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Whey Protein Genetic Polymorphism and Dairy Traits in Goats of South India

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ABSTRACT

Background: The present study investigated the polymorphism in β-lactoglobulin and α-lactalbumin genes in native Malabari and Attappady black breeds in comparison with Saanen Malabari crossbred goats. The polymorphisms in these proteins and their coding genes were reported to be associated with various dairy traits in ruminants.

Methods: Polymorphism detection was carried out by polymerase chain reaction based restriction fragment length polymorphism method. Chi-square analysis was performed to analyse the status of each population. Association of β-lactoglobulin with dairy traits was performed with univariate analysis of variation. Sanger's sequencing of 426 bp fragment of caprine *β-LG* gene was performed commercially and sequences were compared with those of other breeds of various geographical areas.

Result: It was proved that all three populations under study observed Hardy-Weinberg equilibrium with respect to β-lactoglobulin locus. Statistical analysis revealed significant relationship of β-lactoglobulin with peak milk yield in Malabari population studied. Sequence analysis of exon 7 and 3′ flanking region revealed a novel SNP at (C to A transversion) at 7062 position in the native breeds under study. Phylogenetic tree construction and analysis revealed the native Malabari and Attappady black to different clades. The caprine α-LA gene appeared to be monomorphic in all the three population under study. Genetic selection utilizing the association of β-lactoglobulin with peak yield could be used to improve the genetic makeup of goats for high production potential.

Key words: α-lactalbumin, β-lactoglobulin, Dairy traits, Genetic polymorphism, PCR-RFLP, Phylogenetic analysis.

INTRODUCTION

The whey proteins are unprecipitated soluble proteins (β-lactoglobulin and a-lactalbumin) present in whey of ruminants and some monogastrics. The polymorphisms in these proteins and their coding genes offered new explanations for variations in dairy traits. Polymorphism was found in the open reading frame and noncoding regions. The occurrence of polymorphism in milk protein was first described by Aschaffenburg and Drewry (1955). The bovine and ovine β-lactoglobulin (β-*LG*) was reported to be associated with milk yield, milk composition traits as well as milk fatty acid composition (Ng-Kwai-Hang, 1997). The β-*LG* polymorphism was found to be associated with milk production and composition traits in Spanish, French and some Indian goat breeds (Pena *et al*., 2000; Kumar *et al*., 2006). Although genetic polymorphism at β-*LG* region of some Indian breeds were studied, no study has been reported in Malabari and Attappady black. Niksic *et al*. (2021) suggested that differences in the milk parameters were due to different allelic influences on gene expression.

Goat production plays a key role in the rural economy of India. They possess a significant role for the rural poor and marginalized section of the people with respect to their subsidiary income and also provide them with nutritious milk and meat for their own consumption. The preference for goat milk exceeds cow milk as it is rich in monounsaturated and polyunsaturated fatty acids as well as medium chain triglycerides. It is said to be beneficial for people with cardiovascular conditions (Haenlein, 1992) but the production is insufficient to meet the rising demand. Improvement of production through marker assisted selection will have a great advantage to poor farmers. In the light of hiking necessity to improve goat milk production, the objectives of the present study were to analyse and compare the genetic polymorphism at different whey protein loci in indigenous and crossbred goat populations and to find its association if any with various dairy traits. Analysis was carried out in Malabari, Attappady black and Saanen × Malabari crossbred goat.

Apart from milk these breeds are preferred for quality and flavor of chevon, good skin, resistance to diseases, heat tolerance ability and prolificacy. Native genoplasm of these breeds were evolved over generations to adapt adverse agro-climatic conditions of southern India. These native breeds are facing fast genetic degradation and dilution because of intensive production system. Polymorphism analysis of these indigenous breeds reported here would be helpful in

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better understanding of the population biodiversity and formulating action plans on conservation and management.

MATERIALS AND METHODS

Genomic DNA samples

Genomic DNA samples were obtained from three genetic populations; Malabari, Attappady black and Saanen × Malabari crossbred goats, maintained at the University Goat and Sheep Farm, KVASU, Mannuthy. Individual blood samples of each 3 ml of venous jugular blood were collected from 65 Malabari, 25 Attappady black and 30 crossbreds, using EDTA (Ethylene Diamine Tetra Acetic Acid) coated vacutainers and transported with ice packs and stored at -20°C until processed. Genomic DNA was extracted by standard phenol chloroform method (Sambrook and Russels, 2001) with some modifications.

PCR amplification and sequencing

A 426 bp fragment of caprine *β-LG* gene from exon 7 to proximal 3′ flanking region enclosing polymorphic site was amplified using primers reported by Pena *et al*. (2000) at Centre for advanced studies in animal genetics and breeding. Twenty micro liter of reaction mixture contained 50 ng template DNA, 4 µM of dNTPs (Sigma, Bangalore), 25 mM of $MgCl_2$, 10 pico moles of each primers, 1X PCR buffer (20 mM Tris-HCl; 50 mM KCl) and 0.6 U Taq DNA polymerase. Thermal profile was 93°C for 4 minutes, followed by 35 cycles of denaturation (94°C for 1 minute), annealing (55.4°C for 1 minute), extension (72°C for 1 minute) and final extension at 72°C for 10 minutes. PCR was carried out on a peltier thermal cycler (MJ Research Inc.). A 268 bp fragment of exon 3 of α-lactalbumin (*α-LA*) (1769 to 2036) was amplified using the primers reported by Lan *et al*. (2007). PCR was carried out with some variations from the above reaction, using 15 mM $MgCl_2$ at an annealing temperature of 58.5°C. Amplified *β-LG* PCR products of both Malabari and Attappady black goats were sequenced commercially (Bioserve Hyderabad, Pvt. Ltd.).

Milk sample analysis

Thirty milliliter of milk was collected consecutively for two days from the goats in 60 to 90 days of lactation. The fresh milk collected from respective goat was kept for while, mixed with a glass rode and 30 ml was taken in a measuring cylinder. The same procedure was repeated the next day and mean value was found. Milk fat percentage was estimated by Gerber method (IS: 1224, 1977) and total solids in percentage were determined as per Gravimetric method (IS: 1479, 1961). The solids not fat were obtained by subtracting milk fat percentage from the total solids in percentage.

Restriction endonuclease digestion

The PCR products of *β-LG* were digested with Sac II endonuclease. The reaction mixture, which included 10 µL of digested PCR product, 10 U of Sac II, and 1.5 µL assay buffer, was subjected for digestion at 37°C overnight. Restriction digestion of *α-LA* gene was carried out using 10 U of *Msp* I endonuclease for a period of 3 hours at 37°C in dry bath. Digested fragments were then separated by non-denaturing poly acrylamide gel electrophoresis (PAGE). The samples were then loaded on eight percentage non-denaturing polyacrylamide gels, with 8 percentage acrylamide and 0.5 X TBE and stained with ethidium bromide (0.5 µg/ml of 1X TBE). The restriction pattern was documented in a gel documentation system (BioRad, Gel Doc 2000 TM). The genotypes were unambiguously determined directly from the gel images.

Association of genotype with dairy traits

Allelic and genotypic frequencies were estimated for Malabari, Attappady black and cross-bred separately using standard procedure (Popgene, Version 1.32). Variations of allelic frequencies among the three populations were analyzed by Chi-square test of significance by Popgene (Version 1.32). The distribution of genotypes was checked for Hardy-Weinberg equilibrium by Chi-square test, comparing the observed and expected frequencies. Effect of population and *β-LG* genotypes on peak milk yield, milk fat percentage, total solids and solids not fat were estimated by univariate analysis of variance SPSS (Version 20).

Phylogenetic analysis of *β-LG* fragment

PCR products of both Malabari and Attappady black goats were sequenced commercially (Bioserve Hyderabad, Pvt. Ltd.). The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) databases for homologous sequences. Phylogenetic analyses were performed with MEGA (Version X). Sequences in FASTA format of Malabari and Attappady black were aligned by progressive method of multiple sequence alignment with similar sequences of various other breeds of different geographical location extracted from NCBI nucleotide database. Sequenced region of *β-LG* were subjected to tree construction by character-based method like maximum likelihood (Tamura-Nei model) and maximum parsimony. Support of clusters was evaluated by 500 bootstrapped replications.

RESULTS AND DISCUSSION

PCR-RFLP of *β-LG* gene

The variation in genetic material, as it causes the variation in the performance of animals, is the basis for improving livestock through selection. Dairy traits are important criterion under consideration for improvement of small ruminants. The β-lactoglobulin (*β-LG*) and α-lactalbumin (*α-LA*) were considered as important marker genes influencing dairy traits. Electrophoresis of digested PCR products with restriction enzyme Sac II revealed three restriction patterns with identification of two alleles namely S_1 and S_2. Allele S1 was indicated by the presence of two bands of 349 and 77 bp while S_2 by single band size of 426 bp (Fig 1). The allele S_1 possessed polymorphic site for Sac II while in the case of

the allele S_2 polymorphic restriction site at position 349 was absent. In all the populations studied, the frequency of S_2 allele was higher which is in agreement with the past research work (Gharedaghi *et al*., 2016). It might be due to the selective advantage of this allele in the Indian goat breeds, having linkage to any favorable character. But S1 was reported to be common in Spanish and French (Pena *et al*., 2000) as well as Turkish hair goat breeds (Elmaci, 2009). A 268 bp fragment of caprine *α-LA* gene at exon 3 and its flanking regions was amplified. On PCR-RFLP analysis, with Msp I only A1A1 genotype was found to be present in the entire population under study. Lan *et al*. (2007) reported monomorphic pattern with only A1 allele in most of Chinese breeds and very low frequency of A2 allele in some of them.

H-W equilibrium

Insignificant chi-square value (3.35, df=2) of *β-LG*/Sac II alleles indicated that S_1 and S_2 alleles were homogeneously distributed in all the populations studied (Table 1). Similarly on analysis with *β-LG*/*Sac* II genotypes insignificant chi-square values were obtained for Malabari (0.005, df=2), Attappady black (2.2, df=2) and crossbred population (5.88, df=2). This result indicated that all three populations were under Hardy-Weinberg equilibrium. It could be due to random mating with respect to *β-LG* genotypes over generations leading to an equilibrium state in the populations under study. It further indicated lack of selective advantage for different *β-LG* alleles, resulting in a balanced reproductive and survival rates.

Comparison of dairy traits between breeds

Significant variation existed between breeds ($p<0.05$) where the average peak yield (60-90 days of lactation) was found to be higher in Saanen × Malabari crossbred population (637.21±47.5 ml) compared to Malabari (440.22±35.02 ml) and Attappady black goats (400±58.78 ml). The average value in percentage for milk fat, total solids and solids not fat in 60 to 90 days of lactation did not vary significantly between populations (Table 2).

Association of dairy traits with *β-LG*/Sac II polymorphism

Though several association studies were reported for genetic variants of *β-LG* with milk production traits in other species, no much study have been reported for *β-LG* gene polymorphism with milk production traits in goats. Genotypes of *β-LG/Sac* II polymorphism had significant effect on Peak milk yield in Malabari goat population studied (Table 3). Malabari goats carrying S_2 allele showed a significantly higher average ($p<0.05$) for peak milk yield (420±74.24 ml and 501.79±46.47 ml) compared to animals with S_1 allele (250±13.36 ml and 501.79±46.47 ml). In Spanish and French goat breeds *β-LG/Sac* II were found to be associated with several milk production and composition traits (Pena *et al*., 2000). The *β-LG* genotype AA showed a greater milk production performance than the BB genotype along the entire lactation curve; whereas the heterozygote showed an intermediate performance in tropical dairy goats of Colombia (Cardona *et al*, 2016). The peak milk yield has been chosen for yield parameter for better accuracy in estimation. The variation between genotypes may be due to differences in protein binding activity leading to allele specific differences in gene expression. This result suggested *β-LG/Sac* II to be an important marker in selection for dairy traits in goat.

Several association studies were reported for variants of *β-LG* with milk composition traits in bovine and ovine species (Jacob and Puhan, 1992; Dario *et al*., 2008; Dayal *et al*., 2006; Martin *et al*., 2002). The mean milk fat percentage in Malabari and cross bred goats was higher in genotypes containing S2 allele compared to those with S1 allele but the association was not significant (Table 3). Amigo *et al*. (2000) reviewed that out of the three protein variants (A, B, and C) of ovine *β-LG*, the 'A' variant exhibited higher value for total solids ($p<0.05$). The current study could not prove a significant association of *β-LG* genotypes with total solids and solids not fat.

Sequence analysis of *β-LG*

The sequences obtained were subjected to NCBI nBLAST and confirmed the amplification of right fragment. The nBLAST

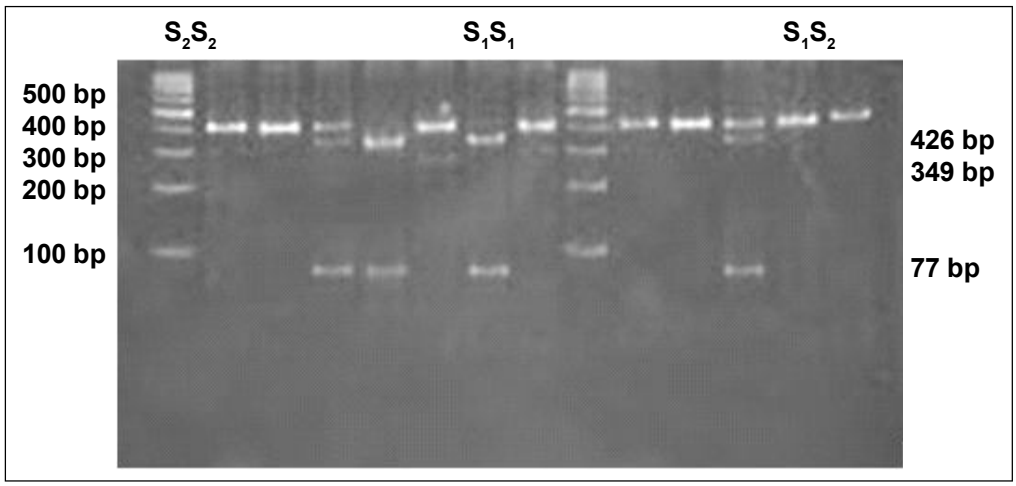

Fig 1: The genotypes of ß-LG/Sac II polymorphism on 8% non-denaturing poly acrylamide gel.

analysis of the nucleotide sequence revealed 99 per cent identity with that of Capra hircus (Accession # Z33881.1) gene encoding *β-LG*. A probable reason for the 1 per cent reduction in identity could be C to A transversion at 7062 position thus pointing to the discovery of a new SNP in breeds under study. This novel polymorphism identified in caprine *β-LG* gene of Malabari and Attappady black if restricted to the indigenous Indian breeds and might be responsible for any special dairy features of native breeds. They differ between each other by a few base substitutions that cause one or two amino acid changes in protein making them functionally different.

Phylogenetic analysis

There is considerable variation among the *β-LG* sequences examined. None of the nucleotide sites among the aligned

Table 1: Genotypic frequency and allelic frequency of Malabari, Attappady black and crossbred population groups.

Population	No. of observations		Genotypes			Chi-square value	Alleles		Chi-square value
			$S_1 S_1$	$S_1 S_2$	$S_2 S_2$		S_1	S_2	
Malabari	65	Obs.	9 (0.14)	0.46 (30)	0.40 (26)	0.005^{NS}	48 (0.37)	82 (0.63)	
		Exp.	8.86	30.28	25.86				
Attappady	25	Obs.	1 (0.04)	15 (0.60)	9 (0.36)	2.20^{NS}	17 (0.34)	33 (0.66)	3.35^{NS}
Black		Exp.	2.89	11.22	10.89				
Crossbred	30	Obs.	3 (1.00)	5 (0.17)	22 (0.73)	5.88^{NS}	11 (0.18)	49 (0.82)	
		Exp.	1.01	8.98	20.01				

NS indicates that the values were not significantly different; Figures in parenthesis are genotypic and allelic frequencies; Chi-square values given are calculated; Obs: Observed genotype number; Exp: Expected genotype number.

Table 2: Mean value of dairy traits in Malabari, Attappady black and crossbred populations.

Dairy traits	Population mean of dairy traits			F-value
	Malabari (47)	Attappady black (11)	Crossbred (30)	
Peak yield (ml)	440.22±35.02	400±58.78	637.21±47.50	22.30*
Fat percentage	4.10±0.14	3.76±0.26	4.14±0.22	0.59^{NS}
Total solids	14.15±0.25	13.7±0.38	14.32±0.31	0.66^{NS}
Solids not fat	10.05±0.18	10.04±0.29	10.18±1.75	0.095^{NS}

*(P 0.05); NS indicates that the values were not significantly different; Numbers of observations are in parenthesis; F-values given are calculated.

Table 3: Mean value of dairy traits in each genotypic group of *β-LG*/Sac II polymorphism.

Dairy traits	Genotype	Population		
		Malabari	Attappady black	Crossbred
Peak yield (ml)	$S_1 S_1$	250±13.36^{b}	-	800±115.47
	$S_1 S_2$	501.79±46.47^{a}	-	780.0±198.4
	$S_2 S_2$	420±74.24^{a}	-	602.86±50.08
	F-value	4.02*	-	1.156^{NS}
Fat percentage	$S_1 S_1$	3.99±0.25	5.60±0.0	3.20±0.25
	$S_1 S_2$	4.21±0.26	3.71±0.20	4.66±0.54
	S_2 S2	4.02±0.18	3.50±0.65	4.16±0.27
	F-value	0.243^{NS}	-	1.373^{NS}
Total solids	$S_1 S_1$	13.69±0.45	15.95±0.0	15.3±1.88
	$S_1 S_2$	14.16±0.42	13.43±0.09	14.57±0.72
	$S_2 S_2$	14.38±0.37	13.66±2.17	14.13±0.32
	F-value	0.464^{NS}	-	0.69^{NS}
Solids not fat	$S_1 S_1$	9.71±0.34	10.35±0.0	12.1±1.75
	$S_1 S_2$	9.96±0.30	9.85±0.25	9.91±0.30
	$S_2 S_2$	10.35±0.28	10.6±1.51	9.98±0.29
	F-value	0.901^{NS}	-	2.841^{NS}

^{NS}- Not significant; *(P 0.05); F-values given are calculated.

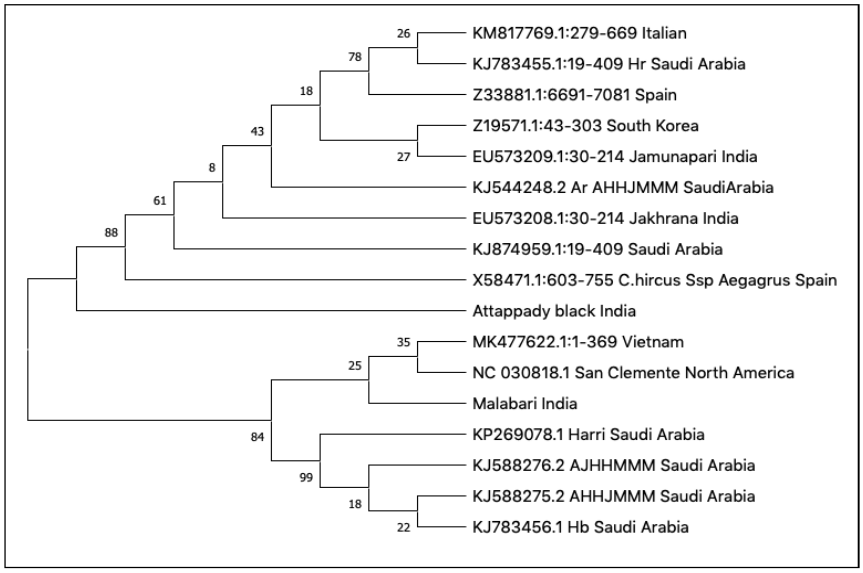

Fig 2: Maximum likelihood analysis of *β-LG* Malabari and Attappady black with other breeds.

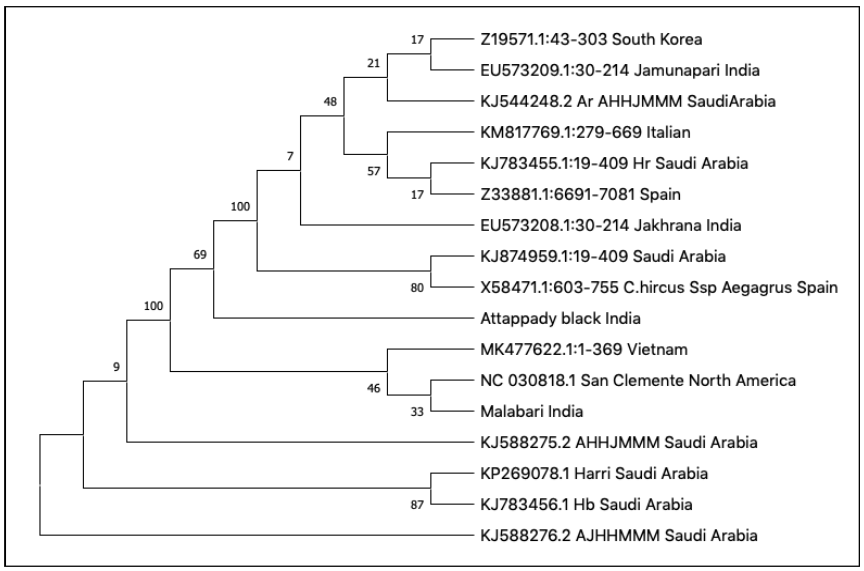

Fig 3: Maximum parsimony analysis of *β-LG* Malabari and Attappady black with other breeds.

sequences were conserved. The sequences contain numerous indels. Most of them appear to have resulted from single evolutionary events. Two phylogenetic analyses, based on maximum likelihood (Fig 2) and maximum parsimony (Fig 3) were performed by Tamura-Nei model. Both phylogenetic methods produced near identical topologies. Clades were strongly supported with bootstrapping. Malabari was found to be an ortholog of San Clemente of North America and Vietnamese chicken. The clade containing the above mentioned breeds diverged from clade containing Harri, AJHHMMM, AHHJMMM and Hb of Saudi Arabia. Attappady black breed possessed near homologous sequence to other Indian breeds such as Jamunapari and Jakhrana.

Genetic divergence and gene flow among Malabari and Attappady black populations with this classical marker proved separate origin as they belonged to separate clades. Attappady black shared a distant common inheritance with other Indian breeds such as Jamunapari and Jakhrana but Malabari showed totally different inheritance. Malabari shared orthology with Arabian breeds pointing to Arabian roots of Malabari breed (Kaura, 1952).

CONCLUSION

The S_2 allele of the candidate gene *β-LG* linked to high peak milk yield was found be abundant in Indian population of indigenous goats. This association with peak yield revealed from this study, suggested feasibility of *β-LG* as a molecular marker. Result of the present study can definitely be used to complement the present breeding program in goats and thereby improve the production potential of the goat population.

The new polymorphism at 7062 position of caprine *β-LG* gene in Malabari and Attappady black indicated sufficient variability in the genoplasm studied which is the raw material for genetic improvement of breed. The Information obtained from present analysis would be helpful in formulating plans on conservation and management of these indigenous breeds.

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