Molecular Characterization of Growth Differentiation Factor 9 (GDF9) Gene Associated with Fertility in Egyptian Baladi Goat

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ABSTRACT

The growth differentiation factor 9 (GDF9) gene (exon 1) is the subject of this study's investigation into polymorphisms concerning fertility in Egyptian Baladi goats. Fifteen females that produce single kid and 55 produce twins kids were used in this study. Methods: blood samples were taken, and genomic DNA was extracted. By using specific primers, a 770 bp region of the GDF9 gene was amplified; the amplified products were subjected to sequence analysis and examined to determine the phylogenetic relationship. The sequence of GDF9 gene of Baladi goats was compared with eight *Capra hircus* accession numbers found in Gen Bank. The results of sequence comparison indicated that the Baladi goat is closely related to the breed of accession number KY780296. In addition, polymorphisms were covered by the PCR-RFLP technique using the *Msp1* restriction enzyme to confirm twinning molecular markers. The banding patterns of *Msp1* enzyme digestion showed three bands of approximately 770, 470 and 300 bp, for does producing twins and one band of approximately 770 bp for does producing single kid.

Knowledge of the mutation in the GDF9 gene (exon 1) early on can be used to create a flock that specializes in producing twins without waiting for maturity and thus reducing production costs and increasing profitability.

Keywords: Baladi goat, Prolificacy, GDF9 gene, Polymorphism, Restriction enzymes.

INTRODUCTION

Goats (*Capra hircus*) are one of the earliest livestock species that were domesticated approximately 10,000 years ago **(Franklin, 1997 and Simm, 1998)**. Early goat domestication may be due to their ability to survive in adverse climatic conditions (ranging from cold mountain regions to hot deserts) and utilizing low-quality pastures. It is well known that the goat genome consists of two sex chromosomes and 29 pairs of autosomes, giving a diploid number of 60 chromosomes **(Evans, 1965)**. Goats have been bred to produce milk, meat, fur, and skins around the world. In Egypt, the breeding of goat aims for the production of meat only, but its milk is used for feeding the newborn offspring. Egypt has three predominant goat breeds, Barki (Sahrawi), Nubian (Zraibi), and Baladi. The productive performance of the Baladi goat has an important economic value due to its effective contribution to bridging the gap in the red meat shortage.

The selection aims to increase fecundity and enhance the production efficiency and reproduction rate in small ruminants. Due to low heritability estimates of reproduction traits. Therefore, using certain locations on DNA as a genetic marker technology has been expressed as a phenotype, and based on inheritance laws can be inherited in the next generations. So the marker gene can be used as an alternative selection method for highly

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accurate prediction of breeding value **(Mishra, 2014).** Some measurements are very important in reproduction such as fecundity, litter size, and twining rate. Ovulation rate is one of the most fecundity parameters that can be genetically affected by a large number of genes **(Drouilhet *et al*., 2009)**.

There is a lot of data to support the idea that the oocyte is important for the development and growth regulation of follicles **(Su *et al*., 2009)**. GDF-9 is one of essential genes that control oocyte-derived growth factors in ovarian function associated with the transforming growth factor-b superfamily **(Otsuka *et al*., 2011)**.

The GDF9, also known as FecG, is localized to the fifth autosome of sheep and plays a major role in folliculogenesis from the initial stage of follicle growth until ovulation **(Kidder and Vanderhyden, 2010).** GDF9 is crucial for ovulation (folliculogenesis and oogenesis) because it plays a significant role in female fertility **(Castro *et al*., 2015)**. Different point mutations (G1–G8) have been reported in Cambridge prolific and Belclare sheep breeds **(Hanrahan *et al*., 2004)**. The high ovulation rate **(Nicol *et al*., 2009)** mainly depends on the mutation that is induced in one site (heterozygous) of the GDF9 gene, on the other hand, if this mutation occurs in both sites of the gene it will disrupt follicle growth, leading to infertility homozygous. The genetic variants of the GDF9 gene were confirmed to be the major gene markers for the enhancement of the prolificacy in Egyptian sheep and goats **(Aboelhassan *et al*., 2021).**

Studies on genetic diversity of important economic genes are rare in Egyptian Baladi goats. The present study was designed to evaluate the genetic polymorphism of the GDF9 gene that may serve as a twinning marker through sequencing and PCR-RFLP in the Baladi goat breed. These data could be applied in a successful breeding program by Marker-Assisted Selection (MAS) to select individuals that produce twin kids.

MATERIALS AND METHODS

This study was performed at the Animal Private Farm in Sids, Beni Suef Governorate, Egypt, and subjected to the rules of the University Institutional Animal Care and Use Committee (FU-IACUC) (approval code number, 2348) and following with European Union Directive 2010/63/EU for animals. All efforts were made to follow local animal welfare guidelines during blood collection.

Blood samples collection and DNA extraction:

The blood samples were collected from seventy female animals belonging to the Baladi goats breed based on their production of single or twins (15 of them produce single and 55 produce twins) through four repetitive production cycles during the years 2022 and 2023. Two mL of blood was collected from each animal in an EDTA sterilized glass tube to prevent coagulants. They were transferred to the laboratory then preserved at 4°C till used. Genomic DNA was extracted from 150 µl of each sample according to **Tillett and Neilan (2000)** protocol and **El Fiky *et al*. (2017)** modification. Then the isolated DNA was preserved for further processing at −20°C.

PCR amplification of GDF9 gene

The extracted DNA was amplified using PCR assay with specific primers to the GDF9 gene (Exon 1), as shown in **Table** 1, according to **Hartatik *et al.* (2023)**. Amplification of 770 pb product of the GDF9 gene was done by PCR technique, described by **El Fiky *et al.* (2017).** The DNA was amplified in thermal cycler MSLPCR13 (Applied Biosystems) and the conditions of PCR reaction are shown in **Table 2**. The QIAquick Gel Extraction kit (QIAGEN no. 28706) was used to purify the amplified products according to the manufacturer's protocol. The purified products were sent to Macrogen Company (South Korea) for nucleotide sequencing.

Sequencing and phylogenetic analysis

The obtained sequences of each Baladi goat breed based on their production of single or twins were subjected to the SeqMan™ II (Windows 32 SeqMan 4.05) package (DNAStar, www.dnastar.com) to obtain a consensus sequence of the GDF9 gene from each. The two consensus sequences were aligned to sequences of the GDF9 gene from *Capra hircus* breeds registered in the GenBank using the BLASTN 2.2.18 to select the greatest similarity of the reference sequences. The

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Phylogenetic tree was generated based on the two consensus sequences and eight sequences from GenBank using the MEGA version 5.2 program. The relationship among consensus sequences and KY780296, FJ712709, JX513390, JX513391, MN401414, AH014112, FJ712708, and HM462268 sequences was determined.

PCR-RFLP analysis

The PCR products of GDF9 exon I from each female producing a single or twin born were digested using *Msp1* restriction enzymes following the manufacturer's guidelines. The PCR products were digested with 2 µl of *Msp1* enzyme according to **El Fiky *et al.* (2017).**

Table 1. Primer sequences used to amplify of GDF9 gene (Exon 1)

Primer name	Primers sequence	Accession number	Product (bp)	Annealing (˚C)
GDF9	F :5- AGAAGTGAACCTAGCCCACC-3 R : 5-CTAACCTCCAGCAGCACTCT-3	EF446168.2	770	60

Table 2. PCR thermal cycling protocol

Steps	Temperature (°C)	Time (min)	Cycle
Initial Denaturation	94	5	1
Denaturation	94	1	35
Annealing	60	1	
Extension	72	2	
Final extension	72	10	1
Refrigeration	4	forever	

Genotype and allele frequencies

The expected genotype and allele frequencies of GDF9 in Egyptian Baladi goat breed were analyzed depending on the heterozygous carrier mothers exhibited high fecundity traits by producing kids more than homozygous mothers **(Huang *et al.*, 2009)**. Therefore, the allele frequency of + (p) = females' percentage which produce single kids (++) and half percentage of females which produce twin kids (+G); the allele frequency of G (q) = half percentage of females which produce twin kids (+G).

RESULTS

Depending on reproductive efficiency, the Baladi goat breed's kidding rate and litter size of does that give birth to single and twin kids, represented ovulation rate that is an extremely significant economic value. The GDF9 plays a critical role in key oocyte-derived growth factors in ovarian activity, and realization of mutations in the GDF9 gene is the causing factor behind some females' extreme proliferation or infertility**.**

DNA sequencing analysis of the GDF9 gene (exon 1)

From each goat individual (70 samples) of the Baladi goat breed, a single fragment containing 770 bp of nucleotide sequences was amplified (Figure 1). A comparison of the base substitutions A to G and G to C between the consensus sequence from Baladi that produces single kid and the consensus sequence from Baladi that produces twin

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kids showed 99.70% similarity between them (Figure 2).

RFLP analysis and genotyping

The Baladi goat breed was examined for *Msp1* enzyme digestion. Figure (3) showed that a polymorphic type of restriction pattern consisted of three bands with 770, 470, and 300 bp for does producing twin kids and one band with 770 bp for does producing single kids Table (3) displays the genotype and allele frequencies of the GDF9 gene according to *Msp1* restriction enzyme digestion. The Baladi goat breed genotype frequencies Fec^{++}, Fec^{+G}, and Fec^{GG} were 0.348, 0.484, and 0.168 based on the findings of *Msp1* enzyme, respectively. The allele frequencies are 0.59 and 0.41 in Baladi goat breed for Fec^{+} and Fec^{G}, respectively.

Phylogenetic analysis

The UPGMA tree's topology of the Baladi goat breed with eight accession numbers of *Capra hircus* was shown as a monophyletic group (Figure 4). This tree indicated that Baladi goat breeds were found with *Capra hircus* goats in two main clusters. The first cluster consists of seven accession numbers (FJ712709, JX513390, JX513391, MN401414, AH014112, FJ712708, and HM462268), and is extremely diverse with the second cluster. The Egyptian Baladi goat breeds groupedwith a breed of accessio number (KY780296) in the second cluster. Egyptian goat breeds' multiple sequence alignment with accession number KY780296 revealed nucleotide no. 2014 (A), and 2165 (G) were similar in all Baladi does that produce single kids and changed to (G), and (C) in Baladi does that produce twin kids, respectively.

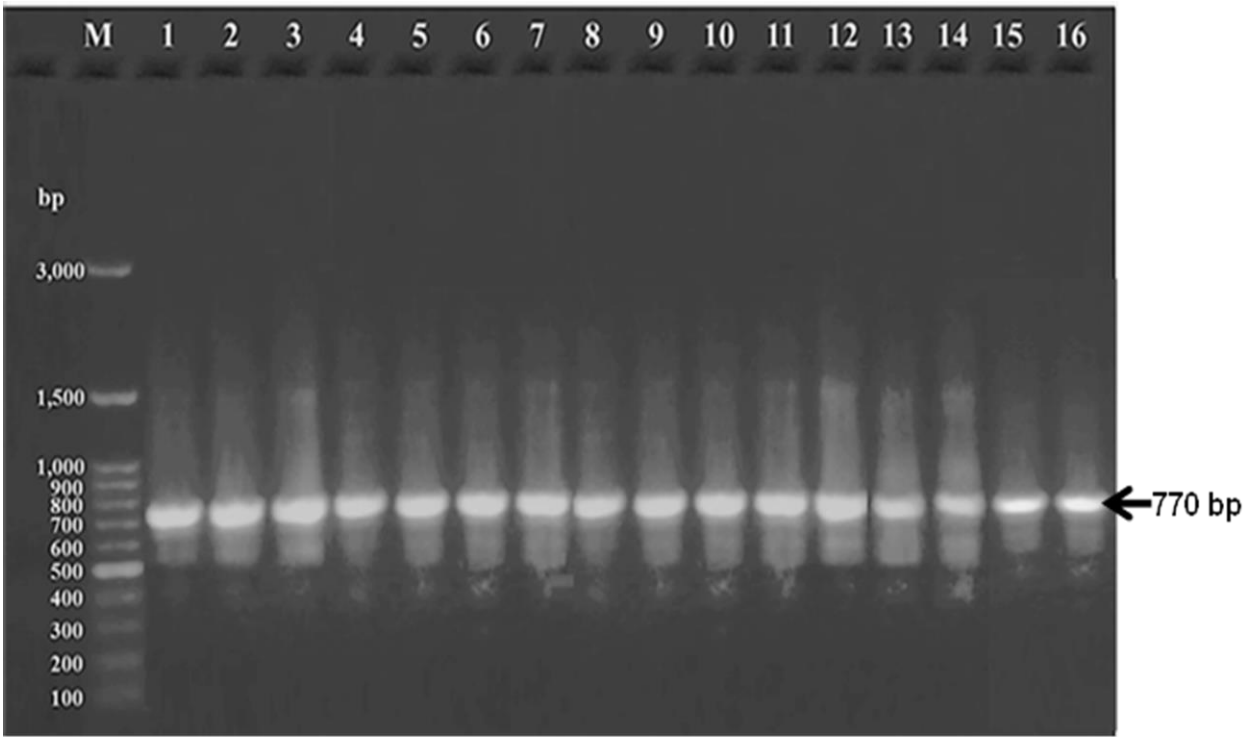

Figure 1: The PCR amplification of the GDF9 (approximately, 770 bp) gene from Egyptian Baladi goat breed. **M**: 100 bp DNA ladder.

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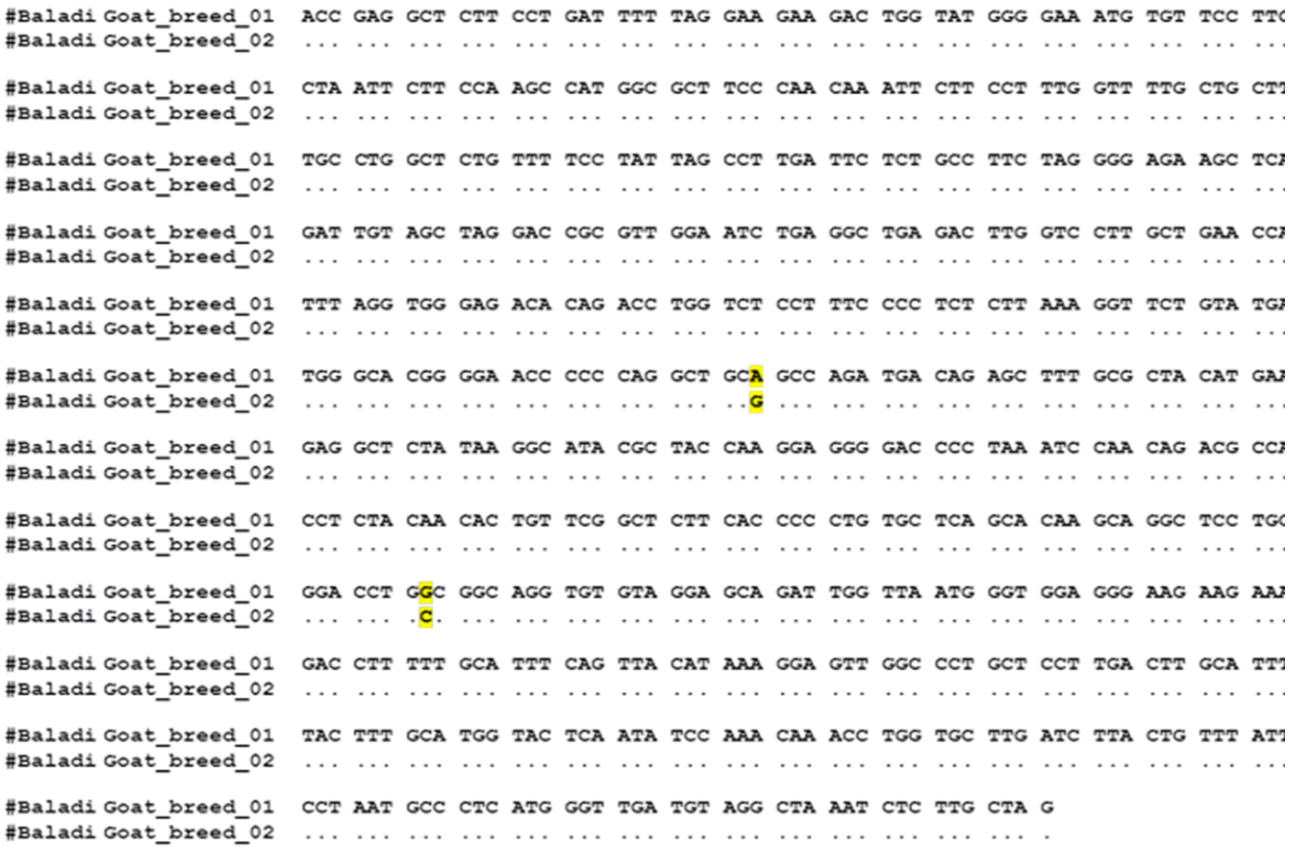

Figure 2: Alignment of nucleotide sequences (770 bp) of the GDF9 gene amplified from Egyptian Baladi goat breeds.

'.' represent identical residues.

Breed 01: does producing single kid

Breed 02: does producing twins kids

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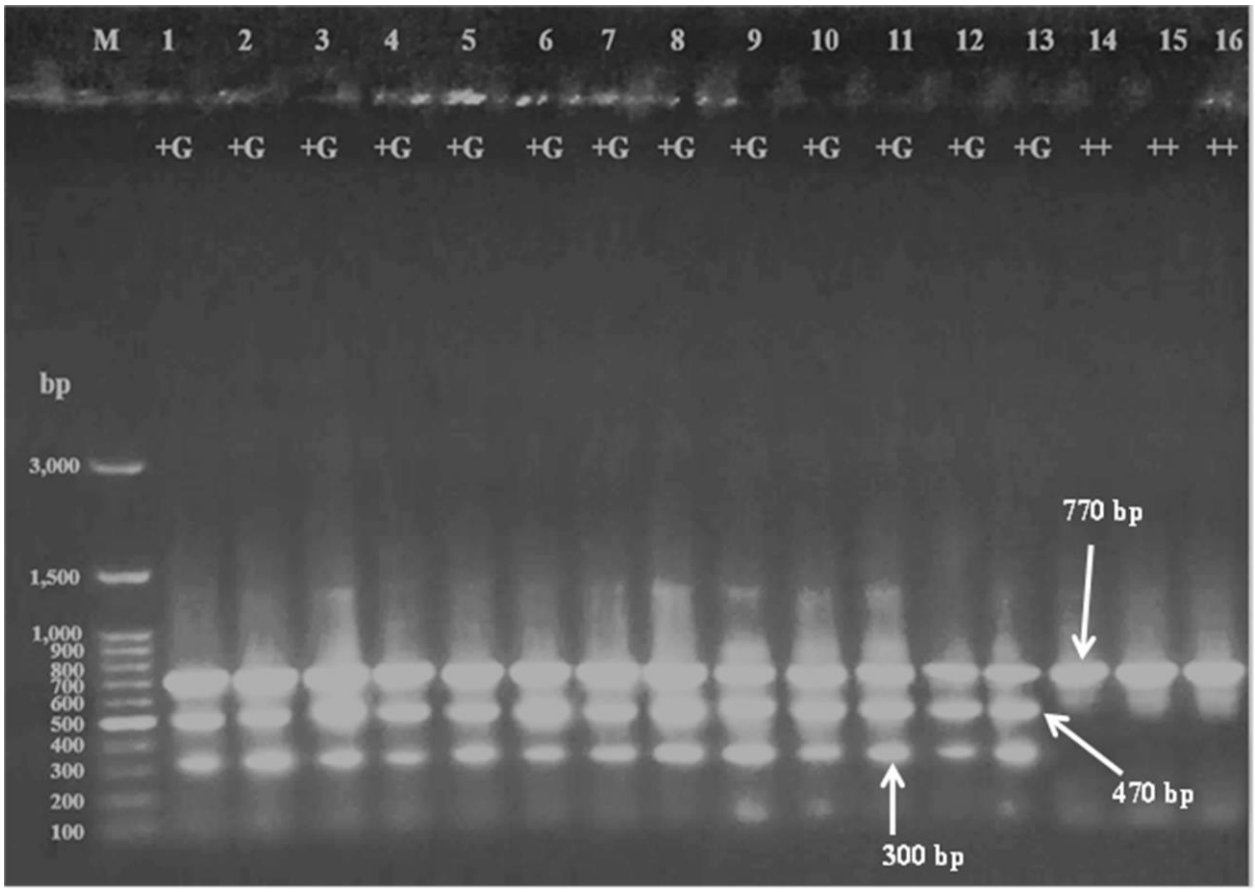

Fig. 3: Digestion product of partial exon 1 of the GDF9 gene from Egyptian Baladi goat with *Msp1* restriction enzyme. *Lanes* 1–13: twin-producing does, *lanes* 14–16: single-producing does, M: 100-bp DNA ladder.

Table 3: Expected genotype and allele frequencies of the GDF9 in Egyptian Baladi goat breed

Restriction enzyme	Goat breed	No. of goat	Expected genotype frequencies			Allele frequencies	
			++	+G	GG	+	G
Msp1	Baladi	70	0.372	0.476	0.152	0.61	0.39

++ = 15 females produce single +G = 55 females produce twins GG = 0.0.

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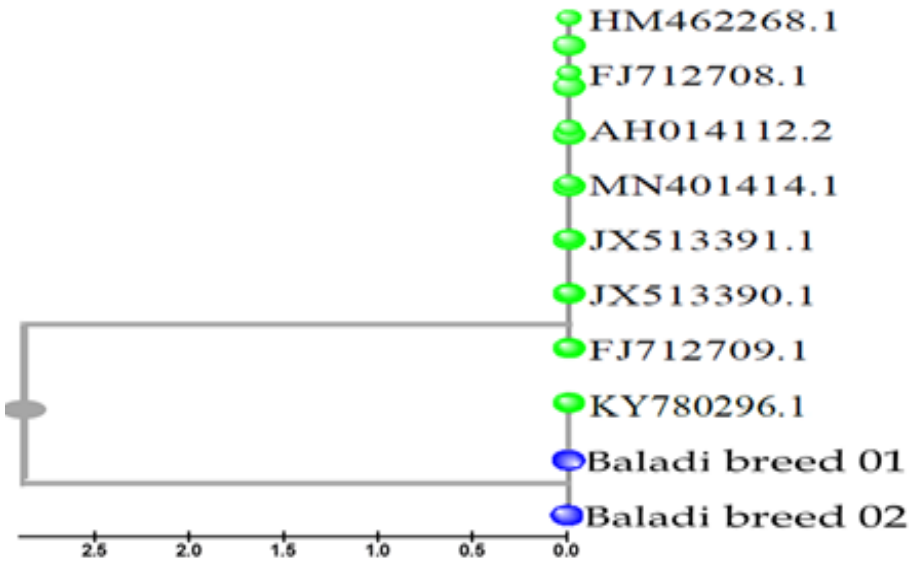

Figure 4: UPGMA dendrogram of 8 *Capra hircus* goat generated based on **Sneath** and **Sokal 1973** distances.Baladi breed 01: does which producing single kid Baladi breed 02: does which producing twins kids

DISCUSSION

In the animal kingdom, several genes and their respective functions are remarkably preserved. It is relatively similar among species, involving cell (metabolism, reproduction, and growth control). Ovulation is a complicated mechanism, which varies between animals and is influenced by environmental and genetic variables. Mammals are able to be multi- or mono-ovulatory, depending on the quantity of mature oocytes and release through ovulation. Ruminants normally release one oocyte each ovulation, as opposed to swine and rats exhibit elevated ovulation rates (**Montgomery *et al.*, 2001**). The ovulation rate varies by breed. In sheep, the ovulation rate varies from 1-10 eggs in Texel & Suffolk sheep and Merino sheep, respectively (**Souza *et al.*, 2001**). Goat breeds vary in the size of their litters, which is determined by the pace of ovulation and the quantity of fertilized eggs. According to the current findings, litter sizes vary both between and within goat breeds and are in line with findings for sheep (**Davis, 2005**). The genetics of goat litter size have been studied by **El-Maaty *et al.* (2022)**. Oocytes contain the growth factor from the beginning of the follicular phase until ovulation.

The gene that codes for oocyte-derived GDF9 is located on chromosome 5 and necessary for normal folliculogenesis (**Hanrahan *et al.*, 2004**). Gene influences on reproductive performance have been determined, with some of the most necessary influencing animal prolificacy. High litter size is an economically essential feature that increases goat productivity by giving more offspring.

Nucleotide sequence ovine GDF9 roughly 2.5 kb long and consists of two exons and single intron. By using the accession number KY780296 and multiple sequence alignment, it was possible to determine that nucleotide numbers 2014 (A) and 2165 (G) matched in all Baladi does that gave birth to a single kid and changed to (G) and (C) in Baladi does that produced twin kids, respectively **(Bodensteiner *et al.*, 1999)**. Polymorphic sequence variants in the GDF9 gene of goats have been found. Fifteen site mutations were discovered; however, only two putative

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genetic markers with transversion mutations in Exons 1 and 2 were detected by restriction enzymes, *BsaI, HapII, and MspI* **(Hartatik *et al.,* 2023)**. Four mutations in exon 2 of the GDF9 have been extensively discovered in many goat breeds **(Wouobeng *et al.*, 2020).** Nucleotide changes were identified (C-T) in $FecG^{H}$ allele (**Hanrahan *et al.,* 2004**), (G-A) in G^{1} allele (**Hanrahan *et al.,* 2004**), (T-G) in $FecG^{E}$ allele (**Silva *et al.,* 2011**), (A-C) in $FecT^{T}$ allele (**Nicol *et al.,* 2009**), (C-T) in $FecG^{V}$ allele (**Souza *et al.,* 2012**), and (G-A) (**Vage *et al.,* 2013**). The transition mutation had no significant influence on prolificacy, whereas deletion mutations (especially the C nucleotide) did.

RFLP is a molecular biology method that distinguishes simple nucleotide sequence differences in identical DNA segments. This method is based on restricted endonucleases, which are extremely sequence-specific. They spilled double-stranded DNA at recognized sites. Electrophoretic techniques can be used to separate the cleaved fragments of digested DNA. The restriction enzymes action produces homologous DNA molecules with varying lengths and limited variations in their sequences. The PCR-RFLP was used to identify the polymorphisms of thee potential genes in five sheep breeds from Egypt and Saudi Arabia (Rahmani, Ossimi, Barki, Harrie, and Najdi). The findings demonstrated that five breeds have significantly disproportionately high polymorphism frequencies of the FecB gene with *Avall* digesting (**Elkorshy *et al.,* 2013**). **Wilson *et al.,* (2001)** employed the PCR-RFLP method; GDF9 gene (exon 1) PCR products digested with *Msp1* restriction enzyme to genotype prolific goats and used it as a molecular marker for twinning. The detection of a single copy of the GDF9 gene improves the prolificacy rate of Egyptian Baladi goat, and the same finding has been observed in many investigations **(Juengel *et al.,* 2004; Davis, 2005**; and **Noshahr and Rafat, 2014).** Does heterozygous for GDF9 mutations exhibit enhanced ovulation rates, however homozygous does are sterile because of the failure of normal ovarian follicular development (**Galloway *et al.,* 2000; Hanrahan *et al.,* 2004**). More than genetic background, age, season, and feed system have an impact on the rate of reproduction and ovulation in goat breeds. Considering these and the high prolificacy of these breeds, it may be inferred that high prolificacy in Egyptian Baladi goats may be influenced by these genetic background or that there may be a main gene.

In conclusion, numerous Egyptian Baladi goat breeds have fecundity genes that significantly influence the ovulation rate and the litter size. To construct the marker-assisted selection strategy, the GDF9 gene was shown to be polymorphic and correlated with producing twining's. While wild-type does have a lower ovulation rate, heterozygous for GDF9 does have a higher kidding rate. The authors declare that they have no conflict of interest.

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الملخص العربي

التوصيف الجزيئي لجين GDF9 والمرتبط بالخصوبة فى سلالة الماعز المصرية البلدية

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دراسة تعدد أشكال جين (GDF9) المرتبطة بالخصوبة في الماعز البلدي المصري هدف هذه الدراسة، والتي إستخدمت ٧٠ أنثى من الماعز البلدي، وأخذ منها عينات الدم، وإستخلاص الحمض النووي الـ DNA. أُستخدم بادئات متخصصة لهذا الجين، تم مضاعفة ٧٧٠ زوج من القواعد في المنطقة exon1 من الجينGDF9 ؛ وناتج التضاعف خضع لدراسة التتابع النيوكليوتيدي لتحديد درجة قرابة سلالة الماعز البلدي المصرية. بالإضافة إلى ذلك، فإن تعدد أشكال جين GDF9 تم دراسته بتقنية PCR-RFLP باستخدام إنزيم القطع *Msp1* لتأكيد الخواص الجزيئية لصفة التوأمية. تم مقارنة التتابع النيوكليوتيدي لجين GDF9 الخاص بسلالة الماعز البلدي بعشر سلالات أخرى مسجلة على البنك القومي للجينات. واستخدم تحليل التتابع النيوكليوتيدي لجين GDF9 لتحديد التشابه بين سلالة الماعز البلدي المصري والسلالات الأخرى. أشارت نتائج مقارنة التسلسل إلى أن الماعز البلدي يرتبط بشكل وثيق بسلالة الماعز المسجلة برقم KY780296 . نجح إنزيم Msp1 في هضم جين GDF9 بكفاءة الذي يتكون من حزمة واحدة إلى ثلاثة حزم بحجم تقريبي ٧٧٠ و٤٧٠ و٣٠٠ زوج من القواعد لإناث الماعز البلدي المنتجة للتوائم، وحزمة واحدة بحجم تقريبي ٧٧٠ زوجًا من القواعد للإناث المنتجة لنتاج واحد. الاستنتاج: يمكن الاستفادة من معرفة الطفرة في جين GDF9 (exon 1) مبكراً لتكوين قطيع متخصص في إنتاج التوائم دون انتظار النضج وبالتالي تقليل تكاليف الإنتاج وزيادة الربحية.

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