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Caprine Myostatin Gene Polymorphism in Domestic and Wild Goat Breeds in Duhok Province/Kurdistan Region of Iraq Using PCR-RFLP and SNP Markers

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ABSTRACT

In Iraq generally and Kurdistan region specifically, goats are an important resource for meat and milk production. It is well known that Myostatin genes (*MSTN*) have the essential role in growth and development due to its crucial involvement in muscle growth. The goal of this research was to reveal the polymorphism of *MSTN* gene in different *Capra hircus* breeds (native, Shami, Meriz and Kamori goat) and in wild mountain goat (*Capra aegagrus)* via PCR-RFLP technique and direct seqencing The *MSTN* (exon 1) polymorphisms were investigated in all breeds, *MSTN-Dra* I/RFLP revealed three genotypes AA and BB were homozygous and AB was heterozygous. The calculated genotype frequencies were 0.270, 0.368 and 0.362 for AA, BB and AB genotype respectively. The allelic frequency was 0.451 for the A allele and 0.549 for the B allele. The average of the observed heterozygosity was 0.362 and the observed homozygosity was 0.638. The sequence data of *MSTN* gene of Native and Shami goats revealed 31 SNPs in nonfunctional region. In addition, in exon region there was 1 SNPs in both breed samples which did not change amino acid sequence and its silence mutation.

In conclusion, the result of PCR-RFLP and SNP experiments obtained in this investigation were evaluated as very useful in genotype analysis of local goat breeds/populations. Thus, DNA polymorphisms in the Myostatin gene could be used as reliable genetic marker in breeding programs in this region.

Keywords: *Capra aegagrus, Capra hircus*, *MSTN* gene, PCR-RFLP, sequencing, SNP.

1-INTRODUCTION

In Iraq, including Kurdistan Region, the domestic goat (*Capra hircus*) is a popular livestock species primarily raised for meat and milk purposes (Scherf, 2000). Therefore, combining trials with emphasis on management and genetic progress such as employing reliable markers may be of great importance to improve animal production (Masoudzadeh *et al*., 2020). The *myostatin* (MSTN) gene has been regarded as a significant candidate gene for domestic animal development and growth due to its crucial involvement in muscle building and its potential utility in animal rearing. According to Schibler *et al.* 1998 and Pinton *et al.* 2000, this gene, which has two introns and three exons, was physically localized to goat chromosome 2q11-q12 (Dominique and Gérard 2006).

Various approaches have been developed to evaluate polymorphism or genetic variation within and between populations (Okumus & Mercan, 2007; Hussain *et al*., 2013; Mahrous *et al*., 2018). Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) is one of most widely utilized procedures. The direct sequence is an effective approach for detecting nucleotide differences in amplified DNA fragments (Akamine *et al*., 2009.). For many plants and animals, single nucleotide polymorphism (SNP) screening has also been the method of choice for identifying and correlating characteristics with sections of the genome (Rafalski, 2002).

The main research study reported in Iraq was related to the two domestic goat breeds (native and Meriz), which were morphologically characterized (Taha 1990; Alkass and Merkhan 2013). At molecular level, Mohammed (2013) reported the use of SSR marker for characterisation of the Meriz and native goat breeds in Duhok province also Abbas and her colleagues (2022) reported the use of PCR-RFLP of growth hormone gene in domestic and wild goat breeds in Duhok province.

To the best of our knowledge, PCR-RFLP and SNPs on MSTN gene in goat breeds in Kurdistan region of Iraq have not been reported. Thus, as a first step towards goat genetic improvement in Kurdistan Region – Iraq, based on the myostatin gene, the goal of this study was to look for genetic polymorphisms in the caprine *MSTN* gene in some domestic goat (*Capra hircus*) and wild goat (*Capra hircus*) breeds in Duhok province.

2-Materials and Methods

2.1. DNA extraction

Blood (3ml) was collected from 71 female goats from different herds in Duhok province (27 Meriz, 26 native, 10 Shami, 5 wild and 3 Kamori goats) in 2.7% EDTA tubes as an anticoagulant and kept at 4 °C until used. Blood genomic DNA was extracted using the phenol-chloroform method (Powell & Gannon, 2002). A Nanodrop spectrophotometer was used to determine the purity and concentration of genomic DNA.

2.2. Polymerase chain reaction:

For amplifying the exon one of goat *MSTN* gene, the unique primer (F. TGGCGTTACTCAAAAGCAAA and R.AACAGCAGTCAGCAGAGTCG) was used which given by Li *et al.,*(2008)..

The master mix reaction contained 1 µL (100 ng) genomic DNA, 1 µL (10 pmol/µL) of each forward and reverse primer, 10 µL of 2×PCR master mix (ADDBIO INC) and 7 µL of deionized distilled water for 20 µL volume. The PCR programmer included 95 °C for an initial denaturation for 5 min, followed by 35 cycles of 94 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 1 min, followed by a final extension at 72°C for 5 min and storage at 4 °C. The PCR products were analyzed using 1.5% agarose gel electrophoresis.

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Red safe stain was used to stain the gels, which were then viewed using a UV trans-illuminator.

2.3. Restriction fragment length polymorphism (RFLP)

The reaction mixture was carried out in a total volume of 25 µL of each sample, which contained 10 µL of PCR amplicons and 10 units of the *Dra*I restriction enzyme (Gena Bioscience) was used for digestion of PCR product then incubated at 37 °C for 6 h. The digested amplicon fragments were separated by 2.5% agarose gel electrophoresis; 100 bp ladder DNA was run with digested PCR products for measuring of the bands. Gels were stained with Red safe stain, then visualized with a UV trans-illuminator and photographed. Data for each locus were analyzed by PopGene program version 1.31 (Yeh *et al*., 1999).

2.4. MSTN gene sequencing

The PCR result products, for each identified genotype of *MSTN* gene in Native and Shami goat breeds were sequenced by Macrogen (Seoul, Korea). To find each single nucleotide substitution between distinct genotypes, the NCBI/BLAST/blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and Clastel W were used to perform sequence analysis and alignment

3. RESULT AND DISCUSSION

The results of amplified PCR products of the specific primer of Caprine Myostatin (*MSTN*) gene revealed a band of 497 bp in the all individual samples of Shami, Meriz, Wild, Kamori and Native goats. This amplified bands represented exon 1 of *MSTN* gene using primer reported by Li *et al.,*(2008) in twenty-seven goat population in China are shown in Figure (1) .

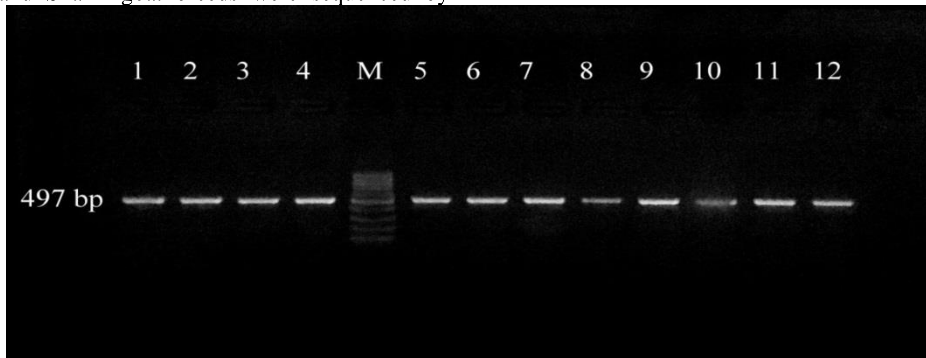

Figure 1: 1.5% Agarose gel electrophoresis, lane M: 100 bp DNA marker, lanes 1 – 12 represent the 497 bp PCR product of *MSTN* gene

The PCR-RFLP results of the *MSTN* gene digested with restriction enzyme *Dra* I produced two different alleles (A and B). The allele A, 497 bp fragments was uncut due to the lack of a restriction site for this enzyme. Allele B, on the other hand, was cleaved into two fragments; one fragment was of 427 bp and the other of 70 bp. In this study, the analysis of 497 bp *MSTN* fragment after digestion revealed polymorphisms with three genotype, AA genotype (497 bp undigested) as homozygous, AB (497, 427 and 70 bp) as heterozygous genotype and BB genotype with only two bands (427 and 70 bp) as homozygous (Figures 2).

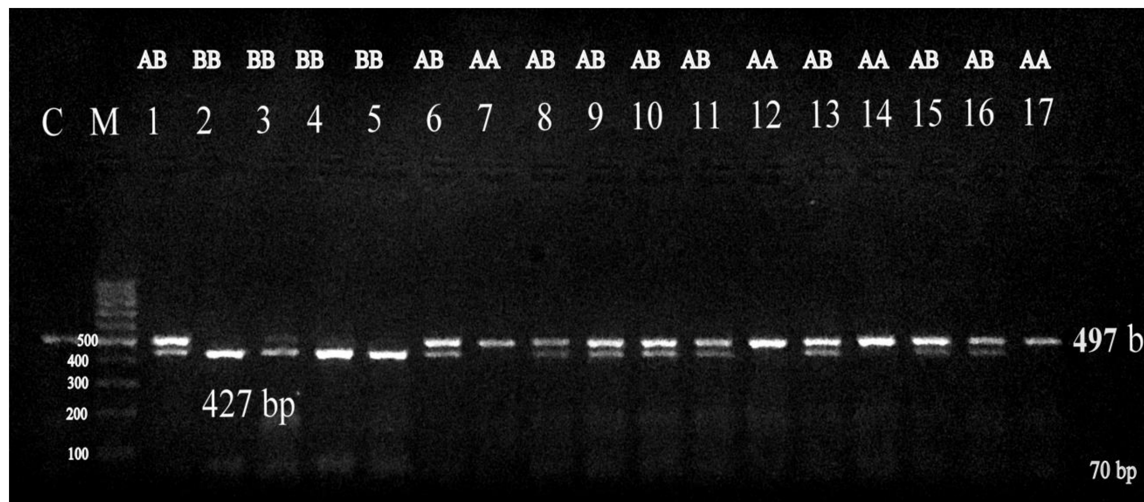

Figure 2: Represent 2% agarose gel electrophoresis of PCR-RFLP patterns of *MSTN* using *DraI*, C: represent undigested amplified PCR product as control. M: represent 100 bp DNA marker, lane7,12,14 and 17 Were AA genotype, Lane 2,3,4 and 5 were BB and the other lane were AB genotype.

Genotype AA was observed due to the presence of a deletion at TTTTA sequence, with the fragment of 497 bp. Genotype BB, containing a fragment of 427 and 70 bp, was established when TTTTA was not deleted. When fragments 70, 427, and 497 bp happened together, the genotype AB was established (Li *et al.,* 2008). According to Li and his colleagues' (2008) findings, only the goat carried this form of loss, and the TTTTA was conserved across species. The TTTTA deletion might only exist in goats (Li *et al.,* 2008).

In this study, the data analysis of allele and genotype frequencies is detailed in Table (1). The genotype frequencies of AB in Shami and Native goats were 0.5 which may be attributed to that half individual carried AB genotype, whereas in the Meriz, Kamori and Wild genotypes, the frequencies represented 0.408, 0.00 and 0.4 respectively. On the other hand, the AA

genotype frequency in Shami, Kamori, Wild, Meriz and Native goats were 0.4, 0.00, 0.2, 0.481 and 0.269 respectively. The BB genotype of the Kamori goats had the highest frequency which was 1.00 and the Shami goats has the lowest frequency 0.1 as well as the BB genotype frequency in Wild (0.4), Meriz (0.111) and Native goats (0.231) were among them.

Table 1: Genotype and allele frequency of *MSTN* gene in five goat breads

Population	Individual Number	Observed AA genotype	Observed AB genotype	Observed BB genotype	Genotype frequency			Allele frequency	
					AA	AB	BB	A	B
Shami	10	4	5	1	0.4	0.5	0.1	0.65	0.35
Kamori	3	0	0	3	0.00	0.00	1	0.00	1
Wild	5	1	2	2	0.2	0.4	0.4	0.4	0.6
Meriz	27	13	11	3	0.481	0.408	0.111	0.685	0.315
Native	26	7	13	6	0.269	0.5	0.231	0.519	0.481
Average		0.352	0.437	0.211	0.270	0.362	0.368	0.451	0.549

In all breeds, genotype AB (0.362) was found to be more common than genotype AA (0.270) and little bit less than genotype BB (0.368). These results were in agreement with Alakilli *et al*. (2012), studying Ardi, Barki, Zaribi, and Masri breeds in Saudi and Egypt. However, these findings contrasted from those published in China by Li *et al* (2008) in 27 goats taken from eleven provinces and autonomous regions in China who reported that AA genotype have the highest frequency than AB and BB.

The average of allelic frequencies in this study revealed that the allelic frequency of allele A (0.451) was lower than allele B (0.549). The highest allelic frequency was recorded in allele A (0.685) in Meriz and the lowest was in the Kamori with 0.00. In the case of allele B, Kamori had the highest frequency (1.00) whereas, the lowest one was in Meriz with 0.315 (Table 1).

Similar results were found in, Zaribi, Barki, Masri and Ardi breeds in Egypt and Saudi by Alakilli *et al*. (2012), These frequencies, however, contrasted from those according to Zhang *et al* (2012) in Boer , Matou , Haimen and Nubi breeds, and Li *et al* (2008) in China who reported that allelic frequency of A allele was higher than B allele .

The degree of heterozygosity found in all goats from these studies was 0.362 for observed heterozygosity and 0.391 for expected heterozygosity. The highest observed heterozygosity was found in Shami and Native goat (0.500) and the lowest was in Kamori (0.00). The average of observed homozygosity (Ho^O) in all breads was 0.638 and expected homozygosity (Ho^E) was 0.609, the highest observed homozygosity was found in Kamori (1.00) and the lowest one was found in both Shami and Native goat (0.500) in Table (2).

Table 2 : Availability, Observed and Expected Homozygosity and Heterozygosity of *MSTN* in 5 goat breeds

Population	A	Ho^O	He^O	He^E	Ho^E
Shami	1.000	0.500	0.500	0.479	0.521
Kamori	1.000	1.000	0.000	0.000	1.000
Wild goat	1.000	0.600	0.400	0.5333	0.4667
Meriz	1.000	0.5926	0.4074	0.4396	0.5604
Native goat	1.000	0.500	0.500	0.5030	0.4970
Average		0.638	0.362	0.391	0.609

In this study the observed heterozygosity (He^O) value was a little bit lower than expected heterozygosity (He^E) which indicates that there were inbreeding within the populations (Coulson *et al.*,1998). This finding may indicate that there is a consistent tendency toward heterozygote deficit as well as the occurrence of inbreeding among the populations because the average observed homozygosity value was greater than the observed heterozygosity (Coulson *et al*.,1998).

In this investigation, the three goats (one Native and two Shami) which had different genotype were prepared for sequencing. Although this data represents a partial sequence of *MSTN* gene (exon 1) whose coding unit is marked in black colour and noncoding unit marked in red colour. The sequence data obtained from the PCR products of these three goats further confirmed the three distinctive genotypes, Native goat (accession number: OP320835) with BB (227 and 70 bp) genotype, Shami1 (accession number: OP346537) with AA (497 bp) and Shami2 (accession number: OP346538) with AB (497, 427 and 70 bp) genotypes that were characterized earlier by PCR-RFLP results obtained in this study. This was evident by the preservation of *DraI* restriction site (TTTAAA in yellow colour) in the sequence of allele B as shown in the sequence data in Figure (3). Thus, it proves that allele B was not changed which was cleaved in to two fragments 427 and 70 bp.

Figure 3: Represent the *MSTN* nucleotide sequence and chromatogram of genotype BB of Native goat showing the intact *Dra*I restriction site in allele B.

Whereas, in allele A the *Dra*I restriction site was changed due to point mutation (SNP) which led to change TTTAAA restriction site to TTGAAA. This mutation led to removal of this enzyme site, leaving the 497 bp band uncleaned (Figures 4 and 5).

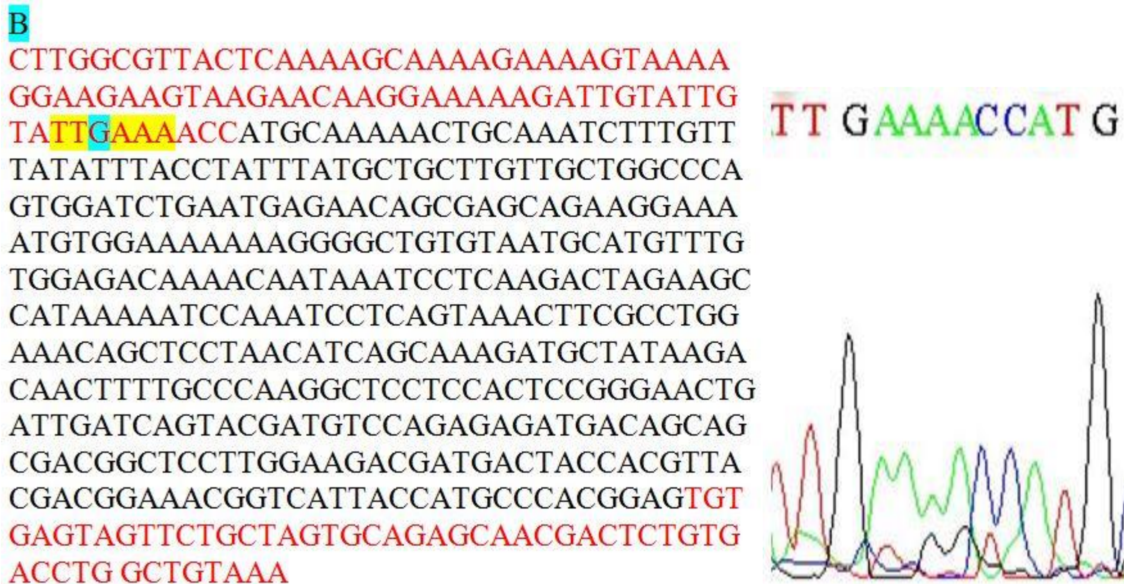

Figure 4: Represent the *MSTN* nucleotide sequence and chromatogram of genotype AA of Shami goat showing the point mutation of *Dra*I restriction site in allele A.

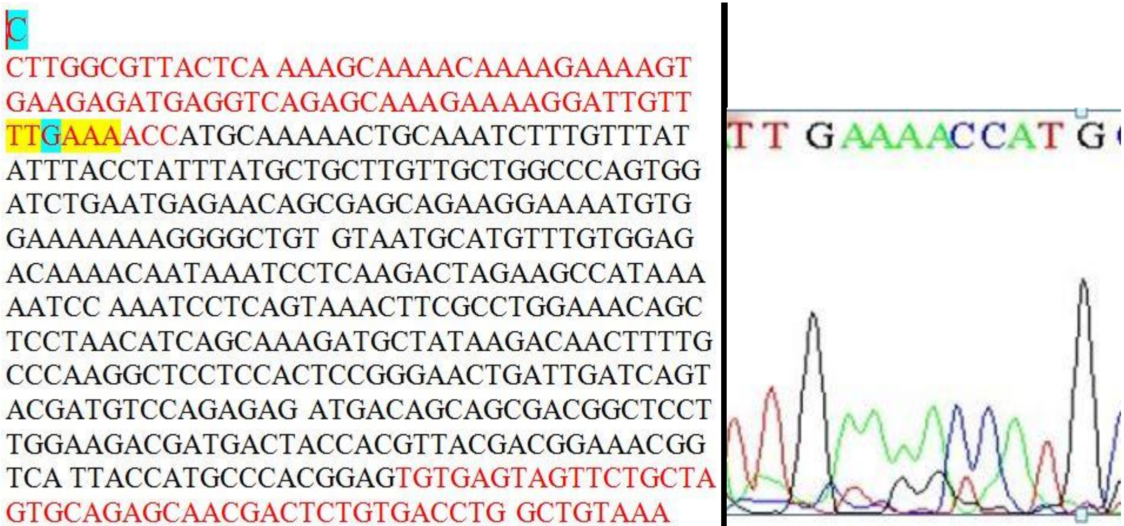

Figure 5: Represent the *MSTN* nucleotide sequence and chromatogram of genotype AB of Shami goat showing the point mutation of *Dra*I restriction site in allele A.

The sequences of three goats sample (one Native and two Shami) of *MSTN* gene were aligned with reference sequence EF591039.1 that is whole myostatin gene of Capra hircus, by direct submission in gene bank, showed different SNPs in noncoding region that showed 18 trans-versions and 12 transitions in all goat sample.

Although the coding region of exon one of *MSTN* gene had one point mutation (T to A) at locus 197, but this mutation was silent in which the amino acid alanine did not change as shown in Figure (6). Our result shows a high degree of conservation in amino acid chain in both Shami and Native goat myostatin gene.

Met, Gln, Lys, Leu, Gln, Ile, Phe, Val, Tyr, Ile, Tyr, Leu, Phe, Met, Leu, Leu, Val, Ala, Gly, Pro, Val, Asp, Leu, Asn, Glu, Asn, Ser, Glu, Glu, Lys, Glu, Asn, Val, Glu, Lys, Lys, Gly, Leu, Cys, Asn, **Ala,** Cys, Leu, Trp, Arg, Gln, Asn, Asn, Lys, Ser, Ser, Arg, Leu, Glu, Ala, Ile, Lys, Ile, Gln, Ile, Leu, Ser, Lys, Leu, Arg, Leu, Glu, Thr, Ala, Pro, Asn, Ile, Ser, Lys, Asp, Ala, Ile, Arg, Gln, Leu, Leu, Pro, Lys, Ala, Pro, Pro, Leu, Arg, Glu, Leu, Ile, Asp, Gln, Tyr, Asp, Val, Gln, Arg, Asp, Asp, Ser, Ser, Asp, Gly, Ser, Leu, Glu, Asp, Asp, Asp, Tyr, His, Val, Thr, Thr, Glu, Thr, Val, Ile, Thr, Met, Pro, Thr, Glu

Figure 6: Represent the amino acid sequence of *MSTN* gene showed the silent mutation that Alanine not changed to another amino acid in all three samples.

The variations in the *MSTN* gene have a considerable impact on growth traits, and genotypes have a significant impact on the majority of attributes. The bigger birth weights and heavier body weights were linked to the AB or CD heterozygous genotypes. Additionally, the heterozygous genotype showed greater effects on superior growth performance when combined than the other genotypes (Zhang et al. 2012).

The strong impact of the 5 bp indel (TTTTA) on goat sizes and early body weights was also documented by Li *et al.* (2008). They found that homozygote animals outperform heterozygotes in terms of growth. This variation could be the result of several breed- and sample-size-related effects. A mixed population with 26 different goat breeds was used for the association study in Li *et al.* (2008).

In view of its crucial function in potential applications in goat breeding and muscle growth, the myostatine gene has been recognized as a significant candidate gene for development and growth of domestic animals (Supakorn, 2009; Zhang et al., 2012). Mutations in the MSTN gene can suppress its expression or result in a non-functional protein, which results in undesirable muscularity (i.e., a sharp increase in both the number of muscle fibers (hyperplasia) and mass (hypertrophy), or the phenomenon known as "double-muscling" in several species) (Grisolia et al.,2009) , such as sheep (Boman *et al.,*2009) goat [Boer goat] (Liu *et al.,*2006) and dogs (Mosher *et al.,*2007).

4-CONCLUSION

The outcome of PCR-RFLP and SNP in this investigation revealed that digestion of amplified fragments with the restriction enzyme (*Dra* I and) was very useful in genotype analysis of the local goat breeds/populations. As a result, growth MSTN gene could be employed as a reliable genetic marker in genetic diversity assessment and in goat breeding programs in Kurdistan Region/ Iraq

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