

Research Article

PGR exon 8 polymorphism coupled with protein modelling in association with reproductive traits in Murrah buffaloes

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Abstract

Progesterone plays a pivotal role in the establishment and maintenance of pregnancy and in the complex regulation of reproductive processes. The present study was undertaken to identify and characterize exon 8 of the progesterone receptor (PGR) gene in Murrah buffaloes using PCR-RFLP with HindIII and MboI restriction enzymes. A total of 65 Murrah buffaloes were selected and categorized into six groups based on their physiological status. Substantial genetic variation was observed in exon 8 of the PGR gene, ranging from 0 to 1.5% at the nucleotide level and from 0 to 4.1% at the protein level. No single-nucleotide polymorphism (SNP) was detected within exon 8; however, two SNPs were identified in the intronic region. The first SNP occurred at position 48 (C→T) and was detected in the multiparous-normal and field-normal groups, whereas the second SNP occurred at position 110 (A→G) and was observed in the multiparous-normal and heifer-normal groups. Entropy analysis further supported the presence of genetic variation, as evidenced by distinct peaks and differences in the corresponding areas. To investigate the potential effects of the identified genetic variations on the overall structure of the translated PGR protein, protein structures were modelled for the different groups and subsequently compared. Pairwise comparison of the predicted protein structures, expressed as root mean square deviation (RMSD), revealed values ranging from 0.247 to 0.891, indicating varying degrees of structural similarity among the groups.

Keywords: Buffalo, PGR exon-8, PCR-RFLP, SNP detection, Protein modelling.

Introduction

Buffaloes constitute an important component of the global livestock sector and make a significant contribution to the agricultural economy of several countries. The global buffalo population is estimated at approximately 194 million, of which nearly 97.13% is concentrated in Asia. South Asia alone accounts for approximately 76.92% of the world's buffalo population. India and Pakistan are the major buffalo-producing countries in the region, accounting for approximately 57% and 43%, respectively, of the buffalo population in South Asia. Buffaloes contribute more than 50% of the total milk production in India despite constituting less than half of the country's bovine population (Mitra *et al.*, 2012). The river buffalo (*Bubalus bubalis*; 2n = 50) is an important livestock species characterized by high lactation performance and its suitability for drafting and ploughing purposes, particularly in dry and plain regions (Amaral *et al.*, 2007).

India is home to some of the most productive river buffalo breeds in Asia, among which the Murrah buffalo is of particular importance. The breed originated in the northwestern region of India and is recognized for its high potential for milk and fat production. In addition to its use as a dairy and draught animal, Murrah buffaloes serve as an important genetic resource for meat production and breed improvement programmes (Sethi, 2003; Rosati and Van Vleck, 2002). Among the buffalo breeds, Murrah, popularly referred to as "black gold," occupies a prominent position because of its superior milk-producing capacity, with an average yield of approximately 2,200 kg per lactation. The breed has been extensively utilized for the genetic improvement of indigenous buffalo populations in India and has also been exported to several countries for crossbreeding and breed improvement programmes. Despite the substantial contribution of buffaloes to India's milk production, the efficiency of milk production remains constrained by the limited identification and systematic selection of genetically superior animals (Mitra *et al.*, 2012). Therefore,

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improving reproductive efficiency is essential for enhancing the productivity and genetic potential of buffalo populations. A comprehensive understanding of the reproductive physiology of female buffaloes, particularly the mechanisms regulated by steroid hormones, is crucial for this purpose. Consequently, elucidating the molecular mechanisms underlying steroid hormone action in the female reproductive tract may contribute to improved reproductive efficiency and genetic advancement in buffaloes.

Progesterone is a key steroid hormone that plays a central role in vertebrate reproductive physiology, particularly in mammals, where it is essential for the establishment and maintenance of pregnancy (Conneely, 2002). It regulates several critical reproductive processes, including the release of mature oocytes, preparation of the uterus for implantation, and maintenance of pregnancy through the stimulation of uterine growth and suppression of myometrial contractility (Graham and Clarke, 1997). The biological actions of progesterone are mediated through specific progesterone receptors (PRs), which are predominantly localized in the nucleus of target cells and belong to the superfamily of nuclear receptor transcription factors (Katsu *et al.*, 2010). Consequently, the progesterone receptor (PGR) gene has been considered an important candidate gene for investigating reproductive traits and progesterone-mediated physiological functions (Gutierrez-Sagal *et al.*, 1993). The PGR gene encodes two major receptor isoforms, PR-A and PR-B, which are generated from a single gene through the use of alternative transcriptional start sites (Conneely *et al.*, 1989; Kastner *et al.*, 1990). The relative expression of these isoforms varies considerably among reproductive tissues and is influenced by developmental stage (Shyamala *et al.*, 1990), hormonal status (Duffy *et al.*, 1997), and pathological conditions such as carcinogenesis (Brandon *et al.*, 1993; Graham *et al.*, 1996; Vang *et al.*, 2023). Alterations in the expression or functional activity of PR isoforms may therefore influence progesterone responsiveness and reproductive function. Polymorphisms and genetic variations within the PGR gene have been investigated across a range of vertebrate species, including chicken (Conneely *et al.*, 1989; Huckaby *et al.*, 1987; Dominguez-Steglich *et al.*, 1992), mouse (Naylor *et al.*, 1989; Schott *et al.*, 1991; Lydon *et al.*, 1995), rabbit (Loosfelt *et al.*, 1986; Peiro *et al.*, 2008), human (Misrahi *et al.*, 1987; Rousseau-Merck *et al.*, 1987; Langmia *et al.*, 2015; Asmarinah *et al.*, 2017), and crocodile (Suwattana *et al.*, 1999). These studies highlight the evolutionary conservation and functional significance of the PGR gene and provide a basis for investigating its genetic variation and potential association with reproductive performance in economically important livestock species such as buffalo.

To enhance the genetic merit of livestock through improved milk production, reproductive efficiency, disease resistance and other economically important

traits, it is essential to identify and characterize the genes and quantitative trait loci (QTLs) associated with these phenotypes. The detection and utilization of genetic polymorphisms through molecular genetic approaches can provide valuable genetic markers for application in animal breeding and selection programmes. Various molecular techniques have been developed for the detection of polymorphisms at structural gene loci, among which polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) is widely used for single-nucleotide polymorphism (SNP) genotyping and mutation detection. The technique offers several advantages including simplicity, rapidity, cost-effectiveness, high reproducibility and the absence of hazardous radioactive materials.

The identification of restriction fragment length polymorphisms (RFLPs) has further strengthened the application of molecular markers in the genetic characterization and selection of superior germplasm. In this context, the present study was undertaken to investigate the occurrence of genetic variation in exon 8 of the progesterone receptor (PGR) gene and to evaluate its potential association with reproductive traits. In addition, the protein structure modelling was also performed in correlation to our earlier study on exon 3 (Sarla *et al.*, 2025) to assess the possible structural implications of the identified genetic variations.

Materials and Methods

Germplasm collection

Sixty-five Murrah buffaloes were used for the present study. Out of which 14 heifers and 21 multiparous buffaloes were selected from the CIRB (Central Institute for Research on Buffaloes) herd and 30 multiparous buffaloes were selected from different regions of Haryana state that were reared on standard farm management conditions at CIRB, Hisar. ANOVA (Analysis of variance) was used to analyse animal physiological data and mean $\pm$ SE were ranked according to SPSS 16.0 (Table 1) using Duncan's multiple range test. Further, all these animals were grouped into six groups on the basis of their conception and calving after parturition as follows: Group: I with 8 animals (Multiparous normal buffaloes; MN), Group: II with 13 animals (Multiparous abnormal buffaloes; MA), Group: III with 10 animals (Multiparous normal buffaloes from the field; FN), Group: IV with 20 animals (Multiparous abnormal buffaloes from the field; FA), Group: V with 7 animals (Heifer normal; HN), Group: VI with 7 animals (Heifer abnormal; HA).

DNA extraction and Primers designing

Fresh blood samples were used for DNA extraction by method of Sambrook and Russel (Sambrook and Russel, 2001) with slight modifications. The quality

and quantity of extracted DNA was analysed by Nano drop spectrophotometer and agarose gel electrophoresis. Primer 3 software was used to design primers (Table 2) from *Bos Taurus* gene sequence (Accession no: NM_001205356.1, Chromosome 14) (Fig 1) for amplification of PGR gene exon 8. The bovine was considered as a reference animal due to the greater percentage similarity of conserved DNA sequences (75%) in Bovidae family which includes cattle, bovine and buffalo (Navani *et al.*, 2002). The region was focused because of its presence on ligand binding site.

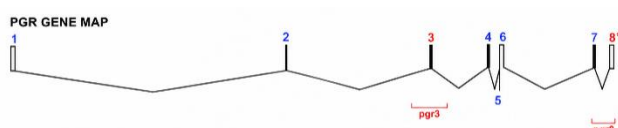


Fig 1 PGR gene map of Bovine

PCR amplification

Table 1 Parameters of animals details (Mean±SE)

Group	Lactation No.	Calving Interval **	Lactation Length *	Total Lactation Yield	305 Days lactation yield	Peak Milk Yield	Dry Period	Open 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

Table 2 Primer sequence and thermal profile for the amplification of PGR gene

Primer	Sequences 5' ----- 3'	Gene	Region/Locus	PCR conditions	PCR product size
PP8f	5'-TATTCTGGAATGGAGAG-3'	PGR Exon-8	Partial intron 7-8, exon 8, 3' down streaming region	94°C 45 sec 55°C C 50sec 72°C 50 sec	393bp
PP8r	5'-ACAGTAAAACTCGTGACCTC-3'				

Restriction Fragment Length Polymorphism (RFLP)

The amplicons were screened for the presence of genetic polymorphism in PGR via PCR-RFLP. The progesterone receptor gene PCR product (exon 8) was digested with *Mbo* I (5'¹⁴GATC3') 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 cleaved fragment were separated by electrophoresis on 2% agarose gel and visualized in Gel-Doc system (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

The PCR product was purified by QIAquick kit (Qiagen). The purified amplified PCR products of PGR gene were sequenced from Xcelris Labs Ltd., Ahmedabad. Both the DNA strands (i.e. forward and reverse) were sequenced and were assembled using

The PCR was carried out for PGR exon 8 in a final volume of 15.0 µl consisted of 80 ng genomic DNA, 1.6 pm each primer, 2.0 mM MgCl₂, 0.15 mM dNTPs, 1X PCR buffer and 1 U Taq polymerase. The thermal profile consisted of initial denaturation (94°C for 5 minutes) followed by 35 cycles of denaturation (94°C for 45 seconds), annealing (55°C for 50 seconds) extension (72°C for 50 seconds) and final extension (72°C for 7 minutes). The PCR amplification was confirmed by restriction digestion of PGR exon 8 amplicons with *Hind* III 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 DNA marker.

Codon Code Aligner 5.0.1. The assembled sequence were then compared among themselves and also with the bovine reference followed by SNP detection using Codon Code Aligner 5.0.1. Further, these sequences were aligned and compared using Bio edit software and multiple alignment programs, Clustal W (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) with DNA weight matrix and multiple parameters like gap opening 10.0, gap extension 0.20 and transition weight 0.50. Pairwise sequence identity, divergence of PGR gene among different groups of buffalo and Entropy plot of PGR gene was calculated using BioEdit software and compared with the reference sequence.

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 [exon 8 and exon 3 (Sarla *et al.*, 2025)] 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/clustalw2/>) 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 constructed phylogenetic tree. BLAST search of translated protein sequence of PGR with PDB data base gives a protein sequence similarity with 67% (PDB ID: P06401, NR3C3).

This homologous protein structure was used as template for homology model building using Prime accessible through the Maestro interface (Schrodinger, Inc.). The modeled structures were evaluated using PROCHECK v3.4.4 (Laskowski *et al.*, 1993) which analyzed for overall and residue by residue geometry. Ramachandran plots provided by program PROCHECK assured very good confidence for predicted protein (Ramachandran *et al.*, 1963). For each protein structure that has been modelled, the binding sites were defined as a set of site points that were predicted using SiteMap (Schrodinger, Inc., version 2.4).

Results

A total of 65 animals (divided into six groups) were used in the present study. The mean conception age of normal buffalo heifers was significantly ($p < 0.01$) lower (35.90 ± 1.46) than the abnormal heifers (54.25 ± 3.16). Calving interval also differed significantly ($p < 0.01$) between groups and within the groups. The lactation length also differed significantly ($p < 0.05$) between different groups. Mean calving interval, lactation length, total lactation yield, peak milk yield, dry period and open days were calculated (Table 1). The animals were genotyped for the presence of any genetic variation and its association with reproductive traits. PGR gene from different buffalo groups was partially amplified using specifically designed primers for PGR (Table 3).

Table 3 Values of most favoured region, generously allowed regions and disallowed regions and Errat score (% age)

Sample	Most favoured regions	Generously allowed regions	Disallowed regions	Errat score
MN	74	3.3	0.4	47.131
FN	74.4	2.9	0.8	64.634
HN	73.6	2.5	0.4	67.480
MA	73.1	3.3	0.4	48.770
FA	70.5	2.1	0.4	49.593
HA	74.7	2.5	0.4	51.600

Amplification and characterization of PGR (exon 8) gene

Amplification of PGR exon 8 resulted into production of amplicon of 393 bp size (Fig 2). The amplified fragment of PGR exon 8 corresponds to part of partial intron 7-8, exon 8 and 3' down streaming region. The amplified products were then confirmed by nucleotide sequencing using the same set of primers and by

comparing with the reference gene of PGR (exon 8) gene from *Bos taurus* (bovine).

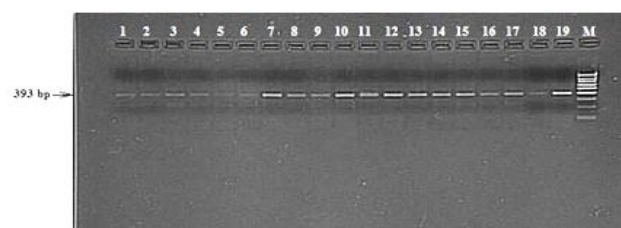


Fig 2 Representative gel pictures showing the 393 bp amplified product of PGR exon 8 from different groups of Murrah buffalo: 1-3 HN, 4-6 HA, 7-9 MN, 10-12 MA, 13-15 FN, 16-20 FA. M: 100 bp ladder

The restriction endonuclease *Hind* III (5'A¹AGCTT3') was used for the digestion of amplicon (393 bp), which cuts at a single site and produced two fragments of 318 bp and 75 bp (Fig 3). When the digested product was electrophoresed on 2.5% agarose gel, it was found in accordance with the data obtained from the analysis of sequence by web cutter. PCR-RFLP of 393bp amplicon was carried out using *Mbo* I (5' ¹GATC3') which produced fragments of 259 bp, 134 bp and the digestion was in accordance with the data from web cutter analysis. Hence, the amplicon was found to be monomorphic for the restriction site of *Mbo* I enzyme (Fig 4).

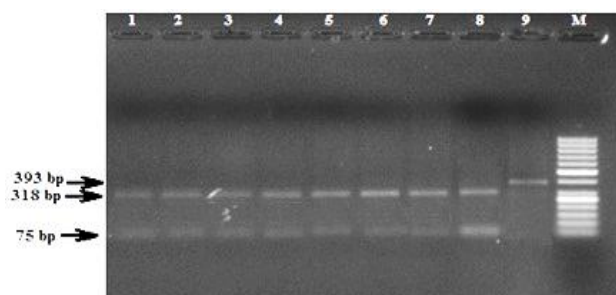


Fig 3 Representative gel pictures for confirmation of PGR exon 8 amplicon by restriction enzyme digestion.

Where 1-2 : HA, 3-4: HN, 5: FN, 6-7: MN and MA groups, 8 shows FA, 9 shows uncut amplicon. M: 50 bp ladder

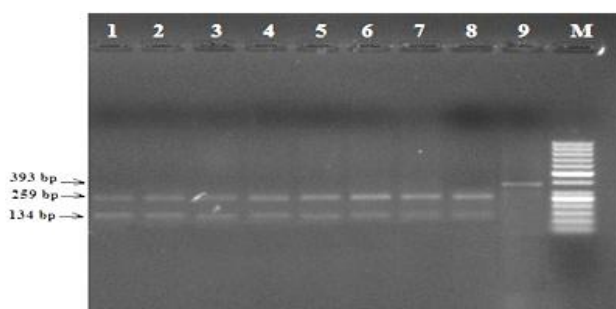


Fig 4 Representative gel pictures showing the *Mbo* I restriction digested fragment of PGR exon 8 from different groups of murrah buffalo, 1-2 : HA, 3-4: HN, 5: FN, 6-7: MN and MA groups, 8 shows FA, 9 shows uncut amplicon. M: 50 bp ladder

Amplified PCR products of representative samples were subjected to custom DNA sequencing from both ends (5' and 3' ends). Nucleotide sequences were visualized using Chromas Lite version 2.0.1. The forward and reverse sequences of each PCR fragment were assembled by Codon Code Aligner to form contigs of the respective region which were then compared with that of bovine (Acc: NM_001205356) sequence to annotate different exonic regions putatively to identify SNPs in respective region. A very few variations were observed among PGR exon 8 region. All the nucleotide sequences of complete amplicon (393 bp) were compared via multiple sequence alignment programs using BioEdit 5.0.1 version and level of variation in nucleotide sequences were detected and represented (Fig 5). Furthermore the exonic nucleotide sequence of PGR exon was compared (Fig 6) and translated and compared with that of the bovine to detect amino acid changes in buffalo PGR region under study (Fig 7).

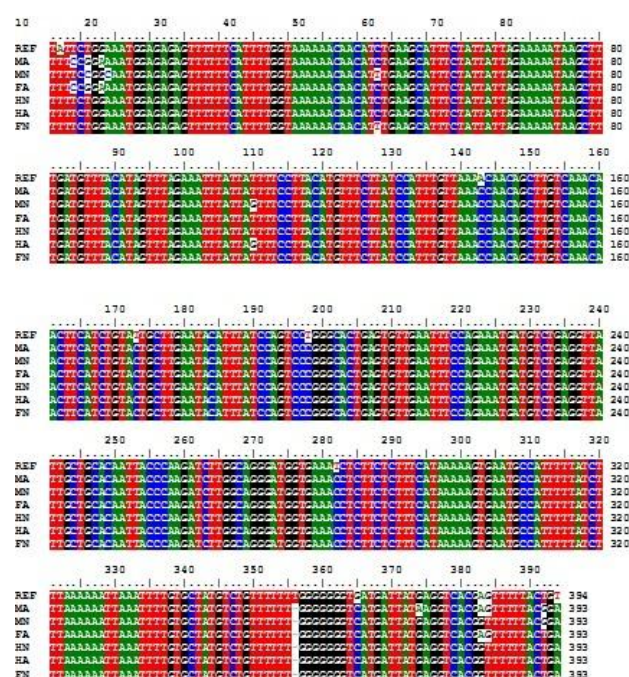


Fig 5 Multiple sequence alignment of PGR-exon 8 (393 bp) complete amplicon obtained from different groups of buffaloes with reference. REF: Bovine, MN, MA, HN, FN, FA, HA

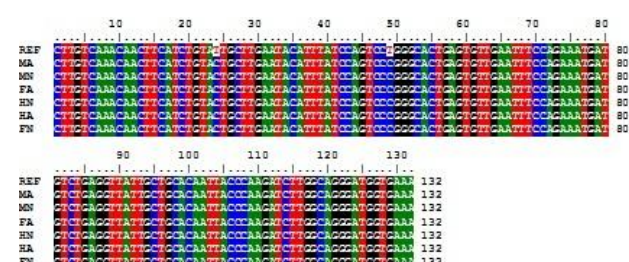


Fig 6 Multiple sequence alignment of PGR exon-8 obtained from different groups of buffaloes with reference. REF: Bovine, MN: multiparous normal, MA, HN, FN, FA, HA



Fig 7 Multiple sequence alignment of PGR protein sequence from different groups of buffaloes with reference. REF: Bovine, MN, MA, HN, FN, FA, HA

When the genetic variation on the basis of PGR gene sequence (exon-8) was calculated among different groups as distance matrix, it was found to vary from 0.0% to 1.5%. All the groups showed very low variation and possess 0.0% value of distance matrix among them indicating that they are much more similar to each other as compared to reference. But all of these groups showed dissimilarity from reference (1.5%). The dendrogram generated based upon DNA sequence similarity showed that HA, HN, FN and FA groups were very much close to each other. MA group was found placed close to MN group. All of these groups differed from reference (Fig 8).



Fig 8 Dendrogram showing similarity between PGR exon 8 gene sequences obtained from different groups of buffaloes. REF: Bovine, MN, MA, HN, FN, FA, HA

Based upon the protein sequence comparison, a very small variation was found among different groups under study and ranged from 0.0% to 4.1%. All the groups showed very low variation and possess 0.0% value of distance matrix among them indicating that they are much more similar to each other as compared to reference. But all of these groups showed dissimilarity from reference (4.1%). Protein sequence similarity based dendrogram showed that MA, MN, FA and HN groups were found very much close to each other whereas HA group was closer to FN group and was distantly placed from reference (Fig 9).



Fig 9 Dendrogram showing similarity between PGR exon 8 protein sequences obtained from different groups of buffaloes. REF: Bovine, MN, MA, HN, FN, FA, HA

SNP Detection

Clustal W multiple alignments with bovine sequence revealed changes in intron 7-8 region only at position

48 C→T in MN and FN groups and second at 110 position i.e. T→G in MN and HN groups. As these variation were present in intron region and no SNP was detected in exonic region (Fig 6) when the nucleotide sequence was compared with reference. Furthermore, a single base deletion was also observed in the all group under study at 356 positions of 3' downstream region (Fig 5).

Entropy plot of PGR exon 8 sequences

The entropy plot showed zero value due to the conserved sequence. The peaks however showed variations in the nucleotide sequence with reference at positions 24 and 49 whereas at position 17 in case of amino acid sequence (Fig 10 and Fig 11).

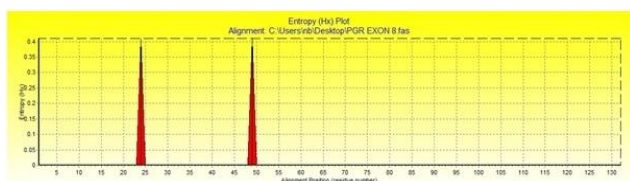


Fig 10 Entropy plot showing variability in PGR exon 8 nucleotide sequence alignments with reference

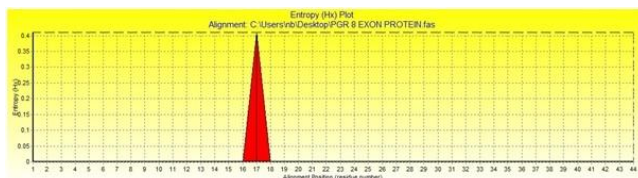


Fig 11 Entropy plot showing variability in PGR exon 8 protein sequence alignments with reference

Percentage identity and divergence of PGR gene exon 8 and proteins among various groups

When percent identity of multiparous abnormal (MA) group was calculated with other groups it was found that this group showed 100% identity with MN, FA, HN and HA and FN groups and showed 98.4% identity with reference.

Percent Identity								
Distance Matrix	MA	MN	FA	HN	HA	FN	REF	
	MA	100	100	100	100	100	98.4	MA
	MN	0.0	100	100	100	100	98.4	MN
	FA	0.0	0.0	100	100	100	98.4	FA
	HN	0.0	0.0	0.0	100	100	98.4	HN
	HA	0.0	0.0	0.0	0.0	100	98.4	HA
	FN	0.0	0.0	0.0	0.0	0.0	98.4	FN
	REF	1.5	1.5	1.5	1.5	1.5		REF
	MA	MN	FA	HN	HA	FN	REF	

Fig 12 Representative of percent identity and distance matrix of DNA of PGR exon 8 among various groups of buffaloes. REF: Bovine, MN, MA, HN, FN, FA, HA

MN group showed 100% identity with FA, HN and HA and FN groups and showed 98.4% identity with

reference. FA group showed 100% identity with HN and HA and FN groups and showed 98.4% identity with reference. HN group was found to show 100% identity with HA and FN groups whereas 98.4% identity with that of reference was observed. HA group showed 100% identity with FN group and 98.4% identity with that of reference. FN group showed 98.4% identity with reference (Fig 12).

When percent identity of MA group was calculated with other groups it was found that this group showed 100% identity with MN, FA, HN and HA and FN groups and showed 97.7% identity with reference. MN group showed 100% identity with FA, HN and HA and FN groups and showed 97.7% identity with reference. FA group showed 100% identity with HN and HA and FN groups and showed 97.7% identity with reference. HN group was found to show 100% identity with HA and FN groups whereas 97.7% identity with that of reference was observed. HA group showed 100% identity with FN group and 97.7% identity with that of reference. FN group showed 97.7% identity with reference (Fig 13).

Modelling of PGR protein structure and comparison among different groups

The template taken for structure modeling was 1A28 (hormone-bound human progesterone receptor ligand-binding domain) with resolution 1.80Å and length amino acids is 256. As the atomic coordinates of PGR protein structure for the organism *Bubalus bubalis* was not available in Protein Data Bank which necessitated for developing a protein model.

Percent Identity								
Distance Matrix	MA	MN	FA	HN	HA	FN	REF	
	MA	100	100	100	100	100	97.7	MA
	MN	0.0	100	100	100	100	97.7	MN
	FA	0.0	0.0	100	100	100	97.7	FA
	HN	0.0	0.0	0.0	100	100	97.7	HN
	HA	0.0	0.0	0.0	0.0	100	97.7	HA
	FN	0.0	0.0	0.0	0.0	0.0	97.7	FN
	REF	4.1	4.1	4.1	4.1	4.1		REF
	MA	MN	FA	HN	HA	FN	REF	

Fig 13 Representative of percent identity and distance matrix of protein of PGR exon 8 among various groups of buffaloes. REF: Bovine, MN, MA, HN, FN, FA, HA

The final model was modelled by compiling exon 4, 5, 6 and 7 from the bovine reference with exon 3 (Sarla *et al.*, 2025) and exon 8 of buffalo which finally consisted of 263 amino acid residue and taken for further analysis. We used both PROCHECK and VERIFY3D software to check the quality of the modeled protein. Ramachandran plot (Fig 14) obtained from the program PROCHECK, which checks the stereo chemical quality of the protein structures, producing a number of postscript plots analyzing its overall residue by

residue geometry, assured the reliability of modeled protein with different values in different regions as shown in 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.

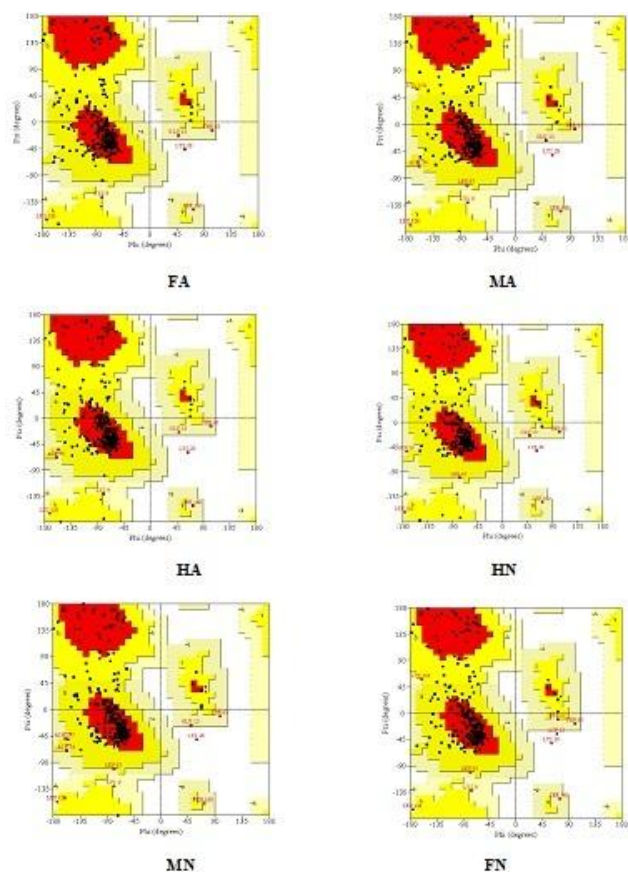


Fig 14 Ramachandran plots assessing the quality of different PGR-A structures of different groups. MN, MA, HN, FN, FA, HA

The residue that deviated from the standard conformational angles of Ramachandran plot was the members of the N-terminal domain of the protein. The distance of these residues to the active site residues also were found to be more than 10Å, which suggested that these residues would interfere little with the binding of ligand in the active site region of PGR. The structural comparison of PGR from different groups revealed small variation among each other (Fig 15). The pair wise comparison of protein structure (expressed as root square mean deviation, RMSD) varies from 0.247 to 0.891. The minimum RMSD value of 0.247 was found between MA and MN, whereas the maximum RMSD value of 0.891 was observed between FA and HN (Table 4).

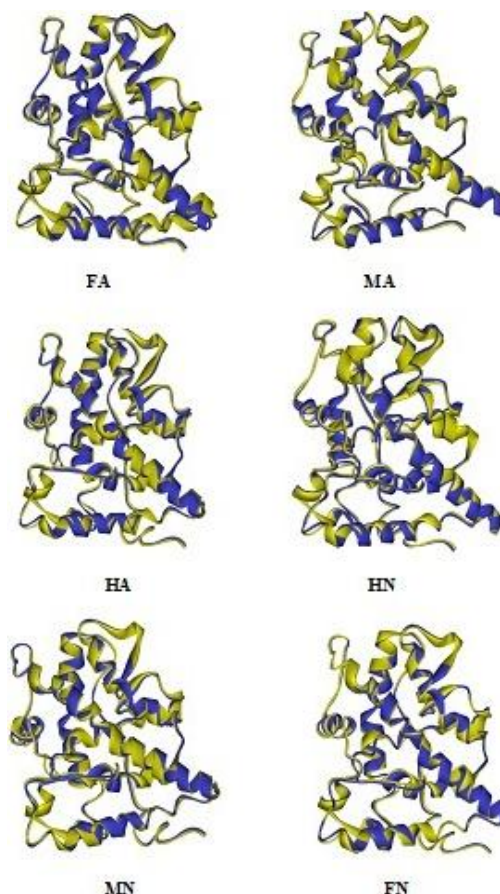


Fig 15 Modelled protein structure of PGR of different groups superimposed with reference. MN, MA, HN, FN, FA, HA

Table 4 The pair wise comparison of modelled PGR protein structure (expressed as RMSD) from different groups

	REF	MN	FN	HN	MA	FA	HA
REF	0						
MN	0.520	0					
FN	0.496	0.489	0				
HN	0.673	0.697	0.595	0			
MA	0.482	0.247	0.412	0.661	0		
FA	0.814	0.594	0.752	0.891	0.583	0	
HA	0.579	0.380	0.509	0.702	0.330	0.545	0
	REF	MN	FN	HN	MA	FA	HA

Various mutations detected in the gene and protein sequence of PGR among different groups of Murrah buffalo have different level of impact in the binding site. We have predicted the binding site using Site Map algorithm (Schrodinger software package). However, we considered only the best binding site (based on Site Score) for the comparatively study. The characteristic features of the binding site analyzed using Site Map is included in Table 5. The significant variation in the binding site among different groups of Murrah buffalo may be associated with reproductive system.

Table 5 The binding site of PGR (predicted using Schrodinger software package) and its physiochemical properties

Proteins	Site Score	size	volume	exposure	enclosure	contact	Hydrophobic	Hydrophilic	balance	don/acc
MN	1.18	153	239.41	0.39	0.81	0.98	1.9	0.51	3.72	1.26
FN	1.19	143	249.7	0.42	0.85	1.02	1.92	0.57	3.4	1.18
HN	1.2	187	343	0.43	0.88	1.06	1.74	0.6	2.88	1.42
MA	1.2	152	248.33	0.36	0.86	1.02	2.05	0.55	3.72	1.24
FA	1.19	155	244.9	0.37	0.85	1	1.72	0.52	3.28	1.48
HA	1.18	96	306.99	0.47	0.85	1.1	2.14	0.54	3.95	1.75

Discussion

Fertility in water buffaloes (*Bubalus bubalis*) is considerably lower than that observed in cattle (*Bos taurus* and *Bos indicus*). Reproductive performance in buffaloes is influenced by several interacting factors, including nutrition, management practices and environmental conditions. The relatively low reproductive efficiency of buffaloes has been attributed to delayed onset of puberty, seasonal reproductive activity and inefficient oestrus detection, which is a critical prerequisite for effective reproductive management and accurate determination of the optimal breeding time.

In the present study, all the experimental groups were found to be monomorphic at the restriction sites investigated. Although no SNP was detected within exon 8 of the PGR gene, two nucleotide variations were identified in the intronic region between intron 7 and exon 8. The first variation was detected at position 48 (C→T) in the multiparous normal (MN) and field normal (FN) groups, whereas the second variation was observed at position 110 (T→G) in the multiparous normal (MN) and heifer normal (HN) groups. These findings, in agreement with previous observations, indicate the possible influence of environmental factors on reproductive performance and associated physiological parameters in buffaloes (Aziz *et al.*, 2001). Nevertheless, several studies have also demonstrated associations between genetic variation in the PGR gene and physiological traits, as well as alterations in gene expression and receptor activity.

Genetic variation in the PGR gene has been reported in several species. Romano *et al.* (2007) identified a 320-bp PV/HS-1 *Alu* insertion in intron G of the *PROGINS* allele along with two SNPs. The SNP G3432T, located in exon 4, resulted in a nonsynonymous amino acid substitution (V660L), whereas the SNP C3764T in exon 5 represented a synonymous mutation (H770H). Among these variants, the V660L substitution was associated with differences in progesterone receptor phosphorylation and degradation following ligand binding. These differences were proposed to arise from alterations in the three-dimensional conformation of the receptor variants, consequently affecting cellular responsiveness to progesterone. Furthermore, the identified genetic variation was associated with reduced transcript abundance and decreased protein activity, highlighting the potential functional significance of PGR polymorphisms.

Similarly, Peiro *et al.* (2008) investigated the promoter region and exons 1–8 of the PGR gene in divergently selected rabbit lines for uterine capacity. The study identified a 2464G>A SNP in the promoter region, three SNPs in the 5' untranslated region (UTR) of exon 1, and one synonymous SNP in exon 7. The promoter-region SNP was significantly associated with the divergently selected high (H) and low (L) lines, with the H line characterized by greater litter size and a higher number of embryo implantations. The association of this polymorphism with litter size suggested a potential regulatory role of the PGR gene in reproductive performance.

The functional significance of the 2464 G>A polymorphism was further investigated by Peiro *et al.* (2010) through analysis of progesterone receptor expression using Western blotting. The GG genotype, which occurred at a higher frequency in the high line, exhibited lower expression of both PR-B and PR-A isoforms than the AA genotype in the oviduct. The GA genotype displayed PR-A expression comparable to that of the other genotypes and PR-B expression in the oviduct similar to the GG genotype. In the uterus, however, the GG genotype exhibited lower PR-B expression than the GA genotype. Both receptor isoforms were expressed in the oviduct and uterus during the early stages of gestation. At 48 and 72 h of gestation, the AA genotype showed higher expression of both PR isoforms than the GG genotype in both tissues, which was suggested to be associated with differences in embryonic survival in rabbits.

Taken together, these findings indicate that genetic variation within the PGR gene can influence receptor structure, expression and functional activity, thereby potentially affecting reproductive performance. In the present study, although no polymorphism was detected within exon 8, the identification of intronic nucleotide variations suggests the presence of genetic diversity in the PGR locus. Further investigation involving a larger population, additional genomic regions, gene-expression analysis and association

studies would be required to establish the functional and reproductive significance of these variants in buffaloes.

Conclusion

The present study provides valuable insights into genetic variation in the progesterone receptor (PGR) gene, a potential candidate gene for reproductive efficiency in buffaloes. However, validation of the identified variants in a larger population is necessary to establish their association with reproductive traits. Integration of molecular genetic approaches with advanced reproductive technologies, including artificial insemination, superovulation, ovum pick-up, *in vitro* maturation, *in vitro* fertilization and embryo transfer, may further enhance reproductive efficiency and accelerate genetic improvement.

The investigated region lies within the 3' region of the PGR gene, encompassing the ligand-binding domain and thus having potential functional relevance to progesterone-mediated reproductive processes. Animals in the field-abnormal group exhibited prolonged calving intervals, extended dry periods and increased open days, which may reflect both environmental influences and underlying genetic variation. The identified polymorphisms therefore warrant further investigation for their potential association with reproductive performance. Given India's valuable buffalo genetic resources, particularly the Murrah breed, systematic genetic characterization and conservation are essential. The combined application of PCR-RFLP and protein structural modelling demonstrated the potential for detecting genetic variation; however, larger-scale studies incorporating genotype-phenotype association and functional analyses are required to establish the utility of PGR variants as molecular markers for improving reproductive efficiency in buffaloes.

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