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Identification of single nucleotide variations in the genes related to reproduction in riverine buffalo

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Microsatellite and single nucleotide polymorphism (SNP) two types of DNA based genetic markers. Genetic markers are defined as identifiable DNA segments that differ in nucleotide sequence from one individual to the other. Genetic markers or DNA based markers are powerful tools for molecular dissection of traits of economic importance and for their potential application in breeding for more productive and efficient livestock. SNP markers are just a single base changes in a DNA sequence which are extremely stable (Sachidandam *et al.* 2001), abundant (Heaton *et al.* 2001) and amenable to high-throughput automated analysis (Lindblad-Toh *et al.* 2000). Due to these advantages SNPs form a preferred DNA marker for genotyping studies.

The livestock industry depends on proper reproduction resulting into optimum production. Reproductive hormones play a very important role in fertilization and maintenance of pregnancy. These hormones trigger broad array of tissue and organ specific physiological responses by binding to their respective receptors. Thus the genes for receptors of the reproductive hormones can be candidate for the production traits. Therefore the identification of DNA based markers in these genes will be economically beneficial. Moreover, in buffaloes, systematic studies to develop DNA based markers are scanty. Hence present study was planned to identify single nucleotide variations in the genes related to the reproduction using PCR-SSCP followed by sequencing.

Genomic DNA was isolated from blood of 25 unrelated animals representing five different buffalo breeds, viz. Murrah, Mehsana, Surti, Jaffrabadi and Toda (5 animals in each breed). Polymerase hain reaction was carried out using gene specific primers – 5'AATCCATCCTACCCCTGGAG3' and 5'GCAATGGA TGGCTAAAGGAG3' for Estrogen receptor (ER), 5'TCTTGGA GGCCGAAAGTTTA3' and 5'TCGGAACTTACATATTGATGACCA3' for progesterone Receptor (PR), 5'GATCCTGATCACCAGCCAGT3' and 5'AGATGGGAAAAAGGGCAACT3' for FSH receptor (FR), 5'TTGGGTAAA ATTCAAATGCAGA3' and 5'ATGATGGTGTGGAGGGGTAA3' for leptin receptor (LR). PCR was conducted in a 20.0 l reaction mix containing 100 ng of genomic DNA for 30 cycles. The annealing temperature was 59°C for ER and 55°C for PR, FR and LR. The sizes of the PCR products obtained were 212 bp, 316 bp, 362bp and 440 bp for ER, PR, FR and LR, respectively.

The PCR products thus obtained were denatured in the SSCP dye (containing formamide and EDTA) at 94°C for 10 min, immediately cooled in an ice bath and used for SSCP analysis. The SSCP analysis was carried out using 15% polyacrylamide gel (49: 1) containing 5% glycerol. Electrophoresis was carried at 25°C, 250 volts for 3 h in 0.5% TBE buffer. The gel was subsequently silver stained (Baseem *et al.* 1991) and difference in SSCP banding pattern was identified. The samples which had produced varied PCR-SSCP pattern were re-amplified, gel extracted and subjected for custom sequencing. The sequences (Accession no. EU662273 to EU662291) obtained were subjected for 'megaBLAST'(Altschul *et al.* 1990) analysis to know the specificity of the amplification. The sequences were further subjected for multiple sequence alignment (ClustalW) (Higgins *et al.* 1994) to identify the single nucleotide variation. The effect of single nucleotide variation on the amino acid sequence and restriction sites was analyzed using 'BLASTx' and 'NEBcutter'.

PCR-SSCP is one of the methods of identification of single nucleotide variation which is based on the conformational change of DNA fragments due to single base change. The change in conformation can be detected by non-denaturing polyacrylamide gel electrophoresis. This is one of the simplest and most widely used methods to identify nucleotide variations (Orita *et al.* 1989). PCR-SSCP can detect 70 to 95% of potential base variations in short 200 or less base

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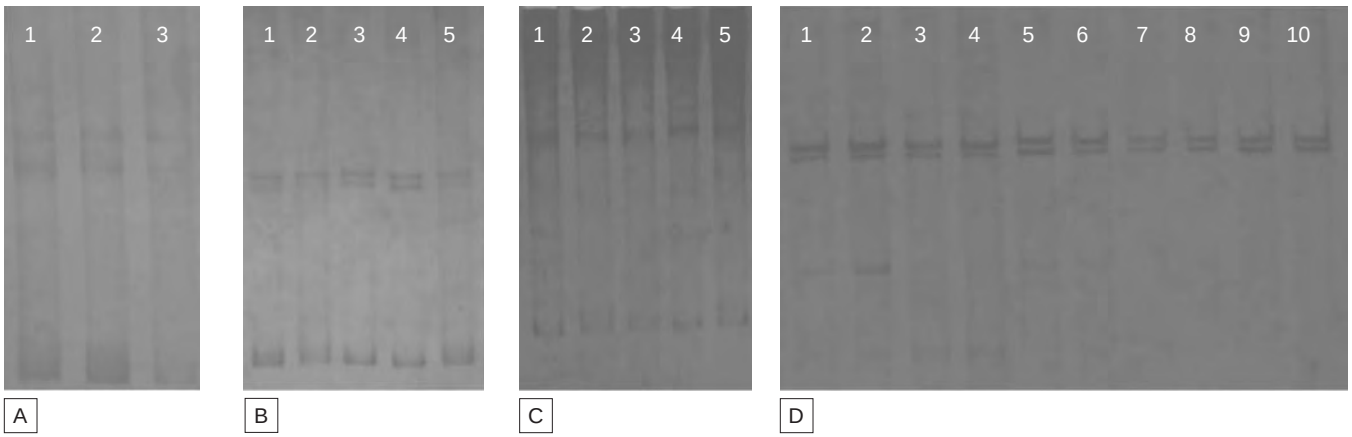
Table 1. Variations identified and their effect on amino acid sequence and restriction site

Gene	Nucleotide variation identified	Breeds of buffalo	Amino acid change	Restriction site
Estrogen receptor (ER) – Coding region	A → G	Toda	Nil	Nil
Progesterone receptor (PR) - Coding region	A → G	Surti	T → A	Creation of site for AciI
	T → C	Murrah and Mehsana	V → A	Creation of site for AciI
	C → T	Jaffrabadi and Mehsana	P → L	Nil
	A → G	Toda	Nil	Nil
FSH receptor (FR) – Coding region	T → C	Surti	I → T	Nil
	A → G	Surti	I → G	Nil
	T → A	Mehsana	L → Q	Nil
	T → C	Toda	C → R	Nil
	T → C	Surti, Jaffrabadi and Mehsana	Nil	Nil
Leptin receptor (LR) – Promoter region	T → A	Mehsana and Murrah	—	Creation of site for SfaNI
	A → G	Jaffrabadi	—	Creation of site for HinfI
	T → G	Toda	—	Nil
	T → A	Murrah	—	Nil

pair products under optimum conditions (Gross *et al.* 1999). However the method can be improved on its efficiency to detect nucleotide variations in the DNA fragments size from 400 to 450 bp by using low cross linker percentage and 5% glycerol. This helps in the successful determination of nucleotide variation in large PCR products (Hamzeiy *et al.* 2002). Therefore, in the present study PCR products of size 212 bp, 316 bp, 362bp and 440 bp of ER, PR, FR and LR, respectively were used for PCR-SSCP analysis and the different SSCP pattern obtained is shown in figure A, B, C, and D, respectively. The PCR products were obtained using the primers designed based on the cattle sequence but they were found to amplify the corresponding gene of buffalo which was confirmed by sequence analysis. The respective PCR products were sequenced and the nucleotide sequences were subjected for 'BLAST" and "clustalW". Alignment of ER gene sequence of five different breeds of buffalo resulted in the identification of one nucleotide variation (A→G) in the Toda breed of buffalo which had correlated with the altered SSCP pattern (Fig. A). However, this nucleotide variation identified neither resulted in amino acid change nor restriction site. PCR-SSCP pattern of PR (Fig. B) was different for each breed of buffaloes. Sequence analysis had also revealed 4 different variations; 2 different nucleotide variations were identified in Mehsana breed of buffaloes. The variations identified are resulted in amino acid change as well as change in restriction site as detailed in the table 2. Analysis of FR and LR sequence had also revealed nucleotide variations which were correlated with PCR-SSCP pattern (Figs C and D). Some of the variations are resulted in amino acid change as well as change in restriction site.

Overall, of the total 14 variations identified, 10 of them



Figs A–D: **A.** PCR-SSCP pattern of estrogen receptor (Template DNA used Lane 1.Jaffrabadi, Lane 2: Murrah and Lane 3: Toda), **B.** PCR-SSCP pattern of progesterone receptor (Template DNA used Lane 1: Jaffrabadi, Lane 2: Mehsana, Lane 3: Murrah, Lane 4: Surti and Lane 5: Toda), **C.** PCR-SSCP pattern of FSH receptor (Template DNA used Lane 1: Jaffrabadi, Lane 2: Mehsana, Lane 3: Murrah, Lane 4: Surti and Lane 5: Toda), **D.** PCR-SSCP pattern of Leptin receptor (Template DNA used Lane 1, 2: Toda, Lane 3, 4: Surti, Lane 5, 6: Murrah, Lane 7, 8: Mehsana, Lane 9, 10: Jaffrabadi).

are transitional in nature. The study on human SNPs from EST traces data bases given transition to transversion ratio of 1.7 (Picoult *et al*. 1999). The variations identified in chicken EST sequence were found 2.3 (Smith *et al.* 2001) and 4 (Kim *et al*. 2002) are higher than mammals. Out of 14 variations identified, 7 variations affected the amino acid sequence and 4 variations resulted in change in restriction sites. The variations which have resulted in amino acid change can be functionally important as these can affect the structure of the protein. The variations which had changed the RE site can be used to develop PCR-RFLP marker.

All the nucleotide variations identified in this study are associated with difference in PCR-SSCP pattern, which demonstrate the potential usefulness of PCR-SSCP analysis for the detection of nucleotide variation. The nucleotide variations identified are found in coding region and promoter region of the genes studied, therefore these variations can be utilized as DNA markers of functional importance. The pregnancy rate, outcome of *in vivo* fertilization, fertility, liter size were shown to be associated with single nucleotide variations identified in the above genes. But the efficacy of single nucleotide variations identified in this study depends on the information content of these variations shown under association studies in the population at large.

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