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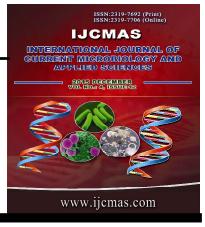

Original Research Article

Genotyping exon 1 and Partial Intron 1 Region of Caprine KISS1 Gene using *Acl I, Sac II* and *Dra III*

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A B S T R A C T

In a view of the importance of KISS1 as a regulator of puberty onset. Polymorphisms of KISS1 was studied to understand the relationship with high prolificacy and sexual precocity of Black Bengal goat breed. The various genotypes of KISS1 gene were identified using RFLP and gene and genotype frequencies were estimated. The maximum gene frequency for Black Bengal goat was observed for G allele (93%) of *Sac II* RE where as it was minimum for T allele (3%) at the same locus. The gene frequency of G allele was 80% and for C allele was 20% for *Acl I* RE. The gene frequency of T allele was 39% and for A allele was 61% for *Dra III* RE. The maximum genotype frequency was found in GG genotype (93%) of Black Bengal goat by *Sac II* RE where as it was minimum for GT allele (7%) at the same locus. The genotype frequency of GG was 60% and for GC was 40% for *Acl I* RE. The genotype frequency of TT, TA and AA was 14%, 50% and 36% respectively for *Dra III* RE. In present investigation it was found that frequency of allele G and T was high and favoured by the natural selection. It can be concluded that three variations in KISS1 gene may play an important role in litter size in native goat breeds of Black Bengal but further research on a large population will be required to confirm the linkage with increased prolificacy in goats.

Keywords

KISS 1, Polymorphism, Black Bengal goat, PCR-RFLP, Genotyping

Introduction

The Black Bengal goat is a highly prolific and major meat producing animal in West Bengal along with the adjoining part of the Jharkhand, Orissa, Bihar, Tripura states of India (Zeshmarani *et al.* 2007). Twins and triplets are common in kidding of Black Bengal goat. An investigation into genetic factors responsible for their high prolificacy, fertility, early sexual maturity has not been much studied. The fecundity genes such as (Chu *et al*. 2011), CART (Wang *et al*. 2011), GDF9 (Feng *et al*. 2011), GNRH1 (An *et al*. 2013), INHA and INHBA (Hou *et al*. 2012), KISS1 (Cao *et al*. 2010, Cao *et al*. 2011 and An *et al*. 2012), KITLG (An *et al*. 2013), POUF1 (Feng *et al*. 2012) and TSHB (Huang *et al*. 2013) genes, amongst others, has been screened and exciting opportunity to add a high level of prolificacy to sheep and goat.

These genes have proved to be traceable and persistent (Abraham and Thomas, 2012). The KISS1 gene encodes a family of

neuropeptides called kisspeptins, which activate receptor G protein-coupled receptor-54 and play a role in the neuroendocrine regulation of GnRH secretion (Smith *et al.* 2006). Kisspeptins are very potent elicitors of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in different mammalian species (Gottsch *et al.* 2004; Dhillo *et al.* 2005). Specifically in the female, the hypothalamic KISS1/ GPR54 system seems to operate as a central conduit for not only the negative but also the positive feedback regulation of gonadotropins, thereby playing a substantial role in the generation of the preovulatory surge of LH (An *et al.* 2013) These findings suggest that the *KISS1* gene could be an excellent candidate gene for reproductive traits in caprine (Ohtaki *et al.* 2001). Genetic improvement of polygenic traits can be enhanced by marker assisted selection which has higher accuracy in estimating the genetic value of animals (Dekker, 2004).

Materials and Methods

Genomic DNA Isolation

Five ml of blood sample was collected from total eighteen Black Bengal goats maintained at goat breeding farm, NDVSU, Jabalpur, where all diets were uniform and health, fertility and production records were maintained. Blood was collected from jugular vein, using EDTA coated vacutainer tube separately for each sample. The collected blood samples were brought to the laboratory on ice. The genomic DNA was extracted from white blood cells using a standard phenol-chloroform extraction protocol (Sambrook et al, 1989). All experiments were performed in accordance with the animal ethics committee of the institution where the experiment was conducted. The quantity and quality (A260/A280 ratio, i.e absorbance at wavelengths of 260 and 280) of DNA was assessed by using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA) and gel electrophoresis. The genomic DNA concentration ranged from 270-2278 ng/µl and diluted upto 50 ng/µl with nuclease free water and stored at -20^0 C for further use.

Polymerase Chain Reaction (PCR) for KISS 1 Gene

In the present study the exon 1 and partial intron 1 region of 1101 bp of KISS1 gene was selected for SNP investigation and genotyping. On the basis of the caprine *KISS1* gene sequence (GenBank accession No. GU142847), pairs of primers (F: 5'-CCT GTG TTT GCT GGA CAG TCT and R: 5'- TGC TCC CTC CCA ACC TTC TT) was selected to amplify the exon 1 and partial intron 1 of the *KISS1* gene (Cao *et al.* 2010). The 25-µL reaction volume contained 50 ng genomic DNA, 12.5 µL 2X reaction mix (Fermentas) and 0.5 µM of each primer. The cycling protocol was 5 min at 95°C and 35 cycles of denaturation at 94°C for 30 s, annealing at 64 °C for 30 s, and extension at 72°C for 35 s, with a final extension at 72°C for 10 min. PCR amplification was confirmed by running, 10 µl of PCR product mixed with 6X gel loading dye from each tube on 1.5 % agarose gel at a constant voltage 80 V for 50 to 60 min in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (Syngene, Gene Genius Bio Imaging).

Restriction Digestion of the PCR Products

The SNPs of the *KISS1* gene were genotyped by polymerase chain reaction-

restriction fragment length polymorphism (PCR-RFLP) using the restriction enzymes *Acl I, Sac II* and *Dra III* (Thermo scientific). The PCR products 10µl from each tube were digested with 1 U restriction enzymes (Acl I /Sac II /Dra III), in the manufacturer's 10 X assay buffer in the final reaction volume of 30µl. The reaction mixture was centrifuged for few seconds for uniform mixing and then incubated at 37˚C for 4 hours in the thermo Cycler and deactivation at 65˚C at 20 min.

Analysis of the PCR-RFLP Products

After restriction digestion, the PCR products were electrophoreses on 1.5 % agarose gel (according to the expected size of fragments) adding 5 µl ethidium bromide in 100ml solution. A constant voltage of 80V for 60 minutes was applied to gel electrophoresis apparatus, using 0.5X TBE buffer. The digested PCR samples, 6X loading dye and GeneRuler™ (Fermentas life sciences) 100bp DNA Ladder (Range, 80-1013 bp) was used as a molecular size marker. The bands were visualized under UV light and documented by gel documentation system. The RFLP product were determined by comparing with molecular size marker and the genotypes were then determined.

Gene and Genotype Frequency

The gene and genotype frequencies were detected by studying the presence or absence of specific size band patterns. Before genotyping these band patterns were compared with available literature and in addition molecular size of the bands was confirmed by comparing with 100 bp molecular marker.

The frequencies of genotypes were calculated.

Genotype frequency =

$$\frac{\text{Total number of individuals of particular genotypes}}{\text{Total number of individuals of all genotypes}}$$

Results and Discussion

PCR amplification of Exon 1 and partial and intron 1 region of KISS1 gene was used as target to reveal the polymorphism. The PCR amplification was carried out and the amplified product of KISS 1 gene was subjected to electrophoreses on 1.5% agarose gel and a single clear band of 1101 bp was obtained (Fig. 1).

Three restriction enzymes used for restriction digestion of KISS 1 gene (1101 bp) were *Acl I, Sac II and Dra III*. The amplified PCR product was digested with *Acl1* enzyme and revealed GG and GC genotypes in Black Bengal goat breed. The band size for GG genotype was 1101 bp and for GC were 1101, 805 and 296 bp. Restriction enzyme, *Sac II* revealed two genotypes, GG and GT genotype. Homozygous genotype GG revealed a band size of 1101 bp whereas heterozygous genotype GT revealed a band size of 1101, 647 and 454 bp. After the digestion with the last enzyme, *Dra III*, the three genotypes TT, TA and AA were obtained. Homozygous genotype TT reveals a band size of 1101 bp whereas heterozygous genotype TA revealed a band size of 1101, 598 and 503 bp and homozygous AA revealed a band size of 598 and 503 bp (Fig.2)

Gene and Genotype Frequency of KISS1 Gene

Gene frequencies and genotype frequencies for KISS1 gene have been summarized in

Table 1 and 2 respectively. The maximum gene frequency for Black Bengal goat was observed for G allele (93%) of *Sac II* RE where as it was minimum for T allele (3%) at the same locus. The gene frequency of G allele was 80% and for C allele was 20% for *Acl I* RE. The gene frequency of T allele was 39% and for A allele was 69% for *Dra III* RE.

The maximum genotype frequency was found in GG genotype (93%) of Black Bengal goat by *Sac II* RE where as it was minimum for GT allele (6.7%) at the same locus. The genotype frequency of GG was 60% and for GC was 40% for *Acl I* RE. The genotype frequency of TT, TA and AA was 14%, 50% and 36% respectively for *Dra III* RE.

PCR-RFLP analysis of KISS1 gene after digestion with *Acl I* revealed two genotypes GG and GC in Black Bengal breed of goat. However homozygous CC genotype was not seen in these breeds of goat. The frequency of GG genotype was higher than GC genotype. The allelic frequency of G allele was higher than C allele. Similar findings were also reported by Cao *et al.* (2010) showing higher frequency of GG genotype in JG (Jining Grey) breed (82%), LC (Liaoning Cashmere) breed (77%) and IMC (Inner Mongolia Cashmere), BG (Boer) and WDG (Wendeng dairy) breed (83%) of goats.

The 1101 bp amplified product after digestion with *Sac II* restriction enzyme revealed two genotypes i.e., GG, GT in Black Bengal breed of goat. However homozygous TT genotype was absent. The frequency of GG genotype was higher than GT genotype. The allelic frequency of "G" allele was higher than allelic frequency of "T". Similar findings in LC goat breed was reported by Cao *et al.* (2010) showing high frequency of GG genotype (49%). However Cao *et al.*, (2010) also reported that the frequency of GT genotype was higher than GG genotype in JG and IMC goat breeds and TT genotype was higher in BG and WDG goat breeds.

The 1101 bp amplified product after digestion with *Dra III* revealed three genotypes. The frequency of TA genotype was higher than AA and TT genotype. The allelic frequency of A allele was higher than T allele. Similar finding was reported by Cao *et al.* (2010) that TA genotype has high genotype frequency in LC and IMC goat breeds.

The lower frequency of GC, GT genotype and absence of CC, TT genotype may be due to unfavorable natural selection. This may also be due to small population size taken under the present investigation. In present investigation it was found that frequency of allele G and T was high and favoured by the natural selection.

Identification of the candidate genes which are responsible for variation in quantitative traits has been a challenge in modern genetics. So far, there are few reports of KISS1 as a candidate gene for reproductive traits in animals, which revealed that KISS1 gene plays an important role in animal reproduction Tomikawa *et al.* (2010).

In the present investigation three polymorphism were detected in KISS1 gene for Black Bengal goat breed by PCR-RFLP using three different REs. An *et al.* (2013a) reported 10 polymorphism in three Chinese goat breeds SN, GZ and BG g.1147T>C, g.1417G>A, g.1428_1429delG, g.2124C>T, g.2270C>T, g.2489T>C, g.2510G>A, g.2540C>T, g.3864_3 865delCA and g.3885_ 3886 ins ACCCC.

Table.1 Gene frequency of KISS I gene of black Bengal breed

RE	Allele	Gene frequency	Percentage
Acl I	G… C	0.8 0.2	80% 20%
Sac II	G T…..	0.97 0.03	97% 3%
Dra III	T A	0.39 0.61	39% 61%

Table.2 Genotype frequency of KISS I gene of black Bengal breed

RE	Genotype	Genotype Frequency	Percentage
Acl I 296bp	$G…G_{(9)}$ $G…C_{(6)}$	0.6 0.4	60% 40%
Sac II 454bp	$GG_{(14)}$ $GT_{(1)}$	0.933 0.067	93.3% 6.7%
Dra III 504bp	$TT_{(2)}$ $TA_{(7)}$ $AA_{(5)}$	0.14 0.5 0.36	14% 50% 36%

Fig.1 PCR amplified product of KISS 1 gene of 1101 bp in Agarose gel electrophoresis (1.5 %)

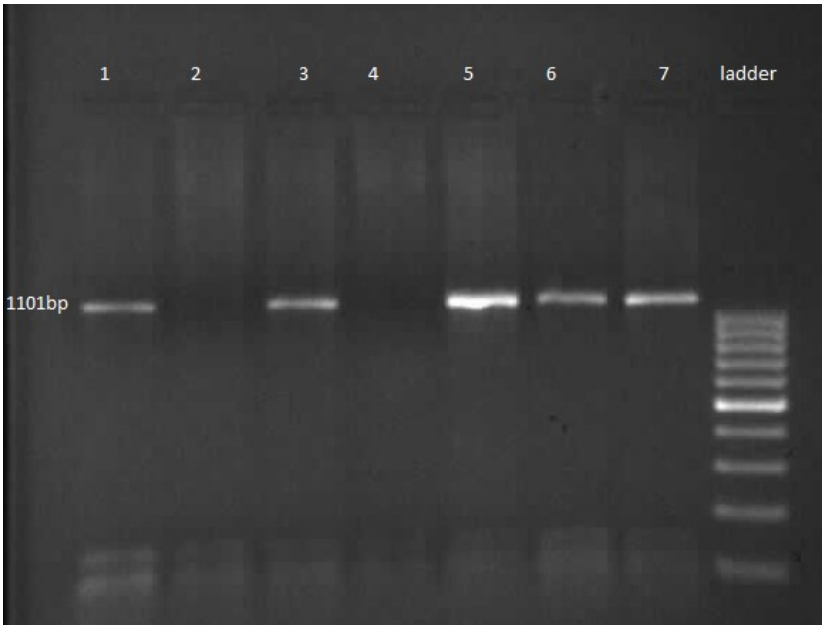
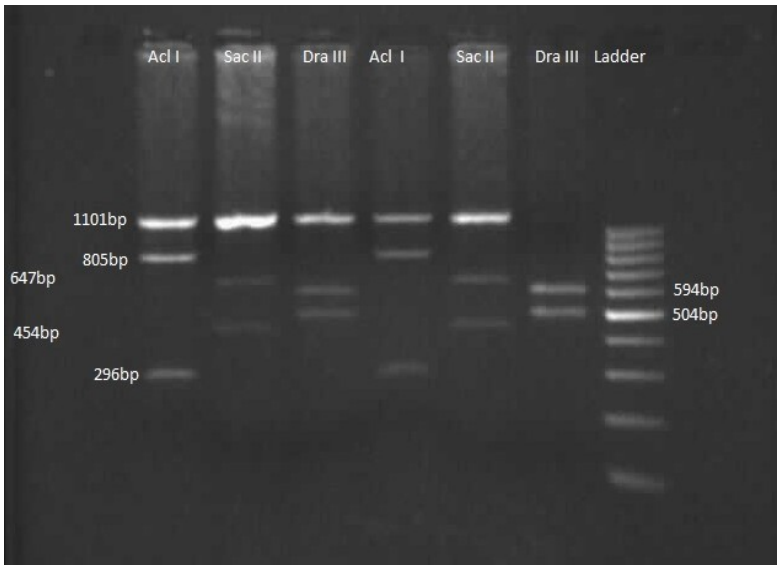


Fig.2 RE digestion (*Acl I, Sac II* and *Dra III)* of KISS 1 gene of exon 1 and partial Intron 1 (1101bp) in agarose gel electrophoresis (1.5 %)



L1 and L4: GCgenotype; L2 and L5: GT genotype; L3: TA genotype; L6: AA genotype
L7: 100bp DNA Ladder

An *et al.* (2013b) also reported two novels SNP in three Chinese goat breeds i.e., Xinong Saanen (SN), Guanzhong (GZ) and Boer (BG) at g.2124 and g.2270. Huijbregts *et al.* (2012) detected three SNP (c.638 insT, c.641C>G and c.645G>CA) in the 5´UTR of human KISS1 gene and found the c.645 G>C mutation was associated with central precocious puberty. Cao *et al.* (2010) reported an association between allele C of the 296 locus and allele deletion of the 1960_1977 locus of the KISS1 gene and larger litter size in Jining goat. Hou *et al.* (2011) identified T2643C and 8_bp base deletions (2677 AGTTCCCC) in intron 2 of the goat KISS1 gene with 82634C showing significant affect on litter size ($p \leq 0.05$).

In conclusion, the maximum genotype frequency was found in GG genotype (93%) of Black Bengal goat by *Sac II* RE where as it was minimum for GT allele (6.7%) at the same locus. The genotype frequency of TT, TA and AA was 14%, 50% and 36% respectively for *Dra III* RE. In present investigation it was found that frequency of allele G and T was high and favoured by the natural selection. From present study it was concluded that three variations in Kiss1 gene may play an important role in litter size in native goat breeds of Black Bengal. Further research on a large size of goat population will be required to confirm the linkage with increased prolificacy in Black Bengal goats.

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