Genetic characterization of three genes associated with fertility performance in Egyptian small ruminant breeds

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Abstract. The fertility and reproduction traits enhancement is considered one of the main targets in livestock breeding programs. This work aimed to indentify RFLPs and SNPs variations among three fertility genes in Egyptian small ruminant breeds. RFLP analysis of the amplified fragments at 462-bp from exon 1 of *GDF9* using *Hpa*II endonuclease showed the presence of two genotypes, GG with the nucleotide G at position 209 and AG genotype with a SNP (A/G) at this position**.** The frequencies of GG and AG genotypes as well as G and A alleles were 83.6%, 16.4%, 91.8% and 8.2%, respectively in Egyptian small ruminants. Depending on the presence of the restriction site of *Taq*I endonuclease (T^CGA) at position 100^101 in the 348-bp amplified fragment from exon 5 of *GPR54* gene, the results showed the presence of two alleles, C and T with three genotypes, CC, TT and CT. There was a SNP (C→T) between the two different alleles at position 100. The total frequencies for CC, CT and TT genotypes in all sheep and goat animals were 33.6%, 62.1% and 4.3%, respectively and the frequencies of C and T alleles were 64.6% and 35.4%, respectively. The PCR amplified fragments of 190-bp from *FecB* gene were digested with *Ava*II restriction enzyme and the results showed that all tested animals have the same homozygous non-carrier genotype (++) with uncut 190-bp fragments. The SNP (G→A) at position 160 resulted the destruction of G^GACC restriction site at position 160^161.

Keywords. *GDF9* – *GPR54* – *FecB* – PCR-RFLP – DNA sequencing – Small ruminants.

I – Introduction

The reproduction traits improvement is considered one of the breeding programs targets in small ruminants. Maker assisted selection depending on genetic and DNA markers associated with reproduction traits became the most effective tool for genetic improvement of economically important traits in different livestock (Kolosov *et al*., 2015). The ovulation rate (Hanrahan *et al*., 2004) and litter size (Cao *et al*., 2011) are two important indicators for the fertility and reproduction performances in farm animals especially sheep and goat (Tang *et al*., 2012 and Dinçel *et al*., 2015). The detection of genes associated with fertility and reproduction traits and the identification of their genetic variation effects on these traits phenomena will help in the reproduction enhancement of sheep and goat breeds. Growth differentiation factor 9 (*GDF9*) gene is expressed in the developing oocytes in the ovaries of ruminants (Bodensteiner *et al*., 1999 and 2000) and plays an essential role in ovarian follicular development, ovulation rate and prolificacy in different mammalian species (Chung and Davis, 2012 and Tang *et al*., 2013). *GPR54* is one of the G protein-coupled receptors and the endogenous receptor of *KISS-1* peptide (Chu *et al*., 2012). *GPR54* gene is highly expressed in placenta, pancreas and in brain whereas it expressed at low level in adrenal glands, testes and spleen (Funes *et al*., 2003 and Cao *et al*., 2011). The kisspeptin/GPR54 pathway has an essential role in puberty process and is considered the key for GnRH secretion regulation. This pathway stimulates LH and FSH secretion to initiate the puberty (Kuohung and Kaiser, 2006 and Tena-Sempere, 2006). Many reports focused on the role of the Booroola fecundity (*FecB*) gene in reproductive endocrinology, ovary development, ovulation rate and litter size (EL-Hanafy and El-

Saadani, 2009). This gene increases the ovulation rate and litter size in small ruminants and the identification of the *FecB* mutation is of great interest in the studies of mammalian fertility (Wilson *et al*., 2001). The small ruminants produced about 9.1% of meat production (MoA, 2004) in spite of they are exposed to compromised and marginalized production system in Egypt. Due to the shortage in domesticated meat and milk production, the reproduction improvements of these livestock must have a top priority in any developing plan. Toward this target, the present study aimed to identify the genetic and single nucleotide polymorphisms of three genes associated with fertility and reproduction traits in Egyptian small ruminant breeds.

II – Material and methods

The blood samples were collected from one hundred and forty animals belonging to three sheep breeds, Barki (32 animals), Ossimi (28 animals) and Rahmani (22 animals) in addition to three goat breeds, Baladi (16 animals), Barki (20 animals) and Zaraibi (22 animals). Genomic DNA was extracted from the whole blood according to the method described by Miller *et al*. (1988) with minor modifications. Briefly, blood samples were mixed with cold 2x sucrose-triton and centrifuged at 5000 rpm for 15 min at 4°C. The nuclear pellet was suspended in lysis buffer, sodium dodecyl sulfate and proteinase K and incubated overnight in a shaking water bath at 37°C. Nucleic acids were extracted with saturated NaCl solution. The DNA was picked up and washed in 70% ethanol. The DNA was dissolved in 1X TE buffer. DNA concentration was determined, using Nano Drop1000 Thermo Scientific spectrophotometer, and then diluted to the working concentration of 50 ng/µl, which is suitable for polymerase chain reaction. The DNA fragments from the tested genes were amplified using polymerase chain reaction technique developed by Mullis *et al*. (1986). A PCR cocktail consists of 1.0 µM upper and lower primers (Table 1), 0.2 mM dNTPs and 1.25U of *Taq* polymerase. The cocktail was aliquot into PCR tubes with 100 ng of sheep or goat DNA. The reaction was cycled with the following conditions, initial denaturation for 5 min at 94°C followed by 30 cycles of denaturation at 94°C (1 min), annealing at optimum temperature for each tested gene (1 min) and extension at 72°C (2 min) and the final extension for 10 min at 72°C. The amplification was verified by electrophoresis on 2% agarose gel in 1x TBE buffer using GeneRuler^{TM} 100-bp ladder as a molecular weight marker for confirmation of the length of the PCR products. The gel was stained with ethidium bromide and visualized on UV trans-illuminator. Ten µl of PCR products were digested with 1 ul of FastDigest restriction enzyme specific for each tested gene (Table 1) at 37°C for 5 min. The restriction fragments were subjected to electrophoresis in 2% agarose/ethidium bromide gel (GIBCO, BRL, England) in 1× TBE buffer (0.09 M Tris-boric acid and 0.002 M EDTA). Gels were visualized under UV light and documented in FX Molecular Imager apparatus (BIO-RAD). The PCR products representing detected genotypes of each tested gene were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite. Results of endouclease restriction were carried out using FastPCR.

Table 1. The sequences and information of primers used in this study

Gene	Primer sequences 5'———— -3'	Anneal. temp.	PCR product size	Restriction enzyme	Ref.
GDF9	GAAGACTGGTATGGGGAAATG CCAATCTGCTCCTACACACCT	63°C	462-bp	*Hpa*II	Kolosov *et al.* (2015)
GPR54	ACCTGGCATCCGCGCAGTT CTCAGAGGGGCCCGTCTTGAT	58°C	348-bp	*Taq*I	Cao *et al.* (2011)
FecB	CCA GAG GAC AAT AGC AAA GCA AA CAAGATGTTTTCATGCCTCATCAACAGGTC	60°C	190-bp	*Ava*II	Wilson *et al.* (2001)

III – Results and discussion

1. Growth Differentiation Factor 9 (*GDF9*) gene

The restriction process of the amplified fragments from *GDF9* at 462-bp using *Hpa*II resulted the presence of two different genotypes GG and AG according to the restriction sites (CC^GG) in these fragments, AG genotypes with four fragments at 410-, 254-, 156-bp and 52-bp and GG genotypes with three fragments at 410-, 254-, 156-bp and 52-bp (Fig. 1).

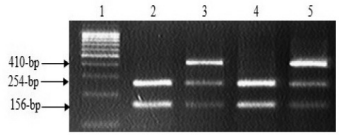

Fig. 1. Electrophoretic pattern after digestion of *GDF9* PCR products with *Hpa*II endonuclease
Lane 1: 100-bp ladder marker
Lanes 2 and 4: GG genotype with 3 digested fragments at 254-156- and 52-bp
Lanes 3 and 5: AG genotype with 4 digested fragments at 410-, 254-, 156- and 52-bp
* The small fragment at 52-bp did not show in the figure.

The total frequencies for GG and AG genotypes were 85.4% and 14.6% in 82 tested sheep animals, 87.5% and 12.5% for Barki, 82.1% and 17.9% for Ossimi and 86.4% and 13.6% for Rahmani, respectively. In 58 goat animals, the frequencies for GG and AG genotypes were 83.6% and 16.4, respectively, for Baladi (81.3% and 18.7%), Barki (85.0% and 15.0%) and for Zaraibi (77.3% and 22.7%), respectively. The total frequencies for GG and AG genotypes as well as G and A alleles in tested small ruminants were 83.6%, 16.4, 91.8% and 8.2%, respectively. The sequence analysis of GG (Fig. 2) and AG (Fig. 3) genotypes showed the appearance of a SNP (G/A) at position 209 in AG genotypes which leads to the presence of four digested fragments at 410-, 254-, 156- and 52-bp in these genotype.

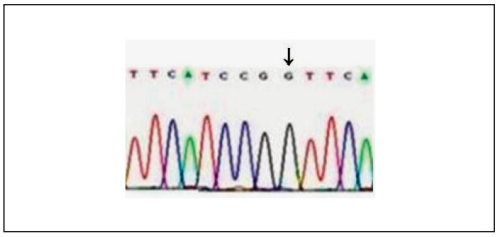

Fig. 2. Genotype GG of *GDF9* with the nucleotide G at position 209.

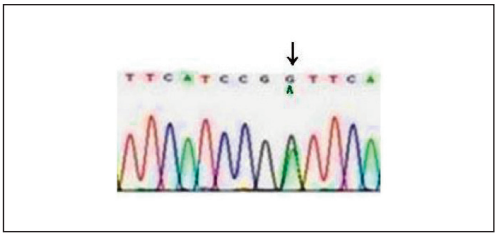

Fig. 3. Genotype AG of *GDF9* with the nucleotide A/G at position 209.

Kolosov *et al*. (2015) determined *GDF9* polymorphism in two Russian sheep breeds-Salskaya and Romanov- using PCR-RFLP technique. They reported the appearance of GG and AG genotypes in exon 1 which was tested in the present study and AA and AG genotypes in exon 4. At exon 1 which is interested in our study, the frequencies of GG and AG genotypes were 90% and 10%, in Salskaya breed and 60.9% and 39.1% in Romanov breed, respectively. This result declared that our sheep breeds is closer genetically to Salskaya than to Romanov breed, as the frequencies of GG and AG genotypes in our sheep breeds were 85.4% and 14.6%, respectively. The PCR amplified fragments from exon 1 of *GDF9* (462-bp) in Iranian Sangsari sheep breed were digested using *Hha*I and the results showed a G to A substitution in *GDF9* locus with allele frequencies for G and A at 80.16% and 19.84%, respectively. The results showed that this Iranian sheep breed possess a rare genotype AA which is not appear in Egyptian or Russian sheep breeds suggesting that

the domestication of sheep breeds may be occurred in Iran and surrounding area of Fertile Crescent. Some reports declared that the sheep animals with GG genotype of *GDF9* possess high fertility than animals with AG genotypes where the animals with AA genotypes are low fertility (Kasiriyan *et al*., 2011). This finding showed that our animals have high fertility rate where most of them possess GG (85.4%) and AG (14.6%) genotypes of *GDF9* with the absence of AA genotype.

2. G Protein-Coupled Receptor 54 (*GPR54*) gene

A fragment of 348-bp from *GPR54* exon 5 was amplified using PCR and the restriction analysis of these fragments using endonuclease *Taq*I declared three genotypes, CC, CT and TT. The appearance of these genotypes resulted from the presence of T^CGA restriction site at position 100^101.

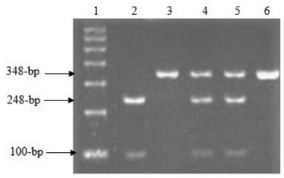

Fig. 4. Electrophoretic pattern after digestion of *GPR54* PCR products with *Taq*I endonuclease
Lane 1: 100-bp ladder marker
Lane 2: TT genotype with 2 digested fragments at 248- and 100-bp
Lanes 3 and 6: CC genotype with undigested fragment at 348-bp
Lanes 4 and 5: CT genotype with 3 digested fragments at 348-, 248- and 100-bp

In sheep breeds, the frequencies of CC, CT and TT genotypes were 31.25%, 62.5% and 6.25% in Barki, 32.1%, 64.3% and 3.6% in Ossimi and 27.3%, 68.2% and 4.5% in Rahmani, respectively with the total frequencies of 30.5%, 64.6% and 4.9% for CC, CT and TT genotypes, respectively. In goat breeds, the frequencies of CC, CT and TT genotypes were 37.5%, 62.5% and 0.0% for Baladi, 35.0%, 60% and 5.0% for Barki and 41.0%, 54.5% and 4.5% for Zaraibi, respectively with total frequencies of 37.9%, 58.6% and 3.5% for CC, CT and TT genotypes, respectively. The total frequencies for CC, CT and TT genotypes in all sheep and goat animals were 33.6%, 62.1% and 4.3%, respectively. The total frequencies for C and T alleles in all tested animals were 64.6% and 35.4%, respectively. The sequence analysis of the two different alleles, C (Fig. 5) and T (Fig. 6), showed the presence of a SNP (C→T) at position 100 in allele T yielding two digested fragments at 248- and 100-bp in this allele.

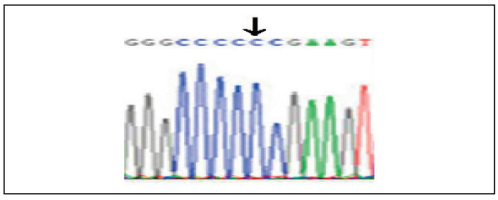

Fig. 5. The nucleotide C at position 100 in allele C of *GPR54* gene.

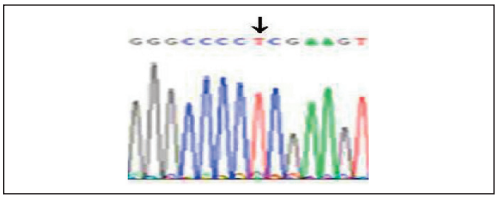

Fig. 6. The nucleotide T at position 100 in allele T of *GPR54* gene.

Tang *et al.* (2012) reported the presence of different mutations in *GPR54* gene in four Chinese sheep breeds, Small Tail Han, Chinese Merino, Hu and Corriedale. In first sheep breed, there are three genotypes AA with frequency of 0.25, AG (0.50) and GG (0.25) with negative effect on the litter size. On the other hand, the frequencies of CC (0.175), CD (0.125) and DD genotypes (0.700) were reported in this breed with positive effect where sheep ewes with genotype CC had lambs more than those with genotype DD or CD. These results declared that allele C of *GPR54* gene may be considered as a candidate marker for improving litter size in sheep.

3. *FecB* gene

A 190-bp fragment from *FecB* gene of sheep and goat was amplified using PCR. The digestion process of these fragments by *Ava*II restriction enzyme revealed the absence of the restriction site (G^GACC) at position 160^161 in tested animals yielding the presence of uncut fragments at 190-bp. This result showed that all tested Egyptian sheep and goats have the same homozygous non-carrier genotype (++) (Fig. 7).

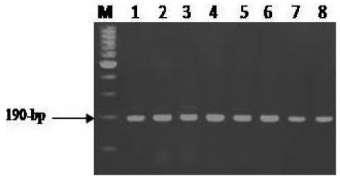

Fig. 7. The electrophoretic pattern obtained after digestion of PCR amplified fragment of *FecB* gene from sheep and goat DNA with *Ava*II restriction enzyme.
Lane M: 100-bp ladder marker
Lanes 1-8: ++ non-carrier homozygous genotype with uncut fragment at 190-bp.

The sequence analysis of the purified PCR products (Fig. 8) representing the detected monomorphic ++ non-carrier genotype showed the presence of a SNP (G→A) at position 160 (Fig. 9) which is responsible for the absence of restriction site (G^GACC) at position 160^161 and consequently the presence of undigested fragments at 190-bp in all tested animals.

CCAGAGGACAATAGCAAAGCAAATTCAGATGGTGAAA
CAGATTGGAAAAGGTCGCTATGGGGAAGTTTGGATGG
GAAAGTGGCGTGGCGAAAAGGTAGCTGTGAAAGTGT
TCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGA
CAGAAATATATCAGACCGTGTTGATGAGGCATGAAAA
CATCTTG

Fig. 8. The nucleotide sequence of ++ non-carrier genotype (190-bp). The nucleotide A at position 160.

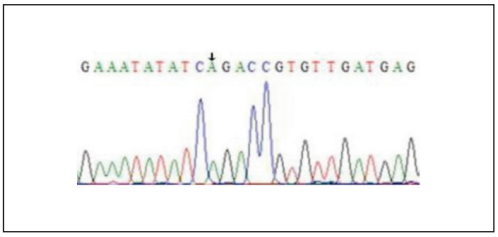

Fig. 9. The nucleotide A in the monomorphic ++ non-carrier genotype.

Two important reproduction parameters with economic importance in small breeding programs are litter size and lamb growth. Souza *et al*. (2003) reported the presence of genetic polymorphism in *BMPR-IB* gene and its association with the *FecB* gene and the high prolificacy in Booroola Merino sheep using PCR-RFLP technique (Souza *et al*., 2001 and Davis *et al*., 2002). The genetic polymorphism in *FecB* gene and its association with some economically important growth parameters was identified by Guan *et al*. (2007). They reported that Hu sheep are homozygous carriers (BB) whereas in Merino prolific meat breed, the three genotypes, BB, B+ and ++ were appeared with different frequencies. In Merino prolific sheep breed, the animals with genotypes BB and B+ have higher mean litter sizes of ewes, the heart girth and chest width than those with genotype ++. The association between *FecB* gene and some reproduction and fertility parameters like reproductive endocrinology, ovary development, litter size, organ development and body mass was reported (Smith *et al*., 1993, Smith *et al*., 1996 and Cognie *et al*., 1998). The effects of *FecB* gene on these parameters are different where it has positive effects on litter size and ovulation rate and negative effects on fetal growth and development and body mass (Wang *et al*., 2003 and Liu *et al*., 2003).

IV – Conclusions

In conclusion, the detection of genes and the identification of their favorable genotypes associated with production and reproduction traits are considered the first step towards the genetic improvements of these economically important traits in different livestock. *GDF9*, *GPR54* and *FecB* genes are considered as three promising markers associated with different fertility trait parameters like ovulation rate, ovarian follicular development, puberty and litter size in small ruminant breeds.

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