Research Article

Association of κ -casein Gene (CSN3) Polymorphism with Milk Production Traits and Protein Structure Changes in Iranian Mahabadi Goat

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Abstract

The κ-casein protein plays a fundamental role in milk production and its composition synthesis in mammary gland. This protein has an important role in the formation, stabilization and aggregation of the casein micelles. In this study, we investigated the genetic variability at the exon 4 of the κ-casein gene (also known as CSN3) using PCR-SSCP analysis and DNA sequencing. Then a protein sequence and structural analysis were performed in order to predict the possible impact of amino acid substitutions on physicochemical properties and structure of the κ-casein protein. Blood samples from 167 Iranian Mahabadi goats were collected DNA extracted and then the then 458bp fragment of the CSN3 gene was amplified using PCR-SSCP. Sequencing analysis showed that there were 8 single nucleotide polymorphisms (SNPs) in the CSN3 exon 4 (g.83C >T, g.85G >A, g.122A>G, g.147A>G, g.222G>A, g.309A>G, g.388T>C and g.429C>T). From the 8 identified polymorphic sites, six sites were non synonymous substitutions and the other two were synonymous substitutions. Also, association analysis showed that genotypes of the SNPs 1, 6, 7 and 8 were significantly associated with fat percentage in the analyzed population ($P<0.05$), but there were not significant association between genotypes and milk production, percentage of protein, lactose, solid-not-fat SNF, protein and fat yield. In addition, the non-synonymous SNPs substitutions do not have affect in the physicochemical properties (hydrophobicity, isoelectric point and net charge) of κ-casein protein. These results suggest that polymorphism at the CSN3 gene could be used as a molecular marker for goat milk's fat percentage.

Key words: CSN3; Gene, Trait; Goat; Polymorphism

Introduction

Goat products have a favorable image in the world; thus, goat farming is practiced worldwide (1, 2). The goat population has increased globally despite major changes in the agriculture due to industrial mergers, globalization, and technological advances in developed countries. The goat production is one of the key elements contributing to the economy of farmers living in the arid and semi-arid regions including most areas of Iran (3). Thus, traits affecting economic viability include those associated with growth, milk, meat and cashmere yield (4). Exploiting the genes associated with economic characteristics of farm animals in the marker assisted selection programs (5) can help with the selection of animals with the most desirable breeding values. Choosing the best genotypes based on the phenotypic values of the animals for quantitative characters is difficult (3). In other words, phenotypic values do not always reflect the genotypic values, especially additive genotypic values of the animals. The improvement of any trait in a population primarily depends on its economic gain (6).

The studied milk production traits of Mahahadi breed goats are presented in Table 1. Up to

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now, no information had been reported about appearance characteristics and milk production traits in Mahahadi goats breed. The average weight of males and females at puberty was 90-100 kg and 38-58 kg, respectively. According to Table 1, average daily milk production, percentage of fat, protein and SNF were 1.03kg, 2.29%, 3.74% and 9.96%, respectively.

Table 1: Statistical characteristics of the data

Trait (daily production)	number	mean	standard deviation	CV
Milk production (kg)	2096	1.03	0.58	56.98
Fat yield (kg)	1649	0.022	0.02	88.94
Fat percent (%)	1664	2.29	1.26	54.99
Protein yield (kg)	1647	0.035	0.02	56.4
Protein percent (%)	1662	3.74	0.39	10.33
SNF percent (%)	1570	9.96	1.18	11.82
Lactose percent (%)	1561	5/45	0.55	10.05

The SSCP technique is most applicable as a diagnostic tool in molecular biology. It can be used in genotyping to distinguish homozygous individuals of different allelic statuses, as well as heterozygous individuals that should each demonstrate distinct patterns in an electrophoresis experiment (7).

The study of genetic polymorphism of casein proteins in dairy goats milk has gotten more attention for their close relationship with milk quality, milk composition and technological processing (8). The caseins are including: αs-1, β, αs-2 and κ (9), which are coded by CSN1S1, CSN2, CSN1S1, and CSN3, respectively, and all of them, except for κ, are considered as calcium-sensitive caseins. Moreover, they are located in a DNA fragment of about 250kb and mapped on the chromosome 6 in cattle, sheep and goat (10). These proteins are the major nutrients resources for baby in the mammalian placenta(11). Although, Threadgill et al. (1990) and Yu-Lee et al. (1986) suggested that genes of calcium-sensitive caseins may have evolved from a common ancestral gene by events such as exon shuffling and gene duplication. The κ-casein gene has evolved from Υ-chain of fibrinogen (12-14). The *κ-casein* (CSN3) has an important role in the formation, stabilization and aggregation of the casein micelles. Therefore, they alter the manufacturing features and digestibility of milk, and also they are involved in the size and specific function of milk micelles (15). The total size of CSN3 is about 13 kb divided into 5 exons and presents two common genetic variants, A and B and these alleles differ by substitutions in 2 amino acids at positions 136 Thr(A)/Ile(B) and 148 Asp(A)/Ala(B) (9).

Mature κ-casein protein has an unstable peptide band which was divided in two parts by rennin activity in gut, containing an insoluble peptide (Para κ-casein or PKC) and a soluble hydrophilic glycopeptide (caseinomacropeptide or CMP). The PKC causes milk clotting which increased milk digestibility by increasing retention time of milk in the gut. The CMP plays a role in decreasing immune response in newborn kids and thus it reduce sensitivity of digested proteins in gut (16). The κ-casein gene was widely analyzed at DNA level in the majority of goat breeds around the world and up to now, 16 alleles related to this gene have been detected (17).

Nilsen et al. (2009) separated casein cluster into two haplotype blocks in cattle, one of them included αs-1, β and αs-2 genes, and the other one was *CSN3* gene (17). The highest significant effect was found with protein and milk yield in single SNPs and within haplotypes of the first block. In contrast, no significant relationship was found for single SNPs and haplotypes with protein and milk yield within the second block. Shekar et al. (2006) were failed to see lactation in their mouse after elimination of *CSN3* mutations (18). Mohammadi et al.

(2009) studied Kappa Casein gene in local and Holstein dairy cattle in Kerman province using PCR-RFLP method. They estimated gene frequencies 0.70 and 0.30 for the A and B alleles, respectively, with an average heterozygosity of 0.43 in local cattle and were 0.75 and 0.25 for the A and B alleles, respectively, with an average heterozygosity of 0.36 in Holstein cattle. Shannon's and Nei's indexes were 0.63 and 0.44 for local caws and 0.56 and 0.37 for Holstein cattle respectively. Based on their information at this locus, no evidence was found of disequilibrium in the populations. Comparison with allele frequencies

in other cattle breeds indicated that frequencies in these animals are within the range of published results for Bos taurus and Bos indicus breeds. Local and Holstein dairy cattle in Kerman showed a high degree of genetic variability for the kappa casein locus, with a frequency of the B allele of 0.30 and 0.25 respectively. Furthermore, determination of gene polymorphism is important in farm animals breeding (5, 19-23). Yet so far, no study concerning the polymorphisms of CSN3 gene in Iranain Mahabadi goat and its association with milk production has been published. Hence, in the present study, we evaluated the genetic variability in the exon 4 of the CSN3 gene in Iranain Mahabadi goats using PCR-SSCP and DNA sequencing methods. Moreover we evaluated the association between this genetic variability and protein structure changes with milk production traits in Mahabadi goat breed.

Materials and Methods

Ethics Statement

All experiments with animals were done based on the Guide for the Care and Use of Laboratory Animals of the Research Station of Department of Animal Science, University of Tehran, Iran.

Animals

The Mahabadi goat is an indigenous breed reared in the rural areas of the east Azerbaijan and west Azerbaijan provinces (Iran). A total number of 167 Mahabadi goats (2-6 years old) were used in this study. These goats were reared under same environmental conditions in order to improve milk production in the experimental farm of animal science department at the University of Tehran, Karaj, Iran.

Milk samples and data collection

The milk production data from 2011 to 2013 were utilized in the association analysis. But milk composition data were recorded only in 2013. In each year, milk yield and milk composition data were weekly collected within a period of 17 weeks, until milk yield dropped significantly (150-200 g/day). Goats were milked with portable milking machine. The first milk recording data for each goat, was done between 10 and 25 days after kidding (24). Before data recording, the kids were penned separately overnight for 12h. The goats and kids were kept together except in recording days. Recording of each goat was performed separately and before sampling, whole milk of each goat was fully stirred. Then a milk sample of 50 mL was taken from each goat, and the samples were transferred to the ice box. Afterwards the samples were transported to the central laboratory of animal science department at the University of Tehran. Before analysis, the samples were heated to 38°C. The milk composition percentage of fat, protein, lactose, and SNF (milk solids-not-fat) were measured using MilkoScan (Milko-Scan 133B; Foss Electric, Denmark).

Blood sample collection and DNA preparation

The blood samples (6 mL per goat) were collected from Jugular vein and then they were kept in a tube containing EDTA as an anticoagulant. All blood samples were placed on ice, immediately transferred to the laboratory and stored at −20°C. The genomic DNA was extracted from leukocytes using salting out procedure and then dissolved in water (25).

PCR amplification

In order to amplify a 458-bp fragment of CSN3 gene, genomic DNA were subjected to PCR using the primers reported by Coll et al. (1993)(26). The PCR was conducted in 25µl reaction mixture containing 1x PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 µM of each primer, 50-100 ng genomic DNA, and 1 U Taq DNA polymerase (SinaClon Company, Iran). The cycling protocol was 5 min at 94°C followed by 33 cycles of 95°C for 1 min, annealing at 57.5°C for 1 min, 72°C for 1 min, final extension at 72°C for 4 min.

Single-strand conformation polymorphism (SSCP) analysis

The PCR-SSCP method was used to detect all mutations within the amplified fragment. Aliquots of 6µl of PCR products were denatured at 96°C for 10 min in 14 µL of denaturing solution (99%

formamide, 25 mM EDTA, 0.025% xylenecyanole, and 0.025% bromophenol blue), and then immediately chilled on ice before loading. Samples ran for 15h at 300V in 4°C on 12% acrylamide: bisacrylamide gel (37.5:1). The bands were visualized submerging the gels in a 0.1% silver nitrate and NaOH solution (containing 0.1% formaldehyde) (27). The PCR fragments from different SSCP patterns were sequenced in both directions. Nucleotide sequence alignments, translations and comparisons were carried out using the BioEdit 7.2 software.

Statistical analysis

The MIXED procedure (SAS, 2007) was used for the association analysis using 2116 edited records from 130 goats. The compound symmetry, autoregressive and unstructured models were compared according to Akaike's information criterion in order to choose the optimum model. The best model was the autoregressive model which assumes that all repeated measures have heterogeneous variance and covariance. Therefore, the REPEATED instruction of the mixed linear model procedure of SAS was implemented with the AR (1). Using following equation model:

$$Yijklmn=\mu + Ai + Mj + Nk + Gl + b1(BWijkl-) + b2(DIMijkl-) + Animalijklm + eijklmn$$

Where Yijklm: the records of milk yield, fat percentage, fat yield, protein percentage, protein yield, SNF percentage and lactose percentage of milk Ai:the effect of ith animal age, Mj: the effect of jth recording month, Gl: the effect of lth genotype, Nk: the effect of kth kidding years, BWijk: the effect of body weight of each animal at kidding, DIMijkl: the effect days in milk, Animalijklm: random effect of animal, eijklm: the effect of residual factors and b1 and b2 are linear regression coefficients of covariate variables.

Protein structure

Initially, to predict the protein structure, the sequence of Capra hircus mRNA of CSN3 was retrieved from NCBI GenBank database (GenBank ID: 406034769) and then, using blastx in NCBI, open reading frame (ORF) detected for CSN3 protein. Later, using BioEdit 7.2 software, mutations were induced; it was back translated to protein by translate tool available in the ExPASy website.

After conversion coding sequence to protein sequence, three-dimensional structure of protein was predicted via I-TASSER.4 software. There are five predicting models for three-dimensional structure of protein by I-TASSER.4 software. The C-score is a confidence score for estimating the quality of predicted models by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5, 2], where a C-score of higher value signifies a model with a high confidence and vice-versa. After this step, predicted protein tertiary structure in PYMOL.1 was comprised for the absence and presence of mutation. In order to analyze the possible impact of amino acid substitutions on physicochemical parameters (hydrophobicity, isoelectric point and net charge) of the CSN3 protein we used GPMAW and ProtParam tool available on the ExPASY website.

Results and Discussion

Polymerase Chain Reaction (PCR)

The PCR resulted in amplification of a 458-bp DNA fragment belonging to the exon 4 of the CSN3 gene. The results showed that amplification fragment sizes were constant with the target ones and had a good specificity, which could be directly analyzed by SSCP (Figure 1).

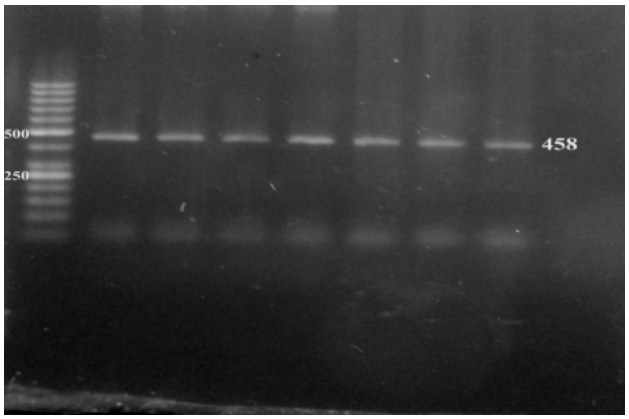

Figure 1: Electrophoresis on a 1.5% agarose gel of the PCR products of the Mahahadi breed goats CSN3 gene exon 4. Line 1: 100bp marker; Line 2-8 PCR products.

Single strand confirmation polymorphism (SSCP) analysis

Detected patterns were nominated as A, B, C and D (Figure 2).

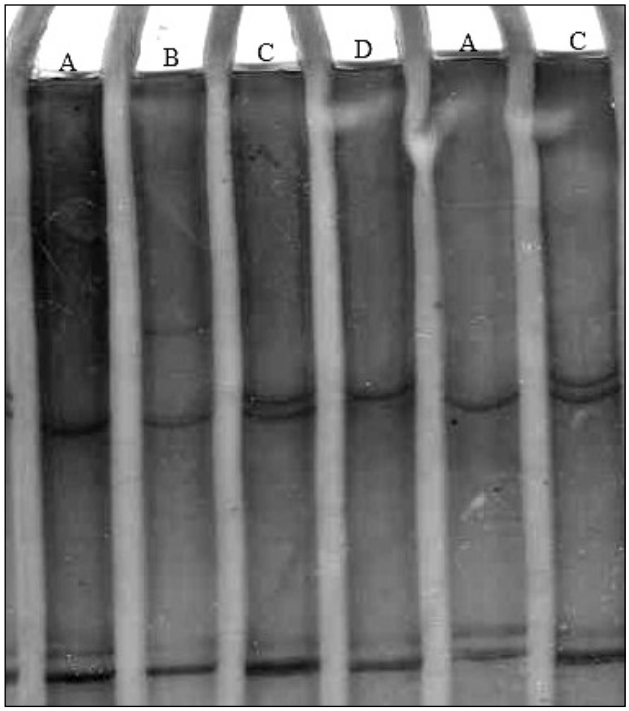

Figure 2: SSCP pattern of the amplified fragment of the Mahahadi breed goats CSN3 gene exon 4, on 12% acrylamide:bisacrylamide gel

DNA sequence analysis and protein structure changes

Sequence analysis of the capra hircus *CSN3* locus allowed the identification of 8 single nucleotide polymorphisms (Figure 3 and Figure 4). These SNPs were g.83C>T, g.85G>A, g.122A>G, g.147A>G, g.222G>A, g.309A>G, g.388T>C and g.429C>T, identified all in the exon 4. Detected mutations in these sequences were compared with NCBI reference sequence; Gene ID: 406034769 (28).

Among the above mentioned polymorphic sites, six were non synonymous and the other two were synonymous substitutions. All the detected substitutions were A to G or T to C and vice versa. The nucleotide substitutions and amino acid changes in CSN3 gene exon 4 are shown in Table 2. None of SNPs, wild type homozygote genotypes of *CSN3* gene were observed that might be the reason small samples size and genetic drift. The SNPs positions, allele frequencies and genotypic frequencies for each SNP are shown in Table 3.

The *CSN3* gene was analyzed by PCR-SSCP to identify different alleles in studied population (29). After SSCP and sequencing analysis were identified A, B, D and M alleles in the Mahabadi breed that A and D alleles had the highest frequency (Table 4). Vacca et al. (2014) found that A and B alleles were the most frequent alleles in the Sarda breed (30). Also, the researchers reported D and M alleles exhibited the lowest frequency in *CSN3* gene (8, 15). In the study of Zepeda-Batista et al. (2015) frequency of A and B alleles were 0.26 and 0.69, respectively (31).

SNP4 and SNP6 had lower frequency (6%) in comparison with the other SNPs. Also, according to the study of Prinzenberg et al. (2005), frequency of these SNPs were low in Sokoto breed (15).

Table 2: Nucleotide substitutions and amino acid changes in the CSN3 gene exon 4 sequences of Mahabadi goat breed

SNP .No	Nucleotide substitution	Nucleotide position (accession No. (406034769	Codon .No	Amino acid change	Type of SNP
1	C/T	83	-	-	Synonymous
2	G/A	85	28	*R to Q	Non- synonymous
3	A/G	122	-	-	Synonymous
4	A/G	147	49	I to V	Non- synonymous
5	G/A	222	74	D to N	Non- synonymous
6	A/G	309	103	I to V	Non-synonymous
7	T/C	388	129	V to A	Non-synonymous
8	C/T	429	143	P to S	Non-synonymous

R: Arginine; Q: Glutamine; I: Isoleucine; V: Valine; D: Aspartic acid; N: Asparagine; A: Alanine;P: Pro-* line; S: Serine

Table 3: Genotypic and gene frequencies for each SNP of *CSN3*-exon-4 in Mahabadi breed goat.

SNP	Position (accession No. 406034769)	Genotype	.NO	frequency	Alleles (low in parenthese)	allele with low Frequency
1	83	TC	76	0.46	C/(T)	0.23
		TT	88	0.54		
2	85	AA	107	0.65	G/(A)	0.17
		AG	57	0.35		
3	122	GG	164	1	A/(G)	-
4	147	GA	57	0.35	A/(G)	0.17
		GG	107	0.65		
5	222	GA	19	0.11	G/(A)	0.06
		GG	145	0.89		
6	309	GA	90	0.55	A/(G)	0.27
		GG	74	0.45		
7	388	TC	19	0.11	T/(C)	0.06
		TT	145	0.89		
8	429	TC	76	0.46	C/(T)	0.23
		TT	88	0.54		

Table 4: Frequency of *CSN3* variant in Mahabadi goat breed

Patterns	*CSN3* variant	Frequency
A	A	0.45
B	M	0.12
C	D	0.35
D	B	0.08

The effect of amino acid substitutions on physicochemical parameters of Capra hircus κ-casein protein

The three-dimensional structures predicted for exon 4 of *CSN3* gene (Figure 5). Furthermore, the non-synonymous SNPs substitutions did not affect in the physicochemical properties (hydrophobicity, isoelectric point and net charge) the κ-casein protein, that these SNPs might be not influence on its structure and function κ-casein protein. Forces between amino acids in proteins are one of the factors that are involved in protein folding, because both amino acids respect to physical-chemical properties they have transactions with each other. The arginine was changed (positively-charged polar) to glutamine (between uncharged and negatively-charged polar) and aspartic acid (negatively-charged polar) to asparagine (between uncharged and negatively-charged polar), namely the forces between amino acids are unchanged. The side chain attached to the amino acids can be polar or non-polar. The non-polar (hydrophobic) side chains in a protein, belonging to such amino acids as phenylalanine, Threonine, valine, and tryptophan, tend to cluster in the interior of the protein molecule. This enables them to avoid contact with the water that surrounds them inside a cell. In contrast, polar side chains such as those belonging to arginine, glutamine, and histidine, tend to arrange themselves near the outside of the molecule, where they can form hydrogen bonds with water and with other polar molecules. Therefore, hydrophobic property of side chains have essential role in the formation of proteins and protein function. Based on Table 5, mutations created in the *CSN3* gene cause a very slight change in the hydrophobicity and isoelectric point of the κ-casein protein. Bonds among the atoms of the protein are very stable and almost do not change at different conditions. Factors affecting protein charge are such as amino acids side chains in protein structure, terminal carboxyl groups and amino (32). Researchs suggest that the protein charge plays a key role in the location and protein activity (33). Based on results of Table 5, protein charge for κ-casein protein was zero in mutant and wild mode. Therefore, probably SNPs in this position have no effect on the protein function. Probably change of valine to alanine and isoleucine, has no effect on protein function, because these amino acids are non-polar and aliphatic and all of them have the same biochemical functions within the protein. Also as regards change of serine to proline has happened approximately at the end of the chain, it seems that this change has no role in breaking the chain and consequently in the change of the protein function.

Table 5: The effect of amino acid substitutions on physicochemical parameters of Capra hircus κ-casein protein

	Amino acid substitutions (Codon no.)					
Physicochemical parameters	*R > Q(28)	I > V (49)	D > N (74)	I > V (103)	V > A (129)	P > S (143)
Isoelectric point	0.23 < 0.24	0.23 < 0.24	0.23 < 0.24	0.23 < 0.24	0.23 < 0.24	0.23 < 0.24
Hydrophobicity index	1.05 < 1.06	1.04 < 1.06	1.05 < 1.06	1.04 < 1.06	1.05 < 1.06	1.04 < 1.06
Protein charge	0 < 0	0 < 0	0 < 0	0 < 0	0 < 0	0 < 0

R: Arginine; Q: Glutamine; I: Isoleucine; V: Valine; D: Aspartic acid; N: Asparagine; A: Alanine;P: Proline; S: Serine*

Association of polymorphism of CSN3 with milk production traits in Mahabadi goat

We chose to study the *CSN3* gene for different reasons: first, because k-casein is a mammalian milk protein involved in a number of important physiological processes; second, because several studies have reported the presence of QTL affecting milk production traits on bovine chromosome 6 (BTA6). In this respect, two distinct regions on this chromosome affect milk traits (including protein yield, protein percentage, fat yield, fat percentage and milk yield), and one of this region, on BTA6, associated with milk traits maps to the casein cluster (CSN1S1, CSN2, CSN1S2 and CSN3) (34).

Association between traits and genotypes at the *CSN3* gene are reported at Tables 6. Significant difference was found among genotypes of SNPs 1, 6, 7 and 8 for fat percentage ($P< 0.05$). However, in the case of milk production, protein and fat yield, and protein, lactose and SNF percentage,

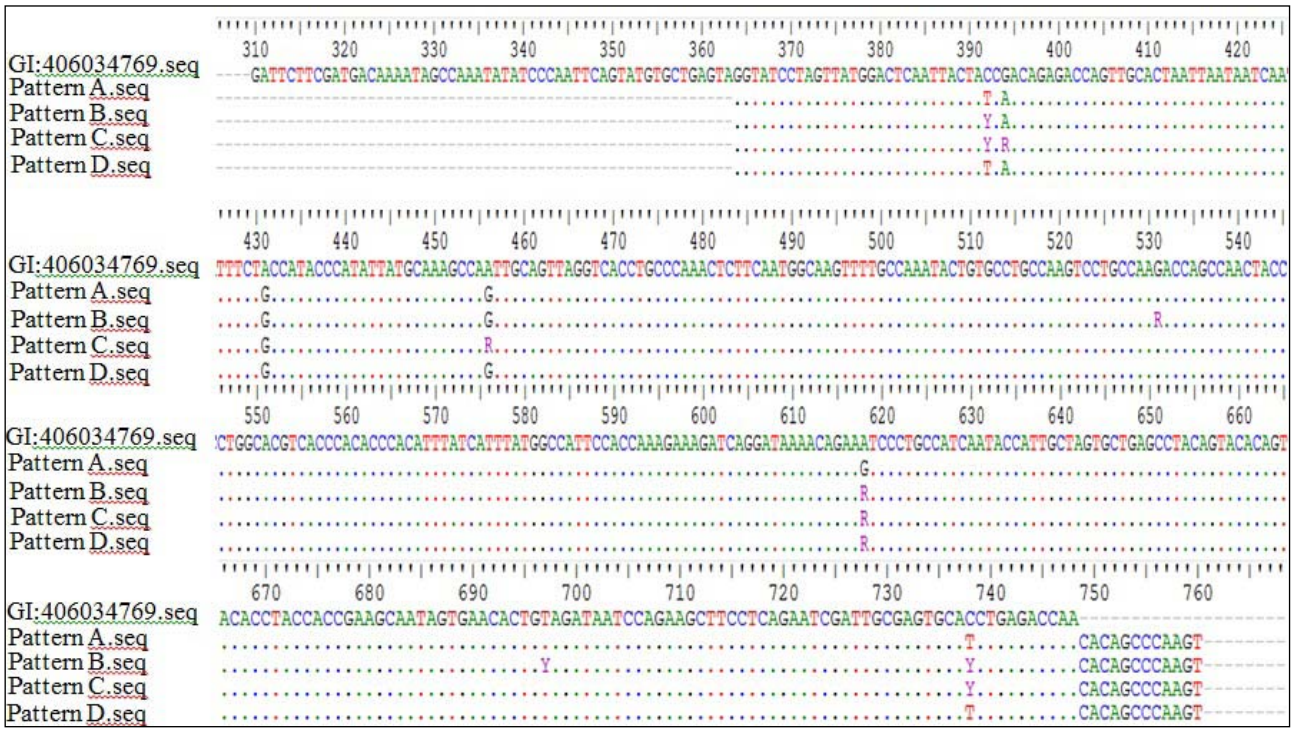

Figure 3: Nucleotide sequence alignment of the *CSN3* gene in Mahabadi goat with using of BioEdit 7.2 software

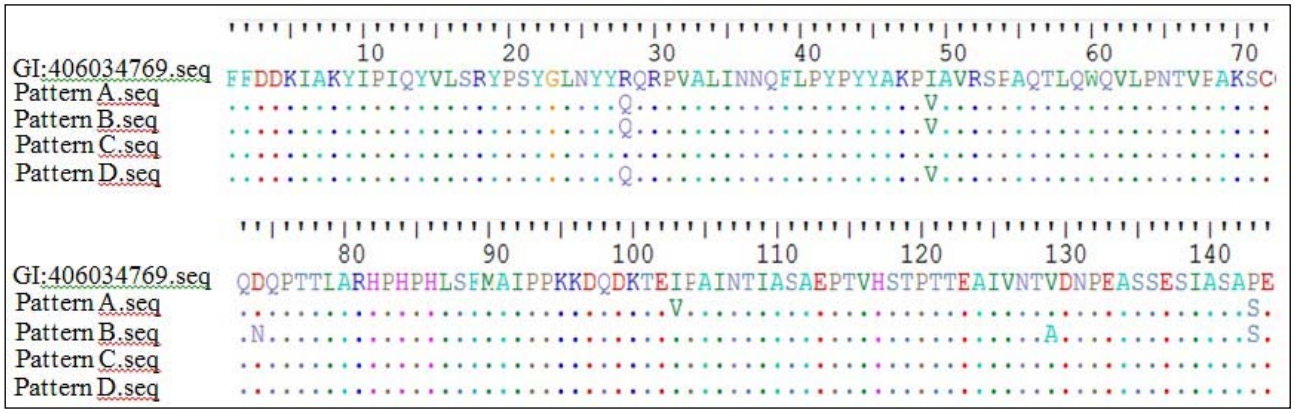

Figure 4: Protein sequence alignment of the *CSN3* gene in Mahabadi goat with using of BioEdit 7.2 software

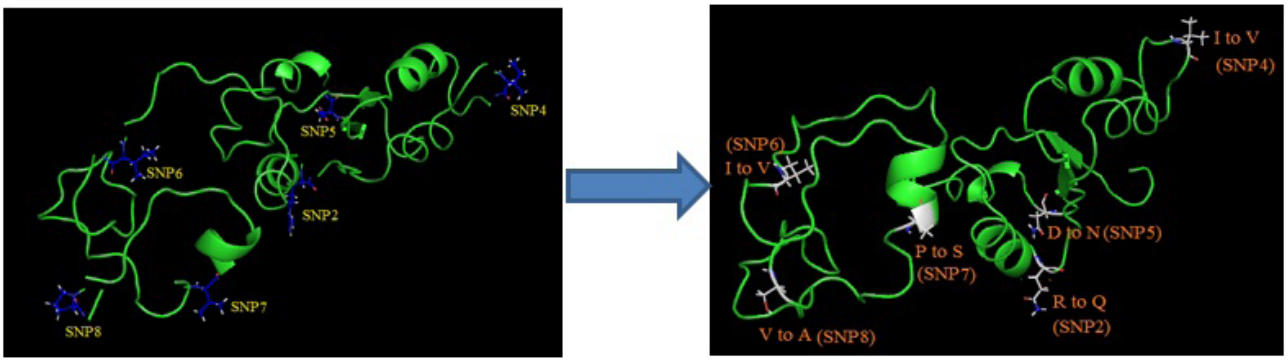

Figure 5. The changes of amino acids and resulting changes in *CSN3* protein structure in Mahabadi goat

there was not found significant difference among the genotypes of all SNPs (P>0.05). The SNPs studied here may not be a causal mutation. Therefore, it seems that eight-SNPs are in linkage equilibrium with QTLs related with milk production, protein and fat yield, and protein, lactose and SNF percentage. Caravaca et al. (2009) and Chiatti et al. (2005) showed that there was an association between polymorphisms at the CSN3 gene with milk protein content in the Murciano-Granadina and Orobica breeds, respectively (11). Conversely, Vacca et al. (2014) reported no association among SNPs of CSN3 with milk production traits. Therefore, our results differ from those of Caravaca et al. (2009) and Chiatti et al. (2005), but are in agreement with Vacca et al. (2014) (11, 30, 35).

Furthermore, Nilsen et al. (2009) stated that the SNPs of *CSN3* gene had no association with milk production, protein yield and protein percentage in cow(34). In addition, Hayes et al. (2006) reported that mutations in the *CSN3* gene promoter region have significant effect on protein and fat percentage in goat (36). Alim et al. (2014) identified three non-synonymous SNPs in exon 4 of bovine *CSN3* gene (37). They showed significant associations between identified SNPs and yield traits (milk, protein and fat) and composition traits (fat and protein percentages), whereas they found no significance for fat percentage in haplotypes association.

Conclusion

It can be assumed that the *CSN3* gene exon 4 in Mahabadi goat exhibited high genetic diversity and the A and C alleles were the most frequent. Also, the SNPs 1, 6, 7 and 8 has associated with fat percentage trait, whereas no association was found between the found SNPs and the other studied traits. The genetic variations of the goat *CSN3* gene may be benefit for fat percentage trait selection and breeding through marker assisted selection (MAS).

markers is one of the best choices for faster and better accomplishment of animal breeding programs (1).

Inter Simple Sequence Repeat (ISSR) is the genome region between microsatellite loci. The ISSR is a molecular marker method which does

Table 6: Association of the single SNPs with milk production traits in Mahabadi goat (LSMean±S.E.)

SNP	Genotype	NO. of animal (NO. of records)	Milk (Kg/ (day	Fat percentage	Fat Yield (kg)	Protein percentage	Protein Yield (kg)	SNF Percentage	Lactose percentage
1	TC	59 (916)	0.86±0.06	2.36±0.11[a]	0.0216±0.002	3.78±0.03	0.035±0.002	10.12±0.07	5.46±0.04
	TT	71 (1200)	0.93±0.05	2.57±0.1[b]	0.0219±0.002	3.77±0.03	0.034±0.002	10.08±0.07	5.44±0.04
2	AG	45(674)	0.84±0.06	2.39±0.12	0.021±0.002	3.81±0.02	0.035±0.002	10.16±0.07	5.48±0.04
	AA	85(1442)	0.92±0.05	2.44±0.1	0.021±0.001	3.79±0.03	0.037±0.001	10.07±0.06	5.43±0.04
4	GA	45 (674)	0.84±0.06	2.39±0.12	0.021±0.003	3.81±0.03	0.035±0.002	10.16±0.07	5.48±0.04
	GG	85 (1442)	0.92±0.05	2.44±0.1	0.022±0.001	3.79±0.03	0.035±0.001	10.07±0.06	5.44±0.04
5	GA	16 (242)	0.91±0.09	2.13±0.2	0.021±0.003	3.76±0.04	0.038±0.003	10.03±0.08	5.43±0.05
	GG	114(1874)	0.92±0.04	2.48±0.07	0.022±0.001	3.77±0.02	0.035±0.001	10.13±0.06	5.46±0.03
6	GA	72 (1138)	0.88±0.06	2.34±0.1[a]	0.0217±0.002	3.78±0.03	0.035±0.002	10.13±0.06	5.46±0.04
	GG	58 (978)	0.92±0.05	2.54±0.1[b]	0.022±0.002	3.76±0.03	0.034±0.002	10.07±0.07	5.44±0.04
7	TC	16 (242)	0.91±0.09	2.13±0.2[a]	0.021±0.003	3.76±0.04	0.038±0.003	10.03±0.08	5.43±0.05
	TT	114(1874)	0.92±0.04	2.48±0.07[b]	0.022±0.001	3.77±0.02	0.035±0.001	10.13±0.06	5.46±0.03
8	TC	59 (916)	0.86±0.06	2.36±0.11[a]	0.0216±0.002	3.78±0.03	0.035±0.002	10.12±0.07	5.46±0.04
	TT	71 (1200)	0.93±0.05	2.57±0.1[b]	0.0219±0.002	3.77±0.03	0.034±0.002	10.08±0.07	5.44±0.04

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