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Expression study on butyrophilin gene and its association with milk production trait in goats

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Abstract
Goats are recognised as important components of livestock farming system and they play a very useful role in providing a steady income to the people of economically backward sections throughout the year. Goat milk is usually recommended for aged persons, infants and convalescent people due to its better digestibility, which can be attributed to smaller diameter of fat globules found in goat milk when compared to cow milk. In Kerala, two native goat breeds, Malabari and Attappady Black vary significantly in milk production. Recent advances in molecular techniques provide opportunities for genetic improvement at a faster pace. Double digest Restriction Associated DNA Sequencing (ddRADseq) is a modern approach for identification of Single Nucleotide Polymorphisms (SNP) across populations. The ddRADseq technique was performed in three pooled samples (ten each) of high and low milk producing Malabari and Attappady Black goats. By using bioinformatics tools, the SNPs were discovered and analysed. A total of 42 candidate genes affecting milk quantity and quality were selected from literature and the entire data was screened for variants in these candidate genes. Out of these candidate genes, gene Butyrophilin (*BTN1A1*) obtained from ddRADseq, was selected for expression study in the present study as researchers showed that *BTN1A1* was significant associated with growth trait and milk yield in Saanen goats. *BTN1A1* is a transmembrane glycoprotein, specifically expressed on the epithelial cells during lactation. In the current study, *BTN1A1* was selected for quantitative polymerase chain reaction (qPCR). In this method, RNA was isolated from milk of late stages of lactation from six each of Malabari and Attappady Black goats using TRIzol LS reagent. All 12 samples of RNA were converted to cDNA using Verso cDNA synthesis kit. For qPCR, target gene (*BTN1A1*) and reference gene (*β-actin*) were standardized. The relative quantification of gene expression was carried out using Illumina Eco® Q-RT PCR system using SYBR green chemistry. In the current study, expression of *BTN1A1* was comparatively higher in Malabari than Attappady Black goats and this difference was found to be statistically significant ($p \leq 0.05$). The relative expression of *BTN1A1* was 6.74 fold higher in Malabari than Attappady Black goats.

Keywords: Goats, milk production, ddRADseq, SNP, qPCR and *BTN1A1*

Introduction
Goats are main components of world- domestic animal bio diversity, as they can be reared with low investment. Goat milk was an alternative source of animal protein for people and it showed better digestibility, because average diameter of fat globules found in goat milk (2.76 µM) was smaller than that of cow milk (3.51 µM). Presence of caproic, caprylic and capric fatty acids in goat milk enhanced its quality and it is recommended for infants, aged people and convalescent persons. Goat milk is supposed to exhibit good medicinal properties which may be attributed to the patients suffering from many ailments. Native goat breeds of Kerala; namely Malabari and Attappady Black apparently varied in milk production capacity. Milk production in goats, is accepted as an important economic trait as far as goat rearing systems are concerned. Recent advances in molecular techniques provide opportunities for genetic improvement of this trait at a faster pace. Sequencing methods were important for SNP discovery and genotyping. Most modern sequencing method is called Double digest Restriction Associated DNA sequencing (ddRADseq). This ddRADseq method is important role in SNP detection. Double digest Restriction Associated DNA Sequencing (ddRADseq) is a modern approach for identification of Single Nucleotide Polymorphisms (SNP) across populations. The ddRADseq technique was performed in three pooled samples (ten each) of high and low milk producing Malabari and Attappady Black goats.

Materials and Methods

Selection of animals for DDRADSEQ

Blood samples were collected from 10 each of high milk producing Malabari, low milk producing Malabari and Attappady Black goats in University Goat and Sheep Farm, Mannuthy, Thrissur. Genomic DNA was extracted from whole blood using standard phenol chloroform method (Sambrook and Russell, 2001). The concentration, purity and quality of DNA were checked by Nanodrop TM 1000 spectrophotometer (Thermo Scientific, USA). The purity of DNA was verified by measuring absorbance at 260 nm and 280 nm. The DNA samples with value between 1.7-1.8 were used for further downstream processing. Library preparation was done with *SphI* and *MIucI* restriction enzymes and by using suitable bioinformatics software, SNPs were identified. Based on ddRADseq results, a total of 4141 variants were identified in Malabari and 2192 variants in Attappady Black Goats. From the SNPs, 42 candidate genes affecting milk production were screened. In current study, Gene *BTN1A1* obtained from ddRADseq, was selected for expression study.

RNA Isolation

Isolation of RNA from milk samples of Malabari and Attappady Black goats (six animals from each group), cDNA synthesis and expression profiling of *BTN1A1* using qPCR technique. Total RNA was isolated from milk samples using TRIzol® LS reagent (Life Technologies, USA). The RNA isolated was treated with Deoxyribonuclease (DNase) enzyme amplification grade (Sigma-Aldrich) to remove DNA contamination in total RNA, if any. To every 24 µl of RNA samples three microlitre of 10X DNase reaction buffer and three microlitre of DNase I enzyme was added and incubated at 25 °C for 15 min. Stop solution (3 µl) was added in order to inactivate DNase I. The samples were heated at 70 °C for 10 min to inactivate DNaseI and then chilled on ice. The yield and purity of isolated RNA samples were measured by Nanodrop TM 1000 Spectrophotometer (Thermo Scientific, USA). The purity of RNA stock was estimated by finding the ratio between OD readings at 260 nm and 280 nm. The samples showing OD value 260/280 around two were chosen for further studies. The quality and integrity of RNA were assessed electrophoretically using one per cent agarose gel prepared in 1X Tris Acetate EDTA (TAE) buffer. RNA samples and 2X RNA loading dye (Fermentas) mixed in equal quantity were denatured for 10 min at 70 ºC which were then snap chilled on ice and loaded into wells of agarose gel. Electrophoresis was performed at 2V/cm for 40 mins. The gel was visualised using gel documentation system (BioRad, USA).

Complementary DNA (CDNA) Synthesis

Complementary DNA was synthesised using Verso cDNA Synthesis Kit (Thermo Scientific, USA). The reactions were set up in 0.2 millilitre PCR tubes (Table 1). Master Mix was prepared excluding template RNA sample which was added individually to each reaction tube. The contents of the tube were mixed gently and centrifuged briefly. The reaction mix was incubated at 42 °C for 30 minutes followed by 72 °C for five minutes. After synthesis cDNA was stored at -40 ºC until further use.

Table 1: Composition of cDNA master mix for cDNA synthesis

Item	Volume(µl)	Final concentration
5X cDNA synthesis buffer	4	1X
Verso enzyme	1	
dNTP mix	2	500 µM each
Oligo dt	1	
RT enhancer	1	
Template RNA	1	1000 ng
Nuclease free water	10	
Total	20	

Quantitative PCR (qPCR) FOR *Btn1a1*

In the present study, qPCR was used to find out the relative expression of *BTN1A1* in milk samples of Malabari and Attappady Black goats (six animals from each group). The relative quantification of gene expression was carried out using Illumina Eco® Q- RT PCR system using SYBR green chemistry. For the present study, *β-actin* was selected as internal control gene. Primers to amplify a short fragment (120bp) of target gene, *BTN1A1* was designed according to caprine (GenBank accession no. NC_030830.1) Primers for endogenous reference gene, *β-actin* was designed from published literature (Naicy *et al*., 2016). Designing and checking of primers were done with Primer3 software. The primers selected were custom synthesized from Eurofins Genomics and were diluted to a concentration of 10 p*M*/µl. The primer sequences used for qPCR for *BTN1A1, β-actin* are listed in table 2

Table 2: Sequence details of primers designed for qPCR

Primer	Sequence(5'-3')	Product size (bp)
BTN1A1 F	CCAGAAAGGTTGGAGATGGCA	108
BTN1A1 R	CACGTCAAAGGGAGCAGAATC	
β-actin F	GCATTGTTGGGTTCCTGT	100
β-actin R	TCACGAAGATCTGCATTTTG	

Concurrent qPCR reactions were set up for *BTN1A1* and *β-actin* using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA). Ten cDNA samples from Malabari and Attappady Black goats were amplified with three technical replicates. Reactions were carried out in Eco Real-Time PCR System (Illumina) using Eco 48-well plates which were sealed by Eco adhesive seals. Reaction mix and thermal cycling parameters are given in tables 3 and 4.

Table 3: qPCR mix to amplify *BTN1A1* and *β-actin*

Sl. No.	Components	Volume (µl)
1	Maxima SYBR Green qPCR Master Mix	5.0
2	Forward primer (10 p*M*/µl)	0.3
3	Reverse primer (10 p*M*/µl)	0.3
4	Template (cDNA)	1.0
5	Nuclease free water	3.4
	Total	10.0

Table 4: qPCR conditions to amplify *BTN1A1* and *β-actin* genes

Sl No	Step	Temperature (°C)	Time
1	Initial denaturation	94	3 min
2	Denaturation	94	30 sec
3	Annealing	62	15sec
4	Extension	72	30 sec

Steps 2 to 4 repeated 40 times.
Data acquisition was performed during extension step of each cycle

Dissociation (melt) curve analysis was done after each PCR. The protocol for melt curve analysis was 95 °C for 15 sec, 55 °C for 15 sec followed by 95 °C for 15 sec.
Reactions were set up for *BTN1A1* and *beta actin* genes. Each sample was amplified in triplicate (technical replicates). The controls set for each run were, non-template control (NTC) for each gene and a negative control (Nuclease free water). The thermal profile for RT-qPCR reaction and plate layout were set in the computer attached to Illumina Eco® Q- RT PCR system. The master mix was prepared for each primer pairs separately. The template (cDNA) was loaded separately into the designated wells. For loading samples, the plate was placed into the Eco sample loading dock, the dock light was turned on and the dock was inclined to a comfortable angle for pipetting. The cDNA samples and qPCR master mix were pipetted into the plate and sealed. The plate adapter with sealed plate was placed into a plate centrifuge along with a second plate adapter for balancing and centrifuged for one minute. The Eco lid was opened and the plate was placed on the block, aligning the notch against the top left corner. The data were analysed for relative quantification by $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001) [3].
The qPCR was normalized to the expression of control gene *Beta actin*. For comparison of *BTN1A1* expression between breeds, Attappady Black was taken as the reference/calibrator sample.
ΔC_T = Average C_T (Target gene) - Average C_T (Reference gene)
$\Delta\Delta C_T = \Delta C_T$ (Test sample) - ΔC_T (Reference sample)
Relative Quantification (RQ) = $2^{-\Delta\Delta C}$T
Statistical comparison between samples was performed using ANOVA (Analysis of variance) and independent sample t-test (SPSS V.24) and subsequent Duncan's multiple range test (p-value of ≤0.05 was considered statistically significant).

Results

Relative expression of *Btn1a1* in Malabar and attappady black goats

In qPCR study, *β-actin* was used as reference gene. The qPCR conditions were standardized for both target gene (*BTN1A1*) and reference gene (*β-actin*). Quantitative PCR was performed for *BTN1A1* and *β-actin* in six Malabari and six Attappady Black goats. The amplification plots and melt curves generated during real time PCR assays in the Illumina Eco® Q- RT PCR are depicted in Fig. 1 and 2. The mean with standard errors C_T, ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta C}{}_T$ of Malabari and Attappady Black goats are given in table 5. After normalizing the relative expression of *BTN1A1* with reference gene *β-actin*, fold change was calculated by considering Attappady Black goats as control. In the current study, expression of *BTN1A1* was comparatively higher in Malabari than Attappady Black goats and this difference was found to be statistically significant ($p \leq 0.05$). The relative expression of *BTN1A1* was 6.74 fold higher in Malabari than Attappady Black goats (Fig. 3).

Table 5: Mean CT values and fold change in *BTN1A1* expression

Group	Mean C_T±SE		ΔC_T	$\Delta\Delta C_T$	Fold chain ($2^{-\Delta\Delta C}{}_T$)
	BTN1A1	*B-actin*			
Malabari	23.45 ± 0.34	26.39± 0.70	-2.93 ± 0.78	-2.75 ± 0.78	6.74^a
Attappady Black	26.55 ± 0.68	26.83 ± 0.58	-0.18 ± 0.90	0 ± 0.90	1^b
				p-value	0.03

Means having different superscript within a column differ significantly (*p-value≤0.05)

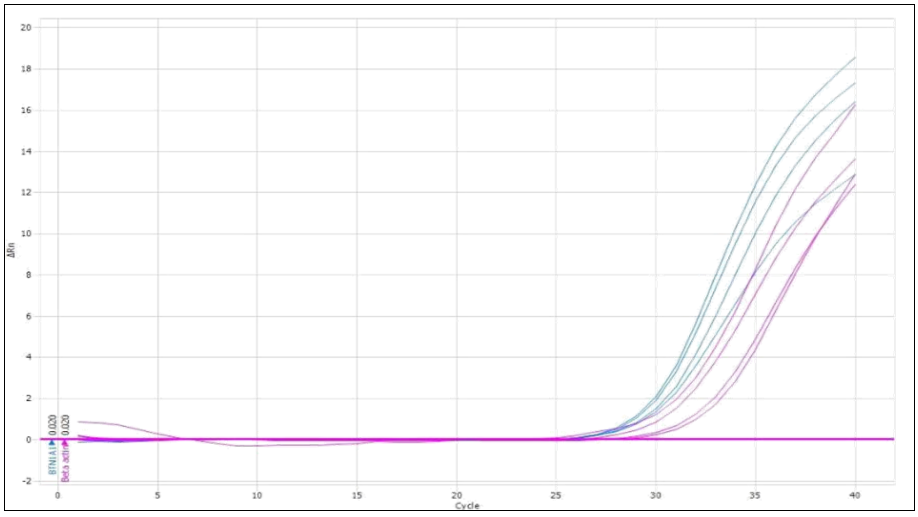

Fig 1: Amplification plot for *BTN1A1* and *β-actin*

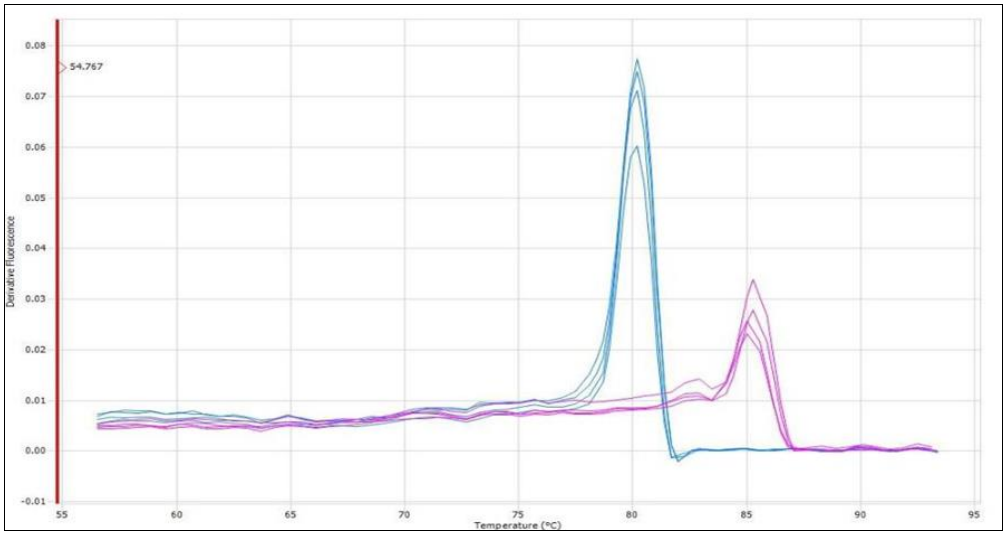

Fig 2: Derivative melt curve of *BTN1A1* and *β-actin*

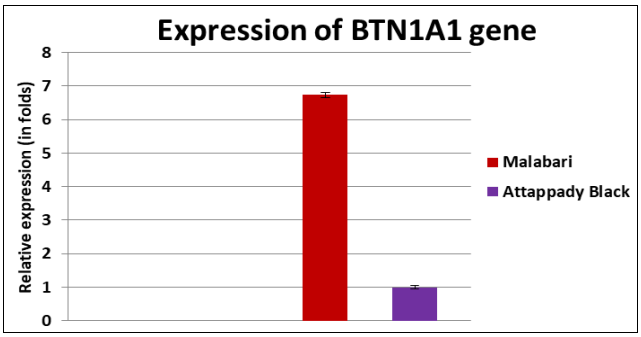

Fig 3: Relative expression of *BTN1A1*

Discussion

Validation of btn1a1 by qPCR

Peterson *et al*. (2012) [4] discovered ddRADseq protocol by modifying the most successful reduced representation sequencing of RAD sequencing method. Two restriction enzymes were used in ddRAD for skipping the random shearing step of RAD Sequencing. ddRADseq acquired important role in high density SNP discovery and genotyping, in model and non -model organisms with high efficiency and low cost. In the current study, 42 candidate genes identified affecting milk production in domestic animals by using ddRADseq results. From this, in present study BTN1A1 gene was selected for qPCR. According to Wang and Brown (1999) [7] for measuring gene expression, Real-time PCR (RT-PCR) is most efficient, sensitive and quick method. This is the most efficient quantification method. This method was used to compare the mRNA levels in different sample population, RNA expression pattern characterisation and analysing RNA structure.

Butyrophilin is a transmembrane glycoprotein, specifically expressed on the epithelial cells of mammary gland during lactation. This protein was mainly associated with fat droplets and involved in milk fat globules secretion, in epithelial cells of mammary gland (Jack and Mather, 1990) [2]. The BTN1A1 played a role in the budding and release of fat globules, from the mammary gland during lactation (Banghart *et al*., 1998) [1]. Qu *et al*. (2011) [5] reported that BTN1A1 coded for fat globule membrane proteins of milk. This protein played a role in the milk lipid secretion and showed significant association with milk yield in Saanen goat. Sztankoova *et al*. (2017) [6] observed that variation analysis confirmed an effect for genetic polymorphism in BTN1A1 on milk yield in goat milk. Considering the importance of this gene and its association with milk yield, this gene was selected for comparative expression studies between high milk producing Malabari and low producing Attappady Black goats. In the current study, expression of BTN1A1 was comparatively higher in Malabari than Attappady Black goats and this difference was found to be statistically significant ($p \leq 0.05$). The relative expression of BTN1A1 was 6.74 fold higher in Malabari than Attappady Black goats [Fig 3].

Conclusion

Double digest Restriction Associated DNA Sequencing (ddRADseq) is a modern approach for identification of Single Nucleotide Polymorphisms (SNP) across populations. The ddRADseq technique was performed in three pooled samples (ten each) of high and low milk producing Malabari and Attappady Black goats. SNPs discovered using bioinformatics tools. Gene Butyrophilin (BTN1A1) obtained from ddRADseq was selected for quantitative polymerase chain reaction (qPCR) in the present study as researchers BTN1A1 was showed significant associated with growth trait and milk yield in Saanen goats. In this qPCR, RNA was isolated from milk of late stages of lactation from six each of Malabari and Attappady Black goats using TRIzol LS reagent. The relative quantification of gene expression was carried out using Illumina Eco® Q-RT PCR system using SYBR green chemistry. In the current study, expression of BTN1A1 was comparatively higher in Malabari than Attappady Black goats and this difference was found to be statistically significant ($p \leq 0.05$). The relative expression of BTN1A1 was 6.74 fold higher in Malabari than Attappady Black goats.

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