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Milk Protein Genes Polymorphism in Indigenous and Crossbred Cattle from a private Dairy Farm in Ethiopia

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ABSTRACT: Kappa casein and beta lactoglobulin genes are major milk proteins which have a direct effect on protein content in dairy cattle. Molecular-based selection through the identification of genetic polymorphism of major protein genes can be used to gain genetic improvement of milk protein yield. The objective of this study was to identify kappa casein (CSN3) and beta lactoglobulin (LGB) genes polymorphisms in indigenous and crossbreed cattle. A total of 90 whole blood samples were collected from individual animal in a private dairy farm. DNA extraction and quality assessment were done using salting out procedure and gel electrophoresis, respectively. Polymerase chain reaction (PCR) was performed with gene specific primers. For genotyping, PCR products of CSN3 gene was digested with *HinfI* and *HindIII* while LGB gene was digested with *HaeIII* restriction enzymes. Two haplotypes A and B; three genotypes, AA, AB and BB were observed at CSN3 of *HinfI* site and LGB *HaeIII* site, but only AA and AB were observed at CSN3 of *HindIII* site in crossbred and indigenous cattle populations. However, at CSN3 locus, A allele was found to be more common (0.65) in indigenous cattle than the B allele (0.35), while B (0.51) allele and AB genotype (0.81) were more frequent in crossbred cattle. But, LGB B allele was higher in indigenous cattle (0.67) compared to Allele A (0.33). LGB BB genotype (0.57) is higher followed by AA genotype in indigenous cattle while both allele and genotypic frequencies are equal in crossbred cattle. Both CSN3 and LGB loci were polymorphic in studied populations. Expected heterozygosity was higher in crossbred (0.49, 0.50) than in indigenous (0.38, 0.33) cattle at CSN3 and LGB locus, respectively which might be due to breed variation. The current findings showed that both CSN3 and LGB genes could be promising diagnostic markers in selecting dairy cattle breed. However, further investigations with large sample size and association study with milk composition is required to substantiate the current result.

Key words/ phrases: Genetic variation, Haplotype, Milk protein, Mutation, Restriction enzyme

INTRODUCTION

Ethiopia is believed to have the largest and home to a huge number of livestock populations in Africa (CSA, 2021) due to its diverse agro-ecology and the significance of livestock in subsistence strategies. There are about 70 million cattle in Ethiopia out of which 56 % are female and 44 % are male according to statistics carried out by the CSA (2021) and 2.3% and 0.31% of this total population are hybrid and exotic breeds, respectively. Local breeds make up the majority of the remaining cattle populations (97.4%) (CSA, 2021).

The livestock industry is a significant contributor to the economy, accounting for 19% of GDP and 16% to 18% of the nation's foreign exchange profits. About 35% of agricultural GDP is contributed by livestock and 45% if indirect contributions are taken into account (Shapiro *et al.*, 2017).

Dairy farming in Ethiopia is primarily a subsistence smallholder business with limited small and medium commercial dairy farms. Approximately 6.7 million dairy cows nationwide produced 3.6 billion liters of milk in 2019, with over 95% coming from regional breeds (Gebregziabher Gebreyohanes et al., 2021). However, there is a critical challenge in milk supply due to low milk yield of local indigenous cattle. Crossbreeding is the most common strategy used in the dairy sector to improve the productivity of the dairy cattle and milk production in the country (Berhane Hagos, 2020). Increasing the productivity of cattle will improve the performance of dairy sector and which will contribute to poverty reduction and

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create more job opportunities for youth (Gezu Tadesse and Zelalem Yilma, 2018).

Milk with high protein content is required as sources of good nutrition especially for children under five and school ages to provide better growth and intelligence. Milk protein with a good composition is useful to overcome health problems, such as hypoalergen on milk (Grenov *et al.,* 2020). High protein content in milk is also required during milk processing to produce good quality of dairy products. Better milk quality through the increasing protein and other components will give higher selling prices of fresh milk for small farmers, so it can ensure the sustainability of their dairy cattle business (Olanrewaju *et al.,* 2020).

Milk protein content in dairy cattle is basically controlled by two groups of major protein genes, namely casein and whey protein globulin. Kappa casein and beta lacto globulin genes are identified to be important of having direct effects for high milk protein content especially in dairy cattle (Azevedo *et al.,* 2008; Soheir *et al.,* 2013). Molecular based selection through the identification of genetic polymorphism of major protein genes can be used to gain genetic improvement of milk protein yield (Anggraeni *et al.,* 2017; Olanrewaju *et al.,* 2020).

Recent research on important milk protein genes have been known to influence both milk yield and composition has improved the efficiency of selecting superior animals (Čítek *et al.*, 2021; Albazi, *et al.*, 2023). It has been demonstrated that the genotypes of the kappa casein and beta lactoglobulin genes in particular have a considerable impact on the fat and protein levels as well as the manufacturing characteristics of milk (Özdemir and Doğru, 2005; Rachagani and Gupta, 2008). Such genes that explain a portion of the genetic variance and can enhance the assessment of breeding values during selection which enhancing traditional breeding programs and connected with performance measures (Caroli et al., 2009). A genetic polymorphism of kappa casein and beta lactoglobulin genes has been identified in different dairy cattle breeds (Azevedoet al., 2008; Rachagani and Gupta, 2008; Anggraeni et al., 2010; Gurses et al, 2016). However, there is limited information on genetic polymorphism of kappa casein and beta lactoglobulin genes except the report of genetic polymorphism of two genes on most indigenous cattle in Ethiopia (Roble Getachew, 2010). Understanding the genetic polymorphism of kappa casein and beta lactoglobulin genes among indigenous and crossbred cattle will serve as baseline information in designing a sustainable genetic improvement breeding program for dairy sector in Ethiopia. Therefore; the objective of the study was to identify Milk protein (kappa casein and beta lactoglobulin) gene polymorphisms in indigenous and crossbred dairy cattle from selected dairy farms.

MATERIAL AND METHODS

Description of sample population

Three indigenous cattle namely, Begait, Borana and Fogera and two crossbred (Born x Jersey and Fogera x Jersey) cattle breeds were considered for the study. The study animals were managed under Project Mercy, a Non-Government Development Organization, and the dairy farm is located 110 km away from Addis Ababa in North Shoa zone of Amhara National Regional state at 9°32'07.3"N 39°26'24.7"E and has an elevation of about 2773 meters above sea level. The study animals, Begait, Fogera and Boran breeds, have been directly procured by project mercy from Humera Tigray region, Bahir Dar Zuria Amhara region and Yebelo Oromia region, respectively. The farm is located at 9°32'07.3"N 39°26'24.7"E and has an elevation of about 2773 meters above sea level. Animals were selected purposively based on their recording history to avoid closely related animals.

Blood samples collection and DNA extraction

Blood samples were collected from Begait (15), Boran (15) and Fogera (15), Jersey Boran (20) and Jersey Fogera (25) crossbred by trained professionals, i.e 45 individual animals from each cattle breed. Blood was drawn from the jugular vein of cattle into 10 ml vacuum tubes containing Ethylene Diamine Tetra Acetic Acid (EDTA) as an anticoagulant and then kept on ice until transported to the laboratory. DNA extraction and quality assessment were done at Addis Ababa University and Bio and Emerging Technology Institute Laboratories. Genomic DNA was extracted from whole blood using salting out procedure. Blood samples were thawed to room temperature and 1.5 ml of blood was mixed with 4.5ml of cold EL buffer (0.155M NH4Cl, 10mM KCO3, and 1mM EDTA, pH 7.4) in a falcon tube and placed on ice for 15 minutes while mixing occasionally. After the tube was centrifuged for 10 minutes at 800 g, the

supernatant was removed retaining the pellet. The pellet was then washed twice with EL buffer until no sign of hemoglobin remained and then re-suspended in 3 ml lysis buffer (KL buffer: 10mM Tris, 2mM EDTA, 0.4 M NaCl, pH 8.2). The resulting cell lysates was incubated overnight at 37°C with 100 µl proteinase K [20 mg/ml] and 150 µl 20% SDS while shaking gently. After digestion was completed, 1.5 ml of saturated NaCl (6M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 1700 g for 15 minutes at room temperature. The precipitated protein pellet was then left at the bottom of the tube and 600 µl of the supernatant containing the DNA was transferred to a 2 ml microcentrifuge tube. Two volumes (1200 µl) of absolute ethanol maintained at room temperature were added and the tubes were inverted for several times until the DNA precipitated. The precipitated DNA was then spooled out with a plastic pipette, rinsed in 70% ethanol a few times and finally transferred to a 1.5 ml microcentrifuge tube containing 0.5 ml TE buffer [10 mMTris-HCl, 0.1 mM EDTA, pH 7.4]. The DNA was then allowed to dissolve overnight at 4^0c (Bruford et al., 1992).

The intactness of the DNA was checked on 0.6% agarose gel electrophoresis. The purity and concentration of DNA was determined by taking spectrometric readings using nanodrop. Samples having optical density ratio (OD260/OD280) between 1.6 - 2.0 were used for Polymerase Chain Reaction.

Polymerase chain reaction (PCR) amplification and Restriction Fragment Length Polymorphism (RFLP) assay

For the detection of kappa casein (CSN3) genotypes, a 633 bp DNA fragments was amplified by PCR using primers described by Rachagani and Gupta (2008). Forward: 5′- CAG CGC TGT GAG AAA GAT GA -3′ and Reverse: 5′- CCC ATT TCG CCT TCT CTG TA -3′. The PCR amplification reactions contained 80 to 100 ng of genomic DNA, 0.5 µM of each primer, 5X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of *Taq* DNA polymerase and sterilized ddH_2O to a final volume of 50 µl. The amplification was carried out in a thermal cycler with the following steps: pre-denaturation for 3 min at 94°C followed by 30 cycles of 94°C for 30 seconds, 58°C for 1 min, 72°C for 2 min, and a final extension of 10 min at 72°C. To confirm amplification of the target region, PCR products were then separated by agarose gel (1.75% w/v) electrophoresis using a 100 bp DNA marker. The gel was run using a 1X TAE buffer and stained with ethidium bromide (1.75µg/ml) for 30 minutes. 633bp CSN3 band was visualized on 1.75% agarose gel using a gel documentation system (Figure 1).

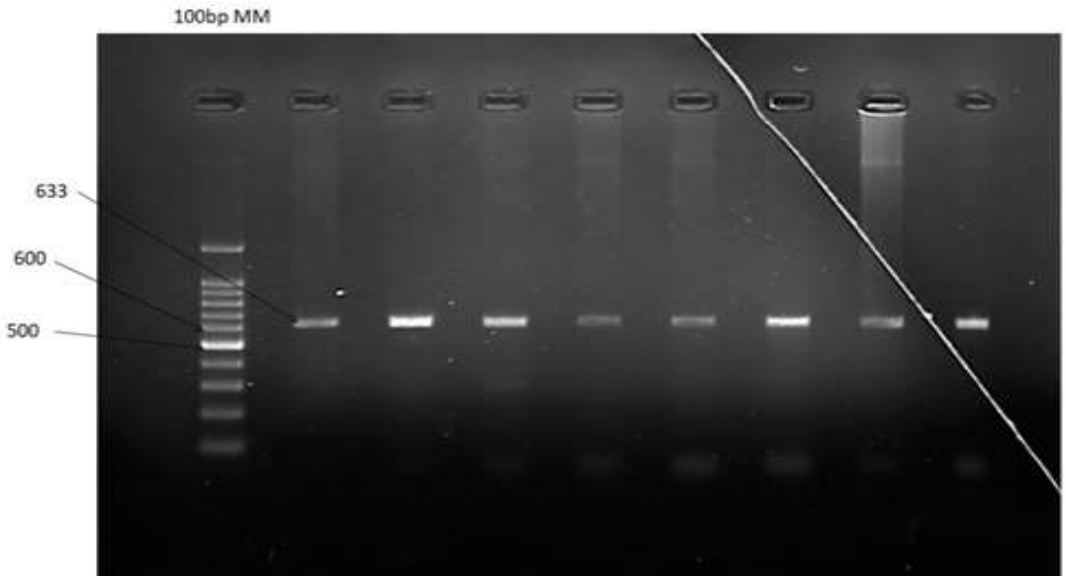

Figure 1. Electrophoretogram of the 633 bp CSN3 fragments generated by PCR amplification of genomic DNA using CSN3 specific primers.

Restriction digestion of the CSN3 PCR products was carried out using two restriction endonucleases: *HinfI* and *HindIII*. The digestion reaction for *HinfI* was set up with 4.65 µl of ddH_2O, 2.25 µl of restriction endonuclease buffer and 0.6 units of *HinfI* and 10 µl of PCR product which was incubated at 37°C for about 3 hours. The digestion reaction for *HindIII* was set up with 2 µl ddH_2O, 2.5 µl of restriction endonuclease buffer and 0.6 units of *HindIII* and 10 µl of PCR product and incubated at 37°C for about 3 hours. The digested PCR products were loaded and separated on 2% agarose gels using a 100bp DNA marker. The gels were stained with ethidium bromide and photographed using a gel documentation system. The different fragments were scored manually by direct counting.

To detect beta lactoglobulin (LGB) genotypes, a 529bp, DNA fragments was amplified by PCR using primers described by (Robel Getachew, 2010). Forward: 5'- CCT GCT GGA ACT CAC TTT CC-3 and Reverse: 5'-ACC TGC CAT TTG TTT TCA GG -3'. PCR amplification of the LGB gene, gel electrophoresis and band visualization were performed in the same manner as that of *CSN*3 gene mentioned above. A clear band pattern of 529bp LGB fragment was identified on 2% agarose gel using gel documentation system (Figure 2).

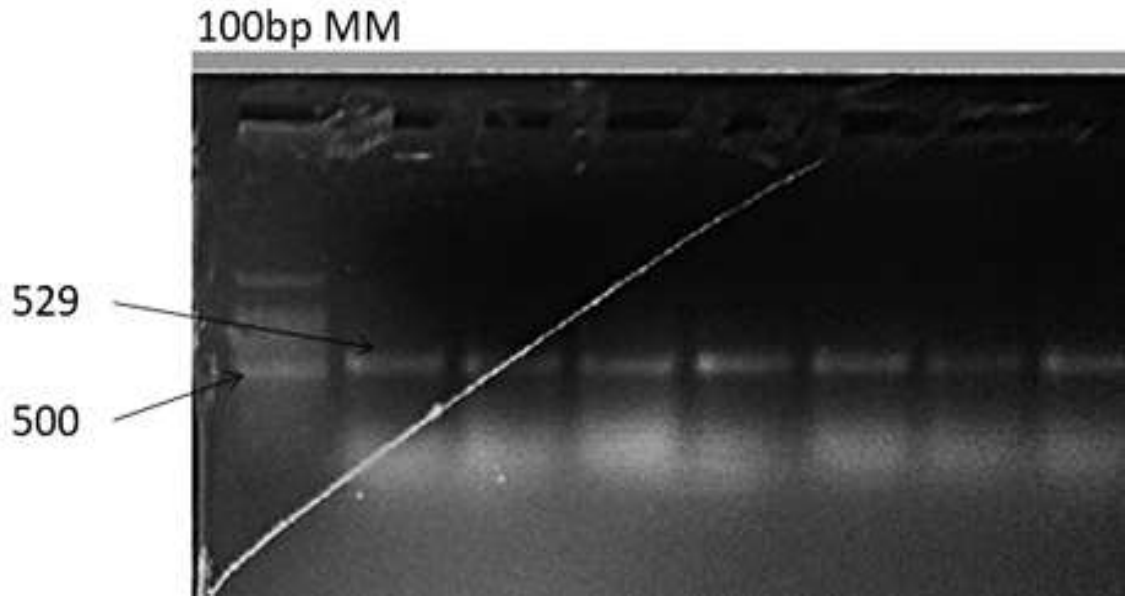

Figure 2. Electrophoretogram of the 529bp LGB fragments generated by PCR amplification of genomic DNA using LGB specific primers.

Restriction digestion of LGB PCR product was undertaken using *HaeIII* restriction enzyme. The digestion reaction for *HaeIII* was set up with 4.89 µl of ddH_2O, 2.25 µl of restriction endonuclease buffer and 3.6 units (0.36 µl) of *HaeIII* and 10 µl of PCR product and incubated at 37°C for about 3 hours. The digested PCR products were loaded and separated on 2% agarose gels in 1XTAE buffer using a 50bp DNA marker. The gels were stained with ethidium bromide and photographed using a gel documentation system. The different fragments were scored manually by direct counting.

Data Analysis

Allele and genotype frequencies and chi-square test of CSN3 and LGB locus was done were estimated and tested using PopGene 1.31 (Yeh et al., 1999). Observed (H_O) and expected (H_E) heterozygosity, effective number of alleles (Ne), Shannon information index, Proportion of polymorphic loci and Nei genetic distance were also calculated with the same software PopGene 1.31 (Yeh et al., 1999).

RESULTS

Restriction Fragment Length Polymorphism (RFLP) of CSN3 gene

Restriction digestion of the PCR products for the CSN3 gene which was carried out using two restriction enzymes, *HinfI* and *HindIII* in indigenous and crossbred cattle is presented in

Figure 3 and Figure 4. Out of 72 samples amplified, only 61 samples, 30 from indigenous and 31 for crossbred, were successfully digested with respective restriction enzymes. Three genotypes, AA (326, 131, 84 and 69bp), AB (457, 326, 131, 84 and 69bp) and BB (457, 84 and 69bp) were identified. All the three band partners were clearly identified on 2% of agarose gel except 23bp.

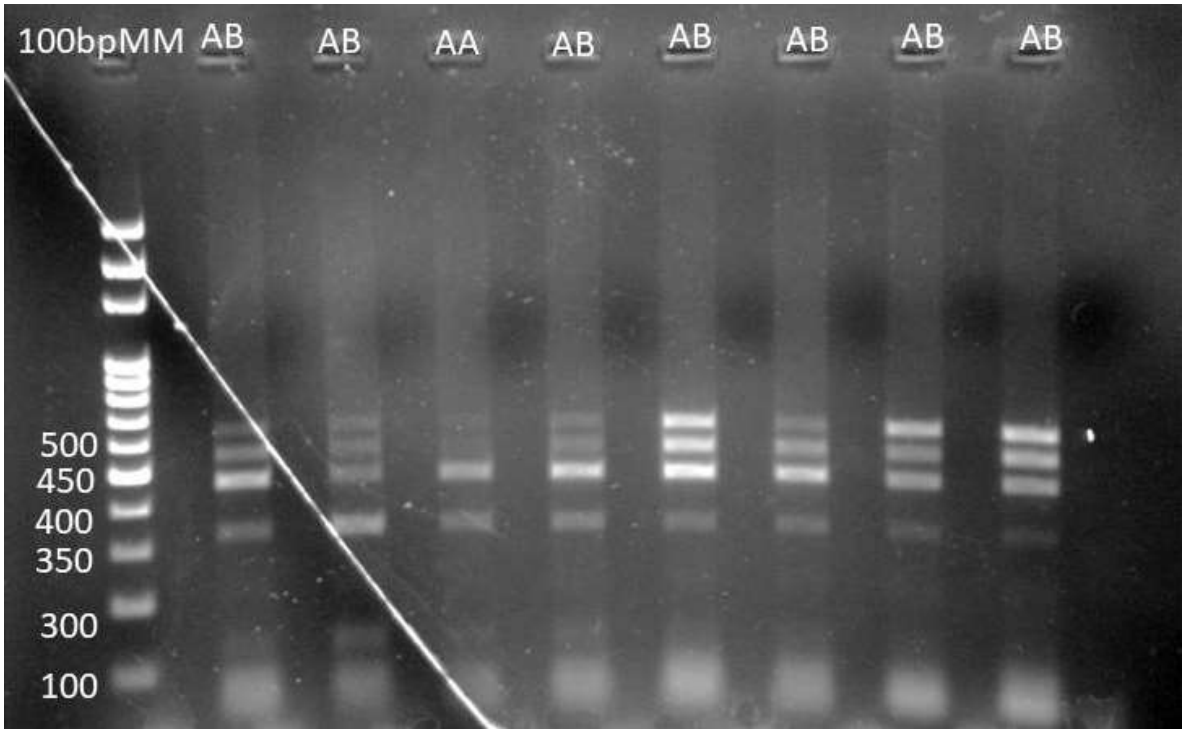

Figure 3. Electrophoretogram of the Restriction digestion of CSN3 gene of cattle using *HinfI* enzyme.

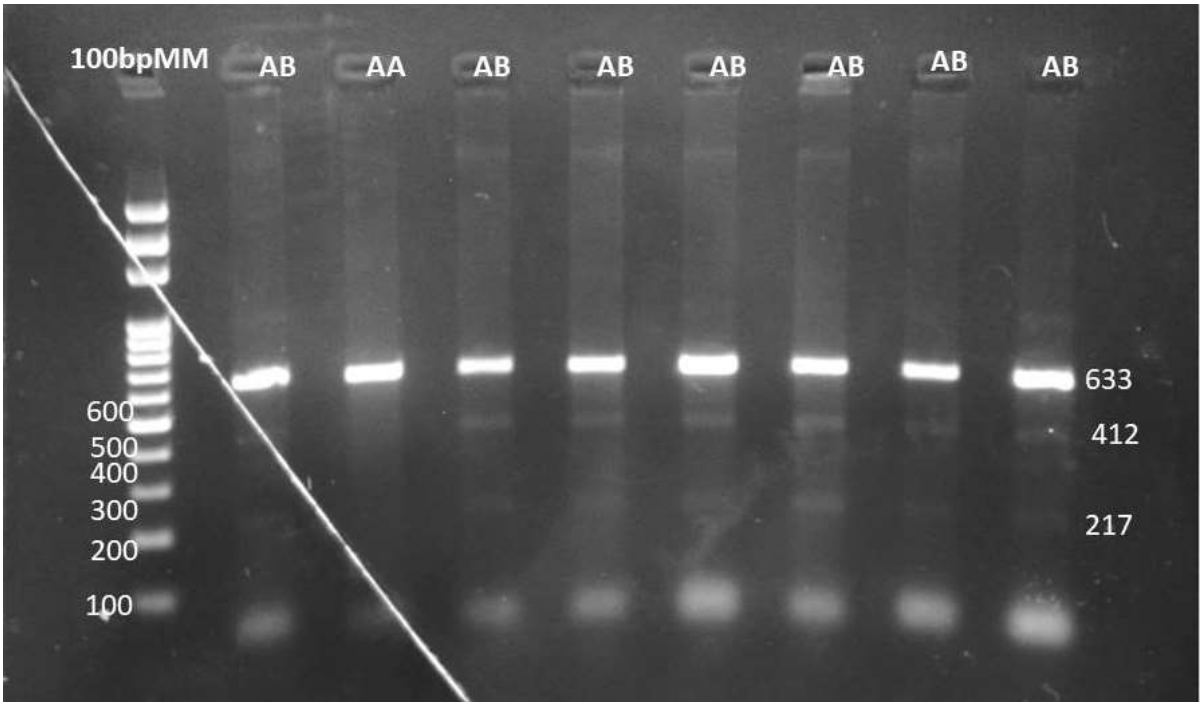

Figure 4. Electrophoretogram of the Restriction digestion CSN3 gene of cattle using *Hind*III enzyme.

Besides, the restriction digestion of CSN3 with *HindIII* endonuclease also revealed two distinct genotypes. The genotypes were AA (undigested, 633bp) and AB (633, 416 and 217bp) though band size 416 and 217 were not clearly seen compared to band size 633.The allelic and genotypic frequencies of CSN3 gene at *HinfI* marker variants varied across the breeds examined. Both indigenous and crossbred cattle showed A and B Allele with different allelic frequencies. The B allele frequency was higher in crossbred (0.60) than in indigenous (0.55) cattle while allele A had a frequency of 0.40 and 0.45 in crossbred and indigenous cattle, respectively. The same trend was also observed in genotypic frequency AB which was higher than AA and BB in both breeds. However, AB is higher in indigenous (0.77) than crossbred (0.74) cattle while BB genotype is higher in crossbred (0.23) than indigenous (0. 17) cattle as shown in Table 1. The genotypic frequency of CSN3 locus at *HinfI* site was deviated from Hardy-Weinberg equilibrium in both cattle breeds ($p<0.05$).

Table1. Allele and Genotypic frequency of CSN3 gene at *HinfI* restriction site in indigenous and crossbred cattle.

Cattle Breed	Alleles	Allele Frequency	Genotypes	Genotype Frequency	Genotype Observed	Genotype Expected	χ^2 value	*P* value
	A	0.45	AA	0.06	2	6.08	9.04	0.00*
Indigenous	B	0.55	AB	0.77	23	14.85		
			BB	0.17	5	9.08		
	A	0.40	AA	0.03	1	5.04	9.04	0.00*
Crossbred	B	0.60	AB	0.74	23	14.92		
			BB	0.23	7	11.04		

**** P<0.05***

The allele and genotypic frequencies of CSN3 gene at *HindIII* variants varied across the breeds examined. Both indigenous and crossbred cattle showed A and B Allele with different allelic frequencies. B allele frequency was higher in indigenous (0.85) and crossbred (0.56) cattle compared to allele A with its frequencies of 0.15 and 0.44 crossbred and indigenous cattle, respectively. AB genotype was higher (0.87) in crossbred while AA genotype was higher (0.77) in indigenous cattle. However, no BB genotype was observed in indigenous and crossbred animals at the *HindIII* restriction site as shown in Table 2. The genotypic frequency of CSN3 locus at *HindIII* site was deviated from Hardy-Weinberg equilibrium ($p<0.05$) crossbred cattle.

Table 2. Allele and Genotypic frequency of CSN3 gene at *Hind*III restriction site in indigenous and crossbred cattle

Cattle Breed	Alleles	Allele Frequency	Genotypes	Genotype Frequency	Genotype Observed	Genotype Expected	χ^2 value	*P* value
	A	0.85	AA	0.70	21	21.68		
Indigenous	B	0.15	AB	0.30	9	7.65	0.93	0.33
			BB	0.00	0	0.68		
	A	0.56	AA	0.13	4	9.87	18.45	0.00*
Crossbred	B	0.44	AB	0.87	27	15.24		
			BB	0.00	0	5.88		

ns=not significant, * P<0.05

The overall allele and genotypic frequencies of CSN3 locus are presented in Table 3. Both indigenous and crossbred cattle showed A and B Allele with different allelic frequencies. Allele A was dominant in indigenous (0.65) compared to crossbred (0.48) cattle while frequency of allele B was higher in crossbred (0.51) than in indigenous (0.35) cattle. A frequency of AB genotype is higher in crossbred (0.81) compared to in indigenous (0.54) cattle while AA genotype is relatively higher (0.38) in indigenous than in crossbred cattle (0.11). However, the genotypic frequency of CSN3 locus was deviated from Hardy-Weinberg equilibrium ($p<0.05$) in crossbred cattle but not in indigenous cattle ($p<0.05$).

Table 3. Allele and genotype frequency of CSN3 locus in indigenous and crossbred cattle.

Cattle Breed	Alleles	Allele Frequency	Genotypes	Genotype Frequency	Genotype Observed	Genotype Expected	χ^2 value	*P* value
	A	0.65	AA	0.38	23	25.35	1.78	0.00^{ns}
Indigenous	B	0.35	AB	0.54	32	27.30		
			BB	0.08	5	7.30		
	A	0.48	AA	0.08	5	14.52	23.42	0.00*
Crossbred	B	0.51	AB	0.81	50	30.97		
			BB	0.11	7	16.52		

ns=not significant, * p<0.05

Genetic variation within cattle breeds at CSN3 locus

The levels of genetic variation within cattle breeds are presented in Table 4. CSN3 locus was polymorphic in both indigenous and crossbred cattle populations. Expected heterozygosity was higher in crossbred (0.49) than indigenous (0.38) cattle. The same trend was also observed in Shannon index value, 0.68 and 0.56 in crossbred and indigenous cattle, respectively. However, the observed heterozygosity was higher than the expected heterozygosity in both breeds. A positive relation was observed between the effective numbers of alleles per locus and mean expected heterozygosity (Table 4).

Table 4. Genetic variation at Kappa Casein (CSN3) locus of indigenous and crossbred cattle.

Genetic diversity indices	Breed		Overall
	Indigenous	Crossbred	
No. of Effective Alleles	1.66±0.32	1.95±0.02	1.80±0.02
Proportion of Polymorphic Loci	100	100	100±0.02
Shannon's Information index	0.56±0.13	0.68±0.01	0.62±0.02
Observed Heterozygosity	0.53±0.23	0.81±0.07	0.67±0.02
Expected Heterozygosity	0.38±0.12	0.49±0.01	0.42±0.02

Nei's (1972) standard genetic distances and similarities at CSN3 locus among indigenous and crossbred cattle were estimated in order to assess the presence of genetic similarity and dissimilarity among cattle breeds at CSN3 locus. The shortest genetic distance (0.07) and highest (0.93) identity was observed between indigenous and crossbred cattle.

RFLP of LGB gene

Restriction digestion of the PCR products for the LGB gene which was carried out using *HaeIII* restriction enzymes in indigenous and crossbred cattle is presented in Figure 5. Out of 72 samples amplified, only 60 samples, 30 from each population were successfully digested with respective restriction enzymes. Two haplotypes, A and B, and three genotypes, AA (211, 181 and137 bp), AB (211, 181, 137, 125 and 86bp) and BB (181, 137, 125 and 86bp) were identified through restriction digestion of the PCR product of LGB locus with *HaeIII* enzyme.

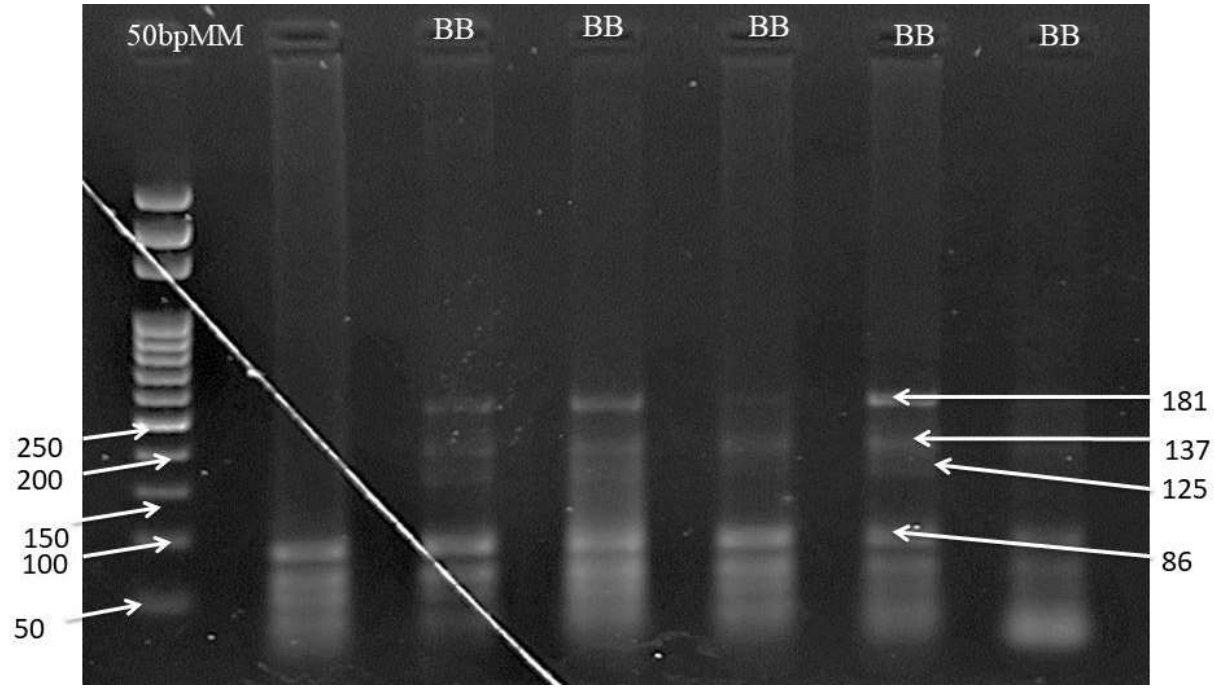

Figure 5. Electrophoretogram of the restriction enzyme patterns of LGB gene of cattle breed using *HaeIII* enzyme.

The allele and genotypic frequencies at LGB locus in both breeds are shown in Table 5. Both indigenous and crossbred cattle showed A and B allele with different frequencies. Frequency of B allele was higher in indigenous cattle (0.67) compared to allele A (0.33). Frequency of LGB BB genotype (0.57) is higher followed by AA genotype in indigenous cattle. However; both allele and genotypic frequencies were equal in crossbred cattle. There was a significant difference between observed and expected genotype at LGB locus ($p<0.05$) in indigenous cattle which showed that indigenous cattle population in the farm was deviated from Hardy-Weinberg equilibrium while there was no significance difference ($p>0.05$) observed in crossbred cattle at the same locus (Table 5).

Table 5. Allele and genotype frequency of LGB locus in indigenous and crossbred cattle.

Cattle Breed	Alleles	Allele Frequency	Genotypes	Genotype Frequency	Genotype Observed	Genotype Expected	χ^2 value	*P* value
	A	0.33	AA	0.23	7	3.33	9.07	0.00*
Indigenous	B	0.67	AB	0.20	6	13.33		
			BB	0.57	17	13.3		
	A	0.50	AA	0.33	10	7.5	3.33	0.68^{ns}
Crossbred	B	0.50	AB	0.33	10	15.0		
			BB	0.33	10	7.5		

*ns=not significant, * P<0.05*

Genetic variation within cattle breeds at LGB locus

The levels of genetic variation within cattle breeds are given in Table 6. LGB locus was polymorphic in both indigenous and crossbred cattle. Expected heterozygosity (H_E) was higher in crossbred (0.50) than indigenous (0.44) cattle. The same trend was also observed in Shannon index value, 0.69 and 0.64 in crossbred and indigenous cattle, respectively. However, the observed heterozygosity was higher than the expected heterozygosity in both breeds. A positive relation was observed between the effective numbers of alleles per locus and mean expected heterozygosity (Table 6).

Table 6. Genetic variation at Beta Lactoglobulin (LGB) locus of indigenous and crossbred cattle.

Genetic diversity indices	Breed		Overall
	Indigenous	Crossbred	
No. of Effective Alleles	1.800	2	1.90±0.00
Proportion of Polymorphic Loci	100	100	100
Shannon's Information Index	0.64±0.13	0.69±0.01	0.67±0.03
Observed Heterozygosity	0.20±0.23	0.33±0.07	0.27±0.07
Expected Heterozygosity	0.44±0.12	0.50±0.01	0.42±0.03

Nei's (1972) standard genetic distances and similarities at LGB locus among indigenous and crossbred cattle breeds was estimated in order to assess the presence of genetic similarity and dissimilarity among cattle breeds at LGB locus. The shortest genetic distance (0.05) and highest (0.95) identity was observed between indigenous and crossbred cattle.

DISCUSSION

High protein content in milk is required during milk processing to produce good quality dairy products. Better milk quality through the increasing protein and other components will give higher selling prices of fresh milk for small farmers, so it can ensure the sustainability of their dairy cattle business (Olanrewaju *et al.*, 2020). CSN3 and LGB are milk protein genes that are identified as having direct effects on high milk protein content especially in dairy cattle (Azevedo *et al.*, 2008; Soheir *et al.*, 2013). Polymorphism of Bovine CSN3 and LGB genes had been used as useful DNA markers for selecting dairy cows with better milk protein content (Anggraeni *et al.*, 2010). Understanding the genetic polymorphism of CSN3 and LGBgenes will pave the way for marker assisted selection in dairy cattle genetic improvement. In the current study, we identified a genetic polymorphism at CSN3 and LGB locus both in indigenous and crossbred cattle.

Genetic polymorphism at Kappa casein (CSN3) gene in cattle

Kappa casein constitutes about 80% of total milk protein among the four casein gene identified in cattle (Soheir *et al.*, 2013). Kappa casein polymorphism in cattle has been extensively investigated. Thirteen protein variants and 1 synonymous variant have been reported in cattle CSN3 gene (Gallinat *et al.*, 2013). Point mutations in exon IV of the bovine kappa-casein (CSN3) gene determine two allelic variants, A and B (Alipanah *et al.*, 200). The A and B variants differ in the amino acids number 136 and 148. At position 136, threonine is replaced by isoleucine, while at position 148, aspartic acid is replaced by alanine, for A and B, respectively (Alexander *et al.*, 1988).

Higher frequency of CSN3B allele was observed in indigenous and crossbred cattle at *HinfI* markers though the number is higher (0.60) in crossbred than indigenous (0.55) cattle which was inagreement with the result of Anggraen *et al.* (2010) who reported that the CSN3 gene was dominated by B allele at the same markers. However, CSN3A allele was the most frequent allele at *HindIII* site in in indigenous and crossbred cattle groups though there was a variation in frequency among indigenous (0.85) and crossbred (0.56) cattle. The same result was reported by (Gurses *et al.*, 2016; Anggraeni *et al.*, 2010). Both cattle groups were not in Hardy-Weinberg equilibrium at *HinfI* sites but only indigenous cattle were in Hardy-Weinberg equilibrium at *HindIII* marker which might be due to small population size and inbreeding. The same result reported by (Robel Getachew , 2010) for Boran and Sheko cattle in Ethiopia.

At both sites, the CSN3A allele was found to be more commonly distributed (0.65) in indigenous cattle than the CSN3B allele (0.35). These results were in accordance with the previously published studies in local breeds of Indian (Rachagani and Gupta, 2008) and in Ethiopia (Roble Getachew, 2010). On the other hand, Hallén *et al.*, (2008) reported that CSN3 gene allele A was predominately higher in Holstein, Jersey and Brown Swiss cattle in Turkey and in Holstein-Friesian cattle of Macedonian (Adamov *et al.*, 2020). However, CSN3B allele and CSN3AB genotype were more

frequently appeared (0.51 and 0.81, respectively) in crossbred cattle population. The same result was reported by (Gurses *et al.,* 2016) who showed that CSN3B allele was more common in Jersey and the Brown Swiss cattle. This difference might be due to breed difference. Kappa casein polymorphism was reported in different dairy cattle breeds (Zepeda-Batista *et al.,* 2015; Gurses *et al.,* 2016; Adamov *et al.,* 2020). High frequency of CSN3A allele observed in indigenous cattle in the current study might be due to the fact that indigenous cows known for their high fat content compared to crossbred cows. Different authors reported that casein A haplotype were associated with high fat yield in dairy cattle (Ng-Kwai-Hang *et al*., 1984; Azevedo *et al.,* 2008; Gurses *et al.,* 2016). Allele B is of practical importance, since the B allele is found to correlate with commercially valuable parameters of cheese yielding efficiency (Azevedo *et al* 2008). Higher frequency in CSN3 B allele in crossbred cattle might be associated with both milk yield and protein content. Adamov *et al.* (2020) reported that the B allele of CSN3 promotes an increase in cheese yield and improves cheese quality, but also correlates with protein content and milk yield.

Kappa casein AB genotype was more frequently observed in both breeds (0.77 and 0.74 in indigenous and crossbred cattle, respectively). However, at *HindIII* site, AA genotype is more frequent (0.77) in indigenous cattle while it was AB genotype in crossbred cattle (0.87) which might be due to better no of A allele and breed variation at *HindIII* site. The favorable effect of the AA genotype in the kappa-casein gene on proteins might be explained by amino acid differences in the mature protein, which may affect the biological properties of kappa casein and its interactions with the other fractions in the casein micelles which will help as a candidate marker to identify breeds for kappa casein of milk and related traits (Debi et al., 2014).

Hence, Better polymorphism at kappa casein gene found the current result might which will help as a candidate marker to identify breeds for kappa casein content of milk and related trait. At the CSN3 locus, AB genotype is more dominant (0.81) in crossbred than indigenous (0.54) cattle in which AA genotype has better share (0.38) than crossbred (0.05) cattle. The same results were reported by (Anggraen *et al.,* 2017; Adamov *et al.,* 2020). Lower number of BB is found in both cattle breed found in the current study was in line with the report of (Anggraini *et al.,* 2010) who reported few BB genotype Holstein Feresian cattle despite of the relatively high frequency of the B allele over the A allele. Kappa casein constitutes about 80% of total milk protein among the four-casein gene identified in cattle (Soheir *et al.,* 2013). Association of CSN3 casein genotype and milk product processing properties has been reported in different cattle breeds Anggraen *et al.,* 2010; Adamov *et al.,* 2020). For example, CSN3 AA genotype was associated with lower concentration of casein in milk protein and not favorable for cheese production while it will be good for milk yield and fat production. Milk with CSN3 BB genotype had higher amount of casein concentration and most favorable genotype for cheese production (Eenennaam and Medrano, 1991) while it was an intermediate amount in AB genotype. It is associated with better milk rennet coagulation properties in terms of shorter rennet clotting time, higher curd firmness and higher cheese yield (Eenennaam and Medrano, 1991; Jensen et al. 2012). These differences are related to the micelle size and the glycosylation degree of the coded protein (Ikonen et al., 1999). More frequent A allele and AA genotype at CSN3 locus in indigenous cattle found in the current study might be associated with fat production. It is supported by the result of (Anggraini *et al.,* 2010) who reported that AA genotype dairy cows significantly associated with higher fat content compared to AB and BB genotype in west Java. The effects of CSN3 polymorphisms on milk production traits were found different among breeds. Protein and solids-not-fat content were affected by the genotypes in Holstein and Brown Swiss, whereas in Jerseys, genotypes had an effect on fat content of milk (Gurses *et al.,* 2016; Azevedo *et al.,* 2008). Alipanah *et al.* (2005) also reported that fat and protein content were affected by CSN3 polymorphism in Russian Black Pied, whereas Russian Red Pied genotypes had an effect on milk, fat yield and fat content. On the other hand, allele B and AB genotype were observed more frequently in crossbred cattle in the current study might be due to crossing of indigenous with Jersey dairy cattle. Gurses *et al* (2016) indicated that the B allele was more frequent in Jersey and it is because Jersey cattle is well known in its potential to transform milk to cheese, and it presents a higher frequency of B variant (Zepeda-Batista *et al.,* 2015). The genotype frequency variation in both cattle breed showed that there will be an opportunity to select dairy cattle with desired genotype and include it in the genetic improvement program.

Estimates of mean expected heterozygosity at CSN3 locus obtained in the present study were within the recommended range. An average heterozygosity should be between 0.3 and 0.8 in the population to be used in measuring genetic variation (Nei, 1996). There was high diversity within crossbred cattle compared to indigenous breed. Effective and mean number of alleles at each locus gives information about the predominance of certain alleles in the populations. The average mean number of alleles reported for the current study is in line with the report of (Gurses *et al.,* 2016) for different dairy cattle. CSN3 locus was polymorphic both in indigenous and crossbred cattle. Alleles of polymorphic loci can be used as diagnostic marker to discriminate between cattle breeds (Chu *et al.,* 2011).

Higher values of observed heterozygosity and expected genotype at CSN3 locus in crossbred cattle might be due to reproductive management and genetic improvement program of the dairy farm. The current finding is in agreement with research finding of (Ren *et al.,* 2011) who indicated that planned mating and artificial selection for an economic trait is expected at commercial farm.

Genetic polymorphism at LGB gene in cattle

More frequent allele B at LGB locus was observed in indigenous (0.67) than crossbred (0.50) cattle while Allele A was higher in crossbred (0.50) than in indigenous (0.33) cattle on the same locus. This was in agreement with the result of (Rachagani *et al.,* 2006; Roble Getachew, 2010; Olanrewaju *et al.,* 2020) who reported that more frequent allele of B at LGB was observed in local cattle (*Bos indicus*) of India, Ethiopia and Nigeria. On the other hand, AB genotype was more frequent in crossbred (0.33) than indigenous (0.20) cattle. The same result was reported by (Rachagani *et al.,* 2006; Anggraini *et al.,* 2010) who indicated that AB was more frequent genotype in local dairy cattle. Association of variants with its LGB content, whey protein and milk product processing properties has been reported in different cattle breeds (Zepeda-Batista *et al.,* 2015; Laisin *et al.,* 2021). For example, LGB A allele was associated with higher LGB content in milk while allele B with lower LGB content in milk. Milk with LGB AA genotype associated with increase milk yield and whey protein content (Anggraini *et al.,* 2010) while BB genotype associated with increase casein and fat content and favorable for cheese production (Eenennaam and Medrano, 1991; Mir *et al.,* 2014; Laisin *et al.,* 2021). An increase of the B allele in the indigenous breeds can help increase their milk yield. Indigenous cattle groups were diverted from Hardy-Weinberg equilibrium at HaeIII site of LGB locus which might be due to selction bressure at *HaeIII* site. The same results were also reported for Boran and Sheko cattle in Ethiopia (Robel Getchew, 2010). Selection pressure, inbreeding and small size of the population will be the responsible factors for the deviation form Hardy-Weinberg equilibrium (Triantaphyllopoulos et al., 2017; Laisin *et al.,* 2021).

Estimates of mean expected hetrozygosity obtained in the present study were within the recommended range. An average heterozygosity should be between 0.3 and 0.8 in the population to be used in measuring genetic variation (Nei, 1996). There was high diversity within crossbred cattle. Effective and mean number of alleles at each locus gives information about the predominance of certain alleles in the populations. The average mean number of alleles reported for the current study is in line with the report of (Zepeda-Batista *et al.,* 2015) for dairy cattle. LGB locus was polymorphic both in indigenous and crossbred cattle. Alleles of polymorphic loci can be used as diagnostic marker to discriminate between cattle breeds (Chu *et al.,* 2011).

CONCLUSION

Two haplotype A and B; three genotypes, AA, AB and BB were observed at kappa casein of *Hinf*I site and LGB loci but only AA and AB were observed at kappa casein of *Hind*III site in both cattle populations. Kappa casein B allele and AB genotype were more frequently appeared (0.50 and 0.81, respectively) in crossbred cattle while CSN3A allele and AB genotype were more frequently appeared (0.65 and 0.51, respectively) in indigenous cattle population. However, Lactoglobulin B allele and BB genotype were more frequently appeared (0.67 and 0.57, respectively) in indigenous cattle while both allele and genotypic frequencies were equal (0.50 and 0.33, respectively) at LGB locus in crossbred cattle. Expected heterozygosity was better in crossbred (0.49, 0.50) than indigenous (0.38, 0.33) cattle both at CSN3 and LGB loci, respectively. Chi-square test revealed genetic equilibrium in indigenous at CSN3 and LGB loci in crossbred cattle whereas crossbred cattle was not in Hardy-

Weinberg equilibrium at CSN3 locus and indigenous cattle at LGB locus. The polymorphism in kappa casein and lactoglobulin genes found both in indigenous and cross bred cattle will be an opportunity for selection of dairy cattle for milk protein and related traits.

Generally, variation in allele and genotypic frequencies observed and expected hetrozygosity, Hardy-Weinberg equilibrium test within and among indigenous and crossbred cattle at both loci in the current study showed that CSN3 and LGB genes will be a promising diagnostic marker in selecting dairy cattle for the desired milk protein of the farm. However, further investigations with large sample size and association study with milk composition is required to substantiate the current results.

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