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Molecular characterization of luteinizing hormone receptor (*LHR*) gene in Murrah bulls

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Abstract

Semen quality of bulls is commonly affected due to multitude inherent genetic disorders. Gonadotropins, interacting with their gonadal receptors, play a key role in sexual development, reproductive functions and metabolism. In this study, we performed molecular characterization and detection of genetic polymorphism in Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5 regions of Luteinizing Hormone Receptor (*LHR*) gene in Murrah bulls. Genomic DNA was isolated from the blood samples within 24 hours of collection by high salt method. Polymorphism was studied using Polymerase Chain Reaction-Single Stranded Conformational Polymorphism (PCR-SSCP) technique followed by Sanger Sequencing. PCR-SSCP and sequence analysis revealed monomorphism within the studied population that is coding region as well as exon-intron boundaries of Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5 of Luteinizing Hormone Receptor (*LHR*) gene is highly conserved among Murrah bulls. Consequently, we could not perform association studies with fresh and frozen semen quality parameters in Murrah bulls.

Keywords: *LHR*, Murrah bulls, PCR-SSCP, Monomorphism

1. Introduction

Murrah is one of the best dairy, water buffalo breed, in the world. Its home tract lies in Rohtak, Hisar, Jind, and Gurgaon districts of Haryana. This particular breed is being used for upgrading local buffaloes in many parts of Asia and other parts of the world. The demand for the elite buffalo males is expanding day by day in order to accomplish the requirement of frozen semen. Bull fertility is an economically important complex trait. Therefore, direct selection for this trait is not feasible because of low heritability. Recently many reports revealed the use of genetic variants in candidate genes as a marker for semen quality (MAS) in swine [1-3] and goats [4]. Genetic markers could be an aid for selection of breeding bulls through which genetic up gradation can efficiently be achieved.

Gonadotropins, the actions of which are mediated at the level of their receptors, play a pivotal role in sexual development, reproductive functions and metabolism. In particular, spermatogenesis is regulated by FSH acting on Sertoli cells and LH acting on Leydig cells [5]. *LHR* is a member of the G-protein coupled receptor and expressed in testicular leydig cells, which activates the cAMP and play a crucial role in initiation and maintenance of gametogenesis [6]. The *LHR* has a key role in testicular development and function [7]. *LHR* is activated only in meiotic spermatocytes and highly abundant in human sperms [8]. Upon binding to luteinizing hormone or its placental counterpart, chorionic gonadotropin (CG), the receptor stimulates adenylyl cyclase activity, cyclic adenosine 3', 5'-monophosphate production [9] and consequently, activation of the Leydig cells and testosterone production. Mutations can be activating or inactivating. Inactivating mutations of *LHR* could cause Leydig cells hypoplasia and decreased production of testosterone. This gives rise to hypogonadism or male pseudo-hermaphroditism, called Leydig cell hypoplasia or agenesis [10].

In men, inactivating *LHR* mutations are associated with severe phenotypes, such as the presence of a 46, XY karyotype correlated with disorders of sex development [11]. In addition, an LH receptor splice mutation responsible for male hypogonadism with subnormal sperm production has been reported [12]. So, the role of *LHR* gene in male reproduction has made it a potential candidate gene for sperm quality traits. Consequently, the present study has been undertaken with the objective to identify sequence variation in initial five exons (Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5) of *LHR* gene in Murrah bulls.

2. Materials and Methods

The experimental plan of the study was duly approved by the Institution Animal Ethics committee of ICAR-NDRI, India. Blood samples were collected from 109 Murrah bulls maintained at three different organized semen stations viz., Centralized Semen Collection Centre of Livestock Breeding and Training Centre, Dharwad; Nandini Sperm Station and State Livestock Breeding and Training Centre, Hessarghatta, Bengaluru, Karnataka, India.

About 10 mL of blood was collected from each animal aseptically by jugular venipuncture in a vacutainer tube containing 0.5 per cent of EDTA and the samples were stored at 4 °C for further use. The genomic DNA was isolated from the blood samples within 24 hours of collection by high salt method (13). Agarose gel (1.5%) was run to determine quality of DNA. The purity and concentration of DNA was determined using spectrophotometer. Optical density (OD) value was determined at 260 nm and 280 nm. DNA samples with OD260/OD280 ranging between 1.7 and 1.9 were of good quality. The genomic DNA was diluted to a final concentration of 100 ng/μl and stored at -20 °C.

Five sets of forward and reverse gene-specific oligonucleotide primers (Table 1) (Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5) were designed based on *LHR* reference sequence of *Bubalus bubalis* (NCBI Reference Sequence: NW_005785820.1) by using primer 3 (<http://primer3.ut.ee/>) online software and were procured from Sigma Aldrich Pvt. Ltd. Bengaluru, India. The Polymerase Chain Reaction (PCR) was performed on about 50-100 ng of genomic DNA in 12.5 μl per reaction volume. The PCR reaction mixture consisted of 200 μM of each dNTPs, 10X Taq Pol assay buffer, 1U Taq polymerase enzyme and 20 pM of each primer. The thermocycler conditions included an initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 30 seconds with varying annealing temperatures based on primer set (Table 1), extension at 72 °C for 30 seconds followed by a final extension at 72 °C for 5 min. The PCR products were electrophoresed at 100 V in 1.5% agarose gel with 1X TBE buffer containing 0.5 μg/ml ethidium bromide along with a DNA molecular size marker of 100 bp. The gels were visualized and documented using Gel documentation system (Gel doc 1000, Bio-Rad, USA) (Figure 1-5).

The genetic variants were determined by single strand conformation polymorphism (SSCP) analysis. Ten microliters of amplified PCR products were further diluted in ten microlitres denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA) and denaturation was carried out at 95 °C for 2 min followed by rapid chilling on an ice block for 20 min and loaded on 12% acrylamide:bisacrylamide (29:1) in 1X TBE buffer for 8 h (200 V) at 4 °C. The gels were silver-stained as described by Sambrook and Russell [14]. Band patterns were characterized by the number of bands and mobility shifts, and each pattern was scored manually. To confirm the mobility shift in each pattern, PCR products of each SSCP pattern in duplicates were chosen and custom

sequenced using automated ABI DNA Sequencer (Medauxin Pvt. Ltd., Bengaluru, India) for detecting SNPs. Sequence data were analyzed using DNA Baser and Clustal W multiple sequence alignment software for detecting SNPs by comparing the observed sequence of the *LHR* gene in Murrah bulls with NCBI reference sequence for *Bubalus bubalis*.

3. Results and Discussion

Inactivating mutations of *LHR* result in subnormal male sexual development or male pseudohermaphroditism [10]. So, in this study we first investigated SNP's in the 109 Murrah bulls. PCR-SSCP analysis exhibited monomorphism in the studied population with respect to Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5 of *LHR* gene (Figure 6, 7, 8, 9 & 10). SSCP band pattern observed to be similar within the Murrah bulls as well as compared to reference sequence of *Bubalus bubalis*. Monomorphism in the studied population with respect to initial five exons of *LHR* gene was validated by sending samples in duplicate for sequencing. Sequencing result analysis revealed absence of variation within Murrah bulls as well as compared to reference sequence indicating that coding region as well as exon-intron boundaries of first five exons (Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5) of *LHR* gene is highly conserved among these buffalo bulls.

As this is a novel research work, explorations of polymorphism in these exonic regions were carried out for the first time in Murrah bulls. Published literature is not available to compare and contrast the findings in Murrah bulls. Our findings are not in agreement with Sun *et al.*, 2012 [15] who found mutations in *LHR* gene in Chinese Holstein bulls. Their least square analysis showed that genotype of *LHR* gene had slight effect on FSM and SD. And genotype AG and TT could be favorable markers for sperm quality traits of FSM and SD, respectively. In addition to this, similar results to present investigation have been reported in Murrah/Graded Murrah buffaloes [16]. In these buffaloes, the *CC* and *CT* genotypes were found absent. Similarly, *CC* and *CT* genotypic frequencies were found as zero in buffaloes [17, 18]. The frequency of *T* allele was 1.0 and for *C* allele was 0.0 in the screened buffalo population. We could not perform association study with semen quality traits of bulls in the present study, because all the screened animals were found monomorphic for *LHR* exon 1, exon 2, exon 3, exon 4 and exon 5.

4. Conclusion

LHR gene polymorphism in Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5 was investigated using PCR-SSCP assay in 109 Murrah bulls and found as monomorphic pattern in the screened population. Consequently, we could not establish any association between SSCP patterns and semen quality traits in fresh and frozen semen. But, the study with more number of animals may lead to genetic variation and that can be used for selection of superior males at an early age.

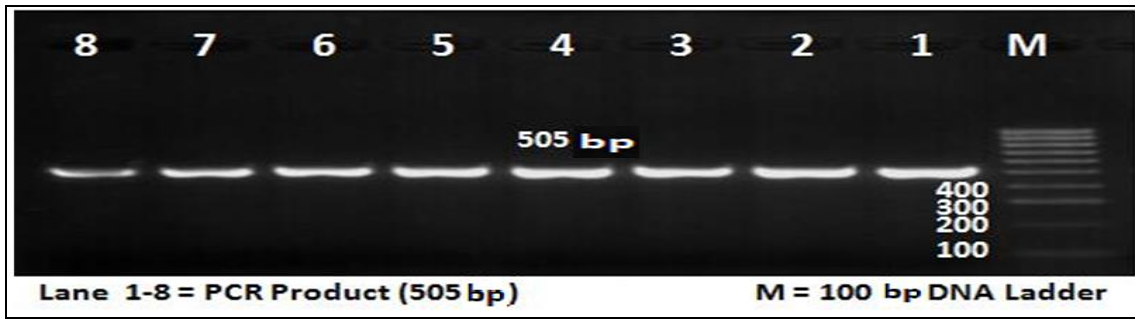


Fig 1: Resolution of polymerase chain reaction (PCR) amplified product of Exon 1 on 1.5% agarose gel. Lane 1-8=PCR product (505 bp), M=100 bp DNA ladder.

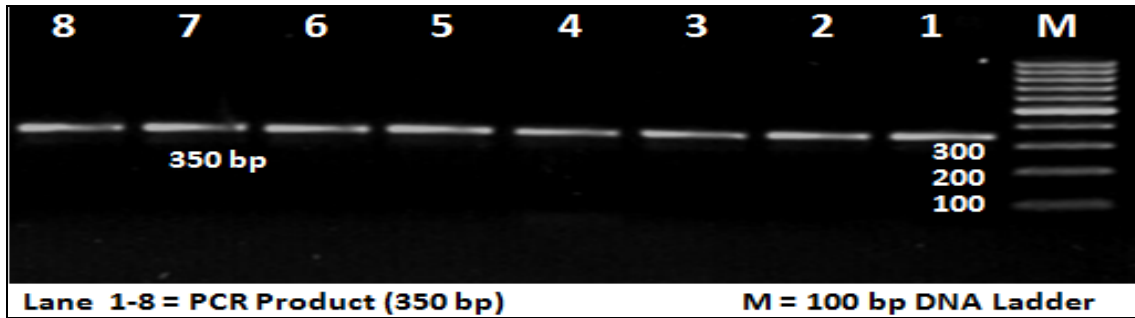


Fig 2: Resolution of polymerase chain reaction (PCR) amplified product of Exon 2 on 1.5% agarose gel. Lane 1-8=PCR product (350 bp), M=100 bp DNA ladder

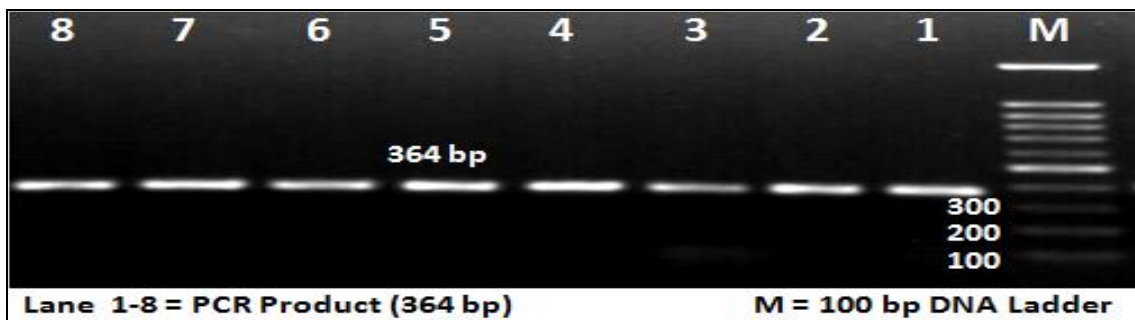


Fig 3: Resolution of polymerase chain reaction (PCR) amplified product of Exon 3 on 1.5% agarose gel. Lane 1-8=PCR product (364 bp), M=100 bp DNA ladder

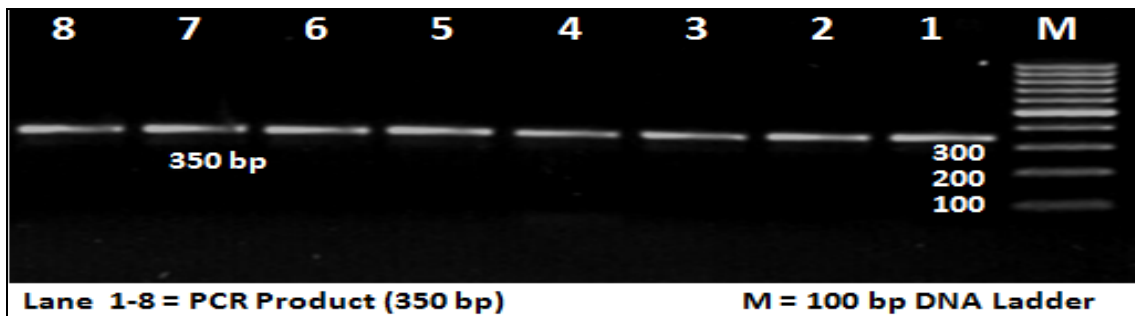


Fig 4: Resolution of polymerase chain reaction (PCR) amplified product of Exon 4 on 1.5% agarose gel. Lane 1-8=PCR product (350 bp), M=100 bp DNA ladder

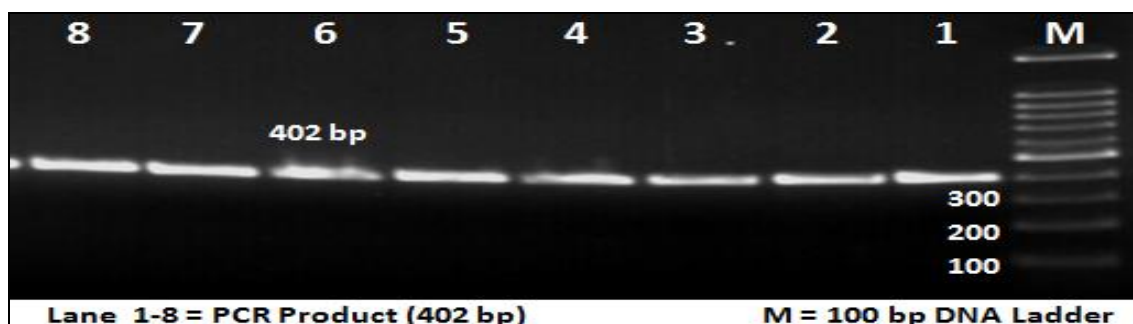
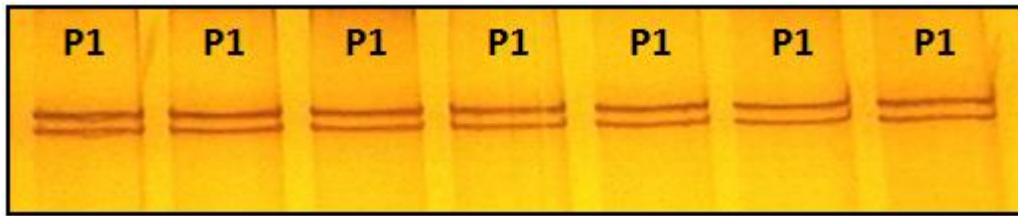
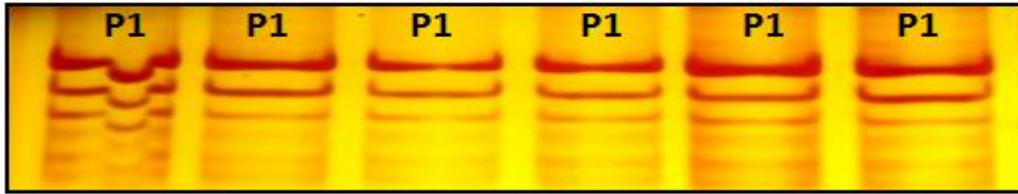
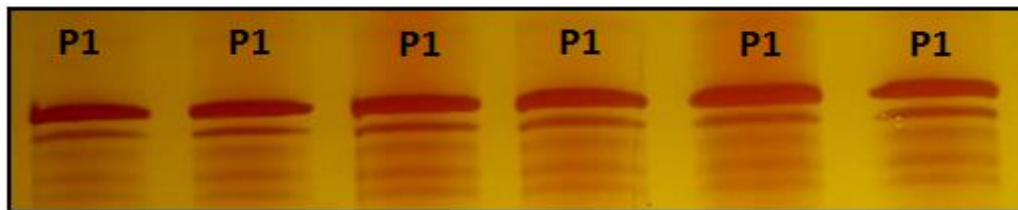
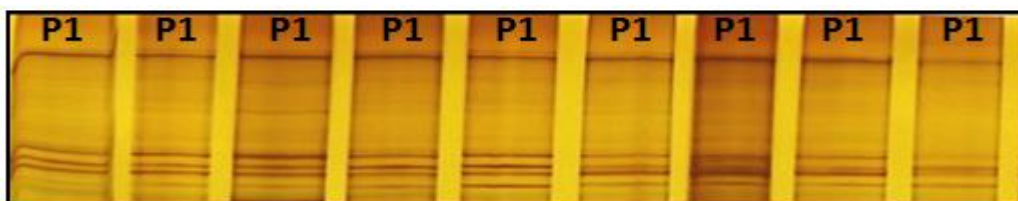


Fig 5: Resolution of polymerase chain reaction (PCR) amplified product of Exon 5 on 1.5% agarose gel. Lane 1-8=PCR product (402 bp),

M=100 bp DNA ladder

Fig 6: PCR-SSCP patterns (P1= pattern 1) of exon 1 of *LHR* geneFig 7: PCR-SSCP patterns (P1= pattern 1) of exon 2 of *LHR* geneFig 8: PCR-SSCP patterns (P1= pattern 1) of exon 3 of *LHR* geneFig 9: PCR-SSCP patterns (P1= pattern 1) of exon 4 of *LHR* geneFig 10: PCR-SSCP patterns (P1= pattern 1) of exon 5 of *LHR* gene**Table 1:** Details of primer sequences (5' to 3') used for amplification of Exon 1, Exon 2, Exon 3, Exon 4 and Exon 5 of *LHR* gene

Primer code	Sequence (5'-3')	Amplicon size (bp)	T _a (°C)
Primer 1	F CTCGGAGTGCAGTTTGAGAA R AAATAAACAAACGGAGTTGT	505 bp	61
Primer 2	F GTCTGAGCTCGGTTACTTCC R CCCCCTGGCCTGCCCTCCCT	350 bp	59
Primer 3	F AGGGAGGGCAGGCCAGGGG R GCTTCTCATCACCCGAGAG	364 bp	61
Primer 4	F GCTATTGGGAAGTCTGCGGTC R TTTTCTTCAAAGGTCCCTGGA	350 bp	59
Primer 5	F GCTCCTAGAGGGAGGTGTGA R GTGCCTGCCAGACCTAGTA	402 bp	60

T_a = Annealing Temperature, bp = base pair

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