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Molecular characterization of the partial coding sequence (CDS) of the luteinizing hormone receptor (*LHR*) gene in Indian cattle breeds

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ABSTRACT

Luteinizing hormone receptor (*LHR*) gene codes for LH receptors in ovaries that are important site for binding of luteinizing hormone (LH), which affects the reproductive physiology in dairy animals. In the present study, partial coding sequence (CDS) including exon 11 (>1100 bp) of *LHR* gene was cloned and characterized in Sahiwal and Hariana cattle breeds. Sequencing and chromatogram study of LHR Exon 11 sequence showed two heterozygous condition revealed A→G and G→A non-synonymous substitutions at position 107 and 524, respectively, which resulted into substitution of asparagine (N) to aspartic acid (D) and valine (V) to methionine (M) at 36 and 175 amino acid position, respectively. Therefore these two heterozygous conditions identified as NV and DM genotypes in Sahiwal breed. Sequence comparison of *LHR* exon 11 of Sahiwal (NV and DM) and Hariana showed 98.7% to 100% homology with exotic cattle breeds at nucleotide and amino acid level, respectively. DNA polymorphism was also investigated using *Hha*I/ PCR-RFLP assay. It showed three types of genotypes namely, TT (303 bp), TC (303, 155, and 148 bp) and CC (155 and 148 bp), where TT genotype was more frequent (45.0%) than TC (42.5%) and CC (12.5%) genotype and frequency of T allele was higher (0.66) than C allele (0.34) in all the screened animals. Chi square ($\chi 2$) analysis indicated that all the screened animals were found in Hardy-Weinberg equilibrium. Association analysis revealed no significant association with milk production and reproduction traits.

Key words: cattle; cloning; PCR-RFLP; LHR/*Hha*I; association study

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Introduction

Infertility is a major reproductive problem in dairy animals worldwide. It is a multifactorial problem, and difficult to diagnose and treat. Clinical symptoms include delayed onset of estrous, lack or suboptimal manifestation of heat signs, and an irregular pattern of follicular cycling (HASTINGS

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et al., 2006). Various hormones influence the reproductive physiology of dairy animals, including the luteinizing hormone (LH). LH acts by interaction with its corresponding receptors (Luteinizing Hormone Receptors; LHR) located on the plasma membrane of the specific target tissues, that is, the ovaries and testes, causing ovulation (KAWATE et al., 1996; KAWATE et al., 2000a), growth of the corpus luteum (KAWATE et al., 2000b), and development of androgens in the male testes (HAFEZ and HAFEZ, 2013). LH acts by stimulating adenylyl cyclase activity and production of cyclic AMP, causing steroidogenesis in these cells (DAVIS et al., 1996). LH helps in the production of androgens by theca cells (MAGOFFIN et al., 1989; IVELL et al., 2000) by stimulating the expression of steroidogenic enzymes, including CYP11A1 (cytochrome P450 side-chain cleavage), STAR (steroidogenic acute regulatory protein), and CYP17A1 (cytochrome P450 17α hydrolase). It also regulates the mRNA concentrations for numerous genes that function in steroidogenesis, autocrine and paracrine signaling, intracellular signal transduction, cytoskeletal regulation, and apoptosis in granulosa cells (SEKAR et al., 2000; SASSON et al., 2004). LH receptor is a G-protein coupled receptor that consists of an extracellular hormone-binding domain, transmembrane domains, and a small intracellular domain (JI et al., 1994). LH receptors are coded by the *LHR* gene located on chromosome 11, and have a total of 11 exons and 10 introns. All the introns and exon 1-10 are found in the extracellular domain, while exon 11 is the largest, and codes for the entire transmembrane, cytoplasmic tail and 3′-UTR regions (DUFAU et al., 1995). Identification of single nucleotide polymorphism (SNPs) in genes using molecular markers and selection of animals on the basis of these SNPs will be of great importance in animal breeding. Any variations in the nucleotide sequence of the *LHR* gene may cause reproductive problems that lead to infertility. The *LHR* gene has been characterized in different species, including rats (MCFARLAND et al., 1989), pigs (LOOSFELT et al., 1989; YERLE et al., 1992), sheep (MONYGOMERY et al., 1995), bovine (MAMLUK et al., 1998; KAWATE et al., 2002) and Indian riverine buffalo (SINGH et al., 2006). Molecular markers, such as restriction fragment length markers (RFLP), are codominant markers, show a high degree of polymorphism and are widely used to find the genotypes associated with a particular gene in a population. They are helpful in observing a increased response towards a trait, thus helping in marker assisted selection (MAS) (ALMEIDA et al., 2000; MACHADO et al., 2003). LHR/*Hha*I PCR-RFLP has been previously employed in various cattle breeds, including the European zebu (MARSON et al., 2005), Kankrej and Gir cattle (CHANDRAN, 2006), Turkish cattle breeds (ARSALAN et al., 2015), Sudanese cattle (OMER et al., 2016), Sahiwal, Rathi and Kankrej (KUMAR et al., 2018), and in buffalo breeds, including Surti, Mehsana, Jaffrabadi (CHANDRAN, 2006), Egyptian buffalo (OTHMAN et al., 2013; SOSA et al., 2016) and Murrah (KATHIRAVAN et al., 2018). We took exon 11 for characterization and PCR-RFLP study because it is the largest exon (>1100 bp) that helps in signal transduction initiated in the extracellular domain, and couples the G-protein (DUFAU, 1998). Furthermore, while some studies have explored various aspects of the LHR gene in cattle, the specific characterization of its exons remains largely unexplored within Indian cattle breeds, with the exception of the Hariana breed. Notably, the LHR/*Hha*I PCR-RFLP of 303 bp has been extensively documented across different species, including various Indian cattle breeds, underscoring its potential as a valuable genetic marker. Within the realm of dairy production, traits such as milk yield and composition hold significant importance. As global population growth continues, the demand for milk is expected to rise correspondingly. Therefore, enhancing dairy cattle herds for these crucial traits is not only in line with the immediate needs of the dairy industry, but also addresses the growing global demand for high-quality milk products (ARSALAN et al., 2015). So, there is a need for an increase in per animal milk production to fulfill the large demand globally. Moreover, genetic characterization of local breeds is important to prevent them from becoming extinct due to low yield, and for their enhancement. As this gene is involved in ovulation and the growth of the corpus luteum, this may be a suitable candidate for MAS. Any nat-

urally occurring mutations in the gene may result in a lower response towards LH, that leads to reproduction failure. Detailed sequence analysis and detection of SNPs allowed us to track any changes in the structural conformation of the gene as they may lead to ineffective binding with hormones, resulting in decreased hormonal expression. Therefore, the objectives of the present study were to clone and characterize *LHR* exon 11, to identify the partial coding sequence (CDS) in Sahiwal and Hariana cattle breeds, and also to analyze the associations between the *Hha*I/PCR-RFLP genotypes and milk production and reproduction traits.

Materials and methods

Source, sample collection and genomic DNA isolation. Venous blood was collected from three adult females from both breeds (3 Sahiwal and 3 Hariana) for characterization and PCR-RFLP. The blood samples were taken randomly from a total of 200 animals (100 Sahiwal and 100 Hariana) maintained at the Livestock Farm Complex (LFC), DUVASU, Mathura, Uttar Pradesh, India. The animals were kept in identical environmental conditions and were fed a standard diet on the farm. Data relating to these animals were recorded, such as: date of birth (DOB), date of successive calving, date of successive drying and date of successful artificial insemination (AI), and from these the reproduction and milk production traits were derived. Milk production records were obtained by totaling the daily milk yield of individual cows after completion of lactation. The cows were milked manually using the full hands method twice a day in the morning and evening shifts, and the milk was weighed on digital scales, in liters. Incomplete lactations, for any recorded reason or pregnancies ending with abortion or other anomalies were deleted. Therefore, only 127 cows (70 Sahiwal +57 Hariana) completed their first and second lactation.

Genomic DNA was isolated using a standard phenol-chloroform DNA isolation protocol (SAMBROOK and RUSSELL, 2001). The concentration and purity of the genomic DNA were determined spectrophotometrically at OD_{260} and OD_{280}. The integrity of the DNA was examined by agarose gel (0.7%) electrophoresis, and the gel was visualized under a UV light after staining with ethidium bromide (EtBr).

PCR amplification cloning and sequencing. Exon 11 of the *LHR* was amplified using a specifically designed primer pair (F; 5′-TGAACTGAGTGGCTGGGATTATG-3′ and R; 5′-CATTCCTTATAGCAAGTCTTGTCC-3′) from the available bovine *LHR* sequence (GenBank Acc. No. U20504; 984-2108) using VNTI software, and synthesized commercially (Imperial life sciences, Gurugram, Haryana). PCR reactions were carried out in a 25 μl reaction mixture containing 1x PCR buffer (NEB, USA), 2 mM $MgCl_2$, 2.5 mM of dNTPs, 5 pmole of each primer and one unit of *Taq* DNA polymerase (NEB, USA). The cycle conditions used were: initial denaturation of one cycle at 95ºC for 3 min; 35 cycles of exon specific amplification denaturation at 95ºC for 30 sec; annealing at 60ºC for 30 sec; extension at 72ºC for 1 min, followed by final extension of 72ºC for 10 min. The amplified product was run on agarose (1.0%) gel electrophoresis in 1x TBE buffer including EtBr. The gel was visualized under a UV light and photographed with an automated gel documentation system. The amplified products were cloned into pTZ57R/T cloning vector (PUREGENE, Quick clone PCR-cloning kit). The positive recombinant clones were identified from the transformed bacterial colonies of *E.coli* DH5α strain, using blue and white colony selection. The positive clones were sequenced commercially (Eurofins genomics India Pvt. Ltd., Bengaluru, Karnataka) by an automated sequencer, using the standard cycle conditions by Sanger’s dideoxy chain termination method.

Sequence analysis. All the 6 sequences obtained of the *LHR* gene, exon 11 were subjected to NCBI BLAST analysis to ascertain whether the obtained sequence was *LHR,* and the nucleotide, as well as the amino acid sequence of the *LHR* gene, the partial CDS of the Indian cattle breeds, were aligned with *Bos taurus* (U67230, AF491303), Holstein (NM_174381), Murrah buffalo DQ858168, Mehsana buffalo DQ858169, Nili Ravi buffalo (DQ858170), Nagpuri buffalo (DQ858171), Surti buffalo (DQ858172), goats (NM_001314279), sheep (NM_001278566), hors-

es (XM_005599992), donkeys (XM_014847909), camels (XM_031466466) and pigs (NM_214449) available in the GenBank database using the Clustal W method of the MegAlign programme of Lasergene software (DNASTAR, USA) and BioEdit software. We employed the Maximum Likelihood (ML) method using MEGA version 4.0 software for phylogenetic tree construction (TAMURA et al., 2007). The ML method was chosen due to its widespread use and demonstrated effectiveness in inferring evolutionary relationships from molecular data. Before constructing the phylogenetic tree, multiple methods were evaluated to determine the most suitable approach. This involved testing various algorithms available in MEGA version 4.0 and assessing their performance in accurately representing the evolutionary relationships among the sequences. The ML method was selected due to its ability to estimate the best-fit model of nucleotide substitution, accounting for evolutionary rate variation among sites, and providing robust branch support values through bootstrapping.

HhaI/PCR-RFLP assay. The primers used for amplification of the 303 bp fragment comprising exon 11 (F; 5′-CAAACTGACAGTCCCCCGCT-TT-3′ and R; 5′-CCTCCGAGCATGACTGGAAT-GGC-3′) were according to the available bovine *LHR* sequence (GenBank Acc. No. U20504; 1179-1481) (MARSON et al., 2005). The PCR reaction mixture components were described earlier. The following cycle conditions were used: one cycle of initial denaturation at 95ºC for 3 min; 35 cycles of exon specific amplification denaturation at 95ºC for 30 sec; annealing at 60ºC for 30 sec; extension at 72ºC for 30 sec, followed by final extension of 72ºC for 10 min. The restriction digestion was carried out at 37°C for 4 h in a total volume of 7.75 µl containing 5.0 µl of PCR product, 0.75 µl of 10X RE buffer, 10 units of 0.5 µl *Hha*I enzyme, and 1.5µl nuclease free water. The digested products were then run through 2.5% agarose gel electrophoresis to visualize the bands, and photographed using a gel documentation system. Sequence analysis was conducted after restriction digestion for the presence of *Hha*I sites in the PCR product (Fig. 1).

Statistical analysis and association study. The genotypic and allelic frequencies of different LHR/*Hha*I genotypes were assessed by the standard procedure (FALCONER and MACKAY, 1996). The chi square (χ^2) test (P≤0.05) was also performed to test whether the distribution of the genotypic frequencies was in the Hardy-Weinberg equilibrium (SNEDECOR and COCHRAN, 1989).

The association study of obtained different LHR/*Hha*I genotypes was performed with the following reproduction traits: age at first calving (AFC = date of 1st calving - date of birth; DOB), service period (date of successful artificial insemination (AI) - date of calving) and calving interval (CI = the difference between two successive calving) and milk production traits including total milk yield (TMY = calculated by totaling the daily milk records of individual cows after completion

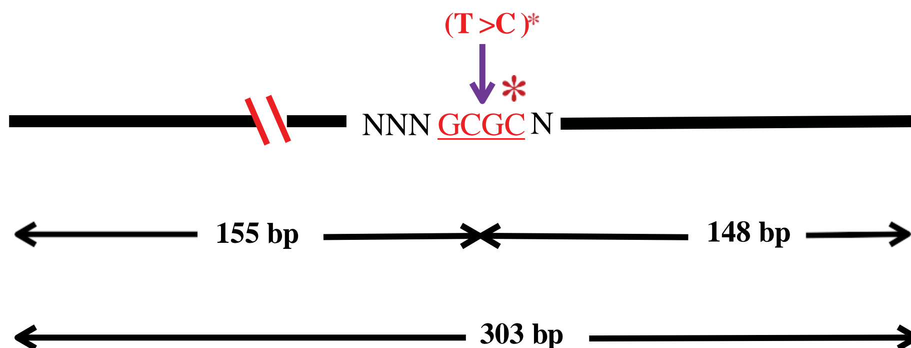

Fig. 1. Schematic representation of the *Hha*I/PCR-RFLP assay of 303 bp LHR amplicons

The recognition sequence of *Hha*I is underlined: (↓) indicates restriction cutting site: (*) (T>C) indicates substitution of C with T from the *Hha*I site

of their lactation), lactation period (LP = date of drying - date of calving). Association analysis was carried out by one way ANOVA with the help of SPSS (version 16.0) statistical software, using the General Linear Model (GLM) with following linear model:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where: Y_{ij} – observed trait value in animal; μ – mean trait value; G_i – effect of genotype; e_{ij} – random error. Significant differences between least square means of the different genotypes were calculated using Duncan's multiple-range test, and P values of 0.05 were considered statistically significant.

Results

Characterization of Exon 11 of LHR, partial CDS. Agarose gel electrophoresis (1.0%) revealed an amplified product of 1125 bp encoding exon 11 of Sahiwal and Hariana cattle breeds. After sequencing, chromatogram study of Sahiwal LHR exon 11 showed mixed peaks (heterozygous condition) and revealed A→G substitution (36 N→D) at nucleotide position 107 (Fig. 2A) and G→A substitution (175 V→M) at nucleotide position 524 (Fig. 2B), which resulted into substitution of asparagine (N) to aspartic acid (D) and valine (V) to methionine (M) at 36 and 175 amino acid position, respectively. Therefore these two type of genotypes are being named as NV and DM genotypes in Sahiwal breed (Fig 2). Three sequences of Sahiwal showed two variations/genotypes and no variation was observed in the three sequences of Hariana. So therefore, partial CDS encoding exon 11 sequences of Sahiwal NV, Sahiwal DM and Hariana have been submitted in GenBank database with accession no. MT681114, MT681115 and MT681113, respectively.

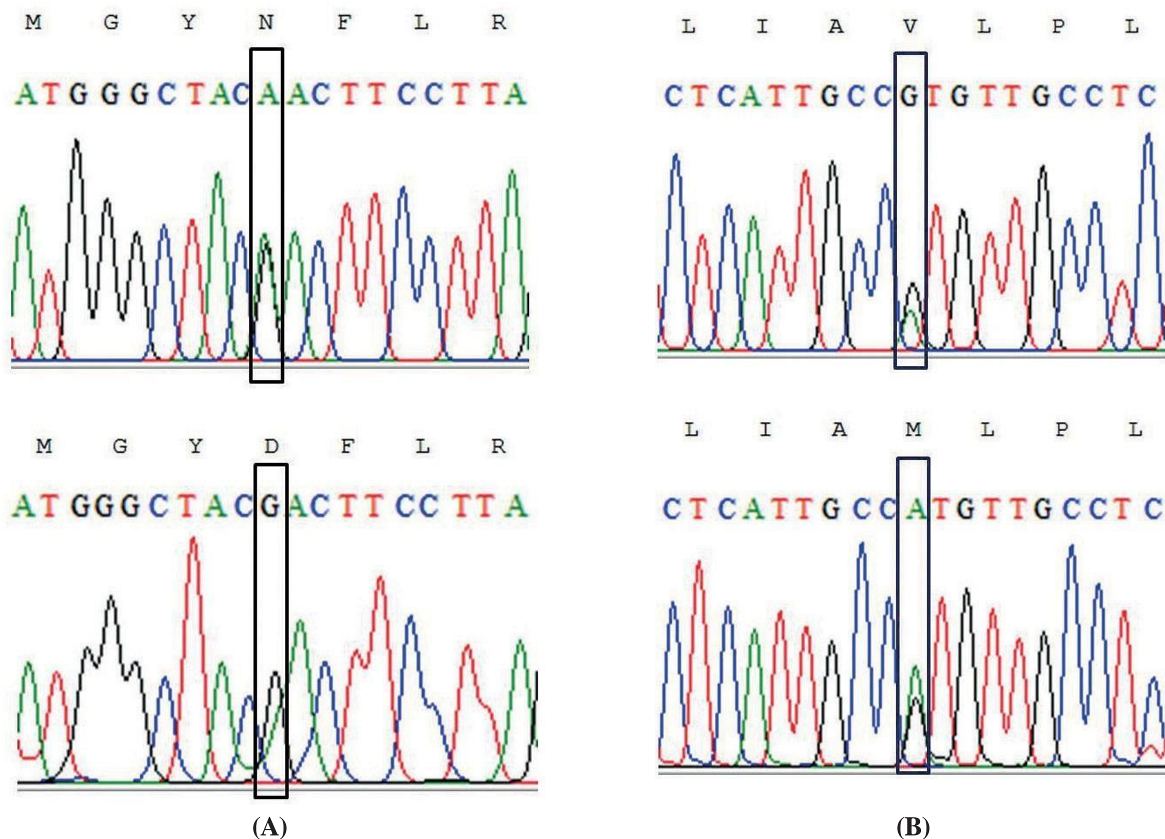

Fig. 2. Chromatogram of the LHR Exon 11 sequence showing that the heterozygote condition revealed A→G substitution (36 N→D) at position 107 (A) and G→A substitution (175 V→M) at position 524 (B)

The nucleotide, as well as amino acid sequence of studied Indian breeds, was compared with the homologous sequence of other breeds and species as shown in Table 1. At nucleotide and amino acid level, Sahiwal DM sequence was 99.8% and 99.5% similar to Sahiwal NV genotype, respectively. While, Hariana amino acid sequence was more closely related to Sahiwal NV (>99.7%) than Sahiwal DM (>99.2%) genotype. Studied Indian cattle breeds showed 99.5% to 99.6% and 98.4% to 98.7% similarity with buffalo breeds at the nucleotide and amino acid level, respectively. With small ruminants, they revealed more sequence identity i.e., ≥97.0% at amino acid level than nucleotide sequence and with pig, they revealed 91.3% to 93.9% homology. Mutations in partial CDS of LHR gene of studied Indian cattle breeds relative to exotic cattle breeds has been presented in Table 2. A G→T substitution was observed at nucleotide position 845 only in Hariana breed, which resulted in amino acid change from valine (V) to phenylalanine (F) at amino acid position 282. All cattle breeds have G→A transition at nucleotide position 107 (except Sahiwal DM) and 921, which resulted in amino acid substitution of D36N, R307Q, respectively. Bos taurus has non synonymous mutations at N231D (692A→G), I251T (753T→C) and F263S (789T→C), respectively.

Phylogenetic tree analysis. The phylogenetic tree (Fig. 3) clearly shows the splitting of two major clusters, *viz.,* ruminant and non-ruminant species. The ruminants cluster divides into the two major bovinae and caprinae groups. In the bovinae group, buffalo form a separate clade/subgroup leaving a cattle subgroup. Furthermore, in the cattle subgroup, Hariana are more closely related to

Table 1. Percentage identity of *LHR* gene exon 11 nucleotide and amino acid of Indian cattle breeds with other exotic cattle breeds, buffalo and other species

Species and Accession number	Nucleotide (%)			Amino acid (%)		
	Sahiwal DM*	Sahiwal NV*	Hariana	Sahiwal DM*	Sahiwal NV*	Hariana
Sahiwal DM_MT681114*	***	99.8	99.7	***	99.5	99.2
Sahiwal NV_MT681115*	99.8	***	99.9	99.5	***	99.7
Hariana_MT681113*	99.7	99.9	***	99.2	99.7	***
*Bos taurus*_U87230	99.5	99.6	99.6	98.7	99.2	98.9
*Bos taurus*_AF491303	99.8	100	99.9	99.5	100	99.7
Holstein_NM_174381	99.8	100	99.9	99.5	100	99.7
Murrah_DQ858168	99.6	99.6	99.5	98.7	98.7	98.4
Mehsana_DQ858169	99.6	99.6	99.5	98.7	98.7	98.4
Nili Ravi_DQ858170	99.6	99.6	99.5	98.7	98.7	98.4
Nagpuri_DQ858171	99.6	99.6	99.5	98.7	98.7	98.4
Surti_DQ858172	99.6	99.6	99.5	98.7	98.7	98.4
*Capra hircus*_NM_001314279	97.2	97.0	96.9	98.4	97.9	97.6
*Ovies aries*_NM_00127856	97.2	97.0	96.9	98.1	97.6	97.3
*Equus caballus*_XM_005599992	90.1	90.0	90.0	91.2	90.9	90.6
*Equus asinus*_XM_014847909	90.3	90.1	90.0	91.7	91.2	90.9
Camel_XM_031466466	93.4	93.4	93.2	96.3	96.3	96.0
*Sus scrofa*_NM_214449	91.7	91.5	91.3	93.9	93.3	93.0

* represents the same accession number of Sahiwal DM, Sahiwal NV and Hariana in rows and columns

Table 2. Nucleotide and amino acid substitutions identified in *LHR* gene exon 11 of Indian and exotic cattle breeds

Nucleotide	107	493	524	692	753	789	845	921
Amino acid	36	164	175	231	251	263	281	307
Majority codon	CGA	CGG	CGT	AAA	CAT	TTT	GGT	CCG
Codon Indian cattle breeds	CAA	CGG	CAT	AAA	CAT	TTT	GTT	CCA
Codon exotic cattle breeds	CAA	TGG	CGT	AGA	CAC	TTC	GGT	CCA
Majority AA	D	L	V	N	I	F	S	R
AA Indian cattle	N	L	M	N	I	F	S	Q
AA exotic cattle	N	L	V	D	T	S	S	Q
Breed/Accession number	Hariana (MT681113), Sahiwal NV (MT681114) *Bos taurus*, Holstein	Bos taurus	Sahiwal DM (MT 681115)	*Bos taurus*	*Bos taurus*	*Bos taurus*	Hariana	All cattle breeds

the Sahiwal NV genotype and other exotic cattle breeds than to the Sahiwal DM genotype. Sahiwal NV and DM genotypes are far apart from other Indian and exotic cattle breeds.

HhaI/PCR-RFLP assay. As anticipated, the PCR amplification targeted the 303 bp region of exon 11 of the LHR gene. Subsequent *Hha*I/PCR-RFLP analysis identified three distinct genotypes: TT (303 bp), TC (303 bp, 155 bp, and 148 bp), and CC (155 bp and 148 bp), confirming the presence of polymorphism in this gene across both cattle breeds (Figure 4). The frequencies of alleles and genotypes were computed and are detailed in Table 3. The TT genotype was more frequent (45.0%) in all the screened animals, followed by TC (42.5%) and CC (12.5%). The frequency of the T allele

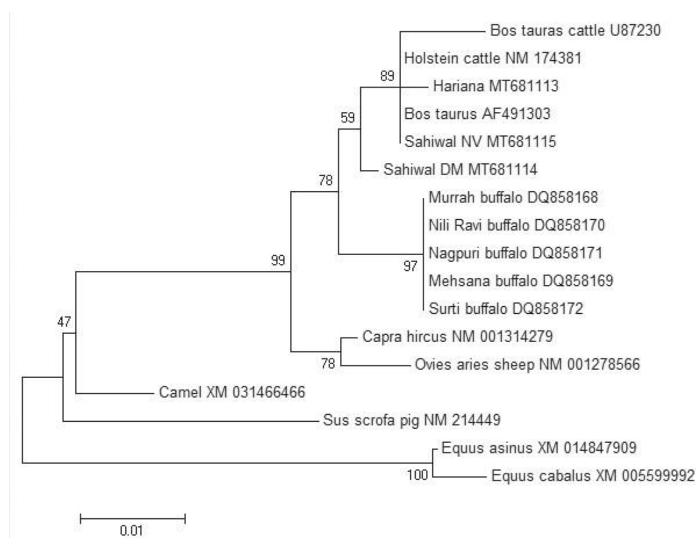

Fig. 3. Phylogenetic tree based on the deduced amino acid sequences of *LHR* exon 11 for the Indian cattle breeds, buffalo and other related species

The percentages of replicate trees (bootstrap value) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

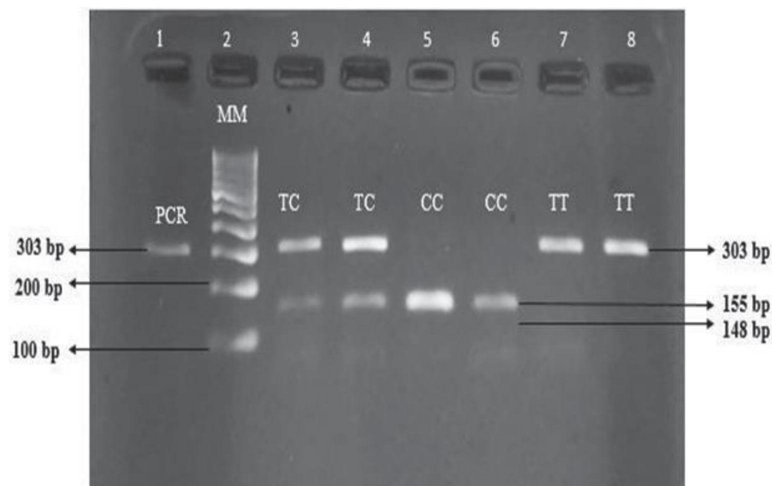

Fig. 4. LHR/*Hha*I exon 11 PCR-RFLP pattern of Sahiwal and Hariana cattle run on 2.5% agarose gel electrophoresis

Lane 1 (303 bp PCR product), Lane 3-8 (genotypic pattern in two breeds), Lane 2 (100 bp Marker). *Note*- Bands at 155 bp and 148 bp looked intact due to limitation of agarose gel electrophoresis to separate bands with a difference less than 10 base pairs

Table 3. Genotypic and allelic frequencies of the LHR/*Hha*I gene in Sahiwal and Hariana cattle

Breed	Genotypic frequency (%)			Allelic Frequency		Chi-square (χ2)	Chi-square (χ2)
	TT	TC	CC	T	C	Calculated value	Tabulated value
Sahiwal (n=100)	54.0 (n=54)	33.0 (n=33)	13.0 (n=13)	0.705	0.295	4.27 (P<0.05)	3.841 (*P<0.05*) at 1 degree of freedom
Hariana (n= 100)	36.0 (n=36)	52.0 (n=52)	12.0 (n=12)	0.62	0.38	1.07 (P<0.05)	
Total (N= 200)	45.0 (n=90)	42.5 (n=85)	12.5 (n=25)	0.66	0.34	0.49 (P<0.05)	

N= Sample size, n= Number of animals in particular genotype

(0.66) was greater than that of allele C (0.34). Chi square analysis revealed that the Hariana breed population was in Hardy-Weinberg equilibrium while the Sahiwal breed deviated from the H-W equilibrium. However, all the screened animals of both breeds were found to be within the H-W equilibrium.

Association Study. The overall mean ± SEM of AFC, TMY, LP, SP and CI in the first and second lactation is presented in Table 4. No significant difference was observed in terms of genotypes and milk production and reproduction traits.

Discussion

We cloned and characterized exon 11 of the *LHR* gene in Sahiwal and Hariana cattle. The nucleotide sequence was determined and the amino acid sequence was deduced. To the best of our knowledge, this is the first study to report the nucleotide and amino acid sequences of any exon of the *LHR* gene in Indian cattle breeds. The bovine *LHR* transmembrane domain consisted of 266 amino acid residues and the intracellular domain consisted of 71 amino acid residues (KAWATE et al., 2002). As reported by other authors (MAMLUK

Table 4. Association study of LHR/*Hha*I exon 11 genotypes with milk production and reproductive traits in Sahiwal cattle and Hariana cattle breeds

Lact	Breed	Geno	n	AFC (days)	TMY (ltrs)	LP (days)	SP	CI
I	Sahiwal (n=70)	CC	8	2170.7±72.2	1825.6±214.2	333.1±28.9	259.5±65.1	540.9±113.5
		TC	23	2191.0±66.3	1953.3±130.6	374.2±20.2	225.4±29.8	507.5±33.3
		TT	39	2166.3±40.5	1672.6±107.7	358.9±17.2	262.3±19.6	532.4±18.1
	Total (N=127)	CC	12	2262.0±113.4	1766.2±159.4	332.2±25.6	225.3±59.9	509.0±94.6
		TC	52	2249.5±102.2	1806.2±94.9	345.4±11.7	197.5±28.7	469.2±23.5
		TT	63	2321.0±84.5	1592.2±84.5	333.7±12.8	233.6±23.8	507.9±25.1
II	Sahiwal (n=70)	CC	8	---	1997.2±256.9	357.2±29.4	255.2±45.4	539.6±90.4
		TC	23	---	2089.6±118.4	374.5±19.0	223.2±32.5	508.4±34.3
		TT	39	---	1887.9±85.8	346.1±11.7	280.6±20.3	560.5±85.4
	Hariana (n=57)	CC	4	---	1736.9±275.2	334.0±43.3	146.6±46.3	426.5±36.5
		TC	29	---	1652.9±91.1	309.6±12.7	166.4±32.4	435.0±15.5
		TT	24	---	1604.6±107.2	294.6±10.8	175.0±34.4	442.0±34.5
	Total (N=127)	CC	12	---	1910.5±190.0	349.5±24.6	219.0±45.6	498.6±72.4
		TC	52	---	1846.1±78.4	338.3±11.8	191.2±32.4	467.7±23.8
		TT	63	---	1779.8±68.7	326.3±8.9	240.5±25.7	520.5±66.0

Lact- lactation, n- number of animals in particular genotype, Geno- Genotype, AFC- age at first calving, TMY-total milk yield, LP- lactation period, SP- service period, CI- calving interval N= Total sample size of both the breeds, n= Number of animals in particular breed

et al., 1998; KAWATE et al., 2002) bovine *LHR* showed 96.9% and 93.6% homology with the pig amino acid sequence. Exon 11 of the *LHR* gene in *Bubalus bubalis* revealed 99.0% and 98.0% homology with the bovine nucleotide and amino acid sequence (SINGH, 2006). A total of eight conserved cysteine residues at positions 75, 83, 115, 190, 219, 221, 257 and 293 in the transmembrane, and three conserved cysteine residues at 319, 320 and 361 in the intracellular domain were reported. A similar pattern was also reported in bovines (Holstein) (KAWATE et al., 2002). The human *LHR* gene exhibits >90.0% homology with porcine and rat LHR (MINEGISHI et al., 1990). Multiple serine, threonine and tyrosine residues were found in the intracellular domain and the cytoplasmic loop that are potential sites for phosphorylation (KAWATE et al., 2002). Many researchers reported homology among G-protein coupled receptors, including LHR, FSHR in terms of transmembrane domain (LOOSFELT et al., 1989; DUFAU et al., 1995; KAWATE et al., 2002). The *LHR* gene has been extensively studied in humans and rats, and researchers have found many mutations causing reproductive problems (SEGALOFF, 2009). Asp578Gly is the most common mutation of LHR (LAUE et al., 1995). Asp578His is the somatic mutation (SEGALOFF, 2009) responsible for hyperplasia and leydig cell adenoma (BOOT et al., 2011). There is insufficient literature available depicting SNPs or mutations in farm animals. The *Hha*I/PCR-RFLP assay we carried out, where we reported the higher frequency of the TT genotype (54.0%) observed in Sahiwal cattle, was in accordance with the findings in Kankrej (52.1%), Gir (68.0%) and Sahiwal (48.0%) (CHANDRAN, 2006; KUMAR, 2018).

The researchers found a higher TT frequency in the respective indigenous cattle breeds. The reason for the similar pattern of genotype frequency may be due to them sharing the same native tract and population size. However, in exotic cattle, such as Kenana, Erashy (OMER et al., 2016) and Turkish cattle breeds (ARSALAN et al., 2015), the frequency of the TT genotype was lowest (ARSALAN et al., 2015). In the current study, the frequency of heterozygotes (TC) was greater in Hariana cattle (52.0%), which is in accordance with the results in European Zebu (MARSON et al., 2005), Turkish cattle breeds (ARSALAN et al., 2015), Kankrej (48.0%) and Rathi (50.0%) (KUMAR, 2018). The frequency of the CC genotype was lowest in all the indigenous cattle breeds in the current study as in others (CHANDRAN, 2006; KUMAR, 2018), except in Rathi and Kankrej (KUMAR, 2018). We observed the high frequency of the T allele (0.70 and 0.62) in Sahiwal and Hariana cattle, respectively. A high frequency of the T allele was also reported by others (CHANDRAN, 2006; KUMAR, 2018) in Kankrej (0.74), Gir (0.82) and Sahiwal (0.66). However, in exotic cattle the frequency of the C allele was highest (ARSALAN et al., 2015). The Chi square test revealed that both the breeds were in the Hardy-Weinberg equilibrium, which means these breeds face less pressure in selection. Similar findings were presented for populations of Sahiwal, Rathi and Kankrej (KUMAR, 2018). The association study of genotypes with milk production and reproduction traits revealed no significant differences, which is in accordance with previous findings (KUMAR, 2018).

In the present study, we reported the cloning and characterization of *LHR* exon 11, partial CDS in Indian cattle breeds. Moreover, the nucleotide and amino acid sequences of Sahiwal and Hariana cattle breeds was compared with the homologous sequences of other species. We identified eight SNPs in Indian and exotic cattle. Furthermore, *Hha*I/PCR-RFLP in exon 11 was also conducted and we found the polymorphic pattern of this gene in Sahiwal and Hariana cattle breeds. An association study revealed no significant differences in genotypes in relation to milk production and reproduction traits. Further investigations need to be undertaken to find the best performing genotypes which may be helpful in marker assisted selection.

Declaration of competing interest

No potential conflict of interest was reported by the authors

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KUMAR, A., S. PAL SINGH, D. SHARMA, A. KUMAR, V. UTTAM: Molekularna analiza dijela kodirajuće sekvencije (CDS) gena za receptor luteinizacijskog hormona (*LHR*) u indijskih pasmina goveda. Vet. arhiv 95, 135-148, 2025.

SAŽETAK

Gen za receptor luteinizacijskog hormona (*LHR*) kodira LH receptore važne za vezanje hormona u jajnicima i na taj način utječe na reproduktivnu fiziologiju životinja koje proizvode mlijeko. U istraživanju je dio kodirajuće sekvencije (CDS), uključujući ekson 11 (>1100 bp) gena *LHR*, kloniran te je provedena njegova analiza u pasmina goveda Sahiwal i Hariana. Sekvenciranje i kromatogram sekvencije eksona 11 gena *LHR* otkrili su dva heterozigotna stanja koja su bila posljedica nesinonimne supstitucije A→G i G→A na poziciji 107, odnosno 524, što je rezultiralo supstitucijom asparagine (N) asparaginskom kiselinom (D) na poziciji aminokiseline 36, te valina (V) metioninom (M) na poziciji aminokiseline 175. Stoga su ova dva heterozigotna stanja otkrivena u ovom istraživanju, identificirana kao genotipovi NV I DM u pasmini Sahiwal. Usporedba sekvencija eksona 11 gena *LHR* u pasmine Sahiwal (NV i DM) i Hariana pokazala je homolognost od 98,7% do 100% s egzotičnim pasminama goveda na razini nukleotida, odnosno aminoksielina. Istražen je i DNA polimorfizam upotrebom testa *Hha*I/PCR-RFLP. Otkrivene su tri vrste genotipova: TT (303 bp), TC (303, 155 i 148 bp) i CC (155 i 148 bp), pri čemu je genotip TT bio učestaliji (45,0%) od genotipa TC (42,5%) i CC-a (12,5%). Učestalost alela T bila je veća (0,66) od alela C (0,34) u svih pretraženih životinja. Hi-kvadrat (χ^2) pokazao je da su pretražene skupine životinja bile u Hardy-Weinbergovoj ravnoteži. Analiza povezanosti nije pokazala znakovitu povezanost s proizvodnjom mlijeka i reproduktivnim svojstvima.

Ključne riječi: goveda; kloniranje; PCR-RFLP; LHR/*Hha*I; istraživanje povezanosti