



Novel PCR-RFLP for detecting single nucleotide polymorphism in exon1 of GPR54 gene and its association with age at first kidding in goats*

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KiSS peptides are considered as upstream regulators of gonadotropin releasing hormone (GnRH) neurons which regulate reproduction. KiSS peptides, peptide products of *KiSS1* gene act as ligands of the G-Protein Coupled Receptor 54 gene [GPR54] otherwise termed as *KiSS1R*. Studies in human and mice confirmed the role of *KiSS1* and GPR54 genes in reproductive maturation and function. Possible associations between variations in *KiSS 1* and GPR54 genes with litter size and sexual precocity in goats were reported (Feng *et al.* 2010, Hou *et al.* 2011).

Malabari and Attappady Black are two native goat breeds of Kerala, India and have definite difference in reproductive function. Malabari goats have high prolificacy with higher twinning percentage (Anonymous 2004). Age at first kidding (AFK) was also significantly lower in Malabari goats compared to Attappady Black (Stephen *et al.* 2005). Hence an effort was made to explore the polymorphism of exon 1 of GPR54 in Malabari and Attappadi Black goats and to analyse the association between SNP, if any, with AFK and litter size (LS) in these animals.

Goats (120) including Attappady Black (50) and Malabari (70) were used for study. Genomic DNA was isolated from venous jugular blood using standard phenol-chloroform extraction procedure (Sambrook *et al.* 1989). Primers were chosen from available literature (Feng *et al.* 2010) and custom synthesized commercially to amplify exon 1 of GPR54 gene.

Forward primer : 5'GGCAATGCAGCCCTTGTC 3'

Reverse primer : 5' ACCGATGTAGAAGTTGGTCAC 3'

Polymerase chain reaction (PCR) was performed with a total volume of 20 µl, containing 150 ng of DNA template, 2 µl 10X PCR buffer, 1.2 mM MgCl₂, 10pM of each primer, 200µM of dNTP and 1 unit of Taq DNA polymerase. The

cycling conditions for DNA amplification included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57.8°C for 30 sec and extension at 72°C for 30 sec. This was followed by a final extension at 72°C for 3 min. Amplification was checked in 2% agarose gel and an amplicon of 250 bp obtained was sequenced commercially by dideoxynucleotide sequencing method using an automated sequencer from randomly chosen representatives of Malabari and Attappadi Black goats. BLAST analysis of sequenced amplicons was performed followed by identification of restriction enzymes at site of mutation using suitable software. Restriction fragment length polymorphism (RFLP) of PCR product was performed using endonuclease *BCeA1*. Digestion of 250 ng of amplified product was done with 2 U of restriction enzyme *BCeA1* at 37°C for 1 h and PCR-RFLP products were visualised in 2% agarose gel to reveal the polymorphism in the population. Association of GPR54 genotypes with AFK and LS were analysed by SPSS software. General linear model for Univariate analysis used in case of AFK is given below

$$Y_{ijklm} = \mu + B_i + W_j + R_k + G_l + (BW)_{ij} + (BR)_{ik} + (BG)_{il} + (WR)_{jk} + E_{ijklm}$$

where, Y_{ijklm} is the trait (AFK) measured on $ijklm^{th}$ animal, B_i is the effect of i^{th} breed, W_j - effect of j^{th} adult body weight, R_k - effect of k^{th} birth weight, G_l - effect of l^{th} GPR54 genotype, $(BW)_{ij}$, $(BR)_{ik}$, $(BG)_{il}$ and $(WR)_{jk}$ are interactions and E_{ijklm} is the random error.

General linear model for Univariate analysis used in case of LS is given below

$$Y_{ijklm} = \mu + B_i + W_j + P_k + G_l + (BW)_{ij} + (BG)_{il} + E_{ijklm}$$

where, Y_{ijklm} is the trait (LS) measured on $ijklm^{th}$ animal, B_i is the effect of i^{th} breed, W_j - effect of j^{th} adult body weight, P_k - effect of k^{th} parity, G_l - effect of l^{th} GPR54 genotype, $(BW)_{ij}$ and $(BG)_{il}$ are interactions and E_{ijklm} is the random error.

PCR amplified products were commercially sequenced and BLAST analysis with available sequences in NCBI database ascertained the identity of the fragment. Malabari and Attappady Black goats were sequenced and paired

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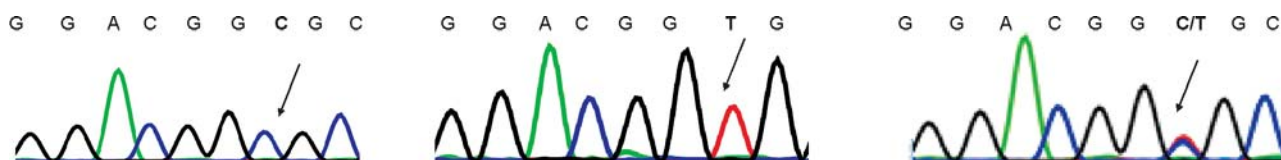


Fig. 1. Snapshots of chromatograms showing C, T, (C and T) at 100th position.

BLAST analysis between these revealed the existence of an SNP at 100th bp of 250 bp amplicon (Fig.1), which was a 'C→T transition at 96th bp of exon 1'. This SNP changed the codon from GGC to GGT, but both codons are coding for same amino acid, glycine and hence, this is a synonymous mutation producing no change in amino acid sequence of the protein.

The software NEBCUTTER identified *BceAI* as a restriction enzyme which recognized ACGGC nucleotides in CC genotype and cuts the 250 bp amplicon into 112 and 38 bp restriction fragments and TT genotype remained uncut. Thus *BceAI* enzyme was utilized to establish a novel PCR-RFLP technique to detect polymorphism in Malabari and Attappady Black goat populations. CC, CT and TT genotypes could be identified and scored as 1, 2 and 3. Both the sequences CC and TT were submitted to GenBank, NCBI and Accession numbers obtained (KF533108.1 and KF533109.1).

Genotypic and allelic frequencies for GPR54 locus presented in Table 1. TT genotype was highest in AB goats, whereas CC genotype was highest in Malabari goats. Significant association was found between GPR54 genotypes and AFK ($P < 0.05$), where CC genotype had significantly lower AFK than CT genotype (Table 2). Attappady Black goats exhibited more AFK (568.75 ± 17.16 days) than Malabari goats (453.21 ± 12.91 days). Litter size was significantly high for Malabari goats (1.67 ± 0.07) when compared to Attappady Black (1.33 ± 0.09), whereas no

significant association was seen between GPR54 genotypes and litter size in the present study.

Results agreed with the findings of Feng *et al.* 2010, who established an association between exon 1 and 5 of GPR54 gene with sexual precocity and high litter size in Jining Grey goats. But in the present study, new SNP showed no significant association with litter size in goats. Cao *et al.* (2010) preliminarily indicated associations between allele C of the 296 locus (intron 1) in *KiSS 1* gene and high litter size in Jining Grey goats. Hou *et al.* (2011), reported that a particular genotype (TC) obtained by a mutation T2643C in the intron 2 of *KiSS 1* gene was associated with superior litter size in Xinong Saanen and Guangzhong dairy goat breeds. The reproductive role of GPR54 gene was first established by Seminara *et al.* (2003) and study on mice and humans concluded that mutations in GPR54 caused autosomal recessive idiopathic hypogonadotropic hypogonadism in humans and mice. A homozygous single nucleotide variant (443T>C) in exon 3 of GPR54 was reported by Seminara *et al.* (2003), which substituted a serine for normal leucine at position 148 (L148S). They further stated that this variant did not appear to be a polymorphism, since it occurred only in reproductively affected individuals. Further reports by Colledge (2004), Popa *et al.* (2005), Aparicio (2005) and Li *et al.* (2009) confirmed the reproductive role of this gene. Maitra *et al.* (2014a) scanned exon 1, 2 and 3 of GPR54 gene in 9 Indian breeds of goats and 2 novel SNPs were recorded in exon1 and they recorded that the SNPs were synonymous. In another study, Maitra *et al.* (2014b) reported that nine SNPs were identified in these breeds of which 4 were novel and none of them were found to be associated with reproductive traits, but difference in litter size and age of sexual maturity was reported for different genotypes. The present study provided further evidence about reproductive role of this gene in goats and pointed towards the need for detailed work in this area.

SUMMARY

KiSS peptides are considered as upstream regulators of gonadotropin releasing hormone (GnRH) neurons which regulate reproduction. They act as ligands of G-Protein Coupled Receptor 54 gene [GPR54]. Malabari and Attappady Black, native goat breeds of Kerala, India, show definite difference in reproductive function. Malabari goats have high prolificacy and lower age at first kidding (AFK) compared to Attappady Black. Hence an effort was made to explore the polymorphism of exon 1 of GPR54 in Malabari

Table 1. Distribution of genotype and allele frequencies in Malabari and Attappady Black

Category		Malabari goat		Attappady Black goat	
		No:	Frequency %	No:	Frequency %
Genotypes	CC	37	52.86	9	18.00
	CT	15	21.43	15	30.00
	TT	18	25.71	26	52.00
Alleles	C	89	63.57	33	33.00
	T	51	36.43	67	67.00

Table 2. Effect of GPR 54 genotype on age at first kidding

Genotypes	No of animals	AFK (LSM±SE)
CC	46	483.81±15.19 ^a
CT	30	533.59±16.29 ^b
TT	44	515.54±14.28 ^{ab}

Values with different superscripts are significantly different ($P < 0.05$).

and Attappady Black goats and to analyse the association between SNP, if any, with AFK and litter size (LS) in these animals. Amplicon of 250 base pair enclosing exon 1 of GPR54 was obtained from genomic DNA, and sequenced amplicons from Malabari and Attapady goats revealed a mismatch C>T 100. (Accession no: KF 533109.1, KF 533108.1). This mutation resulted in the disappearance of recognition site ACGGC for the restriction enzyme *BceA1*. Hence a new restriction fragment length polymorphism (RFLP) was designed using *BceA1* which resulted in 3 genotypes- CC [112,138], CT [250,112,138] and TT [250]. Screening of goats (120) revealed that out of 3 genotypes observed CC genotype and C allele frequencies in Malabari goats were higher than Attappady Black goats. Least square means for AFK revealed that it was least for CC genotype and it was significantly different from CT genotype.

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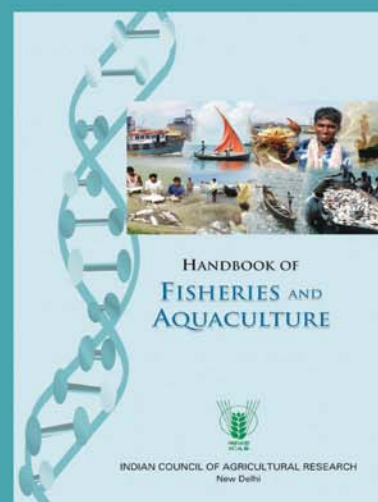
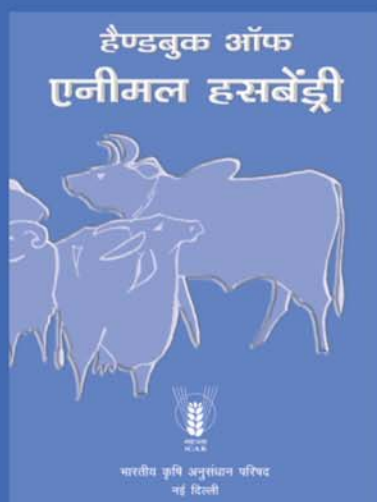
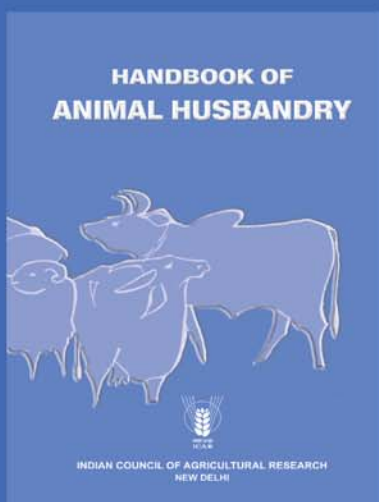
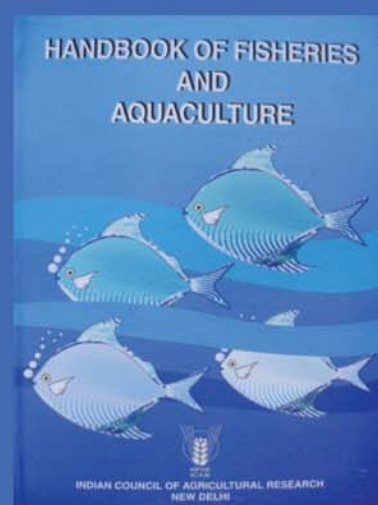
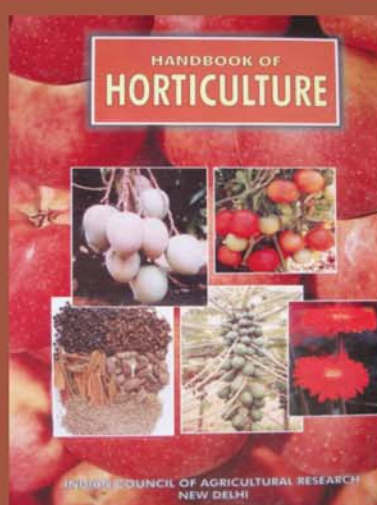
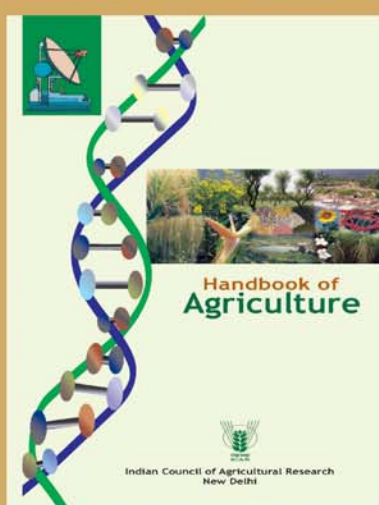
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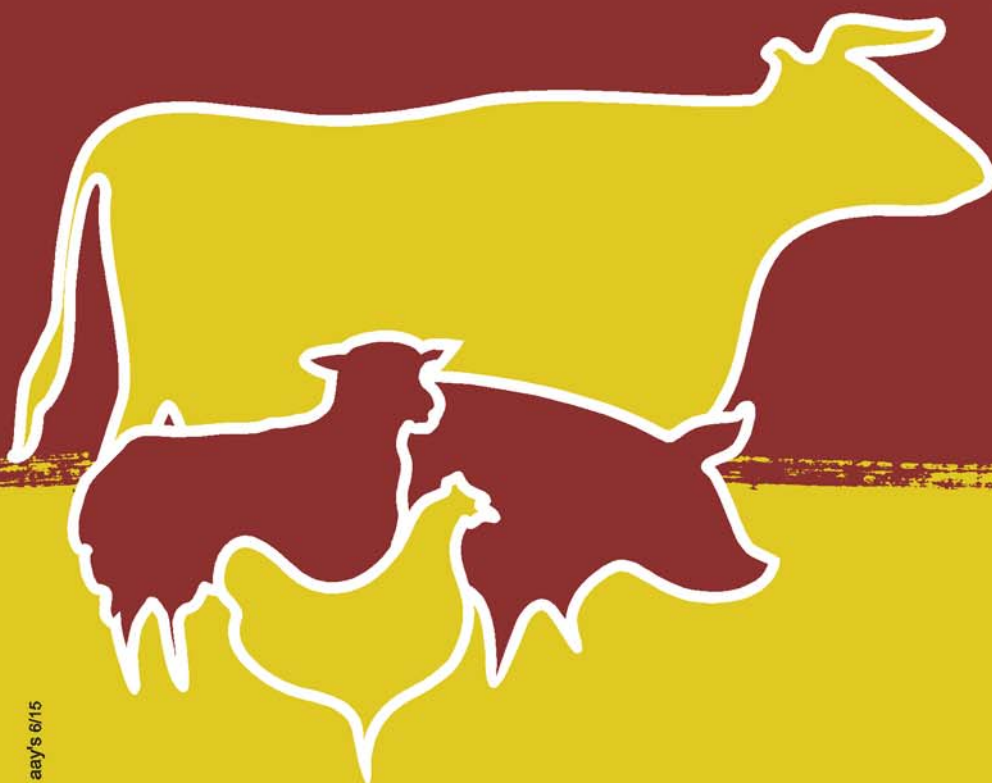
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