

Genetic polymorphism of candidate genes for fecundity traits in Egyptian sheep breeds.

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Abstract

In reproductive planning, intervals between lambings, season, age of ewe, heat stress, nutrition state or breed are some of the factors which have a great effect on sheep fertility. Different mutations in the growth differentiation factor 9 (*GDF9*) and the bone morphogenetic protein 15 (*BMP15*) genes cause an increase in the ovulation rate in sheep. The aim of the current study was to determine the mutations in *GDF9* and *BMP15* genes and the probability of their effect on fertility in the major Egyptian sheep breeds. Blood samples were collected using EDTA from the Barki, Ossimi and Rahmani sheep breeds at different locations in Egypt, and DNA was then isolated using the salting out method. The PCR products of the genes were digested with *MseI* and *DdeI* restriction enzymes for the *GDF9* gene, and with *HinfI* and *DdeI* for the *BMP15* gene. The results showed that the PCR products digested with restriction enzymes exhibited a substitution in the G7 locus of the *GDF9* gene in the Ossimi and Rahmani sheep breeds, and a substitution in the FecG^H locus of the *GDF9* gene in all sheep breeds. On the other hand, while the *BMP15* gene loci digested only with *HinfI* showed mutations, this did not occur in all samples of the three Egyptian breeds. In conclusion, the *BMP15* gene loci showed no polymorphism, while only the *GDF9* gene loci were polymorphic in Barki, Ossimi and Rahmani sheep breeds.

Keywords: PCR- RFLP, Polymorphism, *GDF9*, *BMP15*, Sheep.

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Introduction

There are more than 900 different strains from sheep (*Ovis aries*) that differ greatly in the fertility and physiological traits, including ovulation rate [1]. In some cases, the difference in ovulation rate may be attributed to the influence of case setting genes [2]. Actually, mutations having great effects on ovulation rate were detected in the genes expressing different proteins, including the growth differentiation factor 9b (*GDF9*), BMP receptor-IB (ALK6), transforming growth factor beta (TGFβ) superfamily, bone morphogenetic protein (*BMP15*) and TGFβ receptor [3-7]. Moreover, the additional mutations in some of these genes, or in another gene, are similar to be attended in other fertile mammal's strains [2]. Therefore, the useful sheep largely in that it demonstrates that ability to produce two or three offsprings instead of one [8].

Concerning DNA testing for main genes related to fertility and inheritance patterns, scientific research demonstrated that there are main genes have significant possibility to reproductive

performance elevation of sheep worldwide [2]. Three genes related to fertility were discovered in sheep, Growth differentiation factor 9 (*GDF9*) known as FecG, bone morphogenetic protein receptor type 1B (BMPR1B) known as FecB and bone morphogenetic protein 15 (*BMP15*), known as FecX [4,6]. In the Rasa aragonesa sheep strain, six various mutations were discovered for *BMP15* gene; FecX^L, FecX^H, FecX^L, FecX^G, FecX^B and FecX^R [3,4,9,10], and each one of them has a main effect on fertility. The sterile ewes have two inactive copies from *BMP15* gene (homozygous animals) [3,4]. While, ewes that have inactive single copy from the same gene (heterozygous animal) were fertile and have higher ovulation rate and lambing [3,4,11,12]. As for the *GDF9* gene, it has been identified on eight different mutations (G1-G8). Three of them (G2, G3 and G5) are nucleotide changes which did not lead to change in the amino acids. Other remaining nucleotides (G1, G4, G6, G7, and G8) resulted in changes in amino acids. The first polymorphism (G1), that changes arginine to histidine in exon 1, replaces a basic charged polar group with another

group for mature peptide at a position of furin cleavage. So, it does not affect the activity of mature protein [4]. Ewes containing one allele (heterozygote) from two copy of mutated *GDF9* (*FecG^H*) gene is fertile and has high rate of ovulation [4]. On the other side, ewes containing the same alleles (homozygote) for this mutation are infertile in addition to the elementary ovary failure. Moreover, the heterozygous ewes for mutation of *GDF9* and *BMP15* genes are fertile and these mutations have additional effects on ovulation rate [4].

In Egypt, Barki, Ossimi and Rahmani are major sheep breeds. They are characterized by fat tail, coarse wool, and small to medium size. Body size varies between 51-53 kg adult weight for the Ossimi and Rahmani breeds, and 44 kg for the Barki breed. The annual milk yield ranges between 65 kg for the Ossimi and Rahmani breeds and 59 kg for the Barki breed [13]. Until now, no selection programmes have succeeded in increasing the litter size of Egyptian sheep breeds because of the low possibility of genetic transition. The genetic decline for litter size trait indicates that the addition of genetic variation is not the main component of genetic variation phenotypic [13]. Assistance and promotive molecular genetic studies and biological aspects associated used to study and understand the basic genes. There are reports about the effect of genes on the litter size in other strains of sheep, indicating the ability to search for individual mutations that may affect the characteristics of sheep. Therefore, the objective of this study was to detect the presence of polymorphism in the *FecX^G* and *FecX^B* loci for the *BMP15* gene, and the *FecG^H* and *FecG⁷* loci for the *GDF9* gene in the Barki, Ossimi and Rahmani Egyptian sheep breeds, to determine their role in the enhancement of the fertility rate of these breeds.

Materials and Methods

Animals

Multiparous Rahmani (3-4 years), Barki (3-4 years) and Ossimi (2-3 years) ewes were used for this experiment. The Barki breed was grown at faculty of Agriculture, Cairo University. The Ossimi breed was grown in different places in the Nile Valley and Delta, and Rahmani breed was sourced from Beheira province in the northern part of the Nile Delta.

Chemicals

All chemicals that were used to isolate DNA were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted. Primers and restriction enzymes were purchased from Fermentas Co., Germany.

Sample collections

One hundred and twenty six unrelated female Egyptian sheep with a range of ages (Barki; n=50, Ossimi; n=36 and Rahmani; n=40) were used to collect the blood samples for DNA isolation.

DNA isolation

The blood samples collected from sheep vein were put in a sterile tube containing 0.5 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA). Salting out method was used to isolate DNA from whole blood [14]. Isolated DNA was kept on at -20°C until used after dissolving in TE buffer. Spectrophotometer and agarose gel electrophoreses were used to evaluate the quality and quantity of isolated DNA. The concentration of the evaluated DNA was adjusted at 100 ng/μl and was used as a template in polymerase chain reaction (PCR).

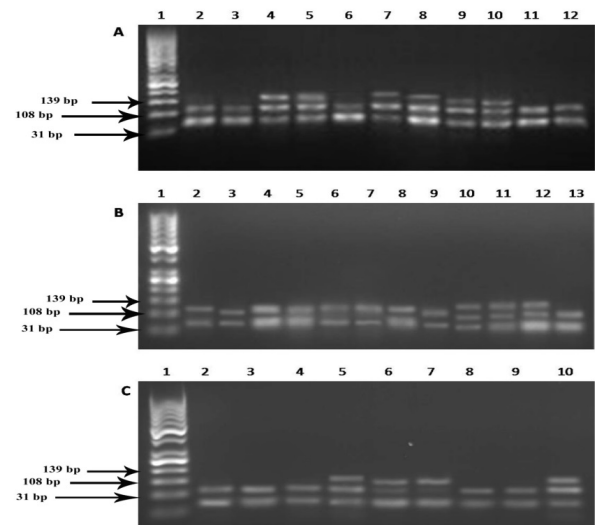


Figure 1. DNA electrophoretic pattern of *FecG^H* locus of the *GDF9* gene amplicon after digestion with *DdeI* endonuclease. Lane 1: DNA ladder; lanes: 4, 5, 7, 8, 9 and 10, in Barki (A), lanes: 10, 11 and 12 in Ossemi (B) and Lanes: 5, 6, 7 and 10 in Rahmani (C) were represented the genotype AB (139, 108 and 31 bp). Lanes: 2, 3, 6, 11 and 12 in Barki (A), Lanes: 2, 3, 4, 5, 6, 7, 8, 9 and 13 in Ossemi (B) and Lanes: 2, 3, 4, 8 and 9 in Rahmani (C) was showed the genotype BB (108 and 31bp).

Polymerase chain reaction (PCR) process

PCR master mix (MM) that consists of 0.2 mM dNTPs (Biotechnology, Cairo, Egypt), 50 mM KCl (Ran Baxy, New Delhi, India), 10 mM Tris (pH 9), 1.5 mM MgCl₂ (Sigma), 0.1% Triton X-100 (Merk), 1.25 units of Taq polymerase (Bioron, Germany), 0.01% gelatin (Merk) and 1.0 μM forward and reverse of specific primers (Table 1). The MM was divided into PCR tubes each containing 100 ng of DNA. The PCR cycle was adjusted at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 58°C for 40 s, 72°C for 1 min and a final extension at 72°C for 10 min. The PCR product was subjected to electrophoresis for detection amplified DNA.

Restriction fragment length polymorphism analysis (RFLP) technique

The PCR products were digested with restriction enzymes specific for each gene (Table 1) at 25 μl final reaction volume.

The RFLEP was carried out using 20 µl from digested PCR product and was incubated overnight at 37°C. After digestion time, the restricted product was analysis by electrophoresis and photographed using gel documentation system. Labimag

software downloaded from the company web page: www.labimage.com was used to detect the fragment sizes and alleles.

Table 1. Primer sequence of candidate genes for prolificacy in Egyptian sheep breeds.

Gene	Site	Size (bp)	Sequences (5'-3')	R. E	References
<i>BMP15</i>	FecX ^G	141	CACTGTCTTCTTGTTACTGTATTTCAATGAGAC GATGCAATACTGCCTGCTTG	<i>HinfI</i>	[4]
	FecX ^B	153	GCCTTCCTGTGTCCCTTATAAGTATGTTCCCTTA TTCTTGGGAAACCTGAGCTAGC	<i>DdeI</i>	[4]
<i>GDF9</i>	FecG ^H	139	CTTTAGTCAGCTGAAGTGGGACAAC ATGGATGATGTTCTGCACCATGGTGTGAACCTGA	<i>DdeI</i>	[4]
	G7	158	CAGTATCGAGGGTTGTATTTGTGTGGGGCCT GCCTCTGGTTCACGCTTCAGTC	<i>MseI</i>	[4]

breeds. One hundred thirty nine and one hundred fifty eight base pair DNA fragments from exon 1 of the *GDF9* gene, also 141 and 153-bp from exon 2 of the *BMP15* gene were successfully amplified. Genotypes of each individual sheep breed were detected using horizontal electrophoresis (Figures 1-4).

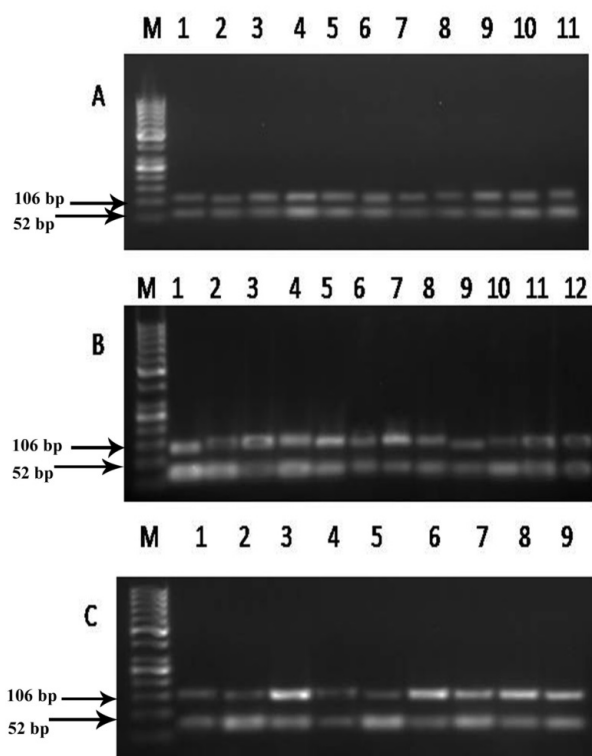


Figure 2. DNA electrophoretic pattern of G7 locus of the *GDF9* amplicon after digestion with *MseI* endonuclease. Lane M, 50 bp DNA ladder; all lanes (1→11) in Barki (A), all lanes (1-12) in Ossemi (B) and lanes (1-9) in Rahmani (C) were represented the genotype AA (52 and 106bp).

Results

DNA fragments of the *GDF9* and *BMP15* genes were digested using four different restriction enzymes (*MseI* and *DdeI* for *GDF9* and *HinfI* and *DdeI* for *BMP15* loci genes) in order to detect the genetic variability between the three Egyptian sheep

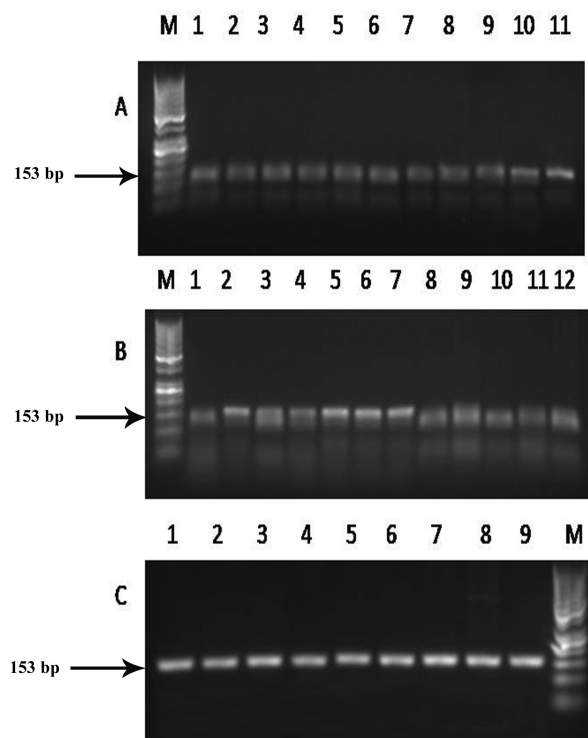


Figure 3. DNA electrophoretic pattern of FecX^B locus of the *BMP15* gene amplicon after digestion with *DdeI* endonuclease. Lane M, DNA ladder; all lanes (1-12) in Barki (A) and Ossemi (B), respectively, and lanes: 1-9 in Rahmani (C) were represented the genotype AA (153bp).

Genetic polymorphism of the *GDF9* gene

The PCR products of the *GDF9* (FecG^H) gene digested with *DdeI* are shown in Figure 1. The results for genotype AB found that all sheep breeds showed mutations in which the restriction enzyme *DdeI* digested the sequences of *GDF9* (FecG^H) to three fragments (139, 108 and 31bp). For genotype BB, however, the restriction enzyme *DdeI* digested the sequences of *GDF9* (FecG^H) to only two fragments (108 and 31bp) in Barki, Ossemi and Rahmani breeds. Through different Egyptian strains (Table 2); the present results showed that strains just contained two genotypes (AB and BB), with the Barki breed having a high frequency of the BB genotype, followed by Rahmani and Ossimi. While, the Barki breed had lower frequency of A allele and AB genotype when compared with the Ossimi and Rahmani breeds.

Table 2. Allele and genotypes frequencies of the *GDF9* (FecG^H) gene among tested Egyptian sheep breeds.

Breed	No. of Animals	Allele frequency		Genotype frequencies		
		A	B	AA	AB	BB
Barki	50	0.11	0.89	0	0.22	0.78
Ossimi	36	0.23	0.77	0	0.46	0.54
Rahmani	40	0.18	0.82	0	0.36	0.64

Table 3. Allele and genotypes frequencies of the *BMP15* (FecX^G) gene between the different Egyptian sheep breeds.

Breed	No. of animals	Allele frequency		Genotype frequencies		
		G	+	GG	G+	++
Barki	50	0.34	0.66	0	0.68	0.32
Ossemi	36	0.36	0.64	0	0.72	0.28
Rahmani	40	0.38	0.62	0	0.76	0.24

The high frequency of allele B and A was found in Barki and Ossimi breeds, respectively. The PCR products of the *GDF9* (FecG⁷) gene digested with *MseI* showed a mutation which enabled the restriction enzyme to digest the gene (Figure 2). The Barki breed alleles of this gene, however, had no restriction site and lead to one fragment 158 bp of DNA (Figure 2A). The Ossimi breed allele types of the same gene, meanwhile, had one restriction site and resulted in two DNA fragments of 52, 106 bp (Figure 2B). The same trend was observed in the Rahmani sheep breed, in that the alleles of this gene had one restriction site and lead to two DNA fragments of 52 and 106 bp (Figure 2C).

Genetic polymorphism of the *BMP15* gene

The results revealed that there was no polymorphism of the *BMP15* (FecX^B) gene in any of the surveyed breeds of Egyptian sheep (Figure 3), the same previous results were found in all digested PCR products with the *DdeI* restriction enzyme (one band, 153 bp). The digested gene products of

PCR *BMP15* (FecX^G) with the *HinfI* restriction enzyme did show mutation, however, but not in all samples (Table 3 and Figure 4). Most of the PCR products of Barki, Ossimi and Rahmani breeds digested with *HinfI* restriction enzyme resulted in 141bp and 111bp fragments (Figures 4A-4C, genotype G+), but, in the majority of the samples from the Ossimi breed, genotype ++ of the *BMP15* (FecX^G) gene showed as only one band in the agarose gel electrophoresis (Figures 4A and 4C). This outcome was much less common, however, with the Barki and Rahmani breeds. Of the different tested breeds (Table 3), the results revealed that only two genotypes (++ and G+) were found in all breeds. A high frequency of genotype ++ was found in the Barki breed, while a frequency of G+ was found in the Rahmani breeds. There was not much variation between the breeds, however, in the frequency of the ++ allele. Moreover, the frequency of the + and G allele was high in the Barki and Rahmani sheep breeds, respectively, although the Ossimi breed had middle values for allele and genotype frequency.

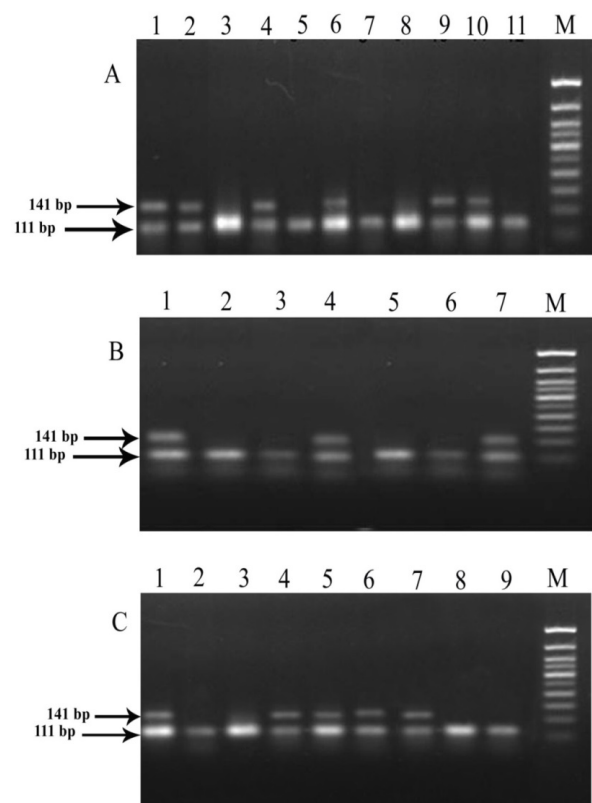


Figure 4. PCR product of the FecX^G locus mutation of the BMP-15 gene digested with *HinfI*. The wild-type allele (+) is 111 bp and the mutant allele (G) is 141 bp. Lane M: DNA ladder; lanes: 1, 2, 4, 6, 9 and 10 genotype G+ (141bp-111bp) in Barki (A), lanes: 1, 4 and 7 genotype G+ (141bp-111bp) in Ossemi (B) and Lanes: 1, 4, 5, 6 and 7 genotype G+ (141bp-111bp) in Rahmani (C). While, lanes: 3, 5, 7, 8 and 11 genotype ++ (111bp-111bp) in Barki (A), Lanes: 2, 3, 5 and 6 genotype ++ (111bp-111bp) in Ossemi (B) and Lanes: 2, 3, 8 and 9 genotype ++ (111bp-111bp) in Rahmani (C).

Discussion

Genotyping was carried out using PCR-RFLP, because it's a rapid, exact and simple technique, to prolife sheep and goats [15-17]. In the present study, this technique was used to detect the genetic polymorphism according to Hanrahan et al. [4]. Polymorphism results concerning the *GDF9* (FecG⁷ and FecG^H) gene confirmed previous observations that had been reported by Hanrahan et al. [4]. The present study in Barki, Ossimi and Rahmani sheep showed same results to those obtained previously [4,18-22]. Most of the tested animals in the present study were of a heterozygous genotype and had a high fertility rate, which is in agreement with Kasiriyani et al. [23], who reported the increased fecundity rate of the *GDF9* gene mutant in Sangsari sheep strain. *GDF9* mutations increased the ovulation rates in heterozygous ewes, while the homozygous ewes were sterile as a result of failure of follicular development [3,4,11]. Polley et al. [24] have been identified 8 different mutations in exons 1 and 2 of *GDF9* gene. Mutation named Thoka gene in *GDF9* gene was found in Icelandic sheep [25]. Another mutation detected by Silva et al. [26] in the Brazilian Santa Inês sheep was called Embrapa. There is a mutation in allele A (G to A), called G1 which was detected in Iranian sheep breeds such as Mehraban breed [27], Kordi and Arabi breeds [28], and Baluchi breed [29]. No homozygous genotype (AA) was found [30], because this genotype may be lethal or has sterility effects, while was found in other breeds such as Mehraban and Baluchi sheep breeds as reported by Abdoli et al. [27] and Moradband et al. [29], respectively. On the other hand, Yadollah et al. [31] showed that there is no any mutation and polymorphism in the *GDF9* gene loci. In general, Ala and Rafat [32] reported that many different loci are under investigation between sheep breeds, more than genetic background. Our results were similar to those of Borhan [33] who found that is no genetic polymorphism in *BMP15* gene in 200 goat breeds. Also, Hua et al. [34] showed that there is a 99% similarity with goat for *BMP15* gene. Moreover, no mutation detected in Chios, Awassi, Kivircik and Imrose sheep breeds for FecX^H, FecX^B and FecX^L gene loci [35]. On the other hand, different goat breeds showed two polymorphic genotypes in exon 2 of *BMP15* gene [36-39]. The detected mutations for *BMP15* (FecX^G) and *BMP15* (FecX^B) in our study had no effect on litter size (genotype GG) in Barki, Ossimi and Rahmani sheep breeds. The same previous results were reported by Moradband et al. [40] in Baluchi Sheep, Davis et al. [21] in East Friesian, Blueface Leicester, Romanov, D'Man, German White-headed Mutton, Loa, Barbados Blackbelly, Teeswater, Finn, Chios, Mountain, Lley and Galician sheep breeds, Kumar et al. [15] in Mulpura sheep and Guan et al. [16] in Charolais, Romney Hills, Suffolk, Chinese Merino and Dorset sheep breeds. In all these breeds the allele of FecB mutatnt was not segregated. In addition, the genetic polymorphism analysis showed that there is no relationship between twining rates and mutated alleles at the *BMP15* FecX^G and *BMP15* FecX^B genes loci in Baluchi sheep breed [24]. In agreement with these findings, the fertility rate in Ossimi, Rahmani and Barki sheep breeds are not related to the mutations of the *BMP15* (FecX^G) and *BMP15*(FecX^B) loci,

which were absent in their genome. It should be attempted to study the other SNPs for these genes loci which may be responsible for the fertility in Egyptian sheep breeds.

Conclusion

The *BMP15* gene loci showed no polymorphism, while only the *GDF9* gene loci were polymorphic in Barki, Ossimi and Rahmani sheep breeds. These findings were accompanied with the fertility rate of the Egyptian breeds, where the fertility rate in Ossimi, Rahmani and Barki sheep breeds are related to the *GDF9* (FecG⁷ and FecG^H) gene but not related to the mutants of the *BMP15* (FecX^G) and *BMP15* (FecX^B) loci, which were absent in the genome of these breeds.

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