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SNPs association studies in promoter III of the acetyl coenzyme-A carboxylase-α gene (*ACACA*) in Munjal–A threatened sheep population of India

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The abounding treasure of genetic diversity of Indian sheep (*Ovis aries*) lies in the 42 registered breeds (NBAGR 2017) distributed across different agro-climate zones of the country. Systematic attempts to characterize and conserve sheep breeds in India were initiated with the establishment of ICAR-NBAGR, Karnal (Bhatia *et al.* 2005). Several indigenous sheep breeds exist in India, but farmers prefers high milk and meat yielding breeds over indigenous breeds, which have severely affected the population dynamics of indigenous breeds. This has further moved the research interest to meat and milk production from elite breeds in sheep industry.

Munjal, dual purpose (milk and meat) breed is popular among the farmers of Punjab, Haryana and Rajasthan maintained primarily for mutton production due to its better reproductive efficiency (Kushwaha *et al*. 1999, Poonia 2008). Animals of Munjal sheep are quite large in size, tall, rectangular and massive with long tail. Head is long with Roman nose and narrow forehead, both sexes are polled. Ears are large and leaf like hanging down with flat cheeks. Fleece is white and coarse. Udder is well developed and medium sized with medium teats. Rearing under organized farm conditions, Munjal sheep is very economical due to its earlier maturity, faster growth and shorter lambing interval as compared to Magra, Malpura and Muzaffarnagri breeds (Poonia 2008). The scarcity of proven rams and existing genetic dilution in the farmer's flocks due to intermixing is posing serious threat to its identity and population status. In spite of these favourable traits, the security of the breeds is threatened due to an alarming decrease in their population size (Kumar *et al.* 2006, Yadav *et al.* 2010), which is also reflected by their genetic analysis (Arora *et al.* 2004, Yadav *et al*. 2011).

However, there is substantial shrinkage in its breeding tract which has now reduced to small pockets of Karnal (Haryana) and Bathinda (Punjab) districts. Yadav *et al*. (2009) reported threatened status of Munjal sheep in its breeding tract. Munjal breed is not included in the list of descript sheep breeds of India, and is least discussed. Morphological characterization, production and reproduction status of Munjal has been studied exhaustively by Yadav *et al.* (2011).

There is no doubt in saying that sheep farming is the main component for farmers doing dryland farming. It is the only source of income for many people in rural areas. Benefits of sheep farming is increasing day by day. Sheep milk is preferred for cultured dairy products like cheese. With enumerable type and origin of high quality cheese, many of which are labelled as protected designation of origin (PDO) products. The differences in cheese yield from cow/buffalo milk and sheep are primarily due to the high fat content (Jandal 1996). Milk products manufactured from sheep such as ice cream, flavoured milk and milk powder are preferred as alternative food products for young and sick people due to its rich nutritional and antiallergenic properties.

ACACA is important enzyme and it regulates fatty acid biosynthesis by catalyzing the ATP-dependent formation of malonyl-coenzyme A in the mammary gland (Garcia *et al.* 2010). The transcription of the *ACACA* gene is performed in a tissue-specific fashion, with the expression of site specific promoters in different tissues (Travers *et al.* 2005). Structural and functional features of Sheep *ACACA* were studied by Barber and Travers (1995). *ACACA* gene has been sequenced and studied by Garcia (2010); they have identified 22 synonymous SNPs in the 6.6 kb length of the coding sequence. Ten SNPs in the promoter complex region of the gene have been reported by Moioli (2005a, b) in sheep.

Since it plays an important role in the cheese making process it is very important to know the sequences of these genes and to identify the possible polymorphism. For this study, a data set with milk phenotypes recorded for Munjal sheep breed was made available from LUVAS, Hisar. The analysed DNA region included the *ACACA* gene promoter. Since genetic variability in promoters could affect expression of gene, the variation in PIII region might affect the variation in fat and protein content in sheep milk.The PIII region of the *ACACA* gene was directly sequenced in

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sheep samples, and 3 identified SNPs were analysed in sheep population from Hisar (Haryana).

For characterizing the Munjal sheep, random sheep flocks were assessed in their breeding tract from Karnal (Haryana) and Bathinda (Punjab) and data were collected by interviewing farmers. The trials were carried out on the experimental farm of LLRU, Hisar in Haryana. Data was collected from 180 animals on parity, stages of lactation, date of lambing, weight of eve at current lambing, litter size (current) and sex with sire, dam details.

Milk sample (10 ml) was collected from 60 ewes with sodium azide and stored at –4°C. Blood samples (10 ml) were collected using traditional method and stored at –20°C temperature until DNA extraction. The genomic DNA was isolated from the samples using the standard protocol (Shashikanth 1999).

Milk sample were collected by hand milking and transported in standard condition for further analysis in laboratory. Most of the animals were in IInd and IIIrd lactation. Sample were collected during mid lactation period. Milk fat and protein extraction was carried out by standard procedure as described in ISO-IDF (2001). Yields were described in grams of fat and protein and as percentage of fat and protein.

Data were collected from pedigreed farm animal records and analysis was performed on the average daily milk parameters. Allelic substitution effect on milk production traits was estimated by regressing the phenotype on the number of copies each allele using the General Linear Model (GLM) in Statistical Analysis Systems version 9.1.3 (SAS 2007).

DNA sequence was amplified by using primer pairs designed based on the DNA sequence of the ovine *ACACA* gene (GeneBank AJ292286). PCR primers used were forward primer: 5′CGACTGCTTTCCCTCTTGAC3′ and reverse primer: 5′ATTACAATGGGCTCCCACAC3′. The PCR reaction was carried out in a volume of 25 µl, containing 100 ng genomic DNA, 0.5 µl of each primer, 0.3 µl of *Taq* DNA polymerase, 0.5 µl $MgCl_2$, and 0.5 µl of dNTPs. Amplification was carried out in a Biorad thermal cycler with following conditions: 94°C for 2 min; 35 cycles at 60°C for 45 sec, final extension at 72°C for 5 min. The amplified PCR product was assessed by Agarose gel electrophoresis using 1% EE Agarose using 1 × TAE buffer and visualized under UV light. All SNPs identified were custom genotyped by direct sequencing of DNA samples.

Average body weight and body biometry of adult Munjal sheep were collected during the study period. The main lambing season in Munjal is October–November. Age at first lambing is 15–18 months, while age at first breeding of rams is 12–15 months. Since quantity of milk and its composition are economically valuable in domestic animals, researcher have been studying genes associated with or could affect milk production and its composition in sheep. The initial aim was to sequence the target region in *ACACA* gene in sheep and to study the presence of polymorphism and its effect on the economic traits.

An amplicon of 392 base pairs (bp) of *ACACA* gene PIII was obtained after direct sequencing of the sheep DNA fragment. In order to determine if there is any polymorphism in sequences obtained, we compared it with the existing sequences from GenBank. The *ACACA* gene sequence was about 99% identical with its ovine and bovine orthologous sequences after NCBI Blast search. No significant differences in allele frequency ($P<0.01$) were found at any positions.Three SNPs were identified in the analysed samples at nucleotide position 1330: G or T; position 1338: C or T and at position 1430: C or T. For the detected SNPs, allelic frequencies were obtained through direct count and controls were performed to test for Hardy Weinberg equilibrium (HWE). Overall nine genotypes were detected in the analysed samples.

In silico analysis with GenBank sequence AJ292286 confirmed studied region (PIII) fall in the core sequence of putative binding sites of transcription factors so its transcription might be influenced in different genotypes. This could further affect the fat content of sheep milk and its properties thereof. As *ACACA* gene is known to influence the synthesis of fatty acids, several studies have confirmed its role in goat (Badaoui *et al.* 2007) and in other domestic animals (Moioli *et al.* 2005, Zhang *et al.* 2010). Previous studies by Moioli (2005a, b) have reported 10 SNPs in the promoter region of *ACACA* gene in sheep. Since expression of *ACACA* gene is tissue specific manner with transcription initiated and controlled from three different promoters which activates in specific tissues only. Travers (2005) confirmed that PIII transcript regulates mammary gland transcription. Many researchers have studied the regulation and expression of different promoters of *ACACA* gene in various tissues and found out that promoter II and III transcription increases during lactation (Ponce-Castaneda *et al.* 1991). The *in-silico* analysis of the studied fragment showed that region is associated with the core sequence of conserved transcription factor binding site using AJ292286 sequence from GenBank (Van Waveren *et al.* 2008).

The association of the three identified SNP in the PIII region of *ACACA* gene with milk production traits (fat and protein quantity) was studied using pedigree record collected from LUVAS, Hisar. Milk samples from experimental animals were collected during mid lactation period and samples were analysed using standard protocols. The average daily milk yield of fat and protein is presented in Table 1. Average milk yield, fat (%) and protein (%) was 0.97 litre/day, 6.93% and 5.21% respectively.

The allele substitution effects on the milk production trait of one lactation period were estimated (as in Sherman *et al.* 2008) and are presented in Table 2 by regressing the milk fat percentage and protein percentage in total lactation on the number of copies of allele of the SNP. SNP 1330 (G<T) and SNP 1338 (C< T) showed some positive relation with fat (%) and protein (%). Whereas the SNP 1430 (C< T) showed positive effect on fat (%) as well as with fat and protein yield. The estimated value was statistically significant only for fat yield and fat (%) in studied breed

Table 1. Statistics of the average daily milk yield of fat and protein in Munjal sheep

Trait	Mean	SD
Fat (g)	42.9	21.2
Protein (g)	33.6	16.2
Fat (%)	6.93	0.67
Protein (%)	5.21	0.37
Milk yield (litres)	0.97	0.48

SD, Standard deviation.

Table 2. Estimate of the allele substitution effect on the average milk parameters

Trait	1330 bp	P value	1338 bp	P value	1430 bp	P value
Fat (g)	–3.74	NS	–3.27	NS	+11.34	0.11
Protein (g)	–5.03	NS	–4.01	NS	+5.67	NS
Fat (%)	+0.18	NS	+0.12	NS	+0.27	NS
Protein (%)	+0.10	NS	+0.06	NS	–0.15	NS
Milk yield (litres)	–0.145		–0.121		+0.137	

NS, non significant.

(Table 2). Because the SNP is located in the promoter which regulates the transcript that are expressed in the mammary gland, it is possible to infer that, due to a differential transcription of the *ACACA* gene, milk fat content are influenced.

Earlier studies in SNP association analysis with milk traits, performed for one SNP of PIII (g.1330G>T), showed a significant allelic substitution effect (+ 0.33%, P<0.0001).There are no reports of significant associations between this gene and milk fatty acid composition in ruminants. However, several studies have identified relationships between the *ACACA* gene and meat fatty acid composition. Badaoui (2007) and Signorelli (2009) have reported relationships between *ACACA* gene SNPs and milk traits in goats. Zhang (2009) identified several SNPs in the promoter I of the bovine *ACACA* gene, having significant associations with the milk fat composition. *ACACA* gene product is rate limiting enzyme, involved in the biosynthesis of palmitic acid and long chain fatty acids. The dietary intake of palmitic acid, which represents approx 22% of sheep milk fat content, increases LDL levels and probable chances of developing cardiovascular disease. Thus studying the expression of *ACACA* gene could be a step in candidate gene approach towards improving sheep milk composition. However, a comparative study on milk quality parameters with other sheep breeds and populations may also serve as critical input in setting out conservation priority amongst them.

SUMMARY

The coding region of the *acetyl Co-A carboxylase alpha* (*ACACA*) gene plays an important role in *de novo* fatty acid synthesis, we have analysed SNPs in the promoter III (PIII) region of *ACACA* gene, with milk fat and protein percentage in the Indian native sheep breed Munjal. The 180 pedigree sheep from LUVAS, Hisar (Haryana) were analysed for candidate gene association studies. The General Linear Model (GLM) procedure of the SAS was used to determine the genetic parameters. The results revealed three polymorphic patterns at position 1330, 1338 and 1480 in the PIII region, total nine genotypes were detected in studied samples. Milk fat and protein yields obtained showed some relationship, but milk fat and protein percentages were not statistically. Each conformation pattern affect milk production traits, differently. The study shows the relationship of polymorphism in the *ACACA* gene promoter which can be exploited as an indicator in marker assisted selection.

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