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Effect of Genetic Variations in BMP 4 Gene (Exon 2 Plus Part Of Intron 2) on Infertility in Egyptian Buffalo Cows

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Abstract

Bone morphogenetic proteins (BMPs) are growth factors belonging to the transforming growth factor beta (TGF-β) superfamily. BMPs are intra-ovarian factors expressed in mammalian ovaries by oocytes, granulosa and theca cells. The present study was designed to isolate and detect polymorphism in BMP 4 (exon 2 plus part of intron 2) gene associated with infertility in Egyptian buffalo cows using PCR-RFLP and nucleotide sequencing techniques. PCR amplification of DNA from 35 Egyptian water buffalo (5 normal fertile, 14 anestrum and 16 repeat breeders) with 482 bp expected size was purified and sequenced. There was no variation in the sequence of amplified region between fertile and infertile group. PCR product was digested using Taqα1 and Hinf1 restriction enzymes gave one type of the restriction banding pattern with no polymorphism. Concluded that BMP 4 is highly evolutionarily conserved gene.

Key words: BMP 4 gene, Egyptian buffalo, Infertility, PCR-RFLP, Sequence.

1. Introduction

The folliculogenesis is a continuous process regulated by a variety of endocrine and intra-ovarian factors. One of these factors is the bone morphogenetic protein (BMP) that is belonging to TGF-β superfamily. Until now, 15 BMPs were described, and only seven (BMP-2, -3, -3b, -4, -6, -7 and 15) expressed in mammalian ovaries [1-3]. Although BMPs were originally named for their ability to induce bone formation, they are pleiotropic proteins that regulate cell fate determination, proliferation, apoptosis, and differentiation during both embryogenesis and adulthood [4].

Several BMPs have been suggested as autocrine/ paracrine regulators of bovine ovarian follicular development, demonstrated by the expression of BMP4 and BMP7 in granulosa and theca cells [5].

BMP 4 controls numerous events of embryonic, fetal and even adult development in vertebrates [6]. BMP 4 expression is very high in healthy follicles but rarely detectable in follicles undergoing atresia, which can act directly on granulosa cells and cause important changes in FSH (follicle stimulating hormone) action [7]. BMP 4 can inhibit progesterone production by granulosa cells and decrease basal granulosa cell progesterone secretion and totally abolish FSH-stimulating action both in cattle and sheep [8]. So, BMP 4 could have relation to reproductive functions in mammals. The aim of the present study was to isolate BMP 4 gene (exon 2 plus part of intron 2) and then to detect any polymorphisms associated with incidence of infertility problems in Egyptian buffalo cows by direct sequencing for detection of single nucleotide polymorphism and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

2. Material and methods

2.1 Animal source and grouping

The present study was conducted on a total of 35 Egyptian water buffalo cows (Bubalus bubalis). Animals were selected from a buffalo nucleus herd kept in Nataff-Gedeed Station, Mahalet-Mousa Farm, Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture. Thirty five buffalo cows were identified to be either normal fertile or infertile (anestrus and repeat breeder) based on pedigree information and confirmed by the rectal palpation and ultrasonography.

2.2 Genomic DNA extraction

Blood samples were collected in EDTA containing vacutainer tubes (kept in ice box) from jugular veins of 35 animals (5 normal fertile, 14 anestrum and 16 repeat breeders). The genomic DNA was extracted from the leucocytes using TIANamp Genomic DNA Kit following the manufacturer protocol (www.tiangen.com/en). The concentration of total extracted DNA was spectrophotometrically determined at 280 and 260.

2.3 Polymerase chain reaction (PCR)

BMP 4 (exon 2 plus part of intron 2) locus was amplified by PCR using primers F: ATTTATTCTTTACCCTTCCACCTC and R: AACTCCTCGCCTTCCCACAG. The reaction was carried out in a reaction volume of 25 µL, containing 3.0 µL DNA template (approximately 100 ng), 12.5 µl Taq Green PCR master mix 5x (Fermentas, #K1071, European Union), 0.5 µL (10 µmol/L) forward primer, 0.5 µL (10µmol/L) reverse primer, and 8.5 µL nuclease free water. Amplification condition was as follow: Initial denaturation at 940c /1 mint., denaturation at 940c

/45sec, annealing at 570c /45 sec, extention at 720c /45 sec, final extention at 720c /5 mint., the number of cycles were 30 and final hold at 40c. The PCR product was visualized on 2% agarose gel on the UV transilluminator and photographed by gel documentation system (Gel DOC TMXR+ Imaging System) fig 1. PCR products for ten animals (3 normal, 3 repeat breeder and 4 anestrum) were purified according to Zymoclean TM Gel DNA Recovery Kit (Zymo Research, catalog no. D4001.D4002, D4007 &D4008) and then sequenced.

2.4 DNA sequencing

The sequences were done in forward direction in 3500 Genetic analysers (Applied BiosystemR). The Sequences were analyzed using the Chromas Lite 2.1 program (http://technelysium.com.au/?page_id=13) and the identity of the sequenced PCR product was examined using Blast search against Gene bank database of buffalo and cattle (Bos Taurus) http://blast.ncbi.nlm.nih.gov/Blast.cgi). The MEGA 6 [9] and Bioedit 7.0.5.3 [10] Softwares were used for sequences alignment and polymorphism detection.

2.5 Polymerase chain reaction- Restriction Fragment Length Polymorphism (PCR-RFLP)

After sequencing of DNA samples, NEB cutter V2.0 [11] software program was used to determine the restriction enzyme that recognized specific restriction site.

A. Using *Taq$^{\alpha}$I* restriction endonuclease enzyme

TaqαI restriction endonuclease enzyme (CutSmart®, Time- Saver enzyme) recognize site with sequence 5' T↓CG A 3'. PCR product was digested according to the following reaction condition: 8 ul PCR products, 1ul of TaqαI restriction enzyme, 5 ul of 10XNE buffer and 6 ul of Nuclease free water with incubation time 15 mints. at 65 °C.

b. Using *Hinf I* restriction endonuclease enzyme

Hinf I restriction enzyme has 5' G↓AN TC 3' recognition site. PCR product for BMP 4 (exon 2 plus part of intron 2) was digested according to the following reaction condition: 10 ul PCR products, 1ul of Hinf I restriction enzyme, 2 ul of 10XNE buffer and 7 ul of Nuclease free water with incubation time 15 mints. at 37 °C. The digestion products were electrophoresed at 100 Volt for 30 min on 2% agarose gel. The banding pattern was determined under ultraviolet light by using gel documentation system.

2.6 Data analysis

Gene and genotypic frequencies were calculated according to [12] by simple counting of the alleles based on the electrophoresis results.

3. Results

Nucleotide sequences of *BMP 4* (exon 2 plus part of intron 2) of representative buffalo cow were presented in fig (2) No variations were detected in nucleotide sequences of normal fertile and infertile group. Nucleotide sequences alignment of *BMP 4* (exon 2 plus part of intron 2) with *bubalus bubalis* showed 99% homology fig (3) and 99% with *bos taurus* fig (4) Genotyping of the amplified samples was performed in this study using *Taq $^{\alpha}$1* restriction enzyme gave product with fragments 270 and 212 bp fig(5). Using *Hinf 1* gave product with fragments 279, 157 and 46 bp fig(6).

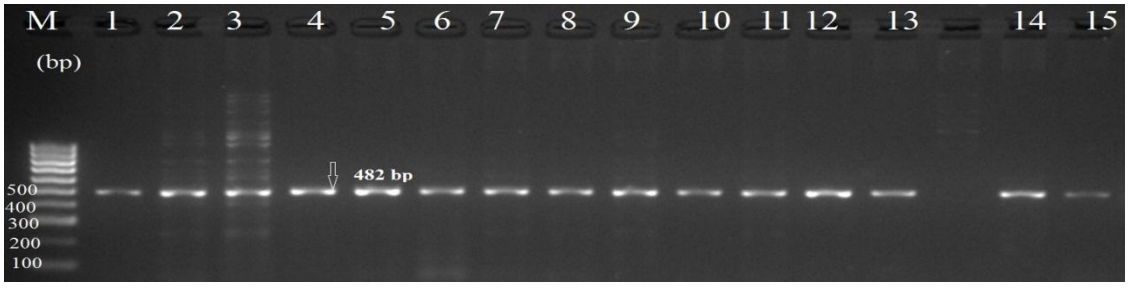
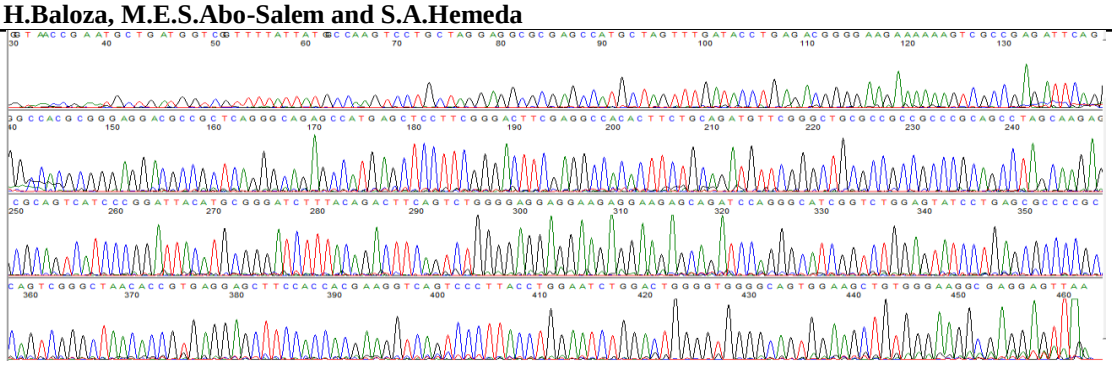
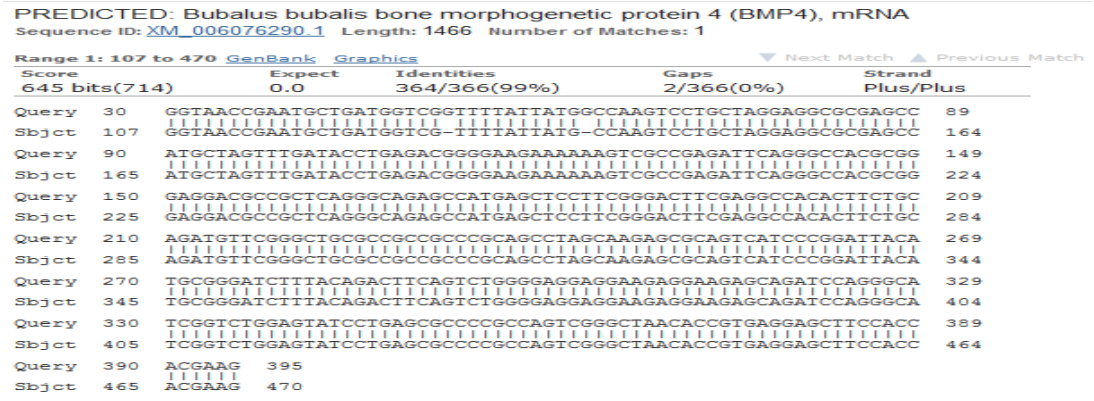


Fig (1) Representative samples of ethidium bromide-stained gel of PCR products; 1- 5 normal, 6- 10 repeat breeder and 11- 15 anestrum of amplified BMP 4 gene (exon 2 plus part of intron 2, 482 bp) in Egyptian water buffalo. Lane M: 100 bp ladder marker.



GGTAACCGAATGCTGATGGTCGGTTTTATTATGGCCAAGTCCTGCTAGGAGGCGCGAGCCATGCTA
GTTTGATACCTGAGACGGGGAAGAAAAAAGTCGCCGAGATTCAGGGCCACGCGGGAGGACGCCG
CTCAGGGCAGAGCCATGAGCTCCTTCGGGACTTCGAGGCCACACTTCTGCAGATGTTCGGGCTGCG
CCGCCGCCCGCAGCCTAGCAAGAGCGCAGTCATCCCGGATTACATGCGGGATCTTTACAGACTTC
AGTCTGGGGAGGAGGAAGAGGAAGAGCAGATCCAGGGCATCGGTCTGGAGTATCCTGAGCGCCC
CGCCAGTCGGGCTAACACCGTGAGGAGCTTCCACCACGAAGGTCAGTCCCTTACCTGGAATCTGG
ACTGGGGTGGGGCAGTGGAAGCTGTGGGAAGGCGAGGAGTTAA(**432 bp**).

Fig (2) Nucleotide sequences of BMP 4 gene (exon 2 plus part of intron 2), representative of normal fertile buffalo cow.



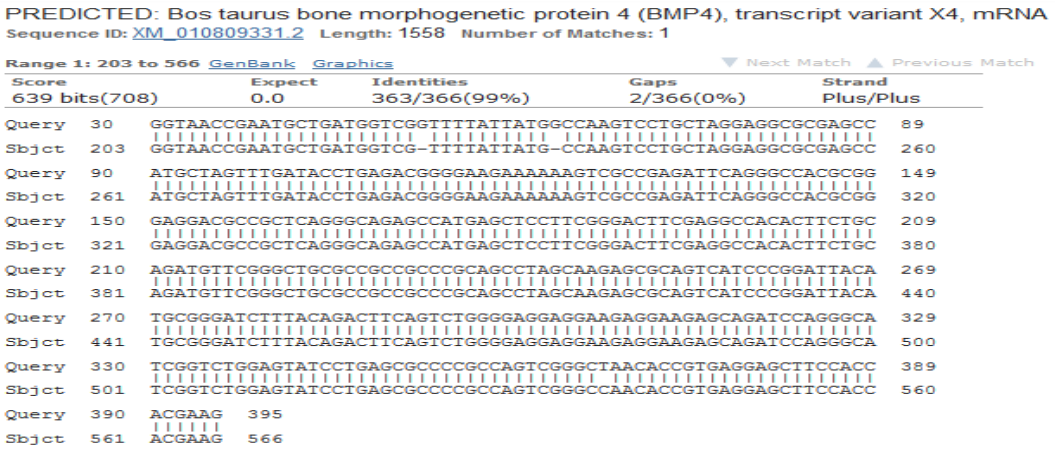
PREDICTED: Bubalus bubalis bone morphogenetic protein 4 (BMP4), mRNA
Sequence ID: XM_006076290.1 Length: 1466 Number of Matches: 1

Range 1: 107 to 470 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
645 bits(714)	0.0	364/366(99%)	2/366(0%)	Plus/Plus

```
Query  30   GGTAACCGAATGCTGATGGTCGGTTTTATTATGGCCAAGTCCTGCTAGGAGGCGCGAGCC  89
Sbjct  107  GGTAACCGAATGCTGATGGTCG-TTTTATTATG-CCAAGTCCTGCTAGGAGGCGCGAGCC  164
Query  90   ATGCTAGTTTGATACCTGAGACGGGGAAGAAAAAAGTCGCCGAGATTCAGGGCCACGCGG  149
Sbjct  165  ATGCTAGTTTGATACCTGAGACGGGGAAGAAAAAAGTCGCCGAGATTCAGGGCCACGCGG  224
Query  150  GAGGACGCCGCTCAGGGCAGAGCCATGAGCTCCTTCGGGACTTCGAGGCCACACTTCTGC  209
Sbjct  225  GAGGACGCCGCTCAGGGCAGAGCCATGAGCTCCTTCGGGACTTCGAGGCCACACTTCTGC  284
Query  210  AGATGTTCGGGCTGCGCCGCCGCCCGCAGCCTAGCAAGAGCGCAGTCATCCCGGATTACA  269
Sbjct  285  AGATGTTCGGGCTGCGCCGCCGCCCGCAGCCTAGCAAGAGCGCAGTCATCCCGGATTACA  344
Query  270  TGCGGGATCTTTACAGACTTCAGTCTGGGGAGGAGGAAGAGGAAGAGCAGATCCAGGGCA  329
Sbjct  345  TGCGGGATCTTTACAGACTTCAGTCTGGGGAGGAGGAAGAGGAAGAGCAGATCCAGGGCA  404
Query  330  TCGGTCTGGAGTATCCTGAGCGCCCCGCCAGTCGGGCTAACACCGTGAGGAGCTTCCACC  389
Sbjct  405  TCGGTCTGGAGTATCCTGAGCGCCCCGCCAGTCGGGCTAACACCGTGAGGAGCTTCCACC  464
Query  390  ACGAAG  395
Sbjct  465  ACGAAG  470
```

Fig (3) Nucleotide sequence alignment of *BMP 4* gene (exon 2 plus part of intron 2) using BLAST of representative samples showing 99% identity with *Bubalus Bubalis.*



PREDICTED: Bos taurus bone morphogenetic protein 4 (BMP4), transcript variant X4, mRNA
Sequence ID: XM_010809331.2 Length: 1558 Number of Matches: 1

Range 1: 203 to 566 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
639 bits(708)	0.0	363/366(99%)	2/366(0%)	Plus/Plus

```
Query  30   GGTAACCGAATGCTGATGGTCGGTTTTATTATGGCCAAGTCCTGCTAGGAGGCGCGAGCC  89
Sbjct  203  GGTAACCGAATGCTGATGGTCG-TTTTATTATG-CCAAGTCCTGCTAGGAGGCGCGAGCC  260
Query  90   ATGCTAGTTTGATACCTGAGACGGGGAAGAAAAAAGTCGCCGAGATTCAGGGCCACGCGG  149
Sbjct  261  ATGCTAGTTTGATACCTGAGACGGGGAAGAAAAAAGTCGCCGAGATTCAGGGCCACGCGG  320
Query  150  GAGGACGCCGCTCAGGGCAGAGCCATGAGCTCCTTCGGGACTTCGAGGCCACACTTCTGC  209
Sbjct  321  GAGGACGCCGCTCAGGGCAGAGCCATGAGCTCCTTCGGGACTTCGAGGCCACACTTCTGC  380
Query  210  AGATGTTCGGGCTGCGCCGCCGCCCGCAGCCTAGCAAGAGCGCAGTCATCCCGGATTACA  269
Sbjct  381  AGATGTTCGGGCTGCGCCGCCGCCCGCAGCCTAGCAAGAGCGCAGTCATCCCGGATTACA  440
Query  270  TGCGGGATCTTTACAGACTTCAGTCTGGGGAGGAGGAAGAGGAAGAGCAGATCCAGGGCA  329
Sbjct  441  TGCGGGATCTTTACAGACTTCAGTCTGGGGAGGAGGAAGAGGAAGAGCAGATCCAGGGCA  500
Query  330  TCGGTCTGGAGTATCCTGAGCGCCCCGCCAGTCGGGCTAACACCGTGAGGAGCTTCCACC  389
Sbjct  501  TCGGTCTGGAGTATCCTGAGCGCCCCGCCAGTCGGGCCAACACCGTGAGGAGCTTCCACC  560
Query  390  ACGAAG  395
Sbjct  561  ACGAAG  566
```

Fig (4) Nucleotide sequence alignment of *BMP 4* gene (exon 2 plus part of intron 2) using BLAST of representative samples showing 99% identity with *Bos taurus.*

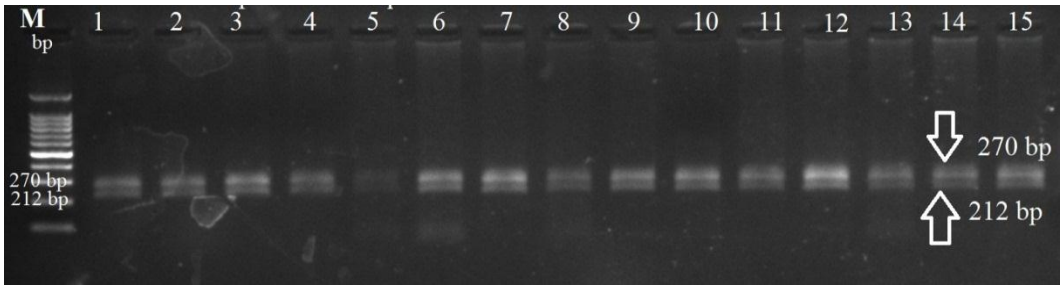


Fig (5) Representative samples of Ethidium- bromide gel showed the restriction banding pattern of BMP 4 (exon 2 plus part of intron 2) using Taqα1 gave product with fragments 270 and 212 bp, 1-5 normal fertile, 6- 10 repeat breeder and 11- 15 anestrous buffalo cows. M lane is 100 bp DNA ladder.

Restriction banding pattern of *BMP 4* (exon 2 plus part of intron 2) using *Hinf 1* gave product with fragments 279, 157 and 46 bp revealed only one type of monomorphic banding pattern fig (6)

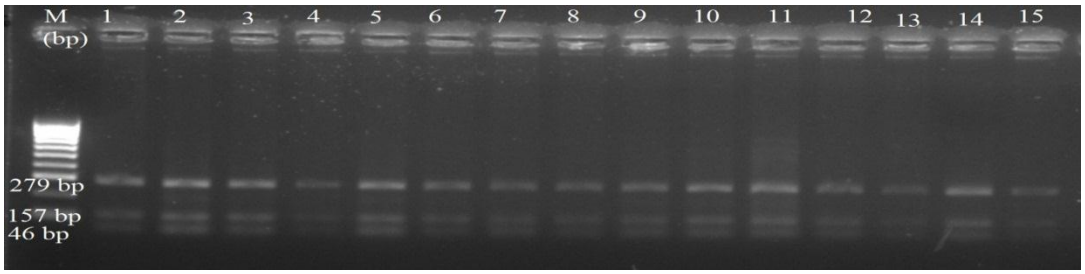


Fig (6) Representative samples of Ethidium- bromide gel showed the restriction banding pattern of *BMP 4* (exon 2 plus part of intron 2) using *Hinf1* restriction enzyme gave product with fragments 279, 157 and 46 bp. 1-5 normal fertile, 6- 10 repeat breeder and 11- 15 anestrous buffalo cows. M lane is 100 bp DNA ladder.

4- Discussion

Alignment of the obtained sequences from all group (normal cyclic, anestrum and repeat breeder) resulted in common sequences between them and no variation was found. Single Nucleotide Polymorphism (SNP) T387C was present at position 387 bp in all obtained sequences when compared with gene sequence of *bos taurus*. This SNP may be associated with the evolution between buffalo and cattle and can be utilized as a maker for species differentiation/ identification.

There is no mutation observed between tested animals. This result is similar to [13] that revealed that, there was no polymorphism in the *BMP 4* gene of goats. But they discovered two new SNPs (EU104684: g.1986A/G, 2203G/A) in the intronic region. Also, Polymorphism was not detected for exon 2 of *BMP 4* gene in four goat breeds in the study of [14].

In contrast to, [15] used the same primers (that used in our study) of cattle *BMP 4* (Gene Bank accession no.AC_000167.1) to amplify the goat *BMP 4*.They identified a novel SNP (G1534A) in exon 2. It was a non-synonymous mutation resulting in an arginine to lysine change in a corresponding protein sequence. They concluded that *BMP 4* is highly evolutionarily conserved, so this SNP gains further importance.

Also, [16] showed G>T mutation in *BMP 4* gene (SNP *rs109778173)* in Holstein cows. They identified 3 genotype TT, GG and GT cows. The G>T mutation at SNP *rs109778173* is classified as silent or synonymous, does not alter the amino acid sequence in the protein. Moreover, [17] analyzed *BMP 4* gene in four Chinese indigenous cattle breeds for any mutation in microsatellites present at 230 bp downstream from the termination site of *BMP 4* gene. Individuals with the AA genotype showed a CA dinucleotide with 19 repeat number and individuals with the AB genotype showed a CA dinucleotide with 19 and 18 repeat numbers.

In our study PCR-RFLP analysis, *Hinf1* restriction enzyme gave product with fragments 279, 157 and 46 bp. In all tested animal, no polymorphism was detected in exon 2 of *BMP 4*.

Hinf I used in cutting BMP 4 in such studies; [16] ***and*** [18] they performed PCR-RFLP on *BMP 4* in cattle using Hinf1 restriction enzyme. Three genotypes recognized on the banding pattern (GG, TT and GT) with genotypic frequencies of 0.64, 0.32, and 0.04, respectively.

Hinf1 restriction enzyme was used [19] to detect the polymorphism of *BMP 15* gene in goat. The restriction banding pattern revealed one fragments of 112 bp for AA.

PCR-RFLP analysis of *BMP 4* using *Taq$^{\alpha}$I* restriction endonuclease enzyme revealed only one type of restriction banding pattern yielding two fragments 270 bp and 212 bp in all samples.

RFLP banding pattern analysis after digestion with *Taq $^{\alpha}$1* and *Hinf1* restriction enzymes are monomorphic, no polymorphism was found in *BMP 4*, these confirm the previous obtained result of nucleotide sequencing. This agree with the concept of BMP4 is one of the best evolutionary conserved growth factors [20] and [20].

Due to the crucial role of *BMP 4* in follicular growth and differentiation, further studies should be performed with more number of buffaloes and other parts of *BMP 4* in order to validate the association between polymorphism of *BMP 4* and infertility problems in buffalo.

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