

FSHR (EXON 10) GENE POLYMORPHISMS AND ITS ASSOCIATION WITH FERTILITY TRAIT IN EGYPTIAN OSSIMI SHEEP

Salah Abdel-Rahman^{1*}, Yehia Mustafa², Hagar Abd Errasool², Hanim Heikal³, Ayaat Elmaghraby¹

¹Department of Nucleic Acid Research, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt

²Department of Genetics, Faculty of Agriculture, Alexandria University, Egypt

³Department of Animal Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, Sadat City University, Egypt

*Corresponding author: salahmaa@gmail.com

Original scientific paper

Abstract: For the association between of Follicle stimulating hormone receptor (*FSHR*) gene (partial part of exon 10) polymorphisms and litter size trait in Egyptian Ossimi sheep, polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) and DNA sequencing techniques were developed. Fifty female Ossimi sheep reared under Egyptian conditions were selected according to their litter size. DNA from blood samples of these animals was isolated to amplify 250-bp of the *FSHR* gene influencing litter size production trait in sheep. Based on litter size, 50 animals were selected from the highest to the lowest litter size productivity during three seasons. PCR-SSCP analysis of the *FSHR* gene (250-bp) showed two various genotypes AA and AB with frequencies 0.64 and 0.36, respectively. The frequencies of the A and B alleles were 0.82 and 0.18, respectively. PCR fragment of *FSHR* gene (191-bp) was sequenced only in the high and low litter size productivity animals (GenBank accession numbers from MG973191 to MG973207, sequentially). The result indicated that 6SNPs (G/71, G/72, G/77, A/110, A/111, A/191) in high fertile animals, while, 10 SNPs (T/1, C/2, T/14, A/69, A/70, A/71, A/74, G/74, A/75, A/136) have found in low fertile animals. Statistically, AA and AB genotypes have no significant differences ($p > 0.05$) on litter size trait in Ossimi sheep. *FSHR* (exon 10) locus was moderate polymorphic (PIC= 0.25) and it can be used for high litter size productivity in Ossimi sheep as a marker-assisted selection (MAS).

Key words: Ossimi sheep, Fertility, *FSHR* gene polymorphisms, PCR-SSCP, DNA sequencing

Introduction

Sheep (*Ovis aries*) have positive economic benefits for human. Where, this kind of animals is preferable as a result of low maintenance cost, adaptation in many environments in the world and resourcefulness for many products such as meat, milk, wool and hides (Abulyazid et al., 2011). In Egypt, sheep meat production is more important than fiber production (6% of the total animal meat production). Ossimi sheep (under study) is an Egyptian Nile Valley breed, which is one of the three major Egyptian sheep breeds besides Barki and Rahmani which represent 65% of the total population of sheep in Egypt (ICARDA annual report, 2007). Ossimi sheep is a most popular and environmentally adaptable in Lower Egypt. In addition, it is a carpet wool breed (Mason, 1996). The mean of Ossimi Kidding rate production under Egyptian condition is 1.22 (lamb/ewe) (Elshenawy, 1995).

Improvement of fertility trait in sheep has become desiring interest for breeders, where moderate increases in litter size can equal outsized gains in profit (Abraham and Thomas, 2012). This can be achieved by improving the genetic worth of the stock by proper selection methods for improving reproduction rate and production efficiency. Therefore research community constantly searches some affecting genes that can act as marker influencing fertility in animals by marker assisted selection (Mishra, 2014). Fertility trait can be improved using marker assisted selection (MAS) which make improvement by detecting variations of fertility or fecundity genes linked with high and low fertile animals (Abdoli et al., 2016).

Many genes affect fertility in sheep such as Boroola gene, growth differentiation factor 9 gene and follicle stimulating hormone receptor gene. This study is concerned with *Ovis arias's* FSHR gene which located in chromosome 3 (3:75470485-75470694) in sheep (n=54). Sequence version Oar_v3.1 in www.ensemble.com has determined that chromosome 3 has 1994 coding genes and 523 noncoding genes, one of these coding genes is FSHR gene (size= 2088bp) consists of 10 exons coded into FSHR protein (656 amino acids). FSHR gene has a large number of variants which differentiated into two categories missense and synonymous. FSH (Follicle stimulating hormone) secreted by an anterior pituitary regulates gonadal functions in male and female, it is under regulation of gonadotropin releasing hormone (GNRH) as well which activates its receptor in granulosa cells (GCs) in the ovary.

FSHR is a member of the family of G-coupled protein activated FSH which secreted by anterior pituitary regulates gonadal functions in males and females, as well as, it is under regulation of gonadotropin releasing hormone (GNRH) (Richards and Midgley, 1976). Thus, the level and the target of hormone response are controlled by mechanisms that determine FSHR levels and cell

specific expression, which are supported by transcription of its genes (*George et al., 2011; Dias et al., 2002; Bogerd, 2007*).

Exon 10 of FSHR gene is large and encodes the C-terminal part of the extra cellular domain (ECD) (hinge region), the transmembrane domain (TMD) and the intracellular domain of the receptor (*Fan & Hendrickson 2005; Jiang et al., 2012*). The objectives of the present study are to detect SNPs (mutations) in a partial region of exon 10 of FSHR gene in a high fertile Egyptian sheep breed (Ossimi sheep) using PCR-SSCP and sequencing techniques. Also, to investigate the relation between the FSHR gene and fertility trait in Ossimi sheep breed under Egyptian conditions.

Materials and Methods

Animals. Blood samples were collected from the jugular vein of 50 Ossimi ewes (5ml/ewe) puncture into tubes containing an anticoagulant disodium EDTA. The samples have stored at -20°C until needed for DNA isolation.

DNA isolation. Genomic DNA was isolated from whole blood samples using a commercially available kit (EZ-10 Spin Column Genomic DNA kit for Blood samples, Bio Basic, Canada). Isolated genomic DNA was separated on agarose gel electrophoresis using 1% (w/v) agarose in 0.5X TBE buffer. The gel was photographed using the gel documentation system (Syngene, UK) to visual genomic DNA band.

PCR amplification and genotyping of FSHR gene. 250-bp fragment of exon 10 of FSHR gene in 50 Ossimi sheep was amplified by PCR using forward (5'-ATCACGCTGGAAAGATGGCATAACC-3') and reverse (5'-ACATTGAGCACAAGGAGGGAC-3') primers (*Li et al., 2010*). PCR was performed in a reaction volume of 25 µl using 200ng of genomic DNA of each sample, 25 pmol of each primer and 2X Taq DNA polymerase Mix (Bioline, UK). Thermal cycling (Peltier-based Thermocycler, Long Gene) was carried out by initial denaturation at 94°C for 5 min, followed by 30 cycles each at 94°C for 1 min, annealing temperature at 60°C for 30 sec, polymerization temperature at 72°C for 30 sec and final extension at 72°C for 10 min, then the samples were held at 4°C. The amplified DNA fragments were separated on 2% agarose gel, stained with ethidium bromide, visualized on a UV Transilluminator and photographed by Gel Documentation system (Alpha Imager M1220, Documentation and Analysis System, Canada).

Single stranded conformational polymorphism (SSCP). Aliquots of 5 µl PCR products were mixed with denaturing solution (98% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue and 10 mM EDTA) and incubated at 98°C for 10 min and then chilled on ice rapidly. Denatured DNA was loaded on 10% PAGE gel (10X 10 CM) in 1X TBE buffer and constant voltage 65V for 5 hours.

For staining DNA bands and visualizing, the gel was stained with ethidiumbromide and photographed by using gel documentation system.

Sequencing. DNA sequencing for purified FSHR amplicon had performed by Genetic Analyzer (Applied Biosystems, Hitachi, Japan). Where, the highest eight (2, 4, 5, 6, 7, 13, 16 and 17) and the nine of lowest (21, 22, 23, 24, 25, 26, 28, 40 and 41) litter size animals were sequenced (one direction, forward) at Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt.

Statistical analysis. For litter size, each ewe has three seasons' records (winter, spring and next winter). Random selection of three parities ewes was done and documented in Sakha farm, Agricultural Research Station, Kafer Elshiekh, Egypt.

Polymorphism information content (homogeneity). PIC (polymorphism information content) is a measurement that gives an indication how much the locus is highly, moderate or poorly mutated by knowing the alleles frequencies of this locus: $PIC = 1 - \sum (p_{ij})^2$, where, p_{ij} is the frequency of different ij^{th} allele of studied locus. The calculation of PIC was processed by PIC Calculator (www.liverpool.ac.uk).

Q-Q Plot. We put the assumption of litter size values of the 50 animals are normal distributed, so we should test this assumption by run the Q-Q plot using (*IBM SPSS Statistics, Version 22, 2013*). A Q-Q plot is a scatterplot created by plotting two sets of quantities against one another, one of it is litter size values of tested animals and normal disrupted expected value that the program put it. If both sets of quantities came from the same distribution, we should see the points forming a line that's roughly straight.

Independent t-test. The Independent samples t-test compares two genotypes means to determine whether they are significantly different or not. The two independent samples (AA and AB genotypes) are assumed to be drawn from populations with unequal variances (i.e., $\sigma_1^2 \neq \sigma_2^2$), the test statistic t is computed as:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

Where: \bar{X}_1 = Mean of first genotype AA; \bar{X}_2 = Mean of second genotype AB; n_1 = Sample size (i.e. number of observations) of AA genotype; n_2 = Sample size (i.e., number of observations) of AB genotype; s_1 = Standard deviation of AA genotype; s_2 = Standard deviation of AB genotype,

Least mean squares analysis. Least mean square test was used to examine the relationship between different genotypes and the litter size. The least squares means were used for multiple comparisons in litter size among the different genotypes. Analysis was performed using the general linear model procedure of (*IBM SPSS Statistics, Version 22, 2013*). The litter size trait of Ossimi ewes was analyzed using the following fixed effects model:

$$Y_{ijklm} = m + A_i + KS_j + P_k + G_l + e_{ijklm}$$

Where: y_{ijklm} = phenotypic value of litter size; m = population mean; A_i = fixed effect of the i th age of ewe ($i = 3, 4, 5, 6, 7, 9, 13$); KS_j = fixed effect of the j th kidding season ($j = 1, 2, 3$); P_k = fixed effect of the k th parity ($k = 1, 2, 3$); G_l = fixed effect of the l th genotype ($l = 1, 2, 3$); e_{ijklm} = random residual effect of each observation.

Results and Discussion

PCR-SSCP of FSHR gene. A partial part of exon 10 of FSHR gene have amplified by PCR and yielded 250-bp in length (Figure1) and screened for polymorphism by PCR-SSCP. The analysis of PCR-SSCP showed only two genotypes AA and AB (Figure 2). Genotype AA (homozygous) was in thirty-two animals, while AB genotype (heterozygous) found in eighteen animals. The calculated frequencies of AA and AB genotypes were 0.64 and 0.36, respectively, and the frequencies of the A and B alleles were 0.82 and 0.18, respectively (Table 1). The results indicated that the Ossimi sheep with AA or AB genotypes have no statistically significant differences ($p \leq 0.05$) on litter size in these animals (50 Ossimi sheep). The PIC and heterozygosity show indications how much this locus mutated. So, by entering data of allele frequencies (A and B) of studied locus of partial part of exon 10 of FSHR gene in PIC calculator (www.liverpool.ac.uk) the heterozygosity and PIC are 0.295 and 0.25, respectively. In general, PIC is an ideal index to evaluate polymorphism on the fragment of the gene, as follows: $PIC > 0.50$ (high polymorphism), $0.25 < PIC < 0.50$ (moderate polymorphism) and $PIC < 0.25$ (low polymorphism). In our results, PIC indicates that polymorphism in this fragment (exon 10 of FSHR gene) has moderate polymorphism and suitable to be candidate gene marker for fertility trait breeding. On the other hand, the heterozygosity of this locus indicates high heterozygosity and genetic diversity in Ossimi sheep; hence, it can be selected as a genetic breeding marker.

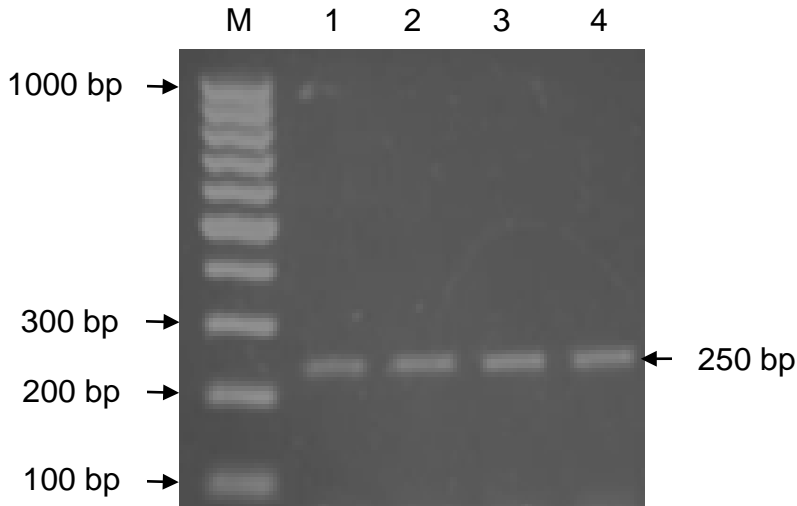


Figure 1. PCR products (250-bp) generated by the FSHR gene (part of exon10) primers. Where, lane M is a molecular weight marker (100 bp) and lanes 1-4 are female Ossimi sheep (as an example)

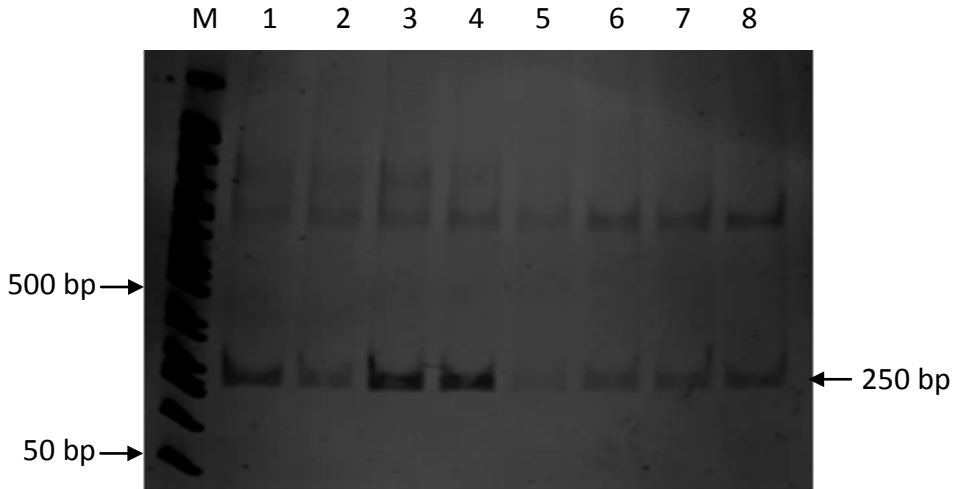


Figure 2. PCR-SSCP analysis of FSHR gene (250-bp). Lanes 1-4 represent AB genotype and lanes 5-8 represent AA genotype. Lane M is DNA marker (50-bp)

Independent t-test. An independent t-test was conducted to compare differences on litter size mean in AA and AB genotypes by knowing the significance. According to this test AA genotype and AB genotype ($M= 1.24$, $SD= 0.33$) and ($M= 1.2$, $SD= 0.28$); $t(125)= 0.685$, $p= 0.495$. This result suggested that no

significance differences between two genotypes mean and genotype does not have an effect on litter size.

Least square analysis. A least mean square test has performed based on data to determine if there was a significance relationship between genotypes, age, parity and season on litter size. The litter size of studied 50 animals of Ossimi sheep was significantly affected by age and not influenced by the other factors. The t-statistics for the slope was significant at the 0.05 critical alpha level (Table 1), $t(149) = -0.07$, $p = 0.9$. So, there was a negative significant relationship between Genotypes and litter size. Furthermore, only 4% of the variability of litter size could be explained by these tested factors.

Table 1. Genotypic and allelic frequencies of *FSHR* gene, least mean squares and estimated standard errors of litter size in Ossimi sheep

Gene	Genotypes & alleles	Number of animals	Genotype and allele frequency	PIC	Heterozygosity	Litter size \pm SE
FSHR	AA	32	0.64	0.25	0.295	0.74 ± 0.67^a
	AB	18	0.36			1.4 ± 0.36^a
	A	-	0.82			-
	B	-	0.18			-

Least squares means followed by the same letter means no significant differences at $P < 0.05$.

Sequencing. For SNP's detection, the fragment 250-bp of FSHR gene was sequenced, aligned and accessioned (Figure 3). Where, the eight highest twins production animals (2, 4, 5, 6, 7, 13, 16 and 17) and the nine lowest (21, 22, 23, 24, 25, 26, 28, 40 and 41) were sequenced and aligned (<https://www.genome.jp/tools-bin/clustalw>) and compared with reference sequence (*Ovis aries* FSHR gene (transcript ID) ENSOART00000004728.1). These 17 sequences were accessioned (MG973191-MG973207) by www.ncbi.nlm.nih.gov (191-bp). Table 2 shows the SNPs in the sequenced fragment (191-bp) in these animals which highlighted in green color for high fertile animals (2, 4 and 16), orange color for low fertile animals (24, 25, 26, 28 and 41).

Anim.2 (accession no. MG973191)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.4 (accession no. MG973192)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.5 (accession no. MG973193)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.6 (accession no. MG973194)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.7 (accession no. MG973195)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.13 (accession no. MG973196)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.16 (accession no. MG973197)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.17 (accession no. MG973198)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.21 (accession no. MG973199)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.22 (accession no. MG973200)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.23 (accession no. MG973201)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.24 (accession no. MG973202)	1	TCGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.25 (accession no. MG973203)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.26 (accession no. MG973204)	1	CAGCTCGAATGCATAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.28 (accession no. MG973205)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.40 (accession no. MG973206)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
Anim.41 (accession no. MG973207)	1	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	60
ref.seq (FSHR gene sequence)	1426	CAGCTCGAATGCAGAGTGCAGCTCCGCCATGCTGCCAGCATCATGTTGGTGGGCTGGGTC	1485

Anim.2 (accession no. MG973191)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.4 (accession no. MG973192)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.5 (accession no. MG973193)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.6 (accession no. MG973194)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.7 (accession no. MG973195)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.13 (accession no. MG973196)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.16 (accession no. MG973197)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.17 (accession no. MG973198)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.21 (accession no. MG973199)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.22 (accession no. MG973200)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.23 (accession no. MG973201)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.24 (accession no. MG973202)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.25 (accession no. MG973203)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.26 (accession no. MG973204)	61	TTTGGCTTTAAAGAGGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.28 (accession no. MG973205)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.40 (accession no. MG973206)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
Anim.41 (accession no. MG973207)	61	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	120
ref.seq (FSHR gene sequence)	1486	TTTGGCTTTGGAGGTGCCCTCTTTCCATCTTTGGCATCAGCAGCTACAAGAGGTGAGC	1545

Anim.2 (accession no. MG973191)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.4 (accession no. MG973192)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.5 (accession no. MG973193)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.6 (accession no. MG973194)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.7 (accession no. MG973195)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.13 (accession no. MG973196)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.16 (accession no. MG973197)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.17 (accession no. MG973198)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.21 (accession no. MG973199)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.22 (accession no. MG973200)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.23 (accession no. MG973201)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.24 (accession no. MG973202)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.25 (accession no. MG973203)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.26 (accession no. MG973204)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.28 (accession no. MG973205)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.40 (accession no. MG973206)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
Anim.41 (accession no. MG973207)	121	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	180
ref.seq (FSHR gene sequence)	1546	ATCTGCCTGCCCATGGACATTGACAGCCCTTGTCACAGCTCTATGTTATGTCCTCTCT	1605

Anim.2 (accession no. MG973191)	181	GTGCTCAATGT	191
Anim.4 (accession no. MG973192)	181	GTGCTCAATGT	191
Anim.5 (accession no. MG973193)	181	GTGCTCAATGT	191
Anim.6 (accession no. MG973194)	181	GTGCTCAATGT	191
Anim.7 (accession no. MG973195)	181	GTGCTCAATGT	191
Anim.13 (accession no. MG973196)	181	GTGCTCAATGT	191
Anim.16 (accession no. MG973197)	181	GTGCTCAATGT	191
Anim.17 (accession no. MG973198)	181	GTGCTCAATGT	191
Anim.21 (accession no. MG973199)	181	GTGCTCAATGT	191
Anim.22 (accession no. MG973200)	181	GTGCTCAATGT	191
Anim.23 (accession no. MG973201)	181	GTGCTCAATGT	191
Anim.24 (accession no. MG973202)	181	GTGCTCAATGT	191
Anim.25 (accession no. MG973203)	181	GTGCTCAATGT	191
Anim.26 (accession no. MG973204)	181	GTGCTCAATGT	191
Anim.28 (accession no. MG973205)	181	GTGCTCAATGT	191
Anim.40 (accession no. MG973206)	181	GTGCTCAATGT	191
Anim.41 (accession no. MG973207)	181	GTGCTCAATGT	191
ref.seq (<i>Ovis aries</i> FSHR gene)	1606	GTGCTCAATGT	1616

Figure 3. DNA sequence alignment of *FSHR* gene (191-bp) among the 17 female Ossimi sheep and *Ovis aries* *FSHR* gene (transcript ID) [ENSOART0000004728.1](#). The asterisks represent the similarity

Table 2. Genotypes and nucleotide sequence variation of the eight animals ordered from high to low litter size

Animal no.	Litter size	Genotype	Nucleotide sequence variations													
			Amplicon nucleotide no.													
			1	2	14	69	70	71	72	74	75	77	110	111	136	191
2	2	AA	C	A	G	T	G	G	A	T	T	C	A	A	G	T
4	1.6	AA	C	A	G	T	G	C	G	T	T	G	A	G	G	T
16	1.3	AB	C	A	G	T	G	C	A	T	T	C	T	G	G	A
24	1	AA	T	C	G	T	G	C	A	T	T	C	T	G	G	T
25	1	AB	C	A	G	T	G	C	A	T	T	C	T	G	A	T
26	1	AA	C	A	T	T	A	A	A	A	A	C	T	G	G	T
28	1	AA	C	A	G	T	G	C	A	G	T	C	T	G	G	T
41	1	AB	C	A	G	A	G	C	A	T	T	C	T	G	G	T
Gene nucleotide no.			1426	1427	1439	1494	1495	1496	1497	1499	1500	1502	1535	1536	1561	1616

Results of virtual (in silico) translated FSHR gene showed 13 amino acids variants in FSHR peptide which may be occurred, as a result of SNP's, in high (2, 4 and 16) and low (24, 25, 26, 28 and 41) litter size production animals by Mega software version 7 (Kumar *et al.*, 2016). One amino acid variant is synonymous and the other 12 amino acids variants are non-synonymous (Table 3).

Table 3. Reference codon, mutated codon, amino acids variation and synonymous/non-synonymous variants

Animal no.	Genotype	Base number	Reference codon	Mutated codon	Peptide amino acid no.	Amino acid	Synonymous or Non-synonymous
2	AA	71	GCA	GGA	460	Ala/Gly	Non-syn
		110, 111	ATG	AAA	473	Met/Lys	Non-syn
4	AA	72	GCA	GCG	460	Ala/Ala	Synonymous
		77	GCC	GGC	462	Ala/Gly	Non-syn
		110	ATG	AAG	473	Met/Lys	Non-syn
16	AB	191	GTC	GAC	500	Val/Asp	Non-syn
24	AA	1, 2	CAG	TCG	437	Glu/Ser	Non-syn
25	AB	136	GAC	AAC	482	Asp/Asn	Non-syn
26	AA	14	AAA	ATA	441	Lys/Ile	Non-syn
		70,71	GCA	AAA	460	Ala/ Lys	Non-syn
		74,75	GTT	GAA	461	Val/ Glu	Non-syn
28	AA	74	GTT	GGT	461	Val/ Glu	Non-syn
41	AB	69	TTT	TTA	459	Phy/Leu	Non-syn

In this study, fifty Ossimi sheep animals were divided into two groups according to their litter size (1.2 lamb/ewe); high fertile group (litter size= 2, 1.6 or 1.3) and low fertile group (litter size= 1). For understanding the relationship between polymorphism in FSHR gene locus (part of exon 10) and fertility trait in Ossimi sheep, SSCP and sequencing techniques were used, and then this relationship was statistically analyzed. However, we found then this locus is moderate polymorphic (PIC=0.25) and has two alleles (A and B) and two genotypes (AA and AB) in all animals. AA genotype frequency was 0.64 and AB genotype was 0.36, hence, A allele frequency was 0.82 and B allele was 0.18.

For 191-bp FSHR fragment sequencing, we found 6 SNPs in high fertile animals (G/71, G/72, G/77, A/110, A/111, A/191). While in low fertile animals, we found 10 SNPs (T/1, C/2, T/14, A/69, A/70, A/71, A/74, G/74, A/75, A/136). Statistically, AA and AB genotypes have no significant differences ($p > 0.05$) in litter size, as well as, regression statistics considered that 10% of genotype differences could describe the differences of litter size in this sample (fifty animals). The results have a valuable meaning in choosing this polymorphic locus of FSHR as a candidate gene for research and breeding programs of fertility trait. These results preliminary showed that FSHR gene is a major gene that influences the prolificacy of experimental animals or a molecular marker in close linkage with prolificacy trait. Consequently, FSHR gene has considered as a possible candidate gene for increasing litter size in Ossimi sheep.

In the previous related studies, FSHR gene polymorphisms and its association with litter size in animals classified to two categories according to regulatory and their translated regions of FSHR gene. An example for this, the 5' regulatory region of FSHR gene had a significant effect on fertility trait and proofed as a marker-assisted selection (MAS) in the improvement programs. Where, *Chu et al.*(2012) found that in 5' regulatory region of FSHR gene 2 mutations (-681 C/T and -629 C/T) in Hu sheep and 3 mutations (-200 G/A, -197 G/A and -98 T/C) in small tail Han sheep. In the same study, the author found that the heterozygous Small tail Han sheep (EG and EF) had 0.89 lambs more than the homozygous. In another study on sheep, *Wang et al.*,(2015) found that the CC genotype of FSHR gene in small tailed and Han sheep had lamb production more than those the TC and TT genotypes with 0.52 ($p < 0.01$) and 0.72 ($p < 0.01$), respectively.

The study in goat, Xiangdong Black, NanJiang Brown and Guizhou Black litter size was affected significantly by FSHR gene ($p < 0.05$) only in Guizhou Black goat and BB genotype was significantly higher than AA and AB genotypes (*Zhu et al.*, 2007). On the other hand, in these three goat breeds, the author found the same five SNPs (-93C/A, -80 G/C, -63 C/A, -56C/G and -55 T/C) of 5' regulatory region of FSHR gene which were not necessarily affect significantly on litter size. In another study in three goat breeds (Jining Grey, Boer and Inner Mongolia Cashmere), *Guo et al.*(2013) found three genotypes CC, CD and DD. Only in Jining Grey goat, the author noticed that the CC genotype had 0.46 and 1.3 kids more than the other CD and DD genotypes, respectively. While CD genotype had 0.57 kids more than DD genotype. On the other hand, the two transversions in 70T/A and 130G/C positions of amplified sequenced region of FSHR gene, are found in DD and CC genotypes in the three previous goat breeds. In Yunling Black and Boer goat breeds, *Cui et al.*, (2009) found four mutations in the coding region of FSHR gene 486C/A (162Arg/Ser), 1042C/G (348Pro/Ala), 1930T/A (644 Phe/Ile) and 2036T/C (679Thr/Ile). In Yunling Black goat only, the author recognized that the FSHR mRNA and protein expression levels were significantly

and positively correlate with fecundity, as well as reduction levels may be associated with the fewer observed oocytes and fewer follicles.

In cows (*Bostaurus* and *Bosindicus*), *Marson et al.*, (2008) evaluated the effect of polymorphisms in exon 10 of FSHR gene using PCR-RFLP (*AluI* restriction enzyme) and the results showed three genotypes GG, GC and CC. Genotype GC revealed higher pregnancy rate (66%) than the two other genotypes (GG and CC). According to the author, has observed no significant effect of the three genotypes (GG, GC and CC) in both *Bostaurus* and *Bosindicus* beef populations. In another research, *Yang et al.*, (2010) found a mutation (-278 G/A) and three genotypes (CC, CD and DD) in Chinese Holstien cows. Genotype CC had a significant increase in the total number of ova and transferable embryos than the other two genotypes, which had an absence of super ovulation response. Also, *Sharifiazdi et al.*, (2018) emphasized that this mutation (-278 G/A) may affect some reproductive variables in Holstein dairy cows.

Lastly in Erhualian and Yorkshire sows, *Zhang et al.*, (2002) detected a transition mutation (566 C/T) in exon 7 of FSHR gene. Consequently, a prediction of Ala/Val substitution has done at residue 189 in ECD and this polymorphism significantly associated with the total born number and number born alive. However, our results with the other previous mentioned studies confirm that the FSHR gene in Ossimi sheep will be a strong candidate gene for further applications in marker-assisted selection (MAS) and breeding programs for fertility trait improvement.

Conclusion

PCR-SSCP genotyping and DNA sequencing techniques were developed to study the association between FSHR gene polymorphism and fertility trait in fifty Ossimi sheep. The results indicated two genotypes (AA and AB) which have no significant differences ($p > 0.05$) on litter size trait, but this locus (partial part of exon 10) was moderate polymorphic (PIC= 0.25). Using DNA sequencing, in the high fertile animals 6 single nucleotide polymorphisms (SNP's) at 6 different positions have observed G/71, G/72, G/77, A/110, A/111, A/191. While in the low fertile animals, 10 SNPs (T/1, C/2, T/14, A/69, A/70, A/71, A/74, G/74, A/75, A/136) were observed. Thus, these findings can be used as marker-assisted selection (MAS) for high fertility trait in Ossimi sheep reared under Egyptian conditions.

Polimorfizmi gena FSHR (ekson 10) i njegova povezanost sa osobinama plodnosti ovaca rase osimi u Egiptu

Salah Abdel-Rahman, Yehia Mustafa, Hagar Abd Errasool, Hanim Heikal, Ayaat Elmaghraby

Rezime

Za povezanost između polimorfizama gena receptora za stimulaciju folikula (Follicle stimulating hormone receptor - *FSHR*) (delimični deo egzona 10) i osobine veličine legla ovaca egipatske osimi rase, razvijene su tehnike lančana reakcija polimeraza - jednocevni konformacioni polimorfizam (PCR-SSCP) i tehnike sekvenciranja DNK. Pedeset ženskih grla rase osimi uzgajanih u egipatskim uslovima odabrano je prema veličini njihovog legla. DNK iz uzoraka krvi ovih životinja je izolovana da amplifikuje 250 bp gena *FSHR* gena koji utiču na proizvodnu osobinu veličina legla kod ovaca. Na osnovu veličine legla, 50 životinja je izabrano od najvećih do najnižih vrednosti veličine legla tokom tri sezone. PCR-SSCP analiza *FSHR* gena (250-bp) pokazala je dva različita genotipa AA i AB sa frekvencijama 0,64 i 0,36, respektivno. Frekvencije A i B alela su bile 0,82 i 0,18, respektivno. PCR fragment *FSHR* gena (191-bp) je sekvencioniran samo u životinjama sa visokom i niskom produktivnošću veličine legla (GenBank pristupni brojevi od MG973191 do MG973207, sekvencijalno). Rezultat je pokazao da je 6SNPs (G/71, G/72, G/77, A/110, A/111, A/191) utvrđen kod visoko plodnih životinja, dok je 10 SNPs (T/1, C/2, T/14, A/69, A/70, A/71, A/74, G/74, A/75, A/136) utvrđen kod nisko plodnih grla. Statistički, genotipovi AA i AB nemaju značajnih razlika ($p > 0,05$) na osobini veličine legla ovaca rase osimi. Lokus *FSHR* (ekson 10) bio je umereno polimorfan ($PIC = 0,25$) i može se koristiti za visoku produktivnost veličine legla osimi ovaca kao metoda selekcije uz pomoć markera (MAS).

Ključne reči: Osimi ovce, plodnost, polimorfizmi gena *FSHR*, PCR-SSCP, sekvenciranje DNK

References

- ABDOLI R., ZAMANI P., MIRHOSEINI S.Z., GHAVI HOSSEIN-ZADEH N., NADRI S. (2016): A review on prolificacy genes in sheep. *Reproduction in Domestic Animals*, 51, 631-637.
- ABRAHAM A., THOMAS N. (2012): Role of fecundity genes in prolificacy of small ruminants. *Journal of Indian Veterinary Association*, 10, 34-37.
- ABULYAZID I., ABDALLA M.S., SHARADA H.M., HASSANIN W.F. (2011): Prolificacy detection in Egyptian sheep using RFLP-specific PCR. *Egyptian Academic Journal of Biological Sciences*, 3, 1-4.

- BOGERD J. (2007): Ligand-selective determinants in gonadotropin receptors. *Molecular and Cellular Endocrinology*, 260–262, 144–152.
- CHU M. X., GUO X. H., FENG C. J., LI Y., HUANG D.W., FENG T., CAO G. L., FANG L., DI R., TANG Q. Q., MA Y. H., LI K. (2012): Polymorphism of 5' regulatory region of ovine *FSHR* gene and its association with litter size in Small Tail Han sheep. *Molecular Biology Report*, 39, 3721–3725.
- CUI H. X., ZHAO S. M., CHENG M. L., GUO L., YE R. Q., LIU W. Q., GAO S. Z. (2009): Cloning and expression levels of genes relating to the ovulation rate of the Yunling black goat. *Biology of Reproduction*, 80, 219–226.
- DIAS J. A., COHEN B. D., LINDAU-SHEPARD B., NECHAMEN C. A., PETERSON A. J., SCHMIDT A. (2002): Molecular, structural, and cellular biology of follitropin and follitropin receptor. *Vitamins and Hormones*, 64, 249–322.
- ELSHENNAWY M. (1995): Sheep development program in Egypt, Strategies for sheep and goat breeding. Zaragoza, 27-32. <http://om.ciheam.org/article.php?IDPDF=96605538>.
- FAN Q.R., HENDRICKSON W.A. (2005): Structure of human follicle-stimulating hormone in complex with its receptor. *Nature*, 433, 269–277.
- GEORGE J.W., DILLE E.A., HECKERT L.L. (2011): Current concepts of follicle-stimulating hormone receptor gene regulation. *Biology of Reproduction*, 84, 7-17.
- GUO X., YANG L., MINGXING C., FENG C., DI R., LIU Q., FENG T., CAO G., HUANG D., FANG L., TANG Q., MA Y., LI K. (2013): Polymorphism of 5' regulatory region of caprine *FSHR* gene and its association with litter size in Jining Grey goat. *Turkish Journal of Veterinary and Animal Sciences*, 37, 497-503.
- IBM Corp. Released (2013): IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.
- ICARDA. ICARDA Annual Report 2006 (2007): International Center for Agricultural Research in the Dry Areas. Aleppo, Syria; p.130.
- JIANG X., LIU H., CHEN X., CHEN P.H., FISCHER D., SRIRAMAN V., YU H.N., ARKINSTALL S., HE X. (2012): Structure of follicle-stimulating hormone in complex with the entire ectodomain of its receptor. *Proceeding of The National Academy of Sciences*, 109, 12491–12496.
- KUMAR S., STECHER G., TAMURA K. (2016): MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870-1874.
- LI Y.J., ZHANG L., SHANG L.Q., WANG H.F., ZOU H., ZHANG H., JI D.J. (2010): Genetic polymorphisms at three loci of PRLR and FSHR Gene correlate with litter size in Chinese Haimen goat. *Journal of Animal and Veterinary Advances*, 9, 2835-2838.
- MARSON E.P., FERRAZ J.B., MEIRELLES F.V., BALIEIRO J.C., ELER J.P. (2008): Effects of polymorphisms of LHR and FSHR genes on sexual precocity in

- a *Bostaurus* × *Bosindicus* beef composite population. *Genetics and Molecular Research Journal*, 7, 243–251.
- MASON I.L. (1996): *A World Dictionary of Livestock Breeds, Types and Varieties*. Fourth Edition. C.A.B International, 273.
- MISHRA C. (2014): Genetic basis of prolificacy in sheep. *International Journal of Livestock Research*, 4, 46-57.
- RICHARDS J.S., MIDGLEY A.R. (1976): Protein hormone action: a key to understanding ovarian follicular and luteal cell development. *Biology of Reproduction*, 14, 82–94.
- SHARIFIYAZDI H., ABDOLAH M., ZAHRA G. (2018): Characterization of polymorphism in the FSH receptor gene and its impact on some reproductive indices in dairy cows. *Animal Reproduction Science*, 188, 45-50.
- WANG W., SHIJIA L., FADI L., XIANGYU P., CHONG L., XIAOXUE Z., YOUJI M., YONGFU L., RUI X., TINGFU L. (2015): Polymorphisms of the Ovine BMPR-IB, BMP-15 and FSHR and Their Associations with Litter Size in Two Chinese Indigenous Sheep Breeds. *International Journal of Molecular Sciences*, 16, 11385-11397.
- YANG W.C., LI S.J., TANG K.Q., HUA G.H., ZHANG C.Y., YU J.N., HAN L., YANG L.G. (2010): Polymorphisms in the 50 upstream region of the FSH receptor gene, and their association with superovulation traits in Chinese Holstein cows. *Animal Reproduction Science*, 119, 172–177.
- ZHANG S.J., XIONG Y.Z., DENG C.Y., ZHENG R., JIANG S.W., XIAO S.M., XIA Y., XU J.X., LIU X.H., WANG C.F., YUAN Z., GONG S.Y. (2002): Study on follicle stimulating hormone receptor as candidate gene for litter size in pigs. *Journal of Huazhong Agricultural University*, 21, 506–508.
- ZHU J., YANG S.L., OUYANG X.X., LIU H.X., HU S.G. (2007): Relationship between polymorphism of follicle stimulating hormone receptor gene and litter size traits in goats. *Grassfeeding Livestock* 1, 12–15.