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Full Length Research Paper

Allele mining in β-lactoglobulin gene of *Capra hircus*

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β-Lactoglobulin (β-LG) genetic polymorphisms are important and well known due to their effects on quantitative traits and technological properties of milk. At the DNA level, polymerase chain reaction (PCR)-single-strand conformation polymorphism (SSCP) allows for the detection of unknown polymorphisms at β-LG loci. Here we describe the usefulness of the PCR-SSCP technique for β-LG typing. In the present study, we amplified and sequenced the part of promoter region and all the seven exons containing the entire coding and untranslated region for the β-lactoglobulin gene in best dairy goat breeds of India namely: Jamunapari and Jakhrana. Nine polymorphisms were detected, one in the l promoter region, four in the introns and four in the exons of the β-lactoglobulin gene. All polymorphisms were single nucleotide substitutions. The polymorphisms in the coding region did not produce any amino acid change.

Key words: β-Lactoglobulin gene, dairy goats, polymorphism, single nucleotide polymorphism (SNP), single strand conformational polymorphism (SSCP).

INTRODUCTION

B-Lactoglobulin is a major whey protein in the milk of ruminants. It is also found in the milk of other mammals, but is absent from the milk of rodents, lagomorphs, humans, and probably camels (Hambling et al., 1992). In ruminant milk, the native protein was found as a dimmer with a molecular weight of 36.4 kDa, 162 amino acids. In most other species in which it has been found β-LG appears to be monomorphic (Hambling et al., 1992). β-LG was among the first proteins to be crystallized and its secondary and tertiary structures are well characterized (Papiz et al., 1986; Monaco et al., 1987).

The β-LG transcription unit spans 4.7 kb arranged in seven small exons (42 to 178 bp in goat) and six introns ranging from 213 bp to 1116 bp. The first exon contains part of the 5' untranslated region and the sequence encoding for the signal peptide and the first 14 amino acids. The 3' untranslated region is included in exon 6 and 7. The goat β-LG gene has been assigned by in situ hybridization to chromosome 11q23 (Hayes and Petit, 1993) and is located in a GC-rich region of the genome (Bernardi et al., 1985).

Bovine β-LG is the first milk protein in which polymorphism was detected (Aschaffenburg and Drewry, 1955). The protein is highly polymorphic in bovine species (Godovac-Zimmerman et al., 1996). The most common genetic variants in *Bos taurus* and *Bos indicus*

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Abbreviations: BLG, β-Lactoglobulin; **SNP,** single nucleotide polymorphism; **SSCP,** single strand conformational polymorphism.

are A and B forms that differ by two amino acid substitutions at positions 64 (Asp-gly) and 118 (Val-Ile) (Ng-Kwai Hang and Grosclaude, 1992). These variants are associated with quantitative effects on milk composition and cheese making properties (reviewed in Ng-Kwai Hang and Grosclaude, 1992). Eleven isoforms of β-LG have been identified in *Bos taurus* (Doosti et al., 2011)

In sheep, the β-LG is polymorphic in all analyzed breeds with two variants, A and B, variant A is normally predominant (King, 1969; Chiofalo and Micari, 1987). The two variants differ by a single amino acid substitution (Tyr-His) at position 20 of the protein (Gaye et al., 1986). A third variant (β-LG C) was detected in German and Spanish merino breeds (Erhardt, 1989; Recio et al., 1997) which differ from A variant by a single exchange Arg - Glu at position 148. A protocol for genotyping variants A and B by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with *Rsal* endonuclease have been described (Schlee et al., 1993).

In goat, the electrophoretic pattern of the β-LG gene has been analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by Grosclaude et al. (1987). β-LG gene polymorphism at the DNA level has been analyzed by PCR-RFLP (Pena et al., 2000) and they also reported two novel genetic variants in the 3' untranslated region (exon 7) of the β-LG gene. A single nucleotide polymorphism in the proximal region of the gene has been reported (Yahyaoui et al., 2000). Ballester et al. (2005) amplified and sequenced the proximal promoter and the first six exons containing the entire coding region for the β-lactoglobulin gene in eleven goat breeds from Spain, France, Italy, Switzerland, Senegal and Asia and detected fifteen polymorphisms, nine in the promoter region and six in the exons of the β-LG gene. In India, Kumar et al. (2006) reported genetic variant in Indian goat breeds as per the already reported variants by Pena et al. (2000) but no nucleotide sequence and thus no single nucleotide polymorphisms (SNPs) are reported in β-LG gene in Indian goat breeds.

The aim of this work was to study the genetic polymorphism in the caprine β-lactoglobulin gene. We describe new polymorphisms in the promoter region, in exon and introns of the goat β-LG gene.

MATERIALS AND METHODS

Animals, sample collection and DNA extraction

Blood samples of 100 animals belonging to two Indian goat breeds (Jamunapari n = 50 and Jakhrana n = 50) were randomly collected from their native breeding tract, avoiding narrow genetic relationships among the sampled animals, representing gene pool for SNP discovery. Blood samples (5 to 6 ml) were obtained by jugular venipuncture, using vacuum tubes treated with 15% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. DNA was extracted from blood as described by Sambrook et al. (1989) with minor modifications and checked for quality and quantity and was diluted to a final concentration of 50 ng/µl.

Primer designing

In silico data mining was done and all β-LG sequences were aligned to obtain longest sequence, which was used further as template (GenBank accession no. Z33881) for primer designing. Seven single-strand conformation polymorphism (SSCP) primer pairs were designed which cover all seven exon with part of their flanking regions (Table 1) using Primer3 (Razen et al., 2000). The primers were checked for their thermodynamic properties and secondary structures using Primer select (DNA Star-Lasergene® v7.2 software). The designed primers were re-analyzed by basic local alignment search tool (BLAST) analysis (www.ncbi.nlm.nih.gov/BLAST) to ensure their respective homology over caprine genome.

DNA amplification by PCR

Seven fragments containing exon 1 to 7 of goat β-LG gene was amplified by PCR using seven pairs of designed primer. The PCR reaction was performed in a 25µl reaction mixture containing 2 µl of DNA solution (100 ng), 200 mM each of dATP, dCTP, dGTP and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.6 unit Taq DNA polymerase and 4 ng µL-1 of each primers (IDT, USA).

The following conditions were used: an initial denaturation step of 95°C for 4 min was followed by 30 cycles of 94°C for 1 min, 66°C for 30 s, and 72°C for 30 s, and concluded with a final extension step of 72°C for 10 min using a PTC-200 DNA engine thermal cycler (MJ Research Inc., Waltham, MA). Amplification was verified by electrophoresis on 2% (w/v) agarose gel in 1x Tris acetate EDTA (TAE) buffer using a 100 bp ladder as a molecular weight marker for confirmation of the length of the PCR products. Gels were stained with ethidium bromide (1 µg/ml).

Single strand conformation polymorphism analysis

The PCR products were resolved by SSCP analysis. Several factors were tested for each fragment in order to optimize factors such as the amount of PCR product, denaturing solution, acrylamide concentration, percentage cross linking, glycerol, voltage, running time and temperature. The SSCP analysis was carried out as follows: each PCR product was diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA) denatured at 95°C for five min, chilled on ice and resolved (10 h, 375 V, 4°C) on 12% acrylamide:bisacrylamide gels (29:1). The electrophoresis was carried out in a BioRad Protean II xi vertical electrophoreses unit using 1X Tris-borate-EDTA (TBE) buffer. Gels were silver-stained (Sambrook and Russell, 2001) and dried on cellophane using a Biorad Model 583 gel dryer. The gel phenotypes were identified and scored manually to obtain PCR-SSCP allele frequency (Table 2).

Sequencing

The DNA samples showing different patterns on SSCP gels were selected for sequencing. For sequencing, the PCR products was either used directly in the reaction or firstly cloned into the plasmid vector T4 (Cloning kit, Banglore Genei). Primers (IDT, USA) used in sequencing are same as for SSCP shown in Table 1. PCR products were purified from all the SSCP variants in the duplicate using PCR cleaning kit (Biogene). Single pass sequencing was carried out using the big dye terminator chemistry version of ABI as per the manufacturer's protocol. The quality of the sequence was assured by the PHRED (Ewing and Green, 1998; Ewing et al., 1998).

Table 1. Sequences of primers designed (GenBank accession number Z33881 as template) for PCR-SSCP of β-LG gene of *Capra hircus.*

Primer		Primer sequence	Size (bp)
Exon 1	1F	5'- CGGGGATGAGCCAAGTAGGA-3'	20
(Fragment 1- 334 bp)	1R	5'- AACCCGACGTCACAGCCTCT-3'	20
Exon 2	2F	5'- CCGGAGTGCAACTCAAGGTC-3'	20
(Fragment 2- 298 bp)	2R	5'- AGCGCCGGGTACCAGTAAAC-3'	20
Exon 3	3F	5'- CTGGCCCTCAGTTCATCCTG-3'	20
(Fragment 3- 281 bp)	3R	5'- GGCAAGTGAAGCAGGTGTCA-3'	20
Exon 4	4F	5'- CGCCGTTGGAACTCACTTTC-3'	20
(Fragment 4- 209 bp)	4R	5'- TGTTGCAGCAAGGACCACAC-3'	20
Exon 5	5F	5'-GGAGGAAGGGAAGGGAACT-3'	19
(Fragment 5- 376 bp)	5R	5'-GTCACCATGGGAGGAGGAG-3'	19
Exon 6	6F	5'- GGTGTCCAGTCCCATCCTGA-3'	20
(Fragment 6- 278 bp)	6R	5'- GAGGCCAGGACACAGGTCAC-3'	20
Exon 7	7F	5'- GTGCCCCAGGAATCACAG-3'	18
(Fragment 7- 250 bp)	7R	5'- CCGTTGTCCAGGAAAGACTC-3'	20

Table 2. Polymorphisms in *Capra hircus* β-LG gene.

Location	Nucleotide position[+]	Polymorphism	Breed
Promoter	-66	C/T	Jakhrana
Exon 3	1806 (3E+9)[#]	C/T	Jamunapari
Exon 6	4122 (6E+32)	T/C	Jamunapari
Exon 7	4601 (7E+82)	G/A	Jamunapari/Jakhrana
	4603 (7E+84)	G/C	Jakhrana
Intron 2	962 (2I+11)	C/T	Jamunapari
	986 (2I+35)	T/C	Jamunapari
Intron 3	1907 (3I+37)	C/T	Jakhrana
Intron 6	4143 (6I+12)	G/A	Jamunapari/Jakhrana

[+]Position of polymorphic sites relative to goat β-LG gene (GenBank accession no. Z33881). [#]Position in the parentheses indicate standard nomenclature of exons and introns

***In silico* sequence analysis and allele mining**

The nucleotide sequences generated after sequencing for each primer pair was edited using Chromas software and in order to check the identity of our sequence, BLAST analysis was also performed. The sequence was aligned with Clustal W program with other already deposited nucleotide sequences of β-LG gene of *Capra hircus*. The nucleotide sequences and the deduced amino acid sequences were analyzed with Sequin software and deposited in Gen Bank (NCBI, USA) under accession numbers EU573196 - EU573209.

Extensive allele mining of β-LG is done and nucleotide variations were found between species and within breeds of *C. hircus* using Megalign (DNA star-Lasergene® v7.2 software). Two algorithms of alignment, Jotun Hein method and Clustal method, were used.

RESULTS AND DISCUSSION

Jamunapari goats are well known for milk production, probably highest amongst the Indian goat breeds

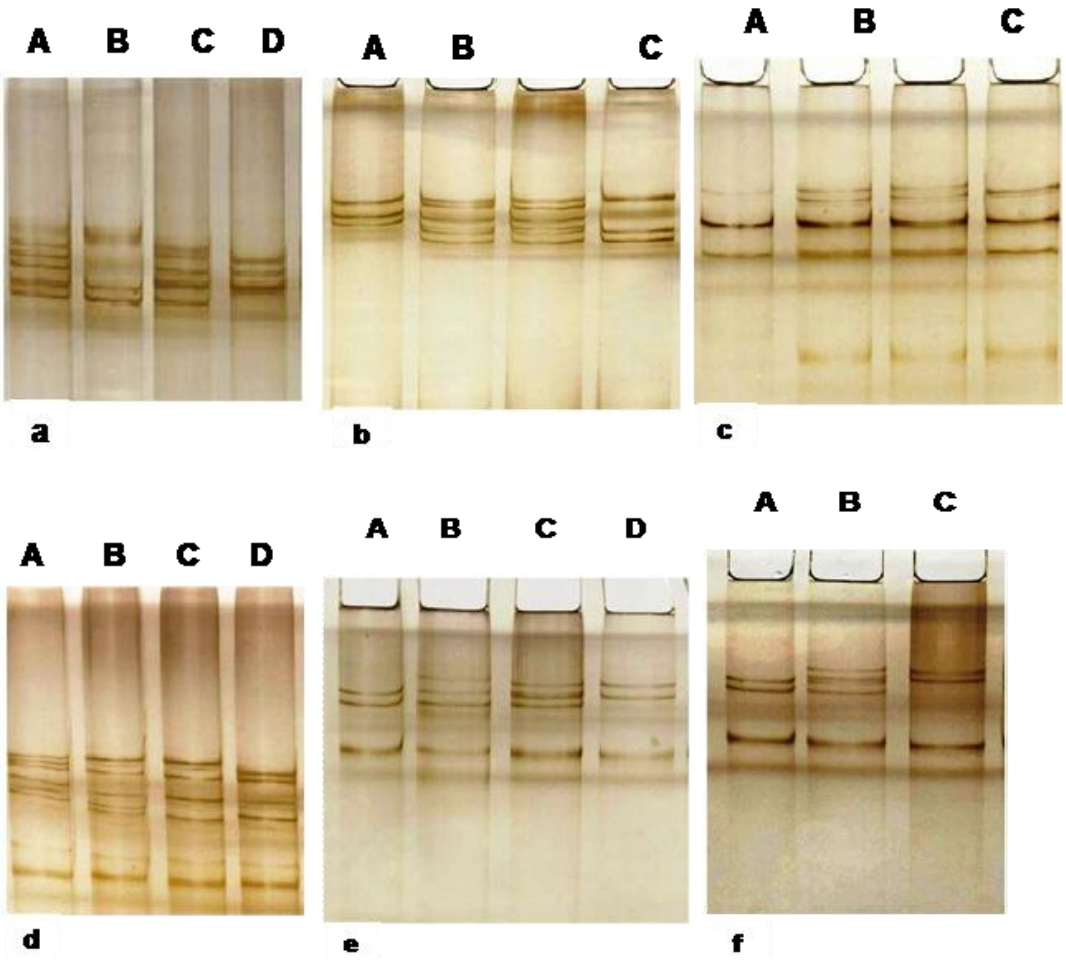

Figure 1. (a and b) showing the SSCP patterns of BLG exon-3 of Jamunapari and Jakhrana goats respectively. (c and d) SSCP patterns of BLG exon-6 of Jamunapari and Jakhrana goats respectively. (e and f) SSCP patterns of BLG exon-7 of Jamunapari and Jakhrana goats respectively.

producing 201 kg/lactation (Acharya, 1982). The breed has an excellent milking ability and growth potential prolific and non-seasonal breeder (Devendra, 1985). Though Jamunapari goats have been considered the best milch breed among goat breeds of India but Jakhrana goats are not less than that (Verma et al., 2004). The average daily milk yield has been reported to be 3.25 kg varying from 2.0 to 4.5 kg (Verma et al., 2004). Higher milk yield (5 to 6 kg per day) has also been reported by earlier workers (Rai et al., 2000). β-Lactoglobulin is a major whey protein in the milk of ruminants. β-Lactoglobulin genetic polymorphisms are important and well known due to their effects on quantitative traits and technological properties of milk. Therefore, we screen the β-lactoglobulin of these breeds for the single nucleotide polymorphism.

Single strand conformational polymorphism analysis

In the optimization of PCR-SSCP of this gene for greater sensitivity and better resolution the PCR product and denaturing solutions ratio 1:4; acrylamide concentration 12%; percentage cross linking 3.3%; 8 volt/cm; running time 10 h and running temperature 4°C were found with improved resolution of the gel, except glycerol

In the present study, genetic variability in β-LG genes was assessed by SSCP technique, which allows detection of changes in the nucleotide sequence of a PCR product affected by single base substitution. Figure 1 shows SSCP banding pattern for β-LG gene exons. The PCR-SSCP of β-LG gene fragments exhibited 39 different conformations or gel phenotypes, identified in seven fragments which represent seven exons. The genotype and allele frequencies of β-LG gene are presented in Table 3.

In our study, a total of 39 gel phenotypes were observed in different fragments of β-LG exons, but in sequencing many of them did not revealed any SNPs. Such observations are not uncommon. In a study by Barracosa (1996), such unusual patterns of gel phenotype is reported which is because of existence of

Table 3. Genotype frequency of SSCP variants of β-lactoglobulin exons.

Specified region of the gene	Alleles and their frequency	
	Jamunapari	**Jakhrana**
Exon 1	A=0.84 , B=0.14, C=0.02	A=0.98, B=0.02
Exon 2	A=0.76, B=0.18, C=0.06	A=0.66, B=0.34
Exon 3	A=0.42, B=0.30, C=0.22, D=0.08	A=0.10, B=0.60, C=0.30
Exon 4	A=0.80, B=0.20	A=0.90, B=0.08, C=0.02
Exon 5	A=0.65, B=0.35	A=0.84, B=0.16
Exon 6	A=0.34, B=0.26, C=0.30	A=0.48, B=0.22, C=0.12, D=0.18
Exon 7	A=0.18, B=0.30, C=.20, D=0.32	A=0.36, B=0.56, C=0.08

more than one confirmation of even exactly similar DNA sequence (PCR-SSCP products) especially when SSCP gel is more optimized having highest sensitivity. Comparing with other methods, for instance denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, chemical and ribonuclease cleavage (Fischer and Lerman, 1980; Myers et al., 1985; Cotton et al., 1988; Riesner et al., 1989), SSCP has several advantages: it does not require specific equipment, it is technically simpler and faster, it can be used in most laboratories and is not very expensive (Orita et al., 1989). Several authors have pointed out that SSCP analysis is a reliable and reproducible technique for the detection of structural gene polymorphism which is primarily due to point mutations (Neibergs et al., 1993; Sheffield et al., 1993; Barroso et al., 1999; Jain et al., 2008).

Sequence analysis and SNP identification

Comparison between nucleotide sequence of goat β-LG gene (accession no. Z33881) using MEGALIGN revealed complete homology except SNPs at specific nucleotides. Figure 2 shows alignment of the coding region of caprine β-LG, as per CLUSTAL method.

To detect polymorphisms in the coding region of the goat β-LG gene, we amplified and sequenced seven fragments corresponding to all the β-LG exon (coding and non-coding region) with part of their flanking region from two highly milk producing goat breeds of India. Comparative sequence analysis revealed C/T substitution at -66 position in the promoter region. Yahyaoui et al. (2000) reported polymorphism at -60 position in the promoter region, while nine polymorphism including deletion/insertion has been reported in different goat breeds (Ballester et al., 2005). Thus, after our study a total of 11 polymorphic locations have been located in the promoter region of goat β-LG gene. However, further studies are required to evaluate the effects of these variants in the expression of the β-LG gene.

Four single nucleotide substitution were found in intron 2, 3 and 6 at position 962 (C/T), 986 (T/C), 1907 (C/T) and 4143 (G/A). Before this study, no intronic region variants have been reported yet in BLG gene of goats. These intronic SNP might be employed as a marker in association studies to determine whether genetic variation at the goat BLG locus has any quantitative effect on BLG synthesis

In exon of BLG, four variants were found in exon 3, 6 and 7. In exon 3, single nucleotide polymorphism at position 1806 (C/T) leads to synonymous substitution while the single nucleotide substitution in exon 6 at position 4122 (T/C) lies on the non-coding region.

Two Single nucleotide substitution were found in exon 7 at position 4601 (G/A) and 4603 (G/C). The polymorphism at position 4601 (7E+82) has been reported previously in Spanish and French goats (Pena et al., 2000) and the polymorphism at position 1806 (3E+9) and 4122 (6E+32) has been characterized recently in different goat breeds (Bellester et al. 2005). These same three polymorphisms in the exons of Indian goat breeds suggest a selection pressure at this DNA region. Ju et al. (2011) reported that SNP in Transferrin (Tf) gene affects milk production, as well as mastitis-resistance traits, in Chinese Holsteins. Kumar et al. (2006) analyzed the polymorphism at both DNA and protein level in Indian goat breeds to observe the effect of the BLG genotype at position 4601 as described by Pena et al. (2000) and was found that BLG AA genotype had a higher milk yield than BLG AB genotype in both Jamunapari and Barbari breeds. However, they did not report any new nucleotide polymorphism in the Indian goat breeds. In our study a novel single nucleotide substitution has been found at exon 7 besides the SNP reported by Pena et al. (2000).

Conclusion

Full coding region of β-lactoglobulin gene of *C. hircus* was analyzed to detect single nucleotide variations and corresponding changes in amino acids and to provide a baseline data of Indian goats. A total of nine single nucleotide substitutions were found throughout the gene, of which six variants were new. Further, on the basis of these finding, real time PCR base allelic discrimination test can be developed. Before any association study is

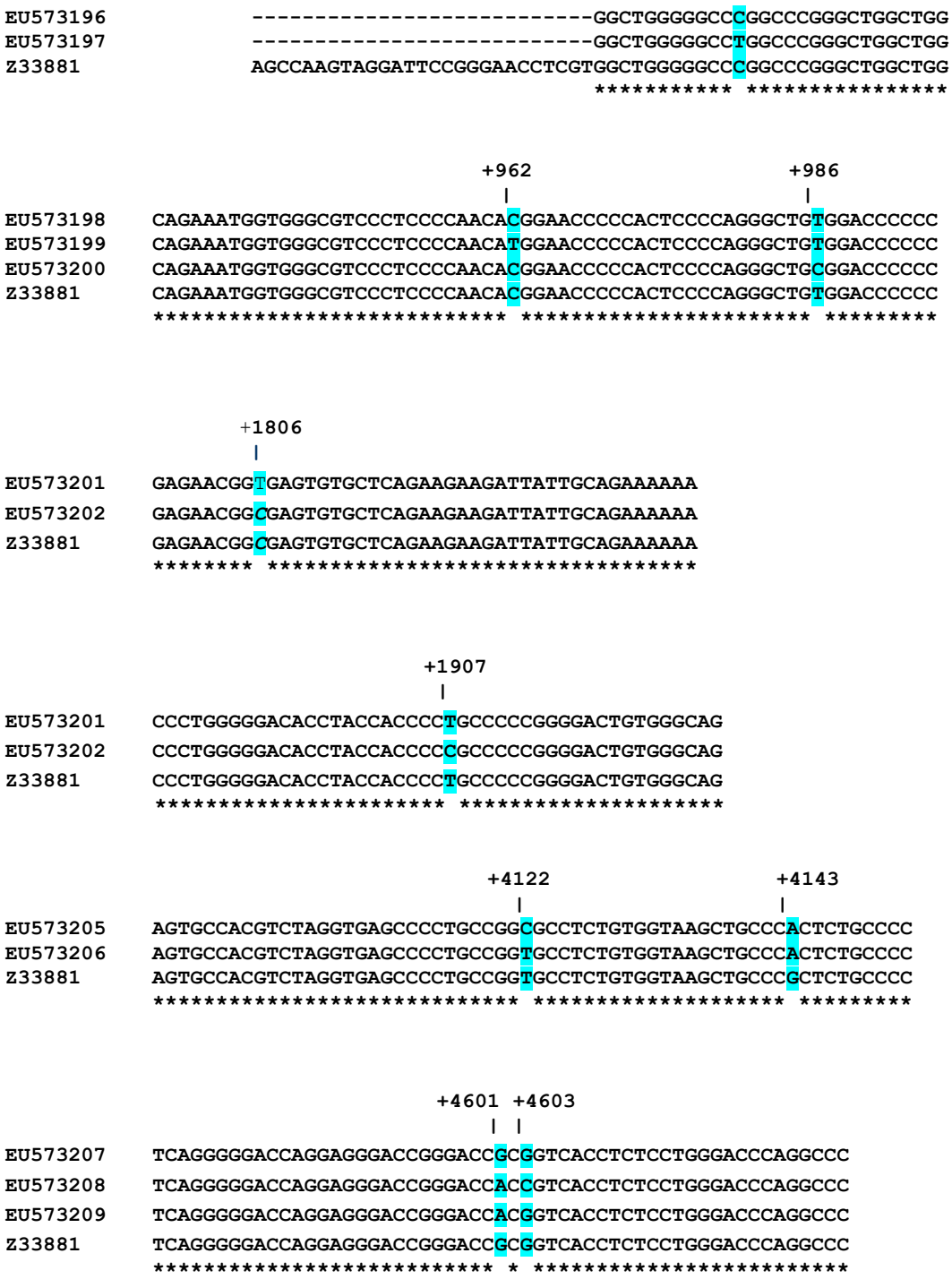

Figure 2. Comparative nucleotide sequence alignment of PCR-SSCP haplotypes of BLG gene (with GenBank accession numbers) showing total of nine nucleotide variation at respective positions with GenBank reference sequence Z33881.

taken up, there is a great need to have base line data of existing gene variants in our indigenous high milk producing goat population. In the modern context the gene variants (SNPs and INDELs) of even un-translated region (introns, 5' UTR, 3' UTR, promotor regions etc.) of a gene, are also found associated with expression of the traits/regulation of traits. Thus the identification of variations/variants across the entire gene in the existing gene pool is mperative. Such molecular data shall be pivotally critical in future association studies and thus

molecular markers may be developed. These developed genotyping techniques of these milk related genes can be used in substantially large number of samples covering many generations for future association studies. Such attempt to increase the milk production through marker assisted selection especially in low input system of the country, where feed efficiency is equally critical for milk production becomes very relevant.

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