MOLECULAR ANALYSIS OF FECG^{H} GENE IN HAMDANI SHEEP BREED IN IRAQI KURDISTAN REGION

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ABSTRACT

The objective of this study was to investigate association between FecG^{H}-GDF-9 gene polymorphism and litter size in Hamdani fat-tailed sheep. The genomic DNA was extracted from eighty-seven blood samples of Hamdani ewes. The FecG^{H}-GDF-9 locus was detected by PCRs, and identified DNA genotyped by DNA sequencing. The evaluate several parameters included fertility (%), conception (%), letter size at birth, twining rate (%), triple rate (%), barrenness (%) and productivity (%) were measured and arrived 91.95, 95.40, 1.825, 150, 11.25, 8.05 and 159.77, respectively. The blast tree view results in NCBI blast show Hamdani sheep is more closely to Norway white face sheep, Han sheep and Pelibuey Sheep breeds which have high litter sizes than the Iranian Ghezel sheep. Hamdani ewe's genome have three changed nucleotides point at 1273 bp (C to G) changed Alanine to Arginine, 1281 bp (A to T) changed lysine to Isoleucine and 1344 bp (C to A) changed Proline to Glutamine. Also three nucleotides deletion were detected at 1279 bp (C), 1283 (T) and 1376 bp (G) position with two inserted nucleotides at 1319 bp (T) and 1357 bp (G) position. The 5'….C/TNAG…3' sequence cite of mutated FecG^{H} locus was detected by *Ddel* restriction enzyme which have significant effect on litter size in sheep breeds.

Keywords: blood, titter size, FecG^{H} –GDF9 gene, DNA sequencing

مجلة العلوم الزراعية العراقية -2022 :53 (1):1- 8 البرزنجي

التحليل الجزيئي للمورث FecG^{H} في الأغنام الحمدانية في أقليم كوردستان العراق

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المستخلص

تهدف هذه الدراسة بيان وجود العلاقة مابين جين (FecG^{H}–GDF9) مع زيادة عدد الولادات من البطن الواحد في النعاج الحمدانية. تم استخلاص الدنا من عينات دم ماخوذة من 87 نعجة حمدانية. تم الكشف عن وجود جين (FecG^{H}–GDF9) باستخدام تقنية البلمرة الحرارية وتم تعيين تسلسل النيوكليدات لهذا الجين. تقييم عدة مقاييس تضمنت الخصوبة (٪) ، الخصب (٪) ، عدد الحملان من البطن الواحد ، معدل التوأمة (٪) ، المعدل الثلاثي للولادات (٪) ، العقم (٪) والإنتاجية (٪) حيث بلغ 91.95 ، 95.40 ، 1.825 ، 150، 11.25 ، 8.05 و 159.77 على التوالي. تظهر نتائج شجرة العلاقة في NCBI أن الأغنام الحمدانية أقرب إلى سلالات الأغنام (Norway White Face) وأغنام (Han) التي تتميز بالولادات العالية منه الى الأغنام الإيرانية (Ghezel).أظهرت نتائج التسلسل وجود ثلاث نقاط استبدال في تسلسل نيوكليوتيدات هذا الجين في النعاج الحمدانية عند التسلسل 1273 (C إلى G) أذ غيرت الألنين إلى الأرجنين ، وعند التسلسل 1281 (A إلى T) حيث غيرت الليسين إلى أيزوليوسين و عند التسلسل 1344 (C إلى A)غيرت البرولين إلى كلوتامين . كما تم الكشف عن وجود حذف في ثلاثة نيوكليوتيدات عند التسلسل 1279 (C) و 1283 (T) و 1376 (G) مع أظافة اثنين من النيوكليوتيدات عند التسلسل 1319 (T) والتسلسل 1357 (G).تم الكشف عن تسلسل TNAG / C لموقع FecGH الطافر الذي له تأثير كبير على عدد الولادات من البطن الواحد في سلالات الأغنام بواسطة إنزيم تقييد (*Ddel*). .

كلمات مفتاحية : الأغنام الحمدانية، عدد الولادات، جين FecGH، تسلسل الدنا.

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INTRODUCTION

The Hamdani breed is adapted fat-tailed sheep breed in Kurdistan region of Iraq. It is a breed used to produce a range of products, meat, wool, milk and give higher twining rate at birth. Furthermore, Hamdani sheep has a potential milk yield of 83.998 kg per lactation (1), growth rate of 148 gm per day (2), litter size 1.36 (3) and wool production of 2.159 kg per year (4). Genetic variations for the above mentioned economic traits exist within and across flocks. Breeding program based on identifying best animals (males and females) will improve the productivity of animals. Traditional animal breeding through appreciation of breeding values requires a lot of effort, money and time. Detection of major genes, DNA profile and sequencing or quantitative trait loci (QTL) it can accelerate the genetic gain for important traits of Hamdani sheep in Kurdistan. Procedures for multiple trait genetic evaluation of animals require accurate calculate of genetic material and environmental (Temporary and permanent) parameters. Genetic characterization of farm animal breeds is importance to identify the genetic materials and also to prioritize breeds for conservation and improvement. DNA or RNA characterization of sheep breeds for economic trait are necessary for analyzing complete herd structure. The complete herd structure helps to plan strategies for conservation and improvement of any breed. (5). Molecular variation is the best base for the breeders, which is used to mold domestic farm animal species to people's needs. The increasing information on genetics of animal breeds using different molecular markers (RAPD, RFLP, SSR, DNA sequencing, etc.) will help to understand the evolutionary history of animal farm better. Also it will help to improve of breed quickly (6 and 7). Recently, the use of molecular genetic markers, specially polymorphism at the DNA level, has been playing an increasing role in animal breeding programs. Among others, the DNA sequencing has been the most widely used for detect polymorphism at the DNA level. The mostly important question to be considered in the survival and improve of any species of farm animals in breeding program is the basis for determining ovulation rate and litter size (8). Over the year's breeders have carefully breeding and maintained breeds of sheep for their high level of litter size and ovulation rate. Typically, such ewes have an increased rate of twin pregnancies (9). Recently, growth differentiation factor-9 (GDF-9), was found to play importance role in specifying ovulation rate and litter size (10 and 11). Therefore, the objective of our study is molecular characterizations of litter size depending of specific gene High prolific Fecundity Growth ($FecG^{H}$) sequencing in exon 2 of GDF-9 on chromosome number 5 in the Hamdani breed.

MATERIALS AND METHODS

Blood Sample collection and DNA extraction : Blood samples were taken from 87 Hamdani ewes using EDTA tube as an anticoagulant along with data on litter size of each ewes in commercial flock Sebardani Ado-Qushtaba, Erbil, Iraqi Kurdistan Region. Genomic DNA was extracted from 200 µL of blood, using the AddPrep Genomic DNA Extraction Kit (ADD BIO INC, Korea), according to the manufacturer's protocol. The DNA concentration was calculated with a Nanodrop Lite (Thermo Scientific®, Wilmington, DE, USA), and quality was visualized on a 1% agarose gel by added ethidium bromide to the gel and used 1X Tris-Borate-EDTA (TBE) buffer (pH 8.0) for 1.5 h with 70 V. DNA samples were stored at -20 C for use (12).

Primer sequence and PCR conditions

The primer pair of the GDF9 ($FecG^{H}$) gene was obtained from Macrogen company (Korea) according to (12) with R: 5'-ATGGATGATGTTCTGCACCATGGTGTG AACCTGA-3' and F: 5'-CTTTAGTCAGCTGAAGTGGGACAAC-3' to amplify a 139-bp PCR product. PCRs were done in 20µl volume containing approximately 10µl of Add Taq Master mix [(50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100) , 2 U Taq DNA polymerase, 2 mM MgCl2, 250µM each dNTP (ADD BIO INC, Korea)], 2µl F and R primer (1.0µM), 5µl Hamdani genomic DNA(50 ng) and and 3µL DNase free water. PCR conditions were as follows: denaturation at 95ºC for 5minutes, followed by 35 cycles of denaturation at 95 ºC for 30 seconds, annealing at 62 ºC for 30 seconds,

extension at 72 ºC for 35 seconds, with a final extension at 72 ºC for 10minutes on Techne Prime PCR thermal cyclers (Techne, Cambridge; UK). PCR products were detected by electrophoresis on 2% agarose gels (12).

DNA purification and DNA sequencing

PCR product (DNA fragment) was excised from the gel using sterile, sharp cutter and Zymoclean Gel DNA Recovery Kits (ZYMO RESEARCH, USA) were used for purified according to the manufacturer's instructions. Purified products were directly sequenced using the F primers of PCR amplification. The sequencing technology process was performed by the Macrogen company (Korean) using Sanger dideoxy sequencing.

Bioinformatics analysis

DNA sequences were analyzed by using the Chromas (V. 2.6.5 Technelysium Pty Ltd, https://technelysium.com.au/wp/chromas/).DN A sequence analysis and alignments were carried out using NCBI BLAST: Nucleotide sequence.

Phenotypic traits

Reproductive performance traits (fertility, conception, Letter size at birth, twining rate, barrenness and productivity) were measured according to (13 and 14) used the following equations:

Fertility (%) = (No. of lambing ewes /No. of ewes available for ram) x 100

Conception rate (%)= ((No. of lambing ewes + aborting ewes)/ No. of ewes available for ram) x 100

Letter size at birth = No. of lambs born/No. of lambing ewes

Twining rate (%) = (No. of twin lambs /No. of lambing ewes) x 100

Triple rate (%)= (No. of triple lambs /No. of lambing ewes) x 100

Barrenness (%)= (No. of non-lambing ewes /No. of ewes available for ram) x 100

Productivity (%) = (No. of lambs weaned/ No. of ewes available for ram) x 100

RESULTS AND DISCUSSION

Phenotypic results

Of the 87 ewes, 80 of them gave birth, 60 of them gave twins and 3 of them gave triple births. Overall the twin lambs, four are abnormal at birth. As in table (1) the reproductive traits in this flock of Hamdani ewes are very high, its mean there are genotypic epistasis in one or more specific locus gene. The fertility and conception are over 90% and litter size at birth is 1.825 (Table, 1). All reproductive results are higher than found by (1, 3, and 15-17), in same sheep breed. While, these results were agreement with reported by (18) in Hu sheep which litter size averaged 1.69. While this results of litter size of Hamdani ewes was lower than reported by (19) in heterozygote Mehraban fat tail sheep and (20) in Han sheep were litter size averaged 1.73 and 2.37, respectively.

Genotypic results:

Few molecular genetic data (using DNA sequencing) is available on Hamdani sheep breed. The results were obtained in our study were the first attempt molecular identification of this breed in Iraqi Kurdistan Region using DNA sequencing.

DNA Extraction

A genomic DNA of 87 ewe's blood samples were extracted and agarose gel electrophoresis (1%) was used to checked quality of DNA (Figure 1). Results show the good quantity (50.65 – 105.1 ng/µL) and quality of genomic samples which have A260/280 ratio of 1.7-2.2.

FecGH PCR amplification

The $FecG^{H}$ gene was amplification in order to detected this gene in Hamdani ewe's samples by PCR technique. The findings as showed in figure (2) revealed the PXR product appeared as single band with molecular base of 139 bp. The results showed high accuracy of optimum condition as appeared in agarose gel electrophoresis.

$FecG^{H}$ gene sequencing

On chromosome No. five of sheep close to the GDF-9 A quantitative trait loci region was identified, which is be strong candidate locus for increased ovulation rate and litter size of sheep breeds. The DNA sequencing of $FecG^{H}$ gene in Hamdani ewes with NCBI blast results show match among Hamdani ewes $FecG^{H}$ locus with ten sheep breeds in world for same litter size ($FecG^{H}$ -GDF9) locus (Table, 2). All ten sheep breeds matched with Hamdani of $FecG^{H}$ gene with above 90% of DNA base pairs. This results indicated that this Iraqi sheep breed have a mutated FecGH locus which have significant effect on reproductive traits of sheep breeds in world.

Table 1. Reproductive traits in Hamdani ewes

Variable	Equation	Value
Fertility (%)	(80/87) *100	91.95
Conception (%)	((80 + 3)/87) *100	95.40
Letter size at	146/80	1.825
birth	(120/80) *100	150
Twining rate (%)	(9/80) *100	11.25
Triple rate (%)	(7/87) *100	8.05
Barrenness (%)	(139/87)*100	159.77
Productivity (%)		

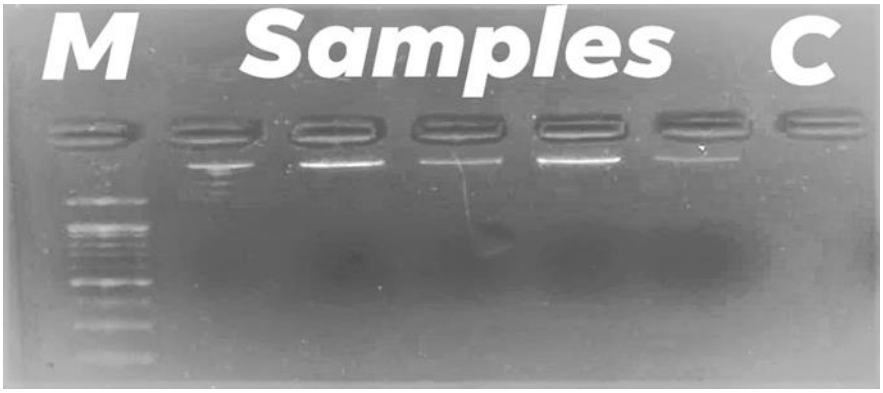


Figure 1. The electrophoresis of total genomic DNA for Hamdani ewes by 1% agarose gel(1.5 h, 70 V, 1X TBE buffer) and visualized by UV light after staining with ethidium bromide. Lane (M) : Molecular size markers (100-1000, 1500 bp), lanes (2-6): Positive results for samples. And lane (7): Negative control.

Figure 2. Agarose gel electrophoresis of PCR reaction for *FecG*^{H} gene (139 *bp*) for DNA samples of Hamdani ewes. Bands were fractionated by electrophoresis on a 2% agarose gel (2 h, 70 V, 1X TBE buffer) and visualized by UV light after staining with ethidium bromide. Lane (M) : Molecular size markers (100-1000, 1500 bp), lanes (2-6): Positive results for samples. And lane (7): Negative control.

Table 2. Sheep breeds and GeneBank number of FecG^{H} -GDF9 matched Hamdani ewes.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — New Select columns — Show 100

select all *10 sequences selected* — GenBank | Graphics | Distance tree of results

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Ovis aries growth differentiation factor 9 (GDF9) gene, GDF9-C allele, partial cds	sheep	161	161	79%	4e-39	92.98%	396	MK675523.1
Ovis aries growth differentiation factor 9 (GDF9) gene, GDF9-B allele, partial cds	sheep	161	161	79%	4e-39	92.98%	396	MK675522.1
Ovis aries growth differentiation factor 9 (GDF9) gene, GDF9-A allele, partial cds	sheep	161	161	79%	4e-39	92.98%	396	MK675521.1
Ovis aries breed Pelibuey growth differentiation factor 9 (GDF9) mRNA, complete cds	sheep	161	161	79%	4e-39	92.98%	1822	KT853039.1
Ovis aries growth/differentiation factor 9 (GDF9) mRNA, complete cds	sheep	161	161	79%	4e-39	92.98%	1852	KR063137.1
Ovis aries GDF9 gene, breed Norwegian white sheep	sheep	161	161	79%	4e-39	92.98%	1681	HE866499.1
Ovis aries growth differentiation factor 9 (GDF9), mRNA	sheep	161	161	79%	4e-39	92.98%	1605	NM_001142888.2
Ovis aries growth and differentiation factor 9 variant FecG-Embrapa (GDF9) mRNA, complete cds	sheep	161	161	79%	4e-39	92.98%	1362	FJ429111.1
Ovis aries growth differentiation factor-9 gene, complete cds	sheep	161	161	79%	4e-39	92.98%	5644	AF078545.2
Ovis aries GDF-9 (GDF-9) gene, partial cds	sheep	158	158	76%	5e-38	93.52%	570	DQ301499.1

Phylogenic tree

The blast tree view results in NCBI blast sheep is more closely to Norway white face sheep, Han sheep and Pelibuey Sheep breeds than the Iranian Ghezel sheep and New Zealand sheep (Figure 3). The reproductive trait of Hamdani ewes in this study are agreement with this results, (21) in Norway white face reported that the $FecG^{H}$ -GDF9 under GenBank (HE866499.1) which have closely related with Hamdani DNA sequence (Figure 4) have significant effect on reproductive traits and litter size. Similar result was found by (20) in Han sheep breed and (22) in Pelibuey sheep (Fig. 5). While (23) in Iranian Ghezel sheep (Which have more distance with Hamdani sheep among this ten sheep breeds, Fiq. 6) explained that $FecG^{H}$ mutation is not found in the Ghezel sheep breed and is not associated with Ghezel breed high prolificacy performance.

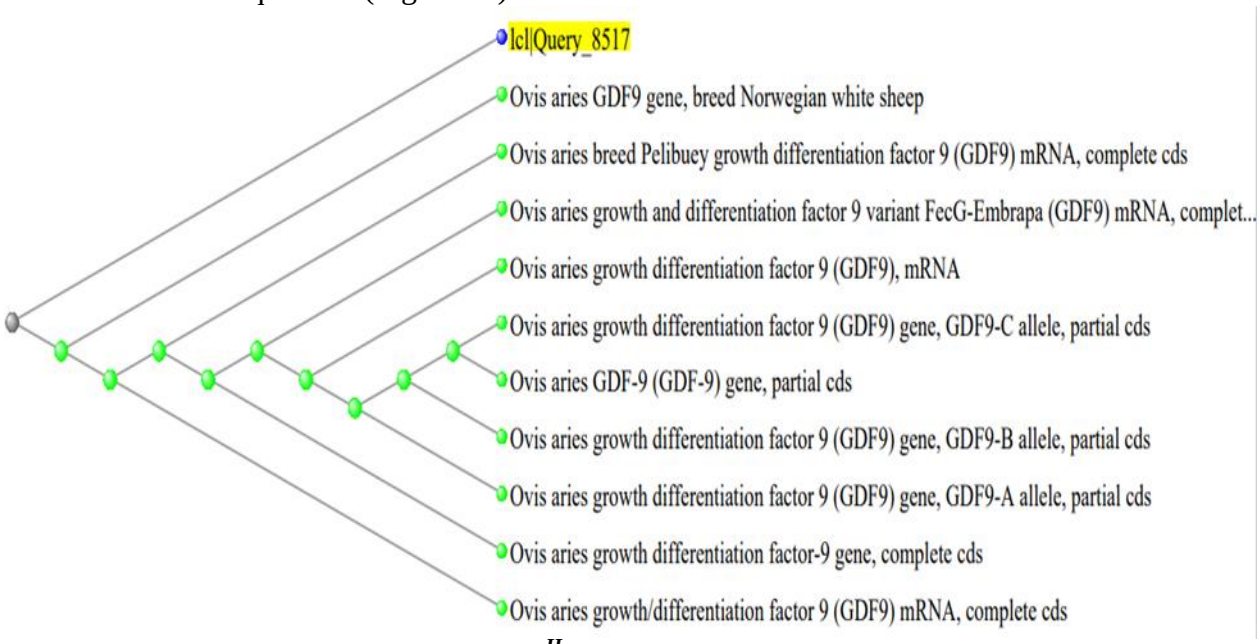
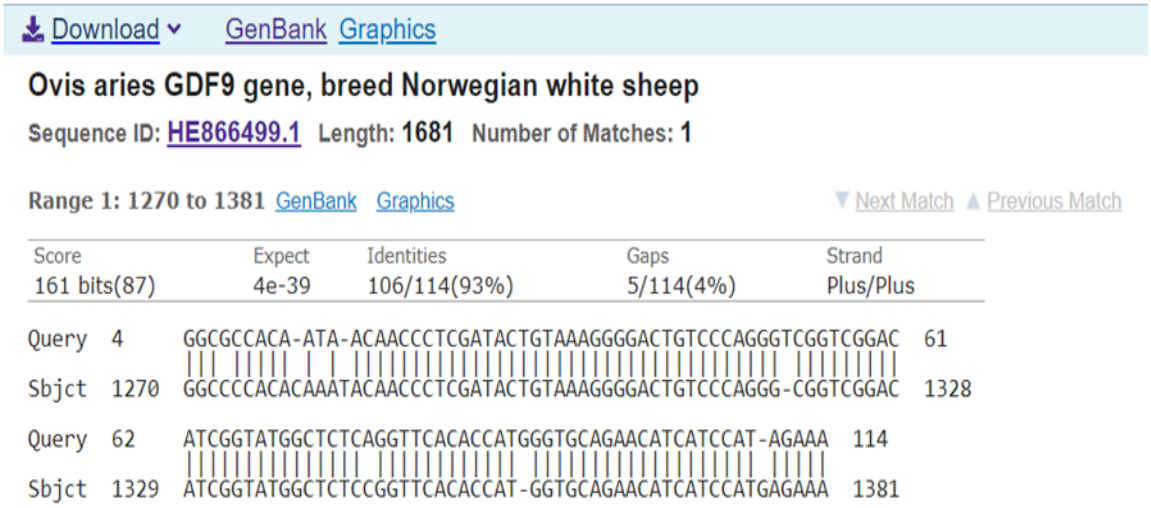


Figure 3. Phylogenetic tree of *$FecG^{H}$* gene among sheep breeds with Hamdani sheep


Download GenBank Graphics

Ovis aries GDF9 gene, breed Norwegian white sheep

Sequence ID: HE866499.1 Length: 1681 Number of Matches: 1

Range 1: 1270 to 1381 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
161 bits(87)	4e-39	106/114(93%)	5/114(4%)	Plus/Plus

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Query  4     GGCGCCACA-ATA-ACAACCCTCGATACTGTAAAGGGGACTGTCCCAGGGTCGGTCGGAC  61
             ||| ||||| | | ||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  1270  GGCCCCACACAAATACAACCCTCGATACTGTAAAGGGGACTGTCCCAGGG-CGGTCGGAC  1328

Query  62    ATCGGTATGGCTCTCAGGTTCACACCATGGGTGCAGAACATCATCCAT-AGAAA  114
             ||||||||||||||| |||||||||||| |||||||||||||||||| |||||
Sbjct  1329  ATCGGTATGGCTCTCCGGTTCACACCAT-GGTGCAGAACATCATCCATGAGAAA  1381
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Figure 4. Sequence alignment of Hamdani sheep *$FecG^{H}$* with Norway white face sheep breed

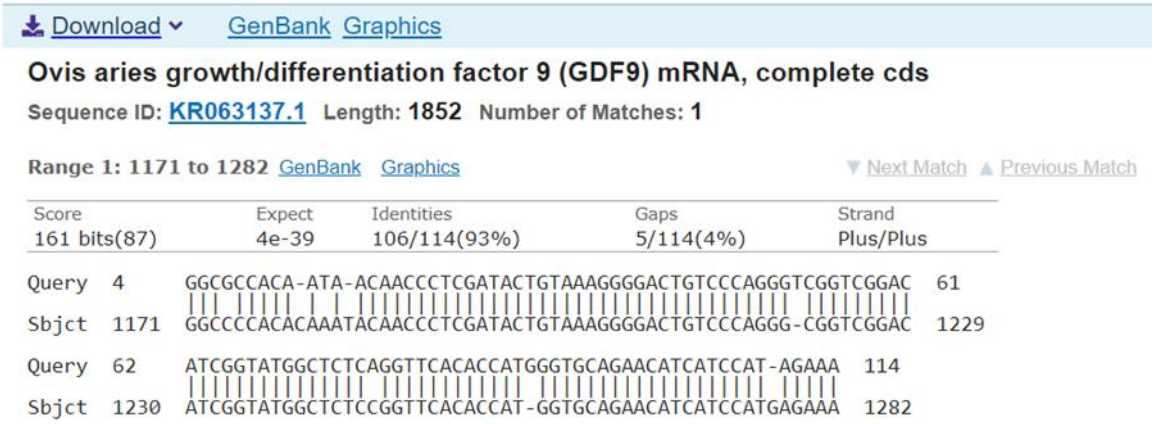

Figure 5. Sequence alignment of Hamdani sheep *FecG^{H}* with Han sheep breed

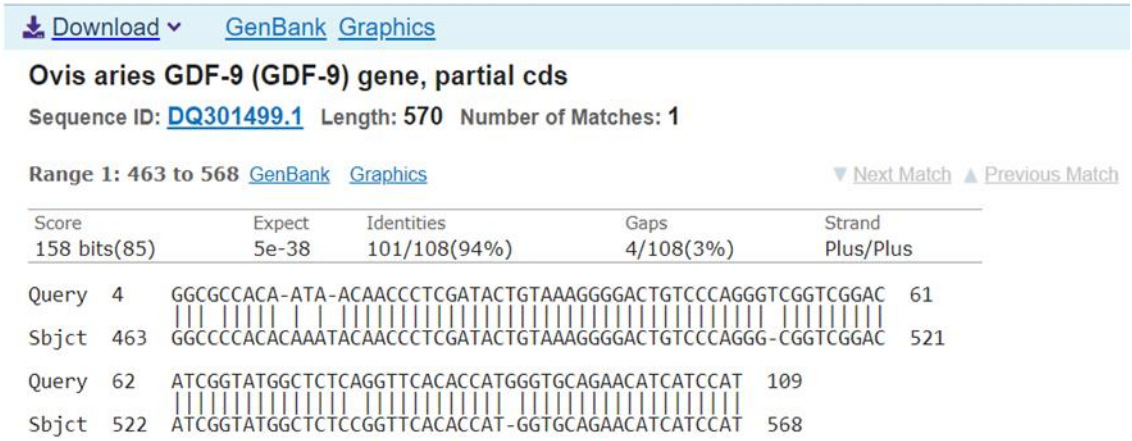

Figure 6. Sequence alignment of Hamdani sheep *FecG^{H}* with Iranian Ghezel sheep breed

DNA code changed

The NCBI sequence Alignment viewer show that FecGH locus (139 bp) of exon 2 of GDF9 of Hamdani ewes genome have three changed nucleotides point at 1273 bp (C to G) changed Alanine to Arginine, 1281 bp (A to T) changed lysine to Isoleucine and 1344 bp (C to A) changed Proline to Glutamine. Also three nucleotides deletion were detected at 1279 bp (C), 1283 (T) and 1376 bp (G) with two inserted nucleotides at 1319 bp (T) and 1357 bp (G) as in (Fig. 6).

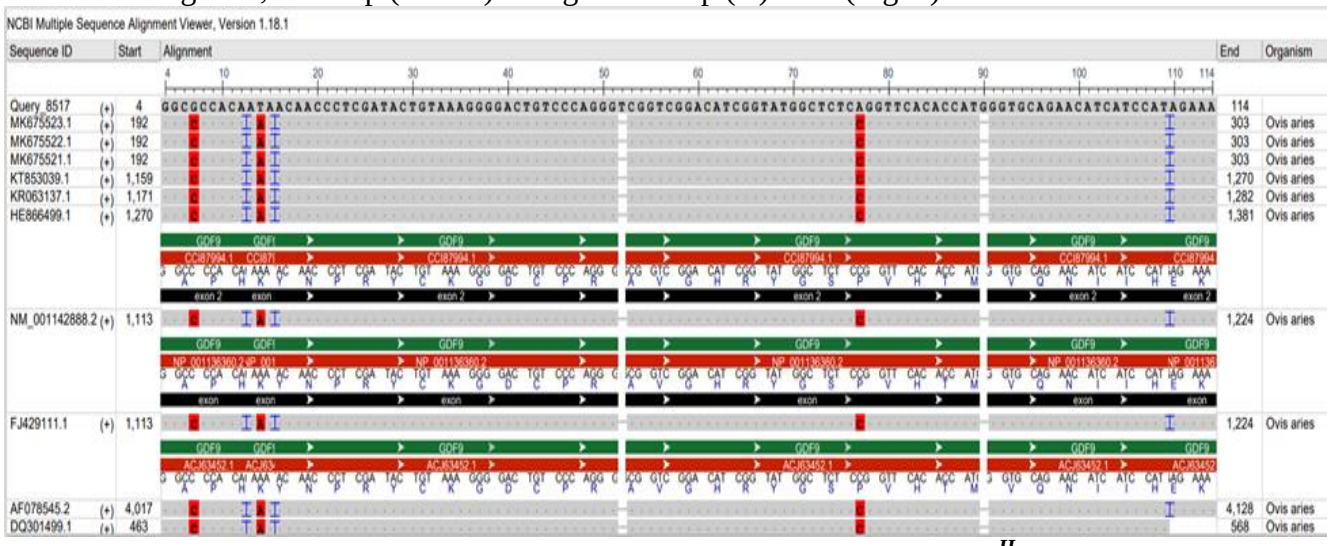

Figure 6. NCBI sequence alignment of Hamdani sheep *FecG^{H} -GDF9*

DNA Restriction Cite

To detect any single nucleotide polymorphism (SNP) two methods used, the 1st one is PCR-RFLP and the 2nd is DNA sequencing. In PCR-RFLP method of FecG^{H} locus PCR product digested with *Ddel* restriction enzyme which cut the mutated FecG^{H} (139 bp) gene at 5'….C/TNAG…3' sequence result two

fragment with 108 and 31 bp, while the wild FecG^{H} remained as its 139 bp (12). In our study the DNA sequencing was theoretically treated with *Ddel* restriction enzyme to detect present of the 5'….C/TNAG…3' sequence. The result showed that the *Ddel* restriction enzyme was cut FecG^{H} of Hamdani ewes at one point (Figure 7), it means the high liter size (1.825) at birth of Hamdani ewes was associated with exon 2 of FecG^{H} -GDF9 locus which have significant effect on ovulation rate in *Ovis aries*. Similar result was found by (24) in Brazilin sheep, (19) in fat tail sheep, (25) by using RFLP-PCR for same gene in Hamdani sheep breed, and (18) in Hu sheep breed.

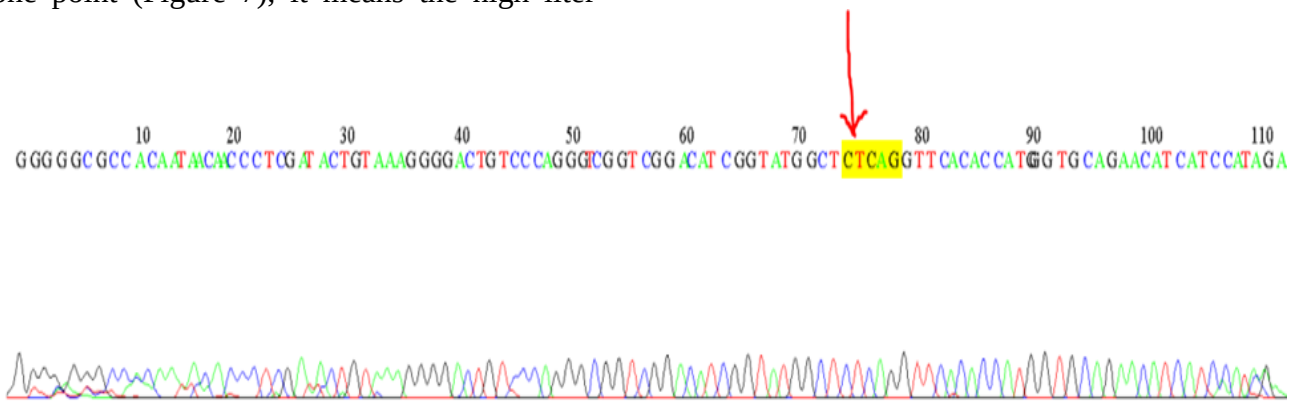

Figure 7. DNA sequence *Ddel* restriction site of Hamdani sheep *FecG^{H} -GDF9*

CONCLUSIONS

This study indicates that are highly correlated between litter size in Hamdani ewes and *FecG^{H} –DGF-9* based on reproductive traits and DNA sequencing blast. The expression level of *FecG^{H} –DGF-9* has a high positive effect on ovulation rate and litter size in Hamdani ewes. Results of our study might provide a theoretical basis for breeding prolific sheep by marker assisted selection(MAS) to increase herd number of this sheep breed in Iraqi KRG.

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