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Genetic variants of beta-lactoglobulin gene and its association with milk composition traits in riverine buffalo

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A study was carried out to determine genetic variants of β-lactoglobulin gene and to explore associations between these and milk composition traits in riverine buffalo. Single strand conformation polymorphism was employed to detect the genetic variants of the gene. Two fragments of this gene i.e. 119 bp of exon I and 400 bp spanning exon IV and intron IV were included in the study. For 119 bp fragment, three alleles namely, A, B and C were observed in all the buffalo breeds whereas four alleles (A, B, C and D) were detected for 400 bp fragment. The frequency distribution of alleles was different in different breeds of buffaloes for both the fragments. For exon I fragment, the milk composition traits such as total SNF, protein, solid, fat and whey protein yield were found to be significantly ($P<0{\cdot}05$) associated with genotypes in Murrah and Bhadawari buffalo whereas in Mehsana breed genotypes were significantly ($P<0{\cdot}05$) co-related with total SNF, solid and fat yield. Genotypes of 400 bp fragment, only total fat yield in Mehsana buffalo was found to be significantly ($P<0{\cdot}05$) associated with genotypes.

Keywords: β-lactoglobulin, buffalo, milk composition, polymorphism.

Riverine buffaloes are mainly concentrated in the Asian countries particularly in the Indian Subcontinent. India possesses excellent breeds of riverine buffalo in terms of germplasm having a sizable proportion of about 53% of total bubaline population of the world. Buffaloes are famous for not only higher quantity of milk production but also for milk solid contents. Besides, animals are generally maintained on agricultural byproducts and crop residues. It is an established fact that buffaloes are resistant to several diseases and environmental stress.

Several methods are available to detect polymorphism of candidate gene, of which, one of the sensitive technique of detection of polymorphism is probably single strand conformational polymorphism (SSCP). This method is simple compared with other techniques like DGGE and TGGE, which need special sophisticated equipment and the techniques are tedious to carry out. Like RFLP, SSCP does not require the use of hazardous chemicals like radioactively labelled probes or specific primers based PCR. SSCP is a better approach to study polymorphism, since it covers the whole gene to detect mutations in alleles that are differing in even one nucleotide as compared with RFLP technique which has a limitation that polymorphism can only be detected within the small restriction sites. The SSCP technique is based on the principle that denatured single strand of DNA takes the different conformations attained by the intra strand pairing (looping & compaction) which in turn has different electrophoretic mobility, which depends upon the composition of nucleotides in that particular strand and is extensively used to detect mutation in candidate genes (Lagziel et al. 1996). No reports are available about the SSCP in buffalo though some reported the association of SSCP typed alleles in sheep and cow with milk proteins (Kaminski & Zabolewicz, 1998).

β-Lactoglobulin locus was reported as polymorphic in cattle (Medrano & Aguilar-Cordova, 1990; Wilkins and Kuys, 1992; Badola et al. 2003; Badola, 2004), but no report of polymorphism of β-lg gene in buffalo is available in the literature. *Hae* III polymorphism in partial exon IV and intron IV of β-lg gene indicates a valine/alanine substitution in amino acid residue 118 (Wilkins & Kuys, 1992; Badola et al. 2003) which influences total milk protein % and fat % in taurine cattle (Bovenhuis et al. 1992). β-Lg gene in dairy cattle revealed a significant influence on cheese yield indicating higher performance by heterozygotes (Aleandri et al. 1990). Jairam & Nair (1983) revealed that cows with β-lg AB genotype had lower age at first calving. Weights at birth to 12 months of age were also found to be influenced by β-lg loci (Singh et al. 1981). However, Ng-Kwai-Hang et al. (1990) found no association of milk protein types with days to attain first breeding, days

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Table 1. Genotype and allelic frequency of two fragments of β-lg gene in various breeds of riverine buffalo

	119 bp fragment								400 bp fragment								
	Genotype frequency					Allele frequency			Genotype frequency					Allele frequency			
Breed	AA	BB	CC	AB	AC	A	B	C	AA	BB	AB	AC	CD	A	B	C	D
Murrah	0·10	0·24	0·20	0·26	0·20	0·33	0·37	0·30	0·24	0·24	0·40	—	0·11	0·44	0·44	0·06	0·06
Mehsana	0·13	0·06	—	0·48	0·32	0·41	0·33	0·26	0·43	0·22	0·20	0·04	0·11	0·59	0·31	0·08	0·02
Bhadawari	—	0·09	0·09	0·48	0·34	0·32	0·30	0·38	0·41	0·27	0·21	—	0·11	0·51	0·39	0·05	0·05
Surti	0·14	0·22	0·28	0·16	0·20	0·33	0·37	0·30	0·40	0·24	0·20	0·06	0·10	0·55	0·34	0·08	0·03

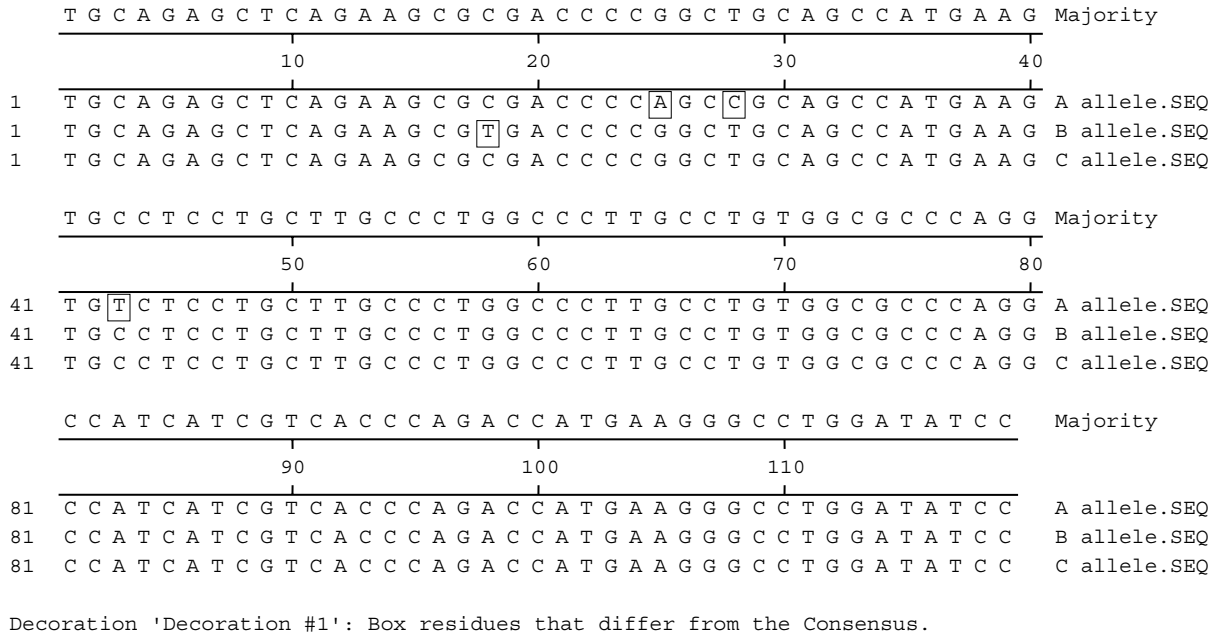

Fig. 1. Multiple alignment of alleles for 119 bp fragment of beta-lactoglobulin gene. (Genbank accession No. AY775796: allele A; AY775797: allele B; AY775798: allele C).

open and number of service per conception. Badola et al. (2003) reported significant association of genotype with milk production traits in cattle.

Keeping these reports in mind, the investigation was undertaken to explore genetic polymorphism of β-lg gene and attempts were also made to determine possible association of β-lg polymorphism with milk composition traits in riverine buffalo.

Materials and Methods

Sample

Blood and Milk samples were taken from 190 buffaloes comprised of 50 Murrah, 44 Bhadawari, 46 Mehsana, and 50 Surti breeds maintained at different organized herds. High molecular weight genomic DNA was isolated from blood according to the method reported by Sambrook & Russell (2001) and was checked for quality, purity and concentration.

Primer designing

The β-lg genomic sequences has not been made available in buffalo, therefore, primers were designed by comparing published NCBI sequences of β-lg gene of cattle, goat and sheep. The polymerase chain reaction (PCR) technique was used to amplify two fragments i.e. 119 bp of exon 1 and 400 bp spanning 105 bp of exon IV & 295 bp of intron IV of β-lg gene. Forward primer used for exon I was 5′-TGC AGA GCT CAG AAG C-3′ and the reverse primer was 5′-GGA TAT CCA GGC CCT TCA-3′. Similarly, the forward and reverse primers for exon IV to intron IV were 5′-CGA GAA CAA AGT CCT TGT GCT-3′ and 5′-CCG GTA ACA AAG GCT CTT AGA-3′, respectively.

Single strand conformation polymorphism (SSCP)

PCR based single strand conformation polymorphism (SSCP) was performed. A volume of 3–4 µl PCR products was mixed with 20–25 µl formamide dye in a 0·2 ml PCR tube. The SSCP solution was denatured at 95 °C for 5 min

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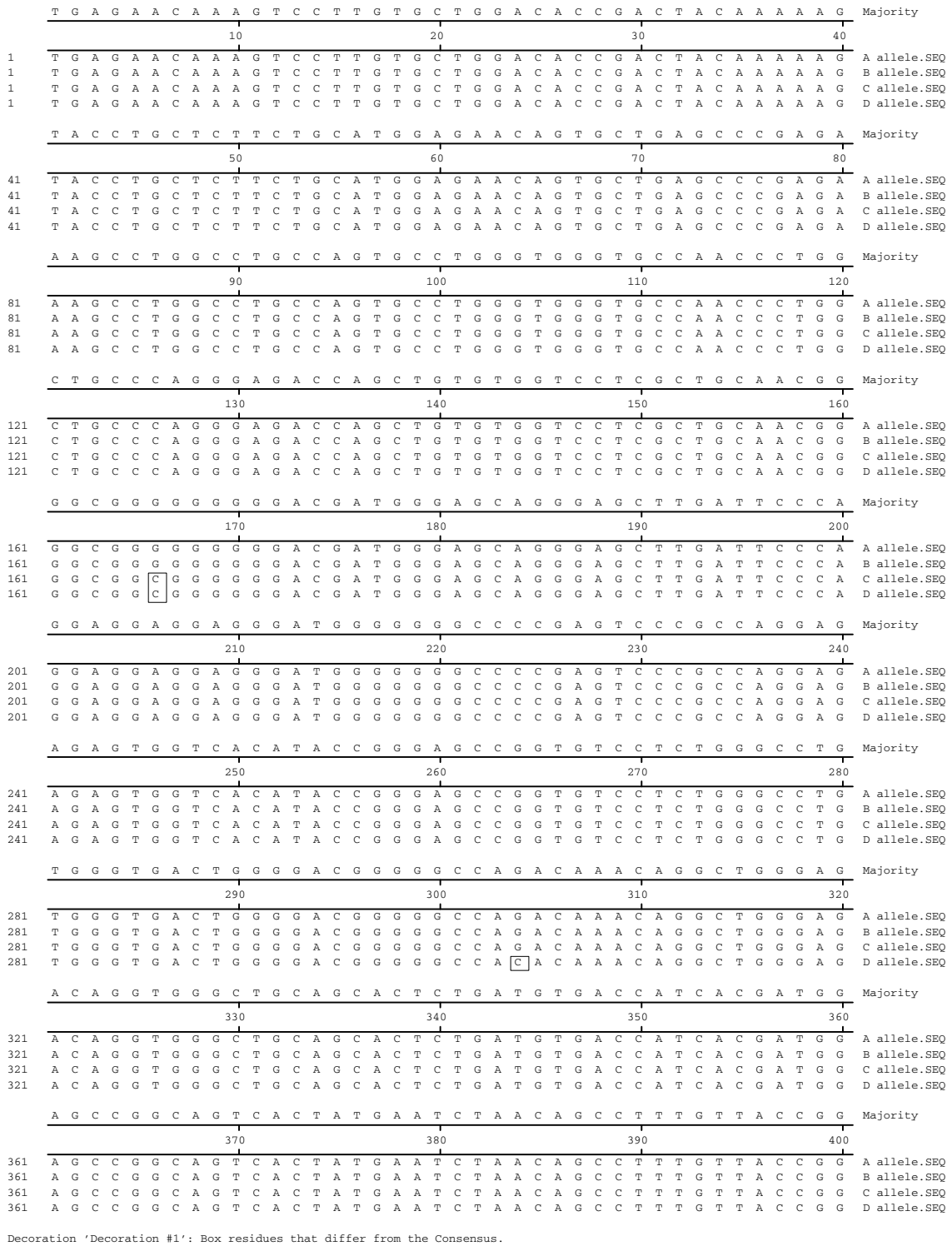

Fig. 2. Multiple alignment of alleles for exon IV and intron IV region (400 bp) of beta-lactoglobulin gene (Genbank accession No. AY775799: allele A; AY775780: allele B; AY775801: allele C; AY775802: allele D). Exon 4 covers first 105 bp and intron IV covers next 295 bp of the 400 bp fragment.

and snapped cool on ice for 15 min. The denatured products of 119 bp and 400 bp were analysed by 12% and 8% PAGE, respectively (acrylamide: bisacrylamide = 50 : 1) containing 50 ml glycerol/l in 1 × TBE in an apparatus of size 16 × 16 cm^2. The electrophoresis was performed at a constant current of 20 mA under controlled gel temperature

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Table 2. Least square means of different milk composition parameters under various genotypic groups (pertaining to exon 1 and exon IV with intron IV of β-lg gene) in different riverine buffalo breeds. Here, TF = Total fat yield, TP = Total protein yield, TS = Total solid yield, TSNF = Total SNF yield, and TWP = Total whey protein yield

	Exon I					Exon IV and intron IV	
Breed /Genotype	TF (Kg)	TP (Kg)	TS (Kg)	TSNF (Kg)	TWP (Kg)	Breed/ Genotype	TF (Kg)
Murrah							
A/A	$88{\cdot}80 \pm 18{\cdot}78^{A}$	$49{\cdot}34 \pm 8{\cdot}98^{A}$	$194{\cdot}94 \pm 35{\cdot}87^{A}$	$106{\cdot}14 \pm 17{\cdot}47^{A}$	$10{\cdot}36 \pm 1{\cdot}88^{A}$		
A/B	$124{\cdot}79 \pm 10{\cdot}87^{B}$	$69{\cdot}69 \pm 5{\cdot}19^{B}$	$274{\cdot}13 \pm 20{\cdot}76^{B}$	$149{\cdot}34 \pm 10{\cdot}11^{B}$	$14{\cdot}63 \pm 1{\cdot}09^{B}$		
B/B	$145{\cdot}14 \pm 11{\cdot}38^{C}$	$75{\cdot}55 \pm 5{\cdot}44^{C}$	$317{\cdot}83 \pm 21{\cdot}74^{C}$	$172{\cdot}69 \pm 10{\cdot}58^{C}$	$15{\cdot}86 \pm 1{\cdot}14^{B}$		
A/C	$151{\cdot}57 \pm 12{\cdot}4^{C}$	$78{\cdot}87 \pm 6{\cdot}18^{C}$	$329{\cdot}40 \pm 24{\cdot}71^{C}$	$177{\cdot}83 \pm 12{\cdot}03^{C}$	$16{\cdot}56 \pm 1{\cdot}29^{B}$		
C/C	$139{\cdot}10 \pm 14{\cdot}51^{B}$	$74{\cdot}67 \pm 6{\cdot}94^{C}$	$298{\cdot}01 \pm 27{\cdot}72^{B}$	$158{\cdot}91 \pm 13{\cdot}50^{B}$	$15{\cdot}68 \pm 1{\cdot}45^{B}$		
Bhadawari							
A/A	$76{\cdot}54 \pm 8{\cdot}92^{A}$	$34{\cdot}65 \pm 3{\cdot}68^{A}$	$158{\cdot}18 \pm 17{\cdot}51^{A}$	$81{\cdot}64 \pm 8{\cdot}79^{A}$	$7{\cdot}27 \pm 0{\cdot}77^{A}$		
A/B	$75{\cdot}99 \pm 9{\cdot}66^{A}$	$34{\cdot}25 \pm 3{\cdot}99^{A}$	$159{\cdot}45 \pm 18{\cdot}98^{A}$	$83{\cdot}45 \pm 9{\cdot}54^{A}$	$7{\cdot}19 \pm 0{\cdot}83^{A}$		
B/B	$94{\cdot}92 \pm 19{\cdot}72^{B}$	$41{\cdot}38 \pm 8{\cdot}13^{B}$	$195{\cdot}34 \pm 38{\cdot}73^{B}$	$100{\cdot}42 \pm 19{\cdot}45^{B}$	$8{\cdot}68 \pm 1{\cdot}0^{A}$		
A/C	$84{\cdot}10 \pm 12{\cdot}03^{B}$	$37{\cdot}13 \pm 4{\cdot}96^{A}$	$175{\cdot}83 \pm 23{\cdot}62^{A}$	$91{\cdot}73 \pm 11{\cdot}87^{C}$	$7{\cdot}79 \pm 1{\cdot}04^{A}$		
C/C	$31{\cdot}34 \pm 24{\cdot}58^{C}$	$13{\cdot}83 \pm 10{\cdot}13^{C}$	$64{\cdot}70 \pm 48{\cdot}26^{C}$	$33{\cdot}36 \pm 24{\cdot}24^{D}$	$2{\cdot}90 \pm 1{\cdot}12^{B}$		
Mehsana						**Mehsana**	
A/A	$83{\cdot}87 \pm 9{\cdot}07^{A}$	—	$203{\cdot}96 \pm 19{\cdot}24^{A}$	$192{\cdot}57 \pm 50{\cdot}34^{A}$	—	A/A	$93{\cdot}66 \pm 6{\cdot}76^{A}$
A/B	$86{\cdot}29 \pm 6{\cdot}92^{A}$	—	$212{\cdot}85 \pm 14{\cdot}69^{A}$	$115{\cdot}34 \pm 38{\cdot}42^{B}$	—	A/B	$92{\cdot}47 \pm 9{\cdot}64^{A}$
B/B	$136{\cdot}73 \pm 14{\cdot}74^{B}$	—	$326{\cdot}28 \pm 31{\cdot}28^{B}$	$208{\cdot}50 \pm 81{\cdot}86^{A}$	—	B/B	$94{\cdot}22 \pm 9{\cdot}18^{A}$
A/C	$78{\cdot}05 \pm 9{\cdot}02^{C}$	—	$196{\cdot}61 \pm 19{\cdot}15^{A}$	$117{\cdot}76 \pm 50{\cdot}09^{B}$	—	A/C	$120{\cdot}12 \pm 10{\cdot}57^{B}$
						C/D	$80{\cdot}70 \pm 13{\cdot}38^{C}$

Different superscripts indicate significant difference at $P \leqslant 0{\cdot}05$

of 4 °C for 10 h and 14 h, respectively. The gels were visualized after silver nitrate staining.

Sequencing

PCR products belonging to different genotypes were eluted from the 1% low melting agarose gel using gel elution kit (GIBCO BRL) for purification. The purified PCR-products were sequenced following the automated dye-terminator cycle sequencing method with Ampli *Taq* DNA polymerase in ABI PRIZM 377 DNA sequencer (Perkin-Elmer).

Estimation of Milk composition parameters

Three milk samples were collected randomly from each individual animal, that is first in stage I (First month of lactation), second in stage II (Second to third month of lactation) and third in stage III (Fourth to last month of lactation). These samples were employed for estimation of fat %, total solid (TS) %, SNF %, total protein (TP) % and whey protein % and mean values were calculated for each parameter. The average values were used to calculate total yield of each animal during the lactation. Least square analysis was performed to estimate the effect of genotypes on milk composition parameters.

Results and Discussion

The genotypes, alleles and their frequencies pertaining to two fragments of β-lg gene are presented in Table 1. Allele-wise nucleotide sequencing was performed in buffaloes both for 119 and 400 bp fragments. All the alleles of 119 bp product have been aligned and depicted in Fig. 1. There was no variation found in exon IV region among different alleles whereas allele-wise differences were detected in intron IV region of 400 bp fragment (Fig. 2). Both the fragments were found to be polymorphic in all the buffalo breeds. The gene as well as genotype frequencies varied from breed to breed. In some breeds, some genotypes were absent whereas in other, some genotypes were predominant. Sometimes, alleles were observed to be absent in some breeds indicating breed specific nature of allelic distribution. These variations are normally used for breed or species characterization, genetic divergence study, phylogenetic relationship and exploring genetic marker for economic traits.

Least square analysis was employed to estimate the effect of genotypes on milk composition traits. For 119 bp fragment, Bhadawari and Murrah breed genotypes were significantly ($P<0{\cdot}05$) co-related to all the composition traits studied and all traits, except total protein and total whey protein yield, were found to be significantly ($P<0{\cdot}05$) associated with genotypes in Mehsana (Table 2). Total protein yield was significantly related to the various genotypes in Murrah and Bhadawari breeds of buffalo. In Murrah, the genotypic contributions of A/C, B/B and C/C, which were not significantly different, were higher than A/B and A/A. Genotype A/C contributed 60% higher yield than the lowest contributor (A/A genotype). In Bhadawari buffaloes the pattern was different. Genotypic contributions to total protein

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yield were in the order B/B>A/A, A/B and A/C>C/C and genotype B/B produced 200% higher yield than the C/C genotype. Similarly, Aleandri et al. (1990) reported that dairy cattle with AA genotype produced higher total protein yield than other genotypes. The effect of β-lg gene variant on total fat yield, in both breeds, followed very similar trends to those observed for total protein yield (Table 2), with B/B and A/C producing the highest yields while C/C and A/A gave the lowest for Bhadawari and Murrah respectively. Comberg et al. (1964) and McLean et al. (1984) also revealed that BB and AB genotype produced higher milk fat production than AA type cattle.

Genotypes of 400 bp fragments were found to be non-significantly associated with all milk composition traits except total fat yield in Mehsana buffalo where it was observed to be significant at 5% level. The genotypes contributed total fat production in the order of A/C > A/A, A/B and B/B > C/D. It was observed that genotype A/C had 50% higher yield than C/D genotype.

Although we have obtained the significant relationship between genotype and traits for milk composition parameters, the analysis showed some limitation due to the small sample size. While analysing data, a number of tangible factors were included in the linear model for partitioning the total variances and their individual contribution showed significant effect. Keeping all these limitations in mind, our findings showed a significant trend of contribution of different genotypes on various milk composition parameters in buffaloes. This may pave an exclusive way to explore the possibility of utilizing potential of genotyping the candidate genes associated with milk composition traits. Basically, riverine buffaloes are the prized biological re sources for obtaining maximum milk solids in terms of protein, fat etc. and they are confined to the tropical Indian subcontinent. However, variants of β-lg gene showing significant differences in various economic traits may be given due consideration when formulating breeding programme of dairy buffaloes to produce milk suitable for product manufacture, as higher solids in milk is a desirable attribute for dairy industry. The B variant of β-lg protein has been preferred for cheese making in cow, as cow of this variant produces higher milk total solids, fat and casein concentrations (Aleandri et al. 1990). Likewise, it can be expected that buffalo with B variant of β-lg may produce milk with required compositional parameters to provide a higher yield of chee se from a given quantity of milk. However, in future biochemical and physiological studies may be followed to confirm the processing qualities of buffalo milk from animals with B variant of β-lg gene.

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