CHARACTERIZATION OF CODING AREAS OF SPAG11B GENE IN MURRAH BULLS

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ABSTRACT

The SPAG11 gene is one of pivotal molecules in reproduction as it takes part in spermatozoa maturation, acquiring motility, capacitation, and egg-sperm interaction as well. The current study was aimed to characterize SPAG11 gene in Indian Murrah bulls through direct DNA sequencing approach. Genomic DNA from Murrah animals were isolated from 130 Murrah bulls and amplified using three sets of forward and reverse primers which were based on reference sequence (Genbank accession no. AC_000164.1) of *Bos taurus* covering entire coding region of SPAG11B gene. The PCR products of 563, 340 and 373, bp covering exons 1 to 3 were subjected to sequencing and subsequently ClustalW analysis revealed the substitution at 34 positions and a single stretch of 22 bp deletion in comparison to the *Bos taurus* reference sequence. Total seven novel SNPs were observed as two in the coding region and five in 5'UTR. However, only one of SNPs resulted in amino acid substitution *viz*. p.1279 arginine to tryptophan in translated protein in Murrah buffaloes. Sequence alignment and homology across species for the targeted nucleotide sequence of SPAG11 gene in Murrah bulls was done by nucleotide BLAST (NCBI) that showed maximum identity of 97% with mRNA of *Bos taurus* and *Capra hircus* followed by 96% homology with *Bos indicus* and *Bison bison* and 95% homology with *Ovis aries and Bos mutus.*

Keywords: *Bubalus bubalis*, buffaloes, SPAG11 gene, characterization, DNA sequencing Murrah bulls

INTRODUCTION

One of the key genes for reproductive traits is Sperm Associated Antigen 11 (SPAG11), which is a peculiar member of β-defensin genes' family. It is responsible for encoding several androgen-dependent, epididymis-specific secretary proteins; those are involved in various reproductive functions *viz*. sperm maturation, acquiring

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forward motility, capacitation and sperm-egg interaction etc. Human epididymis 2 (HE2) in humans, epididymal protein 2 (EP2) in monkeys and Bridging integrator 1b (Bin-1b) in rats are the same genes as SPAG11 gene of bovines. The SPAG11 take in both reproductive and immune tasks in humans (Yenugu *et al*., 2006), rats (Li *et al*., 2001; Zhou *et al*., 2004) and cattle (Avellar *et al*., 2007). The regulation of expression of SPAG11 mRNAs in the bovines is primarily androgen mediated (Hamil *et al*., 2000). Additionally other signaling molecules like hormones, tissue-specific factors, and/or extracellular signals can possibly influence the gene expression present in fetal and adult tissues. Therefore, the SPAG11 gene appears to be a good candidate for mutations associated with altered reproductive performance.

As spermatozoa leaving from the testis are yet to mature, they lack in the fertilizing ability. Subsequently spermatozoa transit through the epididymis, where it gets mature by attaining forward motility and fertilizing capacity (Orgebin-Crist, 1967; Bedford, 1967). The specialized sequent regions of the epididymis *viz*., the head (caput), body (corpus) and tail (cauda) generate epididymal fluid milieu, which are essential for sperm maturation. The SPAG11, which is expressed exclusively in the middle of the caput region, as a region restricted localized pattern coupled with the fact that the microenvironment of the caput region is essential for sperm to acquire their forward motility (Jeulin *et al*., 1996; Xu *et al*., 2010) suggest that SPAG11 have a potential role in sperm maturation.

SPAG11 are cationic molecules with polarity having distinct hydrophobic and charged regions that create channels across membranes by getting embedded into biological (Phospholipid bilayer) membranes. This embedded molecule can modulate other membrane ion channels, including the activation of L-type Ca^{++} channels (MacLeod, 1991; Bateman, 1996). Thus, SPAG11 might enable the acrosome reaction by creating Ca^{++} permeable channels themselves or activating Ca^{++} channels in sperm (Guraya, 2000; Breitbart, 2002). Reportedly during the transit of sperm through the epididymis, a noteworthy change in the Ca^{++} accumulating capacity of sperm occurs (Hoskins, 1983) and the sperm at caput epididymis has about two fold higher Ca^{++} concentration compared to sperm at cauda epididymis (Vijayaraghavan, 1988) that is essential for acquiring motility. However, exact mechanism of action of SPAG11 gene has not yet been reported, the evidence of age specific (Yenugu *et al*., 2006), androgen dependent, localized expression of SPAG11 gene in different parts of epididymis and sperm surface (Hamil *et al*., 2000; Avellar *et al*., 2007) reflects possible role of SPAG11 gene in sperm maturation and acquiring motility. In Boer goats, testicular biometric variables were reported to have association with SNPs in SPAG11 gene (Harighi *et al*., 2019), that further strengthens the hypothesis of the gene playing significant role in male reproductive efficiency.

MATERIALS AND METHODS

The frozen semen samples of 130 Murrah bulls were used for genomic DNA extraction using the phenol-chloroform extraction method described by Green and Sambrook (2012) with requisite alterations followed by assessment of quality and quantity of isolated DNA. A total of three pairs of gene specific oligo-nucleotide primers were designed (Table 2) for Murrah bulls based on SPAG11 gene sequence in *Bos taurus* chromosome 27 (AC_000184.1) with the help of

'PrimerQuest℠' (IDT) and used to amplify the regions spanning around coding exons of SPAG11 gene. The 25 µl PCR reaction mixture containing 50 ng of sample DNA (2 µl), 10 pmol of each primer (1 µl each), PCR master mix (12.5 µl) (Fermentas, Genetix Biotech Asia) and nuclease free water (8.5 µl) was utilized. PCR amplification reactions were carried out in Thermal cycler (BioRad T100™) with 3 minutes of initial denaturation at 95°C followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing for 45 seconds at 60.5°C for primer 1 and 62°C for primer 2 and 3 followed by extension at 72°C for 1 minute. Final extension at 72°C for 10 minutes was performed at the end. The PCR products were visualized on 2% w/v agarose (Low EEO) containing 0.8 µl of 10 mg/ml ethidium bromide in 1X-TBE buffer with 100 bp ladder at 100 volts and 200 mA for 45 minutes. Then gels were examined under UV trans-illuminator and photographed using gel documentation system (BioRad). The primers' sequence, their targeted sequence in *Bos taurus* chromosome, expected and obtained amplicon length and annealing temperature are presented in Table 1. Single, intact sharp DNA bands from amplified PCR products (15 µl) were subjected to direct sequencing from either of the ends (5' end and 3' end) for all sets of primers, after which nucleotide sequences had been visualized and edited in BioEdit software program. The forward sequence and reverse complement of reverse sequences along with primers were assembled for each amplicon to form complete coding region of SPAG11 gene. The Megalign module (Lasergene, DNASTAR version 7.2) was utilized for multiple sequence alignments of the edited sequence with corresponding reference sequences. Translated amino acid sequence were obtained using ExPAsy translate tools from coding sequences. The effect on the protein function by amino acid substitution were predicted with PROVEAN (Protein Variation Effect Analyzer) online tool. Extent of sequence homology of the SPAG11 gene coding fragments with other species was detected with Nucleotide BLAST analysis and phylogenetic tree was constructed using online tool phylogeny.fr.

RESULTS AND DISCUSSIONS

The SPAG11 is a candidate gene with respect to reproduction, immunity as well as for production traits in livestock species. Identification of single nucleotide variations (SNPs and InDels) in coding region of a gene is particularly essential for extending studies to identify their effect on gene expression and protein functions.The location of SPAG11 gene in cattle is on chromosome 27q1.2 on the complementary strand at location 4920221 to 4946628 bases (AC_000184.1), conferring a total length of 34 kb. The configuration of SPAG11 gene consists of a total 6 exons of which 3 are coding (Figure 1). The bovine SPAG11 mRNA has 920 nucleotides, first 210 bases of which form the CDS for the gene (NM_001098154.1). The mRNA translates for sperm associated antigen 11B precursor (NP_001091623.1) having polypeptide length of 69 amino acids which further shows considerable variation among species (Table 2).

All the three primer pairs satisfactorily amplified the desired regions of Murrah genome with specificity. Twenty samples of each amplicon of SPAG11 gene in Murrah bulls were sequenced and aligned, which showed similar amplicon sizes to *Bos taurus* except for third amplicon. The compiled sequence of SPAG11 gene for Murrah bulls was submitted to NCBI GenBank with accession number KY007013.1. Sequence

alignment and homology across species for the targeted nucleotide sequence of SPAG11 gene in Murrah bulls was done by nucleotide BLAST (NCBI); which showed maximum identity of 97% with mRNA of *Bos taurus* and *Capra hircus* followed by 96% homology with *Bos indicus* and *Bison bison* and 95% homology with *Ovis aries and Bos mutus* indicating a highly conserved sequences of SPAG11 gene in various livestock species. Figure 2 shows the phylogeny of various domestic species, constructed using mRNA sequences of homologues genes with help of online phylogeny.fr software. The clustering on dendogram showed that *Bubalus bubalis* was placed on a separate cluster close to *Bos taurus*, *Bos mutus* and *Loxodonta Africana*.

As compared to *cattle* sequence (Gene ID:783025), overall 34 nucleotide variations (including 13 in promoter, 8 in intronic and 13 in exonic regions) were found in the coding areas of SPAG11 gene in buffalo bulls. Among the detected variations, 23 transitions, 8 transversions, 1 insertion and 2 deletions were confirmed. Thirteen variations were found in promoter region and exonic region each and eight were located in the non-coding areas.

Seven novel SNPs were reported in the buffalo bulls under present study (Figure 3, 4 and 5). In the coding region, two nucleotide variations (g.C1279T and g.G2266A) were identified. The alignment of *B. taurus* protein (NP_001091623.1) with translated amino acid sequence of Murrah bulls revealed that g.C1279T as non-synonymous SNP leads to change in amino acid from Arginine (Arg) a basic amino acid to Tryptophan (Trp) an aromatic amino acid, at 43th position of SPAG11 protein. Prediction of the amino acid substitution affecting protein function using PROVEAN tool revealed a significant effect of substitution with a PROVEAN score of -4.308, while default cutoff for neutrality of substitution was -2.5. Two SNPs g. 1674 A>C and g. 2239 C>T in the intron of β-defensin gene in Holstein-Friesian cows were identified by Bagnicka *et al.* (2007) whereas six SNPs including two SNPs in intron 2 (g.1306 G>A and g.1454G>A), one in intron 4 (g.16904 G>T), two in exon 5 (g.16974 C>T and g.17000 A>G) and one SNP in exon 8 (g.22696 T>C) were discovered by Liu *et al*. (2011) in Chinese Holstein bulls. However, none of the corresponding loci were found to be polymorphic in explored Murrah population.

Among the five detected polymorphism present in the 5' regions to exon 1 all of them were found to alter the binding sites for transcription factors at the AliBaba2.1online tool. With respect to the five SNPs -239, -95, -55, -26 and -4; the reference sequence had three sites with potential Sp1 recognition, one each for Da and Erg-1. Whereas the alternate sequence had only one site for Sp1 recognition and one each for MyoD, NF-1, E1, NF-kappa, Da, RAR-alph, T3R-alpha and Erg-1 (Table: 3). Deciphering the DNA sequences bound by Transcription Factors (TFs) has been one of prime targets of genomics research due to its importance in explaining transcriptional regulation of gene expression. Like SP1 transcription factor belongs to the Sp/KLF family of transcription factors that enhances gene transcription (Heckmann *et al*., 2010). Hence, the difference in binding of the Transcription Factors caused by aforementioned SNPs will affect the rate of transcription of the SPAG11 gene, however the specific effects of each of the SNPs on transcription of the gene needs further investigation.

Table 1. Sequence of tested primers.

Name	Primers sequence (Forward/Reverse)	Targeted region	Expected amplicon length	Amplicon length	Ta (°C)
P1	GAAATGACCATCGCAGAGAGA	1-564 (Promoter + Exon 1 + Partial Intron 1)	564	563	60.5
	CGTGTAGAAATAAGTTGCCCATATAC				
P2	CTGCTGTGTGTTTACCCTGAAATA	1184-1522 (Partial Intron 1+Exon2 + Partial Intron 2)	339	340	62.0
	GTCATACTCCTCCCTAACCTTCC				
P3	GGCAGTTTCTTGGGGTCAAT	1978-2372 (Partial Intron 2+Exon3 + Partial Intron 3)	395	373	62.0
	GCACATCGCAGGTGCTTATT				

Table 2. Details of SPAG11 gene in various species.

Species	Accession No.	Chromosome	Exons	Protein
Bos taurus	NM_001098154.1	27	6	69
Homo sapiens	NM_058200.2	8p23	4	108
Sus scrofa	NM_001129977.1	15	3	85
Rattus norvegicus	NM_145087.1	16q12.5	2	68
Macaca mulatta	NM_001110261.1	8	6	131

Table 3. Potential binding of transcription factors with corresponding SNPs at 5'.

Position of SNP		Binding sites for transcription factors	
Gene	Amplicon	Reference	Alternate
-239	213	Sp1	---
-95	357	Sp1	MyoD and NF-1
-55	397	Sp1	Sp1 and E1
-26	426	Da	NF-kappa and Da
-4	448	Erg-1	RAR-alpha, T3R-alpha and Erg-1

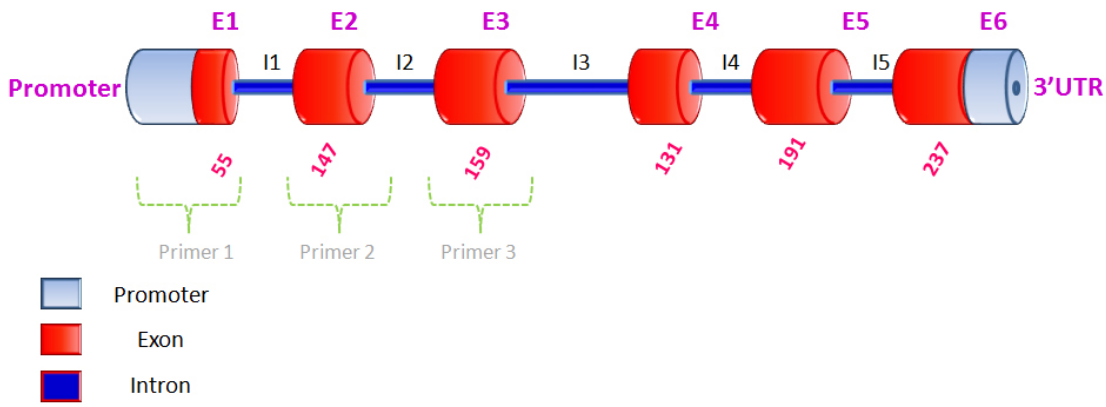

Figure 1. Structure of SPAG11 gene.

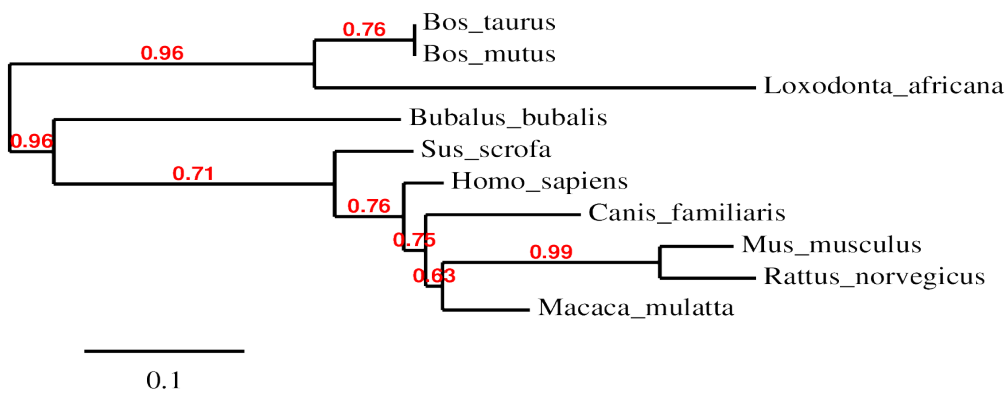

Figure 2. Phylogenetic tree of SPAG11 gene.

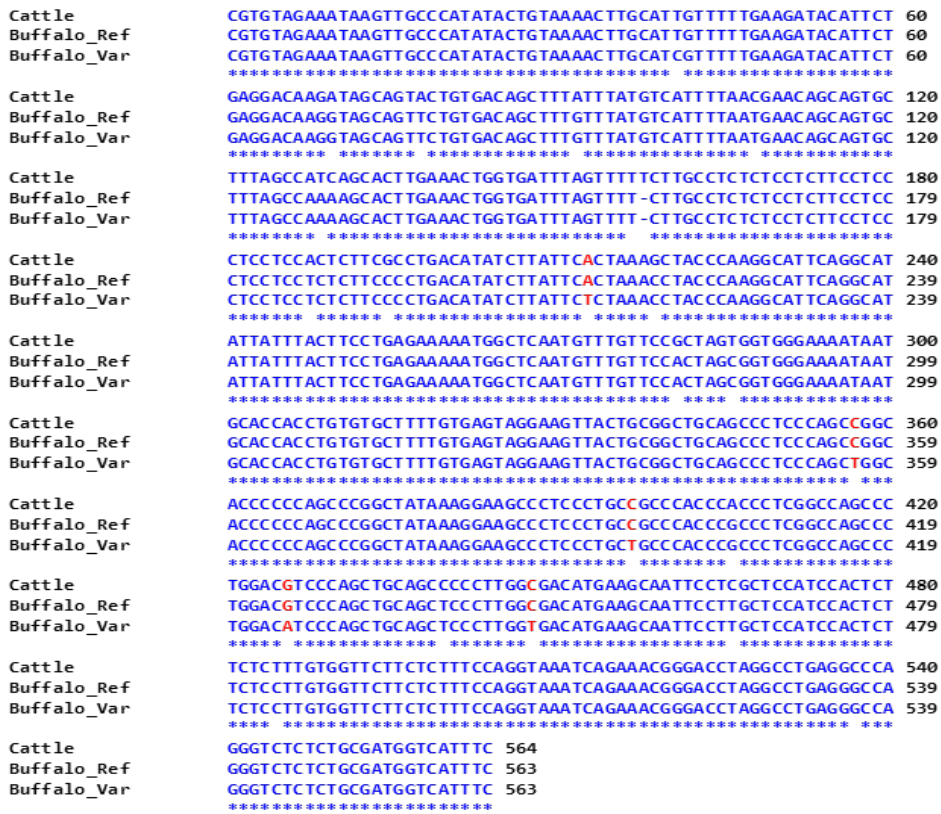

Figure 3. Alignment of first amplicon of SPAG11 sequence in Murrah bulls.

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Cattle          CTGCTGTGTGTTTACCCTGAAATAGGACTTTCCAGAGTCACACATGCTAACCGCCAGGAC 60
Buffalo_Ref     CTGCTGTGTGTTTACCCTGAAATAGGACTTTCCAGAGTCACACATGCTAACCACCAGGAC 60
Buffalo_Var     CTGCTGTGTGTTTACCCTGAAATAGGACTTTCCAGAGTCACACATGCTAACCACCAGGAC 60
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Cattle          CCCAAAGGTCCCAGGAAACAAGAAGAATCTCTGGGACGGGGAACAAACAGGTCTCACCCA 120
Buffalo_Ref     CCCGAAGGTCCCAGGAAACAAGAAGAATCCCCGGGACGGGGAACAAACAGGTCTCACCCA 120
Buffalo_Var     CCCGAAGGTCCCAGGAAACAAGAAGAATCCCCGGGATGGGGAACAAACAGGTCTCACCCA 120
                *** ************************ * **** ************************

Cattle          CTTCACCACCAAGTAAAGCGCTACCTTGTGCCTCGCAAGCCCCCCTTCCCGGGTAAGTGA 180
Buffalo_Ref     CTTCACCACCAAGTAAAGCGCTACCTCGTGCCTCGCGAGCCCCCCTTCCCGGGTAAGTGA 180
Buffalo_Var     CTTCACCACCAAGTAAAGCGCTACCTCGTGCCTCGCGAGCCCCCCTTCCCGGGTAAGTGA 180
                ************************** ********* ************************

Cattle          CAGAGCCCGGGATGGGTCGGTCCATGG-CAGGACCTTCAGAGGGAAAATGGATGGGAGGT 239
Buffalo_Ref     CAGAGCCCGGGATGGGTCGGTCCGTGGGCAGGACCTTCAGAGGGAGAATGGATGGGAGGT 240
Buffalo_Var     CAGAGCCCGGGATGGGTCGGTCCGTGGGCAGGACCTTCAGAGGGAGAATGGATGGGAGGT 240
                ************************ *** ***************** **************

Cattle          AACTGTGGGGGCGGGTGGGCTGAGCGGTGGAGTGTCCTGAGACAATACATGCATGACTTA 299
Buffalo_Ref     GACTGTGGGGGCGGGTGGGCTGAGCAGTGGAGTGTCCTGAGACAATACATGCATGACTCA 300
Buffalo_Var     GACTGTGGGGGCGGGTGGGCTGAGCAGTGGAGTGTCCTGAGACAATACATGCATGACTCA 300
                ************************ ******************************** *

Cattle          GTCTGCTCTGCCTGTGGGGAAGGTTAGGGAGGAGTATGAC 339
Buffalo_Ref     GTCTGCTCTGCCTGTGGGGAAGGTTAGGGAGGAGTATGAC 340
Buffalo_Var     GTCTGCTCTGCCTGTGGGGAAGGTTAGGGAGGAGTATGAC 340
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Figure 4. Alignment of second amplicon of SPAG11 sequence in Murrah bulls.

Figure 5. Alignment of third amplicon of SPAG11 sequence in Murrah Bulls.

CONCLUSION

Present investigation indicate that each of the three primer pairs of SPAG11 gene was successfully amplified in Murrah buffalo bulls. All the seven SNPs identified in SPAG11 gene showed potential to cause changes by predicted differential protein functions or transcription regulation in buffalo population. Thus, the characterization of SPAG11 gene may serve as a base for future research to explore the association of the SNPs in SPAG11 gene with buffalo reproduction and fitness traits, which could be an addition to the existing knowledge for better estimation of genetic breeding values in the genomic selection.

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