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Full Length Research Paper

Six novel PCR-RFLP loci in milk quality candidate genes in *Bubalus bubalis*

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Prolactin receptor (PRLR) and Peroxisome Proliferator Receptor Gamma Coactivator 1 Alpha (PPARGC1A) are candidate genes associated with milk protein and fat yield in dairy cattle. These genes were sequence characterized to identify exonic SNPs in water buffalo (*Bubalus bubalis*). Six novel buffalo specific SNPs were identified and novel PCR-RFLP loci for these SNPs were developed and validated. These loci can effectively be used for genotyping PRLR and PPARGC1A genes in recorded buffalo population for association studies with milk protein and fat yield.

Key words: *Bubalus bubalis,* PRLR gene, PPARGC1A gene, PCR-RFLP, genotyping.

INTRODUCTION

Buffalo have a pivotal and pre-eminent importance in the livestock sector of the country (India). In all the domestic animals, the water buffalo holds the greatest promise and potential for production. Buffalo has star performer status as the premier dairy animal of the country with high potential for milk, meat, fuel, draught power and farm manure production. According to estimates by the "Food and Agriculture Organization of the United Nations" the global water buffalo population has increased 98% in the last decades, from 88 million in 1961 to 188 million in 2009. India has over 105 million buffaloes with 12 well recognized breeds (Murrah, Nili-Ravi, Surti, Jaffarabadi, Bhadawari, Mehasana, Toda, Pandharpuri, Marthwada, Nagpuri, Banni and Chilka) distributed over different agro-climatic zones and they contribute to about 57% of total milk production in the country.

Genetic polymorphisms are playing an increasingly important role as DNA markers in many fields of animal breeding. It has been estimated that there is on an average 0.5 to 1.0 heterozygous SNPs per 1000 base pairs in the human genome (Mark, 2001). In livestock, one expects a slightly lower number of SNPs because of intensive selection in their breeding programmes but the number of SNPs still exceeds other types of genetic markers (Ramesha et al., 2002) . The abundance of SNPs in the genome makes them a powerful tool for genetic studies. It is well established that the quality and quantity of milk protein and fat differ among the species, breeds as well as individuals within a breed. It is essential to identify SNPs in genes responsible for milk quality and quantity like Prolectin Receptor (PRLR) and Peroxysome Proliferators Activated Receptor- Coactivator 1 Alpha (PPARGC1A) genes. PRLR is a candidate gene which is associated with milk protein yield in dairy cattle (Xiucai et al., 2009). The gene coding bovine PRLR is mapped on chromosome 20 corresponding to buffalo chromosome 19 (Amaral et al., 2008). The bovine Peroxysome Proliferators Activated Receptor- Coactivator 1 Alpha gene (PPARGC1A- gene bank accession number AY321517) is a plausible positional and functional candidate gene for a QTL for milk fat yield on BTA6 (Weikard et al., 2005). The role of bovine PPARGC1A gene is energy, fat and glucose metabolism and in regulation of milk fat synthesis in dairy cattle. Present study was undertaken to detect polymorphism at PRLR and PPARGC1A genes and to develop polymerase chain reaction- restriction fragment length polymorphism (PCR - RFLP) protocol for genotyping SNPs in these two genes.

MATERIALS AND METHODS

Samples were collected from the Indian buffalo breeds and DNA was isolated from blood samples using phenol-chloroform-isoamyl

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alcohol (Sambrook and Russell, 2001) and diluted to optimum concentration. The PRLR and PPARGC1A genes were characterized in a panel of 24 animals drawn from 6 diverse Indian water buffalo breeds (Murrah, Bhadawari, Tarai, Pandharpuri, Marathwada and Mehsana). The genomic region corresponding to the putative buffalo PRLR and PPARGC1A genes were amplified by the polymerase chain reaction (PCR) with primers designed from the *Bos taurus* and buffalo gene sequences submitted to NCBI GQ339914 and GU066311 respectively. The gene sequences were aligned taking *B. taurus* as reference using SeqScape v.2 (Applied Biosystems, USA) software. Four SNPs were identified in PRLR gene and two SNPs were identified in PPARGC1A gene and PCR-RFLP protocols were developed for respective SNPs.

Restriction fragment length polymorphism (RFLP) technique

Enzyme restriction sites which recognize the SNPs were identified and primer pairs were designed from buffalo gene sequenced to amplify the flanking regions of SNPs. Six enzyme (HpyCh4IV, BamHI, DraIII, FauI, Eco0109I and AciI) were selected which resulted in only one cutting site in the PCR product recognizing the SNP. PCR products were subjected to digestion by restriction enzyme in a total volume of 10 µl (8 µl PCR product, 1x enzyme buffer, 1 unit enzyme and rest of water) and placed in thermocycler at respective temperatures for 4 h. After digestion, the digested products were quantified to visualize the amplified fragments by Agarose (Promega, USA) gel electrophoresis.

RESULTS

To genotype the SNPs in buffaloes PRLR and PPARGC1A genes using simple PCR- RFLP technique six new PCR-RFLP loci were developed.

For locus PRLR_HpyCH4IV the PCR was carried out using forward (5'TgTgCCTCACCAgACTTTTg3') and reverse (5'gggACTgTgATggATTCTCC3') primers which yielded in product size 250bp, amplifying partial intron 1 complete exon 2 and partial intron 2 region of PRLR gene (Figure 1). A non synonymous SNP G/T at 157 base changes amino acid V19F. The TT (AA) genotype was uncut with HpyCH4IV enzyme and GG (BB) resulted in two bands 154bp and 96bp. The heterozygous GT (AB) samples produced three bands of 250, 154 and 96 bp (Figure 3a).

For locus PRLR_BamHI the PCR was carried out using forward (5'AgCAAggAAgCTCCATACCA3') and reverse (5'CggggATCTATCCCTAAgACA3') primers which yielded in product size 326bp, amplifying partial intron 3, complete exon 4 and partial intron 4 region of PRLR gene. Within this PCR product a synonymous SNP was at position 195. PCR product was digested with BamHI enzyme. The AA (AA) genotype was uncut and GG (BB) resulted in two bands 196bp and 130bp. The heterozygous AG (AB) samples produced three bands of 326bp, 196bp and 130bp (Figure 3b).

For locus PRLR_DraIII the PCR was carried out with forward (5'CAACATTgCTgACgTgTgTg3') and reverse (5'CAATTgAACCCATCCTTCCA3') primers, which yielded in product size 582bp, amplifying partial exon 9 of PRLR gene. Within this region at position 185 a SNP A/G was presented. The AA (AA) genotype was uncut and GG (BB) resulted in two bands 399 and 183 bp when digested with DraIII enzyme. The heterozygous AG (AB) samples produced three bands of 582, 399 and 183 bp (Figure 3c).

For locus PRLR_FauI the PCR was carried out using above primer pair yielding product size 582bp. Within this region another SNP A/G was present at position 434. PCR product when digested with FauI enzyme yielded AA (AA) genotype uncut and GG (BB) genotype resulted in two bands 439 and 143 bp. The heterozygous AG (AB) samples produced three bands of 582, 439 and 143 bp (Figure 3d).

For locus PPARGC1A_Eco0109I the PCR was carried out using forward (5'ggAAAATgTgTCCTggCATT3') and reverse (5'gCggTCTCTCTCAggTAgCA3') primers resulting in PCR product size 529bp, amplifying partial intron 7 and partial exon 8 region of PPARGC1A gene (Figure 2). A non synonymous G/T SNP at 255 position changing amino acid W346G was identified which is recognized by Eco0109I enzyme. The TT (AA) genotype was uncut and GG (BB) genotype resulted in two bands 254 and 275 bp. The heterozygous GT (AB) samples produced three bands of 529, 254 and 275 bp (Figure 3e).

For locus PPARGC1A_AciI the PCR was carried out using forward (5'AggAgCTCCATgACTCCAgA3') and reverse (5'CTTAggCTTTgggTgggTTT3') primers which yielded in PCR product size 625 bp, amplifying partial exon 8 and partial intron 8 region of PPARGC1A gene (Figure 2). Within this region at position 113 a SNP A/G identified with a restriction site of Eco0109I enzyme. The AA (AA) genotype was uncut and GG (BB) resulted in two bands 514 and 111 bp. The heterozygous AG (AB) samples produced three bands of 625, 514 and 111 bp (Figure 3f).

Using these PCR-RFLP genotyping protocols PRLR and PPARGC1A genes were genotyped in 24 samples each of all twelve recognized breeds of Indian water buffalo. The gene and genotypic frequencies in Indian buffalo breeds are given in Table 1. The minor allele frequencies ranged from 18.4 to 30.7%. However, both the alleles were equally present (50.2 and 49.8%) for PRLR_HpyCH4IV locus. Higher frequency of minor allele supports the use of these loci for genotyping these genes in recorded buffalo population for association studies.

DISCUSSION

Milk protein polymorphisms have received considerable interest because of their potential use as an aid to genetic selection and to genetic characterization of bovine breeds (Dei Lama and Zago, 1996). Genotyping of milk protein and milk fat responsible genes such as PRLR and PPARGC1A gene is extremely important for selection practice to improve milk production. The use of

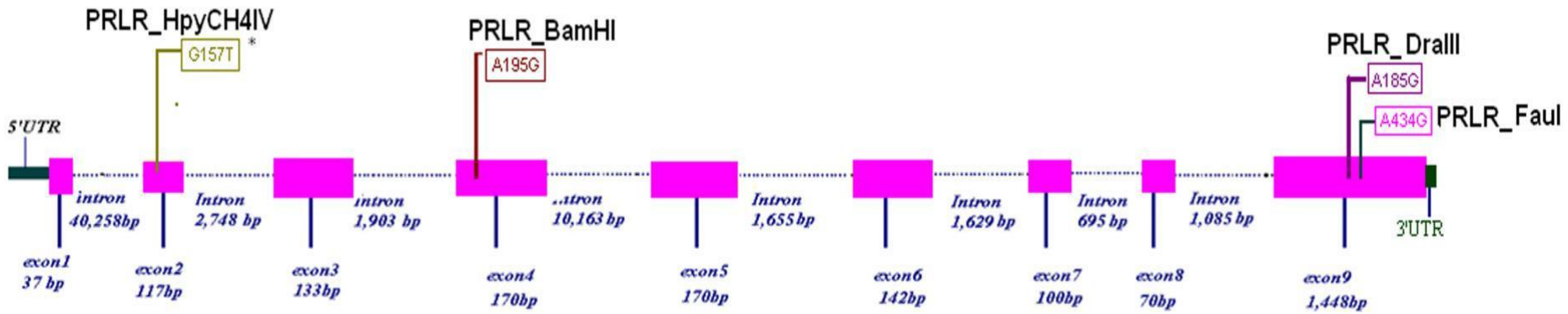


Figure 1. PRLR gene Structure with four loci in exon 2, exon 4 and exon 9.

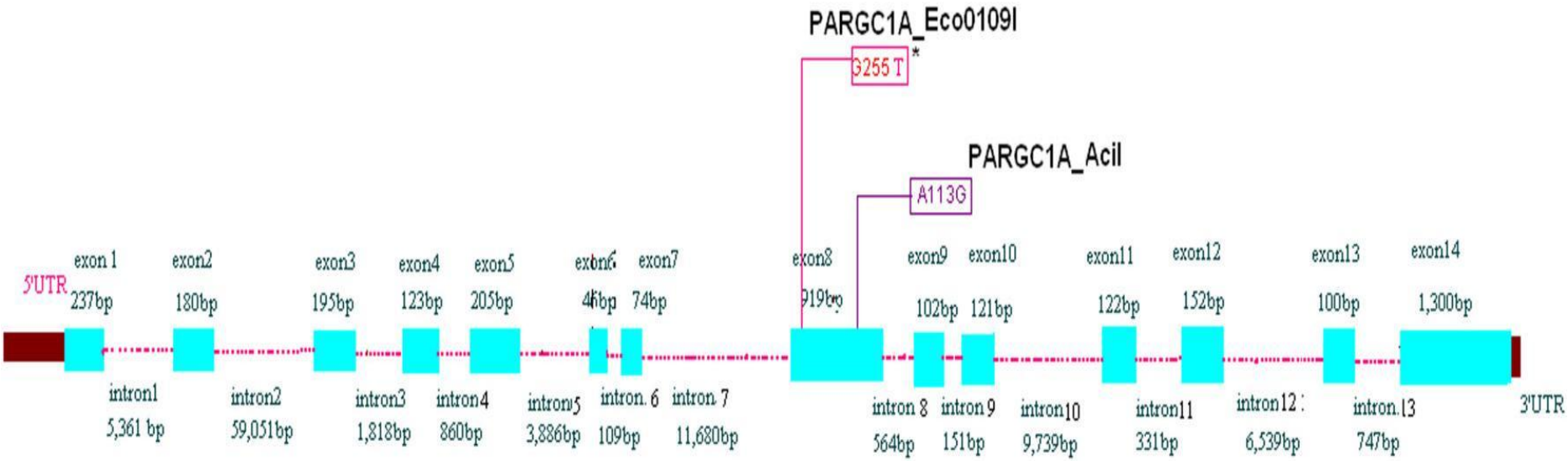
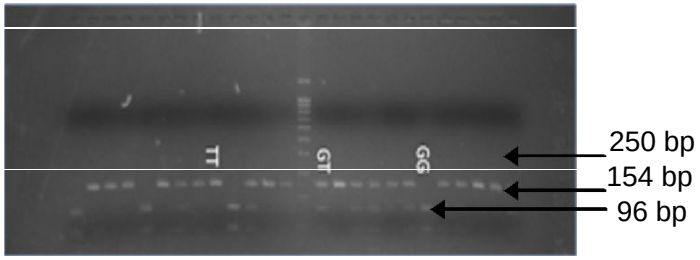
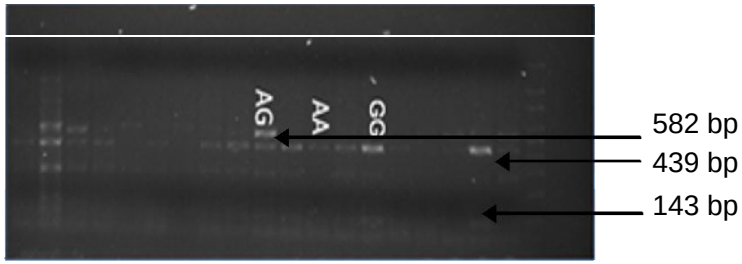


Figure 2. PPARGC1A gene Structure with two loci in exon 8.

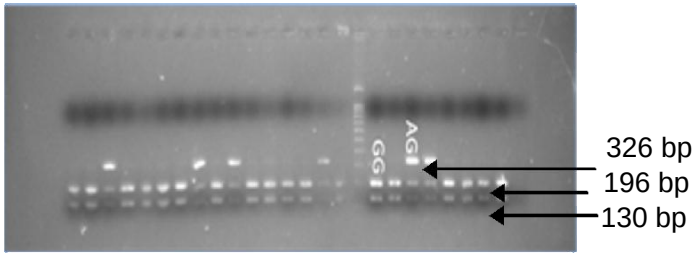
DNA polymorphic markers allows the determination of individual genotypes at many loci and provides information on population parameters such as allele frequencies as well as improving selection by marker assisted selection (Abdel et al., 2009). In the present study, we have established six PCR-RFLP loci for further studying the genetic polymorphism in buffalo. In PCR-RFLP genotype protocol developed in the study



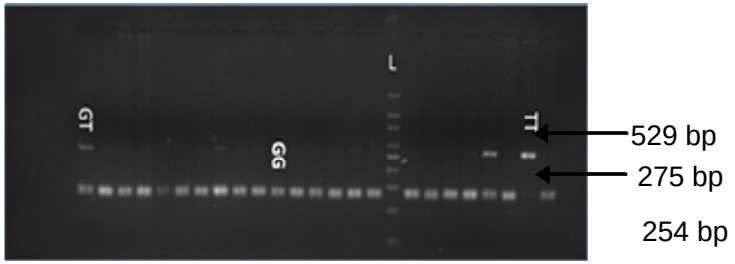
(A) PCR product of PRLR exon2 with HpyCH4IV enzyme with FauI enzyme



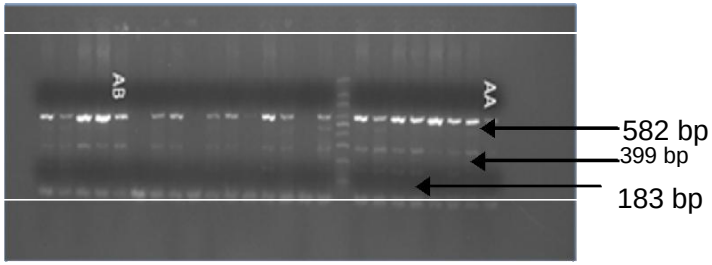
(B) PCR product of PRLR partial exon 9



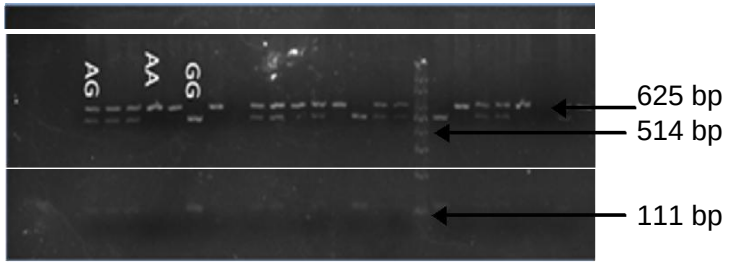
(C) PCR product of PRLR exon4 with BamHI enzyme Eco0109I enzyme



(D) PCR product of PPARGC1A partial exon 8 with



(E) PCR product of PRRL partial exon 9 with DraIII enzyme



(F) PCR product of PPARGC1A partial exon 8 with AciI enzyme

Figure 3. DNA electrophoretic pattern after digestion for different loci (M 100 bp DNA marker).

Table 1. Overall genotype frequencies and allele frequencies of all loci.

Locus	Genotype frequency			Allele frequency	
	AA	AB	BB	A	B
PRLR_HpyCH4IV	0.31	0.38	0.30	0.502	0.498
PRLR_BamHI	0.09	0.31	0.59	0.249	0.751
PRLR_DraIII	0.65	0.30	0.039	0.816	0.184
PRLR_FauI	0.58	0.20	0.17	0.718	0.282
PPARGC1A_Eco0109I	0.20	0.22	0.58	0.307	0.693
PPARGC1A_AciI	0.64	0.30	0.05	0.801	0.199

can effectively be utilized for genotyping. We conclude that time, efforts and funds spent in investigating SNPs and developing PCR- RFLP loci is being well justified, and the results will be of use to other researchers willing to work with genetic markers in buffalo. We also propose to investigate the correlation between the polymorphism of PCR-RFLP loci of two candidate genes and the quality of milk in terms of protein content and fat yield.

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