

GENETIC POLYMORPHISM DETECTION IN BONE MORPHOGENETIC PROTEIN 15 (BMP15) GENE RELATED TO FECUNDITY IN TWO EGYPTIAN SHEEP BREEDS

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Abstract: This study was intended to detect the polymorphism of bone morphogenetic protein 15 (BMP15) gene that can act as marker influencing fertility for increasing litter size in Egyptian sheep breeds (191 Saidi and 145 Ossimi females). In this study, the mean litter size, showed highly significant between Saidi and Ossimi sheep breeds, however, litter size of Saidi white sheep was significantly decreased compared to black and brown. Blood samples were collected from 19 Saidi and 13 Ossimi female and then genomic DNA was extracted. A portion of bone morphogenetic protein 15 (BMP15) gene, 310 bp was amplified using specific primers, and was sequenced and analyzed to clarify the phylogenetic relationship of Egyptian breed sheep. The data suggested that the gene shared a similarity in sequence compared to 9 accession numbers of *Ovis aries* found in GenBank. Molecular phylogenetic analyses were performed based on nucleotide sequences in order to examine the position of the Egyptian breeds among many other sheep breeds. The results indicate that 5 accession numbers of *Ovis aries* are closely related with Ossimi and Saidi female that produce single or twins lamb in UPGMA analysis. In addition, PCR-RFLP method using *Pst*I and *Msp*I restriction enzymes was used to mask polymorphisms of partial exon 2 in 18 female sheep. Results showed that FecX gene was monomorphic and disagreement with litter size, therefore, it is indispensable to survey other gene in order to establish marker assisted selection technique.

Key words: Sheep, Litter size, BMP15 gene, PCR-RFLP, Phylogenetic tree

Introduction

The main objective of sheep breed in the world is one or more of the following: Meat, milk, and wool production, where in Egypt the sheep meat production is more important than fiber production, and the sheep contribute 6% of the total red meat produced (*Abulyazid et al., 2011*). Sheep occupy a special niche in the Egyptian agricultural production system and are important for the rural economy, where the total sheep population in Egypt is 5,488,000 heads (*FAO, 2014*). There are three major breeds in Egypt; Rahmani, Ossimi, and Barki. Rahmani is distributed mainly in north of the Nile delta, Ossimi in mid Egypt and Barki in western Mediterranean coastal region. Minor breeds like Saidi, Sohagi, located in south Egypt, (*ICARDA, 2006*). In Egypt efforts are being made to intensify production systems, primarily through changing reproductive management and crossing native breeds with introduced breeds (*Ibrahim et al., 2010*).

The profitability of sheep farming mainly depends on lamb production per ewe and litter size. Both are important economical traits in sheep breeding and genetics, which mainly depend on breed. Also prolificacy refers to the ability of the female to produce multiple lambs through high ovulation rates and embryo survival. The ovulation rate and litter size are dependent on the interactions of endocrine and paracrine mediators in mammals (*Zhu et al., 2013*). Reproduction is a complex process and fecundity traits such as ovulation rate and litter size can be genetically regulated by many genes with small effects, and sometimes also by single genes with major effects, called fecundity (Fec) genes (*Drouilhet et al., 2009*). Various major genes have been reported to affect prolificacy in sheep, include three related oocyte derived components, namely, bone morphogenetic protein receptor type 1B (BMPR1B), known as FecB on chromosome 6 (*Souza et al., 2001*); growth differentiation factor 9 (GDF9), known as FecG on chromosome 5 (*Hanrahan et al., 2004*) and bone morphogenetic protein 15 (BMP15), known as FecX on chromosome x (*Galloway et al., 2000; Harahan et al., 2004*).

The mutations in the BMP15 gene increase ovulation rate in heterozygous individuals. Heterozygous ewes show multiple ovulations, earlier maturation of granulosa cells and reduced follicle size (*Bodin et al., 2007*). There are several point mutations in BMP15 gene, identified in different sheep breeds (*Galloway et al., 2000; Demars et al., 2013; Shabir et al., 2013; Zamani et al., 2015*). The BMP15 gene significantly affects prolificacy and ewes with two inactive copies of the BMP15 gene (homozygous animals) are sterile and exhibit a similar ovarian phenotype (*Galloway et al., 2000; Hanrahan et al., 2004*). Ewes with a single inactive BMP15 gene (heterozygous animals) are fertile and exhibit an increased ovulation rate and an increased incidence of twin or triplet births (*Davis, 2004; Kasiriyani et al., 2009; Monteagudo et al., 2009*). In Egypt, the genetic diversity of indigenous sheep in respect to these important economic genes has not been

sufficiently studied. Therefore, it becomes essential to make fingerprinting of some genes related to economic traits such as litter size, in order to determine the polymorphism pattern of these genes in the Egyptian sheep breeds. This investigation was carried out to explore the presence of polymorphism in BMP15 gene (exon 2) using DNA sequencing and PCR-RFLP methods in two Egyptian sheep breeds, that can act as marker influencing fertility and helpful in breed selection for genetic improvement programs.

Materials and Methods

Experimental animals

The Saidi and Ossimi sheep breeds used in this study were selected based on their single/twins production in three repetitive production cycles. The data of 191 Saidi and 145 Ossimi female sheep were collected from different farms belonging to the Ministry of Agriculture in Fayoum, Bani-Suef and Minia Governorates. These data were used to study the reproduction traits *i.e.* lambing rate (%), fecundity rate (%), twinning rate (%), triplet rate (%) and litter size. Nineteen Saidi individuals (12 ewes which producing twins and 7 which producing single) and 13 Ossimi individuals (7 ewes which producing twins and 6 which producing single) were used to study the polymorphism.

Statistical analysis

Data were statistically analyzed using the SPSS program, version 16.0 (SPSS, 2007). Means were compared for main effects and their interaction by Duncan's multiple range test (Duncan, 1955), when significant F values were obtained ($P < 0.05$).

Blood sampling

Whole blood samples (5 ml) were collected from Jugular vein for each ewe of 19 Saidi and 13 Ossimi female in vacutainer glass tubes containing EDTA (1 mg/ml). Blood samples were transferred to the laboratory in an ice box kept at 4°C until used. The experimental procedures were performed according to protocols approved by the Biological Studies Animal Care and Use Committee of Egypt. All efforts were made to minimize any discomfort during blood collection.

DNA extraction

Genomic DNA was extracted from whole blood samples using Xanthogenate protocol described by Tillet and Neilan (2000) with some modification. The quantified DNA was stored at -20°C until further processing of PCR amplification of BMP15 gene.

PCR Amplification of BMP15 gene

Specific primers 5´-GCAGGCAGTATTGCATCGGAAG-3´ and reverse 5´-CCTCAATCAGAAGGATGCTAATGG-3´ were used to amplify one region of BMP15 gene (Exon 2) which corresponded to the GenBank accession number AH009593 (*Gholibeikifard et al., 2014*). The primer was synthesized by Invitrogen, Biotechnology Co. Ltd. (USA). The PCR amplification was performed in 25 µl total volume, each PCR reaction mixture containing 12.5 µl Master Mix (onePCR™), 1 µl of each primer, 2 µl of genomic DNA (50 ng/ µl) and 8.5 µl of sterile deionized water. PCR conditions were as follows an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min using thermal cycler 2720 (Applied Biosystems, USA). PCR products were checked by electrophoresis using 1.8% agarose gel in 1X TAE buffer. The products were then purified using QIAquick Gel extraction kit # 28706 (QIAGEN) following manufacturer instructions and sequenced by automated DNA sequencing reactions, which were performed using a sequencing ready reaction kit (Life Technologies) in conjunction with ABI-PRISM and ABI-PRISM big dye terminator cyclers.

DNA Sequence and Phylogenetic analysis

A consensus sequence of BMP15 fragments from both Saidi and Ossimi ewes which producing twins and single was constructed by using the SeqMan™ II 4.05 package for windows 32. These sequences were subjected to alignment with BMP15 sequences of the GenBank, EMBL, DDBJ and PDB from breeds of *Ovis aries* using the BLASTN 2.2.18 and BLASTP 2.2.18 (Basic Local Alignment Search Tool) algorithm at <http://www.ncbi.nlm.nih.gov/>. The MEGA version 5.2 programs (*Tamura et al., 2011*) was used to generate a phylogenetic tree using the UPGMA method according to *Sneath and Sokal (1973)*. The evolutionary distances were computed using the Maximum Composite Likelihood method (*Tamura et al., 2004*).

Restriction Fragment Length Polymorphism (RFLP) analysis

Nineteen PCR products of partial BMP15 gene (exon 2) were digested using *PstI MspI* restriction enzymes (Fermentas, Germany, #ER0611) according to the manufacturer instructions. A final reaction volume of 32 µl containing 10 µl PCR product, 18 µl H₂O free of nuclease, 2 µl of 10X buffer and 2 µl (5 units) of each restriction enzymes. The final volume of mixture was mixed gently and spins down for few seconds then incubated for 18 hours at 37 °C in water bath and stopped at 65 °C for 10 min. Restriction digestion products were checked by electrophoresis using 3% agarose gel in 1X TAE buffer and staining with ethidium bromide. The 100-bp ladder was used as molecular size marker.

Results and discussion

Reproduction traits

Reproductive ability has an important role in profitability of sheep production. The production and fertility traits of 191 and 145 individuals of sheep breeds with different families from Saidi (black, brown and white) and Ossimi sheep, respectively are summarized in Table (1). The data showed that, there is no significant in the average number of ewes mated between Saidi and Ossimi breed sheep. Also no significant between Saidi black and brown in the average number of ewes lambing, ewes lambing twin, ewes lambing triplet and live lambs born, while it's a significantly when compared with Saidi white and Ossimi sheep. The average number of dead lambs born and the average number of total lambs birth for Saidi black, Saidi brown and Ossimi sheep were significantly higher compared with Saidi white sheep. The higher average number of dead lambs born (10) was found in Saidi black sheep and the fewer average number (4.33) was found in saidi white sheep. The Saidi sheep (black, brown and white) had average number of ewes lambing triplet (0.67, 0.33 and 0.17), respectively, where no ewes lambing triplet in Ossimi sheep.

Fertility traits have a major impact on efficiency and profitability in lamb meat production. In this respect twinning rates and litter size are reflected ovulation rate, which they important economic value. As shown in Table (1), the litter size, twinning rate and triplet rate showed highly significant between Saidi and Ossimi sheep breeds, while lambing rate and fecundity rate showed no significant. However, the mean litter size of Saidi white sheep was significantly decreased compared to black and brown. The genetics of sheep litter size has been investigated (*Hanrahan et al., 2004; Mishra, 2014; Wan Samarny et al., 2013; Zamani et al., 2015*). Multiple genes were identified having substantial effects on reproduction traits and some of them are most important being affecting prolificacy in animals. High litter size or twinning is an economically important trait that enhances sheep productivity in terms of producing a higher number of lambs, meat and wool (*Mishra, 2014*).

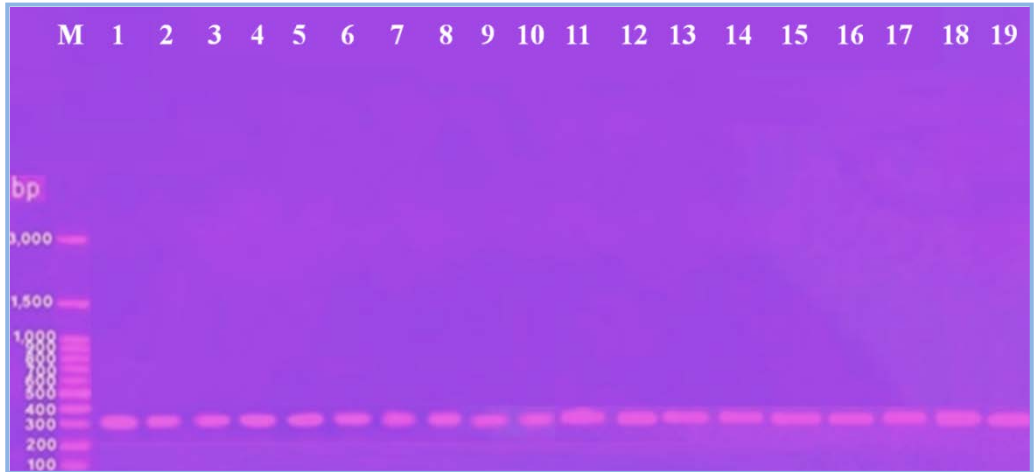
Table 1. Mean traits of Saidi breeds (Black, Brown and White) and Ossimi breeds sheep

Traits	Saidi sheep			Ossimi sheep	± SEM
	Black	Brown	White		
Av. number of ewes mated	28.75	27.08	25.42	28.75	0.64
Av. number of ewes lambing	25.50 ^a	23.08 ^a	11.50 ^b	24.00 ^a	0.99
Av. number of ewes lambing twins	8.17 ^a	7.08 ^a	3.08 ^b	4.75 ^b	0.43
Av. number of ewes lambing triplet	0.67 ^a	0.33 ^{ab}	0.17 ^{ab}	0.00 ^b	0.09
Av. number of live lambs born	25.00 ^a	23.00 ^a	13.08 ^b	24.33 ^a	1.05
Av. number of dead lambs born	10.00 ^a	7.83 ^b	1.58 ^d	4.33 ^c	0.57
Av. number of total lambs birth	35.00 ^a	30.83 ^{ab}	14.67 ^c	28.75 ^b	1.39
Lambing rate (%) ¹	88.85 ^a	85.63 ^a	44.31 ^b	82.75 ^a	2.83
Litter size ²	1.38 ^a	1.28 ^b	1.25 ^b	1.13 ^c	0.02
Fecundity rate (%) ³	86.12 ^a	84.32 ^a	49.30 ^b	84.47 ^a	2.91
Twinning rate (%) ⁴	34.53 ^a	32.81 ^{ab}	25.91 ^{bc}	19.55 ^c	1.57
Triplet rate (%) ⁵	2.68 ^a	1.22 ^{ab}	1.20 ^{ab}	0.00 ^b	0.38

1. Lambing rate (%) = (Number of ewes lambing / Number of ewes mated) X 100.
2. Litter size = Total number of lambs birth / Number of ewes lambing.
3. Fecundity rate (%) = (Number of live lambs born / Number of ewes mated) X 100.
4. Twinning rate (%) = (Number of ewes lambing twins / Number of ewes lambing) X 100.
5. Triplet rate (%) = (Number of ewes lambing triplet / Number of ewes lambing) X 100.

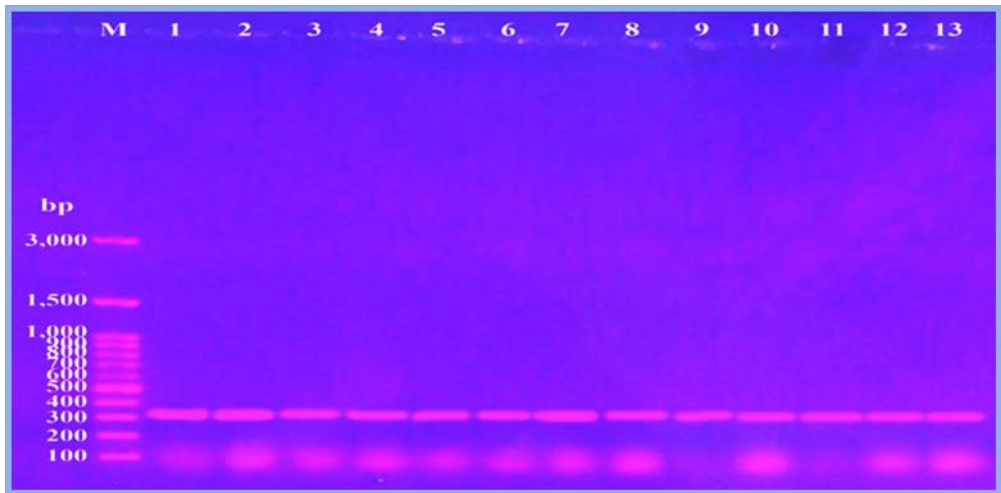
Properties of BMP15 gene (exon 2) sequence

A single fragment of approximately 310 bp nucleotide sequences was amplified from each ewe individual (19 Saidi and 13 Ossimi) sheep breeds (Figures 1 and 2). Alignments of four sequences from Saidi and Ossimi female that produce single or twins lamb revealed 100% similarity between them. The DNA sequence compositions are 54 (A), 70 (C), 66 (T) and 74 (G). The nucleotide frequencies were 23% (A), 28% (T), 29% (C) and 20% (G).



Lanes 3-7, 9, 12, 15, and 17-18: Saidi black color, Lanes 1-2, 8, 11, 14, 16 and 19: Saidi brown color
Lane 10 and 13: Saidi white color, M: 100 bp DNA ladder.

Figure 1. PCR amplification of partial exon 2 fragments of BMP15 gene from Saidi sheep breed



Lanes 1-13: Ossimi white color, M: 100 bp DNA ladder.

Figure 2. PCR amplification of partial exon 2 fragments of BMP15 gene from Ossimi sheep breed

Phylogenetic analysis

The topology of UPGMA tree of Saidi and Ossimi sheep breeds with 9 accession numbers of *Ovis aries* in the GenBank database represented a

monophyletic group (Figure 3). The DNA sequences of BMP 15 gene successfully grouped Egyptian breeds and *Ovis aries* sheep into two main cluster. The first cluster is extremely diverse and consisted of two accession numbers (JN655671 and JN655672). The second cluster had Egyptian sheep breeds and closely related with 5 accession numbers (HM583335, AH009593, KT853038, KT013294 and NM1114767) whereas, two accession numbers (HM583333 and HM583334) were the most distant. The genetic relationship of BMP15 based on nucleotide sequence using UPGMA revealed that accession numbers were closer while accession numbers were farther apart. This is in accordance with *Misra et al.*, (2011); *Bibinu et al.*, (2016).

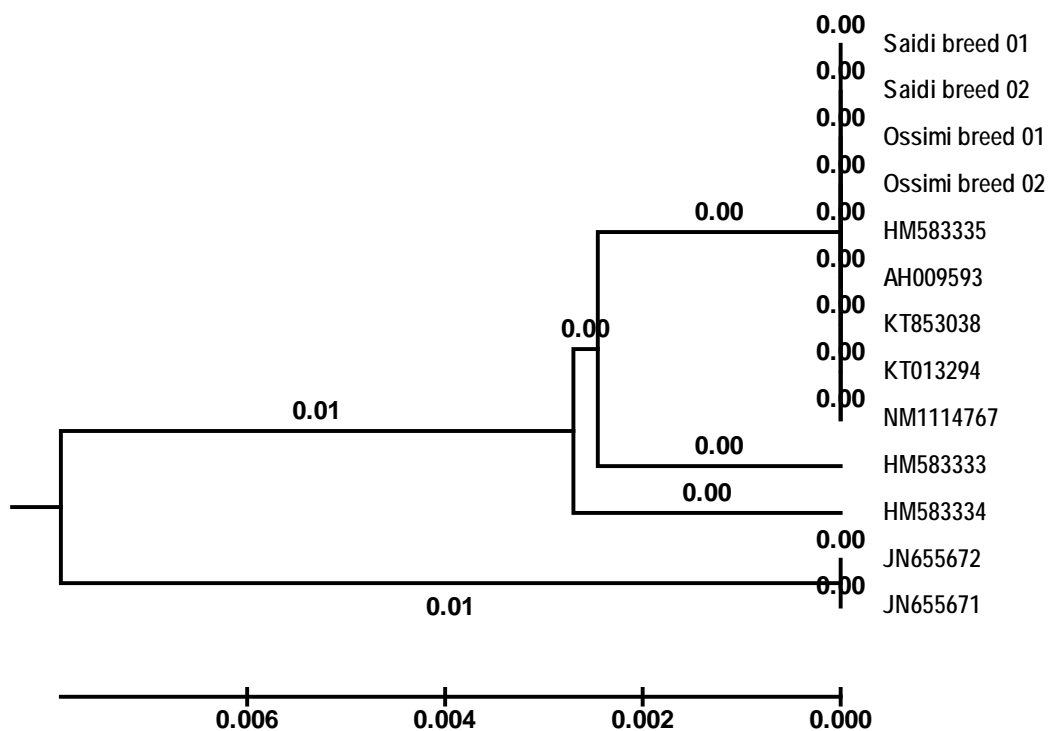
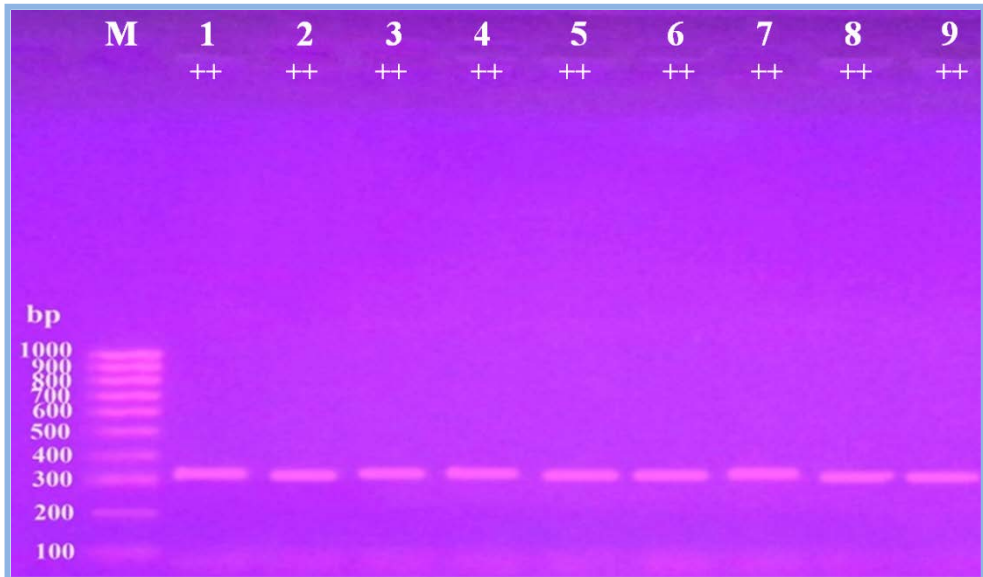


Figure 3. UPGMA dendrogram of 13 *Ovis aries* sheep generated based on Sneath and Sokal distances. Branch lengths are shown above the branches of clades

Saidi breed 01: Black color female which producing twins lamb, Saidi breed 02: Brown color female which producing single lamb, Ossimi breed 01: White color female which producing twins lamb, Ossimi breed 02: White color female which producing single lamb

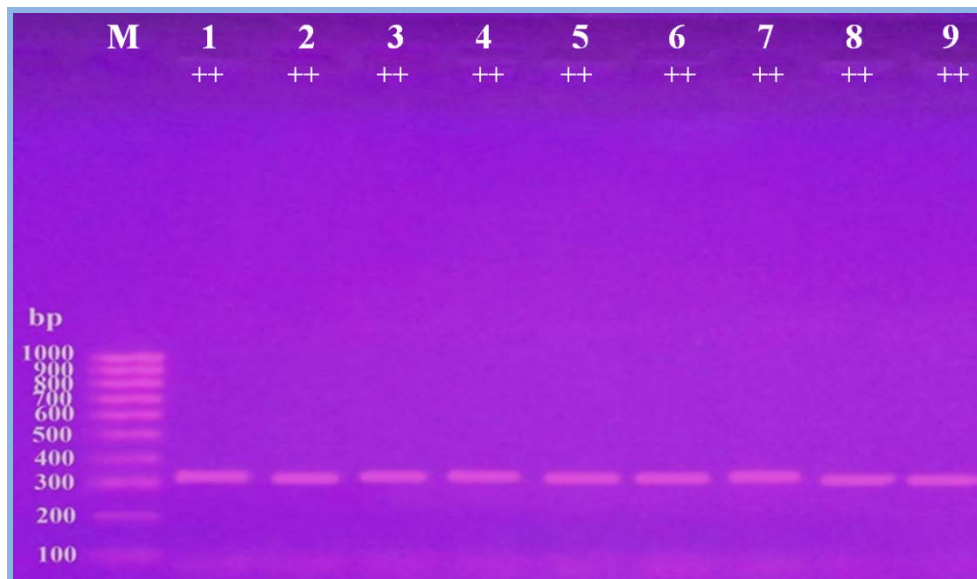
Restriction Fragment Length Polymorphism (RFLP) analysis

PCR-RFLP is a rapid, simple and exact technique for single nucleotide polymorphism (SNP) genotyping. The forced PCR-RFLP approach has been used previously to genotype prolific sheep (*Wilson et al., 2001*). The PCR products of BMP15 gene (exon 2) digested by *PstI* and *MspI* restriction enzymes to survey existence of mutations. Digested products were run on 3% agarose gel electrophoresis, which bands with 310 bp in length were observed, in all 9 Saidi ewes (Figures 4 and 6) and also in all 9 Ossimi ewes (Figures 5 and 7). The results showed that Saidi and Ossimi ewes producing single and twins lamb had a single band at 310 bp position indicating absence of mutation in the *FecX⁺⁺*, Figures (4, 5, 6 and 7). It can be assume that the cause of twinning in Saidi and Ossimi sheep breed might be due to the effect of other fecundity genes that segregate in other prolific sheep breed, or may be a combination of gene products that stimulate/alter the ovulatory cycle. The results found in the present study are in accordance with those obtained for Madras Red, Deccani, Bunnur breeds (*Wilson et al., 2001; Davis et al., 2006*) and Egyptian sheep breeds (*Amr and El-Saadani, 2009*).



Lanes 1, 2, 5, 6 and 9: Saidi black color with genotype ++, Lanes 3, 7 and 8: Saidi brown color with genotype ++, Lane 4: Saidi white color with genotype ++, Lanes 1-3: single producing female, Lanes 4-9: twins producing female, M: 100 bp DNA ladder.

Figure 4. Digestion product of partial exon 2 fragments of BMP15 gene with *PstI* restriction enzyme from Saidi breed



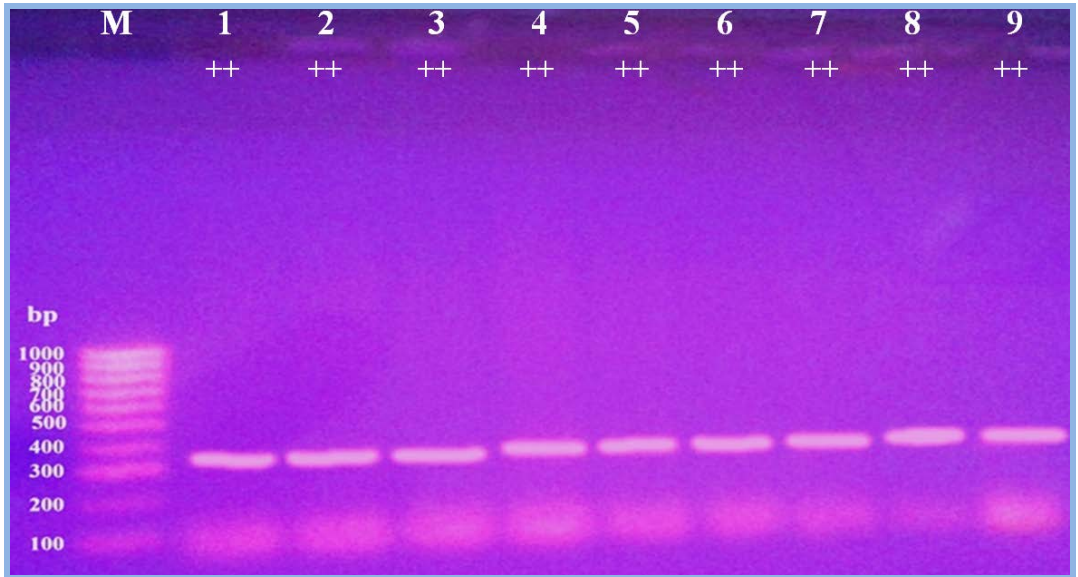
Lanes 1-9: Ossimi white color with genotype ++, Lanes 1-3: single producing female, Lanes 4-9: twins producing female, M: 100 bp DNA ladder.

Figure 5. Digestion product of partial exon 2 fragments of BMP15 gene with *PstI* restriction enzyme from Ossimi breed

At least six different mutations have been identified in the BMP15 gene (Galloway et al., 2000; Hanrahan et al., 2004; Bodin et al., 2007; Martinez-Royo et al., 2008; Monteagudo et al., 2009; Lahoz et al., 2011) wherein ewes heterozygous for the mutations have increased ovulation rates between 0.8 and 2.4 above that of the respective non carrier flocks (Hanrahan et al., 2004). Animals homozygous for each of these mutations are an ovulatory and thus infertile. While all the mutations have similar general effects on fertility, there are subtle differences, as the increases in ovulation rate observed in the heterozygous animals vary from 35 to 100% (McNatty et al., 2004). Heterozygous ewes with mutations in both *FecB* and *FecX* exhibited increased fertility compared with ewes harboring a mutation in only one of these genes (Mishra, 2014).

Davis et al. (2006) reported that none of mutation in BMP15 gene isolated from Hu breed which high prolific sheep in China. Gursel et al. (2011) showed that none of Chios, Kivircik, Awassi and Imrose sheep breeds carries *FecX^H*, *FecX^I* and *FecX^B* mutations. In the other hand, Wang et al. (2011) and Abdel-Rahman et al. (2013) found two polymorphisms in exon 2 of BMP15 in different goat breeds. These findings show that there are differences in prolificacy inheritance patterns between sheep and goat and other species and even among different breeds, possibly. Mutations in *FecB* and *FecX* genes were not the only factors responsible

for high prolificacy (*Guan et al., 2007*) as Malin sheep which lacked mutations in these genes were highly prolific. The highly prolific sheep amongst the Egyptian breeds may indicate a presence of a genomic influence. While this could be related to differences in the background genetics of the various sheep breed.

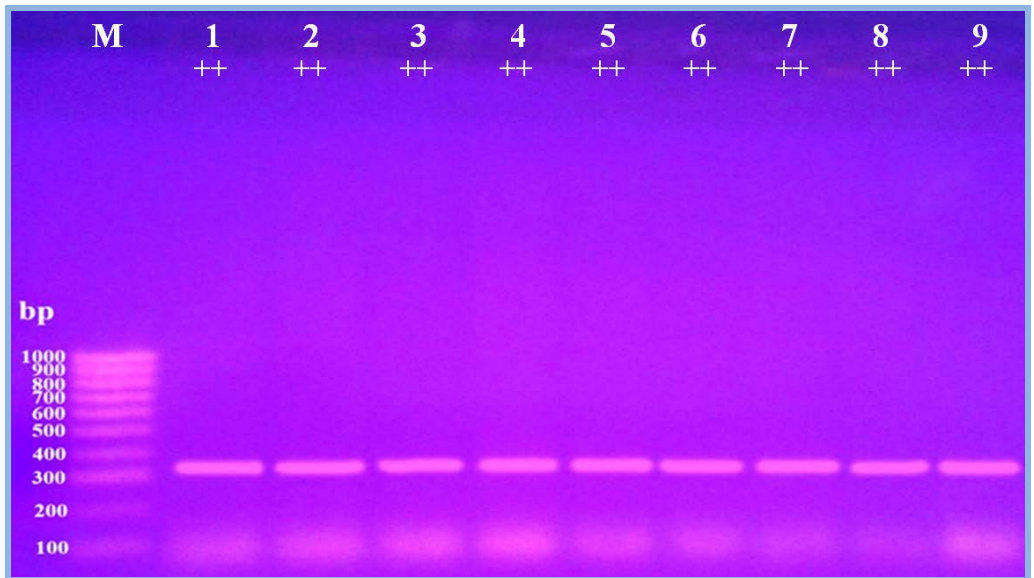


Lanes 1, 2, 5, 6 and 9: Saidi black color with genotype ++, Lanes 3, 7 and 8: Saidi brown color with genotype ++, Lane 4: Saidi white color with genotype ++, Lanes 1-3: single producing female, Lanes 4-9: twins producing female, M: 100 bp DNA ladder.

Figure 6. Digestion product of partial exon 2 fragments of BMP15 gene with *MspI* restriction enzyme from Saidi breed

Conclusion

The sequence analysis and diversity of polymorphism of the isolated BMP15 gene (exon 2) has been studied. It can be concluded that 100% similarity between Saidi and Ossimi female that produce single or twins lamb. PCR-RFLP used for detection of FecX mutations showed that there are not any polymorphisms in tested Saidi and Ossimi sheep. The genetic factor affecting fecundity should be investigated further by other candidate gene due to its higher litter size.



Lanes 1-9: Ossimi white color with genotype ++ , Lanes 1-3: single producing female, Lanes 4-9: twins producing female, M: 100 bp DNA ladder.

Figure 7. Digestion product of partial exon 2 fragments of BMP15 gene with *MspI* restriction enzyme from Ossimi breed

Detekcija genetskog polimorfizma u genu BMP15 u vezi sa plodnošću dve egipatske rase ovaca

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Rezime

Svrha ovog istraživanja je bila da se otkrije polimorfizam koštanog morfogenetskog proteina 15 (BMP15) gena koji može da deluje kao marker koji utiče na plodnost u smislu povećanja veličine legla egipatskih rasa ovaca (191 ženka rase Saidi i 145 ženki rase Ossimi). U ovoj studiji, srednja veličina legla, je bila veoma značajno različita između rase Saidi i Ossimi, međutim, veličina legla Saidi bele ovce je značajno manje u odnosu na crnu i braon. Uzorci krvi su

sakupljeni od 19 ovaca rase Saidi i 13 ovaca rase Ossimi i zatim ekstrahovana genomska DNK. Deo morfogenetski proteinskog 15 (BMP15) gena, 310 bp je pojačan upotrebom specifičnih prajmera, zatim sekvencioniran i analiziran kako bi se razjasnio filogenetski odnos egipatske rase ovaca. Podaci ukazuju da gen deli sličnost u nizu u odnosu na 9 brojeva pristupanja u *Ovis aries* koji se nalaze u GenBank. Molekularne filogenetske analize su izvedene na osnovu sekvence nukleotida u cilju ispitivanja pozicije egipatskih rasa među mnogim drugim rasama ovaca. Rezultati pokazuju da je 5 brojeva pristupanja *Ovis aries* blisko povezano sa ovcama rasa Ossimi i Saidi koje rađaju jedno jagnje ili blizance, kako pokazuje UPGMA analiza. Pored toga, PCR-RFLP metoda, koja koristi *Pst*I i *Msp*I restrikcione enzime, je korišćena da prikrije polimorfizam delimičnog eksona 2 u 18 ovaca. Rezultati su pokazali da FecX gen je monomorfan i u neslaganju sa veličinom legla, stoga, neophodno je da se pronađe drugi gen u cilju uspostavljanja tehnike MAS.

Ključne reči: ovce, veličina legla, BMP15 gen, PCR-RFLP, filogenetsko stablo

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