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Detection of GDF9 Exon2 SNPs and Investigation of their Association with Prolificacy in Two Egyptian Goat Breeds

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THE high prolific Zaraibi and low prolific Barki are among Egypt's most common indigenous goat breeds. The prolificacy of goat herds can be increased by selecting animals having single-nucleotide polymorphisms (SNPs) in fecundity-related genes. Herein, we detailed the use of DNA sequences followed by quantitative polymerase chain reaction (qPCR) and high-resolution melting curve (HRM) analysis to detect SNPs in exon 2 of the prolificacy-linked gene *GDF9* and investigate their association with prolificacy in Zaraibi (n = 100) and Barki (n = 100) goats. The results of DNA sequencing (n = 20/breed) were used as a reference for genotyping the remaining DNA samples using qPCR-HRM. A non-synonymous SNP p.V371M/c.1111G>A in was detected in *GDF9* of Zaraibi goats, however, Barki goats were monomorphic for this locus. The three genotypes of the c.1111G>A SNPs were significantly associated with litter size in Zaraibi goats. Homozygous mutant genotypes (c.1111AA) had significantly higher litter sizes than other genotypes ($P < 0.05$). Based on these findings, we recommend selecting Zaraibi goats with c.1111AA genotypes to increase the prolificacy of this breed.

Keywords: Goat, Prolificacy, GDF9, SNPs, qPCR-HRM

Introduction

Due to their widespread distribution and high market value, goats are among the most economically significant livestock in developing nations such as Egypt. Raising goats is preferable as it requires less money and fewer resources [1]. Based on the last estimate, there are nearly 4.3 million goats in Egypt [2] including several indigenous breeds such as the meat-rearing Baladi and Barki [3] and the milk-rearing Zaraibi (also known as Egyptian Nubian) [4]. The latter is highly prized in Egypt due to its high rate of prolificacy (production of several offspring at once) and is considered the ancestor of the modern Anglo-Nubian standard [5]. In contrast, the Barki breed has a lower prolific rate than Zaraibi [4,6]. These two breeds are found across Egypt, particularly in the Nile River Valley and Delta area, they can withstand harsher conditions with more disease resistance, and produce higher-quality hides [7]. The economic advantages to local farmers from raising these two goat breeds are substantial. Many recessive traits of Egyptian local goats were not sufficiently safeguarded since no scientific selective breeding system had been carried out. Zaraibi and Barki development was hampered by the fact that prolific traits were not determined and selected. As a result, cutting-edge genetic techniques should be used to detect fecundity-related mutations to select Zaraibi and Barki goats based on superior genotypes.

Researchers have found mutations in two members of the transforming growth factor beta (TGFβ), growth differentiation factor 9 (*GDF9*), and bone morphogenetic protein 15 (*BMP15*) genes which are usually referred to as fecundity (Fec) genes, associated with prolificacy in small ruminants. GDF9 is essential for early folliculogenesis, and their mutations could induce ovulation [8]. For its role in folliculogenesis and ovulation, many scientists have documented *GDF9* gene mutations during the last decade and studied

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their association with litter size in small ruminants. Several sheep breeds have shown a correlation between *GDF9* single nucleotide polymorphisms (SNPs) and increased progeny production [9]. The main detected ten fecundity-related *GDF9* SNPs in sheep were G1 (p.R87H/c.260G>A) [10], G2 (c.471C>T) [11], G3 (c.477G>A), G4 (p.E241K/c.721A>G), G5 (p.E326/c.978A>G), G6 (p.V332I/c.994G>A), $FecG^{E}$ (p.F345C/c.1034T>G) [12]; G7 (p.V371M/c.1111G>A) [13,14], G8 ($FecG^{H}$, p.S395F/c.1184C>T) [13], and FecTT (p.S427R/c.1279 A>C) [15]. None of these SNPs were detected in goats so far, except in Egyptian breeds [16-18]. However, some other *GDF9* SNPs, particularly in exon 2, were detected in many goat breeds all over the world as follows: g.3615T>C, g.3760T>C, g.3855A>C/c.909A>C, and p.V397I (g.4135G>A/c.1189G>A) SNPs in Indonesian goats [reviewed in 19], c.818C>T, p.R358K/c.1073G>A, p.V397I, and c.1330G>T in Bangladeshi black Bengal goats [20]; and both nonsynonymous p.Q320P (g.3905A>C/c.959A>C) and p.V397I in cashmere, Chinese, and Iranian Angora goats [17,21-25]. In addition, Song, et al. [17] reported a novel non-synonymous p. A240V/c.719C>T in Tibetan cashmere goats.

In some Egyptian goat breeds, G4 (p.E241K/c.721A>G) and G7 (pV371M/c.1111G>A) SNPs were detected in *GDF9* exon 2 and the association analysis revealed significantly higher fecundity traits in heterozygous G4 and G7 goats than the homozygous animals [18]. However, they failed to precisely define these SNPS due to a lack of DNA sequencing. Nevertheless, the sample sizes on Egyptian goats were rather small, and researchers did not compare breeds with high and low prolificacy. Therefore, the present study aimed to screen *GDF9* exon 2 for SNPs using the qPCR-HRM approach and study their association with prolificacy in Zaraibi and Barki goats.

Material and Methods

Animals

Female goats (n = 200, 2 - 5 years old, and 33 – 37 Kg body weight) enrolled in this study were raised in three stations belonging to the Animal Research Institute of the Egyptian Ministry of Agriculture, which are Sakha Animal Production Research Station, EL-Serw experimental station and Borg El-Arab experimental station. The high prolific Zaraibi (n = 100) and low prolific Barki (n = 100) breeds were selected based on station records of the prolificacy rate in the first three consecutive parities in three successive years. Station records included the kidding date and season as well as litter size for each doe. Based on these records, we selected Zaraibi goats with a large litter size (average litter size 2.16 kids/litter) and Barki goats with a small litter size (average litter size 1.15 kids/litter). To prevent inbreeding, mating between closely related relatives was discouraged. Animals were kept in sheltered semi-open pens and were provided with clover hay and a concentrated mixture in summer and Egyptian clover (*Trifolium alexandrinum*) in winter with free access to water.

Sample collection and DNA extraction

Before blood sample collection from goats, we got ethical approval (VET-IACUC-130) from Animal Care and Use Experimental Committee, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. Five milliliters of blood were drawn from the jugular vein of each goat, placed in sterilized EDTA-treated vacutainer tubes, transferred in an ice box, and frozen at -20°C for later DNA extraction in the lab. The DNAeasy Blood & Tissue Kit (Fermentas, Thermo Scientific, #K0721) was used to extract total genomic DNA from blood samples following the manufacturer's instructions.

Primer design

As previously mentioned, exon 2 of *GDF9* was highly polymorphic and contained the majority of fecundity-related SNPs in goats. Thus, primers were designed to flank SNPs location in exon 2 of *GDF9* based on goat reference genes in GenBank (JN601040.1) and as previously described [18,26]. The sequence of these primers and the size of the PCR products were shown in Table 1.

TABLE 1. **Primers sequences of *GDF9* exon 2 and size of PCR products**

Gene	Forward primer ($^{/}5$ ----- $^{/}3$)	Reverse primer ($^{/}5$ ----- $^{/}3$)	Product size(bp)	References
GDF9	AGAGACCAGGAGAGTGC CAGCTCTGAAT	CGATGGCCAAAACAC TCAAAGGGCTATA	343	[18]

TABLE 2. Comparative analysis of SNPs detected in *GDF9* exon2 from nucleotides c.952 to c.1294 (JN601040.1) between GenBank published sequences of small ruminants. p.

Nucleotides position	**959**	**978**	**994**	**1034**	**1073**	**1111**	**1184**	**1189**	**1279**
SNP	A>C	A>G	G>A	T>G	G>A	G>A	C>T	G>A	A>C
Amino acid (aa) position	240	326	332	345	358	371	395	397	427
aa change	Q>P	E	V>I	F>C	R>K	V>M	S>F	V>I	S>R
Codon change	CAG>CCG	GAA>GAG	GTT>ATT	TTT>TGT	AGA>AAA	GTG>ATG	TCT>TTT	ATT>GTT	AGT>CGT
Egyptian Zaraibi goat [1,2]	CAG	GAA	GTT	TTT	AGA	GTG>ATG	TCT	ATT	AGT
Egyptian Barki goat [1,2]	CAG	GAA	GTT	TTT	AGA	GTG	TCT	ATT	AGT
Cashmere, Chinese, and Iranian goats [3]	CAG>CCG	GAA	GTT	TTT	AGA	GTG	TCT	ATT>GTT	AGT
Indonesian goat [4]	CAG	GAA	GTT	TTT	AGA	GTG	TCT	ATT>GTT	AGT
Bangladeshi black Bengal goat [5]	CAG	GAA	GTT	TTT	AGA>AAA	GTG	TCT	ATT>GTT	AGT
Ovis aries (sheep) [6]	CAG	GAA>GAG (G5)	GTT>ATT (G6)	TTT>TGT (FecG^{E})	AGA	GTG>ATG (G7)	TCT>TTT (G8, FecG^{H})	GTT	AGT>CGT (FecTT)

Red color nucleotides refer to mutant alleles. nucleotide. [1]This study; [2][18]; [3][17,21-25]; [4][reviewed in 19]; [5][20]; [6]Accession number AF078545.2.

TABLE 3. Genotypic and allelic frequencies, value of χ^2 test and diversity parameter of c.1111G>A SNP of *GDF9* in Zaraibi goat

SNP	Genotype frequency (number)			Allele frequency		χ^2 (p value)	He	PIC
c.1111G>A of GDF9	GG	GA	AA	G	A	0.21 (0.90)	0.47	0.36
	0.38 (38)	0.49 (49)	0.13 (13)	0.625	0.375			

He, gene heterozygosity; PIC, polymorphism information content; χ^2, Chi-Square value.

TABLE 4. Association between c.1111G>A three genotypes of *GDF9* and litter size.

Breed (Number)	**Genotype (Number)**	**Single % (Number)**	**Twin % (Number)**	**Triplicate % (Number)**	**Quadruplets % (Number)**	**Litter size (Mean±SEM)**
Zaraibi	GG (38)	78.95 (20)	21.05 (18)	- (0)	- (0)	1.47±0.07 [c]
	GA (49)	28.58 (14)	46.94 (23)	20.41 (10)	4.07 (2)	2.00±0.10 [b]
(100)	AA (13)	- (0)	53.85 (7)	30.77 (4)	15.38 (2)	2.62±0.13 [a]
Barki	GG (100)	85 (85)	15 (15)	- (0)	- (0)	1.15± 0.04 [d]
	GA (0)	- (0)	- (0)	- (0)	- (0)	0
(100)	AA (0)	- (0)	- (0)	- (0)	- (0)	0

Data are expressed as least squares means ± SEM. Different lowercase letters indicate significant differences between genotypes ($p < 0.05$).

PCR amplification and DNA sequencing

Twenty DNA samples from each breed were sequenced to screen for *GDF9* mutation. First, the forty samples were amplified in a thermal cycler. PCR mixture contained 3 µL DNA, 10 pmol primer (1 µL from each primer), 15 µL 2× MasterMix (ThermoFisher, # K0171), and 10 µL DNAase free water (50 – 100 ng). The thermal condition was as follows: one cycle of initial denaturation (94 °C/4 min), 35 cycles of denaturation (94 °C/45 s), annealing (55°C for 40 s), extension (72 °C / 45 s) and one final cycle of extension (72 °C / 5 min). The amplicons were separated on 1.5% agarose gels containing ethidium bromide at 0.25 µg/ml and examined by a UV transilluminator. A 100 bp DNA ladder (Thermo Scientific) was used to quantify the size of the fragments.

Forty PCR products of the required size were purified according to the instructions provided in the PureLink™ PCR Purification Kit (ThermoFisher, # K310001). The amplicons were then sequenced in both directions by an automatic sequencer (ABI 310, Applied Biosystem, USA). Sequence alignment and annotation were performed against goat *GDF9* reference genes in GenBank (JN601040.1) using Geneious 4.8.4 software.

Animal genotyping using qPCR-HRM

For its rapid, accurate, and cost-effective identification, qPCR-HRM was applied to genotype animals as previously described [27,28]. In brief, a PCR mixture (10 µl) containing 5 µl of 2× Maxima SYBR Green/ ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 3 µl of DNA template (20 ng), and 1 µl of each primer (0.5 µM) was inserted in PikoReal 96 thermal cycler (ThermoScientific, USA). We utilized the same *GDF9* primers used in the conventional PCR (Table 1), but with slightly different thermal conditions as described by the manufacturer as follows: initial denaturation (95°C/15 minutes /1 cycle), denaturation (95°C/20 seconds/45 cycles), annealing (55°C for 20 seconds /45 cycles), extension (72°C/30 seconds/45 cycles), and final extension (72°C/5 minutes/1 cycle). Melting curves were obtained by gradually increasing the final cycle temperature from 63 to 95 °C with concurrent observation of the fluorescence intensity every two seconds. The integrated software's HRM Tool (PikoReal 2.2) was used to create differential curves (DC) and normalized melting curves (NMC) from the collected fluorescence data. Comparing the DC and NMC of test samples (n = 160) to those of the reference samples (forty sequenced samples) allowed for animal genotyping for *GDF9* SNPs.

Potential effect of amino acid substitutions

To foresee how amino acid substitutions resulting from the detected non-synonymous SNPs would affect the structure and function of proteins, we used Polyphen-2 software [29].

Statistical analysis

The frequencies of genotypes and alleles were directly determined, while gene heterozygosity (He), Hardy-Weinberg equilibrium (HWE), and the polymorphism information content (PIC) were calculated using PopGene 32 (version 1.32) and GenCal software [30-34]. The association of genotypes resulting from *GDF9* exon 2 SNP with litter size was detected by the following general linear model (GLM, SAS V9, SAS Inst. Inc., Cary, NC, USA): $Y_{ijkm} = \mu + K_i + P_j + G_k + G(K)_{ki} + G(P)_{kj} + e_{ijkm}$

Y_{ijkm} was the litter size phenotypic value; µ was the overall mean, K_i is the fixed effect of the i^{th} (i = 1, 2, 3) kidding year; P_j was the fixed effect of the j^{th} parity (j = 1, 2, 3); G_k was the effect of k^{th} genotype for each gene; $G(K)_{ki}$ and $G(P)_{kj}$ are the interactions between genotypes with kidding year, and parity, respectively; and e_{ijkm} was the random residual effect. Data were presented as least squares means ± standard error of the mean (SEM). After applying the Bonferroni adjustment to the multiple comparisons, we determined that a value of $P < 0.05$ indicated statistical significance.

Results

Analysis of GDF9 exon 2 SNPs by sequencing

Fragments of *GDF9* exon 2 (343bp) were amplified using conventional PCR (Fig. 1). DNA sequencing (n = 20/breed) revealed the presence of a non-synonymous p.V371M/c.1111G>A SNP in *GDF9* exon 2 (Fig. 2A), however no SNPs were detected in Barki goats (Fig. 2B). In the c.1111G>A SNP of *GDF9*, G nucleotide (nt) was replaced by A at nt number 714 of exon 2 which is equivalent to coding nt number 1111 based on reference of black Bengal goat with accession number JN601040.1. This nt substitution changed valine (Val/V) to methionine (Met/M) at position 371 amino acid (aa) of coding sequence (p.V371M) (Table 2, Fig.2).

Genotyping by qPCR-HRM

The results of DNA sequencing (n = 20/breed) were used as a reference for genotyping the remaining DNA samples using qPCR-HRM. We discovered that with both intercalating dyes, NMC and DC, qPCR-HRM could discriminate the different genotypes of *GDF9* (GG, GA, AA) with 99.5% confidence in Zaraibi goats. Based on qPCR-HRM results, *GDF9* locus were polymorphic

in Zaraibi goats with three genotypes (GG, GA, AA). However, Barki goats were monomorphic with only GG.

Analysis of allele/genotype frequencies, Hardy Weinberg's and genetic indices

Zaraibi goats, the frequencies of wild alleles c.1111G(0.625) and its homozygous genotypes c.1111GG(0.38) were higher than the mutant alleles c.1111A(0.375) and its homozygous genotypes c.1111AA(0.13), while the heterozygous genotypes c.1111GA(0.49) exhibited the highest frequencies (Table 3). Genotypes distribution for c.1111G>A SNP were consistent with Hardy Weinberg's law at the level of significance above 0.05 indicating the absence of natural or artificial selection for this SNP among Zaraibi goats. On the other hand, c.1111G>A SNP showed higher mutation frequencies as revealed by medium PIC value (0.36), with high heterozygosity (He, 0.47) (Table 3).

Association between c.1111G>A and c.808C>G SNPs and litter size

The three genotypes of c.1111G>A SNP SNP were significantly ($P < 0.05$) associated with litter size in Zaraibi goats Table 3. Goats carrying homozygous mutant genotypes (c.1111AA) had significantly higher litter size (2.62±0.13 kids) than homozygous wild genotypes (c.1111GG, 1.47±0.07 kids) and heterozygous genotypes (c.1111GA, 2.00±0.10 kids) (Table 4). Since Barki goats had only the wild genotypes (c.1111GG), we compared them with the same genotypes in Zaraibi goats and found significantly lower litter size in Barki goats (1.15± 0.04 kids) (Table 3). We also found higher birth rates of twins (53.85%), triplets (30.77%), and quadruplets (15.38%) in Zaraibi goats with c.1111AA genotypes than other genotypes of c.1111G>A SNP (Table 4).

Data analysis using Ploy phen2 predicted that p.V371M/c.1111G>A SNP in the *GDF9* gene could have a major effect on the function of the GDF9 protein (Fig. 3). Valine (V) occupies this specific position in nine remarkably diverse mammalian species, namely sheep, cattle, buffalo, camel, pig, cat, human, and mouse (Fig. 4).

Discussion

Prolificacy is a desirable feature for the genetic development of small ruminant flocks because of its ability to increase production. When compared to animals that only have one lamb or kid per litter, the prolific species can result in a 2-fold increase in meat production [16]. Litter size, as a quantitative trait, is regulated by many genes and their polymorphisms which could interact together in a complicated way. However, in small ruminant breeds, this trait is considered as a qualitative trait regulated mainly by the fecundity-related gene *GDF9* [35] which is a component of the TGFβ family secreted in the ovary and plays a crucial role as growth factors and receptors in folliculogenesis and ovulation, thereby underlying the high litter size and fertility in ewes and does [36,37]. The majority of mutations associated with fecundity are present in Exon 2 of *GDF9* in both sheep and goats [13,18,38-40].Therefore, this study aimed to screen this locus for mutations and study their association with prolificacy in two Egyptian local breeds with different prolificacy rates, Zaraibi (high prolific) and Barki (low prolific). A non-synonymous SNPs p.V371M/c.1111G>A in *GDF9* was detected in Zaraibi goats however, Barki goats were monomorphic for this locus.

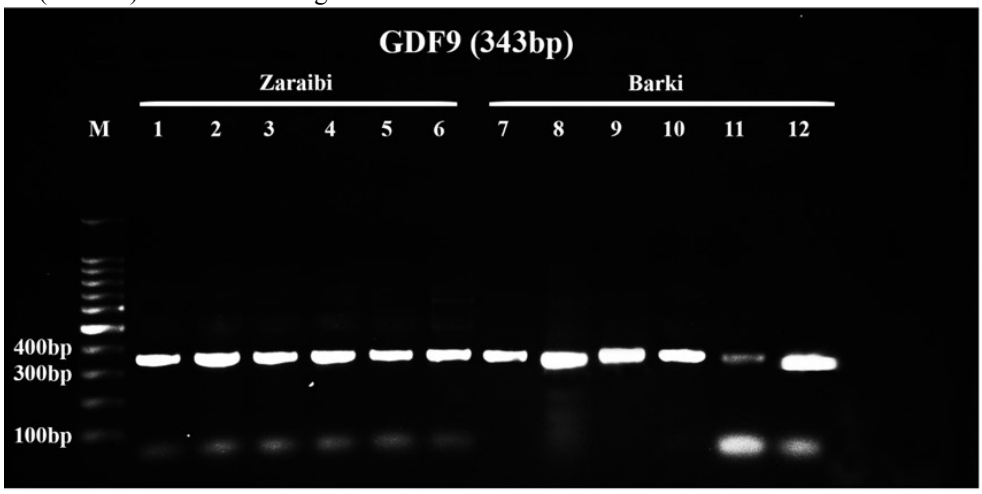

Fig.1. Agarose gel (1.5%) shows PCR products of *GDF9* locus (343bp) from different DNA samples of Zaraibi and Barki goats. M, DNA marker (100 bp).

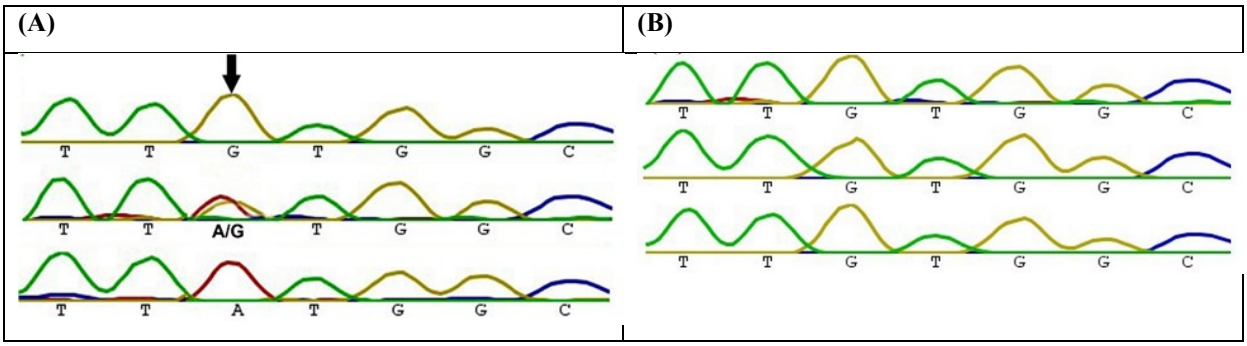

Fig.2. Detection of *GDF9* mutations in Zaraibi goats (A) and Barki goats (B0 using DNA sequencing. A chromatogram of the sequences that cover the site of p.V371M/c.1111G>A SNP in *GDF9* locus (arrow).

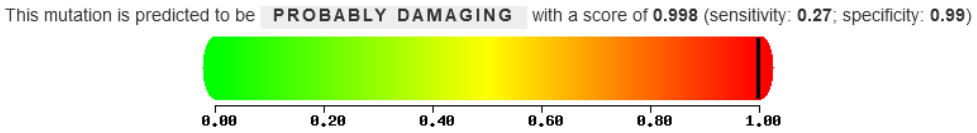

Fig. 3. Effects of p.V371M (c.1111G>A) SNP of the *GDF9* locus on the functional GDF9 protein as predicted by Ployphen2.

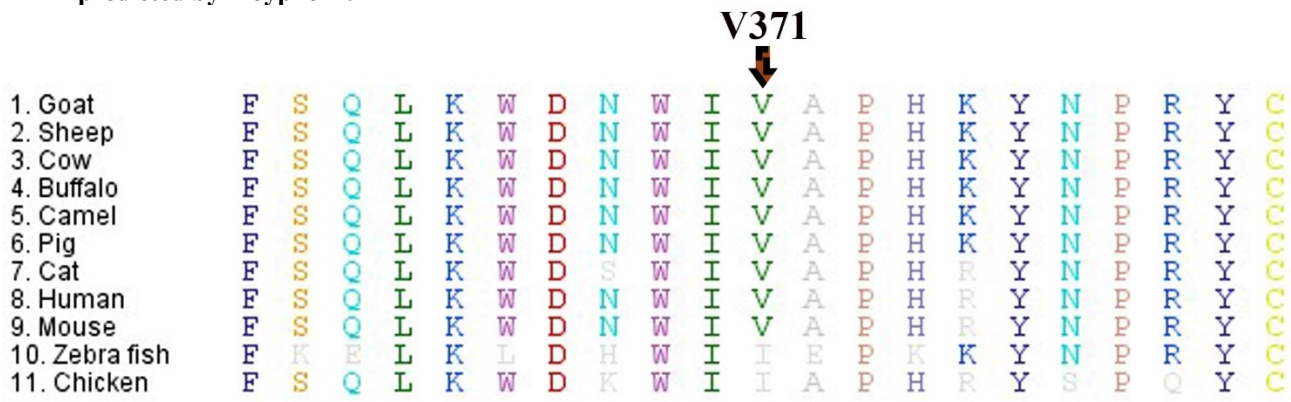

Fig.4. Partial multiple amino acid (aa) sequences of GDF9 among goat (JN601040.1), sheep (NP_001136360.1), cow (NP_777106.1), buffalo (AFH66792.1), camel (XP_006179664.2s), pig (NP_001001909.1), cat (NP_001159372.1), human (NP_005251.1), mouse (NP_032136.2), chicken (NP_996871.2) and zebra fish (NP_001012383.1) shows aa conservation at the position 371 of GDF9.

Our results confirmed the study of Aboelhassan, *et al.* [18] for the presence of p.V371M (c.1111G>A) SNP in *GDF9* exon2 of Egyptian Zaraibi goats. In contrast, this SNP was not detected in Egyptian Barki goat (this study) or other goat breeds such as Indonesian goats [reviewed in 19], Bangladeshi black Bengal goats [20], Tibetan cashmere goats [17], and cashmere, Chinese, and Iranian Angora goats [17,21-25] (Table S1). Among different goat breeds, only Zaraibi goats had the two alleles (c.1111G and c.1111A), while other breeds possessed only the c.1111G allele, suggesting that c.1111G is the wild (ancestral) and c.1111A is the mutant allele (Table S1). To the best of our knowledge, these are the first two reports to determine this SNP in Zaraibi goat at the same position of the fecundity-related G7 (p.V371M/c.1111G>A) *GDF9* SNP in sheep [13,14]. Thus, out of the main naturally ten fecundity-related *GDF9* SNPs in sheep [G1, G2, G3, G4, G5, G6, FecG^{E}, G7, G8 (FecG^{H}), and FecTT], only G7 SNP was found in Egyptian Zaraibi goat. However, Zaraibi and Barki goat lacked two SNPs: p.Q320P/c.959A>C and p.V397I/c.1189G>A SNPs (Table S1) found in Indonesian, Cashmere, Chinese, Bangladeshi black Bengal, and Iranian goats [17,19-25].

The qPCR-HRM technique has been used successfully in diagnostics for the rapid and cost-effective identification of different SNPs in the prolificacy-related *GDF9* and *BMP15* gene in

sheep [27,28]. Similarly, we genotyped the two goat breeds using qPCR-HRM and found *GDF9* locus was polymorphic in Zaraibi goats with three genotypes in *GDF9* (GG, GA, AA), while Barki goats were monomorphic with only GG. In Zaraibi goats, the frequencies of wild alleles c.1111G(0.625) and its homozygous genotypes c.1111GG(0.38) were higher than the mutant alleles c.1111A(0.375) and its homozygous genotypes c.1111AA(0.13), while the heterozygous genotypes c.1111GA(0.49) exhibited the highest frequencies. Similarly, Aboelhassan, et al. [18] found extremely higher heterozygous genotypes c.1111GA(0.91) than c.1111GG(0.09) in some local Egyptian breeds. However, they failed to find the mutant homozygous genotypes c.1111AA and this could be attributed to the small sample size (n = 44) or the less sensitive method T-ARMS-PCR used in genotyping as compared to the highly sensitive and more accurate method, qPCR-HRM, used in the present study. In sheep, the allele frequency of c.1111G(0.75) was higher than c.1111A(0.25), and three genotypes were detected but with higher frequency for c.1111GG followed by c.1111GA [14].

The three genotypes of the *GDF9* c.1111G>A SNP were significantly ($P < 0.05$) associated with litter size with a significantly higher litter size in homozygous mutant genotypes (c.1111AA, 2.62±0.13 kids) than homozygous wild genotypes (c.1111GG, 1.47±0.07 kids) and heterozygous genotypes (c.1111GA, 2.00±0.10 kids). This infers that mutant A allele could be a beneficial allele and the c.1111G>A SNP could be a desirable mutation for prolificacy trait in Zaraibi goats. On the other hand, as Barki goats had only the wild genotype (c.1111GG), we compared it with the same genotype in Zaraibi goats and found significantly lower litter size in Barki goats (1.15± 0.04 kids). We also found higher birth rates of twins (53.85%), triplets (30.77%), and quadruplets (15.38%) in Zaraibi goats with homozygous mutant genotypes than other genotypes. Interestingly, the latter animals had no single birth. In agreement with our findings, Aboelhassan, et al. [18] also found a significant association between c.1111G>A SNP and the mean number of twin production in some Egyptian goat breeds with a higher rate in heterozygous (c.1111GA, 1.93±0.40) than in homozygous (c.1111GG, 0.20±0.10). Moreover, previous studies reported higher prolificacy rate in Zaraibi goats with an average litter size of 2.16 kids/litter and a birth rate of 22.10% for single births, 45.65% for twins, 26.09% for triplets, and 6.16 % for quadruplets [6] and lower prolificacy rate in Barki goats with an average litter size of 1.25 kids/litter and a birth rate of 87.50 % for single births and 12.50% for twins [4]. As previously stated, foreign goat breeds lacked this SNP, so far, but have other SNPs in *GDF9* whose association with prolificacy rate showed contradictory results. Among SNPs discovered in goat *GDF9* exon 2, only the non-synonymous p.Q320P and p.V397I SNPs showed a significant association with litter size in Chinese and Iranian goats [21-25], however in seven native Indian goat breeds no association between these SNPs and litter size was reported [37].

It is well-known that GDF9 regulates the expression of several genes involved in ovarian hormone production in the granulosa cells such as INHB [41]. GDF9 exerts its biological effects only after binding to its receptor, demonstrating that the protein's structure is crucial to its activity [42]. Depending on its context and location, SNP molecular and functional effects can be neutral, detrimental, or beneficial [43]. Indeed, data analysis using Ployphen2 predicted that p.V371M (c.1111G>A) SNP in the *GDF9* gene could have a major effect on the function of the GDF9 protein. Thus, p.V371M (c.1111G>A) SNP could be a beneficial mutation in Zaraibi goats. However, we cannot definitively establish this mutation as the main cause of increased litter size. Our findings just suggest a potential functional link for three compelling reasons. Firstly, this genetic variation denotes an amino acid substitution within the bioactive region of the GDF9 protein [44]. Secondly, valine (V) occupies this specific position in nine remarkably diverse mammalian species, namely sheep, cattle, buffalo, camel, pig, cat, human, and mouse, but in chicken and zebrafish, valine is substituted with another aliphatic amino acid, isoleucine (I). Thirdly, although in the c.1111G>A SNP, a nonpolar amino acid (valine, V) is substituted with another nonpolar amino acid (methionine, M), the two aa have distinct structural characteristics along their side chains. This structural variation may contribute to a reduction in GDF9's binding affinity to its receptors, BMP receptor type II (BMPR2) and TGFβ receptor 1 (TGFBR1) [45]. To gain further validation, it would be beneficial to conduct experiments examining the structural and functional effects of this mutation.

Expression of the *GDF9* gene was oocyte-specific at the early stage of folliculogenesis in sheep and cattle ovaries [46]. However, in goats, *GDF9* is expressed throughout the whole folliculogenesis with abundant levels in small than large antral follicles and in 20 tissues other than the ovary [47]. Another difference between *GDF9* SNPs in sheep and goats is that heterozygotes sheep are more prolific than homozygous sheep, while in goats the reverse is true [13,18,38-40,48,49]. In support, we also found that the mutant homozygous Zaraibi goats had higher litter sizes than the heterozygous goats.

As a limitation, few sample size and a limited number of goats were used in the present investigation. Since the litter size is a quantitative trait including multiple genetic markers, loci, and quantitative trait loci (QTL), we could not presume that p.V371M/c.1111G>A was a causal SNP of litter size. Further investigations including next-generation sequencing (NGS) of the whole genome of Egyptian goats are required in a large population of Egyptian goats and many different local breeds.

Conclusions

The Zaraibi goat is a prolific breed, known for its high litter sizes. We have identified p.V371M/c.1111G>A SNP in *GDF9* that are associated with litter size in Zaraibi goats. Goats that carry both copies of the mutant allele for either SNP (c.1111AA) have the highest litter sizes. In contrast, the low-prolific Barki goat lacks this SNP. These findings suggest that selecting Zaraibi goats with the mutant alleles for this SNP could increase fecundity and production in this breed. However, larger studies with more goats are needed to screen the entire sequences of *GDF9* for novel SNPs and verify the association between these SNPs and increased prolificacy in Zaraibi goats.

List of abbreviations

BMP15: bone morphogenetic protein 15
BMPR2: BMP receptor type II
Fec: fecundity
GDF9: growth differentiation factor 9
He: gene heterozygosity
HRM: high-resolution melting curve
HWE: Hardy-Weinberg equilibrium
INHB: Inhibin B
NGS: next-generation sequencing
PIC: polymorphism information content
qPCR: quantitative polymerase chain reaction
QTL: quantitative trait loci
RFLP: restriction fragment length polymorphism
SNPs: single-nucleotide polymorphisms
SSCP: single-strand conformational polymorphism
T-ARMS: tetra-primer amplification refractory mutation system
TGFβ: transforming growth factor beta
TGFBR1: TGFβ receptor 1

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Author contributions

Aya Kotb, collection of samples, conducting the production experiment, interpretation of data, article preparation; Nasr Nasr and Khaled Kahilo, design of study, interpretation of data; Mohammed EL-Badawy, collection of samples; Mohammed A. El-Magd design of study, conducting the production experiment, data analysis, article preparation. All co-authors approved the final draft of the article.

References

1. Almadaly, E.A., Farrag, F.A., Saadeldin, I.M., El-Magd, M.A., El-Razek, I.M.A. Relationship between total protein concentration of seminal plasma and sperm characteristics of highly fertile, fertile and subfertile Barki ram semen collected by electroejaculation. *Small Ruminant Research*, **144**, 90-99 (2016).
2. Nowier, A.M., Darwish, H.R., Ramadan, S.I. and Othman, O.E. Allele mining in prolactin receptor gene and its association with some economic traits in Egyptian goat breeds. *Animal Biotechnology*, 1-9, (2023).
3. Ashour, G., Neama, A.A., Dessouki, S.M. and Shihab, O. Blood hematology, metabolites and hormones in newborn sheep and goat from birth to weaning. *Int. J. Adv. Res*., **3**, 1377-1386(2015).
4. Anous, M.R.I., Kodit, E., Elhifnawy, H., Rashed, M. and Sadek, M. Assessing Inter and Intra Molecular Genetic Variation of Litter Size in Three Egyptian Goat Breeds Using COI and F-AFLP Markers. *Arab Universities Journal of Agricultural Sciences*, **29**, 341-350 (2021).
5. Shaat, I. and Mäki-Tanila, A. Variation in direct and maternal genetic effects for meat production traits in Egyptian Zaraibi goats. *Journal of Animal Breeding and Genetics*, **126**, 198-208, (2009).
6. Farrag, F.H., Shalaby, N.A., Metawi, H.R., Osman, M.A. and Desoky, A.I. Characterization of Zaraibi goat production systems in delta region of EGYPT. *Journal of Animal and Poultry Production*, **3**, 47-57 (2012).
7. Galal, S., Abdel-Rasoul, F., Anous, M. and Shaat, I. Onstation characterization of small ruminant breeds in Egypt. *In: L. In iguez (ed.),* Characterization of Small Ruminant Breeds in West Asia. *Small Ruminants Research,* **2**,141 - 193.(2005).
8. Ahlawat, S., Sharma, R., Maitra, A. and Tantia, M. Current status of molecular genetics research of goat fecundity. *Small Ruminant Research*, **125**, 34-42 (2015).
9. Abdelgadir, A.Z., Musa, L.M.A., Jawasreh, K.I., Saleem, A.O., El-Hag, F. and Ahmed, M.A. G1 point mutation in growth differentiation factor 9 gene affects litter size in Sudanese desert sheep. *Vet World*, **14**, 104-112 (2021).

10. Abdoli, R., Zamani, P., Deljou, A. and Rezvan, H. Association of BMPR-1B and GDF9 genes polymorphisms and secondary protein structure changes with reproduction traits in Mehraban ewes. *Gene*, **524**, 296-303(2013).

11. Zamani, P., Abdoli, R., Deljou, A. and Rezvan, H. Polymorphism and bioinformatics analysis of growth differentiation factor 9 gene in Lori sheep. *Annals of Animal Science*, **15**, 337 (2015).

12. El Fiky, Z.A., Hassan, G.M. and Nassar, M.I. Genetic polymorphism of growth differentiation factor 9 (GDF9) gene related to fecundity in two Egyptian sheep breeds. *Journal of Assisted Reproduction and Genetics*, **34**, 1683-1690(2017).

13. Hanrahan, J.P., Gregan, S.M., Mulsant, P., Mullen, M., Davis, G.H., Powell, R. and Galloway, S.M. 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). *Biology of Reproduction*, **70**, 900-909 (2004).

14.Våge, D.I., Husdal, M., Kent, M.P., Klemetsdal, G. and Boman, I.A. A missense mutation in growth differentiation factor 9 (GDF9) is strongly associated with litter size in sheep. *BMC Genetics*, **14**, 1-8(2013).

15. Nicol, L., Bishop, S.C., Pong-Wong, R., Bendixen, C., Holm, L.-E., Rhind, S.M. and McNeilly, A.S. Homozygosity for a single base-pair mutation in the oocyte-specific GDF9 gene results in sterility in Thoka sheep. *Reproduction (Cambridge, England)*, **138**, 921-933(2009).

16. Polley, S., De, S., Batabyal, S., Kaushik, R., Yadav, P., Arora, J.S., Chattopadhyay, S., Pan, S., Brahma, B. and Datta, T.K. Polymorphism of fecundity genes (BMPR1B, BMP15 and GDF9) in the Indian prolific Black Bengal goat. *Small Ruminant Research*, **85**, 122-129(2009).

17. Song, T., Liu, Y., Cuomu, R., Tan, Y., A. Wang, C., De, J., Cao, X. and Zeng, X. Polymorphisms Analysis of BMP15, GDF9 and BMPR1B in Tibetan Cashmere Goat (Capra hircus). *Genes*, **14**, 1102 (2023).

18. Aboelhassan, D.M., Darwish, A.M., Ali, N.I., Ghaly, I.S. and Farag, I.M. A study on mutation points of GDF9 gene and their association with prolificacy in Egyptian small ruminants. *Journal of Genetic Engineering and Biotechnology*, **19**, 1-11(2021).

19. Abuzahra, M., Abu Eid, L., Effendi, M., Mustofa, I., Lamid, M. and Rehman, S. Polymorphism studies and candidate genes associated with litter size traits in Indonesian goats. a systematic review [version 1, peer review: 1 approved with reservations]. *F1000Research*, **12**,61 (2023). 10.12688/f1000research.129050.1

20. Shaha, M., Miah, G., Lima, A. Adzhubei, I., Jordan, D.M. and Sunyaev, S.R. Predicting functional effect of human missense mutations using PolyPhen-2. . *Current Protocols in Human Genetics,* **07***, unit,* 7.20.(2013).

Miazi, F.O.D., Ashutosh Identification of Polymorphisms in GDF9 and BMP15 Genes in Jamunapari and Crossbred Goats in Bangladesh. *10 December 2021, PREPRINT (Version 1) available at Research Square [https://doi.org/10.21203/rs.3.rs-1102219/v1]*, (2021).

21. Wang, X., Yang, Q., Wang, K., Yan, H., Pan, C., Chen, H., Liu, J., Zhu, H., Qu, L. and Lan, X. Two strongly linked single nucleotide polymorphisms (Q320P and V397I) in GDF9 gene are associated with litter size in cashmere goats. *Theriogenology*, **125**, 115-121(2019).

22. Feng, T., Geng, C., Lang, X., Chu, M., Cao, G., Di, R., Fang, L., Chen, H., Liu, X. and Li, N. Polymorphisms of caprine GDF 9 gene and their association with litter size in Jining Grey goats. *Molecular Biology Reports*, **38**, 5189-5197(2011).

23. Arefnejad, B., Mehdizadeh, Y., Javanmard, A., Zamiri, M. and Niazi, A. novel single nucleotide polymorphisms (SNPs) in two oogenesis specific genes (BMP15, GDF9) and their association with litter size in Markhoz goat (Iranian Angora). *Iranian Journal of Applied Animal Science*, **8**, 91-99(2018).

24. Zhao, J., Liu, S., Zhou, X., Zhang, R., Li, Y., Wang, X. and Chen, Y. A non- synonymous mutation in GDF 9 is highly associated with litter size in cashmere goats. *Animal Genetics*, **47**, 630-631 (2016).

25. An, X., Hou, J., Zhao, H., Li, G., Bai, L., Peng, J., M Yan, Q., Song, Y., Wang, J. and Cao, B. Polymorphism identification in goat GNRH1 and GDF9 genes and their association analysis with litter size. *Animal Genetics*, **44**, 234-238(2013).

26. Kasiriyan, M.M., Hafezian, S.H. and Hassani, N. Genetic polymorphism BMP15 and GDF9 genes in Sangsari sheep of Iran. *Journal of Genetics and Molecular Biology*, **3**, 31-34(2011).

27. Yao-Jing, Y., Bo-Hui, Y., Xia, L., Jian-Bin, L., Shuo, J., Jian, G., Xiao-Ping, S., Chun-E, N. and Rui-Lin, F. Simultaneous identification of FecB and FecX G mutations in Chinese sheep using high resolution melting analysis. *Journal of Applied Animal Research*, **39**, 164-168 (2011).

28. Escobar-Chaparro, R.A., Guillén, G., Espejo-Galicia, L.U., Meza-Villalvazo, V.M., Peña-Castro, J.M. and Abad-Zavaleta, J. qPCR and HRM-based diagnosis of SNPs on growth differentiation factor 9 (GDF9), a gene associated with sheep (Ovis aries) prolificacy. *3 Biotech*., **7**, 204 (2017).

29. Adzhubei, I., Jordan, D.M. and Sunyaev, S.R. Predicting functional effect of human missense mutations using PolyPhen-2. . *Current Protocols in Human Genetics,* **07***, unit,* 7.20.(2013).

30. El-Magd, M.A., Abbas, H.E., El-kattawy, A.M. Adzhubei, I., Jordan, D.M. and Sunyaev, S.R. Predicting functional effect of human missense mutations using PolyPhen-2. . *Current Protocols in Human Genetics,* **07***, unit,* 7.20.(2013).

31. Mokhbatly, A. Novel polymorphisms of the IGF1R gene and their association with average daily gain in Egyptian buffalo (Bubalus bubalis). *Domestic. Animal Endocrinology*, **45**, 105-110(2013).

32. Abo-Al-Ela, H.G., El-Magd, M.A., El-Nahas, A.F. Adzhubei, I., Jordan, D.M. and Sunyaev, S.R. Predicting functional effect of human missense mutations using PolyPhen-2. . *Current Protocols in Human Genetics,* **07***, unit,* 7.20.(2013).

33. Mansour, A.A. Association of a novel SNP in exon 10 of the IGF2 gene with growth traits in Egyptian water buffalo (Bubalus bubalis). *Tropical Animal Health and Production*, **46**, 947-952(2014).

34. El-Magd, M.A., Abo-Al-Ela, H.G., El-Nahas, A., Saleh, A.A. and Mansour, A.A. Effects of a novel SNP of IGF2R gene on growth traits and expression rate of IGF2R and IGF2 genes in gluteus medius muscle of Egyptian buffalo. *Gene*, **540**, 133-139, (2014).

35. El-Magd, M.A., Saleh, A.A., Abdel-Hamid, T.M., Saleh, R.M. and Afifi, M.A. Is really endogenous ghrelin a hunger signal in chickens?: Association of GHSR SNPs with increase appetite, growth traits, expression and serum level of GHRL, and GH. *General and Comparative Endocrinology*, **237**, 131-139, (2016).

36. EL-Magd, M.A., Saleh, A.A., Nafeaa, A.A., EL-Komy, S.M. and Afifi, M.A. Polymorphisms of the IGF1 gene and their association with growth traits, serum concentration and expression rate of IGF1 and IGF1R in buffalo. *Journal of Zhejiang University Science B*, **18**, 1064-1074 (2017).

37. Gootwine, E. Invited review: Opportunities for genetic improvement toward higher prolificacy in sheep. *Small Ruminant Research*, **186**, 106090 (2020).

38. Abdoli, R., Zamani, P., Mirhoseini, S., Ghavi Hossein- Zadeh, N. and Nadri, S. A review on prolificacy genes in sheep. *Reproduction in Domestic Animals*, **51**, 631-637 (2016).

39. Ahlawat, S., Sharma, R., Roy, M., Tantia, M.S. and Prakash, V. Association analysis of novel SNPs in BMPR1B, BMP15 and GDF9 genes with reproductive traits in Black Bengal goats. *Small Ruminant Research*, **132**, 92-98 (2015).

40. Paulini, F. and Melo, E.O. The Role of Oocyte-Secreted Factors GDF9 and BMP15 in Follicular Development and Oogenesis. *Reproduction in Domestic Animals*, **46**, 354-361 (2011).

41. Islam, M., Basheer, A., Javed, K., Anjum, A. and Zahoor, I. PCR-RFLP-based identification of polymorphisms in BMPR1B, GDF9 and BMP15 genes associated with litter size in Beetal and Teddy goats. *South African Journal of Animal Science*, **49**, 697-708 (2019).

42. Huang, Q., Hu, W., Wang, T., Zhang, C. and Liu, R. Polymorphism analysis on the exon 2 of Guizhou Black goats GDF9 gene. *Chin. Livest. Breed*, **11**, 141-143(2009).

43. Mazerbourg, S. and Hsueh, A.J. Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands. *Human Reproduction Update*, **12**, 373-383 (2006).

44. Mazerbourg, S., Klein, C., Roh, J., Kaivo-Oja, N., Mottershead, D.G., Korchynskyi, O., Ritvos, O. and Hsueh, A.J. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Molecular Endocrinology*, **18**, 653-665 (2004).

45. Das, A., Shaha, M., Gupta, M.D., Dutta, A. and Miazi, O.F. Polymorphism of fecundity genes (BMP15 and GDF9) and their association with litter size in Bangladeshi prolific Black Bengal goat. *Trop. Anim. Health Prod*., **53**, 230(2021).

46. Mcpherron, A.C. and Lee, S.J. GDF-3 and GDF-9: two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. *The Journal of Biological Chemistry*, **268 5**, 3444-3449(1993).

47. Mazerbourg, S., Klein, C., Roh, J., Kaivo-Oja, N., Mottershead, D.G., Korchynskyi, O., Ritvos, O. and Hsueh, A.J. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Mol. Endocrinol*., **18**, 653-665(2004).

48. Bodensteiner, K., Clay, C., Moeller, C. and Sawyer, H. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. *Biology of Reproduction*, **60**, 381-386 (1999).

49. Pan, Z., Di, R., Tang, Q., Jin, H., Chu, M., Huang, D., He, J., Liu, Q., Hu, W. and Wang, X. Tissue-specific mRNA expression profiles of GDF9, BMP15, and BMPR1B genes in prolific and non-prolific goat breeds. *Czech. J. Anim. Sci*., **60**, 452-458 (2015).

50. Bodin, L., Di Pasquale, E., Fabre, S., Bontoux, M., Monget, P., Persani, L. and Mulsant, P. A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. *Endocrinology*, **148**, 393-400 (2007).

51.Galloway, S.M., McNatty, K.P., Cambridge, L.M., Laitinen, M.P., Juengel, J.L., Jokiranta, T.S., McLaren, R.J., Luiro, K. and Dodds, K.G., Montgomery, G.W. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics*, **25**, 279-283 (2000).

الكشف عن طفرات فى اكسون 2 لجين GDF9 والتحقيق من ارتباطها بالخصوبة في سلالتين من الماعز المصري

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الزرابي العالية الخصوبة والبركي القليلة الخصوبة هما من أكثر سلالات الماعز الأصلية شيوعًا في مصر. يمكن زيادة خصوبة قطعان الماعز من خلال اختيار الحيوانات التي تحتوي على الطفرات الفردية (SNPs) في الجينات المتعلقة بالخصوبة. قمنا باستخدام تسلسلات الحمض النووي تليها تفاعل البوليميراز الكمي (qPCR) وتحليل منحنى الانصهار عالي الدقة (HRM) للكشف عن SNPs في اكسون 2 من جين GDF9 المرتبط بالخصوبة ودراسة ارتباطها بالخصوبة في ماعز زرابي (n = 100) وبركي (n = 100) تم استخدام نتائج تسلسل الحمض النووي لعشرين عينة من كل قطيع كمرجع للاستنساخ الجيني لعينات الحمض النووي المتبقية باستخدام qPCR-HRM تم اكتشاف SNP p.V371M / c.1111G> A في GDF9 لماعز زرابي ، ومع ذلك ، كانت ماعز بركي أحادية التشكيل لهذا الموقع. كانت التراكيب الجينية لثلاثة من SNPs c.1111G> A مرتبطة ارتباطًا كبيرًا بحجم القطيع في ماعز زرابي. كان لدى التراكيب الجينية المتحورة المتشابهة (c.1111AA) أحجام قطعان أكبر بشكل ملحوظ من التراكيب الجينية الأخرى (P <0.05) استنادًا إلى هذه النتائج ، نوصي باختيار ماعز زرابية بالتراكيب الجينية c.1111AA لزيادة خصوبة هذه السلالة.

الكلمات المفتاحية: الماعز ، الخصوبة ، الطفرات الوراثية ، تحليل منحنى الانصهار