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RESEARCH ARTICLE

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mtDNA D-loop of Chinese main indigenous sheep breeds using PCR-RFLP

Abstract The polymorphism of mitochondrial DNA (mtDNA) D-loop of 83 individuals from nine Chinese indigenous sheep breeds and two imported sheep breeds was studied with five endonucleases, *Hin*f I, *Msp* I, *Sau*3A I, *Xsp* I and *Taq* I, using PCR-RFLP. The results indicated that there existed two basic haplotypes in the region of mtDNA D-loop. It could be inferred that Chinese indigenous sheep breeds originated from two maternal ancestors. The average polymorphic degree (π value = 0.0421%) of mtDNA D-loop showed that the genetic diversity of mtDNA of Chinese indigenous sheep breeds was very low.

Keywords indigenous sheep breeds, mtDNA, D-loop, PCR-RFLP

1 Introduction

There are still different opinions on the origin, differentiation and genetic relationship among indigenous sheep breeds in China (Compiling Group of Sheep and Goat Breeds in China, 1989; Zhu, 1986; An and Li, 1995; Li, 1997; Xie, 1985; Zou, 1994). Much valuable information has been obtained from the studies on mitochondrial DNA (mtDNA) (Lan et al., 1998; Tu and Zhang, 1998; Li et al., 1998, 2000, 2001; Zhao et al., 2001), RAPD (Gong et al., 2002; Cao et al., 2002; Li et al., 2004b) and microsatellite DNA (Li et al., 2004a), but it was difficult to do comprehensive analysis due to some problems in some studies, such as fewer samples and different restriction enzymes.

Mitochondrial DNA has been proven to be useful markers to study the maternal origin of domestic animals because of its characteristic maternal inheritance. The substitution rate of the D-loop region of mtDNA non-coding region is 5–7 times higher than that of other regions (Liu et al., 2001). So much attention has been paid to the study of origin and differentiation using the D-loop region of mtDNA. The whole length of sheep D-loop region of mtDNA is 1 182 bp (GenBank Accession Number: AF039578). In this paper, the variation of the D-loop region of mtDNA of main Chinese indigenous sheep breeds was studied using PCR-RFLP in order to provide some information on the study of their origin, differentiation and genetic relationship.

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2 Materials and methods

2.1 Materials

Eighty-three samples were taken from nine indigenous sheep breeds and two imported sheep breeds, of which seven were from Mongolian sheep, 11 from Ujumuqin sheep, six from Kazakstan sheep and eight from Altay sheep in Xinjiang Uygur Autonomous Region, seven from Small Tail Han sheep in Shandong Province, eight from Tan sheep in Ningxia Hui Autonomous Region, ten from Hu sheep in Jiangsu Province, ten from Tibetan sheep in Gansu Province, and three from Tong sheep in Shanxi Province. All of the above samples were from kidney samples preserved in 75% alcohol. In all cases, samples were taken from different villages and farms, and owners were questioned in detail to ensure that the samples were not related. Another seven blood samples of Dorset sheep and five blood samples of Merino sheep were from the Inner Mongolia Autonomous Region.

2.2 Primer design and PCR amplification

Primer 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design primers for amplifying part of the D-loop region according to the sheep mtDNA sequence (AF039578). The D-loop region, with a length of 1 055 bp, was amplified by forward (5′-AACTCCCAAACATACAACACGG-3′) and reverse primers (5′-ATTTGA

GTATTGAGGGCGGGAT-3′) at an annealing temperature of 58°C. The sequence of the amplified region and the selected restriction sites are listed in Fig. 1. PCR amplification was carried out with a total reaction volume of 50 μL containing 2 μL DNA (75 ng/μL), 5 μL 10×PCR standard reaction buffer, 4 μL dNTPs (2.5 mmol/L), 2 μL $MgCl_2$ (25 mmol/L), 2 μL each forward and reverse primers (10 μmol/L), and 2.5 U Taq DNA polymerase from Promega (China). After an initial denaturation at 94°C for 3 min, 30 cycles were performed at 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min. The final cycle was followed by an extension at 72°C for 10 min. PCR products were detected on 1.5% agarose gel stained with 0.5 μg/mL ethidium bromide, and photographed under UV light.

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   1 aactcccaaa catacaacac ggacttccca ctccacaaac ccacataaca acccatacaa
  61 gaaaagcaca accatccacc cacggacacg agcgttcata aacccaatat atcttatgtc
 121 tgtcttgaat gtgctaagcg agtacataac attaatgtaa tatagacatt atatgtataa
 181 agtacattaa atgatttgcc ccatgcgtat aagcacgtac ataacattaa tgtaatatag
 241 acattatatg tataaagtac attaaatgat ttgccccatg cgtataagca cgtacataac
 301 attaatgtaa tatagacatt atatgtataa agtacattaa atgatttgcc ccatgcgtat
 361 aagcacgtac ataacattaa tgtaatatag acattatatg tataaagtac attaaatgat
 421 ttaccccatg cgtataggca tgtacattca cttcactgaa gcatgtaggg cattgaactg
 481 cttgaccgta catagtacat gaagtcaaat ccgtcctagt caacatgcat atcccgtcca
 541 ctagatcacg agcttgttca ccatgccgcg tgaaaccaac aacccgcttg gcaaggatcc
 601 ctcttctcgc tccgggccca ttaactgtgg gggtaactat ttaatgaact ttaacaggca
 661 tctggttctt tcttcagggc catctcatct aaaatcgccc attctttcct cttaaataag
 721 acatctcgat ggactaatga ctaatcagcc catgcctaac ataactgtgg tgtcatgcat
 781 ttggtatttt ttaatttttg gggatgcttg gactcagcta tggccgtctg aggccccgac
 841 ccggagcatg aattgtagct ggacttaact gcatcttgag catcctcata atggtaagca
 901 tgggcataat ataattaatg gtcacaggac atacctgctg tatcgtacat ttatatattc
 961 ttttccccc ccttcccctt aaatatttat caccattttt aacacgcttc ccctagata
1021 ttaatataaa tttatcccgc cctcaatact caaat
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Note: Bases in red, blue and green represent restriction and mutation sites of *Sau*3A I, *Xsp* I and *Hinf* I, respectively.

Fig. 1 DNA sequence, restriction and mutation sites of pre-amplified region

2.3 Digestion

Predigestion of the amplified region was carried out using Webcutter 2 (http://www.firstmarket.com/cutter/cut2.html) according to the D-loop region of sheep mtDNA (AF039578). Five suitable enzymes (*Hinf* I, *Msp* I, *Sau*3A I, *Xsp* I, *Taq* I) were selected.

2.4 Data analysis

The distance between haplotypes (P) and nucleotide diversity (π) was estimated according to Nei and Li (1979). The molecular dendrogram of haplotypes was constructed using the UPGMA of Phylip (Version 3.6).

3 Results and discussion

3.1 Restriction morphs and haplotypes

The PCR product with a length of 1 055 bp was consistent with the design. The restriction enzymes, *Sau*3A I, *Xsp* I and *Hinf* I, presented polymorphism (Fig. 2). The restriction morphs and their frequency are listed in Table 1. The sequence variation of some restriction morphs is shown in Fig. 3.

Table 1 Restriction morphs and their frequencies

Morph	Number	Frequency/%	Number of site	Molecular size/kb
*Sau*3A I-A	82	98.80	2	54,346,052
*Sau*3A I-B	1	1.20	1	543,512
Xsp I-A	47	56.63	3	516,473,41,25
Xsp I-B	29	34.94	2	516,498,41
Xsp I-C	2	2.41	4	516,266,207,41,25
Xsp I-D	5	6.02	2	616 (541 + 75),473,41
Hinf I-A	80	96.39	1	811,244
Hinf I-B	2	2.41	2	418,393,244
Hinf I-C	1	1.20	2	468,343,244

Enzyme cutting was theoretically carried out using Webcutter 2 (http://www.firstmarket.com/cutter/cut2.html) according to the sequence of the amplified region of sheep mtDNA D-loop (AF039578). The fragments at 543 bp, 460 bp and 52 bp appeared from *Sau*3A I-A (Table 1 and Fig. 2) due to the enzyme cutting sites of *Sau*3A I at 543 and 595. The *Sau*3A I-B (Table 1 and Fig. 2) was from the mutation of T to C at 598, with GATC becoming GACC (Fig. 3(a)) and forming 512 bp (460 bp + 52 bp).

There were three enzyme cutting sites of *Xsp* I at 516, 541 and 1014 and four fragments of 516 bp, 473 bp, 41 bp and

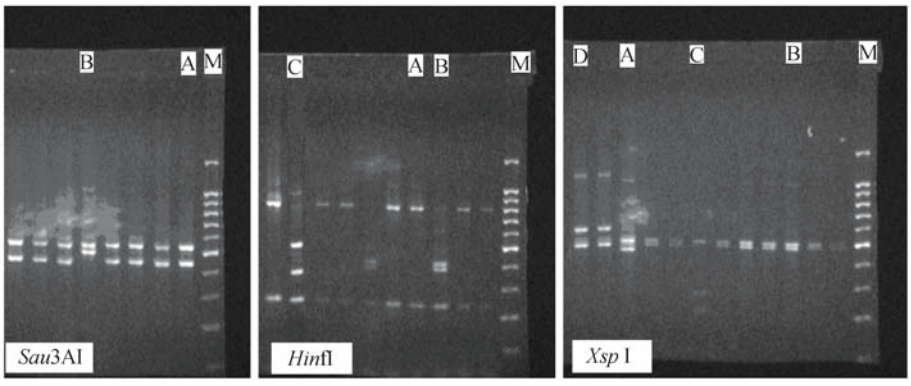
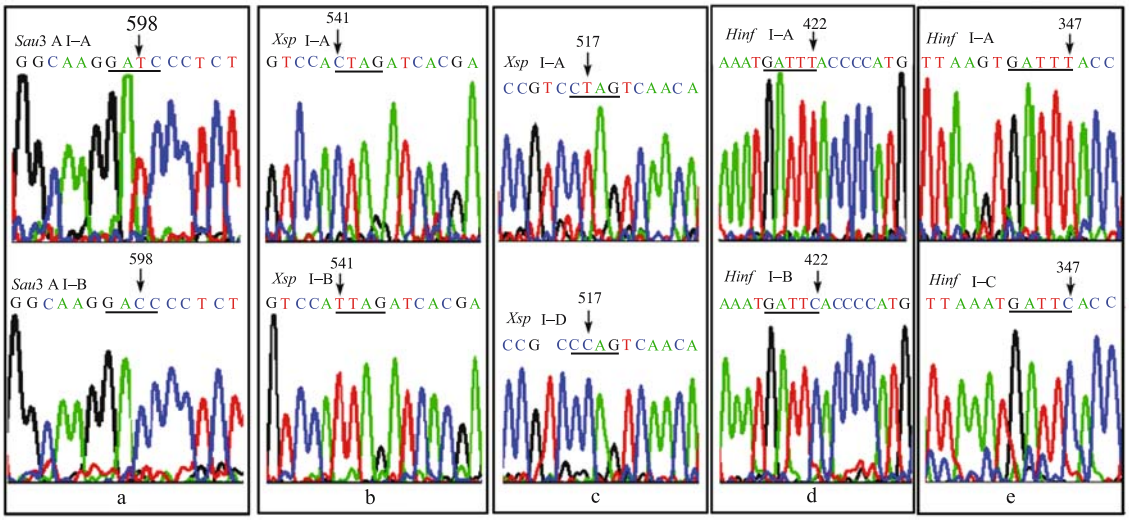


Fig. 2 Digestion results of PCR products of some sheep mtDNA D-loop regions with *Sau*3A I, *Hinf* I and *Xsp* I



M: 100 bp ladder. A, B, C and D represent morphs of each restriction enzyme, respectively.
Fig. 3 Comparison of sequences for different morphs

25 bp made up *Xsp* I-A (Table 1 and Fig. 2). The *Xsp* I-B (Table 1 and Fig. 2) was from the mutation of C to T at 541 (Fig. 3(b)), forming 498 bp (473 bp + 25 bp). The *Xsp* I-C was from the mutation of T to A at 809, forming CTAG from CTTG, and became the new cutting site of *Xsp* I to form 226 bp and 207 bp from 473 bp (Table 1 and Fig. 2). The *Xsp* I-D was from the mutation of T to C at 517, forming 541 bp due to the formation of CCAG from CTAG (Fig. 3(c)). The 616 bp of *Xsp* I-D in Table 1 and Figure 2 includes 541 bp and another 75 bp insertion sequence (TAGTATTAATGTA-ATATAGACATTATATGTATAAAGTACATTAAGTGATT-TACCTCATGCATATAAGCATGTATA).

The *Hinf* I-A was from the 811 bp and 244 bp formed from the cutting site at 811 (Table 1 and Fig. 2). The mutation of T to C at 422 resulted in GATTT becoming GATTC (Fig. 3(d)) and forming *Hinf* I-B, with 418 bp and 393 bp from 811 bp (Table 1 and Fig. 2). The mutation of T to C at 347 resulted in GATTT becoming GATTC (Fig. 3(e)) and forming *Hinf* I-C, with 468 bp and 343 bp from 811 bp (Table 1 and Fig. 2).

Seven haplotypes could be sorted from the restriction morphs (Table 2). The haplotypes I and II were the main and basic haplotypes, which were different mainly from the difference between *Xsp* I-A and *Xsp* I-B.

Table 2 Restriction haplotypes and their frequencies

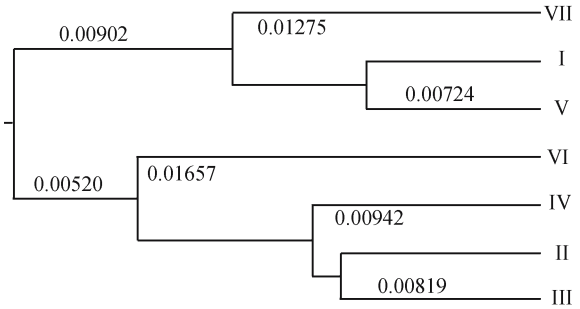
Haplotype	Number	Frequency/%	Morph		
			*Sau*3A I	*Xsp* I	*Hinf* I
I	46	55.42	A	A	A
II	26	31.33	A	B	A
III	2	2.41	A	B	B
IV	1	1.20	A	B	C
V	2	2.41	A	C	A
VI	5	6.02	A	D	A
VII	1	1.20	B	A	A

3.2 Origin inference of Chinese indigenous sheep breeds from haplotypes

It was obvious that haplotypes I and II were the main and basic haplotypes, with a frequency of 55.42% and 31.33%, respectively, from which other haplotypes mutated. It could be inferred that Chinese indigenous sheep breeds originated from two maternal ancestors. This was also demonstrated by the genetic distance among haplotypes (Table 3) and the molecular dendrogram of haplotypes constructed using Phylip (Fig. 4). This conclusion was not only consistent with the results from archaeological and morphological studies

Table 3 The genetic distance between haplotypes

	I	II	III	IV	V	VI	VII
I	0.000 0						
II	0.016 4	0.000 0					
III	0.034 6	0.016 4	0.000 0				
IV	0.034 6	0.016 4	0.021 3	0.000 0			
V	0.014 5	0.021 3	0.039 4	0.039 4	0.000 0		
VI	0.040 3	0.018 9	0.040 3	0.040 3	0.045 6	0.000 0	
VII	0.016 4	0.040 3	0.065 4	0.065 4	0.034 6	0.080 0	0.000 0



The data in the figure represent the length of branches.
Fig. 4 Molecular dendrogram among haplotypes

Table 4 Distribution of haplotypes among and within populations

	I	II	III	IV	V	VI	VII	Total
Aletai sheep	5	3						8
Tibetan sheep	10							10
Polled Dorset	2	4	1					7
German Merino sheep		3	1		1			5
Kazakstan sheep	2	1				3		6
Hu sheep	6	3			1	1		11
Mongolian sheep	5	1		1				7
Tan sheep	1	6					1	8
Tong sheep	3							3
Ujumuqin sheep	10	1						11
Small Tail Han sheep	2	4				1		7
Total	46	26	2	1	2	5	1	83

(Compiling Group of Sheep and Goat Breeds in China, 1989; Zhu, 1986; An and Li, 1995; Li, 1997; Xie, 1985; Zou, 1994), but also with the results of mtDNA (Lan et al., 1998; Tu and Zhang, 1998; Li et al., 1998, 2000, 2001; Zhao et al., 2001), RAPD (Gong et al., 2002; Cao et al., 2002) and microsatellite DNA studies (Li et al., 2004a).

Mongolian sheep distributed in Mongolia Plateau and Tibetan sheep distributed in Qingzang Plateau, with much differences in head type and horn type, might have originated from *O.a. daruini* and *O.a. hadgsoni* of *Ovis ammon* (Compiling Group of Sheep and Goat Breeds in China, 1989). Generally, Chinese indigenous sheep has a close relationship with *Ovis orientalis*, *Ovis ammon* and its subspecies (Xie, 1985). *Ovis ammon arkal* was even sorted into *Ovis ammon* in their classification (Xie, 1985), which was consistent with the results of the main and basic haplotypes representing two different maternal ancestors.

Only one haplotype was found in our previous study on RFLP of mtDNA of Mongolian sheep, Ujumuqin sheep, Small Tail Han sheep and Hu sheep (Li et al., 1998, 2000, 2001). This was consistent with the result of Li (1997), showing that Hu sheep originated from Mongolian sheep based on archaeological evidence. It was also consistent with the results that most of the individuals from Hu sheep, Mongolian sheep and Ujumuqin sheep were of haplotype I (Table 4). The difference of *Hin*d III and *Bgl* I cutting sites in Yunnan sheep (Lan et al., 1998) and Tibetan sheep (Tu and Zhang, 1998) might represent different maternal origins.

3.3 Distribution of haplotypes within and among breeds

The two basic haplotypes I and II were distributed within and among breeds (Table 4), which was consistent with the results of Hiendleder et al. (1998, 1999) based on RFLP of mtDNA. This shows that sheep all over the world had two main types distributed within and among breeds. We noticed that most individuals of Tan sheep and Small Tail Han sheep were of haplotype II. The result of RAPD also showed that Tan sheep had some special bands and a close relationship with Small Tail Han sheep (Gong et al., 2002).

3.4 Diversity of mtDNA of Chinese indigenous sheep breeds

The π value of Chinese indigenous sheep breeds, representing diversity of mtDNA, was 0.0421%, which was in the lower region (0.15%–0.47%) (Hiendleder et al., 1998), showing that the mtDNA diversity of Chinese indigenous sheep breeds was low. The π value of Mongolian sheep, Ujumuqin sheep, Hu sheep and Small Tail Han sheep was 0.0554%, 0.0135%, 0.0653% and 0.0586%, respectively. The π value of Ujumuqin sheep (0.0135%) was slightly lower than the previous results of 0.027% (Li et al., 1998) and 0.018% (Li et al., 2001). The corresponding value of Mongolian sheep (0.0554%) was slightly higher than the previous results of 0.0130% (Li et al., 2000) and 0.013% (Li et al., 2001). Hu sheep and Small Tail Han sheep also had higher π value (0.0653% and 0.0586%) than the previous results of 0% and 0.0450% (Li et al., 2001). These might be related to the highly polymorphic region of mtDNA.

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