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Full Length Article

PCR-RFLP of bone morphogenetic protein 15 (BMP15/FecX) gene as a candidate for prolificacy in sheep

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ABSTRACT

Bone morphogenetic protein 15 (BMP15/FecX) gene is considered one of the major genes and a candidate marker for the reproduction in farm animals, especially sheep. The present study aimed to detect the genetic polymorphisms of BMP15 gene in sheep using PCR-RFLP technique. In the present study, 115 ewes were assigned into high and low prolificacy categories according to their reproductive history. In high prolific group (n = 20), ewes produced twins more than single births. In the low prolific type (n = 95), the ewes produced single births more than twins. DNA was extracted from blood samples of all ewes, subjected to PCR-RFLP analysis and confirmed by sequence analysis. The PCR products of 356 bp size were cut with HinfI restriction enzyme. Three digested fragments of 70, 117 and 169 bp were obtained in both types of sheep. All animals were homozygous with CC genotype. In conclusion, the accessible findings did not detect any mutation in FecX gene in sheep, regardless their prolificacy. Therefore, further attempts are necessary to detect other SNP for BMP-15 gene in Egyptian sheep breeds.

1. Introduction

Prolificacy in sheep is determined basically by the ovulation rate i.e. the number of ova released in each estrous cycle. The number of ovulated eggs reflects the number of pre-ovulatory follicles that are produced in each reproductive cycle [1]. Ovulation rate is under the control of single genes that means a single copy of genes increases ovulation rate as Booroola gene [2]. Mutation studies in different prolific sheep breeds showed that the transforming growth factor beta (TGF-β) super family containing the growth differentiation factor 9 (GDF9/FecG), bone morphogenetic protein 15 (BMP15/FecX) and bone morphogenetic protein receptor (BMPR1B/FecB) are the major determinants of the ovulation rate and litter size [3].

Bone morphogenetic protein-15 gene was described firstly in Romney sheep and named the Inverdale gene (FecXI) [4]. BMP-15 locus is X-linked gene and its expression occurs in the oocytes from the primary stage through ovulation [5]. The role of BMP-15 in early follicle development is species-specific and related to the differences between mono- and poly-ovulatory species [6,7]. BMP-15 gene has a crucial role in granulosa cell proliferation and differentiation during ovarian follicular development [8,9]. It stimulates granulosa cell mitosis and suppresses FSH receptor' s expression [7].

The mutation of BMP15/FecX gene includes *Lacaune* gene *(FecXL)* identified in the French Lacaune meat sheep with large litters [10]. In the Inverdale ewes, $FecX^{I}$ heterozygotes have more differentiated follicles in the ovary, fewer granulosa cell in these follicles, increased granulosa cells sensitivity to LH in the early stage of the follicle and smaller corpus luteum [11]. $FecX^{H}$, $FecX^{G}$ and $FecX^{B}$ have similar characteristics to $FecX^{I}$ [12,13].Two novel non-conservative mutations of BMP-15 called $FecX^{Gr}$ and $FecX^{O}$ have been identified in French Grivette and Polish Olkuska breeds related to hyper-prolificacy. It is noteworthy that the homozygous ewes of these mutations also had an increased ovulation rate without becoming sterile [14]. BMP15 gene looks to be associated with infertility and super fertility mechanisms in a dosage-sensitive manner [8].

Due to the great effect of BMP-15 gene mutations on ovulation rate, litter size, and prolificacy, this gene is considered one of the major genes and a candidate marker for the reproduction in farm animals,

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Table 1
Numbers of low and high prolific sheep.

Sheep type	Barki	Ossimi	Rahmani	Total
Low prolific	62	20	13	95
High prolific	6	7	7	20
Total	68	27	20	115

especially sheep. Therefore, the present study aimed to identify the genetic polymorphisms of BMP-15 gene in Egyptian sheep breeds of different prolificacy using PCR-RFLP technique and provide information about desirable genotypes of genes linked with twining.

2. Material and methods

2.1. Animals and blood sampling

A total number of 115 ewes (20 high prolific and 95 low prolific) kept in a private farm at Gamgara village, Benha, Egypt was used in this study (Table 1). Blood samples (10 mL) were collected from each animal through the jugular vein into EDTA containing tubes and were stored at −20 °C till DNA extraction. All samples were collected from all animals at the same time regardless their pregnant status (pregnant or not).

Animals were classified according to their reproductive history into two types, high and low of prolificacy. In high prolific type, ewes produced more twin birth than a single birth. While, in the low prolific, ewes produced a single birth more than twin birth. All the animals were kept under the same nutritional condition.

2.2. DNA extraction

DNA was extracted from whole blood using DNA preparation kit (Gena Bio Science, Germany), the concentration and purity of extracted genomic DNA were determined by spectrophotometer. DNA samples were diluted (1:50) with deionized water and the optical density (OD) was measured at wavelength 260 nm against deionized water as a blank. The purity of the isolated DNA was confirmed spectrophotometrically by measuring the OD at 260 and 280 nm, and the ratio of OD at 260/280 was calculated.

2.3. DNA amplification by polymerase chain reaction

The DNA fragment of a 356 bp product of the BMP-15 gene was amplified through polymerase chain reaction technique developed by Mullis et al. [15].The following components: 2.5 μL Buffer 10×, 1.5 μL of 25 mM Mg Cl_2, 0.5 μL of 2.5 mM dNTPs mixture, 0.5 μL Primer F,0.5 μL Primer R, 0.3 μL Taq polymerase(5U/μL), 18.2 μL water, and 1 μL DNA were added for each 25 μL reaction mixture.

The primers sequences were F: 5′TTCTCCGTCTAGGGGTATGAG3′ and R: 5′AGGGAACAAGAGCAAAGCGTTAGC3′ according to Shabir and Ganai [16]. The reaction was cycled at 95 °C for 5 min (an initial denaturation), then 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 8 min. PCR products were subjected to electrophoresis in 2.5% agarose gel, 1× TBE buffer with ethidium bromide, at 60 V for 30 min. The bands were visualized under ultraviolet *trans*-illumination and photographed in Gel-Doc equipment (Bio-Radv, USA).

2.4. Restriction fragment length polymorphism (RFLP)

PCR product for FecX gene was digested with Hinf1 restriction enzyme in a total volume of 30 μL (10 μL PCR product, 2 μL enzyme buffers, 1 μL digestion enzymes and 17 μL water) at 37 °C for 15–20 min. After digestion, the samples' fragments were visualized by gel electrophoresis at a concentration of 3% agarose.

2.5. Sequencing analysis

The PCR products were analyzed using direct sequencing by Macrogen Incorporation. Sequence analysis and alignment were carried out using NCBI/BLAST/blast suite.

3. Results

A 356 bp product of FecX gene was amplified using PCR-RFLP technique in all sheep under the study (Fig. 1). The PCR products of 356 bp size were cut with HinfI restriction enzyme into three digested fragments of 70, 117 and 169 bp in all sheep. The fragments were determined and confirmed by sequencing according to the presence of two restriction sites (G/ACTC and G/ATTC). All the animals were homozygous with CC genotype (Fig. 2).

DNA sequence analysis of 356 bp forward (a) and reverse (b) strands of BMP15 gene is presented in Fig. 3a and b.

The sequence alignment of 289 bp out of 356 bp of Egyptian sheep BMP15 with published sequence (accession number: FJ600402.1, Ovis aries & FJ600403.1, Ovisaries) was carried out using BLAST and showed 99% identity (Figs. 4 and 5, respectively).

4. Discussion

Five naturally mutations in exon 2 of the sheep BMP-15 gene have been identified, FecXG (Gallway), FecXB (Belclare), FecXI (Inverdale), FecXH (Hanna), FecXL (Lacaune), and cause infertility in homozygous ewes due to defects in early stages of folliculogenesis [13,17,18].

In the current study, a 356 bp fragment of BMP-15 gene was amplified successfully. The RFLP analysis for Hinf*1* demonstrated the existence of one pattern in all sheep at which homozygous CC genotypes

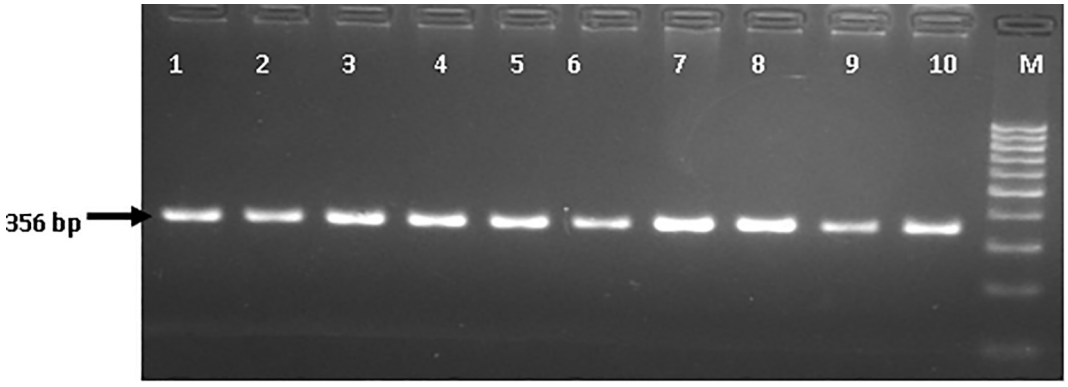

Fig. 1. Ethidium bromide-stained gel of PCR products of amplified BMP-15 gene. **M**: 100 bp ladder marker. **Lanes** 1–10: 356 bp PCR products amplified from Egyptian sheep DNA.

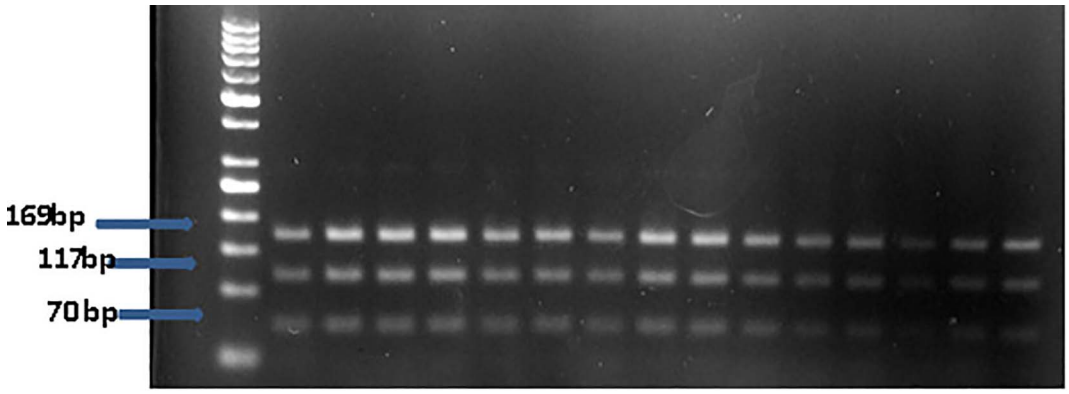

Fig. 2. Electrophoretic pattern of *Hinf*I restriction enzyme-digested PCR amplified sheep BMP-15 gene on electrophoretic gel 3%. **M**: 50 bp ladder marker. **Lanes** 1–15: Homozygous CC genotypes showed three digested fragments at 70, 117 and 169 bp.

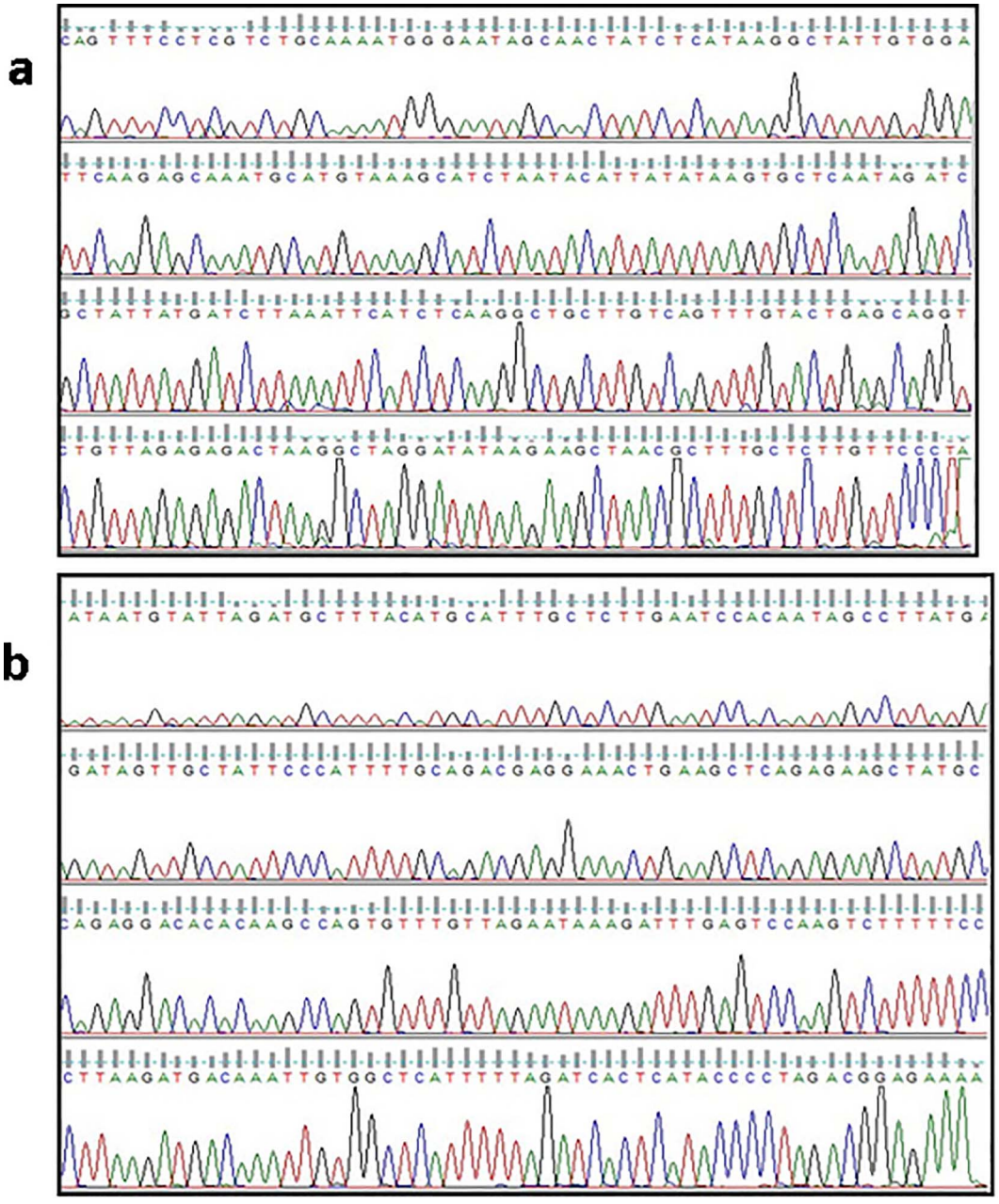

Fig. 3. DNA sequence chromatogram of forward (a) and reverse (b) DNA strand of BMP-15 gene of Egyptian sheep breeds.

Score	Expect	Identities	Gaps	Strand
518 bits(280)	9e-143	286/289(**99%**)	0/289(0%)	Plus/Plus

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Query  36   TGGACTCACATCTTCATTCTAACAAACACTGGCTTGTGTGTCCTCTGGCATAACTTCTCT  95
           |||||||| ||||| ||||||||||||||||||||||||||||||||||| |||||||
Sbjct  68   TGGACTCAAATCTTTATTCTAACAAACACTGGCTTGTGTGTCCTCTGGCATAGCTTCTCT  127
Query  96   GAGCTTCAGTTTCCTCGTCTGCAAAATGGGAATAGCAACTATCTCATAAGGCTATTGTGG  155
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  128  GAGCTTCAGTTTCCTCGTCTGCAAAATGGGAATAGCAACTATCTCATAAGGCTATTGTGG  187
Query  156  ATTCAAGAGCAAATGCATGTAAAGCATCTAATACATTATATAAGTGCTCAATAGATCGCT  215
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188  ATTCAAGAGCAAATGCATGTAAAGCATCTAATACATTATATAAGTGCTCAATAGATCGCT  247
Query  216  ATTATGATCTTAAATTCATCTCAAGGCTGCTTGTCAGTTTGTACTGAGCAGGTCTGTTAG  275
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248  ATTATGATCTTAAATTCATCTCAAGGCTGCTTGTCAGTTTGTACTGAGCAGGTCTGTTAG  307
Query  276  AGAGACTAAGGCTAGGATATAAGAAGCTAACGCTTTGCTCTTGTTCCCT  324
           |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  AGAGACTAAGGCTAGGATATAAGAAGCTAACGCTTTGCTCTTGTTCCCT  356
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Fig. 4. The sequence of Ovis aries bone morphogenetic protein 15 (BMP-15) gene, BMP-15-A allele, intron 1 (alignment by Blast, sequence ID: **FJ600402.1**).

showed three digested fragments. DNA sequence analysis of 356 bp forward and reverse strands of BMP-15 gene declared the absence of any polymorphism in the studied sheep breeds and the ovine coding region of BMP-15 showed 99% similarity with other sheep breeds at the nucleotide level. These results indicated that sheep breeds (Barki, Ossimi and Rahmani) either high or low prolific type lack the FecX locus mutation of BMP-15 gene. In this context, none of the Javanese, Thoka, Woodlands, Olkuska, Lacaune, Belclare, and Cambridge sheep had the *FecXI* mutation despite their high prolificacy [19]. Sequence analysis in former studies revealed that none of the mutations in the BMP-15 were verified in Finn sheep [20], small tailed Han sheep [21], Iranian Baluchi sheep [22] or Sangsari sheep [23].

However, the current results disagree with Elkorshy et al. [24], who identified genetic polymorphism of BMP-15 gene in three native Egyptian breeds (Barki, Rahmani, and Ossimi) as well as two Saudi sheep breeds (Najdi and Harri) using PCR-RFLP with Hinf*I* digestion to identify the genotyping of FecXG loci in exon 2. Also, Hanrahan et al. [13], Davis et al. [25], Guan et al. [26], Chu et al. [27], and Jamshidi et al. [28] documented polymorphism in FecX gene in sheep. Zamani et al. [29] found a new point mutation (G → A) of BMP-15 gene exon 2 in Mehraban and Lori ewes using DNA sequencing methods.

Absence of mutation in our work may be due to the decreased number of ewes and the restriction to one part of the genes. So we need further study with large number of animals and the genetic improvement of ovulation rate through crossbreeding with foreign breed is recommended.

5. Conclusions

The absence of mutation in the FecX locus indicated that it is not the major gene contributes to the large number of lambs per lambing. Further studies using different techniques as genome-wide association are mandatory to delineated the framework governing the twin-lambing performance in Egyptian sheep breeds.

Conflict of interest

The authors declare no conflict of interest.

Score	Expect	Identities	Gaps	Strand
512 bits(277)	4e-141	285/289(99%)	0/289(0%)	Plus/Plus

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Query  36   TGGACTCACATCTTCATTCTAACAAACACTGGCTTGTGTGTCCTCTGGCATAACTTCTCT  95
           |||||||| ||||| ||||||||||||||||||||||||||||||||||| |||||||
Sbjct  68   TGGACTCAAATCTTTATTCTAACAAACACTGGCTTGTGTGTCCTCTGGCATAGCTTCTCT  127
Query  96   GAGCTTCAGTTTCCTCGTCTGCAAAATGGGAATAGCAACTATCTCATAAGGCTATTGTGG  155
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  128  GAGCTTCAGTTTCCTCGTCTGCAAAATGGGAATAGCAACTATCTCATAAGGCTATTGTGG  187
Query  156  ATTCAAGAGCAAATGCATGTAAAGCATCTAATACATTATATAAGTGCTCAATAGATCGCT  215
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188  ATTCAAGAGCAAATGCATGTAAAGCATCTAATACATTATATAAGTGCTCAATAGATCGCT  247
Query  216  ATTATGATCTTAAATTCATCTCAAGGCTGCTTGTCAGTTTGTACTGAGCAGGTCTGTTAG  275
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  248  ATTATGATCTTAAATTCATCTCAAGGCTGCTTGTCAGTTTGTACTGAGCAGGTCTGTAAG  307
Query  276  AGAGACTAAGGCTAGGATATAAGAAGCTAACGCTTTGCTCTTGTTCCCT  324
           |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  AGAGACTAAGGCTAGGATATAAGAAGCTAACGCTTTGCTCTTGTTCCCT  356
```

Fig. 5. The sequence of Ovis aries bone morphogenetic protein 15 (BMP-15) gene, BMP-15-B allele, intron 1 (alignment by Blast, sequence ID: **FJ600403.1**).

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