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

Omar M. Darissa, Raeda Irekat

UNESCO Biotechnology Educational and Research Center (UNESCO BERCEN), Bethlehem University, P.O. Box 9, Bethlehem, Palestine.

Corresponding Author: Omar M. Darissa, UNESCO Biotechnology Educational and Research Center (UNESCO BERCEN), Bethlehem University, P.O. Box 9, Bethlehem, Palestine.
Email: odarissa@bethlehem.edu

How to cite this article: Darissa, O.M. and Irekat, R. (2020). Determination of the Polymorphism of the Booroola Fecundity B, Callipyge and Calpastatin Genes in Assaf Sheep Breed by PCR-RFLP Method. Agricultural Science Digest. 10.18805/ag.D-228
Submitted: 06-01-2020 Accepted: 01-10-2020 Online: 30-12-2020

Notter 2012; Shi \textit{et al.}, 2010). One of these genes, namely FecB was first reported in Booroola Merino breed in Australia and New Zealand (Davis \textit{et al.}, 1982). FecB is located on chromosome 6 of sheep and is one of three fecundity genes belonging to the transforming growth factor \(eta\) (TGF\(\beta\)) superfamily, a group of proteins involved in signaling and regulation 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 \textit{et al.}, 2011). Fecundity B mutation (c.746A>G) which result in a substitution of arginine (R) for glutamine (Q) at position (249 Q \rightarrow 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 inherited muscular hypertrophy where sheep have large muscular rumps. It was first reported in Oklahoma in the 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 tenderness 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 (Bioener Corporation Hyplants). 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 Avall, FaqI (BsmFI) and MspI restriction enzymes (Fermentas), respectively according to the manufacturer instructions. Digestion fragments were resolved on 1% 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 Avall, FaqI (BsmFI) and MspI restriction enzymes (Fermentas), respectively according to the manufacturer instructions. Digestion fragments were resolved on 1% agarose gel stained with 0.5 µg/ml ethidium bromide.

**Data analysis**

Genotypic and allelic frequencies were calculated manually according the Hardy-Weinberg law. FecB RFLP using Avall 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 two alleles were produced by FaqI GGGAC(10/14)^ and made digestion fragments were resolved on 1% 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 Avall, FaqI (BsmFI) and MspI restriction enzymes (Fermentas), respectively according to the manufacturer instructions. Digestion fragments were resolved on 1% agarose gel stained with 0.5 µg/ml ethidium bromide.

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

| Locus      | Primer name/Sequence 5' → 3' | Size   | Tm°C | Reference       |
|------------|-----------------------------|--------|------|-----------------|
| FecB       | FecBfor: CCAGAGGACAATAGCAAAGCAAA | 190 bp | 58   | Davis et al., 2002 |
|            | FecBrev: CAAGATGTTTTCATGCTCATCATCAACAGGTC | 426 bp | 59   | Gapor et al., 2009 |
| Callipyge  | CLPGF: TGAAAACGTGAACCCAGAGCAC | 426 bp | 59   | Gapor et al., 2009 |
|            | CLPGR: GTCTGTAATAGGTCTTCCTCG | 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 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 3: Genotypic percentage and allelic frequencies of CLPG in Assaf sheep from Palestine.

| Genotypic percentage | Allelic frequency |
|----------------------|-------------------|
| AA 94.4%             | A 0.95            |
| AG 0%                |                  |
| GG 5.6%              | G 0.05            |

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

| Genotypic percentage | Allelic frequency |
|----------------------|-------------------|
| MM 61.6%             | M 0.19            |
| MN 38.4%             | N 0.81            |
| NN 0%                |                  |

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 FagI 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.

ACKNOWLEDGEMENT
This work was supported by an Internal Research Grant through the office of the Dean of Research at Bethlehem University. Thanks are also due to the Ministry of Agriculture for their guidance to the farms and help in the collection of the blood samples. The authors are grateful for Dr. Saber Hussein, Lake Erie colleague of osteopathic medicine, PA, USA for reviewing the manuscript.
REFERENCES

Alakilli, S. (2015). Analysis of Polymorphism of Calpastatin and Callipyge Genes in Saud Sheep Breeds Using PCR-RFLP Technique. International Journal of Pharmaceutical Sciences Review and Research. 30(1): 340-344.

Al-Barzinji, Y.M. and Othman G.U. (2013). Genetic polymorphism in FecB gene in Iraqi Sheep Breeds using RFLP-PCR technique. IOSR Journal of Agriculture and Veterinary Science. 2(4): 46-48.

Bradford, G.E., Quirke, J.F., Sitorius, P., Inoumu, I., Tiesnamurti, B., Al-Barzinji, Y.M. and Othman G.U. (2013). Genetic polymorphism in FecB gene in five Egyptian sheep breeds. Biotechnology in Javanese sheep: Evidence for a gene with large effect on ovulation rate and litter size. Journal of Animal Science. 63(2): 418-431. doi:10.2527/jas1986.632418x.

Byun, S.O., Zhou, H., Forrest, R.H., Frampton, C.M. and Hickford, J.G. (2008). Association of the ovine calpastatin gene with birth weight and growth rate to weaning. Animal Genetics. 39: 572-573.

Chu, M.X., Liu, Z.H., Jiao, C.L., He, Y.Q., Fang, L., Ye, S.C., Chen, G.H. and Wang, J.Y. (2007). Mutations in BMPR-IB and BMP-15 genes are associated with litter size in Small Tailed Han sheep (Ovis aries). Journal of Animal Science. 85 (3): 598-603. doi:10.2527/ajas.2006-324 85: 324-330.

Cockett, N.E., Jackson, S.P., Shay, T.L., Farnir, F., Berghmans, S., Snowden, G.D., Nielsen, D.M. and Georges, M. (1996). Polar overdominance at the ovine callipyge locus. Science. 273: 236.

Davis, G.H., Balakrishnan, L., Ross, I.K., Wilson, T., Galloway, M., Lumsden, B.M., Hanrahan, J.P., Mullen, M., Mao, X.Z., Wang, G.L., Zhao, Z.S., Zeng, Y.Q., Robinson, J.J., Mavrogenis, A.P., Papachristoforou, C, Peter, C, Baumung, R., Cardyn, P., Boujenane, I., Cockett, N.E., Eythorsdottir, E., Arranz, J.J. and Notter, D.R. (2006). Investigation of the Booroola (FecB) and Inverdale (FecXI) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. Animal Reproduction Science. 92(1-2): 87-96. doi:10.1016/j.anireprosci.2005.06.001.

Gábor, M., Trakovická, A. and Miluchová, M. (2009). Analysis of polymorphism of CAST gene and Clp gene in sheep by PCR-RFLP method. Lumcroţi ştiintifice Zootehnie şi Biotehnologii. 42: 470-476.

Gootwine, E. (2008). Biological and economic consequences of introgressing the B allele of the FecB (Booroola) gene into Awassi and Assaf sheep. In: Proceeding of the Helen Newton Turner Memorial International Workshop; Pune, India, pp. 119-127.

Gootwine, E., Reicher, S. and Rozov, A. (2008). Prolificacy and lamb survival at birth in Awassi and Assaf sheep carrying the FecB (Booroola) mutation. Animal Reproduction Science. 108(3-4): 402-411. doi: 10.1016/j.anireprosci.2007.09.009.

Guo, F., Lui, S.R., Shi, G.Q., Ai, J.T., Mao, D.G. and Yang, L.G. (2006). Polymorphism of FecB gene in nine sheep breeds or strains and its effects on litter size, lamb growth and development. Acta Genetica Sinica. 33(2): 117-124. doi:10.1016/s0379-4172(06)60303-9.

Jamsheedi, R., Kasirian, M.M. and Rahimi, G.A. (2013). Application of PCR-RFLP technique to determine Booroola gene polymorphism in the Sangaari sheep breed of Iran. Turkish Journal of Veterinary and Animal Sciences. 37: 129-133. doi:10.3906/vet-0806-14.

Jawasreh, K., Jadallah, R., Al-Amareen, A.H., Abdellah, A., Al-Qaisi, A., AlRawashdeh, I.M., Borhan, M., Al-Zghoul, F., KHAIR, M., Ahamed, A. and Obeidat, B. (2017). Association between Mspl calpastatin gene polymorphisms, growth performance and meat characteristics of Awassi sheep. The Indian journal of animal sciences. 87 (5): 635-639.

Jia, C.L., Li, N., Zhao, X.B., Zhu, X.P. and Jia, Z.H. (2005). Association of BMPR-IB gene with litter size traits in sheep. Frontiers in Molecular Biology. 108(3-4): 402-411. doi:10.3906/vet-0806-14.

Jonmundsson, J.V. and Adalsteinsson, S. (1985). Single genes for fecundity in Icelandic sheep. In: Land, R.B., Robinson, D.W. (editors). Major Genes for Reproduction in Sheep. Butterworths, London, pp. 159-168.

Martyniuk, E. and Radomsa, M.J. (1991). A single gene for prolificacy in Olkuska sheep. In: Elena J.M., Bodin, L., Thimonier, J. (editors). Major Genes for Reproduction in Sheep, Paris, Inra, pp. 85-92.

Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research. 16 (3): 1215. doi:10.1093/ nar/16.3.1215.

Notter, D.R. (2012). Genetic improvement of reproductive efficiency of sheep and goats. Animal Reproduction Science. 130 (3-4): 147-151. doi: 10.1016/j.anireprosci.2012.01.008.

Palestinian Central Bureau of Statistics (2014). Livestock Survey (2013). Main Results. Ramallah, Palestine [online]. Website http://www.pcbs.gov.ps/PCBS-Metadata-en/
Determination of the Polymorphism of the Booroola Fecundity B, Callipyge and Calpastatin Genes in Assaf Sheep Breed...

Palmer, B.R., Robert, N., Hickford, J.G.H. and Bickerstaffe, G. (1998). Rapid Communication: PCR-RFLP for MspI and NcoI in the ovine calpastatin gene. Journal of Animal Science. 76 (5): 1499-500. doi:10.2527/1998.7651499x.

Piper, L.R., Bindon, B.M. and Davis, G.H., (1985). The single gene inheritance of the high litter size of the Booroola Merino. In: Land, R.B., Robinson, D.W. (Eds.), Genetics of Reproduction in Sheep. Butterworths, London, UK, 115-125.

Roy, J., Polley, S., De, S., Mukherjee, A., Batabyal, S., Pan, S., Brahma, B., Datta, T.K. and Goswami, S.L. (2011). Polymorphism of Fecundity Genes (FecB, FecX and FecG) in the Indian Bonpala Sheep. Animal Biotechnology. 22 (3): 151-162. doi:10.1080/10495398.2011.589239.

Shi, H., Bai, J., Niu, Z., Muniresha, Fen L. and Jia, B. (2010). Study on candidate gene for fecundity traits in Xingjiang Cele black sheep. African Journal of Biotechnology. 9 (49): 8498-8505. doi: 10.5897/AJB10.1003.

Smit, M., Segers, K., Carrascosa, L.G., Shay, T., Baraldi, F., Gyapay, G., Snowder, G., Georges, M., Cocket, N. and Charlier, C. (2003). Mosaicism of Solid Gold supports the causality of a non-coding A-to-G transition in the determinism of the callipyge phenotype. Genetics. 163(1): 453-456.

Sutikno, A., Yamin, M. and Sumantric, C. (2011). Association of Polymorphisms Calpastatin Gene with Body Weight of Local Sheep in Jonggol, Indonesia. Media Peternakan. 34 (1): 1-6.

Yu, H., Waddell, J.N., Kuang, S., Tellam, R.L., Cockett, N.E. and Bidwell, C.A. (2018). Identification of genes directly responding to DLK1 signaling in Callipyge sheep. BMC Genomics. 19(1): 283. doi:10.1186/s12864-018-4682-1.