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Single Nucleotide Polymorphism in Exon 4 and Promoter Regions of β-Lactoglobulin Gene in Native Cattle (*Bos indicus*) Breeds of India

Kishore A[1], Mukesh M[1], Sobti RC[2], Keviletsu Khate[1], Mishra BP[3] and Sodhi M[1*]

[1]*Cattle Genomics Lab, National Bureau of Animal Genetics Resources, PO Box-129, Karnal-132001, Haryana, India*

[2]*Department of Biotechnology, Panjab University, Chandigarh, India*

[3]*Division of Animal Biotechnology, Indian Veterinary Research Institute, Izzat Nagar, Bareilly, Uttar Pradesh 243122, India*

[*]**Corresponding author:** Sodhi M, Cattle Genomics Lab, National Bureau of Animal Genetic Resources, PO Box-129, Karnal-132001, Haryana, India, Tel: +91 184 2267654; E-mail: monikasodhi@yahoo.com

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Abstract

The present study aims to define the distribution pattern of allelic variants in exon 4 and promoter region of β-lactoglobulin (*BLG*) gene in Indian native cattle (*Bos indicus*). PCR-RFLP analysis of exon 4 using *HaeIII* digestion in 968 animals of 23 Indian cattle breeds, revealed the predominance of BB genotype and B allele with an average frequency of 0.533 and 0.740, respectively. Further, comparative sequence analysis of *BLG* promoter region spanning 579 bp of proximal promoter region and 116 bp of exon1 revealed a total of 15 nucleotide variations with respect to *Bos taurus* sequence. Amongst these, variations at position -204 (C>G)/R5, widely considered to be an important site for milk protein binding factor was further screened by developing genotyping protocol using *Rsa*I enzyme in 779 animals of Indian native cattle. Out of two alleles and three genotypes, G allele and GC genotype was found to be predominantly present with mean values of 0.639 and 0.444, respectively. Chi-square test indicated presence of genetic equilibrium in majority of the breeds with respect to studied variations and revealed no significant difference in distribution of variants across different cattle groups.

Keywords: Single nucleotide polymorphism; *BLG*; Exon 4; Promoter Regions; Bovine milk; *Bos taurus*

Abbreviations

BLG: β-Lactoglobulin; bp: Base-Pair; LD: Linkage Disequilibrium; R: Restriction Site for *RsaI*; '+1': Transcription Start Site; TRE: Thyroid Receptor Element; PRE-RC: Progesterone Receptor Element-Reverse Compliment; AP: Activator Protein; NF1: Nuclear Factor 1; MAF: Mammary Cell Activating Factor; MGF: Mammary Gland Activating Factor; MPBF: Milk Protein Binding Factor; PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

Introduction

Bovine milk contains 3-5% protein, of which 80% is casein and 20% is whey protein [1]. These proteins play a vital role in coagulation and curdling of milk. Among the whey proteins, β-lactoglobulin (*BLG*) forms the major portion in the milk of ruminants. This gene has been mapped to bovine chromosome 11 [2]. Studies of *BLG* gene in taurine cattle have revealed 11 alleles [3] and the most frequent alleles A & B being are observed to be associated with milk composition and cheese making properties [4]. Allele A of *BLG* encodes amino acids aspartic acid and valine, while allele B encodes Glycine and Alanine at positions 64 and 118, respectively [3,5]. In bovine milk, *BLG* protein variants A and B are associated with different amounts of *BLG* protein. The variant A has a higher *BLG* protein concentration than variant B [6,7]. In past, several groups have reported the significant effect of protein variants A and B on milk protein composition or milk production traits [8-15]. These studies revealed that BB genotype is associated with higher casein and lower BLG content.. Comparatively, the B variant of *BLG* was observed to be expressed at relatively lower level in milk with a concomitant increase in casein number [16-18]. In a comprehensive study encompassing 8000 animals and three lactation records, Ng-Kwai-Hang et al. [9] observed that with replacement of allele A by B, there was a significant increase in protein percentage in all the three lactations whereas protein yield increased significantly only in the second lactation [9].

In another study involving 6803 animals with first lactation records, no significant effect on protein percentage was observed in animals with BB genotype [19]. However, the effect of BB genotype on milk yield ($p=0.019$), fat% ($p<0.001$) and protein yield ($p=0.009$) was significant. Sitkowska et al. [20] reported higher milk yield, higher fat and protein content in animals with AA genotype, however the study was based on only 155 cow and also significance of the effect was not provided [20]. Ozdemir and Dogru [21] found significant effect of AA genotype on increased fat percentage and AB genotype on increased milk yield [21]; while Kucerova et al. [22] observed non-significant effect of AA/AB/BB genotypes on milk traits [22].

Efforts have also been made to analyze the variations in the *BLG* promoter and annotate transcription factor binding sites for milk protein gene in taurine cattle [8,23-27]. Wagner et al. [23] characterized the promoter region of Holstein Friesian cattle and identified a total of 16 alleles (R1A/B to R16A/B) [23]. Amongst these, allele R5A/B at position -204 (G>C) from the transcription start site holds importance as it lies within the binding site of milk protein binding factor (MPBF), and might affect the activity of the gene product. The polymorphism analysis of bovine *BLG* promoter region by Lum et al. [24] revealed 10 polymorphic sites [24]. They confirmed functional importance of transversion (G to C) within a consensus binding site for activator protein-2 (AP-2) at position –430 bp from

the transcription initiation site. Wagner et al. [21] also confirmed the association of alleles (A/B) in the coding region with variations in the promoter region [23]. Such associations might explain the difference of protein variant dependent *BLG* synthesis observed *in vivo*.

In contrast to taurine cattle, no such reports are available on polymorphism analysis of *BLG* gene in diverse Indian cattle breeds. Apart from few studies [28,29] wherein protein variants A and B were genotyped using PCR-RFLP in a small number of breeds, no other studies have been carried out to analyze polymorphism in the coding and promoter region. The present study was therefore planned with the objective to analyze distribution pattern of allelic variants in the coding (protein variants) and promoter region across diverse Indian native cattle breeds.

Materials and Methods

Cattle breeds and extraction of genomic DNA

A total of 968 animals belonging to 23 Indian native cattle breeds were genotyped for *BLG* protein variants A, B and R5 or -204 G/C of *BLG* promoter region. Out of these, 779 animals were analyzed for the *BLG* promoter region. These animals were subset of the 968 animals analyzed for exon4 region. Animals that were not genotyped for promoter region were considered missing values and excluded in verification of linkage between the variants of the two analyzed regions. The details of cattle breeds, their geographical location, agro climatic zones, utility and coat color is presented in Table 1. Blood samples were collected from unrelated and true-breeding animals randomly picked from the respective breeding tracts of different cattle breeds using vacutainer tubes containing EDTA as anticoagulant. Genomic DNA was isolated using standard proteinase K digestion followed by phenol: chloroform extraction procedure [30].

PCR-RFLP genotyping of *BLG* protein variants

A total 968 animals representing 23 cattle breeds were genotyped to uncover the status of one of the SNPs associated with *BLG* protein variants A and B. Frequency distribution of A and B alleles was analyzed within a 247 bp fragment, covering exon 4 and flanking intron 4 region of *BLG* gene with restriction site for *HaeIII*, using the primer pairs reported by Shetty et al. [31]. For variant A, the restriction site for *HaeIII* exists within intron 4 only, whereas, for variant B restriction site exists both in exon 4 and intron 4. The PCR reaction was carried out in a reaction volume of 25 µl containing 100 ng of genomic DNA, 5 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM dNTP and 1.0 unit of Taq polymerase (MBI Fermentas). Thermal cycling condition for amplification of the coding region was 95°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 59°C for 40 sec and 72°C for 20 sec with a final extension at 72°C for 3 min. For the excision reaction, 10 µl of each PCR amplified product was digested overnight with 3 units of *HaeIII* restriction endonuclease (MBI Fermentas, USA) at 37°C. Restriction fragments were separated by electrophoresis on ethidium bromide stained 3% agarose gel in 1x TAE buffer and visualized using gel documentation system (UVP, Cambridge, UK).

Sequence and PCR-RFLP analyses of *BLG* promoter region

A 705 bp fragment comprising of 584 bp of the proximal promoter region and 121 bp of exon 1 was amplified using the oligonucleotide primers described by Yahyaoui et al. [32]. The composition of PCR reaction mixture was as described for the coding region. A touchdown PCR protocol was employed with temperature profile of 95°C for 5 min, 10 cycles of 97°C for 15 sec, 63°C for 1 min, and 72°C for 90 sec followed by 25 cycles of 95°C for 30 sec, 63°C for 1 min and 72°C for 90 sec followed by a final extension at 72°C for 5 min. Purified DNA sample was bidirectionally sequenced with ABI Prism® BigDye™ Terminator Cycle Sequencing Kit (version 3.1) (Applied Biosystems, Foster City, CA).

Breed	Utility type	Geographical location[a]	Coat color[b]	AZ[c]	Fat%
Sahiwal	Milch	NR/NWR	R	SAR	4.9[d]
Tharparkar	Milch	NWR	G/W	AR	5.0[e]
Rathi	Milch	NWR	R/B	AR	5.3
Gir	Milch	NWR	R	SAR	4.4[d]
Red Sindhi	Milch	Organized farms	R	AR	4.5[d]
Kankrej	Dual	NWR	G/W	SAR	5.0[e]
Deoni	Dual	CR	G/W	TWDR	4.3[d]
Gaolao	Dual	CR	G/W	HST	5.5[d]
Ongole	Dual	SR	G/W	TWDR	4.2[d]
Mewati	Dual	NR	G/W	SAR	4.9[e]
Hariana	Dual	NR	G/W	SAR	4.9[d]
Red Kandhari	Draft	CR	R	TWDR	4.6[d]
Nimari	Draft	CR	R	TWDR	4.9[d]
Malvi	Draft	CR	G	SAR	4.5[e]
Khillar	Draft	CR	G/W	TWDR	5.1[e]
Dangi	Draft	CR	G/W	TWDR	4.3[d]
Kherigarh	Draft	CR	G/W	HSTR	5.1[e]
Ponwar	Draft	CR	R/B	HSTR	4.7[e]
Malnad Gidda	Draft	SR	R/B	HSTR	NA
Amritmahal	Draft	SR	G/W	TWDR	5.3[e]
Umblachery	Draft	SR	G	TWDR	5.3[e]
Kangayam	Draft	SR	G/W	SAR	3.9[d]
Nagori	Draft	NWR	G/W	AR	5.8[e]

Table 1: List of Indian native cattle breeds included in the study and their categorisation based on utility type, geographical location, coat color and agroclimatic zone along with milk fat%

[a]NR, northern; NWR, northwestern; CR, central; SR, southern; [b]R, red; G, gray; W, white; B, brown; [c]Agroclimatic zones: SAR, semiarid; AR, arid; TWDR, tropical wet and dry; HSTR, humid subtropical; [d](Nivsarkar et al., [41]); [e]http://dad-training.fao.org; NA: Not available.

All sequencing reactions were analyzed using an ABI 3100 DNA capillary sequencer (Applied Biosystems, Foster, CA). Sequence

comparisons were done using Phrap and Gap4 integration from the Staden Package, a suite of sequence handling and analysis software developed at the Medical Research Council Laboratory of Molecular Biology (Cambridge, UK). The sequences were annotated for binding site of transcription factors using program MATCH v1.0, AliBaba2.1, TESS and information available in literature [23,24,27]. The identified SNP (R5A/B, -204 G/C) was further examined using PCR-RFLP on all the 779 animals (subset of 968 animals genotyped for protein variant A and B) analyzed for the promoter region. Restriction digestion was carried out using 10 µl of each PCR product and 3 units of *RsaI* enzyme (MBI Fermentas, USA) overnight at 37°C. The digested products were resolved on 3% agarose gel in 1x TAE buffer and visualized under UV trans illuminator.

Statistical analysis

Gene and genotypic frequency was calculated by direct counting method. Chi-square test was carried out manually to test the populations for Hardy-Weinberg equilibrium as well as for differences between the animals on the basis of geographical location, agro climatic zones, utility and coat color. Linkage disequilibrium (LD) measures, D' and r2, between all variations within promoter region and two protein variants were estimated using Arlequin v3.5 software [33].

Results and Discussion

Detection of BLG protein variants A and B

Screening of the exon 4 region of *BLG* using *HaeIII* detected protein variants A and B with three different genotypes (AA, AB and BB). The restriction fragments corresponding to AA, AB and BB genotypes were: 148 and 99 bps; 148, 99 and 74 bps, and 99 and 74 bps, respectively (Figure 1a). The gene and genotype frequencies of *BLG* protein variants A and B in each of the analysed Indian cattle breeds are depicted in Table 3. The genotype BB was predominant in 15 breeds and ranged from 0.214 (Malnad Gidda) to 0.877 (Kangayam), with an average frequency of 0.531. On the other hand, AB genotype was in higher proportion in 7 breeds with an average frequency of 0.406. The frequency of AB genotype ranged from 0.069 (Ponwar) to 0.755 (Mewati). The least common genotype, AA with an average frequency of 0.064, was absent in 5 breeds *viz.* Hariana, Mewati, Kangayam, Kherigarh and Red Sindhi.

Each of the studied cattle breeds demonstrated predominance of allele B with an average frequency of 0.740. The frequency of allele B ranged from 0.559 (Gir) to 0.938 (Kangayam). On the other hand, average frequency of allele A was 0.260, with Gir showing the highest frequency (0.441). The relatively high frequency of B allele in Kangayam corresponded to higher prevalence of BB genotype in the breed.

The presence of allele B at high frequency and predominance of BB and AB genotypes across the Indian native cattle breeds is similar to earlier reports on Indian cattle. Ganai and Bhat [34] reported high frequency of allele B (82.9%) and BB genotype (71.4%) in Hariana cattle. Similarly, Rachagani et al. [33] reported high prevalence of B allele (0.83 and 0.61) and BB genotype (0.693 and 0.244) in Sahiwal and Tharparkar cattle, respectively. In taurine cattle, comparatively lower frequency of B allele and BB genotype was reported in Holstein (0.647 and 0.399 [11]; 0.471 and 0.198 [34]), Czech Fleckvieh (0.489 and 0.240), Brown Swiss (0.56 and 0.296) [21] and Jersey (0.66 and 0.392) [31]. The allelic distribution revealed the higher frequency A allele in taurine breeds and conversely the naturally evolved Indian zebuine cattle breeds have higher frequency of B allele. The lower occurrence of *BLG* protein variant B and higher occurrence of variant A allele observed in taurine cattle might be attributed due to their intensive selection for higher milk yield, as A allele and AA genotype have been associated with higher milk yield [20,35]. In contrast, the higher frequency of B allele and near absence of AA genotype breeds could be an indication for lack of any selection pressure for milk yield in the naturally evolved Indian cattle breeds.

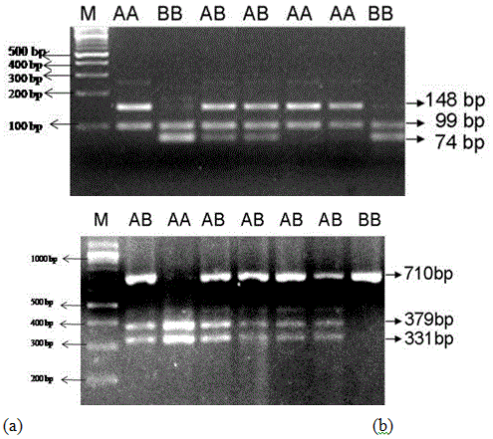

Figure 1: Agarose gel showing BLG (a) exon 4-intron 4 and (b) promoter variants of Indian native cattle breeds; M: 100 bp DNA ladder. PCR-RFLP of the coding region detects one of the SNPs associated with BLG protein variants A and B, while the promoter region detects the R5 or -209 G/C variants.

Till now, no association data using *BLG* variants with dairy traits are available for Indian cattle. However, many such studies have already been reported in different taurine populations trying to evaluate the effect of *BLG* variants on milk production and protein content [9,19,12,20,35-37]. The effect of *BLG* genotypes on milk production trait as different reports indicate is not consistent. Lunden et al. [30] and Ojala et al. [31] observed no effect of *BLG* genotypes on milk production traits, but studies by Bovenhuis et al. [31] and Ikonen et al. [30] observed association of *BLG* genotypes with production traits suggesting AA genotype for higher milk production. Conversely, most of the reports consistently indicate that A variant of *BLG* gene is associated with a greater concentration of *BLG* protein [9-10,24]. In a comprehensive study on approximately 2000 animals, Heck et al. [30], confirmed association of A variant with relatively higher concentration of *BLG* as compared to B variant of *BLG*, however *BLG* variants did not have any effect on milk production trait. The difference in the outcome of these studies could be due to variation in the sample size or cattle types included in the study. Besides, the difference in *BLG* protein level of variant A and B could be due to the differential expression of respective alleles and its linkage with variations within the promoter. Defining the allelic status of A and B protein variants of *BLG* across Indian cattle breeds will be crucial to initiate genotype and phenotype association studies.

Polymorphism at BLG promoter region

The amplicon of the 5'- flanking region of *BLG* gene was sequenced and compared with *Bos taurus* reference sequence [NCBI: DQ489319]. The observed length of the amplicon (705 bp) comprising of 584 bp of promoter region and 121 bp of exon 1 was five base-pair shorter than the observed for goat (710bp) by Yahyaoui et al. [32] as *BLG* promoter in cattle has deletion of six base-pair (between +70 bp and +71bp) and insertion of single base-pair (between -227 bp and -228bp) as compared to goat sequence.

The binding sites for promoter transcription factor of milk protein genes *viz.* thyroid receptor element (TRE), progesterone receptor element (PRE-RC), activator protein-2 (AP-2), nuclear factor 1(NF1), mammary cell activating factor (MAF), milk protein-binding factor (MPBF), c-Rel, Elk-1 and mammary gland activating factor (MGF) of *Bos indicus* were similar to *Bos taurus* (Figure 2). Alignment of the sequences with *Bos taurus* reference sequence (DQ489319) revealed 15 variations at positions +117 (G/A), +99 (T/C), +78 (T/C), +36 (A/G; R1), +32 (C/T; R2), -83 (G/A), -98 (G/C; R4), -170 (A/C), -204 (G/C; R5), -216 (C/T; R6), -287 (G/A), -362 (T/C; R7), -430 (G/C; R10), -457 (C/G; R11) and -573 (A/G). The nomenclature for different SNPs (R1-R11) in the parenthesis is as suggested by Wagner et al. [31].

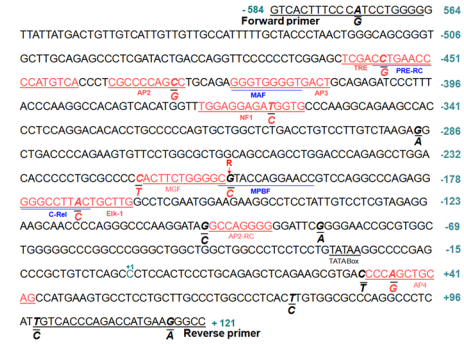

Figure 2: DNA sequence and annotation of the BLG promoter in Indian native cattle breeds. Variations among Indian cattle in reference to *Bos taurus* (DQ489319) are marked with bold and italicized nucleotide. Sequence for primer pair are indicated by underlined oligomers at 5'- and 3'-terminals

Amongst these SNPs, six variations located within the putative transcription factor binding sites (TFBSs) are +36 (A/G; AP4), -170 (A/C; c-Rel and Elk-1), -204 (G/C; MPBF), -362 (T/C; NF-1), -430 (G/C; AP-2) and -457 (C/G; TRE and PRE-RC) possibly affects binding affinity of TFs and thus milk properties [24]. The variations R1-R2, R4-R7, R10-11 observed in the present study have also been reported by Wagner et al. [31] and Ganai et al. [32] (Table 2). Reports are also available for presence of variations at positions +99 (T/C) [27], +78 (T/C) [24,26], -170 (A/C) located within the binding sites for transcription factors (TFs) c-Rel and Elk-1 [27] in taurine breeds. However, variations at positions -424 (T/G; R9) [23], -422 (G/A; R8) [23,24,26] and -22 (G/A; R3) [23] were not observed in the present study.

The other three variations -83 (G/A), -287 (G/A) and -573 (A/G) found in the *BLG* promoter region of Indian cattle have not been described in any other study. Additionally, variation at position +117 (G/A) in the exon-1 has also not been reported previously.

Breed	N	Allelic Frequency		Genotype Frequency			χ^2 value[a]
		A	B	AA	AB	BB	
Sahiwal	46	0.228	0.772	0.022	0.413	0.565	1.37 NS
Tharparkar	50	0.300	0.700	0.080	0.440	0.480	0.11 NS
Rathi	47	0.351	0.649	0.106	0.490	0.404	1.39 NS
Gir	51	0.441	0.559	0.235	0.412	0.353	1.39 NS
Red Sindhi	44	0.250	0.750	0.000	0.500	0.500	4.89*
Kankrej	33	0.288	0.712	0.121	0.333	0.546	1.15 NS
Deoni	28	0.268	0.732	0.036	0.464	0.500	0.94 NS
Gaolao	46	0.336	0.664	0.045	0.586	0.369	3.50 NS
Ongole	39	0.424	0.576	0.179	0.488	0.333	0.00 NS
Mewati	49	0.378	0.622	0.000	0.755	0.245	17.35**
Hariana	48	0.138	0.862	0.000	0.291	0.709	1.57 NS
Red Kandhari	48	0.292	0.708	0.104	0.375	0.521	0.41 NS
Nimari	45	0.200	0.800	0.022	0.356	0.622	0.56 NS
Malvi	43	0.244	0.756	0.047	0.395	0.558	0.22 NS
Khillar	39	0.188	0.813	0.025	0.333	0.666	0.21 NS
Dangi	37	0.203	0.797	0.027	0.351	0.622	0.28 NS
Kherigarh	20	0.150	0.850	0.000	0.300	0.700	0.62 NS
Ponwar	29	0.207	0.793	0.172	0.069	0.759	16.60**
Malnad Gidda	43	0.292	0.708	0.112	0.674	0.214	14.88**
Amritmahal	43	0.193	0.807	0.023	0.341	0.636	0.45 NS
Umblachery	45	0.222	0.778	0.089	0.267	0.644	2.35 NS
Kangayam	57	0.062	0.938	0.000	0.123	0.877	0.24 NS
Nagori	38	0.316	0.684	0.026	0.579	0.395	4.38*
Mean ±SE	42 ±1.76	0.260 ±0.019	0.740 ±0.019	0.064 ±0.014	0.406 ±0.033	0.531 ±0.035	3.39 ±1.17 NS

Table 2: Gene and genotype frequencies of BLG/HaeIII locus (BLG protein variants A and B) in Indian native cattle breeds; [a]: one degree of freedom; NS: non- significant; *: p<0.05; **: p<0.01

Amongst the variations detected in present study, change at position -204 (G/C; R5) creates restriction site for *RsaI* endonuclease enzyme. The frequency of this particular variation was further analysed in 779 Indian native cattle as it is located within the binding site of milk protein binding factor (MPBF), known as signal transducer and activator of transcription-5 (STAT-5). STAT-5 is a transcription activator for *BLG* gene in mammary cells and bind site-specifically in the 5'-flanking region of the gene. It might be postulated that nucleotide change at STAT5 binding site could influence the activity of its gene product, which in turn could result in allele specific differences in expression of *BLG* gene. Similar differential expression

of alleles at *BLG* locus has earlier been proposed to be due to SNPs in the 5'-flanking region (G/C; R5) [23,38-41].

Breed	N	Allele frequency		Genotype frequency			χ^2 value[b]
		A	B	AA	AB	BB	
Sahiwal	38	0.566	0.434	0.289	0.553	0.158	0.59 NS
Tharparkar	32	0.469	0.531	0.281	0.375	0.344	1.95 NS
Rathi	47	0.649	0.351	0.447	0.404	0.149	0.60 NS
Gir	48	0.510	0.490	0.354	0.313	0.333	6.74**
Red Sindhi	31	0.790	0.210	0.581	0.419	0.000	2.18 NS
Kankrej	28	0.643	0.357	0.500	0.286	0.214	4.00*
Deoni	21	0.524	0.476	0.190	0.667	0.143	2.38 NS
Gaolao	34	0.779	0.221	0.588	0.382	0.029	0.43 NS
Ongole	36	0.333	0.667	0.000	0.667	0.333	9.01**
Mewati	39	0.667	0.333	0.436	0.462	0.102	0.06 NS
Hariana	47	0.670	0.330	0.511	0.319	0.170	3.63 NS
Red Kandhari	45	0.611	0.389	0.467	0.289	0.244	6.92**
Nimari	32	0.609	0.391	0.563	0.094	0.344	20.62**
Malvi	23	0.783	0.217	0.565	0.435	0.000	1.78 NS
Khillar	34	0.647	0.353	0.353	0.588	0.059	2.82 NS
Dangi	27	0.778	0.222	0.556	0.444	0.000	2.21 NS
Kherigarh	20	0.550	0.450	0.200	0.700	0.100	3.43 NS
Ponwar	22	0.432	0.568	0.045	0.773	0.182	7.27**
Malnad Gidda	45	0.833	0.167	0.733	0.200	0.067	3.52 NS
Amritmahal	36	0.708	0.292	0.472	0.472	0.056	0.73 NS
Umblachery	25	0.760	0.240	0.560	0.400	0.040	0.23 NS
Kangayam	47	0.723	0.277	0.489	0.468	0.043	1.35 NS
Nagori	22	0.659	0.341	0.409	0.500	0.091	0.28 NS
Mean ±SE	34 ±2	0.639 ±0.006	0.361 ±0.006	0.417 ±0.008	0.444 ±0.007	0.139 ±0.005	3.60 ±0.93 NS

Table 3: Gene and genotype frequencies of BLG promoter/RsaI (R5 or -209 G/C) in Indian native cattle breeds;[b]: one degree of freedom; NS: non- significant; *: p<0.05; **: p<0.01

Rsa1 digestion of the *BLG* promoter amplicon, involving R5 or -204 G/C polymorphism resulted in three genotypes: GG (382 and 323 bps), GC (705, 382 and 323 bps) and CC (705bp), respectively (Figure 1b). The genotype and allele frequencies of *RsaI* variant in *BLG* promoter region across Indian zebu cattle breeds are listed in Table 3. The frequency of predominant allele G ranged from 0.333 (Ongole) to 0.833 (Malnad Gidda), with a mean value of 0.639. The frequency of C allele was lower in all the breeds, except Tharparkar (0.531), Ongole (0.667) and Ponwar (0.568). Among the genotypes, heterozygous GC was observed in all the cattle breeds analyzed with average frequency of 0.444 and ranged from 0.094 (Nimari) to 0.773 (Ponwar). On the other hand GG genotype was predominant in 13 breeds and ranged from 0.000 (Ongole) to 0.733 (Malnad Gidda) with an average frequency of 0.417.

The genotype frequencies of *BLG* protein variants A and B and *BLG* promoter (R5 or -204) G/C polymorphism were also analyzed to test deviation from the expected Hardy-Weinberg equilibrium using Chi-square test. Results revealed presence of genetic equilibrium in majority of the analysed breeds (Table 3). However, 5 breeds (Nagori, Mewati, Ponwar, Red Sindhi and Malnad Gidda) showed deviation from genetic equilibrium for coding region and 6 breeds (Red Kandhari, Kankrej, Gir, Ongole, Nimari and Ponwar) for promoter region. The disequilibrium may reflect events such as selection, genetic drift or population subdivision as animals in these breeds were from either organized farm (Red Sindhi), low population size (Nagori, Red Sindhi) or restricted locations (Malnad Gidda, Ponwar, Mewati).

Further, in order to understand the allelic frequency distribution across different groups of cattle, breeds were classified in different categories on the basis of their utility (milch, dual and draft), geographical location (northern/north-western, central and southern region), coat color (red and grey) and agroclimatic zone (semiarid, arid, tropical wet and dry and humid subtropical) (Figure 3). Results indicated non-significant difference between the groups in both coding (*BLG* protein variants A and B) and promoter region (R5 or -204 G/C) as confirmed by Chi-square test (data not shown).

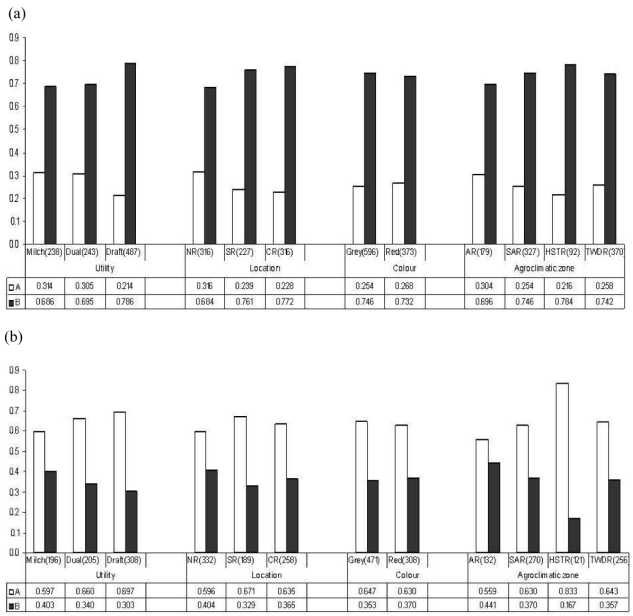

Figure 3: Mean frequencies of (a) BLG protein variants A and B and (b) R5 or -209 G/C variants of BLG promoter region across different groups of Indian native cattle.

Linkage disequilibrium between *BLG* protein variants and variation (R5 or -204 G/C) in the promoter region was also explored. However, no LD was observed between R5 and allele A/B of the coding region. Contrary to the present finding, complete LD between the *BLG* protein variants A and B and the R5 SNP at position -204

(corresponding to position -209 by Wagner et al. 1994 was reported by Lum et al. [24] in Holstein, Brown Swiss and Jersey cattle, and by Ganai et al. [21] in Holstein Friesian cattle. In addition, Lum et al. [24] found complete LD between the *BLG* protein variants A and B and SNPs R1, R2, R3, R4, R5, R6, R7, R10, R11, R13 and R14 in the promoter region.

Phylogenetic relationship for *BLG* promoter region among different mammalian species

Phylogenetic analysis based on UPGMA with 1,000 replicates for flanking region of *beta-lactoglobulin* revealed two major groups. As expectedly, *Bos indicus*, *B. taurus*, *B. grunniens* and *B. bubalis* were grouped together, while, goat and sheep formed another group (Figure 4). Analysis of genetic distance using p-distance method based on pairwise deletion revealed maximum genetic relatedness of Indian zebu cattle with *Bos taurus* (98.96%) followed by *B. grunniens* (98.27%), *B. bubalis* (96.20%), *C. hircus* (92.06%) and *O. aries* (91.88%).

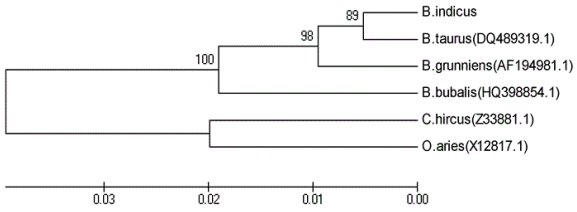

Figure 4: Phylogenetic relationship of beta-lactoglobulin promoter region among different mammalian species.

Conclusions

Utilizing 968 animals representing 23 Indian native cattle breeds, the present study portrays comprehensive frequency pattern of the *BLG* protein variants A and B across naturally evolved Indian native cattle, wherein, systematic information of known variants at this important candidate gene was lacking. In future, correlation analysis of protein variants and variants in promoter region may be carried out to undertake genotype: phenotype association studies for dairy traits across divergent Indian cattle breeds. Further, considering the fact that B variant is predominantly present across dairy, dual and draft cattle breeds and there is no LD between the protein variants and variant R5 (-204 G/C), we might need to look out for new variation in *BLG* locus and or linked gene/QTL as a potential marker with regulatory effect on dairy traits in Indian cattle.

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