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Determination of the Polymorphism of the Booroola Fecundity B, Callipyge and Calpastatin Genes in Assaf Sheep Breed by PCR-RFLP Method

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ABSTRACT

Background: The development of local sheep breeds that have high fecundity levels, high meat content and high growth rates would help satisfy the Palestinian demand of meat and lower its price. It has been reported that mutant alleles of the genes Booroola fecundity (FecB), Callipyge (CLPG) and calpastatin (CAST) are associated with high litter size, larger muscular rumps and higher tenderness of meat after sheep slaughter, respectively. PCR-RFLPs methods have been established to facilitate the survey of sheep breeds for such mutants.

Methods: In this study, 117 blood samples of the most common sheep breed in Palestine, Assaf, were collected from Bethlehem and Jenin districts. Genomic DNA was extracted and purified from the blood samples using the salting out method. The genotypic ratios and allelic frequencies of the FecB, CLPG and CAST genes were determined by PCR-RFLP method.

Result: The results show that the mutant allele B of the FecB gene has a very low frequency among the screened sheep with 0.01 in Bethlehem and 0.07 in Jenin. Also, 94.4% of the tested sheep have the wildtype genotype AA for the CLPG gene with a frequency of 0.95 for allel A. Moreover, the desired heterzygous genotype MN for the CAST gene is 38.4% with an allelic frequency of 0.81 for the mutant allel M. These results would assist establishing sheep breeding programs with the appropriate parental genotypes to improve the livestock sector in the country.

Key words: Assaf, Booroola, Callipyge, Calpastatin, Fecundity.

INTRODUCTION

Local meat production in Palestine is not enough to sustain the demands of the growing population (Food and agriculture organization of the United Nations 2018; Palestinian Central Bureau of Statistics 2014). This is attributed to several reasons including the lack of space due to urbanization and land confiscation, high prices of fodder and lack of proper management plans by the authorities. All of this lead to a huge increase in the meat price in the Palestinian market where poor people depend on the imported, relatively cheap, low-quality frozen meat. One of the feasible solutions to this problem could be the development of sheep breeds that have high fecundity levels, high meat content and high rate of growth. Developement of such breeds requires knowledge of the genetic makeup behind such phenotypes. Fortunately, researchers have already identified mutations in various genes that are associated with the desired characteristics. These natural mutations are restricted to small number of individual sheep and some of them should be inhirted from only one parent to be functional. Therefore, allocation of individual sheep with such mutations (allocation of the desired genotype) among the sheep populations is a prerequisit for the initiation of a breeding program to produce the desired breed.

Several reports showed that the ovulation rate and the litter size of sheep are regulated by a group of genes called fecundity (Fec) genes (Bradford *et al*., 1986; Jonmundsson and Adalsteinsson 1985; Martyniuk and Radomsa 1991; Notter 2012; Shi *et al*., 2010). One of these genes, namely

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FecB was first reported in Booroola Merino breed in Australia and New Zealand (Davis *et al*., 1982). FecB is located on chromosome 6 of sheep and is one of three fecundity genes belonging to the transforming growth factor β (TGFβ) superfamily, a group of proteins involved in signaling and reglation of different cell processes, such as differentiation and proliferation. FecB codes for Bone Morphogenetic Protein Receptor 1B (BMPR1B), also called activin-like kinase 6 protein, which is known to regulate the follicle-stimulating hormone (FSH) which in turn is responsible for ovulation in ewes (Roy *et al*., 2011). Fecundity B mutation (c.746A>G) which result in a substitution of arginine (R) for glutamine (Q) at position (249 Q → R) of BMPR1B causes

attenuation of its signaling and ultimately leads to an increased ovulation rate. The genotypes of FecB in the ewes could be homozygous non-carrier (++), heterozygous carrier (B+) or homozygous carrier (BB). Both carrier genotypes are associated with high ovulation rate per estrous cycle, varying from 3-6. Such high rate of ovulation in the carrier genotypes were attributed to the ovulation of many ovarian follicles with the capacity to ovulate while still small in size (Roy *et al*., 2011).

Callipyge phenotype, also called beautiful buttocks, is an inhirted musclar hypertrophy where sheep have large muscular rumps. It was first reported in in Oklahoma in Dorset breed in 1983. Callipyge gene (CLPG) is located on the telomeric region of the ovine chromosome 18 and follows a pattern of inheritance called polar overdominance (Cockett *et al*., 1996; Smit *et al*., 2003). This means that the callipyge phenotype appears only in heterozygous lambs inheriting the mutant allele (A → G) from their sire and a normal one from the dam. So lambs with two mutated copies of the gene do not express the phenotype. The phenotype is expressed 1-3 months post birth and results in an increase of 35% in muscles of callipygian lambs and an 6-7% decrease in fats (Yu *et al*., 2018).

Calpastatin gene (CAST) is located on chromosome 5 of sheep and is involved in the regulation of the rate of skeletal muscle growth (synthesis/degradation) which is reflected in the average daily weight gain (0-8 month post birth) and meat tenderness after slaughter (Sutikno *et al*., 2011; Byun *et al*., 2008). Calpastatin acts by inhibiting a calcium-dependent cysteine protease called calpain which plays a role in postmortem tenderization of meats. So it is considered as a marker for meat quality. There are two polymorphic variants of CAST; allele M (mutant; A → G) and allele N (normal). Heterozygous lambs (MN genotype) are known for higher weight gain and meat tenderness (Alakilli, 2015).

This study aims at the determination of the polymorphism of the genes; Booroola FecB, CLPG and CAST in the commercially available Assaf sheep in Bethlehem and Jenin districts to select parents suitable for prospective breeding programs toward developing sheep breeds to serve as generous sources of meat.

MATERIALS AND METHODS

Genomic DNA extraction

Blood samples from 117 Assaf sheep (rams and ewes) were collected from Bethlehem and Jenin areas in Palestine. About 5-7 ml of blood were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant from the necks of the sheep and goats. Genomic DNA was extracted and purified from the blood samples using the salting out method (Miller *et al*., 1988). The DNA was eluted with 50µl of TE buffer and stored at -80°C till needed. The quality of the extracted DNA was assessed by gel-electrophoresis in 1% agarose gels and the concentration of DNA was determined using the nanodrop spectrophotometer (NanoDrop ND-1000 spectrophotometer).

PCR-RFLP analysis

The PCR amplifications of the three loci (FecB, CLPG and CAST) were performed using the primers shown in (Table 1). The PCR reaction mixture was prepared using the AccuPower Hot Start PCR premix (Bioneer Corporation Hylabs). The following were added to each reaction; 100 ng of genomic DNA and 0.4 µM of each primer. The total volume of each reaction was adjusted to 20 µl with DNase-free ddH2O. Amplifications were performed in 9600 Perkin Elmer Thermal Cycler as follows: an initial denaturation step of 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing depending on primer (Table 1) for 30 s and an extension at 72°C for 40 s. A final extension step for 5 min at 72°C was conducted. An aliquot of each PCR was electrophoresed in 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide.

The PCR products of FecB (190 bp), CLPG (426 bp) and CAST (622 bp) were digested with 10 units of AvaII, FaqI (BsmFI) and MspI restriction enzymes (Fermentas), respectively according to the manufacturer instructions. Digestion fragments were resolved on 1.5-2.5% agarose gel stained with 0.5µg/ml ethidium bromide in 1X Tris-Borate EDTA buffer (TBE) at 100 mA for 90 min. Gels were photographed using a BioDoc-It imaging system, UVP. Only clear bands were scored and their sizes were estimated by using 100 bp DNA ladder (Invitrogen) and/or Gene Ruler low range DNA ladder (Thermo Scientific).

Data analysis

Genotypic and allelic frequencies were calculated manually according the Hardy-Weinberg law. FecB RFLP using AvaII restriction enzyme G^GWCC (W stands for A or T) produced two alleles; the wildtype + with 190 bp and the mutant allele B with two fragments of 160 and 30 bp. For CLPG RFLP products were produced by FaqI GGGAC(10/14)^ and made up two alleles. The wildtype allele A was digested into three bands of 278, 117, 31 bp while the mutant allele G was

Table 1: List of the primers used in this study.

Locus	Primer name/Sequence 5′→3′	Size	Tm°C	Reference
FecB	FecBfor: CCAGAGGACAATAGCAAAGCAAA			
	FecBrev: CAAGATGTTTTCATGCCTCATCAACAGGTC	190 bp	58	Davis *et al*., 2002
Callipyge	CLPGF: TGAAAACGTGAACCCAGA AGC			
	CLPGR: GTCCTAAATAGGTCCTCTCG	426 bp	59	Gapor *et al*., 2009
Calpastatin	CASTF: TGGGGCCCAATGACGCCATCGATG			
	CASTR: GGTGGAGCAGCACTTCTGATCACC	622 bp	62	Palmer *et al*. 1998

digested into two bands of 395 and 31 bp. Finally, the RFLP products of CAST were produced by MspI (C^CGG) and produced either the normal allele N with 622 bp (wildtype) or the mutant allele M with two fragments of 336 and 286 bp.

RESULTS AND DISCUSSION

The Assaf sheep breed was originally developed by Israeli scientists from a cross of an original Palestinian sheep called Awassi and the East Friesian milk sheep from Germany (Gootwine 2008). It is the most common breed in the Palestinian market. The current study showed that the desired mutant alleles in two of the examined genetic loci which are associated with high litter size (FecB) and larger muscular rumps *i.e.* CLPG (Freking *et al*., 1998) are present with low frequencies in the Assaf breed in Bethlehem and Jenin. PCR amplification of FecB locus produced the desired band of 190 bp (Fig 1). Restriction digestion of the PCR products (Fig 2) showed that 13.3% of the investigated

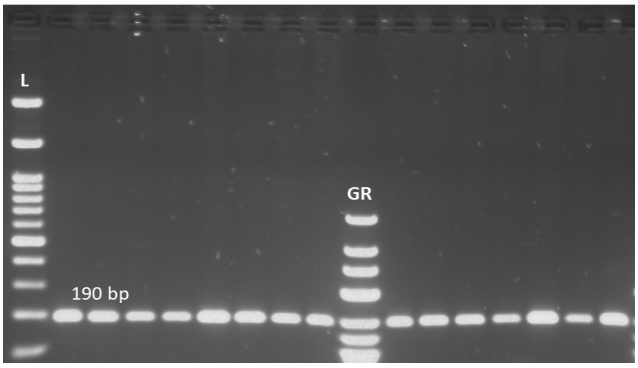


Fig 1: PCR products of FecB gene in part of the screened samples of Assaf sheep breed. Lane L: 100 bp DNA ladder. Lane GR: Gene Ruler low range DNA ladder. Other lanes with 190 bp PCR products amplified from sheep DNA.

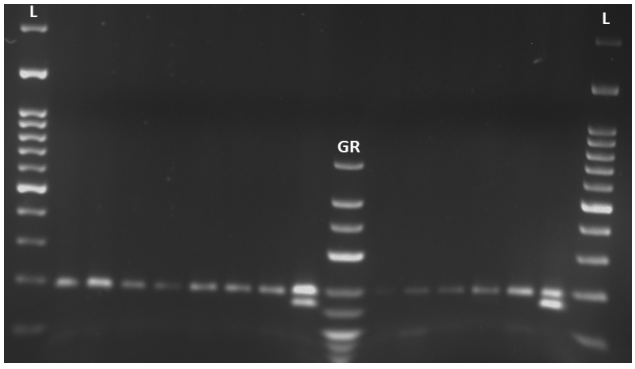


Fig 2: DNA electrophoric pattern of FecB amplicons after digestion with AvaII restriction enzyme. Lane L: 100 bp DNA ladder. Lane GR: Gene Ruler low range DNA ladder.

population in Jenin area is heterozygous for the FecB SNP (B+) and the rest (86.7%) hold the wildtype genotype (++). Moreover, none of the population was homozygous for the SNP (BB). In Bethlehem, the FecB genotypes ++ and B+ were 98% and 2%, respectively (Table 2). Also, the results showed that the allelic frequency of the wildtype allele (+) is 0.93 and 0.99 while that of the mutant allele (B) is 0.07 and 0.01 in Jenin and Bethlehem areas, respectively (Table 2).

The higher percent of B+ genotype in Jenin compared to Bethlehem (Table 2) could be attributed to the fact that many of the Jenin Assaf sheep were included in this study because of their reported history of increased litter size per birth. Among the screened breeds that are associated with large litter size, only few were found to carry the FecB mutation (Davis *et al*., 2006). These breeds include Booroola Merino (Piper *et al*., 1985; Guan *et al*., 2006) Hu and Han from China (Davis *et al*., 2006; Chu *et al*., 2007; Jia *et al*., 2005), Garole from India and Javanese from Indonesia (Davis *et al*., 2002). In most of the surveyed breeds common to the Middle East countries including Iraq, Egypt and Iran, the FecB mutation was not detected (Al-Barzinji and Othman 2013; El-Hanafy and El-Saadani 2009; Jamshidi *et al*., 2013). In Israel, the Booroola mutation has been introduced to the Awassi and Assaf breeds through breeding programs to produce Afec Awassi and Afec Assaf (Gootwine *et al*., 2008). In the Palestinian territories, the FecB genotypes and allelic frequencies of the Assaf sheep have not been revealed before this study.

Amplification of the DNA using CLPG specific primers produced a band of 426 bp (Fig 3) as expected. Digestion with FaqI restriction enzyme produced profiles representing the wildtype AA and homozygous mutant genotypes with percentages of 94.4 and 5.6, respectively (Fig 4 and Table 3). None of the 117 investigated sheep has the heterozygous genotype AG which expresses the phenotype. The allelic frequency of the mutant allele G was 0.05 while that of the normal allele was 0.95 (Table 3). Although the callipyge genotype (AG) was not detected in all of the examined sheep, the mutant allele was detected, albeit with a rather low frequency (0.05). The mere presence of such mutant allele, G, within the local Assaf population would facilitate developing callipyge sheep by inbreeding.

A band of 622 bp was amplified using CAST specific primers (Fig 5). Digestion of the amplified product with MspI restriction enzyme resulted in different RFLP profiles representing two genotypes MM and MN with a genotypic percentage of 61.6 and 38.4, respectively (Fig 6 and Table 4). None of the tested individuals has the NN genotype. The allelic frequencies of alleles M and N were 0.81 and 0.19,

Table 2: Genotypic percentage of and allelic frequencies FecB in Assaf sheep from Bethlehem and Jenin areas.

District	Genotypic percentage			Allelic frequency	
	++	B+	BB	+	B
Bethlehem	98	2	0	0.99	0.01
Jenin	86.7	13.3	0	0.93	0.07

Table 3: Genotypic percentage and allelic frequencies of CLPG in Assaf sheep from Palestine.

Genotypic	Percentage		Allelic frequency	
AA	AG	GG	A	G
94.4%	0%	5.6%	0.95	0.05

Table 4: Genotypic percentage and allelic frequencies of CAST in Assaf sheep from Palestine.

Genotypic	Percentage		Allelic frequency	
MM	MN	NN	N	M
61.6%	38.4%	0	0.19	0.81

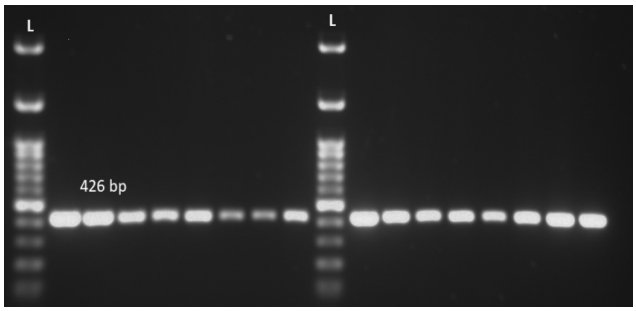


Fig 3: PCR products of CLPG gene in part of the screened samples of Assaf sheep breed. Lane L: 100 bp DNA ladder. Other lanes with 426 bp PCR products amplified from sheep DNA.

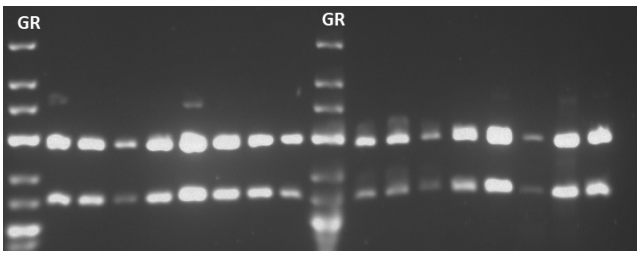


Fig 4: DNA electrophoric pattern of CLPG amplicons after digestion with FaqI restriction enzyme. Lane GR: Gene Ruler low range DNA ladder.

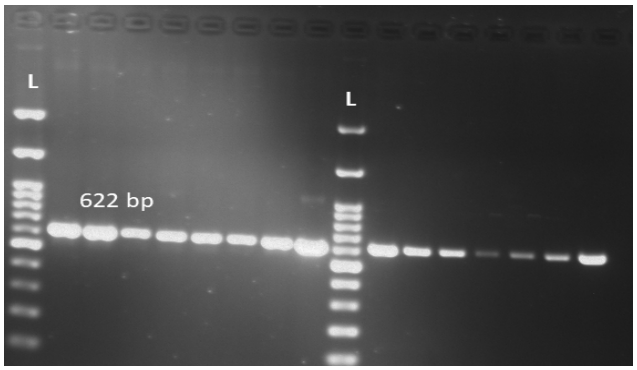


Fig 5: PCR products of CAST gene in part of the screened samples of Assaf sheep breed. Lane L: 100 bp DNA ladder. Other lanes with 622 bp PCR products amplified from sheep DNA.

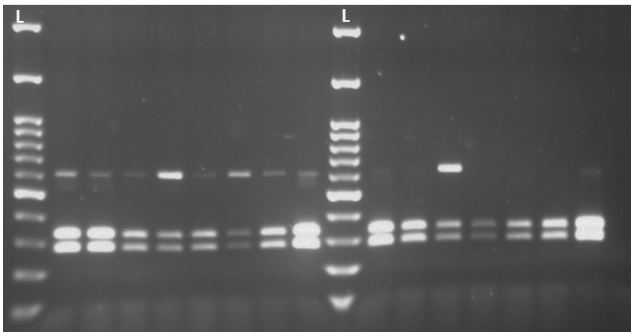


Fig 6: DNA electrophoric pattern of CAST amplicons after digestion with MspI endonuclease. Lane L: 100 bp DNA ladder.

respectively (Table 4). These results show that the desired MN genotype which is associated with satisfactory weight gain in lambs and meat tenderness after slaughter Palmer *et al*., 1998) is already abundant (38.4%) among the Assaf sheep population. The relatively high percentage of the heterozygous genotype MN in the Assaf sheep is expected since this breed has been developed from the Awassi sheep that have a high frequency of the mutant allele N and a large proportion of the MN genotype (Jawasreh *et al*., 2017). It might be worth mentioning that the absence of the undesirable NN genotype and the high frequency of the desirable MN genotype might be a result from the selection process that have been conducted on this breed in Palestine.

The determination of such allelic frequencies and genotypic percentage in the Assaf sheep population would be an important step toward the development of breeding programs aiming at improving the meat industry in Palestine through increasing the number of offspring and their meat content and the quality of the meat after slaughter. Although the Assaf breed has higher growth rates and fecundity than other local Palestinian sheep breeds as Baladi and Mukhla', our results showed that not all the commercially available Assaf individuals can serve as parents in breeding programs aiming at producing a line with the desired phenotypes. This is because of the low frequencies of the desired genotypes and alleles and the requirement for an upfront selection of the individuals with the desired genotypes. This study provides the solution for this obstacle through the selection of the desired parental genotypes. Therefore, we recommend that the Palestinian Ministry of Agriculture should use this study to start a breeding program for the introgression of the desired alleles into the Palestinian Baladi sheep.

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