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ESRα EXON POLYMORPHISM COUPLED WITH PROTEIN MODELLING IN RELATION TO REPRODUCTIVE TRAITS IN MURRAH BUFFALOES

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ABSTRACT

Buffalo plays a pivotal role in livestock and agriculture economy of many countries across the world. Estrogen receptor (ESR) has been known to play crucial role in the development of feminine secondary sexual characteristics as well as in the female reproductive cycle, infertility, and maintenance of pregnancy. The present study was aimed to test the hypothesis that polymorphism at ESR loci has some association with reproductive traits of buffaloes that can be used as a marker for reproductive efficiency in marker assisted selection. Sixty five Murrah buffaloes were selected and divided in to six groups on the basis of their physiological data. We found high genetic variation ranges from 0% to 5.2% in the nucleotide sequence of ESR gene among different groups of buffalos. In contrast 0% to 16.24% variation was found in the translated protein sequence of ESR gene from different groups of buffalos. Only two SNPs were observed at 64 (T→G) and at 68 (T→G) in field abnormal group animals. Entropy plots also confirmed the genetic variation via peaks and the area covered by those peaks. Homology model of protein from different groups of buffaloes were constructed and compared to find out the impact of SNPs in the overall structure of translated protein of ESR. The pair wise comparison of protein structure varies from 0.642 to 1.818 (expressed as root square mean deviation, RSMD). PCR-RFLP coupled with protein modelling showed variation at amino acid level and very little variation was also observed in 3-D protein structures.

KEYWORDS: **Buffalo, PCR-RFLP, ESR, SNP detection, Protein modelling.**

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INTRODUCTION

Buffalo contribute more than fifty percent milk of the total milk production in India but contribute less than half of the cattle population. The current world buffalo population is 194 million and is steadily increasing at the rate of 2% per annum during the last two decades.[1]About 97.13% world buffalo population is present in Asia out of which 76.92% is present in South Asia alone. India and Pakistan have 57% and 43% of the population, respectively. Amongst all the buffalo breeds, Murrah is a distinguished milch breed along with Jaffarabadi and Nili-Ravi close- by.[2]According to phenotypes, karyotypes and recent mitochondrial DNA work the water buffalo has been divided into two subspecies; the river buffalo (2N=50) and the swamp buffalo (2N=48). The riverine buffalo is primarily used for milk production, meat production and for draught purposes. Murrah in India is well known type of river buffalo. Buffalo contributes more than fifty percent milk of the total milk produced in India but constitute less than half of the cattle population.[2-3]Despite having more than fifty percent of milk production, efficiency of milk production remains low due to absence of appropriate selection of genetically superior animals and conventional breeding methods. Steroid hormones play important roles in the reproductive biology of vertebrates, including mammals. The majority of the actions of steroid hormones are mediated by specific receptors that are localized in or near the nucleus of target cells. Steroid hormone receptors form a super family of nuclear transcription factors that include estrogen, progesterone, androgen, glucocorticoid, mineralocorticoid, the vitamin D, and the retinoic acid receptors.[4]Estrogen hormone actions are mediated through intracellular receptors namely estrogen receptor (ESR) which are members of the nuclear receptor (NR) super family.[5]This NR super family consists of 18 receptor members, which are divided into class I and class II NR. Class I NR includes ESR and PGR, which are considered as candidate genes involved in biology of reproductive traits. Estrogen play a central role in normal female reproduction physiology, as well as in the pathology of female reproductive organs. Estrogens exert their actions on target cells through protein receptor (ESR) having two isoforms- ER α and ER β, each of them coded by a separate gene, localised on different chromosomes. Due to the functions that estrogens play in the regulation of reproduction, development of the mammary gland, growth and differentiation of cells, estrogen receptors and their genes are considered candidates for markers of production and functional traits in farm animals, in particular female reproduction.[6-7]The ESR gene has been studied for polymorphism in pig,[8-10] mouse[11]and human.[12- 15]Improvement of reproductive efficiency in female buffaloes requires a better understanding of their reproductive physiology under steroid hormonal control, especially in the oviduct, during estrous cycle. In order to enhance genetic merit of animals with increased milk production, enhanced reproductive efficiency, disease resistance etc, it is important to identify and locate responsible gene quantitative trait loci (QTL) in the genome. Genetic polymorphisms are playing an increasingly important role as genetic markers in many fields of animal breeding. A number of techniques were adopted to detect polymorphism at structural loci, of which polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) is the most preferred one because of its simplicity, quickness, economical, very high repeatability and non-use of hazardous radioactive material. In view of above, the present investigation was planned to detect presence of any genetic variation in ESR gene and its association with reproductive trait in Murrah buffalo.

MATERIALS AND METHODS

Sample collection

A total number of sixty five Murrah buffaloes: thirty five animals (14 heifers and 21 multiparous animals) were selected from ICAR-Central Institute for Research on Buffaloes, Hisar and thirty were from different regions of Haryana state. Fresh blood sample were collected from all the sixty five buffaloes after taking approval from the Institute Internal Ethical Committee. These animals (heifer and multiparous) were divided in to six groups on the basis of their physiological data (lactation period, calving interval, open days and dry period). Correlations between different parameters were determined by analysis of variance (ANOVA) and mean ±SE were ranked using Duncan's multiple range tests. All statistical analyses were performed with SPSS version 16.00(Table 1).

Table 1
Parameters of animals details (Mean±SE)

Group	Lactation No.	Calving Interval ** (Days)	Lactation Length * (Days)	Total Lactation Yield (litres)	305 Days lactation yield (litres)	Peak Milk Yield (litres)	Dry Period (Days)	Open Days (Days)
Multiparous normal (n=8)	3.75± 0.65^{a}	409.26±12.47^{a}	303.15±8.26^{a}	1764.50± 166.27^{a}	1694.00±157.21	9.15±0.63^{a}	121.75±16.58^{a}	149.57±27.10^{a}
Multiparous abnormal(n=13)	3.15± 0.37^{ab}	603.17±26.80^{a}	385.18±23.24^{b}	2555.30±164.96^{b}	2166.40±111.17	9.90±0.53^{ab}	230.26±29.53^{a}	314.02±24.53^{b}
Field - Normal(n=10)	3.90± 0.53^{a}	392.51±15.60^{a}	270.33±13.64^{a}	2271.20±230.65^{ab}	2206.40±215.40	11.91±0.89^{c}	158.31±27.62^{a}	268.16±66.80^{ab}
Field - Abnormal(n=20	2.25± 0.36^{b}	915.08±121.0^{b}	277.97±25.25^{a}	2158.00±221.66^{ab}	2018.40±204.11	11.61±0.45^{bc}	391.74±78.22^{b}	255.15±57.68^{ab}

***** Significant (p<0.01) difference between groups ,* Significant (p<0.05) difference between groups ,a,b,c superscripts differed significant (p<0.05) The age of the heifers was 44.06±2.71 months and multiparous buffaloes were 47.67±1.13 months.***

DNA extraction and primers designing

DNA was extracted from fresh blood samples by the method of Kumar et al [2] with slight modifications.The quality and quantity of extracted DNA was checked by Nano drop spectrophotometer and agarose gel electrophoresis. The genomic region of ESR gene (exon 13 and downstream region) was amplified with primers designed from the *Bos Taurus* gene sequence with Accession no NP_ 001001443 (Fig 1). Due to the greater percentage similarity of conserved DNA sequences (75%) in bovidae family which includes cattle, bovine and buffalo, the bovine was considered as a reference animal.[16]The primers were designed using Primer 3 software (www.genome.wi.mit.edu) for exon 13 and down streaming region (Table 2). This region for polymorphism detection was selected because of its presence at ligand binding site.

Table 2

Primer sequence and thermal profile for the amplification of ESR gene.

Gene	Sequences 5′ -------- 3′	PCR conditions	PCR product size
ESR P_f	5'-AGATCTATACAGCATGAAGTG-3'	94°C 45 sec 60°C 50sec 72°C 50 sec	870bp
P_r	5'-GTGTACTAAGCTGACAAAGTCTTG-3'		

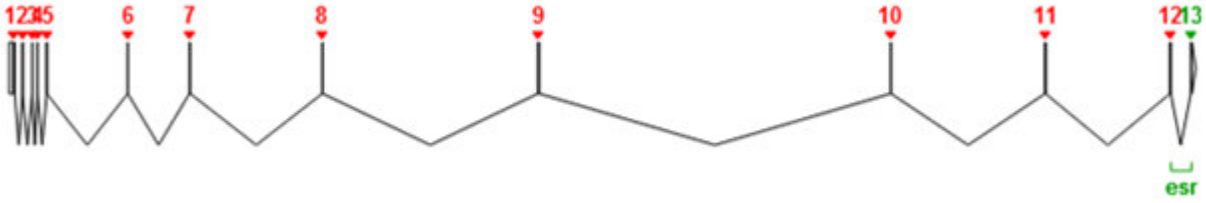

Figure 1

ESR gene map of Bovine.

PCR amplification

The polymerase chain reaction (PCR) was carried out in a final volume of 15.0 µl consisted of 80 ng genomic DNA, 1.7 pm each primer, 2.0 mM $MgCl_2$, 0.15 mM dNTPs, 1X PCR buffer and 1 U Taq polymerase. The PCR thermal profile consisted of initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 50 seconds and extension at 72°C for 50 seconds and final extension at 72°C for 7 minutes. The PCR amplification was confirmed by restriction digestion of amplicons with *Eco*RI (*Escherichia coli* RY 13) enzyme. The final volume of digestion reaction was 15 µl, containing 5 µl reaction solution, 1 µl enzyme buffer, 1 µl enzyme and 8 µl nuclease free water and incubated at 37°C for approximately 3 hours. The reaction was stopped by addition of 4 µl of 6x gel loading dye and freezing the content at -20°C. After digestion, the samples were quantified to visualize the amplified fragments by gel electrophoresis with the 100bp ladder DNA marker.

Restriction fragment length polymorphism (RFLP)

The amplicons were screened for the presence of genetic polymorphism in ESR using PCR-RFLP. The estrogen receptor gene PCR product was digested with *Hpa* II (*Haemophilus parainfluenzae*) (5′C↓CGG3′) for genotyping in a final reaction volume of 25 µl, containing 15 µl reaction solution, 2.5 µl enzyme buffer, 0.8 µl enzyme and 6.7 µl nuclease free water and incubated at 37°C for overnight. The restriction pattern of ESR gene was also studied by using *Stu* I restriction endonuclease enzyme. The estrogen receptor gene PCR product was digested with *Stu* I (*Streptomyces tubercidicus*) (5′AGG↓CCT3′) for genotyping in a final reaction volume of 25 µl, containing 15 µl reaction solution, 2.5 µl enzyme buffer, 0.8 µl enzyme and 6.7 µl nuclease free water and incubated at 37°Cfor overnight. The cleaved fragment were separated by electrophoresis on 2% agarose gel in 1X TAE buffer containing 0.05µg/mL ethidium bromide at 90V for approximately 1 hour. The bands were visualized under ultraviolet light in Gel-Doc system (Syngene, Germany).

Data analysis

The PCR product was purified by QIAquick kit (Qiagen). The purified amplified PCR products of ESR gene were sequenced from Xcelris Labs Ltd., Ahmadabad. Both the DNA strands (i.e. forward and reverse) were sequenced and were assembled using Codon Code Aligner 5.0.1. The assembled sequences were then compared among themselves and also with the bovine reference followed by detection of mutations (SNPs) via CodonCode Aligner 5.0.1. Further, these sequences were aligned and compared using BioEdit software and multiple alignment programs, ClustalW (http://www.ebi.ac.uk/Tools/msa /clastalw2/) with DNA weight matrix and multiple parameters like gap opening 10.0, gap extension 0.20, and transition weight 0.50. Pairwise sequence identity and divergence of ESR gene among different groups of buffalo was calculated using BioEdit software. Entropy plot of ESR gene was also calculated by BioEdit software and compared with the reference sequence. It provides information regarding the amount of variability through a column in an alignment and is measured in bits. Entropy at a column position depends only upon the frequencies of characters that appear in that column. If we have maximum information for any position or especially conserved region, entropy is zero. When the amount of variability increase the entropy at that position also increases, this gives a measure of uncertainty at each position relative to other position.

Entropy can be calculated as

$$H(l) = -\sum f(b,l)\log_{(\text{base } 2)} f(b,l) \quad \text{(measured in bits)}$$

A phylogenetic tree among the different groups of buffalo was constructed based on pairwise similarity coefficients by neighbour-joining/ UPGMA method. Furthermore all DNA sequences obtained were translated into protein sequence using EMBL transseq (http://www.ebi.ac.uk/Tools/st/). These protein sequences were further aligned using multiple alignment programs, Clustal W (http://www.ebi.ac.uk/Tools/msa/clastalw2/) with protein weight matrix and default parameters for gap opening and extension. Pairwise sequence identity and divergence among the protein sequences was calculated using BioEdit software and a phylogenetic tree was constructed. Further the molecular structure of the translated protein sequences were built based on homology technique. BLAST search of translated protein sequence of ESR with PDB data base gives a protein sequence with 60% similarity (PDB ID: P03372, 3ERT). This homologous protein structure was used as template for homology model building of translated protein sequences using Prime accessible through the Maestro interface . The modeled structures were evaluated using PROCHECK v3.4.4[17]Ramachandran plots produced by PROCHECK program assured very good confidence for predicted protein.[18]

RESULTS

Genetic polymorphism using PCR-RFLP

A total of sixty five animals were used in the present study and were further divided into six groups. The mean conception age in normal buffalo heifers was significantly ($p<0.01$) lower (35.90±1.46) than the abnormal heifers (54.25±3.16). Calving interval were significantly ($p<0.01$) differ between groups and within groups. Mean calving interval, lactation length, total lactation yield, peak milk yield, dry period and open days were calculated (Table 1). The lactation length also differed significantly ($p<0.05$) between defferent groups. The animals were genotypes for the presence of any genetic varaiation and its association with reproductive traits. The PCR amplification of ESR gene exon 13 and downstream region produced a clear, distinct and highly reproducible band of 870 bp. The amplified products were confirmed by two-way nucleotide sequencing using the same set of primers and by comparing with the reference gene sequence of bovine. PCR-RFLP of the amplicon was carried out using *Stu* I (5′AGG$^{\downarrow}$CCT3′) and *Hpa*II (5′C$^{\downarrow}$CGG3′) restriction enzymes. Restriction digestion of amplicon by *Stu* Iproduced two fragments of sizes 305 bp and 565 bp(Fig 2) and with *Hpa*II produced two fragments of 759 bp and 111bp (Fig 3).

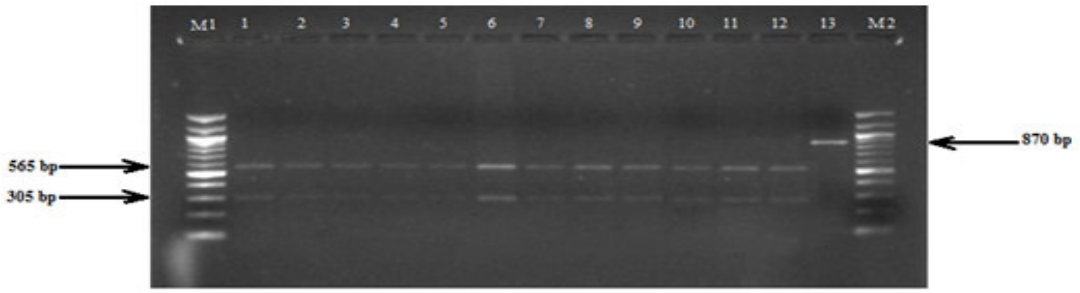

Figure 2

PCR-RFLP pattern of ESR gene. A representative gel pictures showing the Stu I restriction digested fragment of ESR from different groups of murrah buffalo, 1-2 : Heifer abnormal, 3-4: heifer normal, 5-6: field normal, 7-8: multiporous normal and abnormal groups, 9-12 shows field abnormal,13 shows uncut amplicon. M1-M2:1.5 Kb ladder.

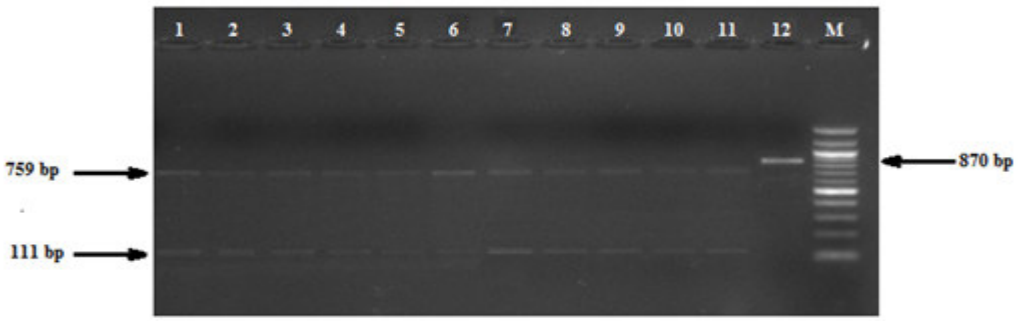

Figure 3

PCR-RFLP pattern of ESR gene. A representative gel pictures showing the Hpa II restriction digested fragment of ESR from different groups of murrah buffalo, 1-2: shows Heifer abnormal, 3-4: field normal, 5-6: field abnormal, 7-8 multiporous normal, 9-10 multiporous abnormal groups and 11 heifer normal,12 shows uncut amplicon. M: 100bp ladder.

However, both of these restriction digestion patterns exhibited monomorphic pattern in all animals under study. But a very few variations were observed when the contigs of ESR sequence of different groups of Murrah buffalo was compared with that of bovine (Acc: NP_001001443) sequence to annotate different exonic regions putatively to identify SNPs in respective region. All the nucleotide sequences of complete amplicon (870 bp) were compared by multiple sequence alignment programs using BioEdit 5.0.1 version and level of variations in nucleotide sequences were detected and represented (Fig 4). Futhermore the exonic nucleotide sequence of ESR gene was also compared (Fig 5) and then the exonic DNA was conceptually translated and compared with that of the bovine to detect amino acid changes in buffalo ESR region under study (Fig 6).

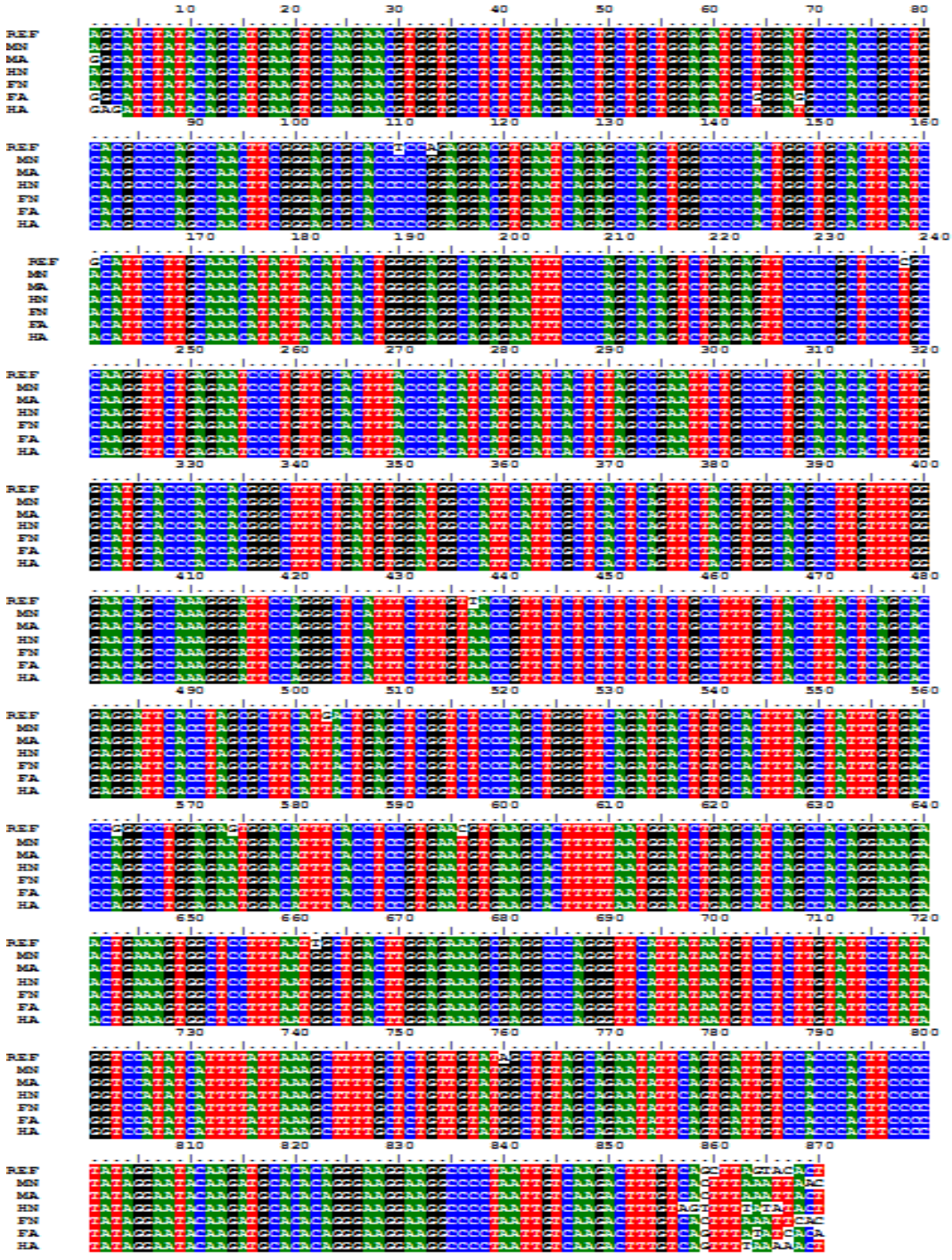

Figure 4

Multiple sequence alignment of ESR (870 bp) complete amplicon obtained from different groups of buffaloes with reference. REF: Bovine, MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal

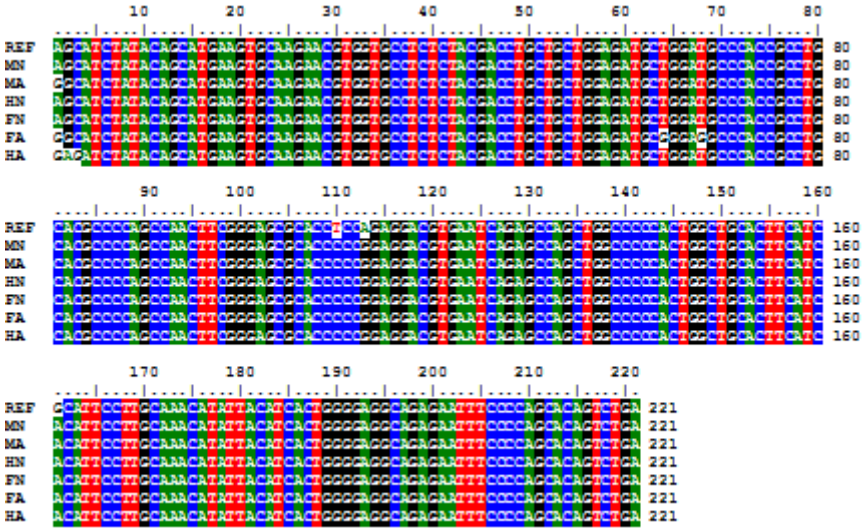

Figure 5

Multiple sequence alignment of ESR exon-13 obtained from different groups of buffaloes with reference. REF: Bovine, MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal

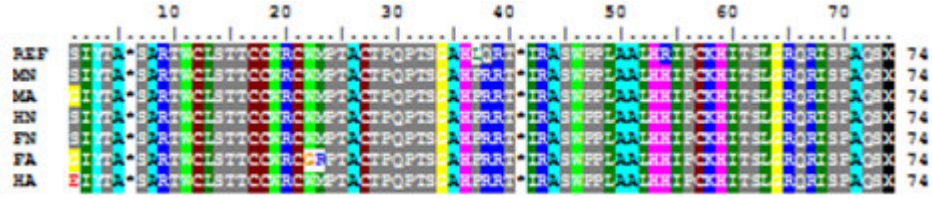

Figure 6

Multiple sequence alignment of ESR protein sequence from different groups of buffaloes with reference. REF: Bovine, MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal

The pairwise genetic variation on the basis of ESR gene sequence was calculated among different groups and was found to vary from 0.0% to 5.2%. Multiparous normal (MN), field normal (FN) and heifer normal (HN) groups showed minimum percentage of variation (0.0%) and hence were more similar to each other. Heifer abnormal (HA) and field abnormal (FA) groups were less dissimilar to each other but were more dissimilar to multiparous abnormal (MA) group. The maximum value of distance matrix was showed by field abnormal (FA) group (5.2%), heifer abnormal (HA) group (5.1%) and multiparous abnormal (MA) group (3.45%) from reference. The dendrogram generated showed genetic relationship between different groups on the basis of DNA sequence similarity. This dendrogram showed that the heifer normal (HN) group was very much close to reference as compared to other groups. Multiparous normal (MN) group was found closer to field normal (FN) group. Heifer abnormal (HA), field abnormal (FA) and multiparous abnormal (MA) groups formed a separate cluster and were distantly placed from reference (Fig 7). Based upon the protein sequence comparison, a small variation was found among different groups under study. Overall variation ranged from 0.0% to 16.24%. Multiparous normal (MN), heifer normal (HN) and field normal (FN) groups showed minimum variations among themselves (0.0%). However, the maximum value of distance matrix was found to be shown by field abnormal (FA) group (16.24%), heifer abnormal (HA) and multiparous abnormal (MA) (10.6%) from reference. When the dendrogram resulted from pairwise protein sequence comparison was studied, it was found to favour the groupings of DNA sequence based dendrogram in case of heifer abnormal (HA), field abnormal (FA) and multiparous abnormal (MA) groups, whereas, multiparous normal (MN) and heifer normal (HN) groups were found very close to each other and to reference along with field normal (FN) group (Fig 8).

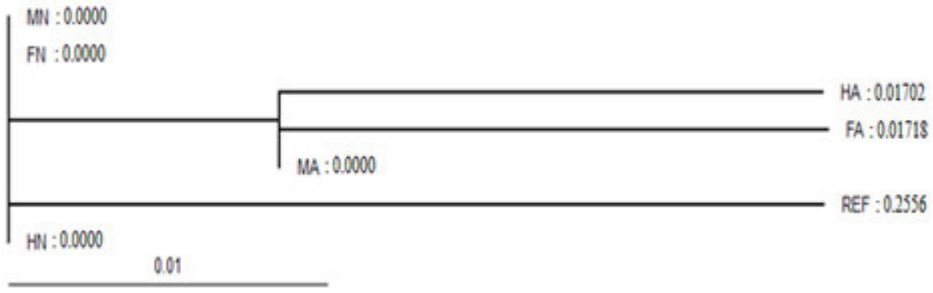

Figure 7
Dendrogram showing similarity between ESR gene sequences obtained from different groups of buffaloes. REF: Bovine, MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal

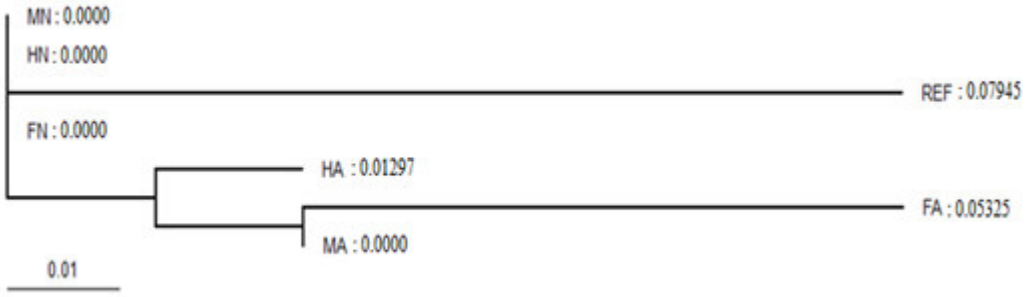

Figure 8
Dendrogram showing similarity between ESR protein sequences obtained from different groups of buffaloes. REF: Bovine, MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal

SNP detection

Clustal W multiple alignments with bovine sequence revealed changes only in 2 bases; one was at position 64 (T→G) and another was at position 68 (T→G) in E (field abnormal group). Any other SNP was not observed in any other sequences. However, at the terminal ends there were some variations present; when these sequences were translated and compared with bovine, the field abnormal group (FA) showed that the changes at 64 position (T→G) lead to change in amino acid at 22th position i.e. Tryptophan (W) → Glycine (G) and at position 68 (T-G) lead to change in amino acid at 23th position i.e. Methionine (M) →Arginine (R). So two non synonymous SNPs were found in field abnormal group only, but any restriction site on these SNPs was not found (Fig 4 and 5).

Entropy plot

The peaks in the entropy plot were present only at that position where any of the sequence (study sample) showed some variation from that of the reference (both in case of DNA and protein) and area of the plot also got increased with the increase in level of variation. The entropy plot showed zero value due to the conserved sequence. The peaks however, showed variations in the nucleotide sequence with reference and also among the groups at positions 1-3, 64, 68, 110, 113 and 161 whereas at position 1, 22-23, 37-38 and 54 in case of amino acid sequence (Fig 9 and 10).

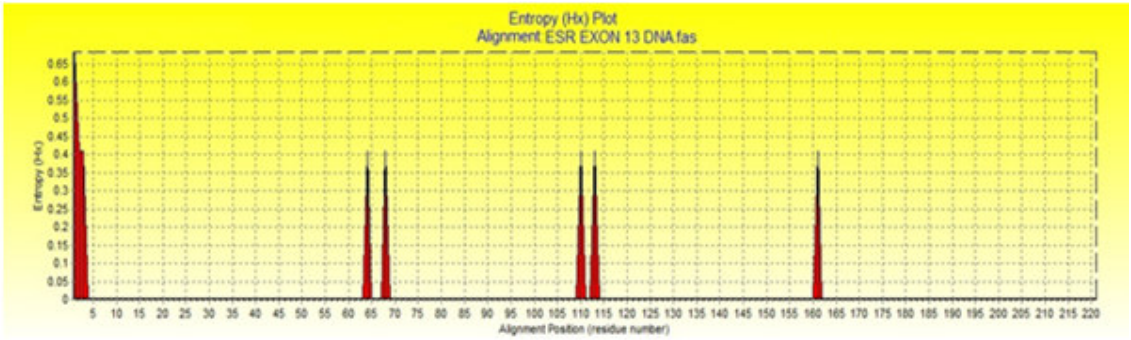

Figure 9
Entropy plot showing variability in ESR nucleotide sequence alignments with reference.

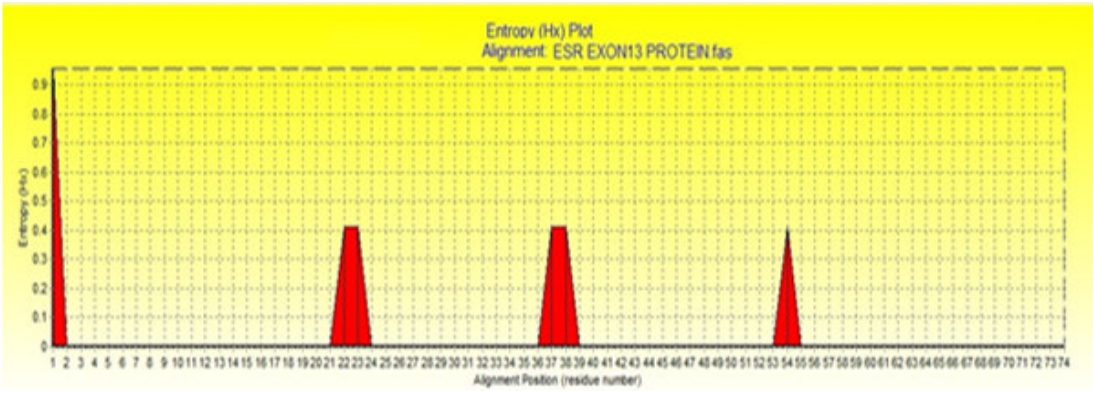

Figure 10
Entropy plot showing variability in ESR protein sequence alignments with Reference.

Percentage identity and divergence of ESR gene and proteins among various groups

Percent identity and divergence was computed by BioEdit software among different groups of animal. When the percent nucleotide identity of multiparous normal group (MN) with other groups was calculated it was found to show maximum identity (100%) with heifer normal (HN) and field normal (FN) group, followed by 99.5 % identity with multiparous abnormal (MA) group and minimum identity (98.6%) with field abnormal (FA), heifer abnormal (HA) group and reference. Percent identity of multiparous abnormal group (MA) showed maximum identity (99.5%) with heifer normal (HN) and field normal group (FN), followed by field abnormal (FA) and heifer abnormal (HA) group (99.0% identity) and showed minimum identity with reference (98.1%). Heifer normal group (HN) showed 100% identity with field normal (FN) group and 98.6% identity with field abnormal (FA) group, heifer abnormal (HA) group and reference. Field normal (FN) group showed 98.6% identity with field abnormal (FA), heifer abnormal (HA) groups and reference. Field abnormal (FA) group was found to possess 98.1% identity with heifer abnormal (HA) and 97.2% identity with reference. Heifer abnormal (HA) group showed 97.2% identity with reference (Fig 11).

Percent Identity

Distance Matrix

	MN	MA	HN	FN	FA	HA	REF	
MN		99.5	100	100	98.6	98.6	98.6	MN
MA	0.9		99.5	99.5	99.0	99.0	98.1	MA
HN	0.0	0.9		100	98.6	98.6	98.6	HN
FN	0.0	0.9	0.0		98.6	98.6	98.6	FN
FA	2.6	1.7	2.6	2.6		98.1	97.2	FA
HA	2.6	1.7	2.6	2.6	3.4		97.2	HA
REF	2.6	3.4	2.6	2.6	5.2	5.1		REF
	MN	MA	HN	FN	FA	HA	REF	

Figure 11
Representative of percent identity and distance matrix of DNA of ESR gene among various groups of buffaloes. REF: Bovine, MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal

Percent Identity

Distance Matrix

	MN	MA	HN	FN	FA	HA	REF	
MN		98.6	100	100	95.8	98.6	95.8	MN
MA	2.6		98.6	98.6	97.2	98.6	94.4	MA
HN	0.0	2.6		100	95.8	98.6	95.8	HN
FN	0.0	2.6	0.0		95.8	98.6	95.8	FN
FA	7.9	5.3	7.9	7.9		95.8	91.6	FA
HA	2.6	2.6	2.6	2.6	7.9		94.4	HA
REF	7.9	10.6	7.9	7.9	16.2	10.6		REF
	MN	MA	HN	FN	FA	HA	REF	

Figure 12
Representative of percent identity and distance matrix of protein of ESR gene among various groups of buffaloes. REF: Bovine, MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal

Comparison of similarity of protein sequences of multiparous normal group (MN) with other groups showed maximum identity (100%) with heifer normal (HN) and field normal (FN) group, followed by 98.6% identity with heifer abnormal (HA) and multiparous abnormal (MA) groups and minimum identity with field abnormal (FA) group and reference (95.8%). Multiparous abnormal (MA) group showed maximum identity (98.6%) with heifer normal (HN), field normal (FN) and heifer abnormal (HA) groups, followed by 97.2% identity with field abnormal (FA) group whereas minimum identity (94.4%) with reference. Heifer normal (HN) group was found to show 100% identity with field normal (FN) group, followed by 98.6% identity with heifer abnormal (HA) group and minimum identity (95.8%) with field abnormal (FA) group and reference. Field normal (FN) group showed 98.6% identity with heifer abnormal (HA) group and 95.8% identity with field abnormal (FA) group and reference. Field abnormal (FA) group showed 95.8% identity with heifer abnormal (HA) group whereas 91.6% identity with reference. Heifer abnormal (HA) group showed 94.4% identity with reference (Fig 12).

Table 3

Values of most favoured region, generously allowed regions and disallowed regions and Errat score (% age) for ESRα protein by PROCHECK and VERIFY3D softwares.

Sample	Most favoured regions	Generously allowed regions	Disallowed regions	Errat score
MN	64.1	3.6	1.2	**73.529**
MA	69.3	3.6	1.8	**72.353**
HN	67.1	3.6	1.2	**71.345**
FN	65.1	4.2	0.6	**65.882**
FA	65.9	4.8	1.8	**74.405**
HA	60.8	4.8	0.6	**73.529**

Modelling of ESR protein structure and comparison among different groups

The atomic coordinates of ESR protein structure of *Bubalus bubalis* was not available in Protein Data Bank which necessitated for developing a protein model. The final model, which was developed and taken for further analysis, consisted of 261 amino acid residue. The quality of the modelled protein was checked by using PROCHECK and VERIFY3D programs. Ramachandran plots (Fig 13) was also obtained via PROCHECK, which check the stereo chemical quality of the protein structures, which assured the reliability of modelled protein with different values in different regions (Table 3). The assessment with VERIFY3D, which derives "3D-1D" profile based on the local environment of each residue, described by the statistical preferences for the area of the residue that is buried, the fraction of side chain area that is covered by polar atoms (oxygen and nitrogen) and the local secondary structure, also substantiated the reliability of the three dimensional structure. The residue that deviated from the standard conformational angles of Ramachandran plot was the members of the N-terminal domain of the protein. This was an ignorable condition since the N-terminal end was not critical in present study. The distance of these residues to the active site residues were also found to be more than 10Å, which suggested that those residue would interfere little with the binding of ligand in the active site region of ESR. The structural comparison of ESR from different groups revealed small variation among each other (Fig 14). The protein structures were more or less similar to each other. The pairwise comparison of protein structure (expressed as root square mean deviation, RSMD) varies from 0.642 to 1.818. The minimum RSMD value of 0.642 was found between multiporous abnormal (MA) and heifer normal (HN) group, whereas the maximum RMSD value of 1.818 was observed between field abnormal (FA) group and reference (Table 4).

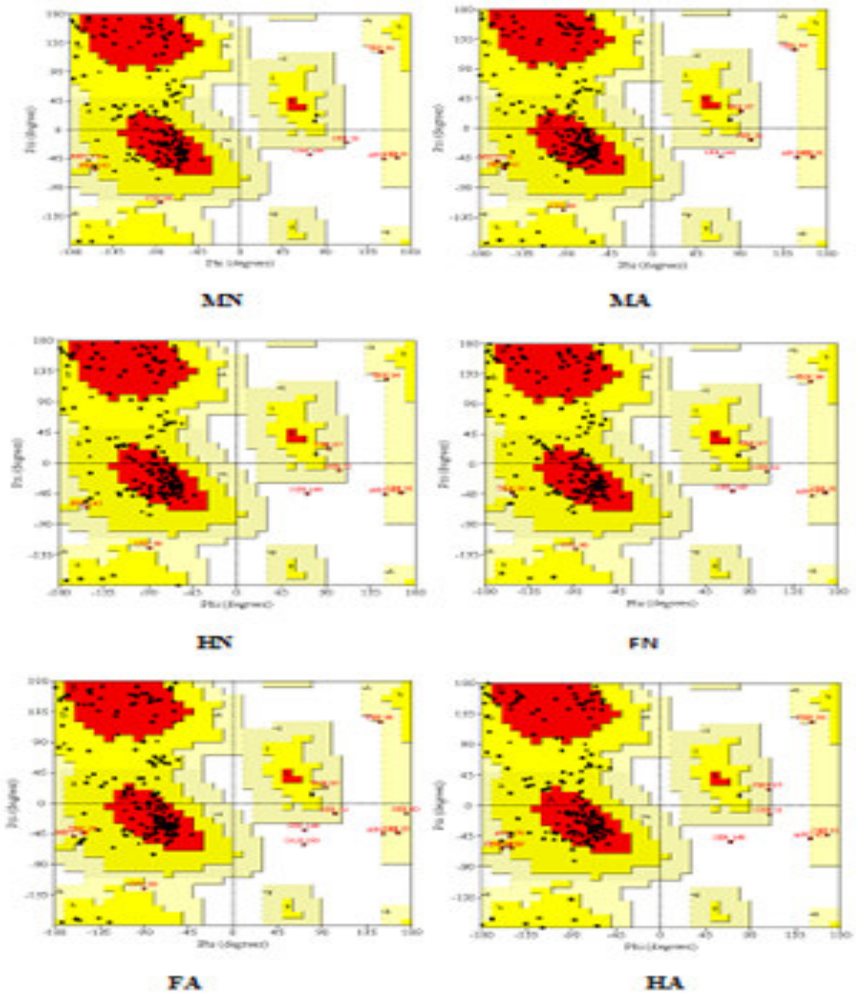

Figure 13
Ramachandran plots assessing the quality of different ESR structures of different groups. MN: multiparous normal, MA: multiparous abnormal, HN: heifer normal, FN: field normal, FA: field abnormal, HA: heifer abnormal.

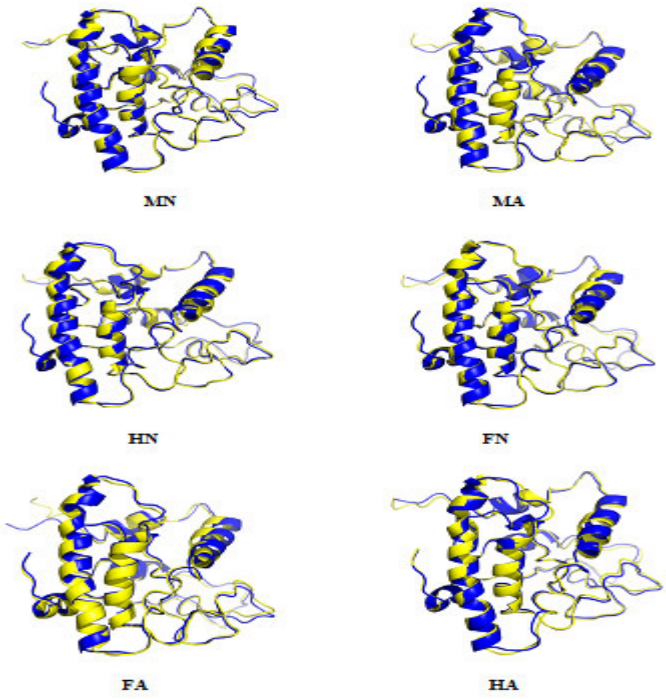

Figure 14
Binding site of ESRα (predicted using Schrodinger software package) and its physio-chemical properties

Table 4
Pairwise comparison of modeled ESRα protein structure (expressed as root square mean deviation, RSMD) from different groups.

	REF	MN	MA	HN	FN	FA	HA
REF	0						
MN	1.467	0					
MA	1.465	0.754	0				
HN	1.477	0.675	0.642	0			
FN	1.528	0.906	0.981	0.752	0		
FA	1.818	1.339	1.273	1.357	1.676	0	
HA	1.476	0.878	0.821	0.851	0.916	1.615	0
	REF	MN	MA	HN	FN	FA	HA

Various mutations detected in the gene and protein sequence of ESR among different groups of murrah buffalo have different level of impact in the binding site. Furthermore; the binding sites have also been predicted using Site Map algorithm (Schrodinger software package). However, only the best binding site was considered (based on SiteScore) for the comparative analysis (Table 5). The significant variation in the binding site among different groups of Murrah buffalo may be associated with reproductive traits.

Table 5
Binding site of ESRα (predicted using Schrodinger software package) and its physio-chemical properties

Proteins	Site Score	size	volume	exposure	enclosure	contact	Hydrophobic	Hydrophilic	balance	don/acc
MN	1.07	236	516.22	0.52	0.73	0.9	0.86	0.79	1.08	0.85
MA	1.04	300	791.3	0.57	0.74	0.92	0.63	0.94	0.67	0.66
HN	1.08	288	767.29	0.5	0.76	0.94	0.87	0.84	1.03	0.76
FN	1.11	247	597.51	0.45	0.79	0.97	1.14	0.73	1.57	0.57
FA	1.07	244	575.9	0.5	0.79	0.95	0.84	0.96	0.88	0.46
HA	1.04	304	775.52	0.59	0.71	0.88	0.9	0.84	1.08	0.75

DISCUSSION

Associations between ESRα gene polymorphism and reproduction were studied in pigs. Rothschild *et al*[19] found one mutation in a non-coding region (intron) of the porcine ESRα gene (RFLP-*Pvu*II) to be significantly associated with the mean number of piglets born per litter. Xu *et al*[20] also showed significant associations for the ESR locus with the number of piglets born alive in later parities and the total number of piglets born of all parities. However, no significant associations of *ESR* α alleles with number of piglets born alive were observed in different German pig lines. [9] Only a little study focused on the association between gene polymorphisms and reproduction traits in cows. Zahmatkesh*et al*[21] studied the polymorphism in ESR α and its association with reproductive traits in cattle. The polymorphism detected was in 5' region but no significant relationship was found between SNP detected among polymorphic genotypes and the reproductive traits under study. It was concluded that the SNP might not have altered the ESRα gene transcription factor's binding site and hence did not caused any significant phenotypic change. However the other earlier studies in cattle [22, 23]also detected SNPs in 5' region of ESRα (promoter binding site) and did not found any association between detected variation and reproductive parameters. Kaminski *et al*[24] identified three ESR/ *Ava* I genotypes and evaluate the relations between ESR/*Ava* I genotypes and daily gain, meatiness and selection index in Polish Large White boars. A significant difference for meatiness was observed but no associations were found between ESR/*Ava* I genotypes and both daily gain and selection index. Santana *et al*[25]evaluate the effect of ESR*Pvu* II polymorphism genotypes on the expected progeny differences (EPD) for litter size (LS), average daily weight gain (DWG) and back fat thickness (BT) in Brazilian Large White, Landrace and Pietrain herds.Brazilian Landrace population studied was found to be polymorphic for the *Pvu*II site (AA, AB and BB). Despite the presence of the polymorphic *Pvu*II site in the Brazilian Landrace animals, there were no differences among ESR genotypes in the expected progeny differences (EPD) for litter size (LS), average daily weight gain (DWG) and back fat thickness (BT) traits. The complete association between ESR polymorphism and reproductive traits was also lacking. Fertility is a key reproductive trait and greatly affected by differences in or changes in the environment, health and nutritional status. [26]Fertility might be considered as two traits, inherited fertility and expressed fertility. Inherent fertility refers to genetic potential for reproduction performance, whereas the expressed fertility can be measured by calving interval, calving rate, service per conception and age at first calving. Among the reproduction traits calving interval is the most important criterion of fertility. [27,28, 29] Long calving interval is considered as a major problem in buffalo breeding.[30]In the present study the exonic region taken was present at 3' region of the gene which is actually the ligand binding site and contributes significantly in the reproductive performance of the animal. As some of the animals belonging to the field abnormal group in the present study possessed significant irregularities in reproductive traits like long calving intervals, long dry periods and large number of open days. Hence the SNPs detected in the field abnormal animals might be associated with reproductive traits. But these observed SNPs are not present in other

abnormal groups (heifer abnormal and multiparous abnormal).Hence these genetic variations might be the epigenetic changes, which implies these nucleotide variations to a large extent be influenced by environmental factors.[31]

Summary

PCR-RFLP coupled with protein modelling showed variation at amino acid level and very little variation was also observed in 3-D protein structures. Hence the ESR gene can be used as the candidate genes in the studies involving reproductive traits. The ESR gene-based selection can be more or less successful also in small herds, real (non-experimental) conditions and during short period. But an examination of a larger sample population could bring a more conclusive evaluation of the ESR-based selection. Furthermore, improvement in veterinary services, especially inbreed improvement when coupled with molecular biological studies, would certainly enhance buffalo production considerably.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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