

Detection of *G1* Point Mutation of Growth Differentiation Factor 9 Gene and its Association with Litter Size in Three Sudanese Desert Sheep Ecotypes

AMANI ZEINELABDEEN ABDELGADIR FRAH^{1,2} LUTFI M.-A. MUSA^{3,4}, SARA T. IBRAHIM⁵, ROMAZ M.-A.OMER⁶

¹ Head department of Animal Production, Faculty of Agriculture, Omdurman Islamic University, Sudan

² Department of Animal Production, Faculty of Agricultural Production and Processing Technology, International University of Africa, Sudan.

³ Department of Animal Breeding and Genetics Faculty of Animal Production, University of Khartoum, Sudan

⁴ Arabs Center for Studies of Arid Zone and Dry Land (ACSAD), Syria

⁵ Ministry of Animal Resources, Sudan

⁶ University of Khartoum, Sudan

* Corresponding author: amanizein@yahoo.com

المستخلص: تم استخلاص الحمض النووي الجينومي من الدم الكامل لثلاثة أنواع فرعية من الأغنام الصحراوية (الدباسي ، والوتيش ، والشقر) باستخدام طريقة كلوريد الجوانيديين بهدف الكشف عن الطفرات المحتملة التي قد تصاحب عدد المواليد في البطن الواحدة في الأغنام الصحراوية في السودان. تم استخدام PCR-RFLP باستخدام إنزيم القطع *HhaI* وتحديد تسلسل الحمض النووي باستخدام تقنيات التسلسل للتمييز الجيني لموضع *GDF9* في الأنواع الفرعية الثلاثة. كان تغير الأحماض الأمينية الناتج عن طفرة *G1* هو أرجينين إلى هيسثيدين عند الموقع 87. كان لأليل النوع البري (A) أعلى تكرارات ، بينما كان لأليل النوع الطافر (a) أقل تكرارات في جميع الأنواع الفرعية المدروسة. كانت تكرارات التركيب الوراثي للنوع البري (AA) أعلى من التكرارات غير المتماثلة (Aa) وتكرارات التراكيب الطافرة (aa) في عشائر الأغنام الثلاثة المدروسة. لم تكن هناك فروق معنوية في تكرارات الأليل بين الأنواع الفرعية الثلاثة. تأثر عدد المواليد في البطن الواحدة بشكل كبير بالتركيب الوراثي لجين *GDF9* ، وترتيب الولادة والسلالة ($P \leq 0.01, 0.01$ and 0.05 على التوالي). سجل نوع الوتيش والولادة الثانية وكذلك الأفراد غير المتماثلين أعلى عدد مواليد في البطن الواحدة. تم تحديد تسلسل أربع عينات من الحمض النووي. أشارت نتائج محاذاة عينات الجينات *GDF9* مع إدخال قاعدة البيانات إلى أن جميع الأنواع الفرعية الثلاثة كانت متشابهة ومطابقة للتسلسل المرجعي. أشارت نتائج محاذاة عينات الجينات *GDF9* مع التسلسل الموجود في قاعدة البيانات إلى أن جميع السلالات الثلاثة كانت متشابهة مع بعضها ومتماثلة مع التسلسل المرجعي. لم تلاحظ أي تغييرات في النوكليوتيدات وجميع الأحماض الأمينية المترجمة ظلت على حالها وفقا للتسلسل المرجعي. كشفت شجرة النشوء والتطور أن سلالة الشقر هي سلف مشترك للسلالات السودانية المدروسة وأن سلالة الوتيش أكثر ارتباطًا بسلالة الشقر مقارنة بسلالة الدباسي. هذه النتيجة قد تلقي بعض الضوء على انخفاض القدرة التناسلية للدباسي.

Abstract: Genomic DNA was extracted from whole blood of three Desert sheep subtypes (Dubasi, Watish and Shugor) using the guanidine chloride method aiming at detecting possible mutation that may associated with litter size in the Desert sheep of Sudan. The PCR-RFLP using *HhaI* restriction enzyme and sequencing techniques sequencing were used for genotyping the *GDF9* locus in the three subtypes. The amino acid change resulting from the *G1* mutation was Arginine to Histidine at residue 87. The wild type allele (A) had the highest frequencies, whereas the mutant type allele (a) had the lowest frequencies in all studied subtypes. The genotype frequencies of the wild type ewes (AA) were higher than the heterozygous (Aa) and the mutant type (aa) frequencies in the studied three populations of sheep. There were no significant differences in the allele frequency between the three subtypes. Litter size was significantly influenced by genotypes of *GDF9* gene, parities and subtypes ($P \leq 0.01, 0.01$ and 0.05 , respectively). Watish ecotype in the second parity and the heterozygous individuals recorded the highest litter size. Four samples of DNA were sequenced. The results of alignment of *GDF9* gene samples with the database entry indicated that all three subtypes were similar and identical to the reference sequence. The phylogenetic tree revealed that, Shugor is the common ancestor of studied subtypes and Watish is more related to Shugor than Dubasi. This result might explain the lower reproductive performance of Dubasi compared to Watish and Shugor.

Keywords: *GDF9* gene, Sudanese Sheep, Common Ancestor, Similarity and Identity

Ethical Approval: According to the Animal Use in Research Committee of the Sudan Veterinary Council no special approval was required for this research. The Council's guidelines regarding the handling of animals and sampling were strictly adhered to.

1. Introduction:

Growth differentiation factor 9 or *FecG* is a member of the transforming growth factor β that is released from oocytes during folliculogenesis. It also inhibits granulosa cell apoptosis and follicular atresia which is essential for normal follicular development from the early stage in sheep (Khodabakhshzadeh et al., 2016). Other functions of *GDF9* gene include cellular processes involved in reproduction, female gamete generation, female gonad development, multicellular organism reproduction, multicellular organismal reproductive process, ovulation cycle process, regulation of reproductive process, sexual reproduction, transforming growth factor beta receptor signaling pathway, transmembrane receptor protein serine/threonine kinase signaling pathway (Mostafavi et al., 2008).

Ovine *GDF9* gene spans approximately about 2.5 kilo bases at chromosome 5 and contains two exons separated by one intron (Figure 1). The two exons spanned about 397 and 965 bp respectively. The first encodes for 1-134 amino acids, and the second encodes for 135-456 amino acids, while the intron spans about 1126 bp (Khodabakhshzadeh et al., (2016); Chu et al., (2011)). Mutations in *GDF9* have different effects on ovulation rate and they can even cause infertility in some cases (Abdoli et al., 2013). There are eight mutations (*G1-G8*) in growth differentiation factor 9 gene (*GDF9*) two of these mutation *G2* and *G3* are located in intron while *G5* located in exon2 but lead to unchanged sequence of amino acids (Roy, et al, 2011) (Figure 2). The remaining five nucleotide substitutions (*G1, G4, G6, G7, and G8*) lead to amino acid substitutions. For *G1* mutation, the heterozygotes ewe had the highest fertility whereas the mutant type ewes had non additive effect on ovulation rate and causing sterility (Hanrahan et al, 2004). The effect of this mutation (*FecG1*) is about 1.4 lambs per ewes. (Barzegari et al., 2010) attributed that to the possibility that the absence of this gene blocks the follicular growth at the primary stage in homozygotes, which results in sterility, while inactivation of only one copy of *GDF9* increases the ovulation rate. *GDF9* gene was known to influence ovulation rate in a dose-responsive manner (Våge et al., 2013).

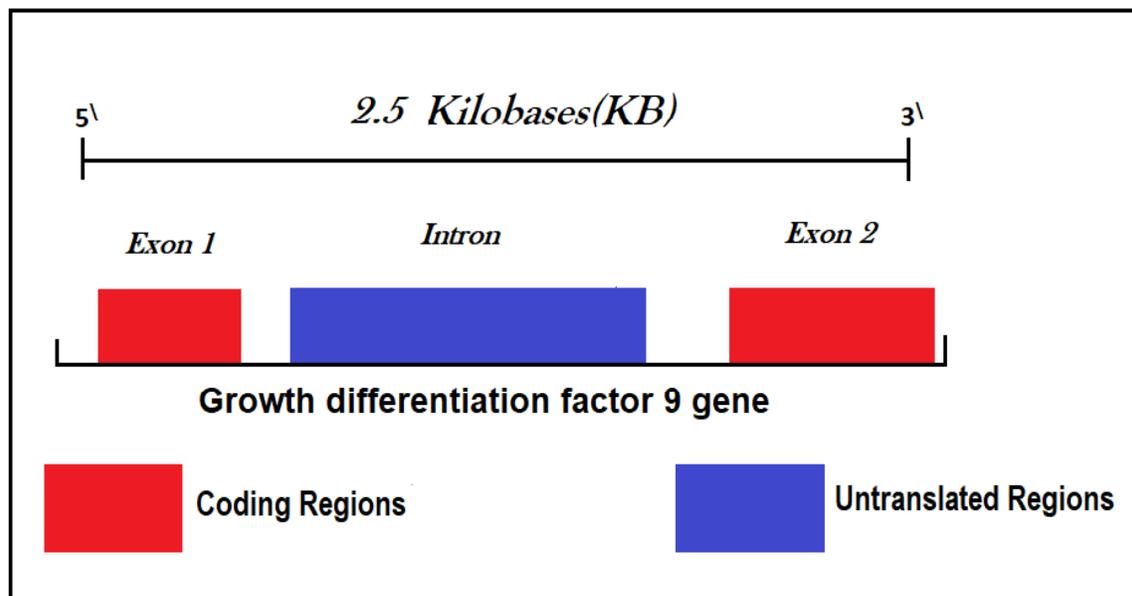


Figure 1: *GDF9* Gene Structure, it contains two exons separated by one intron (Roy et al., 2011)

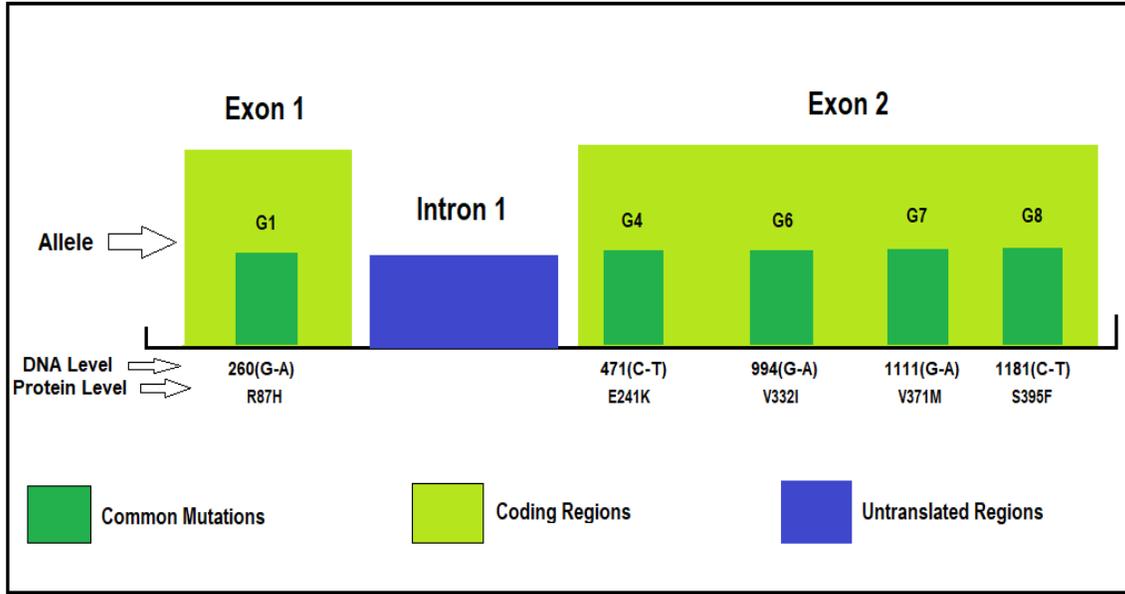


Figure 2: The most reported mutation of *GDF9* gene. Note that G2 and G3 are located in intron while G5 located in exon but lead to unchanged amino acid (Hanrahan et al., 2004)

2. Materials and Methods:

2.1 Genotypic Detection of *GDF9* Gene:

The recent research was carried out in the Molecular Biology and Immunology Unit, Department of Biology, Central laboratory of Veterinary Research, Ministry of Animal Resources and Fisheries.

2.1.1 Samples Collection:

Blood samples were randomly collected from unrelated 100 ewes. The ewes belonged to the three subtypes of Sudanese Desert Sheep, namely Dubasi {n= 30}, Shugor {n= 30}, and Watish {n= 40}. Blood samples were taken from the jugular vein and placed in 10 ml vacuum blood collection tubes containing EDTA. All samples were immediately placed in an ice box and transferred to the laboratory (The Central Laboratory of Veterinary Research, Sudan) where they were stored under refrigeration (-20°C) pending DNA extraction.

2.1.2 DNA Extraction:

Genomic DNA was extracted from whole blood. The extraction was performed and the DNA purified using Guanidine Chloride method as described by (Gassoum *et al.*, 2014) with slight modifications. In this method, 3-5 ml blood was collected in EDTA tubes, then 10 ml red cell lysis buffer (RCLB) was added in a Falcon tube and centrifuged for 5 min at 6000 rpm, this process was repeated until a clear pellet appeared. The supernatant was discarded and 800 µl of white cell lysis buffer (WCLB) and 10 µl of proteinase K (10 mg/ml) plus 1 ml Guanidine Chloride and 300 µl Ammonium Acetate, were added, vortexed and incubated at 37°C overnight. Then, an equal volume of chloroform in new Falcon tube was added, mixed well and centrifuged at 6000 rpm for 5 mins. The upper layer was collected in to a clean Falcon tube and 10 ml of cold ethanol (95%) was added and incubated at -20°C overnight. Thereafter, the sample was centrifuged for 10- 15

minutes carefully at 6000 rpm, the supernatant discarded. Then, the pellet was washed with 4 ml of 70% ethanol and centrifuged for 7 min at 12000 rpm, and the supernatant discarded. The previous steps were repeated till the pellet became clear and was dried for 1-2 hours. Then 100 ml of Tries Acetate (TE) buffer or 20 µl distilled water was added and stored at -20° C.

The quality of the extracted DNA was checked by adding 2µl of template DNA and 3µl of loading dye, and visualized on 1.5% Agarose gel by using buffer containing ethidium bromide. The images of each gel or the DNA band were photographed using Bio-Rad Gel Documentation 2000 system. The good quality samples were kept till use.

2.1.3 Amplification of Growth Differentiation Factor 9 gene:

The amplification reaction was carried out by RFLP-PCR and the conditions were as described by (Hanrahan *et al.*, (2004); Moradband *et al.*, (2011) (Table 1) using 35 cycles at 95° C for 300s at initial denaturation, followed by 94° C for 45s at denaturation, 58° C for 40s at annealing, 72° C for 60s at extension and final extension at 72° C for 10 min. The PCR products (462 bp) were digested by *HhaI* restriction enzyme at 37° C for 3 hours. Then added PCP Products about 7 µl with 2 µl buffer, 0.5 µl *HhaI* enzyme, and 0.5 µl bovine serum albumin (BSA) as enhancer. The mixture was made up to 25 µl by addition of sterilized distilled water, then spinned for 30s, and incubated at 37° C overnight. The amplified samples were loaded on 1.5% agarose gel and visualized by U.V under gel documentation system.

Table 1: The primers and restriction enzyme

Gene	Point mutation	Primer name	Primer Sequences 5'-3'	PCR Product (bp)	Annealing temperature	Restriction Enzyme	Reference
GDF 9	G1	G9-1734F	5'-GAAGAC TGGTATG GGG AATG-3'	462 bp	58° C	HhaI	Hanrahan et al, (2004); Moradband et al, (2011)
		G9-2175R	5'-CCAATC TGCTCC TACAC ACCT-3'				

Source: Modified from Hanrahan et al., (2004); Moradband et al., (2011)

2.1.4 Gene and genotype frequencies estimation:

Gene and genotype frequencies were calculated based on the counting method as described by Falconer and Mackay (1996), which depends on the genotype numbers observed in the population according to the following equations:

$$P = [AA + \frac{1}{2}Aa] / N$$

$$Q = [aa + \frac{1}{2}Aa] / N$$

Where:

P = The wild type allele frequency.

Q = The mutant allele frequency.

AA = The observed numbers of wild type individuals.

Aa = The observed numbers of heterozygous individuals.

aa = The observed numbers of mutant type individuals.

N = The total number of observed individuals.

The Chi square test was used to test Hardy-Weinberg equilibrium.

2.1.5 Association analyses between genotypes, parity number and Litter size in studied breeds:

The association between genotypes of the studied fecundity genes, parity number and litter size in the three sheep breeds under study was analyzed using the General linear model (GLM) method, performed by IBM SPSS Statistics version 21 (Statistical Package for the Social Sciences). The chi-square test was used to check the statistical significance, and a p-value of ≤ 0.05 was considered not significant. Analysis of variance and Duncan multiple range test (DMRT) were carried out as appropriate to assess the statistical significance of various factors affecting litter size. The linear model was:

- Model (1):

$$Y_{ijk} = \mu + B_i + G_j + P_k + E_{ijk}$$

Where:

Y_{ijk} = The litter size record of the ijk^{th} ewe.

μ = The overall mean.

B_i = Effect of the i^{th} breed ($i = 1-3$).

G_j = Effect of the j^{th} genotype ($j = 1-3$).

P_k = Effect of the k^{th} parity number ($k = 1-4$).

E_{ijk} = The random error term.

2.2 Sequencing of *GDF9* gene:

The purification and standard sequencing of the PCR products of *GDF9* gene was performed for the three genotypes of *GDF9* gene by Macrogen Company (Seoul, Korea).

2.3 Bioinformatics Analysis:

2.3.1 Sequences similarity and alignment:

The DNA Chromatograms were presented by the “Finch TV 1.4.0 software” and DNA cleaning process was done by removing nucleotides with sharp quality (Less than 10) then, nucleotides of *GDF9* gene were blasted against databases to get the most similar and identical sequences (Johnson et al., 2008). Sequences with high identities were gotten from NCBI in the FASTA format and they underwent alignment by “Bio Edit 7.0 software” with the reference sequence (Pruitt et al., 2005) which was obtained from NCBI (Coordinators, 2017) (NC_019484). Then, global phylogenetic trees were designed using “Multiple sequence alignment by software CLUSTALW” (McWilliam et al., 2013) to determine the relationship among sheep breeds from different countries. The reference proteins of studied fecundity genes was obtained from ExPASy-Universal protein resource (Research, 2007). Nucleotide sequence was translated to protein by ExPASy translation tool (Gasteiger et al., 2003) and subjected to multiple sequence alignment by using Bio Edit 7.0 software.

3. Result:

3.1 Allele and Genotype frequencies:

3.1.1 Detection of the *G1* Mutation of the *GDF9* Gene:

PCR products were detected by running on a 1.5% agarose gel electrophoresis. The results of amplification of exon 1 of *GDF9* gene showed the desired fragments properly amplified in a product size of 462 bp fragment (Figure 3).

The PCR-RFLP approach was applied to identify genotypes of the *GDF9* gene in the sheep samples under study by using *HhaI* restriction enzyme. The nucleotide substitution G to A in *GDF9* exon 1 region disrupts a *HhaI* restriction enzyme cleavage site (GCGC to GCAC) at nucleotide 260 of the 462bp PCR produce. The amino acid change is Arginine to Histidine at residue 87.

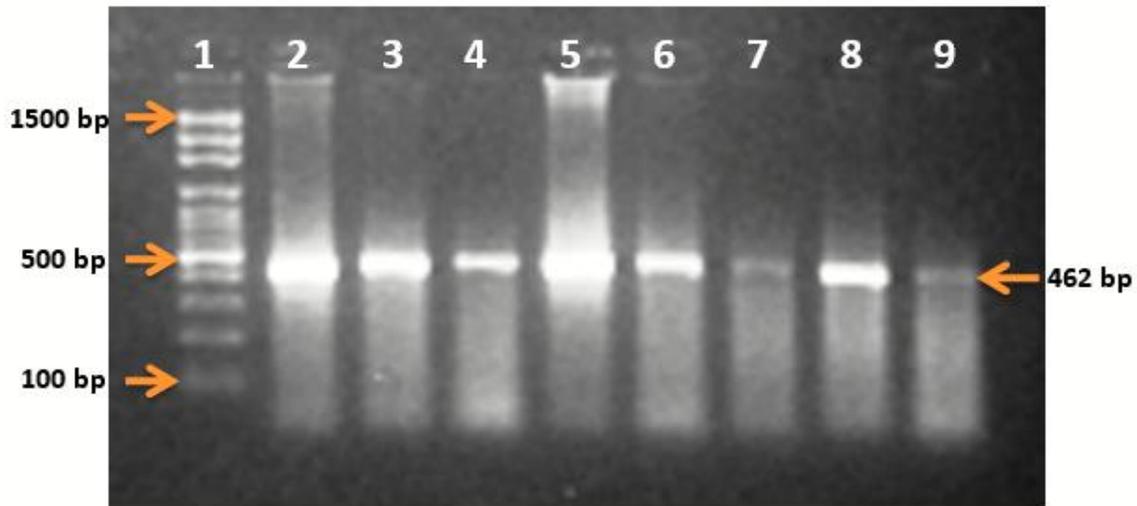


Figure 3: PCR products of exon 1 of *GDF9* gene (462 bp) on 1.5% agarose gel electrophoresis in Dubasi, Shugor and Watish Sudanese sheep breeds. Lane 1, DNA ladder: MW 100-1500 bp fragments. Lane 2, 3, 4, 5, 6, 7, 8, and 9 showing typical band size of (462bp) corresponding to the molecular size of *GDF9* gene.

Restriction digestion of PCR products with *HhaI* restriction enzyme showed three genotypes. The wild type animals (AA) had three fragments 254, 156 and 52 bp, the heterozygous animals (Aa) had four visible bands of 410, 254, 156 and 52 bp (Figure 4) and the mutant type animals had two fragments 410 and 52 bp (Figure 5).

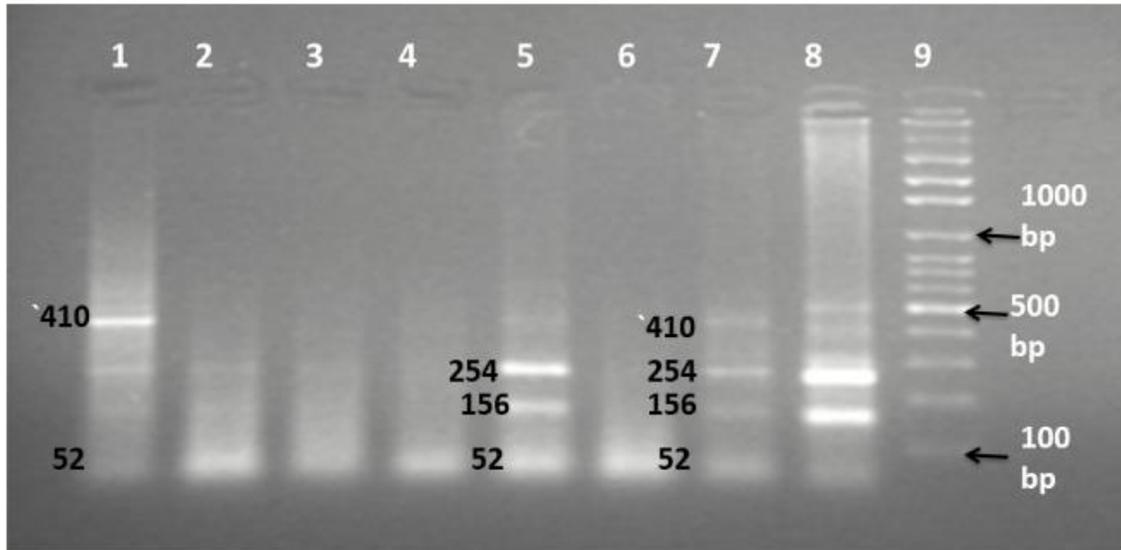


Figure 4: DNA electrophoretic pattern of *G1* point mutation of the *GDF9* gene amplified after digestion with *HhaI* restriction enzyme in three of Sudanese sheep breeds. Lane 1, 7, 8 (Aa): 410, 254, 156 and 52 bp. Lane 2, 3, 4 and 6 undigested PCR product. Lane 5 (AA): 254, 156, and 52 bp. Lane 9 DNA ladder: MW 100-1500 bp fragments.

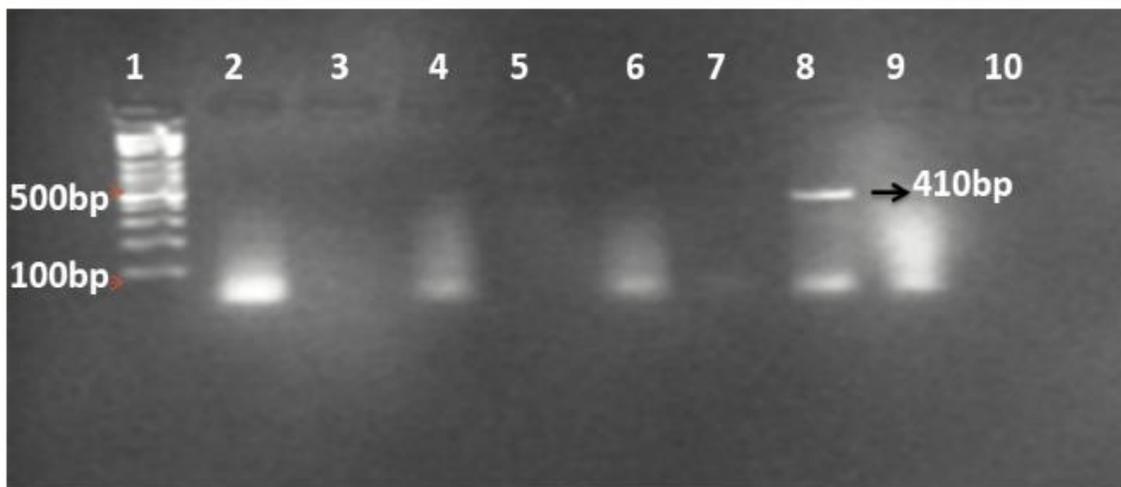


Figure 5: DNA electrophoretic pattern of *G1* point mutation of the *GDF9* gene amplified after digestion with *HhaI* restriction enzyme. Lane 1, DNA ladder: MW 100-1500 bp fragments. Lane 2, 4, 6 and 9 undigested PCR product. Lane 3, 5, 7 and 10 empty. Lane 8 (aa): 410 and 52 bp.

3.1.2 Genetic variability:

Table 2 presents the allele and genotype frequencies of *GDF9* gene. The wild type allele (A) had the highest frequency, whereas mutant type allele (a) had the lowest frequencies in Dubasi, Watish and Shugor breeds. The genotype frequencies of the wild type ewes (AA) was obviously higher than the heterozygous (Aa) and the mutant type (aa) frequencies in the three populations of sheep breeds under study.

Chi-square test was used to assess the Hardy - Weinberg equilibrium. The Chi-square test showed that the examined populations of Dubasi ($\chi^2=0.63$), Shugor ($\chi^2=0.16$) and Watish

($\chi^2=0.04$) were in Hardy-Weinberg equilibrium, while the pooled population of the three breeds was not ($\chi^2=14.00$).

Table 2: Allele and genotype frequencies of the point mutation *G1* of the *GDF9* gene in Dubasi, Shugor and Watish sheep breeds¹

Breed	Number of animals				Allele frequency		Genotype frequency			H.W.E (χ^2 - value)
	Total	AA	Aa	aa	(A)	(a)	AA	Aa	aa	
Dubasi	26	16	8	2	0.77	0.23	0.61	0.31	0.08	0.63 NS
Shugor	28	16	10	2	0.75	0.25	0.57	0.36	0.07	0.16 NS
Watish	34	20	12	2	0.76	0.24	0.59	0.35	0.06	0.04 NS
Total	88	54	30	6	0.78	0.22	0.60	0.34	0.06	14.00 S

¹NS: No significant deviation from HWE, S: Significant deviation from HWE, ($p<0.01$)

3.1.3 Association with litter size:

The least squares means and standard error for litter size of the different *GDF9* genotypes and parities in Dubasi, Shugor and Watish sheep are given in Table 3. The ewes with heterozygous (Aa) and homozygous wild type (AA) genotype had 0.346 and 0.207 lambs more than the homozygous (aa) genotypes, respectively. Moreover, the Watish breed had a higher litter size followed by Shugor and Dubasi breeds. The 2nd parity was higher in litter size followed by the 3rd, 4th and 1st parities.

The analysis of variance results of litter size in the three sheep breeds are presented in Table 4. Litter size was highly significantly influenced by genotypes of *GDF9* gene, parities and breeds ($p<0.01$).

Table 3: Association between *GDF9* genotypes and parity number with litter size trait in Dubasi, Shugor and Watish sheep breeds¹

Variable	Type / Number	Litter Size
Breed	Dubasi	1.134± 0.053 ^a
	Shugor	1.232± 0.053 ^{ab}
	Watish	1.307± 0.051 ^b
Genotype	AA	1.247± 0.033 ^b
	Aa	1.386± 0.046 ^b
	Aa	1.040± 0.094 ^a
Parity Number	1 st	1.130± 0.053 ^a
	2 nd	1.357± 0.053 ^b
	3 rd	1.211± 0.058 ^a
	4 th	1.201± 0.072 ^a
Grand mean		1.224± 0.037

¹Means with same superscripts within each item are not significantly ($P<0.05$) different.

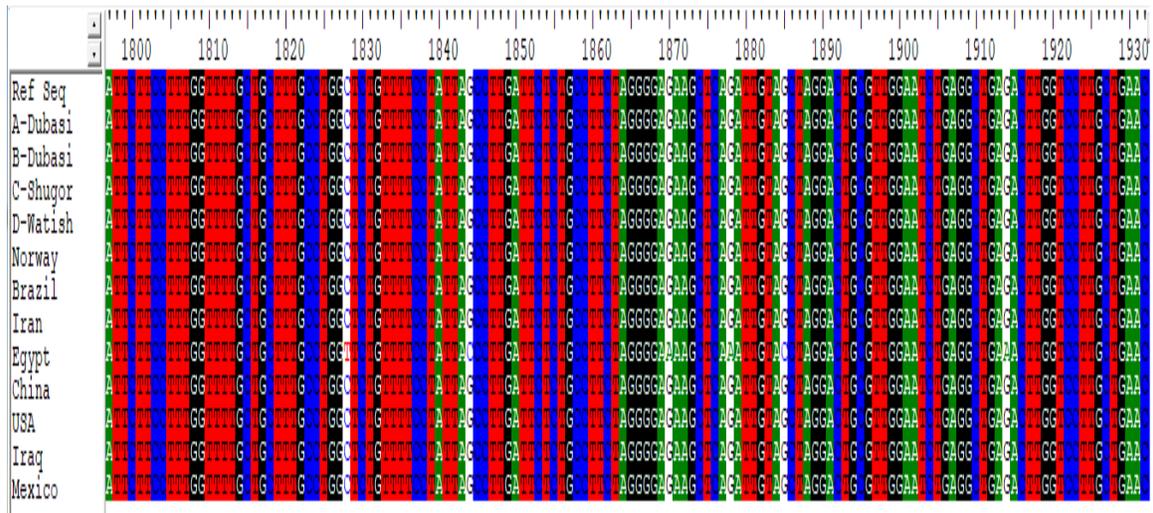
Table 4: Analysis of variance: The effect of breed, *GDF9* genotypes and parity on litter size in the three breeds¹.

Source of variation	DF	SM	MS	F-ratio	Pr> F
Breed	2	1.472	0.736	3.957*	0.020
Parity	3	2.364	0.788	4.234**	0.006
Genotypes	2	2.411	1.205	6.479**	0.002

¹SM = Sum of squares, DF = Degrees of freedom, MS = Mean of squares. *p < 0.05, **P < 0.01.

3.2 DNA Sequencing of *GDF9* gene:

Four different samples of DNA that were not used in the restriction enzyme analysis were subjected to sequencing. The objective was to broaden the sample base [accession numbers were: A-Dubasi (MN862511); B-Dubasi (MN862512); C-Shugor (MN862513); D-Watish (MN862514)]. The results of alignment of *GDF9* samples with the database entry indicated that all three breeds (Dubasi, Shugor and Watish) were similar and identical to Reference sequence. No nucleotide changes were observed and no mutations were detected in these four samples (Figure 6).



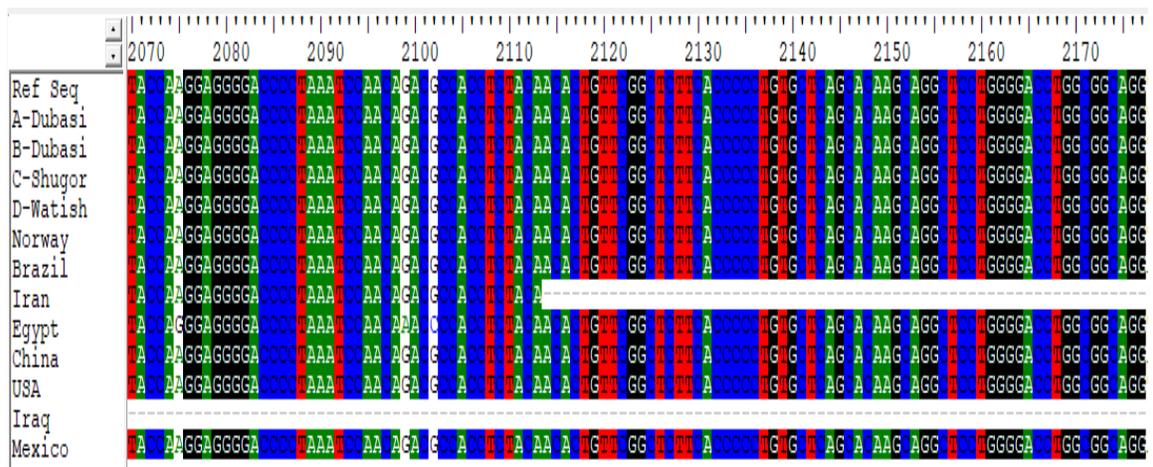
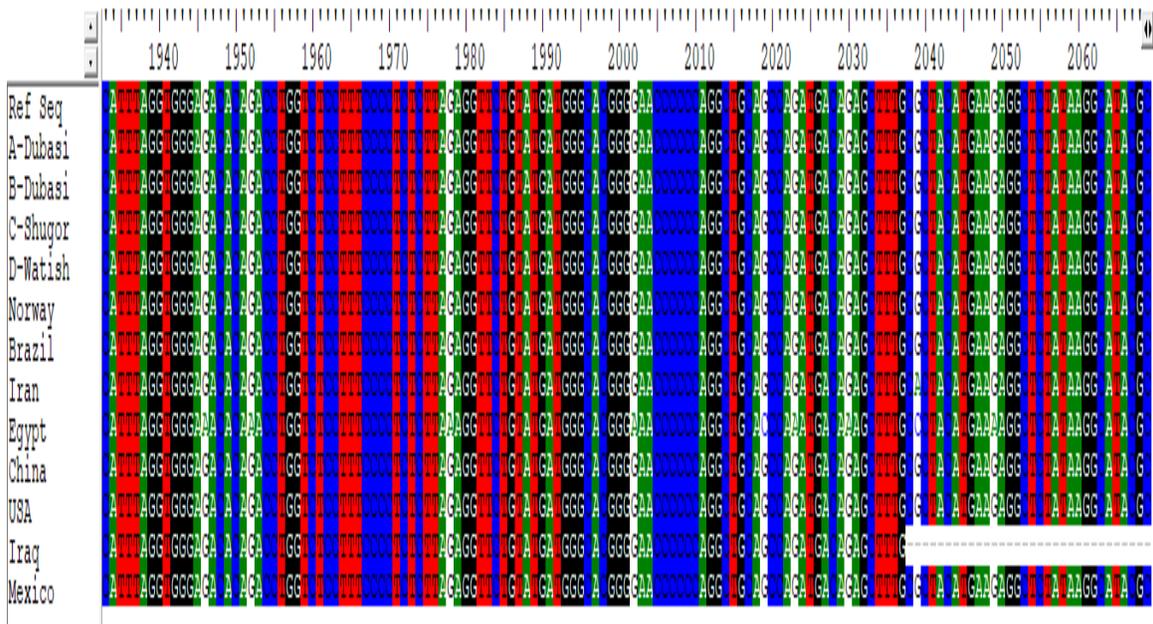
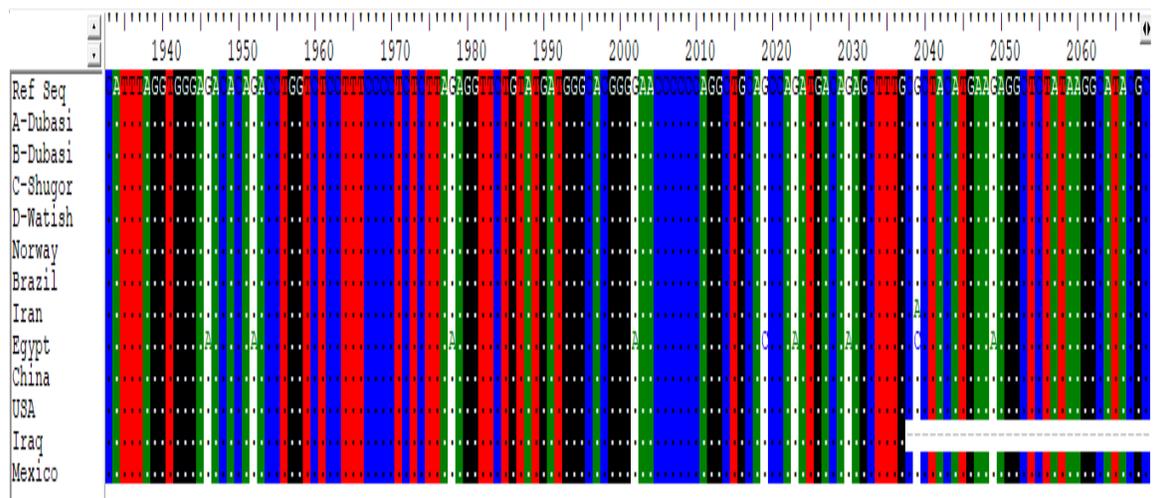
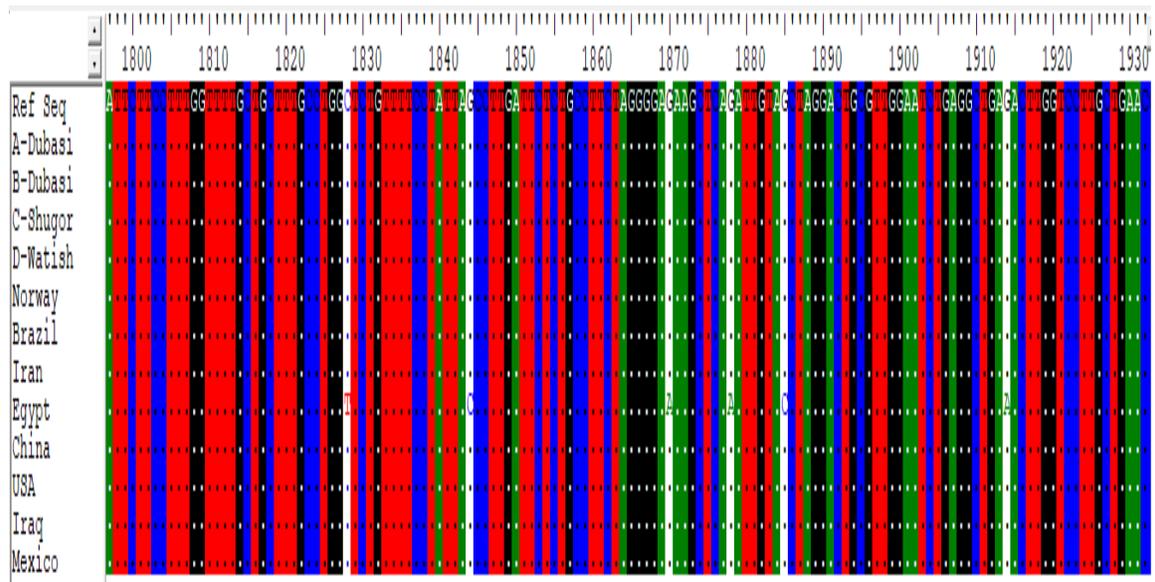


Figure 6: Similarity and Identity of alignment between the GDF9 DNA sampled from the three breeds and the database Ref Seq (Bio-Edit 7.0 software), Iraq (MF416087), Norway (HE866499), Egypt (KT357485), Brazil (FJ429111), Iran (KX377509), China (KR063137), USA (AF078545), and Mexico (KT8530)

On conservation level, all Sudanese breeds were conserved with the Reference sequence as shown in Figure 7.



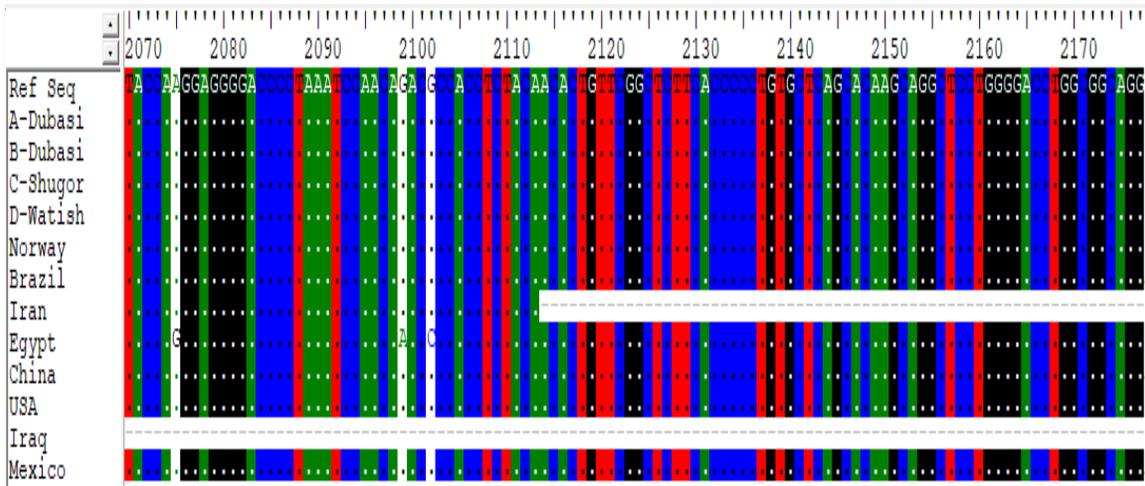


Figure 7: Conserved regions in alignment of *GDF9* gene with database and Ref Sequences (Bio-Edit 7.0 software), Iraq (MF416087), Norway (HE866499), Egypt (KT357485), Brazil (FJ429111), Iran (KX377509), China (KR063137), USA (AF078545), and Mexico (KT8530)

There wasn't any change at the DNA level. All translated amino acids were the same according to reference sequence without any mutation as shown in Figure 8.

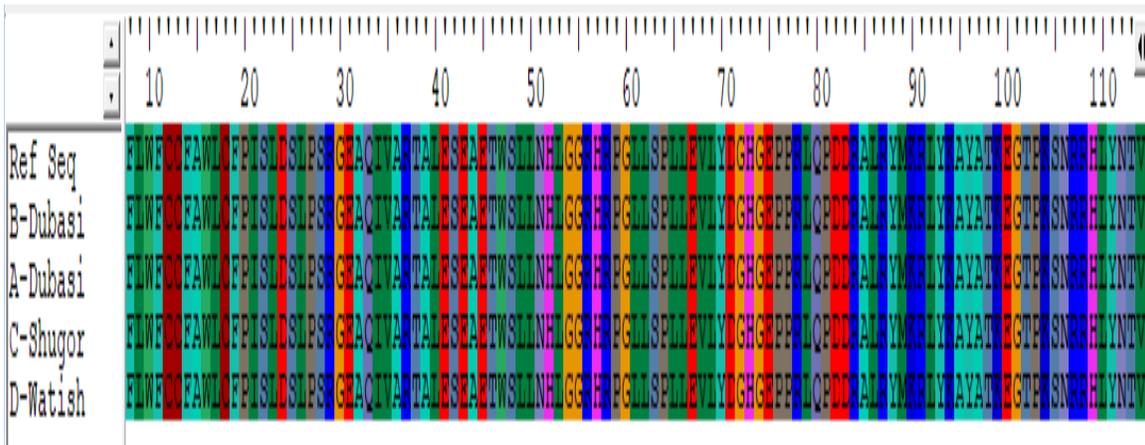


Figure 8: Amino acid alignment of *GDF9* gene with ref sequence using Bio-Edit 7.0 software

Figure 9 shows the phylogenetic tree for the three breeds. The two samples of Dubasi breed (A and B) were the most related to each other, Norway is the most related global sample to Sudanese breeds (Including Watish and Shugor). Brazil, china and Mexico were out grouped. In addition, China and Brazil were more related to each other than Mexican samples.

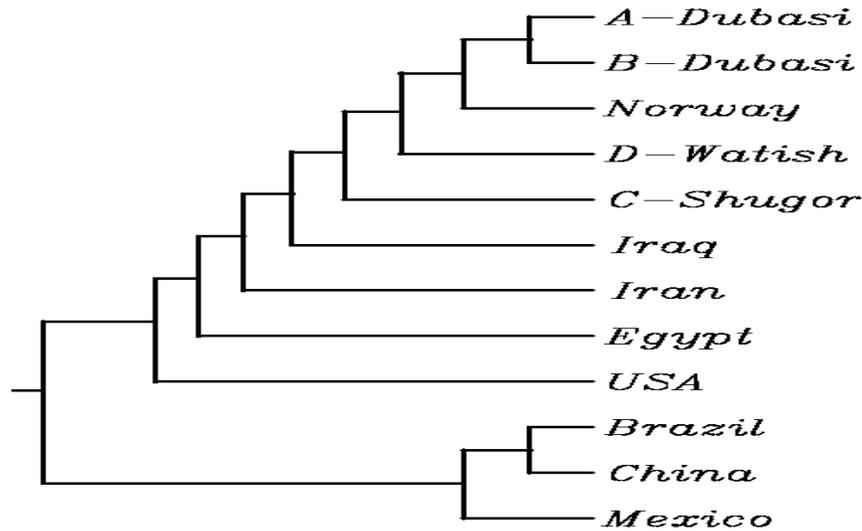


Figure 9: Global Phylogenetic tree (Multiple sequence alignment by CLUSTALW). Iraq(MF416087), Norway(HE866499), Egypt(KT357485), Brazil(FJ429111), Iran(KX377509), China(KR063137), USA(AF078545), and Mexico (KT8530).

4. Discussion:

In the current research, we analyzed the polymorphic variations of the gene coding for the growth differentiation factor 9, a member of the transforming growth factor β superfamily. This superfamily has a vital role in female fertility and the *GDF9* Protein is very essential for ovarian follicular development in sheep especially in the early stages of folliculogenesis (Otsuka *et al*, 2010). This study focused on scanning for the *G1* point mutation located in exon 1 of *GDF9* gene in three Sudanese sheep breeds.

PCR-RFLP is a dependable and simple method used to study the polymorphisms of the *GDF9* gene in many sheep breeds. In this study the scanning for the *G1* mutation was done in Dubasi, Shugor and Watish sheep breeds in Central Sudan. The results showed that the frequency of the mutant allele was low (0.22) compared to wild type allele (0.78) in all three studied populations. However, the results showed that *GDF9* locus was polymorphic in the studied individuals and the frequency might change if a larger sample is used. This result is in agreement with reports in Belclare and Cambridge breeds (Hanrahan *et al*, 2004), Arkha Merino sheep (Farajzadeh *et al*, 2007), Moghani and Ghezded breeds (Barzegari *et al*, 2010), Sangsari sheep (Kasiriyan *et al*, 2011), Baluchi sheep (Moradband *et al*, 2011), Hisari sheep (Yadollah *et al*, 2014), Moghani sheep (Ala and Rafat, 2014) and Saidi sheep (EL, F.Z.A., *et al*, 2017). On the other hand Gorlov *et al*, (2018) reported that, the frequency of the mutant allele was high and there were no observations of wild type genotypes (AA) in Volgograd and Salsk sheep. EL, F.Z.A., *et al*, (2017) reported that all of the observed genotypes were wild type in Ossimi sheep. Whereas, Liandris *et al*, (2012) and Nanekarani *et al*, (2016) reported no observations of the mutant type individuals in Lori sheep breeds population. The low frequency of the mutant genotypes found in the

studied Sudanese sheep suggests the possibility of spreading this important mutation in these breeds by marker-assisted selection (MAS).

The chi-square test results indicated that the populations of Dubasi, Shugor and Watish sheep were in Hardy-Weinberg equilibrium for the *GDF9* locus when considered separately, while they were not in equilibrium when pooled together. This result is probably due to the low sample size used in this study. Similar to this report, Nanekarani *et al*, (2016); Bahrami *et al*, (2014) and Kasiriyani *et al* (2011) observed that populations of Lori, Hisari and Sangsari sheep were in the Hardy-Weinberg equilibrium at the *GDF9* locus.

The inheritance of sheep litter size has been studied by many investigators (Drouilhet *et al*, 2009; Mishra, 2014 and Zamani, 2015). Litter size varies between and within sheep breeds and it is governed by ovulation rate and the number of inseminated oocytes (Davis, 2005). In this study, litter size was significantly affected by *G1* point mutation of *GDF9* gene and heterozygous genotypes presented the higher litter size (1.386 ± 0.046) compared to wild type genotype (1.247 ± 0.033) and homozygous carrier ewes (1.040 ± 0.094) in the studied Sudanese sheep breeds. This result is in agreement with those obtained in Belclare and Cambridge breeds (Hanrahan *et al*. 2004), Baluchi sheep (Moradband *et al*, 2011), Afshari sheep (Javanmard *et al*, 2011). However, Liandris *et al*. (2012) reported an insignificant association between the mutation in the *GDF9* gene and litter size in Karagouniki breed.

The study showed that, the Watish ecotype demonstrated the highest litter size (1.307 ± 0.051) compared to the Shugor (1.232 ± 0.053) and Dubasi (1.134 ± 0.053) ecotypes, which may explain the multiple birth phenomenon usually observed in the Watish ewes as being partly due to the presence of *G1* mutation of *GDF9* gene.

In the present study the average litter size in the 2nd parity (1.357 ± 0.053) was significantly higher than other parities. The mean litter sizes of ewes in the 3rd parity (1.211 ± 0.058) and in the 4th parity (1.201 ± 0.072) were not statistically different and were higher than the litter size of ewes in the 1st Parity (1.130 ± 0.053) ($p < 0.01$). This result might be attributed to the degree of development of ewes in the first parity or to nutrition levels in the breeding season of the selected ewes. The amount of available pasture depends directly on the intensity of the rainy season which varies from one season to the other in these states.

In the sequence analysis, different four samples were used as mentioned previously for the purpose of expanding and generalizing the results. Our findings showed that there was no amino acid change and no nucleotide addition, substitution or deletion was found in the tested samples of Sudanese breeds and therefore the protein function is maintained. Also the results of alignment of *GDF9* gene samples with database sequence indicated that, all the breeds tested (Dubasi, Shugor and Watish) were similar and identical to the reference sequence. On the other hand, the phylogenetic tree analysis (Figure 9) showed that USA sample (AF078545) is the main common ancestor of Egypt sample (KT357485), Iran sample (KX377509), Iraq sample (MF416087), Shugor sample (C), Watish sample (D), Norway sample (HE866499) and Dubasi sub groups (A and B). Whereas Brazil (FJ429111), China (KR063137) and Mexico (KT8530) were out grouped. According to this finding Shugor breed is the common ancestor of studied Sudanese breeds and Watish breed is more related to Shugor than Dubasi breed. This result may explain the lower reproductive potential present in the Dubasi breed compared to Watish and Shugor.

5. Conclusion:

Genotypic characterization together with phenotypic characterization is an important step in any sustainable conservation or breeding programme. The results of the present study

revealed that the presence of one copy of *GDF9* gene increased the litter size in the studied Sudanese sheep. This is in agreement with the litter size effects of this gene found in the prolific sheep breeds in the world.

Acknowledgments

The authors kindly thank the staff member of Central laboratory of Veterinary Research, Ministry of Animal Resources and Fisheries, Sudan. We acknowledge Dr. Mohamed A. Salih and Dr. Aubai O. Saleem the staff members of the Department of Bioinformatics, Africa city of technology. We acknowledge Dr. Hisham N. Altayb from Sudan University of science and technology for their support. And also we acknowledge the sheep owners for their co-operation.

References:

- ABDOLI, R., ZAMANI, P., DELJOU, A. & REZVAN, H. J. G. 2013. Association of BMPR-1B and GDF9 genes polymorphisms and secondary protein structure changes with reproduction traits in Mehraban ewes. 524, 296-303.
- ALA NF, RAFAT A. Genetic Polymorphism of GD1F9 Gene in Iranian MOGHANI Sheep Breed. Iranian J APPL Animal SCI 2014; 4: 887-890.
- BAHRAMI, Y., BAHRAMI, S., MOHAMMADI, H.R., CHEKANI-AZAR, V. and MOUSAVIZADEH, S.A., 2014, July. The polymorphism of GDF-9 gene in HISARI sheep. In *Biological Forum* (Vol. 6, No. 2, p. 46). Research Trend.
- BARZEGARI, A., ATASHPAZ, S., GHABILI, K., NEMATI, Z., RUSTAEI, M. & AZARBAIJANI, R. J. R. I. D. A. 2010. Polymorphisms in GDF9 and BMP15 associated with fertility and ovulation rate in Moghani and Ghezel sheep in Iran. 45, 666-669.
- CHU, M., YANG, J., FENG, T., CAO, G., FANG, L., DI, R., HUANG, D., TANG, Q., MA, Y. & LI, K. J. M. B. R. 2011. GDF9 as a candidate gene for prolificacy of Small Tail Han sheep. 38, 5199-5204.
- COORDINATORS, N. R. J. N. A. R. 2017. Database resources of the national center for biotechnology information. 45, D12.
- DAVIS GH. Major genes affecting ovulation rate in sheep. Genet SEL EVOL. 2005; 37: 11–23.
- DROUILHET L, LECERF F, BODIN L, Fabre S, MULSANT P. Fine mapping of the FECL locus influencing prolificacy in LACAUNE sheep. ANIM Genet. 2009; 40:804–12.
- EL, F.Z.A., Hassan, G.M. and NASSAR, M.I., 2017. Genetic polymorphism detection in bone morphogenetic protein 15 (BMP15) gene related to fecundity in two Egyptian sheep breeds. *Biotechnology in Animal Husbandry*, 33(1), pp.37-53.
- FALCONER, D. S. and T. F. C. Mackay. 1996. Introduction to quantitative genetics, Fourth edition. Longman group Ltd.
- FARAJZADEH M, DEHNAD A, RAHIMI G, AMIRI A (2007) Genetic polymorphisms in oocyte-derived growth factor (GDF-9) gene in ARKHA Merino sheep using RFLP-PCR. The 5th National Biotechnology Congress of Iran. Tehran, Iran. p 503.
- GASSOUM, A., ARBAB, M. A., ALDEAF, S. A., ELHASSAN, L. A., ELSHIBLI, E. & ELHASSAN, A. M. J. I. 2014. Allele Frequency Of P53 Gene Arg72Pro In Sudanese Meningioma Patients And Controls. 6, 243-248.
- GASTEIGER, E., GATTIKER, A., HOOGLAND, C., IVANYI, I., APPEL, R. D. & BAIROCH, A. J. N. A. R. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. 31, 3784-3788.
- GORLOV, I. F., KOLOSOV, Y. A., SHIROKOVA, N. V., GETMANTSEVA, L. V., SLOZHENKINA, M. I., MOSOLOVA, N. I., BAKOEV, N. F., LEONOVA, M. A., KOLOSOV, A. Y. & ZLOBINA, E. Y. J. R. L. S. F. E. N. 2018. GDF9 gene polymorphism and its association with litter size in two Russian sheep breeds. 29, 61-66.

- HANRAHAN, J. P., GREGAN, S. M., MULSANT, P., MULLEN, M., DAVIS, G. H., POWELL, R. & GALLOWAY, S. M. J. B. O. R. 2004. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). 70, 900-909.
- JAVANMARD, A., AZADZADEH, N. & ESMAILIZADEH, A. K. J. V. R. C. 2011. Mutations in bone morphogenetic protein 15 and growth differentiation factor 9 genes are associated with increased litter size in fat-tailed sheep breeds. 35, 157-167.
- JOHNSON, M., ZARETSKAYA, I., RAYTSELIS, Y., MEREZHUK, Y., MCGINNIS, S. & MADDEN, T. L. J. N. A. R. 2008. NCBI BLAST: a better web interface. 36, W5-W9.
- KASIRIYAN, M.M., HAFEZIAN, S.H. and HASSANI, N., 2011. Genetic polymorphism BMP15 and GDF9 genes in Sangsari sheep of Iran. *International Journal of Genetics and Molecular Biology*, 3(1), pp.31-34.
- KHODABAKHSHZADEH, R., MOHAMMADABADI, M., ESMAILIZADEH, A., SHAHREBABA, H. M., BORDBAR, F. & NAMIN, S. A. J. P. J. O. V. S. 2016. Identification of point mutations in exon 2 of GDF9 gene in Kermani sheep. 19, 281-289.
- LIANDRISA, E., KOMINAKISB, A., ANDREADOUA, M., KAPEOLDASSIA, K., CHADIOA, S., TSILIGIANNI, TH., GAZOULID, M., IKONOMOPOULOSA. I., (2012). *Journal of Small Ruminant Research*. 107, 16– 21.
- MCWILLIAM, H., LI, W., ULUDAG, M., SQUIZZATO, S., PARK, Y. M., BUSO, N., COWLEY, A. P. & LOPEZ, R. J. N. A. R. 2013. Analysis tool web services from the EMBL-EBI. 41, W597-W600.
- MISHRA C. Genetic basis of prolificacy in sheep. *Int J Livest Res*. 2014; 4: 46–57.
- MORADBAND, F., RAHIMI, G. and GHOLIZADEH, M. (2011) Association of polymorphisms in fecundity genes of GDF9, BMP15 and BMP15-1B with litter size in Iranian Baluchi sheep. *Asian Aust. J. Anim. Sci.*, 24(9): 1179-1183.
- MOSTAFAVI, S., RAY, D., WARDE-FARLEY, D., GROUIOS, C. & MORRIS, Q. J. G. B. 2008. GeneMANIA: a real-time multiple association network integration algorithm for predicting gene function. 9, S4.
- NANEKARANI, S., GOODARZI, M., KHEDERZADEH, S., TORABI, S. and LANDY, N., 2016. Detection of polymorphism in Booroola gene and growth differentiation factor 9 in Lori sheep breed. *Tropical Journal of Pharmaceutical Research*, 15(8), pp.1605-1611.
- OTSUKA, F., 2010. Multiple endocrine regulation by bone morphogenetic protein system. *Endocrine journal*, 57(1), pp.3-14.
- PRUITT, K. D., TATUSOVA, T. & MAGLOTT, D. R. J. N. A. R. 2005. NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. 33, D501-D504.
- RESEARCH, U. C. J. N. A. 2007. The universal protein resource (UniProt). 36, D190-D195.
- ROY, J., POLLEY, S., DE, S., MUKHERJEE, A., BATABYAL, S., PAN, S., BRAHMA, B., DATTA, T. K. & GOSWAMI, S. L. J. A. B. 2011. Polymorphism of fecundity genes (FecB, FecX, and FecG) in the Indian Bonpala sheep. 22, 151-162.
- VÅGE, D. I., HUSDAL, M., KENT, M. P., KLEMETS DAL, G. & BOMAN, I. A. J. B. G. 2013. A missense mutation in growth differentiation factor 9 (GDF9) is strongly associated with litter size in sheep. 14, 1.
- YADOLLAH B, Sajad B, Hamid RM, Vaheid CA, Seyed AM. The Polymorphism of GDF-9 Gene in Hisari Sheep. *Biol Forum Int J* 2014; 6: 46-52.
- ZAMANI, P., NADRI, S., SAFFARIPOUR, R., AHMADI, A., DASHTI, F., ABDOLI, R. J. T. A. H. & PRODUCTION 2015. A new mutation in exon 2 of the bone morphogenetic protein 15 gene is associated with increase in prolificacy of Mehraban and Lori sheep. 47, 855-860.