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Single nucleotide polymorphism identification and characterization of GnRHR gene in *Bos taurus* and *Bos indicus* cattle

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Abstract: The hypothalamic gonadotropin-releasing hormone receptor (GnRHR) plays an essential physiological role in reproductive function. In this investigation, we studied genetic variation in the entire coding region of GnRHR gene using PCR-SSCP technique in 250 *Bos taurus* (Holstein Friesian, Jersey) and *Bos indicus* (Malnad Gidda, Deoni) bulls. The genetic variants in GnRHR gene were determined by PCR-SSCP technique by using 5 sets of primers. SSCP analysis of fragment 1, comprising exon 1, and fragment 3, comprising exon 2, revealed 2 SSCP patterns in all breeds under study, while fragment 2 of exon 1 and fragment 4, comprising exon 3, revealed 3 patterns in Malnad Gidda; Holstein Friesian and Deoni breeds showed 2 SSCP patterns. In Jersey bulls, fragment 4 showed 3 patterns. The fragment 5 comprising exon 3 was monomorphic in all 4 breeds under study. SNPs were confirmed by direct sequencing. The sequence analysis revealed 7 transitions (T940C, T1021C, T1090C, T1174C, T13940C, T18023C, and G17887A), 3 transversions (G13817C, G17926C, and G17926C), 2 deletions (854DA and 17684DT), and 2 insertions (1009IA and 180100IC) in GnRHR gene. The study revealed high genetic variation in GnRHR gene in cattle.

Key words: GnRHR, PCR-SSCP, single nucleotide polymorphism, cattle

1. Introduction

Gonadotropin-releasing hormone gene receptor (GnRHR) is a member of the rhodospin-like G-protein–coupled receptor (GPCR) family and predominantly couples the Gq/11 family of G proteins in various cellular environments (1). GPCRs are characterized structurally as 7 transmembrane-spanning helices linked by consecutive extracellular and intracellular loops. In general the extracellular domains and/or transmembrane regions are involved in the formation of the ligand-binding pocket, whereas the cytoplasmic regions present sites for interactions with G proteins and other intracellular regulatory proteins. Following binding of gonadotropin-releasing hormone, the associated G proteins activate a phosphatidylinositol-calcium second messenger system. The consequent mobilization of Ca^{2+} and activation of protein kinase C isoenzymes mediates the secretion of gonadotropic luteinizing hormone (LH) and follicle stimulating hormone (FSH) (2), which regulate gonadal function, including steroidogenesis and gametogenesis. The interaction of GnRH and its receptor is a critical event in the endocrine control of reproduction (3). The GnRH signaling pathway has been studied extensively, and it plays a major role in sexual differentiation and reproduction.

GnRHR is expressed on the surface of pituitary gonadotrope cells, as well as in lymphocytes, breast, ovary, and prostate (4). Genes of the hypothalamic–pituitary–testicular axis play a key role in male reproductive performance (5). Presently, many GnRHR mutations have been found to reduce GnRHR binding and/or activation of inositol triphosphate or phospholipase C and are associated with idiopathic hypogonadotropic hypogonadism or Kallmann's syndrome in humans (6). Most mutations in GnRHR that either activate or inactivate their functions were reported to be responsible for several reproductive genetic disorders in humans (7). The interaction of GnRH and its receptor is a critical event in the endocrine control of reproduction. Physiological and developmental disorders during puberty in humans such as hypogonadotropic hypogonadism and adrenal insufficiency are the consequence of mutations in the GnRHR gene (8). GnRHR binds with affinity to GnRH on pituitary gonadotropes to stimulate release of the gonadotropic hormones LH and FSH that in turn regulate production of gametes and gonadal hormones (9). The response of pituitary gonadotropes to GnRH correlates with the concentration of GnRH receptors (GnRHRs) on the cell surface (10). As a result, the GnRHR gene

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appeared to be a good candidate gene for mutation analysis associated with reproductive performance (11). Earlier researchers studied G286A and T340C transitions in bovine GnRHR gene and reported that 1 A allele or C allele had a favorable positive effect on sperm quality traits in bulls (12).

The Malnad Gidda are small sized cattle. They have a compact body frame (around 80–120 kg) and are native to heavy rainfall areas of the Western Ghats in Karnataka, India (13). The word Gidda means dwarf and Malnad means a place receiving heavy rainfall. They are distributed predominantly in Malnad and the adjacent coastal districts of Karnataka in India. Deoni is a dual purpose breed found in parts of Maharashtra, Andhra Pradesh, and Karnataka in India. Holstein Friesian and Jersey cattle breeds are being extensively used in India for crossbreeding and upgradation programs to improve production in native Indian cattle. The objective of this study was to identify SNPs in the coding region of the bovine GnRHR gene by PCR-SSCP technique.

2. Materials and methods

2.1 Animals and DNA extraction

Two hundred and fifty breeding bulls comprising *Bos taurus* bulls (Holstein Friesian and Jersey, n = 100 from each breed) maintained by the organized frozen semen stations in Karnataka; Deoni (n = 25) males maintained at the National Dairy Research Institute, Bangalore, Karnataka; and Malnad Gidda (n = 25) males reared by the farmers of Malnad and the coastal region of Karnataka were used in the study. Blood samples (8–10 mL) were collected by jugular venipuncture into a vacutainer tube containing EDTA as an anticoagulant. Genomic DNA was isolated by the high salt method as described by Sambrook (14), with minor modifications. The quality and quantity of DNA were analyzed by agarose gel electrophoresis and UV spectrophotometer. It was diluted to a final concentration of 100 ng/μL and stored at –20 °C for subsequent analysis.

2.2 PCR-SSCP and sequencing analysis

The PCR-SSCP technique was used to study genetic variation in the entire coding region of GnRHR gene. Based on NCBI reference sequence NC_007304.5 for GnRHR gene, 5 sets of primers were designed to cover the entire coding region using Primer 3 (V.4.0) online software. Primers were procured from Amnion Bioscience Pvt. Ltd., Bangalore. The details of the primers, expected product sizes, and annealing temperature are summarized in Table 1. Genomic DNA was amplified in a final volume of 25 μL containing 20 pmol of primer, 200 μM of dNTP, 10X reaction buffer, 1 U of Taq DNA polymerase, and 100 ng of genomic DNA. After denaturation at 94 °C for 5 min, 35 amplification cycles were performed comprising denaturation at 94 °C for 45 s, with specific annealing temperature, and extension at 72 °C for 1 min followed by a final 5 min extension at 72 °C. The PCR products were electrophoresed on 1.5% agarose gel using 1X TBE buffer along with a 100 bp DNA marker and visualized under Gel Doc system (BIO-RAD, USA). The amplified PCR products were further subjected to PAGE and visualized by silver staining (15). The different SSCP patterns were named AA, BB, and CC, as per international practice (16–18). The genetic variants were determined by single-strand conformation polymorphism (SSCP) analysis. Polymorphisms of bovine GnRHR gene were detected by PCR-SSCP (Figure 1). The SSCP band patterns were characterized by the number of bands and mobility shifts, and each pattern was scored manually. The PCR products giving unique SSCP band patterns were further analyzed

Table 1. Primer sequences, PCR product sizes, and annealing temperature of the exons of GnRHR gene.

Primers	Sequences	Size (bp)	Ta (°C)	Regions	Reference
Fragment 1	F: 5'-GCCCACAAAACAAGTTCACC-3' R: 5'-AGGAGCTCTCCAGCATACCA-3'	531	55	Exon1	Designed based on NCBI reference sequence: NC_007304.5
Fragment 2	F: 5'-TGGAAAGATCCGAGTGACAG-3' R: 5'-TTCCAGAGCCCAAGCTCTTA-3'	533	55	Exon 1	
Fragment 3	F:5'-GTTGCTTTGGGCAACAACAT-3' R:5'-TTCTCTGGTTGGGGTATTGC-3'	410	57	Exon 2	
Fragment 4	F:5'-GTCCTTCAACTCCTCCATCG-3' R:5'-CACCCTGTTTACCATGTCAGG-3'	703	50	Exon 3	
Fragment 5	F:5'-TCTGCTGGACGCCCTACTAT-3' R:5'-GCCTTAGGCCATGGCTATTT-3'	482	55	Exon 3	

F = forward, R = reverse, Ta = annealing temperature.

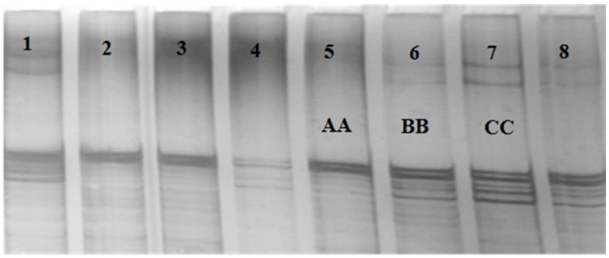

Figure 1. Different SSCP band patterns observed in fragment 4 comprising exon 3 of GnRHR gene.

by direct sequencing (Amnion Bioscience Pvt. Ltd., Bangalore, India). Sequence data were analyzed using Bio Edit software (19) and aligned by using CLUSTAL W Multiple Alignment software for detecting single nucleotide polymorphisms.

3. Results

3.1 SSCP band patterns

The entire exonic region of bovine GnRHR gene consisting of 3 exons was amplified by PCR using 5 sets of primers. The PCR-SSCP analysis of GnRHR gene revealed varying degrees of genetic polymorphism with respect to each of the GnRHR gene fragments analyzed in 4 different breeds of cattle. SSCP band patterns along with frequency for each band pattern are shown in Table 2. PCR-SSCP analysis of fragment 1 revealed 2 band patterns in all 4 breeds under study. Fragment 2 of exon 1 revealed 3 band patterns in Malnad Gidda cattle, whereas in Holstein Friesian, Jersey, and Deoni breeds only 2 SSCP band patterns were observed. In all 4 breeds fragment 3 corresponding to exon 2 revealed 2 band patterns viz., AA and BB, with higher frequency for BB pattern (Figure 2). The fragment 4 corresponding to exon 3 showed 3 band patterns in Malnad Gidda and Jersey, while only 2 SSCP band patterns were observed in Holstein Friesian and Deoni breeds. The fragment 5 corresponding to exon 3 showed a monomorphic pattern in all breeds.

3.2 SNP identification

Representative samples were custom sequenced using automated ABI DNA sequencer (Amnion Biosciences Pvt. Ltd., Bangalore, India) to confirm the mobility shift in each pattern. Sequence data were analyzed using Bio Edit software Clustal W Multiple Alignments (Figure 3) for detecting single nucleotide polymorphisms (SNPs) by comparing the

Table 2. Single-strand conformation polymorphism (SSCP) band patterns and genotype frequency (GF) of GnRHR gene in cattle.

Breed / Fragments	Malnad Gidda		Holstein Friesian		Jersey		Deoni	
Fragments	Band patterns observed	GF	Band patterns observed	GF	Band patterns observed	GF	Band patterns observed	GF
F1	AA	0.50	AA	0.45	AA	0.45	AA	0.65
	BB	0.50	BB	0.55	BB	0.55	BB	0.35
F2	AA	0.35	AA	0.75	AA	0.45	AA	0.55
	BB	0.30	BB	0.25	BB	0.55	BB	0.45
	CC	0.35						
F3	AA	0.30	AA	0.45	AA	0.33	AA	0.33
	BB	0.70	BB	0.55	BB	0.67	BB	0.67
F4	AA	0.60	AA	0.25	AA	0.33	AA	0.53
	BB	0.30	BB	0.75	BB	0.07	BB	0.47
	CC	0.10			CC	0.60		
F5	Mono-morphic	-	Mono-morphic	-	Mono-morphic		Mono-morphic	-

F1 and F2: exon 1 region of GnRHR gene; F3: exon 2 region of GnRHR gene; F4 and F5: exon 3 region of GnRHR gene.

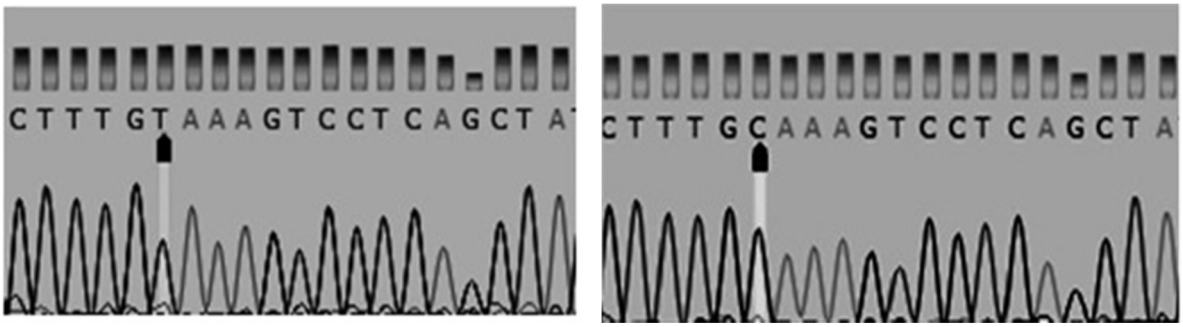

Figure 2. Sequence comparison of AA and BB genotypes of bovine GnRHR gene. The arrow indicates T→C transition at 1021 position in Jersey breed.

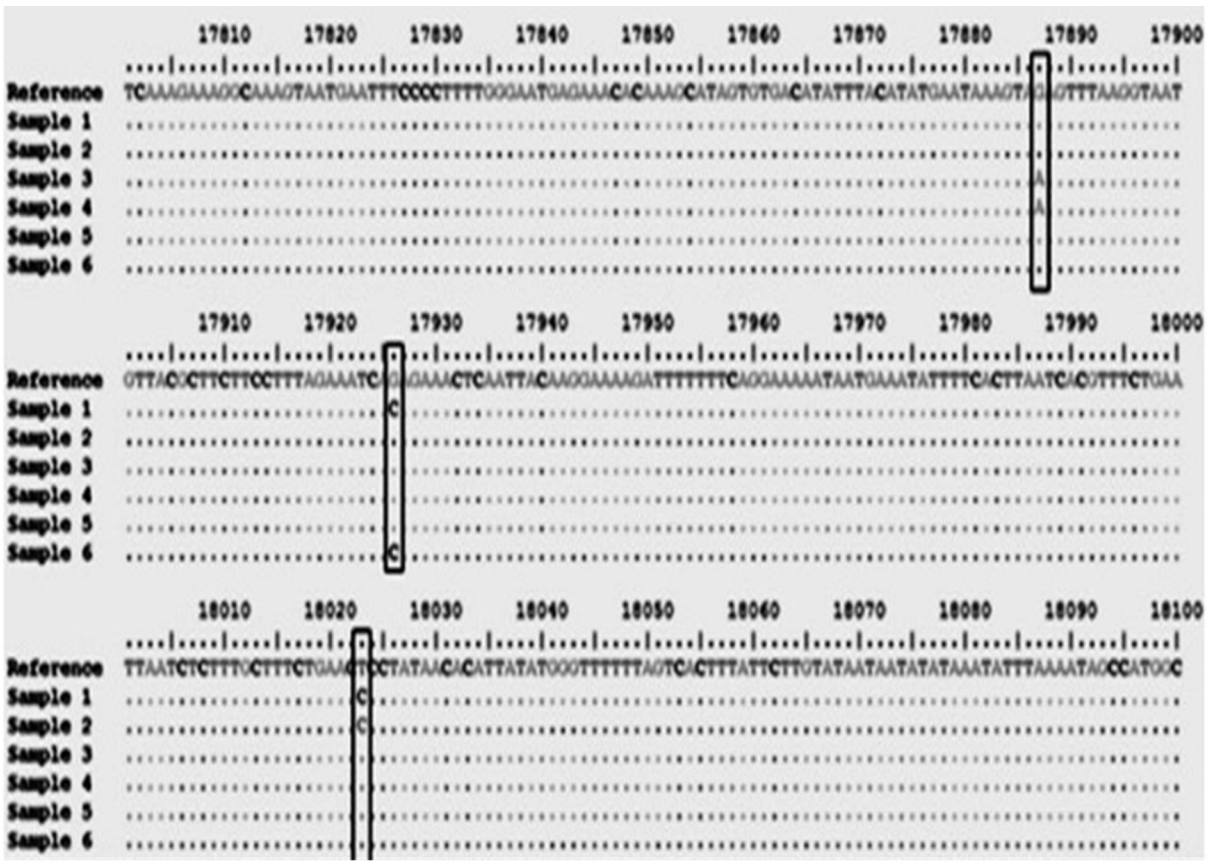

Figure 3. CLUSTAL W Multiple Alignment of fragment 4 (GnRHR gene) showing 6 SNPs in 6 samples in different breeds. Samples 1 and 2: T→C transitions; samples 3 and 4: G→A transitions; samples 1 and 6: G→C transversions; starting sequence corresponds to 17801 of reference sequence (NCBI reference sequence: NC_007304.5).

observed sequence with the bovine GnRHR gene reference sequence (NCBI reference sequence: NC_007304.5). Single nucleotide polymorphisms (SNPs) observed in the GnRHR gene in cattle breeds are given in Table 3. In exon 1, four SNPs and 2 indels were observed across the breeds, including 3 novel variants in Holstein cattle including insertion of adenine at 1009 bp, deletion of adenine at 854, and 1 transition (T→C) at 1090 bp; 2 variants (T940C and T1021C) were observed in Jersey cattle. Only 1 SNP (T1174C) was detected in Malnad Gidda cattle. Two SNPs were detected in exon 2 region of GnRHR gene, T→C transition was exhibited by Holstein Friesian cattle at the 13940 bp positions, and G→C transversion was exhibited by Jersey cattle at 13817 bp positions. The exon 3 region of GnRHR gene showed 4 SNPs and 2 indels; in Holstein Friesian cattle 3 variants including insertion of cytosine at 18099 bp position, deletion of thymine at 17684 bp position, and 1 transversion (G→ C) at 17926 bp were observed. Malnad Gidda cattle exhibited 2 SNPs (G17926C, T18023C), while only 1 SNP (G17887A) was detected in Jersey cattle (Figure 4). The present study

Table 3. Single nucleotide polymorphisms (SNPs) observed in GnRHR gene in *Bos taurus* and *Bos indicus* cattle.

Fragments	Position of SNP in gene	Breed	Variation	Mutation	Amino acid change
F1	1009IA*	HF	CTGGA/AGAGC	Insertion of A	Glu (no change)
	854DA*	HF	GTTGA/-ACTT	Deletion of A	Glu (no change)
	T940C	JY	CCAAT/CCTGC	(T→C) transition	Ile to Thr
F2	T1021C	JY	TTTGT/CAAAG	(T→C) transition	Val to Ala
	T1090C	HF	AGCCT/CGACC	(T→C) transition	Leu to Ser
	T1174C	MG	AGCCT/CGACC	(T→C) transition	Phe to ser
F3	T13940C	HF	GGCTT/CCTCA	(T→C) transition	Phe (no change)
	G13817C	JY	CTCAG/CTGTG	(G→C) transversion	Glu to His
	G17926C	MG	ATCAG/CAGAA	(G→C) transversion	Gln to Glu
	T18023C	MG	GAACT/CCTAT	(T→C) transition	Leu to Pro
F4	G17887A	JY	AGTAG/AAGTT	(G→A) transition	Glu to Lys
	G17926C	HF	ATCAG/CAGAA	(G→C) transversion	Gln to Glu
	17684DT*	HF	TGGTT/-AACA	Deletion of T	Val (no change)
	18099IC*	HF	TGGG/CCCTA	Insertion of C	Thr (no change)

F1 and F2: exon 1 region of GnRHR gene; F3: exon 2 region of GnRHR gene; F4 and F5: exon 3 region of GnRHR gene.
D = deletion; I = insertion; A = adenine, T = thymine, G = guanine, C = cytosine; HF = Holstein Friesian; MG = Malnad Gidda; JY = Jersey.
a = polymorphism residues underlined (the common nucleotide followed by the variant).

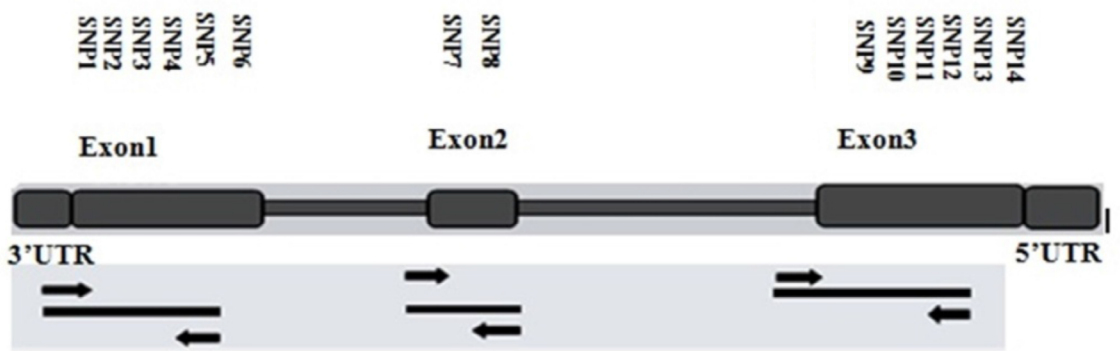

Figure 4. Scheme of the GnRHR gene showing the amplified region and novel variants detected. Vertical and horizontal arrows indicate SNP/indel positions.

revealed a high degree of genetic variation in the coding region of GnRHR gene among Holstein Friesian, Jersey, Malnad Gidda, and Deoni cattle.

4. Discussion

Genetic variation in bovine GnRHR gene in Holstein Friesian, Jersey, Deoni, and Malnad Gidda breeds of cattle was detected by PCR-SSCP technique. The observed variations were confirmed by a direct sequencing approach. A total of 14 SNPs were detected in the coding region of GnRHR gene. It is generally assumed that coding regions are more conserved than non-coding regions. However, some researchers reported similar or slightly lower density of SNPs in coding than in non-coding regions (20). In the present study we observed a total of 10 SSCP band patterns and 14 SNPs in the entire coding region of the GnRHR gene among Holstein Friesian, Jersey, Malnad Gidda, and Deoni cattle.

GnRHR is a key molecule in the hypothalamic–pituitary–gonadal axis that regulates sex steroid hormone production and reproductive processes. Earlier reports also indicated that the GnRHR protein is involved in different developmental and metabolic processes (21). It is possible that the mutations influence sex hormone levels and that this, in turn, leads to disease. Their results showed GnRHR gene has a significant effect on reproduction (22). There is evidence that genetic variation within GnRHR contributes to the regulation of pubertal timing in human and mouse populations (23). However, how they influence

diseases is not exactly known. Earlier research reported that GnRHR gene can be a potential marker for improving sperm quality traits, implying that bulls with GA or CT genotype could be selected for breeding programs (12). In the present study we identify 14 novel variants of the bovine GnRHR gene including 10 SNPs and 4 indels.

PCR-SSCP is a simple and efficient technique for detection of single-base substitutions and can be employed for evaluating genetic variability in large populations. The Malnad Gidda breed has reproductive uniqueness, producing a yearly calf even under a low input system. Further studies on bovine GnRHR gene and its association with reproductive performance using a large sample size would help to explain portions of the genetic variability among different breeds of cattle and also may aid in developing markers for reproductive performance in cattle. The identified gene variants, however, need large population studies and association studies between SNPs and reproductive performance in order to identify markers for use in marker-assisted selection.

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