MOLECULAR GENETIC STUDIES ON BUTYROPHILIN GENE IN MALNAD GIDDA CATTLE AND ITS ASSOCIATION WITH MILK QUALITY TRAITS

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CERTIFICATE

This is to certify that the thesis entitled ***"MOLECULAR GENETIC STUDIES ON BUTYROPHILIN GENE IN MALNAD GIDDA CATTLE AND ITS ASSOCIATION WITH MILK QUALITY TRAITS"*** submitted by **Mr. VISHWANATH, B., ID No. MVHK - 1104** in partial fulfillment of the requirements for the award of the degree of **MASTER OF VETERINARY SCIENCE** in **ANIMAL GENETICS AND BREEDING** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of bonafide research work carried out by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associationship, fellowship or other similar titles.

Bangalore
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(Dr. SHRI KRISHNA ISLOOR)

This thesis is affectionately dedicated

to

My Family, Guide, k. Rahman and

Iqbal Ahmed family, beloved sister

Sujatha. N and

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LIST OF ABBREVIATIONS

BLAST	:	Basic Local Alignment Search Tool
BLASTn	:	Nucleotide BLAST
bp	:	base pair
DNA	:	deoxy ribo nucleic acid
dNTPs	:	deoxy ribo nucleotide triphosphate
EDTA	:	ethylene diamine tetra acetic acid
FAO	:	Food and Agricultural Organization
G	:	Gram
Hr	:	Hour
i.e.	:	that is
KCL	:	potassium chloride
Kb	:	kilo base
M	:	Molar
µg	:	micro gram
$MgCl_2$	:	Magnesium chloride
mg	:	milli gram
µl	:	micro litre
ml	:	milli litre
µM	:	micro moles
mM	:	milli moles
ng	:	nano gram
NCBI	:	National Centre for Biotechnology Information

OD	:	optical density
pmol	:	pico moles
PCR	:	polymerase chain reaction
RFLP	:	Restriction Fragment Length Polymorphism
rpm	:	revolutions per minute
Taq	:	*Thermus aquaticus*
TBE	:	tris- boric acid-EDTA
TE	:	tris-hydrochloride
i.u	:	international units

Introduction

1. INTRODUCTION

The livestock plays a vital role in overall development of agricultural economy of the rural India and is one of the most effective tools for empowerment of the human race in the developing world.

The livestock biodiversity is getting eroded over the years and the greatest single cause for which is the growing trend of global reliance on a limited number of selected breeds known for high productivity under intensive system of management requiring high inputs. The reducing number of breeds in use would eliminate a sizeable amount of variation in the species, besides jeopardizing the present gene combinations in rest of unique gene pool. Livestock development programmes are increasingly promoting the universal use of very few ‘improved’ breeds. Such programmes have had the effects on indigenous animal genetic resources and the neglect of unique cattle. This has necessitated, developing strategies for conservation of indigenous cattle genetic resources, and thereby maintaining the genetic diversity.

In India, as well as in rest of the world, genetic erosion is happening among native farm animal breeds and some highly productive breeds were promoted in the last five decades to meet the rising demand for animal products. The semen of these 'elite' breeds were used extensively for crossing with indigenous breeds, leading to large-scale propagation of a few exotic breeds at the cost of the native breeds, some of which are now truly endangered. Factors like commercialization of dairy sector aggravated the loss of local breeds.

Karnataka state is known for having a rich heritage and diversified natural resources like flora and fauna including livestock wealth. The livestock in Karnataka are well adapted to existing agro-ecological and environmental conditions over a long period of time and withstood the occurrence of enzootic diseases, scarcity of feeds and fodder and local farming systems, indicating their possession of excellent gene combinations for disease resistance and adaptability.

Malnad Gidda is a dwarf, heat and disease resistant cattle breed, native to Karnataka, confined to Western Ghats region. The population of this breed is getting reduced over the years due to increased crossbreeding programme with exotic high yielding germplasm. Local people believe that Malnad Gidda cattle milk has some medicinal properties though not supported by any scientific studies. No systematic genetic studies have been undertaken to characterize this breed. This breed needs immediate characterization and has to be conserved in order to make available this precious germplasm for future generation.

Fat is one of the important components of milk, based on the quality and quantity of which, pricing of milk is determined in India. Fat is the major contributor to the energy density of whole milk and is essential to many of the physical and manufacturing properties of dairy products. Fat are also the most variable component of milk and many environmental and physiological factors affect milk fat secretion in cows.

Most traits of economic importance in livestock are quantitative in nature, and hence the ability to unravel the genetics of quantitative traits is quite complex.

Many molecular techniques have been developed for the identification and characterization of candidate genes that are responsible for production traits. Once the potential candidate genes are identified, incorporation of those genetic markers in the selection experiment would ultimately enhance the genetic gain over a period of time. Selection intensity with respect to a certain trait will be relatively higher through indirect selection (like candidate gene approach) and thus, overall genetic progress can be achieved for the desired trait at a considerably higher rate.

Butyrophilin (BTN1A1) is the major protein in the milk fat globule membrane (MFGM) which plays an important role in secretion of milk fat by promoting final formation of lipid droplet in the apical membrane and the subsequent releasing of fat droplets into the lumen of udder. It also helps in stabilizing and determining the size of lipid droplets after secretion. It has been found that BTN protein increases manifold to about 40 times during lactation stage in mammals (Mather, 2000). The importance of BTN1A1 gene in milk fat synthesis has been established by gene expression studies and knockout studies in rodents. Butyrophilin (BTN1A1) gene, which codes for BTN protein, is one of the candidate genes that affect the milk yield and composition in dairy cattle (Komisarek and Dorynek, 2003).

No systematic study has been carried out in Malnad Gidda cattle with respect to molecular genetic characterization and milk quality traits. Keeping this point in view, the present investigation was carried out with the following objectives.

Objectives of the Study:

1. To amplify Butyrophilin gene in Malnad Gidda cattle
2. To study the polymorphic pattern of amplified Butyrophilin gene by PCR-RFLP and DNA sequencing methods
3. To study the association of Butyrophilin gene polymorphism with milk quality traits -fat, SNF and total ash.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Malnad Gidda cattle

Malnad Gidda cattle are small animals and distributed in Malnad area of Karnataka. Animals of this breed are playing a significant role in the livelihood of farmers in terms of draught, milk and manure. These animals possess capacity of grazing at higher altitudes.

2.1.1 Physical characteristics of Malnad Gidda cattle

Malnad Gidda animals bear a compact body, weighing about 90 to 140 kg. The coat colour varies from predominantly black to red, fawn, brown and white. The eyelids, muzzle and tail switch are predominantly black in animals with black coat colour. However, the muzzle colour may be pinkish in fawn and white coat coloured animals. The ears are horizontal in orientation. The horns are small to medium in length and oriented outward, upward and inward with pointed tips. The limbs are short and comparatively thinner. The hump is small in cows but more prominent in bulls. The dewlap size is small to medium. The udder is bowl shaped and small in size. The teats are funnel shaped with pointed tips (Singh *et al*., 2008). The famous dwarf variety of native cattle found throughout the Malnad region has been recently recognized by National Bureau of Animal Genetic Resources (NBAGR) of the Indian Council of Agricultural Research as a registered breed variety of the country.

Singh *et al*. (2008) studied the production parameters of Malnad Gidda cattle. The performance data is given in Table 2.1

Table 2.1 Production parameters of Malnad Gidda cattle

Trait	Mean ±SE
Birth weight (Kg)	8.56±0.44
Weight at maturity(Kg)	87.29±2.95
Age at first calving (months)	45.41±1.22
Age at first service in males (months)	38.06±1.24
Calving interval (months)	17.02±0.68
Service period (months)	8.38±1.03
Lactation period (months)	8.95±0.40
Dry period(months)	7.17±0.53
Daily milk yield(liters)	2.11±0.17
Peak yield (liters)	3.09±0.24
Lactation milk yield (liters)	569.13±46.24
No of services / conception	1.48±0.07

Fig. 1: Typical Malnad Gidda Bull

Fig. 2: Typical Malnad Gidda Cow

2.2 Molecular markers for genome analysis

A Molecular marker is defined as any stable and inherited variation detectable or measurable by a suitable technique and which can be used to detect presence of specific genotype or phenotype, which is otherwise not measurable or very difficult to detect. A piece of genetic material that bears or produces a distinctive feature is generally considered to be a marker, usually a mutant base, and is either dominant or recessive (Strickberger, 1985).

'Molecular markers' or 'genomic markers' originated during the recent past due to tremendous progress in the field of molecular biology. A large amount of data have been generated since the first demonstration of RFLP (Grodzicker *et al*.,1974) and the proposal to use them as markers in human genetics (Botstein *et al*.,1980), in genetic improvement of plants (Beckman and Soller,1983; Burr *et al.,*1983) and in animals (Beckman and Soller,1983). The use of PCR (Saiki *et al.,* 1988) to amplify a DNA sequence of interest and subsequent restriction enzyme analysis (RFLP) of the amplified product (Pinder *et al.,* 1991) represented a milestone in this endeavor. An unlimited number of genetic polymorphisms at DNA sequence level have provided a number of genomic markers such as RFLP's (Botstein *et al*., 1980), minisatellites or variable number of tandem repeats (VNTR) (Jeffrey *et al*., 1985; Nakamura *et al*., 1987), minisatellites (Litt and Lutty, 1989; Weber and May, 1989; Tautz, 1990; Fries *et al*., 1990) and RAPD markers (Williams *et al*., 1990; Welsh and McClelland, 1990).

The use of molecular markers to define the genetic makeup (genotype) and predict the performance of animal is a powerful aid in animal breeding (Beuzen *et al*.,

2000). The selection based on these markers is known as Marker Assisted Selection (MAS). Several candidate genes for milk qualities have been studied by various authors. BTN1A1 gene is one such candidate genes found to be associated with milk quality.

2.3 PCR – RFLP Technique

2.3.1 Polymerase Chain Reaction (PCR)

Mullis *et al*. (1986) developed the process known as polymerase chain reaction or PCR. This allows the amplification of particular regions of the DNA. In order to initiate the process of replication of the DNA, two informing sequence codes deno primers are required which promote the beginning and reversion of the reaction of the polymerase at particular locations of the genome. A reproduction or amplification of thousands of copies of a chromosomal region or gene of interest is obtained by repeated cycles of synthesis and denaturalization (chain separation) of the DNA using temperature changes. Since the primers are specific sequences to bond to a determined region of the DNA, only the specific amplification of the desired sequence of DNA is obtained.

2.3.2 Deoxy Ribonucleoside Tri Phosphates (dNTPs)

PCR is normally performed with dNTP concentration of around 100 μM, although at lower dNTP concentration (10 - 100 μM) Taq DNA polymerase has a higher fidelity. The four dNTPs at equal concentrations to minimize mis-incorporation errors and lowest possible dNTP concentration appropriate for the length and composition of the target sequence must be used (Innis and Gelfand, 1990).

2.3.3 PCR buffer and magnesium concentration

PCR buffer is an important component of PCR which affects the outcome of amplification. In particular, the concentration of $MgCl_2$ has a profound effect on the specificity and yield of amplification. Generally, excess Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield. Innis and Gelfand (1990) recommended a PCR buffer that contains 10 – 50 mM TrisHCl with pH between 8.3 and 8.8 at 20ºC. A magnesium concentration of 1.5 – 2.0 mM is optimal for most PCR products generated with Taq DNA Polymerase. Optimization normally involves supplementing the magnesium concentration in 0.5 or 1.0 mM increments (Sambrook *et al*., 2001).

2.3.4 Annealing temperature

Annealing temperature is one of the important thermal cycling parameter. It is the temperature at which primer anneals to template DNA. This temperature varies from primer to primer and is to be determined based on number of bases in the primer and percentage of GC content in the primer. A thumb rule formula- $T_m = (G + C) \times 4 + (A + T) \times 2$ is generally used for approximation of this temperature (Sharrocks, 1994).

2.3.5 DNA template

Use of high quality, purified DNA templates greatly enhance the success of PCR reactions. Optimum concentration of template DNA per reaction could vary substantially from typical conditions (100ng per reaction) depending on the use of primer - template combination (Sambrook *et al*., 2001).

2.3.6 Primers used for PCR

Generally the primers used in PCR are between 20 - 30 nucleotides in length with higher GC content, which allow a reasonably high annealing temperature to be used. Ideally, PCR primers should have a 40 – 60 per cent GC content, be similar in size (18-25 bases), T_m values and nucleotide ratios, and be free of repetitive motifs, palindromes, excessive degeneracy and long stretches of poly purines or poly pyrimidines.

Primer - dimer is an amplification artifact often observed in the PCR product, especially when many cycles of amplification are performed on a sample containing very few initial copies of template. It is a double-stranded fragment whose length is very close to the sum of the two primers and appears to occur when one primer is extended by the polymerase over the other primer.

2.3.7 Restriction endonuclease

Restriction endonuclease (RE) is a class of enzymes able to cleave DNA at a specific nucleotide sequence. In some bacteria they form part of defensive mechanism against infection by bacteriophage in which the bacterium will methylate specific sites in its own DNA to prevent cleavage by its intrinsic restriction enzyme which are still able to destroy foreign unmethylated DNA. Different enzymes, as obtained from a different range of organisms, have different specificities, often recognizing four or six base pairs. Because of this specificity, restriction enzymes cleave a sample of DNA into defined polynucleotide fragments which can be separated according to their length. This procedure is sufficiently sensitive to be able to detect a difference of one base pair in

certain circumstances and hence the enzymes are highly essential and a prerequisite for the procedure of genetic manipulation.

2.3.8 Restriction Fragment Length Polymorphism (RFLP)

Changes in the DNA sequence associated with an allelic change at the locus will be visualized by the altered mobility of restriction fragments on gel electrophoresis. Individuals carrying different allelic variants of the gene will show different band distribution patterns. This is the principle of Restriction Fragment Length Polymorphism (RFLP).

RFLPs occur as a result of DNA base changes, deletions, insertions or rearrangements that either create, eliminate or translocate restriction enzyme cleavage sites. RFLP denotes that a single restriction enzyme produces fragments of different lengths from the same stretch of genomic DNA of different strains of a species or from different related species.

If a recognition site is absent, then digestion with the relevant restriction enzyme will generate a long fragment. If a recognition site is present, then digestion with the relevant restriction enzyme will generate a shorter fragment. If the recognition site is in only one of two parental alleles, digestion will produce two different electrophoretic patterns (Botstein *et al*., 1980).

2.3.9 Principles of Restriction Fragment Length Polymorphism

RFLPs occur as a result of DNA base changes, deletions, insertions, or rearrangements that either create, eliminate or translocate restriction enzyme cleavage

site (Theilmann *et al*., 1989). A point mutation, deletion or insertion can create or abolish the recognition site for a particular RE at the locus and there by changes the size of the resulting restriction fragments. Inversion on the other hand, changes the distance between a pair of RE sites and therefore, causes changes in size of restriction fragments. The changes in DNA sequences associated with an allelic change at a locus can be visualized by the altered mobility of restriction fragments on gel-electrophoresis.

Individuals carrying different allelic variants of the gene will show different distribution patterns. These differences in band numbers and sizes result from change in fragment size and are termed as Restriction Fragment Length Polymorphism (RFLP). Such variants are inherited in a Mendalian fashion, since gene expression is not required for RFLP analysis, variation in the flanking regions or introns of genes may also be detected. Consequently, the RFLP approach for analysis of genomic variation is potentially powerful (Theilmann *et al*., 1989).

2.4 Bovine Butyrophilin (BTN1A1) gene

The first finding that linked the Bovine Leukocyte Antigen (BoLA) region to a single milk fat per cent was reported by Hines and Ross (1986). Despite the scanty density of mapped markers, it was the first study that conclusively established a discernible genetic influence of the BoLA region on a milk production trait. To date, the only two genes located in the BoLA region that are candidates to directly influence milk production traits are bBTN (Ashwell *et al*., 1996) and bPRL (Hallerman *et al.,* 1988).The role of BTN was a matter of speculation until the generation of an mBTN1A1 knockout mouse that revealed its importance for milk - fat secretion (Mather, 2000).

BTN1A1 is a candidate gene which is very important for milk yield and composition. The Butyrophilin (BTN) belongs to the immunoglobulin family of transmembrane proteins.

BTN1A1 gene has been found in mammalian species (Jack and Mather, 1990; Taylor *et al*., 1996) like cattle, human and mice. The bovine Butyrophilin gene located to chromosome 23 consists of 8 exon and 7 intron and is 7003 bp long gene fragment (Ashwell *et al*., 1996; Komisarek and Dorynek, 2003).

Table 2.2 Sizes of exons of the bovine BTN1A1 gene

Exons	1	2	3	4	5	6	7	8
Size (bp)	97	137	348	282	150	21	27	1741

Table 2.3 Sizes of introns of the bovine BTN1A1 gene

Introns	1	2	3	4	5	6	7
Size (bp)	598	378	1356	847	511	106	404

(Seyfert and Luthen, 1998)

BTN1A1 is a QTL candidate that affects economically important trait in dairy animal because it is specifically expressed in lactating mammary tissue and the gene product BTN1A1 may function in secretion of milk lipid (Zegeye, 2003). Many gene expression studies have confirmed the definite role of BTN1A1 in lipid secretion (Banghart *et al.,* 1998; Ogg *et al.,* 2004; Robenek *et al.*, 2006) and disease resistance (Jonchere *et al.*, 2010; Moyes *et al.*, 2011).

It has also been proved that the amount of bovine Butyrophilin mRNA expression increases exponentially during last 6 weeks of pregnancy in bovines, specifically in the lactating mammary epithelial tissue (Mather, 2000; Bionaz and Loor, 2008). BTN1A1 was mapped to the long arm of chromosome 23 (Frank *et al*., 1981). This gene expression is regulated at the transcription level by lactogenic hormones (Banghart *et al*., 1998). It is a type 1 transmembrane glycoprotein placed between plasma membrane and surface of fat droplets, synthesized from 526 amino acids and conserved in B30.2 domain in the cytoplasmic tail. It was identified as a main component of milk fat globule membrane (MFGM) that is important in secreting and stabilizing milk- fat droplets in milk.

The genetic variation in the bovine BTN1A1 gene has been exploited as a marker for QTL controlling milk yield and fat percentage (Ashwell *et al.*, 1996) as well as Somatic cell count (Heyen *et al.*, 1999; Komisarek *et al.*, 2006).

Gene BTN1A1 in cattle is located in the region of chromosome 23 q21 - q23, in which the presence of other important QTLs controlling milk yield and composition (Taylor *et al*., 1996; Ashwell *et al*., 1997; Zhang *et al*., 1998) and SCC (Ashwell *et al*., 1996; Heyen *et al*., 1999) has been identified. Most of them like the BoLA complex were found to influence the SCC, whereas, genes like the prolactin (PRL) influence the milk yield and fat composition.

Although several polymorphic forms of BTN1A1 have been identified (Taylor *et al*., 1996; Seyfert and Luthen, 1998; Husaini *et al*., 1999; Zegeye *et al*., 1999), studies on their impact on production traits in cattle are very scanty and have not been confirmed conclusively.

2.4.3 Role of Butyrophilin protein

Milk fat in the lactating cow is secreted in the udder as micro lipid droplets which are encircled by a special membrane composed of lipid bilayer and proteins. This membrane has been designated as Milk Fat Globule Membrane (MFGM) which is important in secreting and stabilizing of milk fat droplets in milk. It comprises 34-43 per cent of the total MFGM proteins in the Holstein- Friesian cows and approximately 20 per cent in the milk of Jersey cows (Mather, 2000).

Substantial increase in knowledge of the intracellular origin of lipid droplets, the identity and potential function of MFGM proteins in milk - lipid secretion and molecular determinants involved in the process of lipogenesis has been advanced in the past decade (Mather, 2000; Keenan, 2001; McManaman *et al*., 2007). Secretion of the fat globule in milk is primarily based on attaining proper shape of the fat droplets in which Butyrophilin protein plays an important role during the process of lactogenesis (Bhattacharya *et al.*, 2004; Robenek *et al*., 2006).

Bovine Butyrophilin consists of 526 amino acids (~56 kDa) and the first 26 of them constitute a signal peptide (Jack and Mather, 1990). Butyrophilin protein exhibits a domain character and is strongly connected with the cell membrane (Jack and Mather, 1990).

Butyrophilin is a type I transmembrane glycoprotein (66 - 67 kDa molecular weight) containing an N - terminal exoplasmic domain with two Ig - like folds and a C - terminal cytoplasmic domain with a highly conserved B30.2 region which is involved in interactions with other proteins like xanthine - oxidase and adipophilin (Henry *et al.*,

1998; Magdalena *et al*., 2008). It has been found that increase of BTN correlates with the onset of milk fat secretion towards the end of pregnancy and is maintained throughout lactation (Banghart *et al.*, 1998).

The unique location of Butyrophilin protein within the cell, the domain structure of the protein and site - specific expression occurring only during lactation indicate that Butyrophilin protein is of great importance in milk lipid secretion process by steering the extrusion process of the milk - fat globules (Ogg *et al*., 2004; Heid and Keenan, 2005; Bauman *et al*., 2006; Anderson *et al*., 2007). Based on the expression profiles of Butyrophilin (BTN1A1) and Xanthine Oxidoreductase (XO) during pregnancy and lactation, and the phenotypes of Btn-/- and Xo+/- mice, it has been postulated that specific interaction between BTN and XO is required to form signalling complex. Formation of this 'supra - molecular complex' among BTN1A1 and XO proteins with other proteins on the surface of cytoplasmic lipid droplets, such as Adipophilin (ARDP) is an essential step in the assembly of the MFGM and subsequent expulsion of lipid droplets from the cell (Valivullah and Keenan, 1989; Jeong *et al.*, 2009). Some researchers have also proposed an alternate view that milk fat globule secretion is controlled by interactions between plasma membrane and Butyrophilin in the secretary granule phospholipid monolayer (Robenek *et al*., 2006).

These results strongly suggest that Butyrophilin protein function in the secretion of lipid droplets and from a practical standpoint, these results highlight the importance of the MFGM in stabilizing lipid droplets in milk and the consequences due to weakening or disruption of this membrane. The BTN1A1 gene is thus an obvious candidate to be

included in genetic screens of dairy cattle for milk quality and production traits (Bauman *et al.*, 2006).

2.5 PCR amplification of Butyrophilin gene

Bhattacharya *et al*. (2006) studied polymorphism of Butyrophilin gene and its association with milk quality traits in cross bred cattle. A 501bp Butyrophilin gene fragment covering part of exon 8 was amplified. The PCR amplification was performed in a total volume of 25µl with 100 ng of genomic DNA. The thermal cycling conditions were, denaturation at 95ºC for 2 minutes, 39 cycles including 30 seconds at 95ºC, 60 seconds at 65º, 30 seconds at 72ºC and final extension of 5 minutes at 72ºC.

Sadr *et al.* (2008) studied polymorphism of Butyrophilin gene in Najdi cattle of Iran. They amplified fragment of exon 8 of Butyrophilin gene in a final volume of 25µl containing 33.3 ng of genomic DNA. The PCR conditions included, an initial denaturation at 95ºC for 5 min, followed by 33 cycles at 95ºC for 30 seconds, 59ºC for 30 seconds and 72º C for 2 minutes, followed by an final extension of 72ºC for 7 minutes. The PCR products were separated by 1.2 percent (w/v) agarose gel electrophoresis.

Magdalena *et al.* (2010) studied the associations between two polymorphisms localized in the seventh exon of the Butyrophilin gene (BTN1A1/Hae*III* and BTN1A1/*SchI*) with milk production traits in 171 Jersey cattle. They amplified a 568bp fragment of seventh exon of Butyrophilin gene in a final volume of 20µl containing a 60ng of genomic DNA. The amplification profile used were, initial denaturation of templates for 5min at a temperature 94ºC, followed by 35 cycles of repeated ,

denaturation at 94ºC for 60 seconds, annealing at 64ºC for 60 seconds , extension at 72ºC for 90 seconds , a final extension at 72ºC for 5 minutes.

Rangarajan (2012) studied the polymorphism of exon 8 and exon 3 of Butyrophilin gene and their association with milk production traits and somatic cell count in Holstein Friesian cross bred and Deoni cattle. They followed the thermal cycling conditions of initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 57ºC for 1 min, extension at 72ºC for 1 min and final extension at 72ºC for 5 min.

Yardibi *et al.* (2013) studied the polymorphism of Butyrophilin gene in East Anatolian and South Anatolian Red cattle breeds. They amplified an 893 fragment of exon 8 of Butyrophilin gene in a final volume of 25µl containing 50 to 100 ng of genomic DNA. For BTN1A1, the amplification conditions followed were, initial denaturation at 95ºC for 5min, followed by 33 cycles at 95ºC for 30 seconds ,annealing at 59ºC for 5min and extension at 72ºC for 2 min followed by a final extension at 72ºC for 7 minutes.

2.6 Milk

Milk is the only diet whose sole function in nature is food. The role of milk is to provide nourishment and protection for the mammalian young. Milk also has been a food source for human beings since the dawn of history. Milk is a very complex food with over 100,000 molecular components.

2.6.1 Milk fat

Fat is the most important constituent of milk as it is used as a basis for fixing the purchase and sale price of milk. Fat is the most variable component in milk in both their concentration and chemical composition, whether inter- or intra-species differences are considered. It ranges from about one per cent in donkey milk to more than 50 percent in aquatic mammals.

Milk fat is the major source of lipid used by the mammalian new born for accumulating body adipose tissue. The major lipids in milk fat are triglycerides, which are composed of three fatty acids covalently bound to a glycerol molecule by ester bonds. The remainder of the lipid fraction consists mainly of diacylgylcerols, cholesterol, phospholipids and free fatty acids. Fat in milk is present as fat globules ranging from 0.1 to 15 mm in diameter. These fat globules or droplets are covered by a thin membrane whose properties are different from both milk fat and plasma. The fat globule membrane helps to stabilize the fat globules in an emulsion within the aqueous environment of the milk.

The bulk of the fat in the milk exists in the form of small globules, which average approximately two to five microns in size. This is an oil-in-water type emulsion. The surface of these fat globules is coated with an adsorbed layer of material commonly known as the fat globule membrane.

This membrane contains phospholipids and proteins in the form of a complex and stabilizes the fat emulsion. In other words, the membrane prevents the fat globules from coalescing and separating from one another.

The emulsion may, however, be broken by agitation (at low temperature), heating, freezing etc. Chemically, milk fat is composed of number glyceride-esters of fatty acids. Milk fat on hydrolysis gives a mixture of fatty acids and glycerol. The fatty acids are saturated or unsaturated fatty acids. Saturated fatty acids are relatively stable. The fat associated substances are phospholipids, cholesterol, carotene and fat soluble vitamins (A, D, E, and K).

2.6.2 Solid –not-Fat

Milk with high solids-not-fat is valuable to the consumer for its flavor and nutritional value and to the manufacturer of milk products, especially relating to cheese yield. Solids-not-fat consists of all solids in milk other than fat.

2.7 Genetic polymorphism of BTN1A1 gene and its association studies with milk quality traits

Pareek *et al*. (2002) associated the allele variants of BTN1A1 encoding gene with milk production traits among Polish Black and White and German HF population.

They found a significant association between the protein sequence polymorphism and milk fat percentage. In a study conducted on 131 Holstein Friesian bulls, genotype LL was characterized by a higher breeding value for milk fat content than animals with LA genotype in exon 8 region (Komisarek and Dorynek, 2003).

Bhattacharya *et al.* (2006) carried out *Hae* III PCR - RFLP of BTN1A1 gene exon 8 in crossbred cattle and observed three genotypes, AA, BB and AB with frequencies of 0.78, 0.17 and 0.04 respectively. The gene frequencies of A and B alleles were found to

be 0.87 and 0.13 respectively. Komisarek *et al.* (2006) analyzed the relationship between two SNPs of the *BTN1A1* gene in exons 3 and 8 and revealed that K468R in Exon 8 is more likely the causative mutation affecting milk traits in cattle. They observed that for K468R mutation, the AA genotype was characterized by significantly higher milk, fat, and protein yields than the AG and GG genotypes.

Sadr *et al.* (2008) studied the DNA polymorphism of the BTN1A1 gene in Iranian Najdi cattle through semi - nested PCR - RFLP method and found that the 893 bp Butyrophilin gene fragment (exon 8) was polymorphic in those cattle population.

Magdalena *et al*. (2010) studied the associations between two polymorphisms localized in the seventh exon of the Butyrophilin gene (BTN1A1/Hae*III* and BTN1A1/*SchI*) with milk production traits of Jersey cattle and found that GG /AG combined genotype was characterized by a significantly lower fat percentage in comparison to other combinations.

Rangarajan (2012) studied the polymorphism of exon 8 and exon 3 of Butyrophilin gene and its association with milk production traits and somatic cell count in 150 Holstein Friesian cross bred cows and 51 Deoni cows. They observed that there was no significant difference between the total daily fat percentage and BTN1A1 genotypes in both the breeds, for exon 3. However, for exon 8 they observed that, in both the breeds, the animals with AA genotype showed a higher milk fat percentage and the animals with BB genotype showed lower values of milk fat percentage.

Yardibi *et al. (*2013) studied BTN1A1, gene polymorphism in East Anatolian red cattle breed and South Anatolian red cattle breed. They observed three genotypes following amplification of a region of BTN1A1 gene. The overall frequency of A allele was significantly higher than that B allele.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental animals

The present investigation was carried out on 50 lactating Malnad Gidda cattle; randomly selected from Uttara Kannada and Chickmagalur districts of Karnataka.

3.1.2 Chemicals and reagents

The chemicals and reagents used in the present study were of molecular biology grade and are presented in Annexure 1 and 3.

3.1.3 Primers

The primers required for the amplification of desired gene in this study were procured from Sigma, Bangalore.

3.1.4 Glassware and plastic-ware

All the glassware were thoroughly cleaned and sterilized as per the standard procedures. Micro-centrifuge tubes and micro tips were autoclaved before use.

3.1.5 Equipment

The major equipment used in the present study is presented in Annexure 2.

3.2 Methods

3.2.1 Blood collection

About 10 ml of blood was collected from each animal aseptically from jugular vein in a vacutainer tube containing 0.5 per cent EDTA. The samples were stored at 4°C and DNA isolated within 24 hrs.

3.2.1.2 Milk collection

About 100 ml of milk was collected from the same Malnad Gidda cattle from which the blood sample collected. The milk was collected in a tube containing a drop of hydrogen peroxide as a preservative.

3.2.2 Isolation of genomic DNA from venous blood

DNA isolation was carried out by adopting High salt method as prescribed by Miller *et al.* (1988). Five ml blood was taken in a 15 ml centrifuge tube. Five ml of RBC lysis buffer was added into it and inverted several times and incubated at 4ºC with occasional mixing for complete lysis of cells. The tubes were centrifuged at 4,000 rpm at room temperature for 10 min and the buffy coat was removed along with small quantity of RBC and plasma and transferred to another centrifuge tube. The buffy coat was resuspended in four volumes of RBC lysis buffer and kept at 4ºC for five minutes with occasional mixing. The mixture was centrifuged at 2000 rpm for five minutes. This step was repeated three to four times until a clear nuclear pellet obtained. The resulting pellet was suspended in five ml of TE (pH 8.0) buffer and carefully disturbed with tip of a pipette. Proteinase-K and Sodium Dodecyl Sulphate (SDS) were added at a final concentration of 0.2µg/ml (15µl) and 0.5per cent (0.5ml), respectively. The mixture was

incubated at 37ºC for overnight with occasional mixing. After the incubation, two ml of 5M NaCl solution was added and mixed thoroughly. One volume of Chloroform: Iso amyl alcohol (24:1) was added and mixed thoroughly by brief vortexing on a cyclomixer for one minute. The mixture was centrifuged at 6000 rpm for 10 minutes. The upper aqueous phase was transferred carefully using a Pasteur pipette to a fresh tube. Finally, two volumes of chilled absolute alcohol (ethanol) was added to the supernatant containing DNA at room temperature and inverted several times until the DNA was precipitated.

Precipitated DNA strands were transferred to a micro centrifuge tube containing one ml of ice-cold, 70 per cent ethanol and centrifuged at 2,000 rpm for five min. Supernatant was discarded and the pellet air - dried. Dried DNA pellet was resuspended in 400 µl TE buffer and kept at 65^0C for 15 min. The samples were stored at -20°C till further use.

3.2.3 Estimation of quality and quantity of genomic DNA

The quality, purity and concentration of DNA were checked using UV - Spectrophotometer. About 30 µl of DNA was dissolved in 2970 µl of triple glass distilled water (1 in 100 dilutions) in a cuvette and optical density (OD) values measured at 260 nm and 280 nm with distilled water as blank. DNA samples having the OD_{260} /OD_{280} ratio between 1.7 and 2.0 were used for further PCR reaction. The concentration of DNA was estimated using the following formula:

$$\text{Concentration of DNA (ng)} = OD_{260} \times 50 \times 100 \text{ (dilution factor)}$$

3.3 Agarose gel electrophoresis

Agarose gel electrophoresis (0.8%) was carried out for confirming the quality of isolated DNA. Appropriate amount of agarose was dissolved in 1X tris boric acid EDTA (TBE) buffer to make a final concentration of 0.8%.The agarose was melted in a microwave oven. Ethidium bromide was added at a concentration of 0.5µg/ml of agarose gel solution and the solution allowed cooling sufficiently. The gel tray was sealed on either side by using adhesive tape and the comb placed in proper position. The melted agarose solution was poured in to the gel tray carefully, avoiding formation of air bubbles.

After the gel had been polymerized, the comb and the seal on either side of the gel tray were removed carefully. The gel tray was kept in an electrophoresis tank and 1X TBE buffer was poured to submerge the gel in the tank.

The DNA samples were mixed with $1/6^{th}$ volume of 6X gel loading buffer and loaded in to the wells using pipette. The gel was electrophoresed in 1X TBE buffer at 100 volts at room temperature for about half an hour. The gel was visualized under UV trans-illuminator and photographed using gel documentation system.

The quality of the DNA was assessed based on the bands developed. Good quality DNA was used for further study.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 PCR amplification of Butyrophilin gene

A 893 fragment of exon 8 of BTN1A1 gene was amplified by Polymerase Chain Reaction.

3.4.2 PCR primers

The details of the oligonucleotide primers (Sadr *et al.* 2008) used for PCR are given in Table 3.1.

3.4.3 Composition of PCR reaction mixture (PCR)

PCR was carried out in a reaction mixture of 25µl. A master mix for a minimum of 10 samples was prepared and aliquoted into 23µl in each PCR tube. One µl of genomic DNA and one µl of Taq polymerase enzyme were added to make a final volume of 25µl.The composition of reaction mixture for PCR are given in Table 3.2.

3.4.4 Preparation of template DNA for PCR

The stock DNA was then diluted with autoclaved distilled water to obtain working solution at a final concentration of 50 ng / µl.

3.4.5 PCR methodology

1. PCR tubes containing the mixture were tapped gently and quickly and spun at 10,000 rpm for a few seconds.
2. Allele specific PCR conditions were optimized for amplification of gene specific products; the thermal cycles were repeated for 35 times to obtain sufficient PCR amplification for PCR-RFLP analysis.

Table 3.1 Sequences of primers used for amplification Butyrophilin gene

Primer	Primer sequence	Expected product size
Forward	BTN-(5'-TCCCGAGAATGGGTTCTG-3')	893bp
Reverse	BTN-(5'-ACTGCCTGAGTTCACCTCA-3')	

Table 3.2 Composition of reaction mixture for PCR

Sl. No.	Components	Quantity
1	Sigma water	19µl
2	Buffer (10X) with $Mgcl_2$	2.5µl
3	2.5 Mm dNTPs (100 µM each)	1.0 µl
4	Forward Primer (20 pmol / µl)	0.25 µl
5	Reverse Primer (20 pmol / µl)	0.25 µl
6	DNA Template (50 ng / µl)	1.0 µl
7	Taq DNA Polymerase (3 i.u / µl)	1.0 µl
	Total Volume	25.0 µl

3.4.6 Agarose gel electrophoresis to check PCR product amplification

After the PCR, the tubes were removed from the Thermal cycler and placed in freezer till further use. The amplified products were checked on 1.5 per cent agarose gel.

1. The correct amount of agarose (1.05 grams) was weighed and placed in a conical flask and 70 ml of 1X TBE buffer added to prepare 1.5 per cent solution. It was placed in a microwave oven until the agarose dissolved completely.
2. The solution was cooled to 60^0C and Ethidium bromide (0.5 µg / ml) added and mixed thoroughly.
3. The comb was positioned 0.5-1 mm above the agarose plate so that a complete well was formed when the agarose was added.
4. As soon as the gel was completely set after 30 - 45 min at room temperature, both comb and tape were removed carefully from the plate and the gel was placed in the electrophoresis tank with TBE buffer.
5. Eight µl of PCR product from each tube was mixed with 5 µl of loading dye and electrophoresed at 100 V for 30 minutes in 0.5 X TBE buffer.
6. DNA marker (100 bp DNA ladder) was also loaded and run along with the samples in the same gel.
7. After half an hour, the power supply was turned off and the lid was removed from the gel tank. The gel was visualized as a single compact band of expected size under the UV trans-illuminator and photographed using gel documentation system.
8. The quality of the DNA was assessed based on the bands developed. Good quality DNA was used for further analysis for PCR - RFLP technique.

Table 3.3 Thermal cycling conditions for amplification of exon 8 of Butyrophilin gene

Sl. No.	Step	Temperature	Time
1	Initial denaturation	94°C	5 min
2	Denaturation	94°C	50 sec
3	Annealing	58°C	40 sec
4	Extension	72°C	1 min
Repeated the steps 2 to 4 for 35 times			
5	Final Extension	72°C	5 min
6	Hold	4°C	-

3.5 RFLP analysis with *Hae* III

*Hae*III is one of the restriction enzymes isolated from the *Haemophilus aegyptius* bacteria. This enzyme cleaves the DNA at the positions where the GGCC sequence is found. The cleavage occurs between the second and the third nucleotides (G and C) are given in Table 3.4. The *Hae*III cuts both strands of DNA at the same location, yielding restriction fragments with blunt ends.

3.5.1 Restriction digestion of PCR products

The PCR products were digested with *Hae* III restriction endonuclease at 37º C for 16 hrs, with the composition of digestive mixture as indicated in Table 3.5.

3.6 Agarose gel electrophoresis of RFLP products

The restriction enzyme digested PCR products were separated on three per cent agarose gel electrophoresis. The restriction pattern resolved by agarose gel electrophoresis was photographed by Gel documentation system and the fragment sizes determined in comparison to DNA molecular weight markers by using the Image Lab Version 4.0 software.

3.7 Determination of Gene and Genotype frequency

The genotype was determined by scoring the bands under the gel doc system. The gene and genotype frequencies were calculated by the formulae for counting the number of heterozygotes and homozygotes.

Table 3.4 The Restriction enzyme used to digest PCR products

S. No	Restriction enzyme	Recognition site
1	*Hae III*	5'...GGCC ...3' 3'...CCGG ...5'

Table 3.5 Optimized RE digestion mixture

Sl. No	Reaction components	Quantity
1	Sigma water	3µl
2	10× assay buffer for RE	1.5µl
3	RE	0.5µl
4	PCR product	10µl
5	Total	15µl

The genotype frequencies were calculated directly by counting the bands appearing in the gels.

$$\text{Genotype frequency} = \frac{\text{Proportion of animals with particular genotype}}{\text{Total number of animals}}$$

$$\text{Frequency of an allele} = \frac{(2 \times \text{no. of homozygotes}) + (\text{no. of heterozygotes})}{2 \times \text{total no. of individuals}}$$

3.8 Sequence analysis

3.8.1 Sequencing of PCR products

The samples which showed different RFLP patterns were selected for sequencing. PCR products were sent to Chromous Pvt Ltd, Bangalore for sequencing and the results analyzed.

3.8.2 DNA sequence analysis

The BTN1A1 sequences were analyzed by BLAST (Basic Local Alignment Search Tool) search at NCBI site for homology using BLAST programme. The BLAST programme compares a nucleotide query sequence against nucleotide sequences in data base.

3.8.3 Establishment of gene identity

The BLAST analysis was used to find variation in the study with the reference sequences in bovines and with sequences of Butyrophilin gene of other species.

3.9 Estimation of milk constituents (Fat per cent, SNF and Total Ash)

About 100 ml of milk samples were collected from lactating cows of Malnad Gidda purebred cattle to estimate the fat per cent, Solid-not-fat and total ash. The milk samples were collected from all those animals from which blood samples were taken.

3.9.1 Estimation of fat in milk Samples (Gerber method)

Fat percentage of milk samples was determined using standard method of butyrometric fat determination in milk (Sulphuric acid process) according to Gerber (1935).

The fat was separated from milk through the addition of sulphuric acid, followed by addition of amyl alcohol and centrifugation. The fat content was read directly on a specially calibrated butyrometer after centrifugation.

Procedure:

1. The milk sample (temperature about 20°C) was mixed thoroughly, taking care to minimize incorporation of air. The sample was allowed to stand for a few minutes to discharge any air bubbles and gently mixed again before pipetting.
2. The following reagents were sequentially pipetted into the butyrometer:
    - 10 ml of 90 per cent sulphuric acid
    - 10.75 ml milk (must not be mixed with the acid)
    - 1 ml amyl alcohol
    - 3 - 4 drops of distilled water

3. The butyrometer was closed with caoutchouc stopper and shaken until the milk got dissolved. The butyrometer was then turned upside - down for five to six times.
4. It was then spun in the Gerber centrifuge for 15 min.
5. The fat column was adjusted by using the stopper, so that it was at the graduated part of the butyrometer. The fat content was read directly on a special calibrated butyrometer after centrifugation.

3.9.2 Estimation of Solid-not-Fat

Milk SNF comprises protein, carbohydrates, vitamins, minerals, etc, in milk other than milk fat. Lactometer reading was taken for all milk samples and SNF per cent was calculated by using the Formula method by calibrating Lactometer at 27º C which had graduation from 20º C to 35º C corresponding to specific gravity of 1.020 and 1.035.The properly mixed sample was poured into a cylinder (100ml) ensuring that some milk over flowed when the lactometer was introduced. The lactometer was given a gentle push at the top and reading was taken at stand till position. Simultaneously, temperature of the milk in the cylinder was noted down. The Solid-not-fat (SNF) content was calculated by using the formula-

$$\mathbf{SNF\% = CLR/4+0.2F+0.14}$$

Where CLR = Corrected Lactometer reading at 27ºC and F is Fat%

3.9.3 Ash content

Ash content of milk was estimated as per the method given in AOAC (2000). A well-mixed sample was accurately weighed into a silica dish and evaporated to dryness. The residue was ignited and then placed in a muffle furnace at a temperature not more than 550º C for 3-4 hours. It was cooled in desiccators and weighed.

3.10 Statistical analysis

The observed genotype frequencies based on RFLP patterns were tested for Hardy – Weinberg equilibrium by Chi-square test. The observed and expected frequencies were analyzed for goodness of fit at probability $p \leq 0.05$.The significance of differences in means observed in milk quality parameters between the genotypes was tested by Student‘t’ test.

Results

4. RESULTS

4.1 Isolation, yield and purity of genomic DNA

The Miller's High Salt method (Miller *et al*., 1988) was utilized for isolating the genomic DNA from blood samples of Malnad Gidda cattle.

About 300 µg of pure genomic DNA was extracted by this technique from 10 ml of blood sample. The ratio of optical density at 260 and 280 nm ranged from 1.72 to 2.0 with a mean of 1.867 ± 0.006, which was indicative of good purity and good deproteinization.

The quality of isolated DNA was confirmed by agarose gel electrophoresis (0.8%) and was of high molecular weight and appeared as single bands without sheared fragments (Fig. 3).

4.2 PCR amplification of Exon 8

The composition of PCR master mix and conditions required for thermal cycling were optimized for PCR amplification of exon 8 of *BTN1A1* gene. The optimum annealing temperature was found to be 58°C.

The amplified products were checked on 1.5 per cent agarose gel. The amplified product size was 893bp in all the samples studied (Fig. 4).

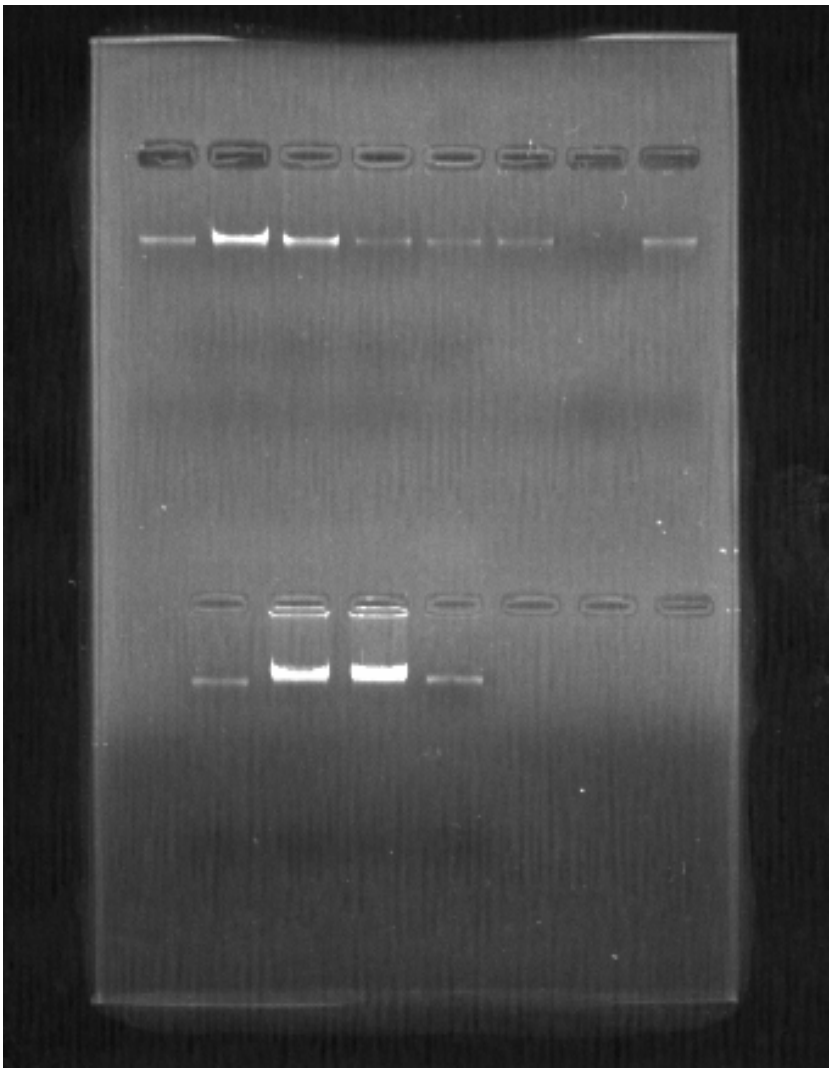

Fig. 3: Genomic DNA extracted from blood samples of Malnad Gidda cattle and run on 0.8% agarose electrophoresis.

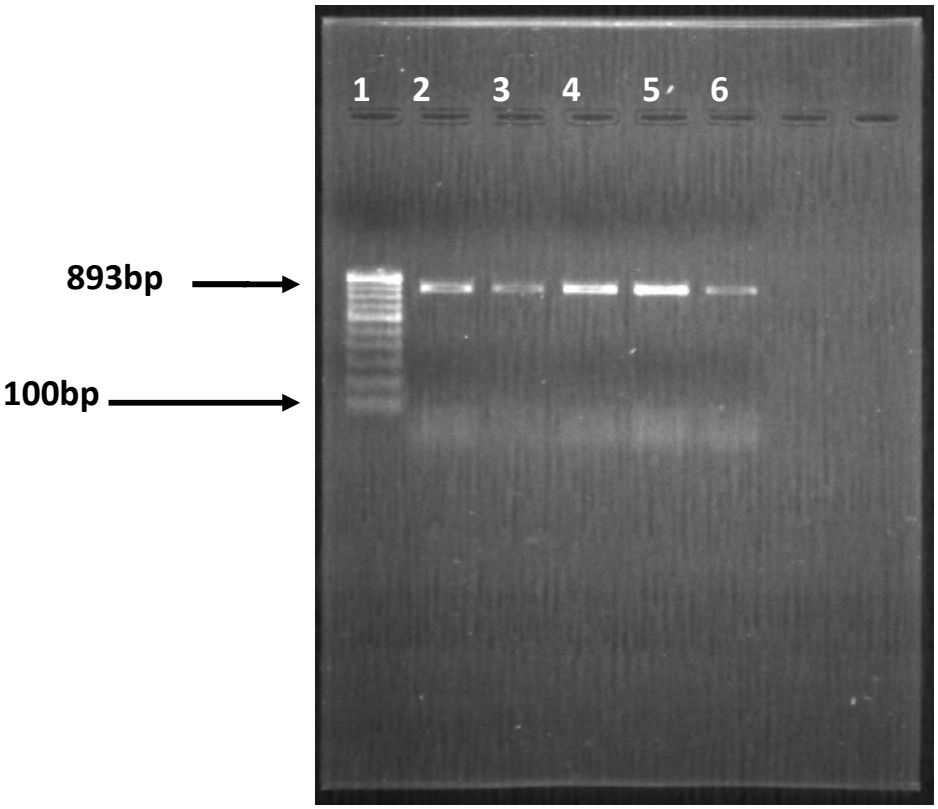

Fig. 4: Agarose gel electrophoresis showing 893 bp fragment of exon 8 of BTN1A1 gene

Lane 1 : 100 bp DNA ladder

Lane 2-6: PCR amplicons

4.3 Genotyping of BTN gene exon 8 in Malnad Gidda cattle

The genetic characterization of exon 8 of BTN1A1gene in Malnad Gidda cattle was carried out using PCR - RFLP technique. The gel photographs showing representative banding patterns and indicating the different genotypes observed are shown in Fig. 5. The allelic and genotypic frequencies of Exon 8 obtained through PCR - RFLP analysis are presented in Table 4.1.

The PCR amplified products of exon 8 were digested with *Hae*III restriction enzyme, followed by separation of restriction digested DNA fragments on three per cent agarose gel. Two different genotypic patterns were obtained for exon 8.

The 893bp PCR amplified product was digested into restriction pattern, which was denoted as AA, had fragments of sizes 371, 231, 185, 83 and 23 bp. The second pattern denoted as AB, had fragments of 338 bp in addition to the fragments specified under AA genotype. Since the sum of the fragments sizes exceeded the PCR product size in the second genotype, this allele pattern was considered as, heterozygous, AB type. No animal showed BB homozygous type.

4.4 Allelic and genotype frequencies

The RFLP data analysis indicated that the frequencies of two genotypes, AA and AB were 0.76 and 0.24 respectively. There were 38 animals with AA genotype and 12 with AB genotype. The expected numbers for AA, AB and BB genotypes to be under Hardy-Weinberg equilibrium were 39, 10 and 1.The Chi-square analysis of observed and expected frequencies revealed that the studied population was under Hardy- Weinberg equation as the Chi-square value (0.1366) was not significant.

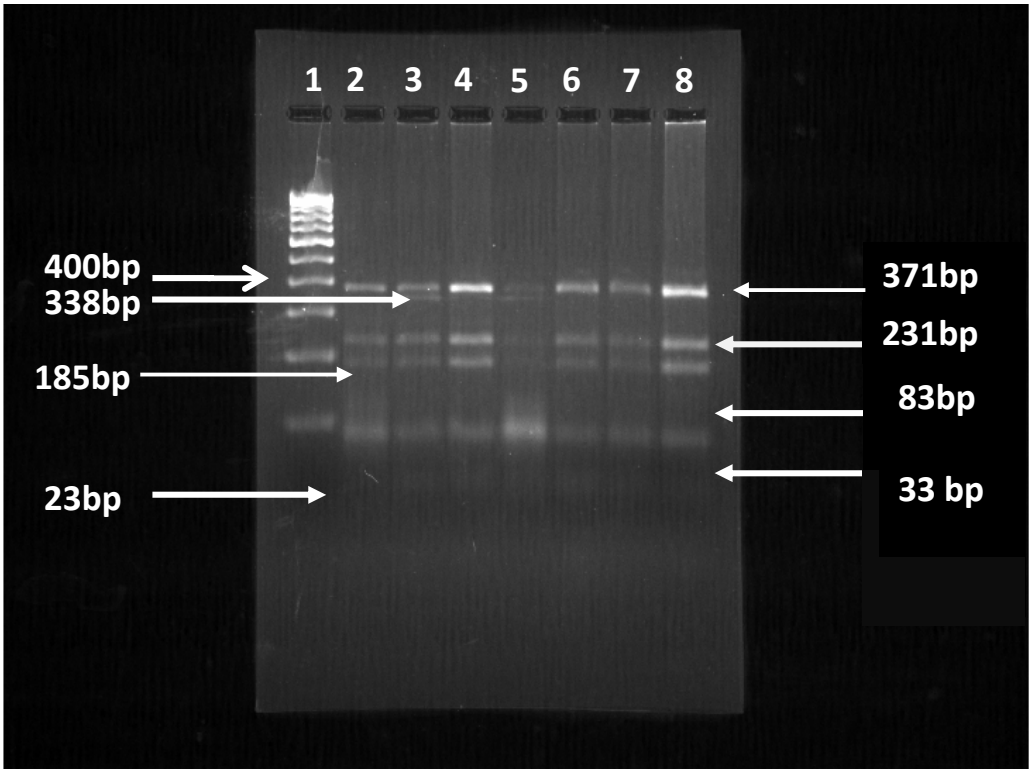

Fig. 4: *Hae* III digested PCR - RFLP patterns of 893 bp fragment of BTN1A1 exon 8 of Malnad Gidda cattle

Lane 1: 100 bp DNA ladder

Lane 2,4,6,7 & 8: AA genotype

Lane 3 and 5: AB genotype

4.5 Milk quality parameters and their association with BTN1A1 alleles

The average fat percentage of animals with AA genotype was 5.69± 0.06 and that of AB genotype was 5.25 ± 0.14. The‘t’ test analysis revealed that the fat percentage was significantly higher in AA genotype than AB genotype.

The SNF values for AA and AB genotype were 8.64 and 8.47 per cent respectively. The SNF percent of AA genotype was significantly higher than that of AB genotype.

The total ash contents were 0.90 and 0.96 per cent, respectively in AA and AB genotype and the difference was non-significant.

Table 4.1 Genotype and allelic frequencies of BTN1A1/*Hae* III polymorphism in Malnad Gidda Cattle

Genotype	Observed frequency	Expected frequency	Alleles	Allele frequency
AA	0.76 (38)	0.58 (39)	A	0.88
AB	0.24 (12)	0.36 (10)		
BB	-	1	B	0.12

Table 4.2 Allelic pattern of BTN1A1 gene digested with *Hae*III restriction enzyme in Malnad Gidda cattle

Sl. No	Allele type	Fragment size (bp)	Number of animals (n=50)
1.	AA	371,231,185,83,23	38
2.	AB	371,338,231,185,83,33,23	12

Table 4.3 Estimated milk parameters of Malnad Gidda cattle

Genotype	**Fat %**	**P value**	**SNF**	**P value**	**Total Ash**	**P value**
AA	5.69 ± 0.06^{a}	0.002	8.64 ± 0.01^{a}	0.000023	0.90±0.017	0.12
AB	5.25 ± 0.14^{b}	0.002	8.47 ± 0.02^{b}	0.000023	0.96±0.055	0.12

Means with different superscript differed significantly ($P < 0.05$)

4.6 DNA Sequence analysis

On the basis of the RFLP patterns the different alleles were selected for DNA sequencing. These alleles were sequenced by Sanger's dideoxy chain termination method in an automated DNA sequencer**.**

The sequence data was subjected to NCBI BLASTn and was compared with data available in the public domain for bovine species and also for other species. It was confirmed that the amplified product was that of BTN1A1 gene fragment. The BLAST analysis of Malnad Gidda Cattle revealed 100 per cent identity with that of *Bos taurus* gene encoding Butyrophilin and 99 per cent identity with Sahiwal cattle breed. The BLAST analysis also had 99 per cent identity with that of *Bubalus bubalis,* 98 per cent with *Ovis aries* and 97 per cent identity with *Capra hircus* (Table 4.4).

Clustal 2.1 software was used to find out the variation between the Malnad Gidda and *Bos indicus* Sahiwal Butyrophilin gene sequence. Multiple sequence alignment showed that there was a difference between the Malnad Gidda and Sahiwal Cattle amino acid sequence. The amino acid alignment revealed that the nucleotide substitution from A to G in the fragment resulted in amino acid change from iso leucine to valine at 475^{th} position (Annexure 5)

No difference was observed on comparison of BTN1A1 exon 8 DNA sequence of Malnad Gidda with that of *Bos taurus* data available in the public domain (Annexure 4).

Table 4.4: Results of BLASTn showing the percentage of identity of the exon 8 region of the BTN1A1 locus of Malnad Gidda with other species

Accession no	Description	Max Score	Total Score	Query coverage (per cent)	Max Identity
XM002697557.3	*Bos taurus* Butyrophilin gene	1648	1648	100	100
Z93323.1	*Bos taurus* gene encoding Butyrophilin gene	1648	1648	100	100
AY 860625	*Bos indicus* Sahiwal BTN1A1 gene	920	920	56%	99
AY860619.1	*Bos indicus* Hariana BTN1A1 gene	920	920	56%	99
AY491468.1	*Bos indicus* Tharparkar BTN1A1 gene	904	904	56%	99
AY491471.1	*Bubalis bubalis* (Murrah)	893	893	56%	99
AY491472.1	*Bubalis bubalis* (Bhadawari)	887	887	56%	99
AY491474.1	*Bubalis bubalis* (Surti)	883	883	56%	98
EF102891.1	*Capra hircus*	1500	1500	100%	97
AY491475.1	*0vis aries*	876	876	56%	98

Discussion

5. DISCUSSION

The present study was undertaken with the perspective of gaining information on genetic polymorphism of Butyrophilin gene and its association with milk quality traits in Malnad Gidda cattle, a native cattle breed of Karnataka. Malnad Gidda is a low milk producing breed which is known for disease resistance and believed to have some medicinal property in its milk. Milk quality is one of the important traits to be considered for improvement of production in cattle. Furthermore, Butyrophilin gene is considered as an important marker gene influencing dairy traits, variation in these loci would be an important tool in selection for dairy traits.

5.1 Isolation of genomic DNA

In the present study, the high salt DNA extraction procedure (Miller *et al.,* 1988) was used for the isolation of high molecular weight genomic DNA from blood samples. Better results were obtained, when the incubation temperature was maintained at 37º C for 12 to 16 hours instead of incubating at 55º C for two hours and again at 65º C for 15 minutes.

Several techniques are available for isolation of genomic DNA from blood and other tissues. Different laboratories follow different techniques, depending upon the facilities available. The high salt method is safer as compared to phenol- chloroform method as it does not involve phenol, which is corrosive. The high salt method gives more yields as compared to phenol- chloroform method (Annapoorani, 1996), which may be due to loss of DNA in phenolic phase.

5.1.1 Yield and quality of DNA

About 300 µg of pure genomic DNA was extracted from 10ml of blood sample by high salt DNA extraction procedure. Mitra (1994) obtained higher yields of 343 to 693 µg with an average of 403 µg per 10 ml of cattle blood. Senthil (1995) reported 450 µg to 800 µg with an average of 625 µg per 15ml of cattle blood using similar protocol.

Aravindakshan (1997) reported pure DNA in the range of 246 µg to 572 µg with an average of 360 µg per 10 ml of cattle blood. By use of same protocol, Nagaraja (1998) extracted about 400 µg of pure genomic DNA from 10-15 ml of cattle blood samples. The quality of the extracted DNA was found to be satisfactory.

5.2 PCR-RFLP of BTN1A1 gene

5.2.1 PCR amplification

The PCR technique as described by Sadr *et al.* (2008) and Yardibi *et al.* (2013) was followed for amplifying an 893bp fragment of exon 8 of BTN1A1gene

5.2.2 Amplification of gene BTN1A1 by PCR

For specific amplification of target DNA sequence and for obtaining specific quantity of desired product, it is essential to optimize the different parameters.

The optimum parameters used for the amplification of 893bp fragment of exon 8 region of Butyrophilin gene are given Table No 3.3. Annealing temperature and time optimized in this study were 58º C for 40 seconds.

Sadr *et al*. (2008) and Yardibi *et al*. (2013) used 59º C for 60 seconds for optimum annealing. Rangarajan (2012) followed 57ºC for 1 minute. The variation in annealing temperature between laboratories indicated that the annealing temperature needs to be standardized for different laboratory conditions.

An 893 bp fragment of the exon 8 of the Butyrophilin gene was amplified by PCR using oligonucleotide primers. The size of amplified product was same for all the animals studied. This was in accordance with the results of Sadr *et al*. (2008) in Najdi cattle of Iran, Yardibi *et al*.(2013) in South and East Anatolian Red cattle breed and Rangarajan (2012) in Deoni and Holstein-Friesian cattle indicating that region BTN1A1 in cattle is conserved.

5.2.2 PCR-RFLP

The PCR-RFLP technique was followed to detect variation within the amplified genomic portion. The restriction enzyme *Hae III* which had a higher potential for detecting polymorphism, was used to digest the PCR amplicon.

Sadr *et al*. (2008) and Yardibi *et al*. (2013) followed 12 hours digestion of PCR product with *HaeIII* restriction endonuclease. However, in the present study satisfactory digestion was not observed with 12 hours digestion, whereas digestion for longer duration of 16-20 hours provided satisfactory restriction products.

The restriction enzyme digested fragments were separated by 3 per cent agarose gel electrophoresis. This allowed the identification of two alleles *viz.,* A and B which

resulted in two genotypes AA and AB. The genotype BB was not observed in the studied population.

The AA genotype exhibited fragments of 371 bp, 231bp, 185bp, 83bp, 23bp, which was considered homozygous as the sum of the fragment sizes added up to the PCR product. The second pattern had an additional fragment of 338 bp over and above the fragments of homozygous genotype AA. This was considered as heterozygous as the sum of the product sizes exceeded the PCR product size of 893bp. Probably the 371 bp fragment of AA genotype was further digested into a 338bp fragment and a smaller unresolved 33 bp fragment. Since there was no BB genotype animal observed in the present study, the AB genotype was confirmed by referring the findings of Sadr *et al*.(2008) and Yardibi *et al*. (2013) who reported BB genotype also in their studies, in which they reported presence of 338 bp fragment and absence of 371 bp fragment.

5.3 Allelic and genotype frequency

The analysis of Malnad Gidda population data in the present study indicated that there was higher frequency of 'A' allele (0.88) and a very low frequency of 'B' allele (0.12). Majority of the animals were of AA genotype (38 out of 50) with a genotype frequency of 76 per cent. The animals with heterozygous genotype (AB) were very few in numbers (12 out of 50) with a genotype frequency of 24 per cent.

The *Hae III* allelic patterns found in the present study were in accordance with the findings of Sadr *et al*. (2008) and Yardibi *et al*. (2013). They also observed significantly higher frequency of A allele than B allele. The AA genotype was predominant in all the breeds (Najdi, South Anatolian and East Anatolian) studied by them.

Sadr *et al*. (2008) also reported no BB genotype in Najdi cattle in many of the regions of Iran except for a small number of cattle of one of the three regions studied.

The high frequency of AA genotype also resulted in high frequency of 'A' allele (0.88) and a very low frequency of 'B' allele (0.12).

Taylor *et al.* (1996), Husaini *et al.* (1999) and Komisarek and Dorynek (2003) also studied part of exon 8 region of BTN1A1 gene and reported higher frequency of 'A' allele in HF cattle.

There is no literature on Indian cattle comparable to the present study. However, Bhattacharya *et al.* (2006) studied a 501 bp Butyrophilin gene fragment covering part of exon 8 in crossbred cattle, and reported a higher frequency of 0.87 for 'A' and a low frequency of 0.13 for 'B' allele

The review of above research findings indicate that irrespective of whether the cattle is high yielding (cross bred cattle of India and Najdi cattle of Iran) or low yielding (Malnad Gidda Cattle), 'A' allele is predominant and hence it can be postulated that there may not be any association between milk yield and exon 8 of BTN1A1 gene. However, significance of absence of or very low frequency of B allele needs further investigation.

5.4 Hardy-Weinberg Equilibrium

The population of Malnad Gidda investigated in the present study was found to be in Hardy-Weinberg equilibrium, for BTN1A1 genotypes. Most of the animals under study were reared by farmers and no selection is practiced and hence there is equilibrium in the population.

5.5 Association of milk quality traits with BTN1A1genotypes

In the present study, milk quality traits such as fat per cent, SNF per cent and total ash were estimated, and their associations with BTN1A1genotypes were analyzed. It was observed that the animals with genotype AA had higher fat and SNF percentages in comparison to AB genotype. However, no association was observed for total ash.

The results of the present study were also in accordance with that of the Bhattacharya *et al*. (2006), who observed association between genotypes of exon 8 of BTN1A1 gene and milk quality, traits in Holstein Friesian×Hariana crossbred cattle. Highest value of Fat and SNF was found for AA genotype and lowest value was found for BB genotype.

5.6 DNA sequence analysis of Butyrophilin gene

The DNA sequence data of exon 8 of Butyrophilin gene was subjected to NCBI nBLAST. The sequence of the present study was compared with that available in public domain. Malnad Gidda DNA sequence had 100 per cent homology with *Bos taurus* BTN gene and 99 per cent with that Sahiwal BTN1A1sequence.

The exon 8 region of BTN1A1 DNA sequence of Malnad Gidda cattle was subjected to multiple sequence alignment analysis for comparing with the *Bos indicus* and *Bos taurus* DNA sequence available in public domain (Appendix 4). A nucleotide transitition from A to G was observed in Malnad Gidda cattle when compared with Sahiwal cattle DNA sequence (Annexure 5).

This base substitution has resulted in amino acid substitution from iso leucine to valine (Annexure 6). The significance of this mutation on milk quality traits needs further detailed study.

CONCLUSIONS:

1. The amplified fragment of exon 8 region of BTN1A1 gene was found to be polymorphic.
2. BTN1A1 gene is conserved in Malnad Gidda cattle also.
3. 'A' allele was predominant in the population studied.
4. The AA genotype of BTN1A1 region was significantly associated with high milk fat and Solid-not-Fat percentage.
5. No association between ash content and genotype was observed.
6. The DNA sequence analysis showed 100 per cent alignment with *B.tarus* and 99 per cent alignment with *B.indicus* cattle.

Summary

6. SUMMARY

The present study was undertaken with the objectives to study the polymorphic pattern of amplified Butyrophilin gene by PCR-RFLP and DNA sequencing methods and to study the association of Butyrophilin gene polymorphism with milk quality traits such as Fat, SNF and Total ash.

The polymorphisms in exon 8 of bovine BTN1A1 gene were studied in 50 Malnad Gidda cows. Exon 8 (893 bp) of BTN1A1 gene were amplified by employing allele specific oligonucleotide primer sets by PCR technique. The PCR products were digested with *Hae*III restriction enzymes. Restriction fragments were resolved on 3 per cent agarose gel and the bands were analyzed. The 893bp PCR amplified product was digested in restriction pattern, denoted as AA, had fragments of sizes 371, 231, 185, 83 and 23bp. The second pattern denoted as AB, had fragment of 338bp in addition to the fragments in AA genotype. There was no animal with BB genotype.

The mean fat percentage of animals with AA genotype was 5.69±0.06 which was significantly higher than mean fat percentage of AB genotype (5.25±0.04). Similarly, AA genotype had higher SNF percent (8.64%) than that of AB genotype (8.47%). The difference in mean milk ash content between genotypes was non-significant.

The DNA sequence data of exon 8 of Butyrophilin gene was subjected to NCBI n BLAST. Malnad Gidda DNA sequence had 100 percent homology with that of *Bos taurus* and 99 per cent with that of Sahiwal DNA sequence. A nucleotide substitution

from A to G was observed in Malnad Gidda cattle resulted in amino acid substitution iso leucine to valine.

The result of the present study revealed polymorphism of exon 8 of Butyrophilin gene in Malnad Gidda cattle and higher milk fat and SNF percentages in AA genotype compared to AB genotype.

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Abstract

8. ABSTRACT

The present work was aimed at studying the Butyrophilin gene polymorphism and its association with milk quality traits in Malnad Gidda cattle. Blood and milk samples were collected from 50 Malnad Gidda cattle from Uttar Kannada district and Chickmagaloor districts of Karnataka. Milk samples were collected at fortnight intervals, for six times from the same animals. Genomic DNA was isolated by Miller's High Salt method and Fat, SNF and Total Ash were analyzed by Gerber method, Formula method and Gravimetric method respectively.

The genetic polymorphism of Butyrophilin gene was investigated by PCR-RFLP technique. An 893bp fragment of exon 8 of Butyrophilin gene was amplified. The amplified products were digested with restriction enzyme *HaeIII*. The restricted products were subjected to three per cent agarose gel electrophoresis and revealed two alleles A and B and genotypes AA and AB. The genotype BB was absent in the population studied. The highest gene and genotype frequencies were observed for A allele (0.88) and AA genotype (0.76) and lowest gene and genotype frequencies were observed for B allele (0.12) and AB genotype (0.24) respectively. The population studied was in Hardy-Weinberg equilibrium. Sequence analysis indicated 100 % homology to *B.taurus* and 99 per cent to *B.indicus.* There was a significant association between milk quality traits like Fat and SNF with genotypes AA and AB where A allele was gene was favorable, whereas total ash was not associated with any genotype. The present study revealed the polymorphism of BTN1A1 gene and its association with milk quality traits.

Appendices

9. APPENDICES

ANNEXURE 1

Chemicals:

Chemical	Source
Absolute alcohol	Merck
Acetic acid	BDH-E, Merck (India) Ltd.
Agarose	SRL, Mumbai.
Ammonium chloride	SRL, Mumbai
Ammonium per Sulphate	SRL, Mumbai
Boric acid	SRL, Mumbai
Bromophenol blue	SRL, Mumbai
Chloroform	SRL, Mumbai
dNTPs	Bangalore Genei Pvt Ltd.
EDTA	SRL, Mumbai
Ethidium bromide	SRL, Mumbai
Isoamyl alcohol	BDH-E, Merck (India) Ltd.
Potassium chloride	SRL
Sodium chloride	Merck
SDS	SRL, Mumbai
Tris base	SRL, Mumbai

Enzymes:

Proteinase-K	Bangalore Genei Pvt Ltd
Taq DNA polymerase	Bangalore Genei Pvt Ltd
RE	Roche Molecular Diagnosis

Molecular size markers:

250 base pair DNA ladder	Bangalore Genei Pvt Ltd
100 base pair DNA ladder	Bangalore Genei Pvt Ltd.

ANNEXURE 2

Laboratory equipments:

Equipments	**Suppliers/Firm**
Bench top centrifuge	Remi, India
Electric balance	Sartorius,Switzerland
Gel Documentation System	Bio Rad
Gel drier	Bio Rad
Microcentrifuge	Eppendorf
Micropipettes	Nichipet EX
pH meter	Global, India
Sequencing Electrophoresis Apparatus	Bangalore Genei Pvt Ltd
Spectrophotometer	Eppendorf
Submarine electrophoresis unit	Bangalore Genei Pvt Ltd
Thermal cycler	Bio Rad
UV Transilluminator	Cleaver Scientific Ltd

ANNEXURE 3

A. Solution or reagents for DNA isolation

EDTA (0.5M)

Disodium Ethylene Diamine Tetra Acetic acid (EDTA)	: 18.61g
Triple glass distilled water to make up to	: 100ml

pH adjusted to 8.0, autoclaved and stored at room temperature

Sodium Dodecyl Sulphate (SDS)

Sodium Dodecyl Sulphate	: 10 g
Triple glass distilled water to make up to	: 100 ml

5M NaCl

Sodium chloride	: 29.27g
Triple glass distilled water to make up to	: 100 ml

Filtered and stored at room temperature

Tris-EDTA buffer (TE)

Tris (hydroxy methyl) aminomethane (10mM) pH 7.6	0.1211g
EDTA (0.1Nm)	0.0372g
Triple glass distilled water to make up to	: 100ml
pH adjusted to	: 8.0

RBC lysing buffer

Ammonium chloride (1.7M)	: 9.1 g
Triple glass distilled water to make up to	1000ml
pH adjusted to 8.0, kept at 4°C	

B. Solutions or Reagents for checking of DNA samples:

Tris acetate EDTA (TAE) buffer (50X)

Triple (hydroxy methyl) amino methane (10mM) pH 7.6	: 48.4 g
Glacial acetic acid	11.42ml
0.5 M EDTA (pH 8.0)	: 20 ml
Triple glass distilled water to make up to	1000ml

Agarose (0.8per cent)

Agarose	0.24 g
Triple glass distilled water to make up to	: 30 ml

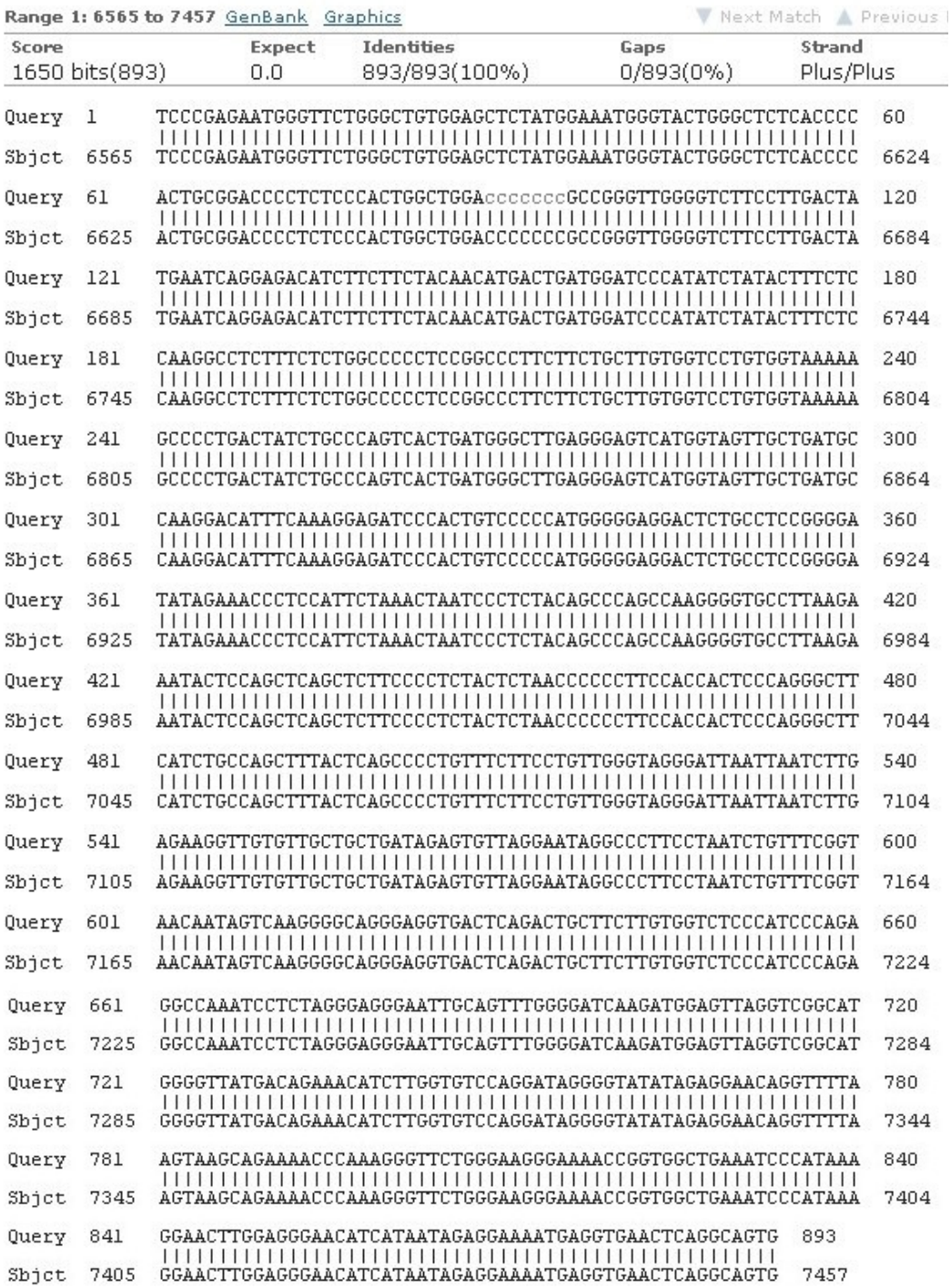
Agarose (1.5per cent)

Agarose	: 0.45 g
TBE (1X) to make up to	: 30 ml

Agarose (3.0per cent)

Agarose	: 0.9 g
TBE (1X) to make up to	: 30 ml

ANNEXURE 4



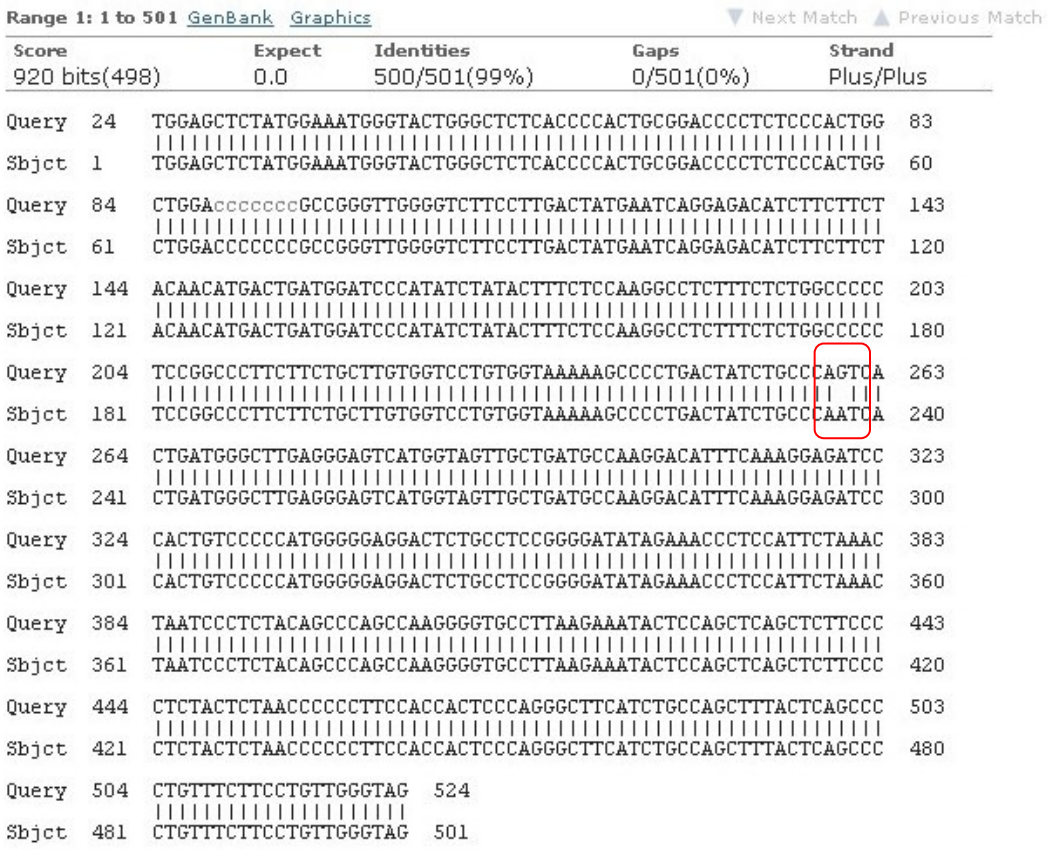
Range 1: 6565 to 7457 GenBank Graphics ▼ Next Match ▲ Previous

Score	Expect	Identities	Gaps	Strand
1650 bits(893)	0.0	893/893(100%)	0/893(0%)	Plus/Plus

```
Query  1     TCCCGAGAATGGGTTCTGGGCTGTGGAGCTCTATGGAAATGGGTACTGGGCTCTCACCCC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6565  TCCCGAGAATGGGTTCTGGGCTGTGGAGCTCTATGGAAATGGGTACTGGGCTCTCACCCC  6624

Query  61    ACTGCGGACCCCTCTCCCACTGGCTGGAcccccccGCCGGGTTGGGGTCTTCCTTGACTA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6625  ACTGCGGACCCCTCTCCCACTGGCTGGACCCCCCCGCCGGGTTGGGGTCTTCCTTGACTA  6684

Query  121   TGAATCAGGAGACATCTTCTTCTACAACATGACTGATGGATCCCATATCTATACTTTCTC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6685  TGAATCAGGAGACATCTTCTTCTACAACATGACTGATGGATCCCATATCTATACTTTCTC  6744

Query  181   CAAGGCCTCTTTCTCTGGCCCCCTCCGGCCCTTCTTCTGCTTGTGGTCCTGTGGTAAAAA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6745  CAAGGCCTCTTTCTCTGGCCCCCTCCGGCCCTTCTTCTGCTTGTGGTCCTGTGGTAAAAA  6804

Query  241   GCCCCTGACTATCTGCCCAGTCACTGATGGGCTTGAGGGAGTCATGGTAGTTGCTGATGC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6805  GCCCCTGACTATCTGCCCAGTCACTGATGGGCTTGAGGGAGTCATGGTAGTTGCTGATGC  6864

Query  301   CAAGGACATTTCAAAGGAGATCCCACTGTCCCCCATGGGGGAGGACTCTGCCTCCGGGGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6865  CAAGGACATTTCAAAGGAGATCCCACTGTCCCCCATGGGGGAGGACTCTGCCTCCGGGGA  6924

Query  361   TATAGAAACCCTCCATTCTAAACTAATCCCTCTACAGCCCAGCCAAGGGGTGCCTTAAGA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6925  TATAGAAACCCTCCATTCTAAACTAATCCCTCTACAGCCCAGCCAAGGGGTGCCTTAAGA  6984

Query  421   AATACTCCAGCTCAGCTCTTCCCCTCTACTCTAACCCCCCTTCCACCACTCCCAGGGCTT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6985  AATACTCCAGCTCAGCTCTTCCCCTCTACTCTAACCCCCCTTCCACCACTCCCAGGGCTT  7044

Query  481   CATCTGCCAGCTTTACTCAGCCCCTGTTTCTTCCTGTTGGGTAGGGATTAATTAATCTTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7045  CATCTGCCAGCTTTACTCAGCCCCTGTTTCTTCCTGTTGGGTAGGGATTAATTAATCTTG  7104

Query  541   AGAAGGTTGTGTTGCTGCTGATAGAGTGTTAGGAATAGGCCCTTCCTAATCTGTTTCGGT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7105  AGAAGGTTGTGTTGCTGCTGATAGAGTGTTAGGAATAGGCCCTTCCTAATCTGTTTCGGT  7164

Query  601   AACAATAGTCAAGGGGCAGGGAGGTGACTCAGACTGCTTCTTGTGGTCTCCCATCCCAGA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7165  AACAATAGTCAAGGGGCAGGGAGGTGACTCAGACTGCTTCTTGTGGTCTCCCATCCCAGA  7224

Query  661   GGCCAAATCCTCTAGGGAGGGAATTGCAGTTTGGGGATCAAGATGGAGTTAGGTCGGCAT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7225  GGCCAAATCCTCTAGGGAGGGAATTGCAGTTTGGGGATCAAGATGGAGTTAGGTCGGCAT  7284

Query  721   GGGGTTATGACAGAAACATCTTGGTGTCCAGGATAGGGGTATATAGAGGAACAGGTTTTA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7285  GGGGTTATGACAGAAACATCTTGGTGTCCAGGATAGGGGTATATAGAGGAACAGGTTTTA  7344

Query  781   AGTAAGCAGAAAAACCCAAAGGGTTCTGGGAAGGGAAAAACCGGTGGCTGAAATCCCATAAA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7345  AGTAAGCAGAAAAACCCAAAGGGTTCTGGGAAGGGAAAAACCGGTGGCTGAAATCCCATAAA  7404

Query  841   GGAACTTGGAGGGAACATCATAATAGAGGAAAATGAGGTGAACTCAGGCAGTG  893
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7405  GGAACTTGGAGGGAACATCATAATAGAGGAAAATGAGGTGAACTCAGGCAGTG  7457
```

Nucleotide sequence comparison of Butyrophilin gene of Malnad Gidda with that of *Bos taurus* cattle

ANNEXURE 5



Range 1: 1 to 501 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
920 bits(498)	0.0	500/501(99%)	0/501(0%)	Plus/Plus

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Query  24   TGGAGCTCTATGGAAATGGGTACTGGGCTCTCACCCCACTGCGGACCCCTCTCCCACTGG  83
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGGAGCTCTATGGAAATGGGTACTGGGCTCTCACCCCACTGCGGACCCCTCTCCCACTGG  60

Query  84   CTGGAcccccccGCCGGGTTGGGGTCTTCCTTGACTATGAATCAGGAGACATCTTCTTCT  143
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTGGACCCCCCCGCCGGGTTGGGGTCTTCCTTGACTATGAATCAGGAGACATCTTCTTCT  120

Query  144  ACAACATGACTGATGGATCCCATATCTATACTTTCTCCAAGGCCTCTTTCTCTGGCCCCC  203
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  ACAACATGACTGATGGATCCCATATCTATACTTTCTCCAAGGCCTCTTTCTCTGGCCCCC  180

Query  204  TCCGGCCCTTCTTCTGCTTGTGGTCCTGTGGTAAAAAGCCCCTGACTATCTGCCCAGTCA  263
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  181  TCCGGCCCTTCTTCTGCTTGTGGTCCTGTGGTAAAAAGCCCCTGACTATCTGCCCAATCA  240

Query  264  CTGATGGGCTTGAGGGAGTCATGGTAGTTGCTGATGCCAAGGACATTTCAAAGGAGATCC  323
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CTGATGGGCTTGAGGGAGTCATGGTAGTTGCTGATGCCAAGGACATTTCAAAGGAGATCC  300

Query  324  CACTGTCCCCCATGGGGGAGGACTCTGCCTCCGGGGATATAGAAACCCTCCATTCTAAAC  383
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CACTGTCCCCCATGGGGGAGGACTCTGCCTCCGGGGATATAGAAACCCTCCATTCTAAAC  360

Query  384  TAATCCCTCTACAGCCCAGCCAAGGGGTGCCTTAAGAAATACTCCAGCTCAGCTCTTCCC  443
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TAATCCCTCTACAGCCCAGCCAAGGGGTGCCTTAAGAAATACTCCAGCTCAGCTCTTCCC  420

Query  444  CTCTACTCTAACCCCCCTTCCACCACTCCCAGGGCTTCATCTGCCAGCTTTACTCAGCCC  503
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CTCTACTCTAACCCCCCTTCCACCACTCCCAGGGCTTCATCTGCCAGCTTTACTCAGCCC  480

Query  504  CTGTTTCTTCCTGTTGGGTAG  524
            |||||||||||||||||||||
Sbjct  481  CTGTTTCTTCCTGTTGGGTAG  501
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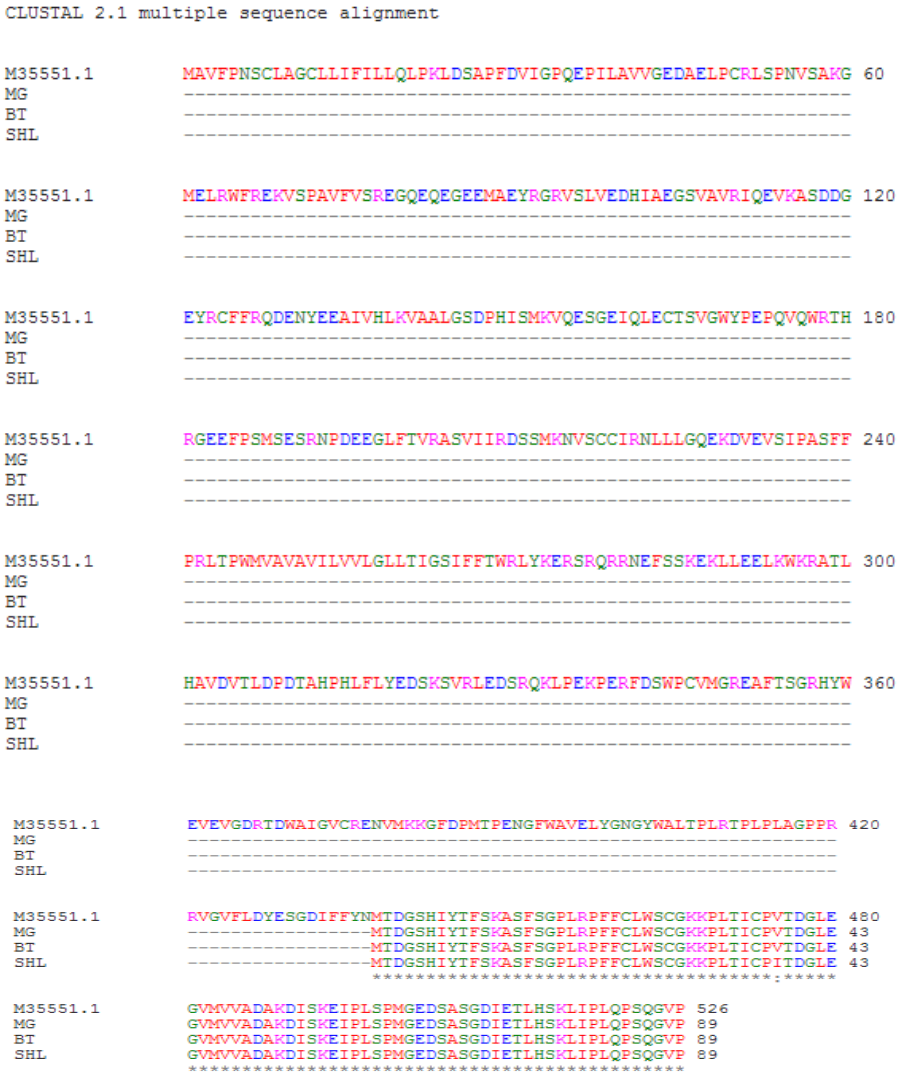
Nucleotide sequence comparison of exon 8 and partial cds of Malnad Gidda (query) with that of Sahiwal cattle (subject)

ANNEXURE 6



Multiple alignment of amino acid sequence of exon 8 region of Butyrophilin gene of Malnad Gidda, *Bos taurus* and Sahiwal cattle