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Original article

Genetic polymorphism of beta-lactoglobulin in kenyan small east african goat breed using pcr-rflp and sequencing

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ABSTRACT

Refinement of coherence and economic gains is an essential target in Dairy industry. This can be achieved by increasing economic returns without increasing the size of the herd. Animal selection is the main method for improvement of livestock production around the world. The determination of candidate genes for economic traits holds a promising future as molecular markers for improving productivity in farm animals. Polymorphism in the beta-lactoglobulin gene has been successfully studied in many goat populations of the world. However, this has not been clarified yet in the local Kenyan goat breeds. The objectives of this study were to screen beta-lactoglobulin gene variants and to identify its polymorphism in Small east African goat breed using Polymorphic Chain Reaction and Restriction Fragment Length Polymorphism and sequencing techniques. A total of 60 goats were genotyped, 30 from each region (Samburu and Narok Counties). Genomic DNA was then isolated using Qiagen QiAmp blood mini kit. The amplified product was observed as 426 bp of exon 7 and the restriction digestion with SacII revealed two alleles, namely A and B and three genotypes, (AA, AB and BB) at the β-lactoglobulin gene locus. Allelic frequencies for goats found in Samburu and Narok were 0.233 and 0.133 respectively for A allele; 0.767 and 0.867 for B allele respectively , while genotypic frequencies were 0.1 and 0.0 for AA, 0.267 and 0.267 for AB, and 0.633 and 0.733 for BB respectively. In the pooled data

for the small east African goat breed, the allelic frequencies were 0.183 and 0.817 for the A and B allele respectively, while genotypic frequencies were 0.05, 0.267 and 0.683 for the AA, AB and BB respectively. No deviations from the Hardy Weinberg equilibrium were observed. After PCR, a 426 base pairs sequence in exon 7 of 60 goat samples were sequenced and variation analyzed. Two point mutations corresponding to base substitutions were identified. The substitutions of G to A were found at both positions 6705 and 6751 as compared to the Capra hircus sequence (Accession number Z33881.1). Further studies on other beta-lactoglobulin gene regions as well as other milk protein genes are necessary to establish associations of all its variations and the effects of the variants in the indigenous goat breeds.

1. Introduction

Indigenous goats of Kenya are very important to poor farmers and pastoralists; despite being faced with challenges of persistent droughts, diseases, conflicts and poor nutrition, goats, especially indigenous in an area, are the most versatile domestic animals in adaptation to arid and humid, tropical and cold, and desert and mountain conditions (Gall, 1991; Quarterium, 1991 and Silanikove, 2000; Muigai et al., 2009). According to the FAOSTAT database for the world population of goats, Africa has the second largest goat population after Asia. Kenya alone has 29 million goats contributing 34,598 tonnes of indigenous goat meat production annually (FAOSTAT, 2011).

The Small east African goat breed is widespread throughout Kenya especially among the pastoralist communities. Pastoralists depend on goats mainly for meat and milk. Milk production per doe is extremely variable. To maximize economic gain from goats, farmers must obtain high milk per goat. Milk production is affected by both environmental and genetic factors. Polymorphism on milk protein genes is used as a marker for improving milk production in livestock (Ng-Kwai- Hang, 1998).

Beta-lactoglobulin (β-LG) is one of the two major whey proteins identified in the milk of most animals including cattle, sheep, goat, dogs, and pigs, but it is not identified in human, mice, lagomorphs and other some mammalian species (Kumar,et al. 2006). β-LG was the first milk protein in which polymorphism was revealed by protein electrophoresis of bovine milk (Aschaffenburg and Drewry, 1955). Since then, genetic polymorphisms in β-LG have been described both at protein level (Kumar et al 2002; Gargi et al., 2009; Baranyi et al., 2010.) and DNA level (El-Hanafy et al., 2010; Elyasi et al., 2010; Oner et al 2011) in livestock. Genetic polymorphisms identified in β-LG gene results to the formation of its different variants within and between various species (Patel.,et al, 2007). The genetic association of different variants of the β-LG and milk production traits has been reported in cow (Rachagani et al, 2006; Karimi et al, 2009)), goat (Kumar,et al. 2006) and sheep (Garzon and Martinez, 1992).In a study by Moili et al., (1998), they revealed two genetic variants (A and B) of β-LG in goat milk at the protein level. Variation in exon 7 of β-LG gene in Spanish and French goats was also investigated using PCR-RFLP and sequencing, and two new genetic variants were reported (Pena et al, 2000). However no study has been reported in Kenyan indigenous goats (Small east African goat) regarding beta-LG polymorphism at the protein or DNA level. This study was aimed to analyze the genotyping of the β-LG in the Kenyan Small east African goat breed using PCR-RFLP technique.

2. Materials and methods

2.1. Sample collection and DNA extraction

The goats undertaken in this study were selected from two counties (Samburu and Narok) which belong to the similar agro-ecological zone but distant from each other and random strategies were employed to choose goats for sampling. To minimize the likelihood of any close genetic relationships, only a maximum of two

individuals were sampled from the same village. Animals sampled in this study were selected based on their phenotype which should represent as much as possible to the known characteristics of the small east African goat breed. Therefore, blood samples from the jugular vein of equal number of males and females were collected using vacuutainer tubes containing anticoagulant (EDTA) from a total of sixty goats; Samburu County (30 goats) and Narok County (30 goats). DNA was extracted from 100 µl of blood, using a commercial Qiagen kit (QIAamp Blood mini kit; Qiagen, Hilden, Germany) following the manufacturer's protocol. The quality of extracted DNA was measured by electrophoresis on 1 % agarose gel stained with ethidium bromide.

2.2. PCR amplification

Genotyping for β-LG-SacII polymorphism was performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) as proposed by Pena et al. 2000). Primer sequences were the following, Forward 5'-CGG GAG CCT TGG CCC TCT GG-3'; Reverse 5'-CCT TTG TCG AGT TTG GGT GT-3'. PCR amplification was performed in a total of 25 µl reaction mixture containing 150 ng goat genomic DNA, 0.5 µm of each primer, 150 µm of each dNTP, 0.5 units of taq polymerase (PHUSION HIGH FIDELITY DNA POLYMERASE, Themoscientific Inc. USA). The amplification reaction was carried out under the following conditions, Initial denaturation step of 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 67 °C for 1 min and 72 °C for 1 min, and concluded with a final extension of 72 °C for 5 min, using a Perkin Elmer cetus DNA Thermal Cycler 480 (Perkin-Elmer Applied Biosystems, Foster City, CA 94404, USA). The reaction products were analyzed by electrophoresis on 2 % agarose gel. Ethidium bromide was added to gels to visualize the analysis results under UV light in the UVP 97-0192-01 MultiDoc-It UV Imaging System (UVP, Cambridge, UK).

2.3. PCR-RFLP genotyping

The PCR products were purified using Thermo Scientific GeneJET PCR Purification Kit.

The resultant purified PCR products (10µl each) were digested with 10 U (1µl) of SacII restriction endonuclease (Themoscientific Inc. USA) at 37°C for 6 hours. Then the digests were electrophoresed on 2.5% agarose gel stained with 1% ethidium bromide.

2.4. Sequencing

Sanger sequencing was performed for β-LG exon VII region using the Primers used for PCR in both Forward 5'-CGG GAG CCT TGG CCC TCT GG-3' and Reverse 5'-CCT TTG TCG AGT TTG GGT GT-3' with an expected size of 426 bp. All the 60 samples were selected for sequencing. Sequencing was carried out using the big dye terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Purified products were then electropherosed on ABI 3730 XL (Applied Biosystems) as per the manufacturer's protocol in the Beckman Coulter Genomics Inc. Facility (UK).

2.5. PCR-RFLP and sequence data analysis

Direct counting was used to estimate genotype and allele frequencies of β-LG genetic variants as observed after PCR-RFLP. Chi-square statistic ($\chi 2$) was used to check whether the populations were Hardy-Weinberg equilibrium. Most other statistical computations were performed using PopGene32 software (Yeh et al., 2000).

BioEdit (Ibis Biosciences) was used for viewing and editing the DNA sequences. Multiple alignments were performed using CLUSTALW program (Thompson et al., 1994) and compared with already deposited nucleotide sequences of β-LG gene of Capra hircus (GenBank sequence Z33881). The sites that were polymorphic were confirmed by visual examination of the electropherograms. The sequence data were subjected to analysis of molecular variance (AMOVA) using the Arlequin 3.5.1.3 program (Excoffier et al., 2005). Goats from the same geographic regions were grouped together to obtain two final groups (Samburu and Narok), i.e. to make Samburu Small East African goats and the Narok Small East African goats.

3. Results

Amplification of exon 7 of goat β-lactoglobulin gene produced single band with molecular size of 426 bp (Figure 1) in all the samples. PCR-RFLP with the SacII enzyme revealed the polymorphic site, which was produced by a single nucleotide substitution in position +4601 (Pena et al., 2000). This revealed two variants A and B and three genotypes AA, AB and BB in the entire population studied. These genotypes were discriminated according to their fragment sizes as viewed after their electrophoresis i.e. AA (undigested 426bp), BB (349bp and 77bp) and AB

(426bp, 349bp and 77bp) as shown in Figure 2). The allelic and genotypic frequencies of the beta-LG gene polymorphism are presented in Figure 3. Allelic frequencies for goats found in Samburu and Narok were 0.233 and 0.133 respectively for A allele; 0.767 and 0.867 for B allele respectively , while genotypic frequencies were 0.1 and 0.0 for AA, 0.267 and 0.267 for AB, and 0.633 and 0.733 for BB respectively. In the pooled data for the small east African goat breed, the allelic frequencies were 0.183 and 0.817 for the A and B allele respectively, while genotypic frequencies were 0.05, 0.267 and 0.683 for the AA, AB and BB respectively. To test genotypic frequencies for fit to Hardy-Weinberg's equilibrium (HWE), the Chi-square test was used which compares the observed and expected genotype counts. The LGB locus was found to be in HWE for both populations examined with P-value ranging from 0.131 in Samburu to 0.434 in Narok indicating that there were no significant deviations at 5% significance level (α=0.05).

The PCR products were subjected to sequence analysis in comparison to each other and to that published in gene bank (accession No. Z33881). CLUSTALW program revealed complete homology except SNPs at specific nucleotides. Two single nucleotide substitution were found in exon 7 at position 6705 (G/A) and 6751 (G/A). The AMOVA revealed that there was a large percentage (98.15%) of total variation within populations and a very small percentage (1.85%) among populations.

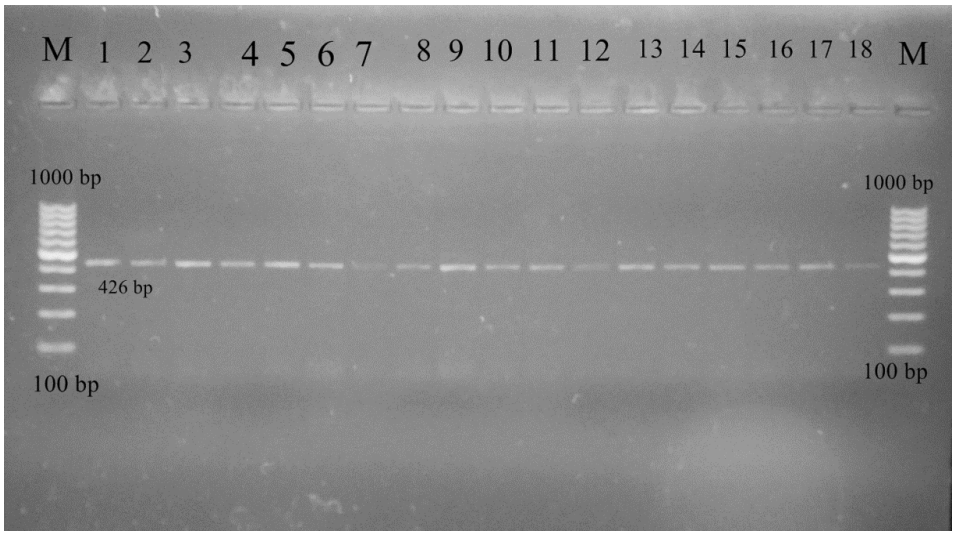

Fig. 1. Electrophoresis pattern of amplified caprine genomic DNA with b-Lg specific primers separated on 2% agarose gel. Lane 1-18, fragments amplified for b-Lg locus showing a 426bp amplicon. Lane M, molecular size marker (100 bp DNA Step Ladder).

Fig. 2. Electrophoresis pattern for b-Lg locus after digestion with sacII restriction enzyme. Lane 1, AA genotype; lanes 2, 6, 10, 12, 13, 16,18, AB genotype; lanes 3,4,5,7,8,9,11,14,15,17, BB genotype. Lane M, molecular size marker (100bp DNA Step Ladder).

Allele and genotype frequencies of β-LG gene for Sac I I site in Small East African goat breed populations.											
Population	n	Genotype						Allele Frequency		X^2 (df=1)	p value
		AA		AB		BB		A	B		
		Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F				
Samburu	30	3 (1.5424)	0.1	8 (10.9153)	0.267	19 (17.5424)	0.633	0.233	0.767	2.277	0.131 Ns
Narok	30	0 (0.475)	0	8 (7.051)	0.267	22 (22.475)	0.733	0.133	0.867	0.612	0.434 Ns
All (Both)	60	3 (1.9412)	0.05	16 (18.1176)	0.267	41 (39.9412)	0.683	0.183	0.817	0.853	0.356 Ns
F: Frequency; NS: Non significant											

Fig. 3. Allele and genotype frequencies of β-LG gene for SacII site in the small east African goat breed.

4. Discussion

The β-LG gene (exon 7 to the 3'flanking region) of the small east African goat was genotyped by PCR-RFLP and sequencing methods and two variants (A and B) identified. Three genotypes namely AA, AB and BB were also detected.

Caprine beta -LG have affected the physicochemical properties of milk, protein contents and cheese making properties (Ng-Kwai-Hang 1998). Bovine beta-LG locus has been studied and eight variants have been reported at the DNA level, however, alleles A and B are most frequent (Ng-Kwai-Hang 1998) and allele B was associated with lower whey protein content and higher casein content in milk (Ng-Kwai- Hang 1998). Three variants (A, B & C) have been reported in the ovine species (Erhardt 1989). The beta -LG locus in the goat had been characterized at the DNA level and two novel genetic variants had been reported (Pena et al. 2000).

Polymorphism have been identified at the beta-LG locus in the small east African goat as identified in other French and Spanish goats (Folch et al. 1999; Pena et al. 2000).

Goat β-LG polymorphism has been investigated with Hair goat breeds by Elmaci et al., (2008) and showed that the frequency of genotype AA (S_2S_2, 0.011) was much lower than AB (S_1S_2, 0.44) and BB (S_1S_1, 0.45),and that of allele A (S_2) was lower than that of allele B (S_1). This was in line with the result of the Small East African goat breed as found in the entire studied sample even though genotype AA was missing in the Narok goat population. Also consistent with the present study, Kumar et al., (2006) reported a higher frequency of S_1S_1 (BB) genotype (ranging from 0.42 to 1.00) and S_2 (B) allele frequency (ranging from 0.59 to 0.97) in 13 different studied breeds. Garg et al., (2009) and Boulanger (1976) also in different studies showed the superiority of variant B over variant A.

Contradictory to the above findings, Sahar et al., (2009) study on polymorphism of beta-lactoglobulin gene on Egyptian goat breeds (Baladi, Barki, Damascus and Zaraibi) revealed higher frequency for A (S_2) allele than the B (S_1) allele. Additionally, BB (S_1S_1) was absent in the sampled Egyptian goats and the frequency of AA (S_2S_2) was higher than the AB except in Barki breed which had only AB (S_1S_2) genotype. In a separate study, the β-LG locus polymorphism for Barki, Damascus and their cross breeds also showed lower frequencies of B allele (ranging from 0.1 to 0.5) in comparison with this study where the pooled data revealed a frequency of 0.7 for the B allele.

Kumar et al., (2006) analyzed the polymorphism at both DNA and protein level in Indian goat breeds (Jamunapari and Barbari) to observe the effect of the beta-LG genotype at position 4601 as described by Pena et al., (2000) and found that beta-LG-AA genotype had a higher milk yield than beta-LG-AB genotype in both breeds. A similar result on the superiority of beta-LG AA over beta-LG AB in relation to milk production has been realized in a different study done by Prakash et al., (2002) on 5 Indian goat breeds.

In another association study, Kahilo et al., (2014) revealed contradictory results showing that, the milk production may vary between different genotypes according to the breed, this is because they found different effects according to the breed and they recorded a significantly higher milk production in the Balady Hybrid and Zarayby breeds with the BLG AB genotype while in the Damascus and Alpine AA genotype were significantly ($p<0.05$) superior in milk production. This means it would be prudent to do association studies in a particular breed

to determine the association of its variants with milk production than relying on available data from a different breed to do selection.

Two single nucleotide substitution were found in exon 7 at position 6705 (G/A) and 6751 (G/A). The polymorphism at position 6751 (4601) has been reported previously in Spanish and French goats (Pena et al., 2000). Kahilo et al., (2014) also revealed a substitution of G with A at nucleotide no. 6705 and no. 6751 in both Balady Hybrid and Damascus breeds in Egypt. This was in consistent with the present study. Besides polymorphism at position 6751 (4601) another substitution (G/C) was characterized in Indian goats (Jamunapari and Jakhrana) at position 4603 (Anubav et al., 2012). This mutation was not shown in the present study and no new polymorphism have been identified in the small east African goat besides those previously revealed in other breeds.

Despite the data being from separate and distant locations, significantly lower percentage of among population variation and extremely low levels of FST (0.01846) show that there is no significant variation among the populations of small east African goat breed in Kenya. This can be attributed to the livestock trade and pastoralism which disrespects administrative borders.

5. Conclusion

Exon 7 and 3' flanking region of the beta-LG was analyzed to detect different variants and single nucleotide variations to provide a baseline data for the small east African goats. A total of two variants and two single nucleotide substitutions were identified. This study also showed the existence of a genetic polymorphism at β-LG gene in small east African goat breed for the first time. Before any association study on this breed, it is paramount to have a baseline data of the existing genetic variation in such an indigenous breed well-adapted to the local environment. Polymorphisms in both the coding and non-coding regions as well as in the promoter and the 3' flanking regions are found to have an impact in expression of traits. Thus the identification of such variations in genetic resources is important for the conservation of desirable traits in livestock.

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