et al., 2008; Shen et al., 2007, 2009; Wang and Wang, 2003; Wang et al., 2006), such as activities, habitat choice, feeding styles, etc. Here, we compared the two groups of blue sheep and found significant differences in their appearance and habitat selection. Firstly, in appearance, the body size of adult dwarf blue sheep is just 50% of that of adult blue sheep, which is especially apparent from their horns. Moreover, as to feeding style, dwarf blue sheep rely on the grass as their main food resource, while blue sheep also browse for part of their food besides grass. In addition, the preferred habitat of these two blue sheep populations is distinct: blue sheep usually inhabit the shady, upper alpine elevations above 4000 m, choosing open rocky slopes as their resting places, whereas dwarf blue sheep prefer to inhabit the lower alpine altitudes ranging from 2400 m to 4200 m and often

* Corresponding author. Tel.: +86 28 8541 2805; fax: +86 28 85414886.
*E-mail address:* fundzou@yahoo.com.cn (F. Zou).

1567-7249/$ – see front matter © 2012 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2012.07.107

dwell in the dense shrub which nearly covers their bodies. However, the molecular basis of body size variations and selection of different ecological niches and between these two groups of blue sheep is still unclear.

Despite substantial work on the physiological, morphological and behavioral characteristics of blue sheep, little research has been done to examine potential adaptations to morphological variation in these two species at the molecular level. It is therefore important to investigate the key genes for aerobic metabolic pathways to understand the molecular mechanisms that underlie morphological variation.

Mitochondria serve as a critical function in the maintenance of cellular energy storage, thermogenesis, and apoptosis (Nisoli et al., 2005). The mitochondrial protein-coding genes, which include 13 essential mitochondrial electron transport system (ETS) proteins (7 subunits of the NADH dehydrogenase complex, the cytochrome b subunit of the cytochrome bc1 complex, 3 subunits of cytochrome c oxidase, and 2 subunits of ATP synthase), are essential for producing the energy used to generate ATP and maintain body temperature (Das, 2006). As the ETS is the primary energy generation system in aerobic metazoans, natural selection would be expected to favor mutations that enhance ETS function, especially, in harsh environments (Blier et al., 2001; Manoli et al., 2007). Hence, the mitochondrial genome represents a particular useful genetic marker for investigating the molecular basis of organismal adaptation to the decreased oxygen pressure and cold temperatures found in high altitude environments. In several mammalian species, that show evidence of morphological and physiological adaptation to life at high altitude, such as alpaca (*Lama pacos*), chiru (*Pantholops hodgsonii*), yak (*Bos grunniens*), and pikas (*Ochotona*), or niche shifts such as subterranean caviomorph rodents (*Caviomorpha*), molecular mechanisms through mtDNA-encoded genes have been analyzed in COX, NADH, CytB and ATP genes (da Fonseca et al., 2008; Da Silva et al., 2009; Di Rocco et al., 2009; Hassanin et al., 2009; Luo et al., 2008; Ning et al., 2010; Tomasco and Lessa, 2011; Xu et al., 2005; Yu et al., 2011). In the present study, we sequenced and compared mitochondrial genome and ATP synthase sequences of blue sheep with dwarf blue sheep to investigate the adaptive evolution of morphological variation between these two groups.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

Three dwarf blue sheep muscle samples were collected from Batang county, Sichuan province, China in 2006 and two blue sheep muscle samples were taken, one each from Ganzi and Kangding counties, Sichuan province. Muscle sample was preserved in 95% ethanol at −80 °C. Mitochondrial DNA was extracted from the muscle according to the protocol detailed in *Genomic DNA Extraction Kit* (Tiangen, China). Total DNA was diluted to approximately 20 ng/μl for polymerase chain reaction (PCR).

### 2.2. Amplification and sequencing

The complete sequence of mtDNA for one dwarf blue sheep was produced from 23 overlapping regions, which were generated by PCR using a set of degenerate primers described by Hassanin et al. (2009) with minor modifications. The substitutions of degenerate sites were as follows: (1)12SL41:Y-C,Y-T,R-G;(3)16SL518:R-A;(5)N1L64:N-G,N-G;(7)N1U840:Y-C,H-A,Y-C,H-C/N2L492:B-T,B-T,K-G,Y-T;(8)N2U354:Y-C,N-T/AsnL:R-A;(9)C1L339:W-A,D-A,D-G;(10)C1U426:N-A,N-A,Y-C,H-C,Y-C/C1L1017:R-G,R-G,R-G;(11)C1U897:Y-T,H-A,H-C,Y-C/C2L15:R-G,R-A,N-G,R-G;(12)SerU:Y-A,Y-T,W-T,R-A,Y-T/A8L1:K-T,Y-T,R-A,Y-T;(13)C2U63:H-A,R-G,Y-C,Y-C/C3L45:N-T,R-G,D-G,N-G;(14)A6U654:Y-C,N-A,Y-C,Y-C,N-C/GlyL:R-G.Y-C;(15)C3U780:H-A,Y-C/N4L27:Y-G,R-G,D-T,D-G;(16)U213M1:Y-C,Y-C/L918M1:K-G,R-G,R-G,R-G;(17)N4U840:H-A,Y-C,Y-C,H-C,Y-C/Leu2L:Y-T,R-G/;(18)Ser2U:Y-T.Y-T/N5L652:D-G,D-G,K-T,D-G;(19)N5U501:R-A,Y-C,H-A,Y-C/N5L1214:D-T,K-T,D-A,D-G;(20)N5U1146:M-C,N-C,N-A,Y-C/N6RL154:D-T,H-A,D-G,D-G;(21)N6RU102:R-G,Y-C,H-A/CBL402:R-A,K-T;(22)CBU162:M-C,R-A,H-A/LTHR:Y-C;(23)L482:W-A.

Amplification was performed in a total volume of 50 μl containing 50 mM KCl, 10 mM Tris–HCl, 1.5 mM $Mg^{2+}$, 200 μmol of each dNTP, 0.2 μmol of each primer, 1 U Taq DNA polymerase (TaKaRa, China) and approximately 10 ng of genomic DNA. Thirty five amplification cycles were carried out on a S1000™ thermal cycler with denaturation at 94 °C for 30 s, annealing at optimal temperature (Hassanin et al., 2009) of each pair of primers for 45 s, and extension at 72 °C for 45 s and 7 min at 72 °C. Both strands of all PCR products were sequenced on the ABI 373/377 following manufacturer's (PE Biosystems) specifications for cycle sequencing reactions.

### 2.3. Sequence analyses

The 23 mtDNA fragments were aligned by DNA BASER Sequence Assembler 3.2.4 (software available at http://www.DNABaser.com). The locations of protein-coding and ribosomal RNA (rRNA) genes were determined by comparison with known sequences from blue sheep. The tRNA genes were identified by the tRNA-SE v.1.21 (Lowe and Eddy, 1997). Some tRNA genes, which were not found by the tRNA-SE, were identified by proposed secondary structures (Kumazawa and Nishida, 1993) and anti-codons.

Thirteen protein-coding genes were aligned by using MEGA 4.0 (Tamura et al., 2007). All ambiguous regions, i.e., involving ambiguity for the position of gaps, were excluded from the analyses to avoid erroneous hypotheses of primary homology. To further confirm the phylogenetic relationships of *Pseudois* within *Caprina*, multiple alignments of the 13 concatenated protein coding genes from dwarf blue sheep and nineteen other species (Table 1) were performed using ClustalX (Thompson et al., 1997).

Three methods were used for tree reconstruction: Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inferences (BI). The neighbor-joining analyses were conducted with 1000 bootstrap replicates implemented by the MEGA3.1 program (Kumar et al., 2004). ML analyses were conducted on PAUP 3.1.1 (Swofford, 2002). The substitution model for ML analyses was selected using the Akaike Information Criterion (AIC) as implemented in ModelTest (Posada and Crandall, 1998). Bootstrap values were determined using 1000 (ML) full heuristic searches. Bayesian inferences were carried out under MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Posterior probabilities (PPs) were

**Table 1**
The taxa and Genbank sequence accession numbers of twenty species of *Caprini* used for phylogenetic analysis.

| Taxon | Common name | Length (nt) | Accession no. |
|---|---|---|---|
| *Ammotragus lervia* | Aoudad | 16,540 | FJ207522 |
| *Budorcas taxicolor* | Takin | 16,588 | FJ207524 |
| *Capra falconeri* | Markhor | 16,640 | FJ207525 |
| *Capra hircus* | Domestic goat | 16,643 | NC_005044 |
| *Capra ibex* | Alpine ibex | 16,716 | FJ207526 |
| *Capra nubiana* | Nubian ibex | 16,705 | FJ207527 |
| *Capra pyrenaica* | Spanish ibex | 16,561 | FJ207528 |
| *Capra sibirica* | Siberian ibex | 16,583 | FJ207529 |
| *Hemitragus jayakari* | Arabian tahr | 16,457 | FJ207523 |
| *Hemitragus jemlahicus* | Himalayan tahr | 16,712 | FJ207531 |
| *Oreamnos americanus* | Rocky mountain goat | 16,604 | FJ207535 |
| *Ovis ammon* | Argali | 16,613 | HM236188 |
| *Ovis aries* | Domestic sheep | 16,616 | NC_001941 |
| *Ovis canadensis* | Bighorn sheep | 16,463 | NC_015889 |
| *Pseudois nayaur* | Blue sheep | 16,737 | FJ207537 |
| *Pseudois schaeferi* | Dwarf blue sheep | 16,663 | JQ040802 |
| *Rupicapra pyrenaica* | Isard | 16,438 | FJ207538 |
| *Rupicapra rupicapra* | Chamois | 16,434 | FJ207539 |
| *Out group* | | | |
| *Ovibos moschatus* | Muskox | 16,431 | FJ207536 |
| *Pantholops hodgsonii* | Chiru | 16,498 | NC_007441 |

calculated using four independent Markov chains run for 2,000,000 Metropolis-coupled MCMC generations, with tree sampling every 1000 generations and a burn-in of 2000 trees.

### 2.4. Selection constraint analyses

The nonsynonymous–synonymous substitution rate ratio ($d_N/d_S$, denoted $\omega$) is used as a measure of selective pressure at the protein level (Li et al., 1985; Miyata et al., 1979), with $\omega>1$ indicating positive selection, $\omega=1$ indicating neutral evolution, and $\omega<1$ indicating purifying selection. Here, we used the codon-based likelihood approach implemented in the CODEML program of the PAML4 package (Yang, 2000) to assess potential adaptive evolution in mitochondrial protein-coding genes. The combined dataset of 13 protein-coding genes from blue sheep and dwarf blue sheep was used. To allow distinct estimates of $\omega$ for different lineages (branch models), we performed (1) a full model in which all branches in the phylogeny have different $\omega$ values; and (2) different intermediate models that permit different $\omega$ values for each clade or branch of interest (either phylogenetic and/or ecological, i.e., blue sheep or dwarf blue sheep taxa) using three approaches of neighbor-joining, maximum likelihood, and Bayesian inferences, respectively.

To detect any significant changes in selective pressure between the blue sheep and the dwarf blue sheep, we applied branch-specific methods to five datasets: (1) all 13 protein-coding genes of the mtDNA; (2) the three subunits of the cytochrome coxidase complex (COX); (3) the seven subunits of the NADH dehydrogenase complex (ND: ND1, -2, -3, -4, -4L, -5, and -6); (4) the cytochrome b subunit of ubiquinol cytochrome c oxidoreductase (CYT B); and (5) the two subunits of the ATP synthase complex (ATP: ATP6 and ATP8). We also analyzed each of the 13 protein-coding genes separately.

## 3. Results

### 3.1. Characteristics of the dwarf blue sheep mitochondrial genome

The general characteristics of the mitochondrial genome of dwarf blue sheep are summarized in Table 2. The complete mitochondrial genomes of 20 species of *Caprini* range from 16,431 bp to 16,737 bp in size, with the newly determined *P. schaefer* mitogenome being 16,663 bp long. Genome length differences are largely due to the variation in tandem repeats of the control region. The control region of dwarf blue sheep has three tandem repeats of 75 bp, which is one fewer than that of the blue sheep referred to in this study. All genomes share not only 13 protein-coding genes, 22 tRNA genes, 2 rRNAs, and a control region, but also the same gene order. The mitochondrial genome of the dwarf blue sheep is apparently AT-biased (32.5% A, 27.6% T, 26% C, and 13.2% G), which is consistent with the base composition of the third-codon position (33.3% A, 26.8%T, 26.8% C, 13.8% G). The sequence divergence between blue sheep and dwarf blue sheep ranges from 0.3% (ND3) to 3.1% (ND6) for the protein-coding dataset (average 1.63%), 0.08% for the rRNA dataset, 1.7% for the tRNA dataset, 7% for the control region, and 1.9% for the complete dataset.

### 3.2. Mitogenomic phylogeny of the subtribe Caprina

The various mtDNA and nuclear genes, such as cytochrome *b*, 12 S rRNA, K-casanin, were used for the analysis of the phylogeny of blue sheep (Hassanin and Douzery, 1999; Hassanin et al., 1998; Ropiquet and Hassanin, 2005a, 2006). The phylogenetic relationships among the 11 genera of *Caprina* were elaborately described by Hassanin et al. (2009). The tribe *Caprini* belongs to the subfamily *Antilopinae*, and it is composed of 11 genera: *Ammotragus*, *Budorcas*, *Capra*, *Hemitragus*, *Naemorhedus*, *Oreamnos*, *Ovibos*, *Ovis*, *Pantholops*, *Pseudois* and *Rupicapra*. Hassanin et al. (2009) analyzed the complete mitochondrial genome of 24 ruminants, including 20 species of *Caprini* and uncovered the existence of a new large clade, named subtribe *Caprina*, containing all genera of *Caprini*, except Pantholopina and Ovibovina. In this study, we explored the phylogenetic relationships of all genera in *Caprina*. The sequences of complete mitochondrial genomes were obtained for 20 species from the GenBank (Table 1), including 18 members of the subtribe *Caprina* and 2 outgroups, *P. hodgsonii* and *Ovibos moschatus*.

**Table 2**
Characterization of mitochondrial genes of *Pseudois schaeferi*. Note: V.A, amino acid variable sites compared to mitochondrial genes of *Pseudois nayaur*; $\omega$ ($d_N/d_S$), nonsynonymous–synonymous substitution rate ratio.

| Genes | Alignment length | Nucleotide composition | | | | Pairwise distance (%) | V.A. | $\omega$ ($d_N/d_S$) |
|---|---|---|---|---|---|---|---|---|
| | | A | T | G | C | | | |
| ND1 | 956 | 31.4 | 26.6 | 12.1 | 29.9 | 1.8 | 0 | 0.061 |
| ND2 | 1042 | 36.8 | 26.6 | 8.4 | 28.2 | 1.8 | 5 | 0.156 |
| COX I | 1545 | 28.7 | 29.4 | 16.4 | 25.4 | 1.6 | 0 | 0.041 |
| COX II | 684 | 34.9 | 26.6 | 13.7 | 24.7 | 1.9 | 2 | 0.236 |
| ATP8 | 201 | 40.8 | 27.4 | 7.0 | 24.9 | 0.5 | 0 | 0.021 |
| ATP6 | 681 | 32.9 | 28.3 | 10.6 | 28.2 | 2.9 | 6 | 0.339 |
| COX III | 784 | 26.5 | 27.7 | 15.2 | 30.6 | 2.1 | 1 | 0.134 |
| ND3 | 346 | 30.4 | 27.8 | 11.3 | 30.4 | 0.3 | 0 | 0.012 |
| ND4L | 297 | 31.0 | 29.0 | 12.5 | 27.6 | 0.7 | 0 | 0.065 |
| ND4 | 1378 | 31.9 | 27.4 | 10.6 | 30.0 | 1.4 | 2 | 0.073 |
| ND5 | 1821 | 33.9 | 25.7 | 10.2 | 30.1 | 1.7 | 9 | 0.166 |
| ND6 | 528 | 21.0 | 41.7 | 29.4 | 8.0 | 3.1 | 2 | 0.069 |
| Cyt b | 1140 | 31.3 | 24.7 | 13.5 | 30.4 | 1.5 | 5 | 0.062 |
| 12 sRNA | 958 | 35.9 | 21.3 | 18.4 | 24.4 | 0.6 | | |
| 16 sRNA | 1575 | 37.5 | 24.3 | 17.5 | 20.9 | 1.0 | | |
| tRNA | 1516 | 36.1 | 27.9 | 15.0 | 21.0 | 1.7 | | |
| D-loop | 1013 | 32.0 | 27.0 | 13.4 | 27.6 | 7.0 | | |
| Combined data | 16,465 | 32.5 | 27.6 | 13.8 | 26.0 | 1.9 | | |

Phylogenetic analysis of the alignment of 13 protein-coding sequences using ML and BI methods produced the trees presented in Fig.1A and B, respectively. Results for ML and BI analyses of 13 protein coding sequence were broadly consistent except for a few topological differences, which were not strongly supported. Three main clades, Clade 1, Clade 2, and Clade 3, were identified in the phylogenetic trees. In the Bayesian tree, three genera—*Capra*, *Pseudois*, and *Hemitragus*, comprised the Clade 1, a "goat-like clade", of which *Capra sibirica* has sister-group relationship with *Hemitragus jemlahicanus* and the relationship between *C. sibirica* and *P. nayaur* is more distant than that between *C. sibirica* and *H. jemlahicanus*. Clade 2 consists of three genera (five species) *Arabitragus*, *Ammotragus* and *Rupicapra*, of which *Ammotragus lervia* has sister-group relationship with *Arabitragus jayakari*. The Clade 3 is composed of three genera *Budorcas*, *Oreamnos* and *Ovis*. This result suggests that the genus *Hemitragus* is polyphyletic, which is in agreement with the data published by Ropiquet and Hassanin (2005b).

### 3.3. Positive selection analyses

Because the CODEML likelihood analysis may be sensitive to the tree topology used, three tree topologies inferred from different tree-building methods were all used in the positive selection analysis. The $\omega$ ($d_N/d_S$) values based on the 13 protein-coding sequences for the dwarf blue sheep and blue sheep were 0.104 and 0.048 (NJ tree), 0.1050 and 0.047(Bayesian tree), 0.329 and 0.296 (ML tree), respectively. The results of the independent analyses of four protein complexes (ND, COX, ATP synthase, CytB) using three different tree-building methods were broadly consistent, and are presented in Table 3. The highest $\omega$ values were found in the ATP complex (0.343, NJ tree) in blue sheep, while the lowest $\omega$ ratio was found in dwarf blue sheep for the COX complex (0.015, Bayesian tree). The $\omega$ ratios of ND, COX, CYTB in the dwarf blue sheep were comparable to those in the blue sheep, however, the $\omega$ ratio of the ATP synthase complex of blue sheep (0.340, ML tree) was almost 20 times that of

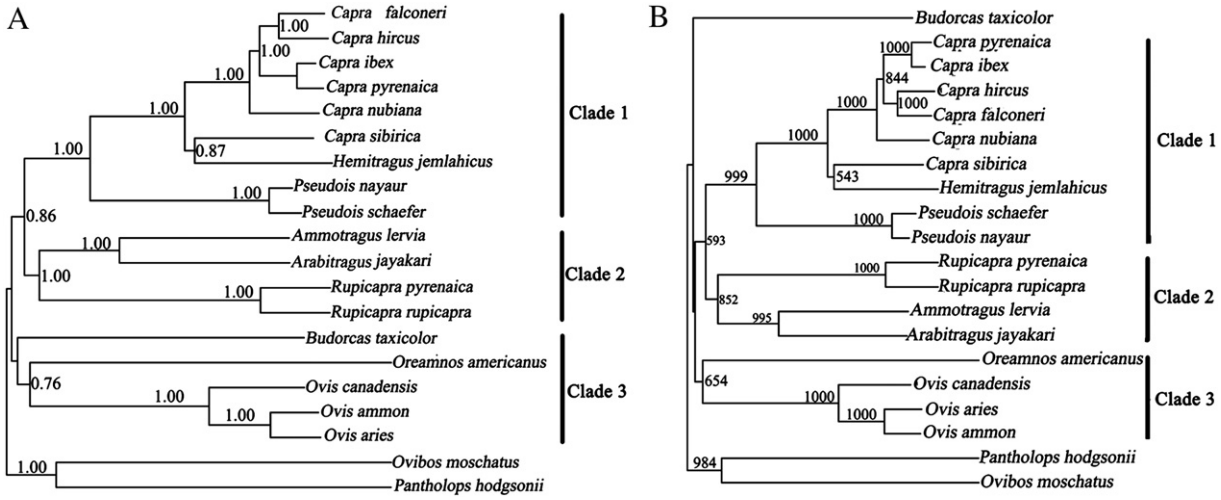


**Fig. 1.** Phylogenetic trees based on the 13 mitochondrial protein-coding genes for the subtribe *Caprina*. A) Bayesian inference, PP = 1; B) Maximum likelihood BP = 1000.

dwarf blue sheep (0.016, ML tree). This result was broadly consistent with the results from the Bayesian and NJ trees.

## 4. Discussion

In mammals, mitochondria serve as the power sources producing ATP through oxidative phosphorylation (OXPHOS). The ability to adapt to energy-intensive conditions, such as high altitude, low temperatures, scarce food supplies, and steep slopes, often depends on the amount of available energy (Guderley, 2004). All 13 protein-coding genes of the mtDNA genome are key subunits of four of the five complexes involved in the OXPHOS machinery, i.e., ND, CytB, COX, and ATP synthase (Wallace, 2007). Therefore, amino acid variations occurring in protein-coding genes of the mtDNA genome may influence the ability to adapt to harsh environments (Grossman et al., 2004). An increasing number of cases of adaptive evolution in mitochondrial genes have been reported in artiodactyls, primates, and humans living in high altitude environments (da Fonseca et al., 2008; Hassanin et al., 2009; Mishmar et al., 2003; Xu et al., 2005; Yu et al., 2011). In the present study, the complete protein-coding genes of the mtDNA of the genus *Pseudois* were analyzed in the first attempt to understand the adaptive evolution of morphological variation between the blue sheep and the dwarf blue sheep. In the mitochondrial genome of the dwarf blue sheep, 32 amino acids have changed when compared with the mitochondrial genome of blue sheep (Table 2), and the ATP synthase complex has the highest amino acid substitute rate (2.04%, 6/293) of four complexes. Furthermore, we found that the amino acid Ala at the site 277 was highly conserved in the mitochondrion of *Caprini* species. It was reported that this site was variable in the alpaca, however, it may result from sequencing error (da Fonseca et al., 2008; Hassanin et al., 2009). To test if the high $\omega$ value identified in the blue sheep sequenced for this study is present in other individuals, we sequenced the ATP6 and ATP8 genes in two additional *P. Schaeferi* individuals (both collected from Batang county, GenBank accession numbers JQ040798 and JQ040800) and two *P. nayaur* individuals (collected from Ganzi and Kangding counties Sichuan province, GenBank accession number JX073032 and JX073033, respectively) and found similar results (Table 4). The $\omega$ value (0.285) of blue sheep was four times of that of dwarf blue sheep (0.076, $P<0.05$).

Mitochondrial oxidative phosphorylation transforms dietary calories to generate ATP to provide energy for normal body function and maintaining homeostasis, like body temperature. The balance between these two functions is determined by the efficiency of coupling the mitochondrial inner membrane electrochemical gradient to synthesize ATP through ATP synthase. Variants that reduce coupling efficiency will reduce ATP production, but increase heat production (Mishmar et al., 2003; Wallace, 2007). Partial uncoupling of the mitochondria increases the basal metabolic rate of the individual and hence requires a higher caloric intake. Thus, mtDNA ATP6 variants that reduce coupling might partially account for the increased basal metabolic rate that has been observed in indigenous, circumpolar, human populations (Leonard et al., 2002). By contrast, our results show that a significant acceleration in amino acid changes occurred in the ATP synthase of blue sheep. This is supported by the previous analyses in *Caprini* (Hassanin et al., 2009), suggesting that the ATP synthase complex is a signature of adaptive variation in the mitochondrial protein-coding genes of blue sheep, even though no $\omega$ ratio was larger than 1. We speculate that the higher $\omega$ value of blue sheep in ATP synthase complexes than dwarf blue sheep accounts for the significant morphological variation between these two high altitude goats. We know that there is a close relationship between the body mass and metabolic rate, in that the mammalian basal metabolic rate is proportional to body mass (White and Seymour, 2003). Compared with blue sheep, the most notable morphological variation for dwarf blue sheep is that its body size is only 50% as large as blue sheep. Based on the above theory, we infer that dwarf blue sheep with a small body size may have a relatively low basal metabolic rate. Moreover, we compared the feeding styles between blue sheep and dwarf blue sheep and found that the former include browsing as a part of their food, in addition to grass, while the

**Table 3**
$d_N/d_S$ ($\omega$) for each of the four complexes (ATP synthase, COX, CytB, and ND) and complete protein-coding genes of the mitochondrial DNA. ND, NADH dehydrogenase (ND1, 2, 3, 4 L, 4, 5, 6,); COX, cytochrome oxidase (COX1, COXII, COXIII); ATP, ATP synthetase (ATP6 AND ATP8); CYTB, cytochrome b; NJ, Neighbor-Joining tree; BT, Bayesian tree; ML, Maximum Likelihood tree.

| Taxon | ND | | | COX | | | ATP | | | CYTB | | | 13 protein-coding genes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NJ | BT | ML | NJ | BT | ML | NJ | BT | ML | NJ | BT | ML | NJ | BT | ML |
| Blue sheep | 0.034 | 0.076 | 0.041 | 0.047 | 0.047 | 0.092 | 0.343 | 0.340 | 0.340 | 0.222 | 0.246 | 0.266 | 0.048 | 0.047 | 0.296 |
| Dwarf blue sheep | 0.127 | 0.100 | 0.046 | 0.016 | 0.015 | 0.057 | 0.012 | 0.021 | 0.016 | 0.261 | 0.288 | 0.281 | 0.104 | 0.105 | 0.329 |

**Table 4**
$d_N/d_S$ ($\omega$) of mitochondrial ATP synthase in blue sheep and dwarf blue sheep.

| | $d_N$ | $d_S$ | $\omega(d_N/d_S)$ |
|---|---|---|---|
| Blue sheep | 0.006 | 0.021 | 0.285 |
| Dwarf blue sheep | 0.001 | 0.013 | 0.076 |

latter rely on grass as their main food resource. However, grass is generally more fibrous and therefore requires longer rumination time and low rumen turnover rates (according to Kleiber's law, Kleiber, 1975; Demment and van Soest, 1985). Therefore, we infer that dwarf blue sheep have lower metabolic rates than blue sheep and propose that the diversity of feeding styles and large body size, which increase the basal metabolic rate of blue sheep, may be factors resulting in a significant acceleration of nonsynonymous mutations of ATP synthase. In addition, blue sheep and dwarf blue sheep are both high altitude goats, but the difference of altitude is insignificant. Therefore, we infer that the high altitude is not a major factor influencing the nonsynonymous mutations of ATP synthase in the blue sheep. Because our data are limited, more information on the structure, function, and evolution of mitochondrial and nuclear genomes is required to reveal the exact molecular mechanisms underlying the morphological variation between these two types of blue sheep.

## 5. Conclusion

Taken together, our analyses point toward the adaptive evolution of morphological variation in two blue sheep using 13 complete protein-coding genes from the mitochondrial genome. We speculate that the high altitude is not a major contributor to the high $\omega$ ratio of the ATP synthase complex in blue sheep, but rather it is the body size and/or feeding style which influences its basal metabolic rates.

## Acknowledgments

We are grateful to Assistant Professor Katherine Neiswanger from University of Pittsburgh for their assistance in this work. This research was funded by the Program for New Century Excellent Talents in University (NCET-10-06-07), National Natural Science Foundation of China (30970335), Scientific Research Foundation for Returned Scholars, Ministry of Education of China (20091001-9-9) and Supporting Program for the Distinguished Young Scholars Subject of Sichuan Province (09ZQ026-044).

## Reference

Blier, P.U., Dufresne, F., Burton, R.S., 2001. Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. Trends Genet. 17, 400–406.
Cao, L.R., Wang, X.M., Fang, S.G., 2003. A molecular phylogeny of bharal and dwarf blue sheep based on mitochondrial cytochrome b gene sequences. Acta Zool. Sin. 49, 198–204.
da Fonseca, R.R., Johnson, W.E., O'Brien, S.J., Ramos, M.J., Antunes, A., 2008. The adaptive evolution of the mammalian mitochondrial genome. BMC Genomics 9, 119.
Da Silva, C.C., Tomasco, I.H., Hoffmann, F.G., Lessa, E.P., 2009. Genes and ecology: accelerated rates of replacement substitutions in the cytochrome b gene of subterranean rodents. Open Evol. J. 3, 17–30.
Das, J., 2006. The role of mitochondrial respiration in physiological and evolutionary adaptation. BioEssays 28, 890–901.
Demment, M.W., van Soest, P.J., 1985. A nutritional explanation for body-size patterns of ruminant and nonruminant herbivores. Am. Nat. 125, 641–672.
Di Rocco, F., Zambelli, A.D., Vidal Rioja, L.B., 2009. Identification of camelid specific residues in mitochondrial ATP synthase subunits. J. Bioenerg. Biomembr. 41, 223–228.
Feng, J., Lajia, C., Taylor, D.J., Webster, M.S., 2001. Genetic distinctiveness of endangered dwarf blue sheep (Pseudois nayaur schaeferi): evidence from mitochondrial control region and Y-linked ZFY intron sequences. J. Hered. 92, 9–15.
Grossman, L.I., Wildman, D.E., Schmidt, T.R., Goodman, M., 2004. Accelerated evolution of the electron transport chain in anthropoid primates. Trends Genet. 20, 578–585.
Groves, C.P., 1978. The taxonomic status of the dwarf blue sheep (*Artiodactyla: Bovidae*). Saugetierk. Mitt. 26, 177–183.
Guderley, H., 2004. Metabolic responses to low temperature in fish muscle. Biol. Rev. Camb. Philos. Soc. 79, 409–427.
Haltenorth, T., 1963. Klassifikation des Sagetiere: Artiodactyla. Handb. d. Zool. Berl. 8, 1–167.
Hassanin, A., Douzery, E., 1999. The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome b gene. Mol. Phylogenet. Evol. 13, 227–243.
Hassanin, A., Pasquet, E., Vigne, J.D., 1998. Systematic relationships within the subfamily Caprinae (Artiodactyla, Bovidae) as determined from cytochrome b sequences. J. Mammal. Evol. 5, 217–236.
Hassanin, A., Ropiquet, A., Couloux, A., Cruaud, C., 2009. Evolution of the mitochondrial genome in mammals living at high altitude: new insights from a study of the tribe Caprini (Bovidae, Antilopinae). J. Mol. Evol. 68, 293–310.
Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Kleiber, M., 1975. The Fire of Life: an Introduction to Animal Energetics. Krieger.
Kumar, S., Tamura, K., Nei, M., 2004. MEGA: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.
Kumazawa, Y., Nishida, M., 1993. Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. J. Mol. Evol. 37, 380–398.
Leonard, W.R., Sorensen, M.V., Galloway, V.A., Spencer, G.J., Mosher, M.J., Osipova, L., Spitsyn, V.A., 2002. Climatic influences on basal metabolic rates among circumpolar populations. Am. J. Hum. Biol. 14, 609–620.
Li, W.H., Wu, C.I., Luo, C.C., 1985. A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes. Mol. Biol. Evol. 2, 150–174.
Liu, G.K., Zhou, C.Q., Yang, Z.S., Liao, W.B., Huang, Y., Li, Q.T., 2010. A primary study on group type and habitat selection of dwarf blue sheep (*Pseudois schaeferi*) in Luoni Mountain. J. China West Nor. Uni. (Nat. Sci.) 31, 114–121.
Long, S., Zhou, C.Q., Wang, W.K., Hu, J.C., Huang, Y.C., Tang, L., 2008. Behavior coding and ethogram of the dwarf blue sheep (*Pseudois schaeferi*). Acta Ecol. Sin. 28, 5632–5640.
Lowe, T.M., Eddy, S.R., 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25, 955–964.
Luo, Y., Gao, W., Gao, Y., Tang, S., Huang, Q., Tan, X., Chen, J., Huang, T., 2008. Mitochondrial genome analysis of *Ochotona curzoniae* and implication of cytochrome c oxidase in hypoxic adaptation. Mitochondrion 8, 352–357.
Manoli, I., Alesc, S., Blackman, M.R., Su, Y.A., Rennert, O.M., Chrousos, G.P., 2007. Mitochondria as key components of the stress response. Trends Endocrinol. Metab. 18, 190–198.
Mishmar, D., Ruiz-Pesini, E., Golik, P., Macaulay, V., Clark, A.G., Hosseini, S., Brandon, M., Easley, K., Chen, E., Brown, M.D., Sukernik, R.I., Olckers, A., Wallace, D.C., 2003. Natural selection shaped regional mtDNA variation in humans. Proc. Natl. Acad. Sci. 100, 171–176.
Miyata, T., Miyazawa, S., Yasunaga, T., 1979. Two types of amino acid substitutions in protein evolution. J. Mol. Evol. 12, 219–236.
Ning, T., Xiao, H., Li, J., Hua, S., Zhang, Y.P., 2010. Adaptive evolution of the mitochondrial NADH6 gene in the domestic horse. Genet. Mol. Res. 9, 144–150.
Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., et al., 2005. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310, 314–317.
Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.
Ropiquet, A., Hassanin, A., 2005a. Molecular phylogeny of caprines (Bovidae, Antilopinae): the question of their origin and diversiWcation during the Miocene. J. Zool. Syst. Evol. Res. 43, 49–60.
Ropiquet, A., Hassanin, A., 2005b. Molecular evidence for the polyphyly of the genus Hemitragus (Mammalia, Bovidae). Mol. Phylogenet. Evol. 36 (1), 154–168.
Ropiquet, A., Hassanin, A., 2006. Hybrid origin of the Pliocene ancestor of wild goats. Mol. Phylogenet. Evol. 41, 395–404.
Schafer, E., 1937. On the dwarf bharal (*Pseudois spec. nov*) and the large bharal (*P. nahhor Hodgson*) of the Tibet. Zool. Garten Leipzig 9, 263–278.
Shen, D.J., Wang, Y., Zeren, J.M., Zeng, G.W., Huang, Y.C., Li, K.J., Tang, L., 2007. Primary study on behavior of dwarf blue sheep (*Pseudois schaeferi*). Sichuan J. Zool. 26, 774–776.
Shen, D.J., Zheng, H.X., Wang, Y., Ge, L., Zeng, G.W., Huang, Y.C., Li, K.J., Tang, L., 2009. Winter habitats of dwarf blue sheep (Pseudois schaeferi) and goral (Naemorhedus gora1) in Batang County Sichuan China. Acta Ecol. Sin. 29, 2320–2330.
Swofford, D.L., 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, Massachusetts. version 4.0b10.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Tomasco, I.H., Lessa, E.P., 2011. The evolution of mitochondrial genomes in subterranean caviomorph rodents: adaptation against a background of purifying selection. Mol. Phylogenet. Evol. 61, 64–70.
Wallace, D.C., 2007. Why do we still have a maternally inherited mitochondrial DNA? Insights from evolutionary medicine. Annu. Rev. Biochem. 76, 781–821.
Wang, Y., Wang, X.M., 2003. Population ecology of dwarf blue sheep ( *Pseudois schaeferi* ). Biodivers. Sci. 11, 59–62.
Wang, Y., Zheng, H.X., Zhao, J.H., Xu, H.F., Zhang, H.F., Zeng, G.W., Huang, Y.C., 2006. Grouping behavior of dwarf blue sheep in Batang County China. J. Henan Univ. (Nat. Sci.) 36, 73–75.
White, C.R., Seymour, R.S., 2003. Mammalian basal metabolic rate is proportional to body mass$^{2/3}$. Proc. Natl. Acad. Sci. 100, 4046–4049.
Xu, S.Q., Yang, Y.Z., Zhou, J., Jing, G.E., Chen, Y.T., Wang, J., Yang, H.M., Wang, J., Yu, J., Zheng, X.G., Ge, R.L., 2005. A mitochondrial genome sequence of the Tibetan antelope (Pantholops hodgsonii). Genomics Proteomics Bioinformatics 3, 5–17.

Yang, Z., 2000. Maximum likelihood estimation on large phylogenies and analysis of adaptive evolution in human influenza virus A. J. Mol. Evol. 51, 423–432.
Yu, L., Wang, X.P., Ting, N., Zhang, Y.P., 2011. Mitogenomic analysis of Chinese snub-nosed monkeys: evidence of positive selection in NADH dehydrogenase genes in high-altitude adaptation. Mitochondrion 11, 497–503.
Zeng, B., Xu, L., Yue, B.S., Li, Z.J., Zou, F.D., 2008. Molecular phylogeography and genetic differentiation of blue sheep *Pseudois nayaur szechuanensis* and *Pseudois schaeferi* in China. Mol. Phylogenet. Evol. 48, 387–395.
Zhou, C.Q., Zhou, K.Y., Hu, J.C., 2003. The validity of the dwarf bharal (*Pseudois schaeferi*) species status inferred from mitochondrial Cyt b gene. Acta Zool. Sin. 49, 578–584.