An *Msp*I PCR-RFLP detecting a single nucleotide polymorphism at alpha-lactalbumin gene in goat

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ABSTRACT: Alpha-lactalbumin (α-*LA, LALBA*) was strongly correlated with the nutritional value and the functional properties of whey and whey products. However, there are not many studies of goat *LALBA* gene and its polymorphisms in literary sources. In this paper, on the basis of PCR-SSCP and DNA sequencing, one M63868:g.1897T>C mutation in exon 3 at *LALBA* locus identified a Single Nucleotide Polymorphism (SNP): p.L100P of the *LALBA* protein, which could be detected by *Msp*I endonuclease. Hence, we described a method based on *Msp*I PCR-RFLP for SNP detection at the *LALBA* locus in 801 goats of China. Frequencies of goat *LALBA*-A_2 allele varied from 0.000 to 0.024 in Chinese populations. Gene heterozygosity, effective allele numbers and PIC (Polymorphism Information Content) of goat *LALBA* locus in Chinese populations varied from 0.000 to 0.0472, 1.000 to 1.0496, 0.000 to 0.0461, respectively, which were lower values than in Italian populations. Therefore, we considered that the low frequency of *LALBA*-A_2 allele at goat *LALBA* gene possibly characterized Chinese native breeds. Moreover, Chinese native populations demonstrated poorer polymorphism at goat *LALBA* locus than Italian populations.

Keywords: goat; alpha-lactalbumin (*LALBA*) gene; polymorphism; Single Nucleotide Polymorphism (SNP); PCR-RFLP

Alpha-lactalbumin (α-*LA*, *LALBA*), one of the two major whey proteins, was strongly correlated with the nutritional value and the functional properties (i.e. gelling, film-forming, foaming and emulsifying) of whey and whey products (De Wit, 1989; Kinsella and Whitehead, 1989). The goat *LALBA* transcription unit is located on chromosome 5 and organized in 4 exons coding for the 123 AA (amino acid) polypeptide chain (Hayes et al., 1993). In contrast to numerous studies that had been carried out on goat casein genes and their polymorphisms (Ramunno et al., 2004, 2005; Moatsou et al., 2005; Prinzenberg et al., 2005), studies of goat *LALBA* gene and its polymorphisms were not so frequent. It was only reported by Cosenza et al. (2003) that the polymorphisms of goat *LALBA* gene showed three mutations in Italian populations with limited samples. However, to date, no studies about the polymorphisms of goat *LALBA* gene in Chinese populations have been published.

In this paper, we described for the first time a method based on *Msp*I PCR-RFLP for SNP (Single Nucleotide Polymorphism) detection at the goat *LALBA* locus in 801 goats of China.

MATERIAL AND METHODS

DNA samples. Genomic DNA samples were obtained from 801 goats belonging to nine ge-

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netic types: Inner Mongolia White Cashmere (IMWC, *n* = 452), Xinong sannen dairy (Sa, *n* = 74), Guanzhong dairy (GZ, *n* = 62), Laoshan dairy (LS, *n* = 80), Leizhou (LZ, *n* = 34), Guizhou Black (GB, *n* = 21), Banjiao (BJ, *n* = 25), Matou (MT, *n* = 22), Guizhou white (GW, *n* = 31) reared in the province of Inner Mongolia, Shaanxi, Shandong, Guangdong, Sichuan, Hubei and Guizhou (China), respectively. DNA samples were extracted from leucocytes and ear tissue according to Mullenbach et al. (1989).

Primer design and genotype determination of PCR-SSCP

The PCR (Polymerase Chain Reaction) was used to amplify the *LALBA* gene fragments from goat genomic DNA. According to the strong similarity between sheep and goat *LALBA* gene, three primer pairs, designed by using as template the sequences of M63868 (Vilotte et al., 1991) and NM_001009797.1 (sheep), were used for the amplification of exon 1 with 5' flanking (P1), exon 3 and part of its flanking region (P2) and exon 4 with 3' untranscription region (P3) of goat *LALBA* gene (Table 1).

The PCR was performed in a 25 µl reaction mixture containing 10 pmol of forward primer and the same amount of reverse primer, 200µM dNTP (dATP, dTTP, dCTP and dGTP), and buffer (including 1.5mM $MgCl_2$), 0.625 units *Taq* DNA polyme-rase and 50 ng goat genomic DNA as template. The amplification was carried out for 33 cycles at the conventional PCR protocol with different annealing temperatures.

SSCP (Single-Strand Conformation Polymorphism) method was used to scan mutations within the amplified regions. Because the optimal size of DNA for SSCP was under 400 base pairs, all DNA fragments amplified by primer pairs were suitable for SSCP. Aliquots of 10 µl PCR products were mixed with 10 µl denaturing solution (95% formamide, 25mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled on ice (Sun et al., 2002). Denatured DNA was subjected to 10% PAGE (Polyacrylamide Gel) in 1 × TBE buffer and constant voltage (200 V) for 16 h at a constant temperature of 12°C, then gels were stained with 0.1% silver nitrate (Sun et al., 2002).

After the polymorphism was detected, the PCR products of different electrophoresis patterns were sent to sequence and analyze. Before sequencing, PCR products were purified with QIAquick column (QIAGEN 40724). Only exon 3 and its flanking region amplified with P2 primer showed polymorphism, namely A pattern with two bands and B pattern with three bands. Hence, in order to explore the genetic variations of exon 3 of goat *LALBA* gene in Chinese populations, ten products from different patterns were sequenced in both directions and their sequences were submitted to the GenBank database (Accession number # DQ629104-629112 and DQ673921). Homology searches, comparison between sequences, and multiple alignments were accomplished by means of DNASIS-Pro software (Hitachi Software Engineering Co., San Bruno CA, USA). According to the nomenclature (www.genomic.unimelb.edu.au/mdi/mutnomen and www.hgvs.org/mutnomen/) and Den Dummen and Antonarakis (2000), one M63868:g.1897T>C mutation was detected in exon 3 at *LALBA* locus. Interestingly, this SNP could be genotyped by restriction enzymes *Msp1*.

Genotyping of *LALBA* alleles by means of *Msp*I PCR-RFLP

By means of PCR, a 268 bp DNA region spanning exon 3 and a part of the flanking regions of the *LALBA* gene were amplified. The PCR was carried

Table 1. The primer pair sequences and their information on *LALBA* gene in goat

Name	Sequence	Annealing temperature	Size (bp)	Note
P1	F: 5' CCCTGAGGCTTTTTCCAC 3' R: 5'-TGGAGGGAAAGAGTGAAGA 3'	60.0°C	268	Exon 1 and flanking regions (721–989 nt)
P2	F: 5' TCATCTAAAAGGCAACAGGTA 3' R: 5' ATAGTGCTGGGGCGAAA 3'	58.5°C	268	Exon 3 and flanking regions (1 769–2 036 nt)
P3	F: 5' TGAACACCTGCTGTCTTTGC 3' R: 5' CATCCCTAGAGATTAGTCCTTAC 3'	56.0°C	165	Exon 4 and 3' UTR (NM_001009797.1.453-617 nt)

out in a 25 µl reaction mixture containing: 50 ng genomic DNA, 10 pmol of each primer, 0.625 units *Taq* DNA polymerase (Promega), 25mM buffer (including $MgCl_2$), each dNTPs (dATP, dTTP, dCTP and dGTP) 200 µm. The amplification protocol consisted of an initial cycle at 95°C for 5 min, then 33 cycles at 94°C for 30 s, 58.5°C for 30 s, 72°C for 40 s, with an extension step at 72°C for 10 min in the final cycle.

Aliquots of 25 µl PCR products were digested with 15 units *Msp*I endonuclease (MBI, Fermentas) for 5 h at 37°C following the supplier's directions. The digested PCR products were analyzed by electrophoresis on 10% PAGE stained with 0.1% silver nitrate.

Statistical analysis

Differences in genotypic and allelic frequencies at goat *LALBA* locus within/between Chinese populations and Italian populations were analyzed using a χ^2-test, which was performed by SPSS software (Version 13.0). Population genetic indexes e.g. gene heterozygosity, gene homozygosity, effective allele numbers and PIC (Polymorphism Information Content) were calculated by Nei methods (Nei and Roychoudhury, 1974; Nei and Li, 1979).

RESULTS AND DISCUSSIONS

We were aware of limited research related to three mutations in intron 1, exon 3 and in the 3' untranscription region at goat *LALBA* locus (Cosenza et al., 2003), respectively. However, in our study, only exon 3 with its flanking region demonstrated polymorphisms (namely A and B patterns) by PCR-SSCP method, which is a technique based on the principle that single-stranded DNA molecules form specific sequence-based secondary structures under nondenaturing conditions (Orita et al., 1989). The alignment among nucleotide sequences of M63868 and the third exon of *LALBA* locus of ten selected from different patterns demonstrated one mutation. An M63868:g.1897T>C mutation in exon 3 at *LALBA* locus identified an SNP: p.L100P of the *LALBA* protein. Here, the allele characterized by the presence of C was designated as *LALBA-*A_2, while that characterized by the presence of T was designated as *LALBA-*A_1. Interestingly, we found for the first time that this SNP could be detected by *Msp*I endonuclease.

The M63868:g.1897T>C mutation of exon 3 added an *Msp*I endonuclease restriction site (C*C*GG). Therefore, the amplified DNA fragment, containing exon 3 and a part of the flanking region, digested with this endonuclease, showed a single undigested 268 bp fragment for *LALBA-*A_1 allele and two fragments of 128 bp and 140 bp for *LALBA-*A_2 allele (Figure 1).

Frequencies of *LALBA-*A_2 allele in the analyzed populations were 0.017, 0.024, 0.024, 0.023, and 0.020 for Inner Mongolia White Cashmere, Guanzhong dairy, Guizhou Black, Matou and Banjiao populations, respectively, while 0.000 was for the other populations reared in China, with genotype distributions in agreement with Hardy-Weinberg equilibrium (Table 2).

Genotypic frequencies for the various polymorphisms at *LALBA* locus were not found to be significantly different between Chinese populations based on a χ^2-test (χ^2 = 8.890, *df* = 8, *P* = 0.352). Moreover, significant differences between allelic frequencies of Chinese populations were not found (χ^2 = 8.771, *df* = 8, *P* = 0.362). However, it was found that genotypic frequencies for the various polymorphisms at *LALBA* locus showed significant differences between Italian populations (χ^2 = 13.054, *df* = 2, *P* = 0.011), as well as allelic frequencies between Italian populations (χ^2 = 13.044, *df* = 2, *P* = 0.001). The frequencies of alleles at *LALBA* locus in Chinese populations showed significant differences when compared with those of

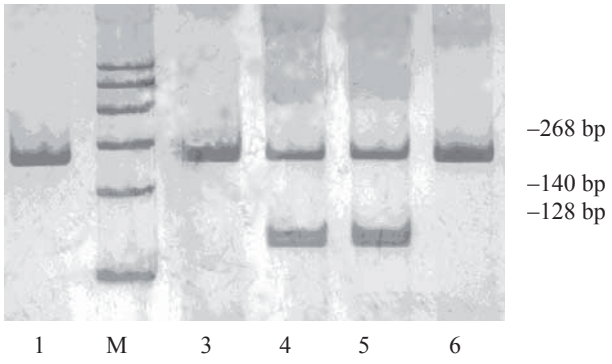

Figure 1. DNA electrophoretic patterns on 10% PAGE after digestion with *Msp*I endonuclease of the DNA region containing exon 3 of goat *LALBA* gene in Chinese populations

Lane 1: undigested PCR product; Lane 2: M = Marker I (Tianwei times, China), six ladders with 100 bp, 200 bp, 300 bp, 400 bp, 500 bp and 600 bp; Lane 3 and 6: *LALBA-*A_1*/LALBA-*A_1; Lane 4, 5: *LALBA-*A_1*/LALBA-*A_2

Table 2. Genotype distribution and allelic frequencies at the goat *LALBA* locus

Breeds	Observed genotypes			Allelic frequencies		
	LALBA-A$_1$/*LALBA-A*$_1$	*LALBA-A*$_1$/*LALBA-A*$_2$	total	*LALBA-A*$_1$	*LALBA-A*$_2$	χ^2 (HWE)*
Inner Mongolia White Cashmere	437	15	452	0.983	0.017	0.132
Xinong sannen dairy	74	0	74	1.000	0.000	0.000
Laoshan dairy	80	0	80	1.000	0.000	0.000
Guanzhong dairy	59	3	62	0.976	0.024	0.038
Leizhou	34	0	34	1.000	0.000	0.000
Guizhou Black	20	1	21	0.976	0.024	0.013
Matou	21	1	22	0.977	0.023	0.012
Banjiao	24	1	25	0.980	0.020	0.010
Guizhou white	31	0	31	1.000	0.000	0.000

χ^2 (HWE)* = Hardy-Weinberg equilibrium χ^2 value. Their *P* values were all above $\alpha = 0.05$

Table 3. Population genetic indexes at the goat *LALBA* locus in Chinese and Italian populations

Breeds	Gene homozygosity	Gene heterozygosity	Effective allele numbers	PIC
Inner Mongolia White Cashmere	0.9666	0.0334	1.0345	0.0328
Xinong sannen dairy	1.0000	0.0000	1.0000	0.0000
Laoshan dairy	1.0000	0.0000	1.0000	0.0000
Guanzhong dairy	0.9528	0.0472	1.0496	0.0461
Leizhou	1.0000	0.0000	1.0000	0.0000
Guizhou Black	0.9535	0.0465	1.0488	0.0454
Matou	0.9556	0.0444	1.0465	0.0434
Banjiao	0.9608	0.0392	1.0408	0.0384
Guizhou Black	1.0000	0.0000	1.0000	0.0000
*Girgentana	0.8074	0.1926	1.2385	0.1741
*Red Syrian	0.6893	0.3107	1.4506	0.2624
*Local goat	0.6226	0.3774	1.6062	0.3062

* data on Girgentana, Red Syrian and Local population are from Cosenza et al. (2003)

Italian populations ($\chi^2 = 216.275$, $df = 1$, $P = 0.001$). Gene heterozygosity, effective allele numbers and PIC (Polymorphism Information Content) of goat *LALBA* locus in Chinese populations varied from 0.000 to 0.0472, 1.000 to 1.0496, 0.000 to 0.0461, respectively, which were lower values than those in Italian populations (Table 3). According to the classification of PIC (low polymorphism if PIC value < 0.25, median polymorphism if 0.25 < PIC value < 0.5, and high polymorphism if PIC value > 0.5), all Chinese populations belonged to a very low polymorphism level. Therefore we considered that the low frequency of *LALBA-A*$_2$ allele at goat *LALBA* locus possibly characterized Chinese native breeds. Moreover, the *Msp*I PCR-RFLP analysis revealed that Chinese native populations demonstrated poorer polymorphism at goat *LALBA* locus than Italian populations.

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