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Complete mitochondrial genome of the Liuyang black goat and its phylogenetic relationship with other Caprinae

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ABSTRACT. In this study, the complete mitochondrial genome sequence of the Liuyang black goat was investigated, and phylogenetic relationships between the Liuyang black goat and other species of Caprinae were analyzed. The total length of the mitochondrial genome was 16,715 bp, which consisted of 33.50% A, 27.27% T, 25.98% C, and 13.25% G. The mitochondrial genome contained a major non-coding control region (D-loop region), two ribosomal RNA genes, 13 protein-coding genes, and 22 transfer RNA genes. Neighbor-joining and maximum-parsimony trees of Caprinae constructed using 13 mitochondrial protein-coding genes showed that the Liuyang black goat is phylogenetically closest to *Hemitragus jemlahicus* (the Himalayan tahr) and Blue sheep to form clade A. Tibetan antelopes clustered separately in clade B and so did sheep in clade C.

Key words: Liuyang black goat; Caprinae; Mitochondrial genome; Phylogenetic relationship

INTRODUCTION

The goat was one of the first animals to be domesticated (MacHugh and Bradley, 2001; Cao et al., 2006; Zeder et al., 2006). It is a source of milk, meat, dung for fuel, and materials for clothing and building, such as hair, bone, and skin (Li et al., 2000; Tang et al., 2016). Ever since Nass and Nass (1963) discovered mitochondrial DNA (mtDNA), molecular biologists, geneticists, and breeders have studied it, and the rapid development of polymerase chain reaction (PCR) and sequencing technology has accelerated progress in studying mtDNA. The mitochondrial genome in animals has a 15-23-kb double-stranded circular structure, and plays an important role in metabolism and apoptosis (MacHugh and Bradley, 2001).

Animal mtDNA is a covalent, closed, circular double-stranded DNA molecule. Because of its low molecular weight, simple and stable structure, and maternal inheritance it is ideal for studying animal origins, evolution, and differentiation (Mariotti et al., 2013). Mitochondria are important organelles within eukaryotic cells, because they are the cells' power plants; 95% of the energy present in cells is derived from mitochondrial oxidative phosphorylation. Except for red blood cells, all somatic animal cells contain mitochondria, which are the only organelles outside the nucleus that contain genes.

Structural analyses of the animal mitochondrial genome have shown that the mtDNA of most mammals consists of 37 genes and a non-coding sequence of variable length (the control region or D-loop). The 37 genes include 13 protein-coding genes, two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes. The 13 protein- coding genes are cytochrome c oxidase subunits I, II, and III (*COI*, *COII*, and *COIII*), cytochrome b (*Cytb*), ATP synthase subunits *ATPase6* and *ATPase8*, and seven subunits of NADH dehydrogenase (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*). The two rRNA genes are *12s rRNA* and *16s rRNA*.

The mitochondrial genome is widely used to study species origins, molecular evolution, and for phylogenetic analysis. Luikart et al. (2001) analyzed hypervariable regions of the 481-bp mtDNA D-loop of 406 goats from 88 breeds, and classified them into three highly differentiated haplotype groups. There is only one haplotype in Central and Southeast Asia. Previous studies have found that goats and other domestic animals (cattle, sheep, and pigs) have a number of maternal origins; it is possible that these goats originated in Central Asia. Ye et al. (1998) studied the DNA of Yunnan goats, and found that Yunnan and Korean goats share the same ancestor, confirming to some extent the homology of Asian goats. Li et al. (2001) used 14 restriction endonucleases to digest the mtDNA of several sheep species (Mongolian, Ujumqin, Hu, and small-tail Han), and detected 32 restriction sites and 16 restriction morphs. Among them, *Bgl* was polymorphic and *Xho*I had no cut-off point. The 16 restriction morphs were sorted into two gene haplotypes, haplotype I and haplotype II. Haplotype I belonged to sheep mtDNA, and its amount of polymorphic mtDNA (π value = 0.0129%) indicated that the genetic diversity of goat mtDNA is low.

MATERIAL AND METHODS

DNA, PCR amplification, and sequencing

Ear samples were obtained from healthy adult Liuyang black goats in Hunan Province, China. Genomic DNA was extracted by the standard phenol-chloroform method. The quality

of the DNA was examined by ultraviolet spectrophotometry after electrophoresis on a 0.5% agarose gel. Based on the mtDNA sequence of the Yunnan black goat (GenBank accession No. KF952601), 23 pairs of primers were designed using the Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) (Table 1).

Table 1. Primer sequences used in this study.

Upstream prime (5'-3')	Downstream prime (5'-3')	Annealing temperature (°C)
cccagccttcctgttaactc	gtgcgtgcttcatggcctaa	52.3
cataaagacgttaggtcaaggtgta	aacgctttcttaattgatggctgct	53.5
gcctggtgatagctggttgt	ggattgcgctgttatcccta	56.5
cgagaagaccctatggagcttta	ttgagttggaggctcagcctgat	53.1
ccttacgacctgccacatcc	gggtatgggcccgatagctt	55.5
tcaagcatccccccacaaac	gatagggtggttgtggttga	56.8
ttggaggctgaggaggacta	tcagcggttgatgaacatgg	53.0
tctacttctcccgccgcgaa	tcagggtgtccaaagaatca	54.2
aactcccctgtttgtgtgat	ggcatccgttcagtcactct	55.4
ccacgacgatactctgatta	gacctggaattgcgtctgtt	54.6
tgaagacttaagcttcgattcct	aatgggcgagtgatgctttagtt	52.8
caccaaaggacaaacatgaaca	tgaggctaggaggacggaggta	56.2
aggccttcgctatggaataa	ttcgtaggctaggcttacag	54.6
cataggatcggctcgccttc	attagttctgtagcggtgaatgt	55.1
taaccttcttctccgactcc	tcatcaggcagccattagtg	53.4
cgttatcgtcgccatcctta	ctatcttgaagctgagcgataa	57.2
ccgcacccatcataataacc	ggtgcagatgtggaggaatg	51.9
ccgctttcatccactaacaga	ttatctgggcttgtgagatgg	56.7
ccgctaacataactcaccac	tgggtgggtctttcggatgt	54.8
tcccaccccaccactcacaa	gtagcatggcgcctaagata	55.9
ggcacaaacctagtcgaatg	ggtgctgatagtgaggctatgg	54.8
aggacagccagtcgaacatc	atctagtggacgggatacgc	52.7
tccacatgcatattaagcacgta	cgtggatgcttgcatgtgta	56.3

The complete mtDNA was amplified by PCR and sequenced by Shanghai Biosune Co. Ltd., Shanghai, China. The PCR volume was 20 μL, and the reaction conditions were as follows: amplification by PCR was conducted under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 5 min.

Phylogenetic analysis

The known mitochondrial genomes of 44 representative different species and breeds of Caprinae (20 domestic goats, 16 domestic and foreign sheep, 4 Blue sheep, 2 *Hemitragus jemlahicus*, and 2 Tibetan antelopes (*Pantholops hodgsonii*)) were used. We obtained the mitochondrial genome sequences of these species by searching the GenBank database (http://www.ncbi.nlm.nih.gov/) and referring to the literature (Table 2).

Sequence alignment, arrangement, and divergence analyses were conducted using the EditSeq and MegAlign programs of DNASTAR 5.02 (DNASTAR Inc., Madison, WI, USA). The base composition of, and the Kimura 2-parameter genetic distance between, the different species was determined using the MEGA4.1 software (Tamura et al., 2007). Phylogenetic tree was constructed using the neighbor-joining (NJ) and maximum-parsimony (MP) methods in MEGA4.1. Support for individual branches was assessed based on bootstrap percentage values computed after 1000 replications with the closest-stepwise-addition option.

Table 2. Experimental samples used in this study.

Species	GenBank No.	Code	Species	GenBank No.	Code
Chuanzhong black goat	KP273589	CH1	Small-tail Hulunbuir sheep	KP702285	SM1
Dazu black goat	KP271023	DA1	Qilian White Tibetan sheep	KP998473	QI1
Xiangdong black goat	KM998968	XI1	Ganjia Tibetan sheep	KP998472	GA1
Boer goat	KM233163	BO1	Huoba Tibetan sheep	KP998471	HU1
Meigu goat	KM244714	ME1	Awang Tibetan sheep	KP998470	AW1
Xinong Shaneng milk goat	KP195269	XS1	Viena sheep	KF938356	VI1
Shaannan White goat	KP195268	SH1	Finnsheep	KF938355	FI1
FuShun black goat	KP662716	FU1		KF938354	FI2
YouZhou Wu goat	KP677511	YO1	Kainuu gray sheep	KF938353	KA1
Jining Qing goat	KP677510	JI1	Oparino sheep	KF938352	OP1
Hechuan white goat	KP677509	HE1	Duolang sheep	KF938332	DU1
Intersexual goat	KP662714	IN1	Hanzhong sheep	KF938329	HA1
Jianyang Daer goat	KM670319	JY1	Jingzhong sheep	KF938328	JZ1
Nanjiang Yellow goat	KM093871	NA1	Hulun Buir sheep	KF938327	HB1
Hainan black goat	KM360063	HA1	Qira Black sheep	KF938326	QB1
Chinese Tibetan goat	KJ940969	CT1	Qinhai Tibetan sheep	KF938325	QT1
Inner Mongolia white cashmere goat	GU068049	IM1	Blue sheep	KP998469	BL1
Jintang black goat	KP231536	JB1		KJ784494	BL2
Liuyang black goat	KR866125	LI1		NC_016689	BL3
Yunnan black goat	KF952601	YU1		JQ040802	BL4
Tibetan antelope	NC_007441	TI1	*Hemitragus jemlahicus*	NC_020628	HJ1
	DQ191826	TI2		FJ207531	HJ2

RESULTS

Characteristics of the Liuyang black goat mitochondrial genome

The mitochondrial genome of the Liuyang black goat is a circular double-stranded DNA that is 16,715 bp long (GenBank accession No. KR866125), and consists of 33.50% A, 27.27% T, 25.98% C, and 13.25% G. The AT content (60.77%) is significantly higher than the GC content (39.23%), which is consistent with the base composition of mitochondrial genomes of other Caprinae species (Parma et al., 2003; Mereu et al., 2008; Zhang et al., 2016). In addition, as with other black goats and sheep (Li et al., 2002; Cañón et al., 2006), the mitochondrial genome contained two rRNA (12S and 16S rRNA), 22 tRNAs, 13 protein-coding genes (NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5, and 6; cytochrome c oxidase subunits I, II, and III; ATPase subunits 6 and 8; and cytochrome b), and one non-coding region (D-loop). Of these genes, only NADH dehydrogenase subunit 6 and eight tRNAs ($tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Glu}$, and $tRNA^{Pro}$) were encoded in the L-strand, while the others were encoded in the H-strand.

Protein-coding genes

The total length of the Liuyang black goat mtDNA that encodes 13 protein-coding genes was 11,760 bp. The longest gene was *NADH5* (1821 bp), and the shortest was *ATPase8* (198 bp; Table 3). In addition to *ND2*, *ND3*, and *ND5* having ATA as initiation codons, the other protein-coding genes initiated with the start codon, ATG. Four types of termination codon were identified (Table 3). *ND2*, *Cytb*, and eight other genes ended with TAG, AGA, and TAA, respectively. *COX3*, *ND3*, and *ND4* ended with an incomplete termination codon ‘‘T-’’, as observed in other Caprinae (Ee et al., 2015a,b). ‘‘T-’’ was at the 5'-terminal of the adjacent gene, and presumably formed the TAA stop codon by post-transcriptional polyadenylation (Anderson et al., 1981). As in other mammals, there were four overlapping reading frames,

ATPase8/ATPase6, *ATPase6/COIII*, *NADH4/NADH4L*, and *NADH5/NADH6*, which overlapped by 40, 1, 7, and 17 bp, respectively (Table 3).

Table 3. Characteristics of the Liuyang black goat mitochondrial genome.

Gene	Position		Size (bp)	Base composition (%)				Start codon	Termination codon	Strand
	From	To		A	C	G	T			
tRNA^{Phe}	1	68	68*	38.24	19.12	19.12	23.53			H
12S rRNA	69	1023	955	36.62	23.82	18.05	21.51			H
tRNA^{Val}	1025	1091	67	38.81	19.40	11.94	29.85			H
16S rRNA	1092	2657	1566	37.33	20.61	17.42	24.63			H
tRNA^{Leu}	2664	2738	75	32.00	22.67	17.33	28.00			H
ND1	2741	3696	956	32.29	28.63	11.08	28.00	ATG	TAA	H
tRNA^{Ile}	3697	3765	69	39.13	10.14	15.94	34.78			H
tRNA^{Gln}	3763	3834	72	36.11	30.56	9.72	23.61			L
tRNA^{Met}	3837	3905	69	27.54	23.19	18.84	30.43			H
ND2	3906	4947	1042	37.26	28.35	7.85	26.53	ATA	TAG	H
tRNA^{Trp}	4948	5014	67	37.31	17.91	16.42	28.36			H
tRNA^{Ala}	5020	5084	65	39.13	21.74	10.14	28.99			L
tRNA^{Asn}	5086	5158	73	30.14	28.77	16.44	24.66			L
tRNA^{Cys}	5191	5259	69	27.94	20.59	19.12	32.35			L
tRNA^{Tyr}	5259	5326	68	27.94	22.06	16.18	33.82			L
COX1	5328	6872	1545	29.00	25.50	16.12	29.39	ATG	TAA	H
tRNA^{Ser}	6870	6940	71	33.33	27.54	14.49	24.64			L
tRNA^{Asp}	6946	7014	69	39.71	14.71	14.71	30.88			H
COX2	7015	7698	684	35.23	23.98	13.30	27.49	ATG	TAA	H
tRNA^{Lys}	7702	7768	67	34.33	19.40	17.91	28.36			H
ATPase8	7770	7967	198	39.90	24.75	7.07	28.28	ATG	TAA	H
ATPase6	7928	8608	681	32.45	28.78	10.72	28.05	ATG	TAA	H
COX3	8608	9391	784	26.66	28.83	14.54	29.97	ATG	T-	H
tRNA^{Gly}	9392	9460	69	34.78	20.29	14.49	30.43			H
ND3	9461	9806	346	31.21	27.75	11.27	29.77	ATA	T-	H
tRNA^{Arg}	9808	9876	69	40.58	10.14	11.59	37.68			H
ND4L	9877	10173	297	31.65	25.59	11.78	30.98	ATG	TAA	H
ND4	10167	11544	1378	31.93	29.17	10.67	28.23	ATG	T-	H
tRNA^{His}	11545	11614	70	41.43	15.71	10.00	32.86			H
tRNA^{Ser}	11615	11675	61	33.33	21.67	15.00	30.00			H
tRNA^{Leu}	11676	11745	70	37.14	14.29	20.00	28.57			H
ND5	11746	13566	1821	33.33	28.67	10.82	27.18	ATA	TAA	H
ND6	13550	14077	528	42.42	28.79	7.20	21.59	ATG	TAA	L
tRNA^{Glu}	14078	14146	69	39.13	21.74	11.59	27.54			L
Cytb	14151	15290	1140	31.93	28.16	13.07	26.84	ATG	AGA	H
tRNA^{Thr}	15294	15363	70	35.71	21.43	17.14	25.71			H
tRNA^{Pro}	15364	15428	65	34.85	27.27	13.64	24.24			L
D-loop	15429	16715	1287	31.27	25.74	14.03	28.96			H

*Numbering starts with the 5'-position of *tRNA-Phe*. TAA, stop codon completed by addition of 3'-adenine residues to mtRNA. H and L denote heavy and light strands, respectively.

In the Caprinae species selected from GenBank, *COI*, *COII*, *NADH4L*, *NADH4*, *NADH5*, *NADH6*, and *Cytb* had the same lengths, initiation codons, and stop codons (Table 4). Some genes exhibited 1- to 3-bp changes in length. For example, *NADH2* and *NADH3* in the Tibetan antelope was 1 and 2 bp longer, respectively, at the 5'-end than in other species, while *Cytb* in *H. jemlahicus* was 1 bp shorter than in other species.

Non-coding region (D-loop region)

The complete length of the D-loop region was 1287 bp, and was located between *tRNA-Pro* and *tRNA-Phe* (Table 3). There were large differences in D-loop lengths between

species of Caprinae, which ranged from 1014 to 1373 bp, probably because of the different number of repetitive motifs between species. The length of the core repeat sequence in the Caprinae species was 75 bp, and was GTA CAT AGT ATT AAT GTA ATA TAG ACA TTA TAT GTA TAA AGT ACA TTA AAT GTG ATT TAC CTC ATG CAT ATA AGC AC, which is repeated differently in different individuals and causes heteroplasmy.

Table 4. Lengths of protein-coding genes in mitochondrial genomes of the Liuyang black goat and other Caprinae.

	Ba	Bb	Bc	Bd	Be	Bf
NADH1	956	955	955	956	956	957
NADH2	1042	1042	1042	1042	1042	1044
COI	1545	1545	1545	1545	1545	1545
COII	684	684	684	684	684	684
ATPase8	198	201	201	201	198	201
ATPase6	681	679	681	681	681	681
COIII	784	784	784	784	784	784
NADH3	346	346	346	346	346	347
NADH4L	297	297	297	297	297	297
NADH4	1378	1378	1378	1378	1378	1378
NADH5	1821	1821	1821	1821	1821	1821
NADH6	528	528	528	528	528	528
Cytb	1140	1140	1140	1140	1139	1140

Ba, domestic goats; Bb, domestic and foreign sheep; Bc, small-tail Hulunbuir sheep, Huoba Tibetan sheep, Awang Tibetan sheep, and Ganjia Tibetan sheep; Bd, Blue sheep; Be, *Hemitragus jemlahicus*; Bf, Tibetan antelope.

The D-loop regions of sheep have 3-4 repeat units (75 bp), and Blue sheep have 2-3 repeat units. Two repeat units are in the D-loop regions of Tibetan antelope and *H. jemlahicus*. No repetitive motifs were detected in D-loop regions of goats. These repeat units are not completely repeated; as is shown in Figure 1, mutations in the repeat units were mainly located at the 8th, 20th, 60th, 66th, and 75th digits, which means that the 75-bp repeat units in the mtDNA D-loop are incompletely repeated, but are all of equal length (75 bp). Base differences led to the differences between these goats.

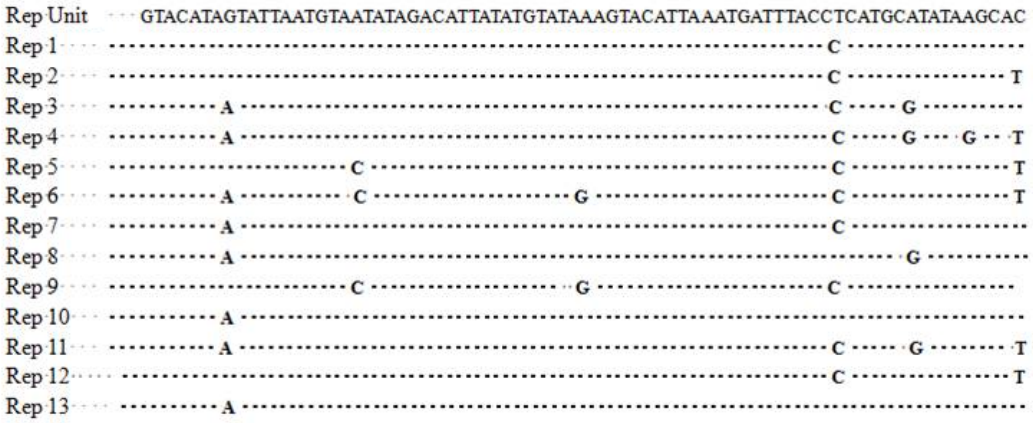

Figure 1. Repeat units of mtDNA D-loop sequence repeat region in sheep.

tRNA and rRNA genes

There were 22 tRNA genes in the mitochondrial genome of the Liuyang black goat. Their lengths ranged from 65 bp (*tRNA-Ala*) to 75 bp (*tRNA-Leu*), and they were distributed in protein-coding genes and rRNA genes. The total length of the Liuyang black goat mtDNA that encodes the tRNA genes was 1512 bp. *12S rRNA* and *16S rRNA* were 955 and 1566 bp in length, respectively, and were located between *tRNA-Phe* and *tRNA-Leu* and separated by *tRNA-Val*, as is the case in other Caprinae. *12S rRNA* in the Liuyang black goat was 2 bp longer than in the Chinese Tibetan goat or the Yunnan black goat, but *16S rRNA* was the same in length and location in all of the goats.

Phylogenetic analysis

Phylogenetic trees for 44 individuals of Caprinae based on the amino acid sequences of 13 protein-coding genes were constructed using NJ and MP methods (Figures 2 and 3). The topological structures of both trees were almost identical. We found that the Caprinae were divided into three groups: all of the goats, Blue sheep, and *H. jemlahicus* were clustered into clade A (99%), Tibetan antelopes were alone in clade B (99%), and all of the sheep were together in clade C (99%). In clade A, goats and *H. jemlahicus* clustered together first, and then clustered with Blue sheep. There were more individuals in clade A (26, 59.09%) than in clade B (2, 4.55%) or clade C (16, 36.36%).

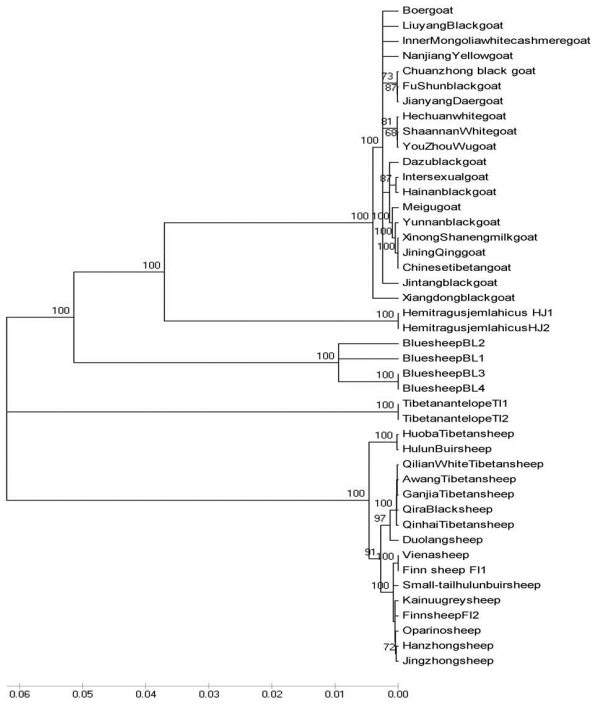

Figure 2. Neighbor-joining tree of Caprinae constructed from 13 mitochondrial protein-coding genes.

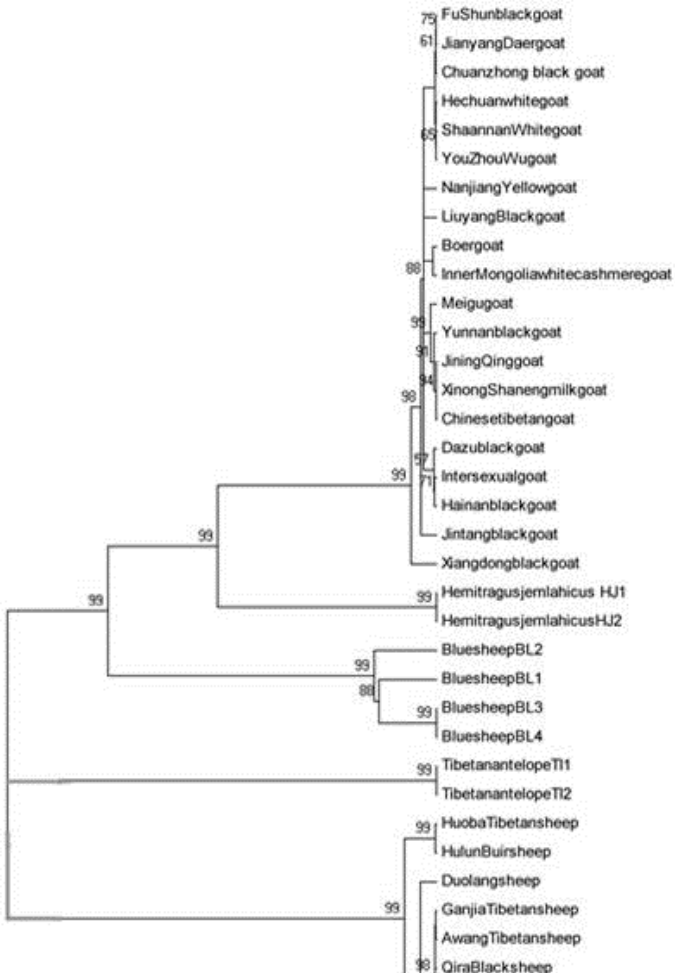

Figure 3. Maximum-parsimony tree of Caprinae constructed from 13 mitochondrial protein-coding genes.

DISCUSSION

The purpose of this study was to detail the characteristics of Liuyang black goat mtDNA, to compare its mtDNA sequence with those of other Caprinae, to determine the length and sequence variations of the genes, and to contribute to the reference mtDNA database of domestic goats. Phylogenetic trees using 13 protein-coding genes were constructed to determine phylogenetic relationships between the species studied.

Regarding gene content and arrangement in the mitochondrial genome, the Liuyang black goat sequence conforms to the mtDNAs of other Caprinae, both in size and composition. The mitochondrial DNA of animals is a small, extrachromosomal genome that is approximately 16 kb in length. Mitochondrial DNA in eukaryotes ranges from 6 to 2000 kb in length, e.g., human, 16,569 bp; Bovidae, 16,338 bp; and Muridae, 16,275 bp (Gardner, 1991; Palmer,

1997). Parma et al. (2003), using cloning and conventional molecular biology techniques, found that the mitochondrial DNA sequence of goats is 16.64 kb long. The Liuyang black goat belongs to the Bovidae and the length of its mitochondrial DNA is 16,715 bp, which is consistent with being in that group.

The D-loop region in the goat is 1212 bp long and located between *tRNA-Pro* and *tRNA-Phe* (Parma et al., 2003; Wang and Chen, 2005). We found that the D-loop in Liuyang black goat mtDNA was 1287 bp long, which generally agrees with the results of previous studies. A few differences in some bases may have been caused by bases being inserted or deleted, or by repeat motifs (75 bp). Sultana and Mannen (2004) found a 76-bp motif in the mtDNA of Pakistani domestic goats, and reported a 17-bp missing fragment in this region. In our study, we also found a motif in the D-loop, but the 17-bp missing fragment was not observed.

The D-loop region is more variable than other regions, and D-loop variation may indicate a history of past isolation, even in the event of the contemporary admixture of groups that evolved in allopatry (Huo et al., 2016). The mtDNA D-loop region has been used extensively in studying relationships between populations (Giuffra et al., 2000; Wu et al., 2014), because variation in this region is higher than in other mtDNA regions or nuclear genes.

Mitochondrial DNA is ideal for studying animal origins and evolution, as well as group genetic differentiation, because it is extranuclear and maternally inherited. Phylogenetic development was once investigated by studying mtDNA using restriction fragment length polymorphism (RFLP). Wilson and Cann (1992) used RFLP on mtDNA to investigate the intraspecific and interspecific evolutionary relationships of several fishes in the Salmonidae family. Most of the results that they obtained were consistent with the conclusions of classical morphological analysis.

In this study, we constructed phylogenetic trees for 44 individuals of Caprinae based on the amino acid sequences of 13 protein-coding genes using the NJ and MP methods (Figures 2 and 3). All of the domestic goats were clustered within a short genetic distance of each other, indicating that they are closely related and have a low level of genetic differentiation and a lack of genetic diversity. This is in agreement with the findings of previous studies in China and elsewhere (Luikart et al., 2001; Huang et al., 2008; Wang et al., 2008; Amills et al., 2009; Liu et al., 2009). After the domestic goats were clustered with *H. jemlahicus*, they were then clustered with Blue sheep to form clade A, and the frequency was 99%. Some researchers hold the opinion that Blue sheep are the wild ancestors of goats (Li, 1993), and we found that goats and Blue sheep had a low level of divergence and were closely related, but cannot be certain whether Blue sheep are the wild ancestors of goats. More advanced sequencing technologies are required to investigate the mtDNA of wild goats. All of the sheep were closely related, and clustered together to form clade C. Among them, there was a short genetic distance between Huoba Tibetan sheep and Hulunbuir sheep and a large genetic distance between them and other sheep species, which may be associated with habitat differences. Tibetan antelopes were clustered together to form an independent clade B, and there was a relatively large genetic distance between them and sheep and goats, which may also be explained by habitat or ecological differences. Seasonal sexual isolation occurs in Tibetan antelopes when females migrate to give birth (Zhou et al., 2006), which may affect gene exchanges between different geographical populations. In addition, Tibetan antelopes live in a harsh, high-altitude environment, so hybridization is unlikely to occur frequently. Therefore, Tibetan antelopes have a close phylogenetic relationship with other Caprinae.

Conflicts of interest

The authors declare no conflict of interest.

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