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ORIGINAL ARTICLE

Investigating mutations in the genes *GDF9* and *BMP15* in Pelibuey sheep through the amplification-refractory mutation system with tetra-primers

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ABSTRACT. Single Nucleotide Polymorphisms (SNP) or mutations are variations with a broad distribution in the genome and, as part of genetic studies, SNP allow the identification of allelic variants related to characteristics of economic importance in sheep production. However, the identification of SNP and their genotypes through sequencing is expensive, as it requires specialized materials and equipment. The objective of this study was to identify polymorphisms and their genotypes in the growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) genes in Pelibuey sheep using the tetra-primer amplification-refractory mutation system through polymerase chain reaction (T-ARMS-PCR). DNA extraction and amplification of *BMP15* and *GDF9* were conducted from blood samples contained in Whatman™ FTA™ cards from 60 multiparous Pelibuey ewes with reproductive records. The T-ARMS-PCR methodology allowed the identification of wild-type genotypes and mutated homozygous genotypes in polymorphisms *G4* and *G6* of *GDF9*, whereas mutations in the *BMP15* gene were not found. These results were confirmed by sequencing. In conclusion, the T-ARMS-PCR methodology allowed the identification of mutated and wild-type genotypes in SNP *G4* and *G6* of *GDF9*, although no mutations were found in *BMP15* in Pelibuey sheep. This technique was found to be reliable, rapid, and easily applied to identify polymorphic genotypes.

Keywords: gene, mutation, T-ARMS-PCR, genotyping, sequencing.

INTRODUCTION

Many studies have reported that the ovulation rate and litter size in sheep are regulated by genes that affect ovarian function. These are known as fertility genes (Davis, 2005; Fabre *et al*., 2006) and include the bone morphogenetic protein receptor (*BMPR-IB*), bone morphogenetic protein 15 (*BMP15*), and growth differentiation factor 9 (*GDF9*), which are intra-ovarian regulators of folliculogenesis (Ahlawat *et al*., 2014; Aboelhassan *et al*., 2021) expressed in follicle oocytes during development (Hanrahan *et al*., 2004; Strauss III & Williams, 2019). The *BMP15*, *BMPR-IB*, and *GDF9* genes belong to a large family of transforming growth factor β (TGFβ) and, with their mutations or Single Nucleotide Polymorphisms (SNP), heterozygous sheep generally increase their ovulation rate and prolificacy, whereas homozygous sheep present infertility and primary failures in folliculogenesis (Hanrahan *et al*., 2004; Nicol *et al*., 2009; Gootwine, 2020).

In *BMP15*, the mutations found in the gene coding region are *FecX*^{I} (Inverdale), *FecX*^{H} (Hanna) (Galloway *et al*., 2000), *FecX*^{B} (Belclare), *FecX*^{G} (Galway) (Hanrahan *et al*., 2004), *FecX*^{L} (Lacaune) (Bodin *et al*., 2007), *FecX*^{R} (Roa) (Martínez-Royo *et al*., 2008), *FecX*^{Gr} (Grivette), *FecX*^{O} (Olkuska) (Demars *et al*., 2013), and *FecX*^{Bar} (Tunisian Bárbara) (Lassoued *et al*., 2017). In most of these mutations, there was an average increase of 0.6 offspring per ewe compared to the wild genotype (Galloway *et al*., 2000; Hanrahan *et al*., 2004; Fabre *et al*., 2006; Bodin *et al*., 2007; Martinez-Royo *et al*., 2008). In contrast, 11 specific mutations have been reported in the gene *GDF9* in the coding region, but not all of these affect ovarian function (G2 and G3) (Hanrahan *et al*., 2004), and only nine affect ovulation rate and litter size: *FecG*^{H} (high fertility or *G8*) (Hanrahan *et al*., 2004), *FecG*^{T} (Thoka) (Nicol *et al*., 2009), *FecG*^{E} (Embrapa) (Silva *et al*., 2010), *FecG*^{1} (Baluchi or G1) (Moradband *et al*., 2011), *FecG*^{V} (Vecaria) (Souza *et al*., 2014), *FecG*^{F} (Finnish or G7) in white Norwegian sheep (Våge *et al*., 2013), *FecG*^{A} (Araucana or G5, *G6*) (Bravo *et al*., 2016), and *G4* (Dash *et al*., 2017).

Polymorphism is the main source of variation in the DNA of an organism and can be used as a molecular marker to identify allelic variants associated with economically important characteristics (Niciura *et al*., 2018). In the recognition of these allelic variants, some methodologies have been used to identify SNP and their genotypes in ewes. These methods

include DNA sequencing, restriction fragment length polymorphism through Polymerase Chain Reaction (PCR-RFLP), single-strand conformation polymorphism (SSCP), hybridization with TaqMan fluorescence probes, and DNA Microarrays, among others (Ahlawat *et al*., 2014; Niciura *et al*., 2018). The disadvantage of these methodologies is the high cost of reagents as well as the requirement for specialized materials and equipment for sample processing (Niciura *et al*., 2018). In this context, Newton *et al*. (1989) developed an Amplification-Refractory Mutation System (ARMS-PCR). This, together with the tetra-primer procedure (Ye *et al*., 2001), was found to be an economical, reliable, and easy-to-use methodology for identifying mutations in genotypes (Nicol *et al*., 2009; Polley *et al*., 2010).

In the Tetra-primer Amplification Refractory Mutation System through Polymerase Chain Reaction (T-ARMS-PCR), a fragment of the gene of interest and each internal primer are combined with an opposing external primer in a single reaction to amplify the wild-type, mutated, and control fragment forms in a single PCR reaction (Polley *et al*., 2010). Thus, the objective of this study was to identify polymorphisms in *GDF9* and *BMP15* in Pelibuey ewes by using T-ARMS-PCR.

MATERIALS AND METHODS

Collection and conservation of samples

Sterile syringes were used to collect three mL of blood from the jugular veins of sixty multiparous ewes of the Pelibuey breed, from the agriculture and livestock production unit "El Gargaleote", property of Universidad Autónoma Chapingo; under the criteria of the Mexican Official Norm (*Norma Oficial Mexicana*, NOM-062-ZOO-1999) [SAGARPA, 2001] on technical specifications for the production, care, and use of laboratory animals, and following the regulations for the use and care of research animals, as approved by the General Academic Council of Colegio de Postgraduados, Mexico (COLPOS, 2016). From the collected blood, a 0.5 mL subsample was placed in a Whatman™ FTA™ mini card (WB 120055, GE Healthcare©, United Kingdom). The cards were dried in the shade according to the manufacturer´s instructions (GE Healthcare©, United Kingdom) until subsequent processing. The laboratory phase was conducted in the facilities of the Molecular Biology Laboratory of common use in the Postgraduate Program in Plant Health, at Campus Montecillo, Colegio de Postgraduados, Texcoco, State of Mexico.

Sample processing

For DNA extraction, sections of approximately one mm^2 were cut from the Whatman FTA mini card impregnated with blood and deposited in an Eppendorf tube of 0.2 mL. They were then washed three times with 200 µL of FTA purification reagent (GE Healthcare©, United Kingdom) at room temperature, for seven minutes each, and the purification reagent was discarded after each wash. Two further washes were then carried out with 200 µL of Tris (hydroxymethyl aminomethane)-EDTA (ethylenediaminetetraacetic acid) buffer or TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) at room temperature for seven min, and the TE buffer was discarded after each wash, according to the manufacturer's instructions and the methodology followed by Muñoz-García *et al*. (2021). On average, the ratio of the absorbance of DNA at 260 and 280 nm was 1.8 with a concentration of 452.98 ng μL^{-1}.

Polymorphisms and genotypes in the *BMP15* and *GDF9* genes by T-ARMS-PCR

This study used the T-ARMS-PCR methodology described by Ye *et al*. (2001) for the detection of polymorphisms and their genotypes in the *BMP15* and *GDF9* genes of Pelibuey sheep. Fragments of these genes were amplified by PCR, using the sequences listed in Table 1, as reported by Polley *et al*. (2010). The reaction mixture for the T-ARMS-PCR of the genes was produced separately; each reaction mixture had a final volume of 13.75 µL, containing 6.25 µL of GoTaq® Colorless Master Mix 2X (Promega© Madison, WI, USA), 0.625 µL of each primer (two Forward and two Reverse; IDT®, Illinois, USA) at a concentration of 10 µM and five µL of trehalose dihydrate at 10% (Merck© KGaA 64271, Darmstadt, Germany). The tubes containing the reaction mixture and fragment of the processed FTA mini card were introduced into a Biometra® thermal cycler.

The amplification conditions programmed for each allele (wild-type and mutated) are detailed below. For exon 2 of the gene *GDF9*: initial denaturation was performed at 93 °C for three min; 35 cycles of denaturation at 93 °C for 40 s; alignment, Table 1, 40 s; extension, 72 °C, 40 s; and a final extension at 72 °C for five min. For exon 2 of *BMP15*, initial denaturation was performed at 95 °C for five min; 35 cycles of denaturation at 95 °C for 40 s; alignment, Table 1, 40 s; extension, 72 °C, 30 s; and a final extension at 72 °C for four min. The amplicons of the *GDF9* and *BMP15* genes were separated by gel electrophoresis on a 2.5% agarose gel in Tris-borate-EDTA or TBE running buffer at 1% (IBI Scientific, Dubuque, Iowa, USA) at 80 volts for 80 min in each case. A 100-1000 bp molecular marker was used (Promega©, Madison, Wisconsin, USA). The gel was stained with Gel Red® (Biotium, Hayward, California, USA), which was then added to the agarose gel. Three µL of amplicon was placed in each well, plus three µL of Green GoTaq® Flexi buffer (Promega©, Madison, Wisconsin, USA) was added. The gel was visualized in a Quantum® photo-documenter using the Vision Capt ® software.

Polymorphisms and genotypes in the *GDF9* and *BMP15* genes by PCR and sequencing

To confirm the mutations and genotypes found in the *BMP15* and *GDF9* genes using the T-ARMS-PCR technique, amplification of exon 2 of *GDF9* and *BMP15* was conducted through a final point PCR for subsequent sequencing. The reaction contained GoTaq® Colorless Master Mix

Table 1. Sequences used to amplify and identify the polymorphisms and genotypes of genes *GDF9* and *BMP15* in Pelibuey ewes using the T-ARMS-PCR methodology.

Gene	Mutation	Primer	Sequence 5´→3´	Product size (bp)
GDF9	*G4*	IF(G)	296 TTCACATGTCTGTAAATTTTACATGTGAGG 325	M= 212, W= 261, C= 417, TA= 50 °C"
		IR(A)	350 GCTGAAGATGCTGCAGCTGGTCGTT 325	
		OF	90 CAACAACTCCATTTCTTTTCCCTTTCCTG 118	
		OR	506 TAGGCAGATAGCCCTCTC TTCTGGTCAG 479	
	G6	IF(A)	573 CAGCTCTGAATTGAAGAAGCCTCGGA 598	M= 193, W= 223, C= 362, TA= 52 °C"
		IR(G)	625 ATTCACTCAGATTGACTGAAGCTGGCAC 598	
		OF	403 TATCTGAACGACACAAGTGCTCAGGCTT 430	
		OR	764 CTGGGACAGTCCCCTTTACAGTATCGAG 737	
	G8	IF(T)	763 AGGGCGGTCGGACATCGGTATGGATT 788	M= 146, W= 108, C= 198, TA= 55 °C"
		IR(C)	817 TGATGTTCTGCACCATGGTGTGAACCGTAG 788	
		OF	710 GGATTGTGGCCCCACACAAATACAACCC 737	
		OR	907 CATCAGGCTCGATGGCCAAAACACTCAA 880	
BMP15	*Fec X^{G}*	IF (C)	363 CTTCTTGTTACTGTATTTCAATGACAATC 391	M= 112, W= 102, C= 158, TA= 48 °C"
		IR (T)	417 GAGAGGTTTGGTCTTCTGAACACTATA 391	
		OF	306 AAGAGGTAGTGAGGTTCTTGAGTTCT 331	
		OR	463 AGAGAAGAGAAGGGTCTTTTTCTGTA 438	
	Fec X^{L}	IF (A)	606 TGCTCCCCATCTCTATACCCCAAACTAATA 635	M= 204, W= 252, C= 398, TA= 52 °C"
		IR (G)	662 TGTAGTACCCGAGGACATACTCCCTGAC 635	
		OF	411 ACCTCTCCCTAAAGGCCTGAAAGAGTTT 438	
		OR	808 ACAAGATACTCCCATTTGCCTCAATCAG 781	

[Nucleotide specificity is indicated in parentheses. F= forward, R= Reverse, O= external (common), I= internal (specific allele), M= mutant, W= wild, C= control fragment and TA= temperature of alignment (Polley *et al*., 2010)].

2X (Promega© Madison, Wisconsin, USA), each primer (*BMP15*, exon 1: For-5'-AATCCTTCTTTGGGGACTGG-3' and Rev-5'-AGCCCTAAAGGGAAGCAAAT-3', and exon 2: For-5'- CCAGAAAAGCCCAACCAATC-3' and Rev-5'-AGTGTAGTACCCGAGGACAT-3', AH009593.2, (Galloway *et al*., 2000); *GDF9*; exon 2: For-5'-GGAGAAAAGGGACAGAAGC-3' and Rev- 5'-ACGACAGGTACACTTAGT-3', (Silva *et al*., 2010); 10 µM; IDT®, Illinois, USA), and trehalose dihydrate at 10% (Merck© KGaA 64271, Darmstadt, Germany). The tubes containing the fragments of the processed card and reaction mixture were placed in a Biometra® thermal cycler. The amplification conditions were as follows: *BMP15* (exon 1): initial denaturation at 95 °C for five min; 35 cycles of denaturation at 95 °C for 30 s, alignment at 54 °C for 40 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for five min; (exon 2): initial denaturation at 94 °C for five min; 35 cycles of denaturation at 94 °C for 30 s, alignment at 54.8 °C for 40 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for four min. *GDF9* (exon 2): initial denaturation at 93 °C for three min; 35 cycles of denaturation at 93 °C for 40 s, alignment at 56 °C for 40 s, and extension at 72 °C for 40 s; and a final extension at 72 °C for five min. The amplicons were separated by electrophoresis on a 1% agarose gel in Tris-borate-EDTA running buffer at 1% (TBE; IBI Scientific, Dubuque, Iowa, USA) at 80 volts for 40 min. A molecular marker of 250-10000 bp was used (Promega©, Madison, Wisconsin, USA). The gel was stained with Gel Red® (Bio-

tium, Hayward, California, USA), which was then added to the agarose gel. Three µL of the amplicon plus three µL of Green GoTaq® Flexi buffer (Promega©, Madison, Wisconsin, USA) were added to each well. The gel was visualized using a Quantum® photo-documenter with the Vision Capt® software. Finally, once the appearance of the genes in the agarose gel was confirmed, the amplicons obtained (10 µL) were sent to MACROGEN© (Seoul, South Korea) for cleaning and sequencing. The primers were the same as those used for the amplification of each gene.

Bioinformatic analysis of sequences

The quality of the sequences was analyzed using the software Sequencher® version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA, http://www.genecodes.com). From the sequences of each gene, a search was performed using Nucleotide BLAST of the National Center for Biotechnology Information, and those that presented the greatest similarity with those of this study were selected: for the *BMP15* gene, AH009593.2 and KT238844.1, whereas for *GDF9*, AF078545.2, and HE866499.1. From the sequences of each exon, together with the reference sequences, alignment was conducted to determine the degree of similarity and to detect mutations with their genotypes, using the MUSCLE® algorithm (Edgar, 2004) in MEGA 7® (Molecular Evolutionary Genetics Analysis, Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Polymorphisms and genotypes in the *GDF9* and *BMP15* genes revealed by T-ARMS-PCR

In this study, the T-ARMS-PCR methodology allowed the identification of two specific mutations known in exon 2 of the gene *GDF9*, since fragments of 261 bp (in five out of 60 sheep) and 212 bp (in 57 out of 60 sheep) were identified for the polymorphism *G4*, and 223 bp (in 30 out of 60 sheep) and 193 bp (in 30 out of 60 sheep) for *G6*, respectively (Figure 1). In addition, none of the sheep presented a heterozygous genotype for these two variants. Polymorphism *G8* was not detected in the present study. On the other hand, in gene *BMP15*, only the wild forms of *FecX*^{G} and *FecX*^{L} polymorphisms were detected when amplifying fragments of 102 and 252 bp, respectively (Figure 1).

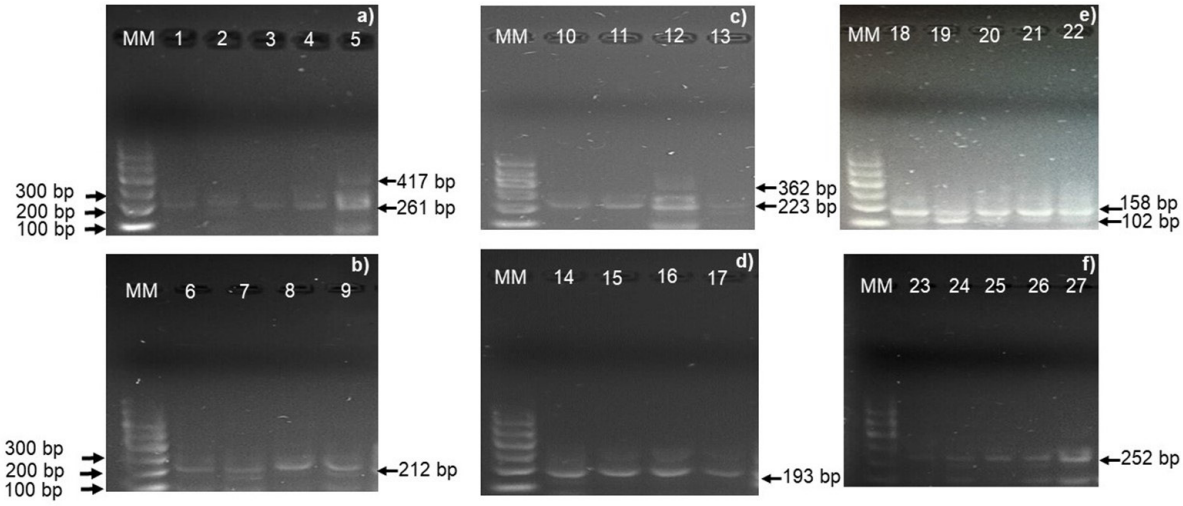

Figure 1. Polymorphisms and genotypes in the genes *GDF9* and *BMP15* identified by T-ARMS-PCR in Pelibuey ewes (MM= molecular marker). Polymorphism G4 with the genotypes a) wild (lanes 1 to 5: ewes with a fragment of 261 bp and control 417 bp) and b) mutated (lanes 6 to 9: ewes with a fragment of 212 bp). Polymorphism G6 with the genotypes c) wild (lanes 10 to 13: ewes with a fragment of 223 bp and control 362 bp) and d) mutated (lanes 14 to 17: ewes with a fragment of 193 bp). Polymorphism *FecX*^{G} in the wild form e) (lanes 18 to 22: ewes with a fragment of 102 bp, control 158 bp), and *FecX*^{L} in the wild form f) (lanes 23 to 27: ewes with a fragment of 252 bp).

Polymorphisms and genotypes in the *GDF9* and *BMP15* genes identified by sequencing

The sequences in this study had a similarity of 99.32 - 99.77% for *BMP15* and 99.67 - 99.45% for *GDF9*, with the sequences AH009593.2 and KT238844.1 for *BMP15*, and AF078545.2, and HE866499.1 for *GDF9*, respectively. Alignment of the sequences of gene *BMP15* from this study with those of the reference (AH009593.2, KT238844.1) did not reveal any change or mutation. The sequences from this study aligned with those of the reference (AF078545.2, HE866499.1) allowed the identification of SNP *G4* and *G6* in exon 2 of *GDF9*. In *G4*, guanine was substituted with adenine at position 721 (721 G→A) while, in *G6*, guanine was replaced with adenine at position 994 (994 G→A) (Figure

2). Likewise, this analysis confirmed the identification of wild-type and mutated genotypes in Pelibuey ewes. It is important to mention that ewes were not found to have the heterozygous genotype in polymorphisms *G4* and *G6* (Figure 2), nor was the mutation *G8* found using this methodology.

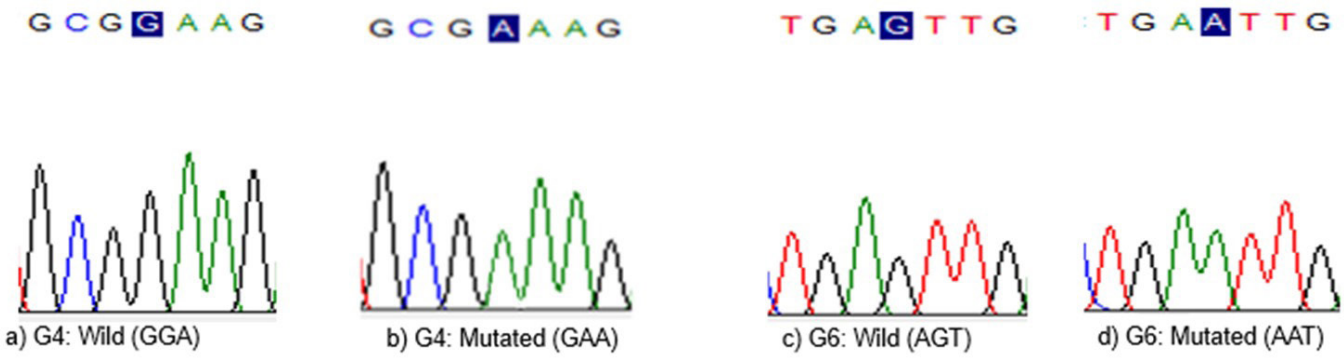

Figure 2. Electropherograms of SNP *G4* and *G6* obtained by sequencing exon 2 of the *GDF9* gene in Pelibuey ewes. Polymorphisms *G4* with a) wild and b) mutated genotypes, and *G6* with c) wild and d) mutated genotypes.

In polymorphism *G4*, mutant and wild alleles were detected with allelic frequencies of 0.08 and 0.92 while, in *G6*, wild and mutant alleles with allelic frequencies of 0.5, and 0.5, respectively, were detected.

SNP are changes or mutations in a nitrogenous base in the DNA sequence that can be used as molecular markers (Ahlawat *et al*., 2014). Currently, SNP are used to identify allelic variants and their genotypes (Ahlawat *et al*., 2014; Niciura *et al*., 2018). In this study, T-ARMS-PCR allowed the identification of polymorphism *G4*, and in the genotyping of the SNP, 5% of the sheep presented the mutant genotype and 95% of the wild-type genotype. Likewise, polymorphism *G6* was found in the genotype of the SNP, where 50% of the ewes had the wild-type genotype and the other 50% had the mutated or carrier genotype. The results obtained using the T-ARMS-PCR methodology in this study are in agreement with those reported by Polley *et al*. (2010), Roy *et al*. (2011), Dash *et al*. (2017), and Aboelhassan *et al*. (2021). The results of this study suggest that Pelibuey ewe is polymorphic in the specific *G4* and *G6* mutations of *GDF9*. It is important to mention that the polymorphism *G8* was not found in sheep in this study, and the absence of this allelic variant suggests that it was not present in ewes.

On the other hand, polymorphisms were not found in the gene *BMP15* using the T-ARMS-PCR methodology; only the wild variants of the SNP *FecX*^{G} and *FecX*^{L} were amplified in Pelibuey ewes. The results of this study support those reported by Polley *et al*. (2010) for Garole sheep, Roy *et al*. (2011) for Bonpala sheep, and Aboelhassan *et al*. (2021) for five Egyptian sheep breeds. However, they differ from those reported by Argüello-Hernández *et al*. (2014), who used the same methodology in sheep of Pelibuey breed. The lack of mutations in the gene *BMP15* in sheep in this study was perhaps because this gene is monomorphic and the wild allele is probably the one that predominates in these sheep (Salazar-Montes *et al*., 2013).

Sequence analysis of *GDF9* and *BMP15* showed an average 99.5% similarity with the sheep sequences reported in NCBI GenBank, suggesting that the sequences obtained in this study are broadly equal to those of other sheep. When comparing the sequences from exon 2 of *GDF9* obtained in this study with the reference sequences (accession AF078545.2 and HE866499.1), polymorphisms *G4* and *G6* were found. In the genotyping of these SNP, only the wild-type and mutated (homozygous) forms were found for each variant. In this study, the *G4* and *G6* polymorphisms identified through sequencing agreed with those reported in sheep of the Belclare, Cambridge, Mehraban (Hanrahan *et al*., 2004; Ahmadi *et al*., 2016), and Barki, Osseimi, Rahmani, Saudanez, and Awassi (Aboelhassan *et al*., 2021) breeds. Mutations were not found in the two codifying regions of *BMP15* (exons 1 and 2), according to the sequence analysis of this study compared to those of the reference (accession AH009593.2 and KT238844.1).

It should be noted that in both the T-ARMS-PCR methodology and sequencing, sheep were not found to have the heterozygous genotype in *GDF9*. The absence of heterosis in polymorphisms *G4* and *G6* in Pelibuey sheep indicates low genetic variation, which could be due to the Wahlund effect, which refers to a sub-population structure, mixture of populations, or possible endogamy, or may also be due to reduced flock (Kumar *et al*., 2007). The results obtained by the T-ARMS-PCR methodology in the search for and genotyping of polymorphisms were confirmed and validated with the results of sequencing DNA samples obtained from the same ewes of the Pelibuey breed. The results obtained by T-ARMS-PCR did not differ from those obtained by sequencing *BMP15* and *GDF9*. Finally, T-ARMS-PCR is a simple, fast, and easy methodology used to search

for SNP and genotypes in sheep, according to Polley *et al*. (2010), Niciura *et al*. (2018), and Aboelhassan *et al*. (2021). T-ARMS-PCR methodology and sequencing both allowed the identification of SNP and grouping of ewes of the Pelibuey breed according to their genotype.

CONCLUSION

The T-ARMS-PCR methodology allowed the identification of polymorphisms *G4* and *G6* and their wild-type and mutated genotypes in *GDF9*; however, sheep with the heterozygous genotype were not found, and no polymorphisms were found in the gene *BMP15*. This methodology was found to be rapid, reliable, and easy to apply for identifying SNP of interest.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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