



Effect of DNA polymorphism in *Butyrophilin* gene on milk yield in Murrah buffalo (*Bubalus bubalis*)

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The name for the mammary specific protein *Butyrophilin* (BTN) was coined from the greek terms ‘*butyros*’ and ‘*philos*’ to reflect its co-localization with milk-fat globules and denoted a possible function in the secretion of milk fat globules (Franke *et al.* 1981). *Butyrophilin* is the major glycoprotein of bovine milk fat globule membrane (MFGM), accounting for over 40 and 50% of the total protein on weight and molar bases, respectively (Jack and Mather 1990). *Butyrophilin* has been shown as a mammary-specific gene located on chromosome 23 (Ashwell *et al.* 1996) and used as genetic marker to investigate allelic substitution effects on several quantitative milk production traits. *Butyrophilin* proved as a good candidate to serve as genetic marker because of its exclusivity to the mammary gland and its purported role in milk fat secretion (Mather 2000). Recently the association of *Butyrophilin* gene polymorphism with milk yield, protein yield and SNF yield has been reported in Korean dairy proven and young bulls (Jang *et al.* 2005).

The PCR-SSCP analysis (Orita *et al.* 1989) is a technique based on the principle that single-stranded DNA molecules form specific sequence-based secondary structures under non-denaturing conditions. Thus genetic markers associated with economic traits can be searched directly using molecular marker techniques which can identify genetic variation at specific loci and analyze the relationship between genetic variation at quantitative trait loci (QTL) and production traits (Bao *et al.* 2010).

Buffalo is the most important farm animal species in Asia, especially India, where it is extensively used for milk, meat, fuel and fertilizer production (from manure), as well as for draught power (Borghese 2005). India is rich in buffalo genetic resource having 97 million in 2003 (Annual Report

2006) which accounts for 59.5% to total world buffalo’s population. Buffalo milk contributes 55.6% to country’s total milk production. Murrah buffalo is the most important dairy breed with superior genetic potential for milk production. Indian buffaloes are hardy, thrive well on poor quality nutrition and are good converter of inputs into milk as compared to cattle (Biswas *et al.* 2003). However, their inherent potential for growth and production has not been exploited due to inadequate information about genetic basis and breeding strategies. River buffalo and domestic cattle are closely related because they belong to same *Bovidae* family, cytogenetically characterized, shares homology in chromosome banding (Gallagher and Womack 1992) and gene mapping (Di Meo *et al.* 2005). Therefore comparative genomics approach with various DNA marker techniques might prove feasible approach in analysing buffalo genome. Therefore the present work has been carried out to analyse *Butyrophilin* gene polymorphism using PCR-RFLP, PCR-SSCP followed by DNA Sequencing in Murrah buffalo (*Bubalus bubalis*). The association study was carried out to find possible relationship (if any) with milk production traits in Murrah buffalo.

The study was conducted on 110 Murrah buffalo animals maintained at Institute’s herd. Blood samples (10 ml) were collected by jugular veinipuncture using vacuum tubes containing acid citrate-dextrose solution (ACD) as an anticoagulant. Besides, 50 ml of milk was collected from each animal to estimate 305 days lactation milk yield, milk fat % and SNF % and total protein %.

Genomic DNA isolation was performed from blood using the phenol-chloroform-extraction protocol (Clamp *et al.* 1993) with some modifications. The integrity of the DNA was assessed following electrophoresis in a 0.8% agarose gel stained with ethidium bromide. In addition, the OD ratio 260/280 nm was measured to check for protein contamination and to calculate the DNA concentration. All stock DNA samples were kept at –80°C for longer storage, and the

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working aliquots were maintained at -20°C .

Fat % of milk samples was estimated by gerber method (ISI 1977) while total protein % was worked out by formaldehyde-titration method (Pyne 1932). Lactometer reading was taken of all milk samples and total solid% (TS%) and SNF% were calculated by using the following formula:

$$\text{TS\%} = \text{CLR}/4 + 1.2\text{F} + 0.14$$

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

where CLR, corrected lactometer reading which equals to lactometer reading + 0.5 + temperature difference from $84^{\circ}\text{F} \times 0.1$; F, fat%.

The *Butyrophilin* gene primers reported in cattle were used to screen the PCR-RFLPs in buffalo (Jang *et al.* 2005). The other PCR primers for PCR-SSCP analysis were designed on the basis of cattle genbank sequence encompassing polymorphic restriction site using PRIMER3 software (Rozen and Skaletsky 1998).

The polymerase chain reaction (PCR) was carried out on about 100 ng/ μl of genomic DNA in a 25 μl reaction volume. The reaction mixture consisted of 2.5 μl of 10 $\times$ PCR assay buffer containing 1.5 mM MgCl_2 , 200 μM each of dNTPs, 0.75 U *Taq* DNA polymerase and 10 pM of each primer. Amplification was carried out in a thermal cycler using PCR cycling conditions as (95°C for 5 min) and 34 cycles of 45 seconds at 95°C , annealing temperature (57°C) and 72°C consecutively, followed by a 5 min final extension at 72°C . The forward sequence of intron 1 primer; 52 - CCTGCTTATTTCCCTAGTCTC-32 and reverse sequence; 52 -CCACCCTAAGGTTAGTCAATC-32. The PCR amplification was verified by electrophoresis of the PCR products with loading dye (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol) on 2% (w/v) agarose gel in 0.5 $\times$ TBE buffer using a 100 bp ladder as marker for confirmation of the length of the PCR products. The agarose gels were stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$). The amplified products (5 μl) were detected on 2% agarose gel using 1 μl of loading dye as a stop dye, electrophoresed and visualized using UV light after ethidium bromide staining.

The PCR-RFLP polymorphism analysis of *Butyrophilin* gene fragments was performed by using *TaqI*, *MboI* and *HaeIII* restriction enzymes to identify PCR-RFLP in buffalo. In this PCR amplified DNA products (15 μl) with distilled water (4.75 μl) were subjected to RE digestion with restriction buffer (2.5 μl) and respective restriction enzymes (0.5 μl). The mixtures were digested at 37°C for 3 h and were separated in 3% agarose gel to visualise bands under UV transilluminator.

The *Butyrophilin* gene PCR products were resolved by SSCP analysis. The various factors were tested for each fragment and optimized such as amount of PCR product (5 μl), acrylamide-bisacrylamide concentration (12%), glycerol (10%), voltage (200 volts), electrophoresis run time (12 hours) and temperature (room temperature with ice

chilled circulating water to electrophoresis assembly). The amplified PCR products were diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA) and heat denatured at 95°C for 10 min. After denaturation, PCR products were immediately transferred to chilled ice pack and kept in -20°C for 10 min. The PCR products were resolved on a non-denaturing 12% acrylamide: bis-acrylamide (49: 1) gel. The vertical gel electrophoresis was carried out in a cell electrophoresis unit using 1 $\times$ TBE buffer at 10–12.5 volts/cm for 12 h at room temperature. Gels were silver-stained (Sambrook and Russell 2000) and photographed using digital camera for *Butyrophilin* SSCP pattern analysis.

The PCR products representing different SSCP patterns in Murrah buffalo were directly got sequenced using DNA sequencing service. The nucleotide sequence alignments were carried out using alignment tools, viz. Geneious software, ClustalW and BLAST to reveal single base variations. These allelic variants nucleotide sequence were analysed and submitted in NCBI genbank. The DNA sequence polymorphism observed were used to genotype buffalo populations.

The frequency of polymorphic alleles, genotypes and their accordance with Hardy-Weinberg law was assessed by POPGENE 1.31 software (<http://www.ualberta.ca/~fyeh>). The association between polymorphic allelic variants of *Butyrophilin* genes and milk production traits were analysed using GLM procedure (SYSTAT). The following model was used:

$$Y_{ijkl} = \mu + g_i + s_j + p_j + h_k + e_{ijkl}$$

where Y_{ij} , observation on j^{th} animal i^{th} genotype; μ , population mean; g_i , effect of i^{th} genotype ($i=1, 2$); s_j , effect of j season; p_j , effect of j parity; h_k , effect of k year; e_{ijkl} , random error.

In the present investigation, a fragment (576 bp) of exon 2–3 of *Butyrophilin* gene was digested using *TaqI* enzyme and a 683 bp gene fragment of exon 5, 6 using *MboI* enzyme which exhibited uncut fragments in buffaloes screened indicating monomorphic nature of the region. But previously, *Butyrophilin* candidate gene polymorphism analysis study conducted in Korean dairy proven and young bulls (Jang *et al.* 2005) reported polymorphic nature of these RFLPs. Similarly, the 501 bp gene fragment of exon 7 of buffalo *Butyrophilin* gene digested using 12U of *HaeIII* enzyme resulted monomorphic pattern in buffaloes. However, in a various studies in bovine *Butyrophilin* gene (Taylor *et al.* 1996, Bhattacharya *et al.* 2006) reported a *HaeIII* PCR-RFLP.

The *Butyrophilin* gene was screened for SSCPs at introns 1, intron 3 and intron 4 using primers BTI1, BTI3 and BTI4 respectively in buffaloes. The SSCP patterns for all the three primers were best resolved in 12% acrylamide-bisacrylamide with other optimized SSCP conditions, viz. voltage (200volts), time (12 h) and SSCP solution with 10% glycerol.

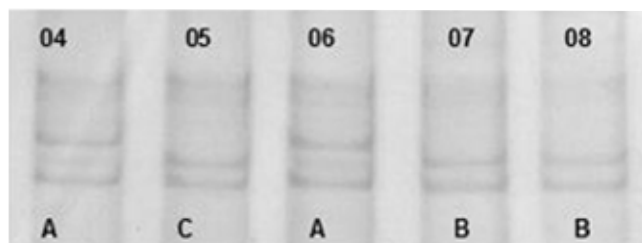


Fig. 1. BTII SSCP within intron1 of Murrah buffalo *Butyrophilin* gene
where A, B and C = SSCP Patterns,
4-8 = Murrah Animal Numbers

The *Butyrophilin* gene fragments amplified using BTII yielded three polymorphic SSCP patterns, viz. A, B and C in 12% non-denaturing PAGE (Fig 1) while fragments amplified using BT I3 and BT I4 primers did not exhibit polymorphic SSCP fragments. The frequency of polymorphic SSCP patterns in BT II amplified fragments of buffalo was found with following frequencies: A = 0.6, B = 0.31 and C = 0.09 in Murrah. This study revealed polymorphic SSCP with 3 patterns in intron 1 region of *Butyrophilin* gene of Murrah buffalo.

The nucleotide sequences of Murrah buffalo *Butyrophilin* gene variants were verified using chromatogram and compared among themselves using geneious alignment tools to reveal BTII SNP (c.184C>T>G) among the experimental population. The SNP position was located at 184th position. The sequencing results confirmed the BTII SSCP polymorphism having 3 patterns in Murrah buffalo. The nucleotide sequences of *Butyrophilin* gene variants of Murrah buffalo arising from this study were submitted in NCBI GenBank (EU194868, EU1997977 and EU199798). The Murrah buffalo *Butyrophilin* variant sequence (EU194868) was found to have 96% pair-wise % similarity with *Bos*

taurus nucleotide sequence (AF005497).

The BT II SSCP polymorphism revealing BT II SNP (c.184C>T>G) was used to genotype populations of Murrah buffalo in which BTII SNP (c.184C>T>G) genotypes were found in Hardy-Weinberg proportions. BT II SNP (c.184C>T>G) frequency for variant C, T and G was 0.6, 0.31 & 0.09 respectively.

The variance analysis indicated BT II SNP (c.184C>T>G) was significantly associated ($P < 0.05$) with 305 days lactation milk yield of Murrah buffaloes. The Murrah buffaloes with BTII TT genotypes had 683.93 kg and 320.48 kg higher milk yield as compared to BT IICT and BT IICG genotypes respectively. The positive association of BT II SNP (c.184C>T>G) polymorphism with milk production traits may be useful for improving milk performance in dairy buffaloes. However, it was observed that, BTII SNP (c.184C>T>G) genotypes were not significantly differing with fat and SNF % trait values of Murrah buffalo. The least squares means for milk yield, fat and SNF % in Murrah buffaloes with different genotypes is given in Table 1.

Presently for improvements in selection and breeding technologies, the selection pressure has been steadily growing. Among the tools at the disposal of modern animal breeders, marker assisted selection (MAS) conceivably seems to be the most direct control, which can hasten the rate of genetic gain of desirable traits in farm animals. The initial step towards this aim is to identify (or locate) genetic markers that will serve as flag posts along the length of the chromosome of interest. Although the potential benefit from these markers is undeniable, without a higher density map, or markers specifically shown to tightly co-segregate with a gene of interest, they will not be of immediate practical use to breeders. In the MAS of dairy animals some genes have been proposed as potential candidates associated with dairy

Table 1. Details of primer sequences used for Murrah buffalo butyrophilin DNA polymorphism analysis using PCR-RFLP and PCR-SSCP

Primer	Primer Sequence	T ^A / RE/Gene region	Reference
BTN1	FP 52 -TGGAGCTCTATGGAAATGGG-32	60°C / Hae III	Jang <i>et al.</i> (2005)
	RP 52 -CTACCCAACAGGAAGAAACAG-32		
BTN3	FP 52 -CTGAAGTTCCTGACAAACTCG-32	60°C / TaqI	Jang <i>et al.</i> (2005)
	RP 52 -CTCTGCATCTTCACCCACCAC-32		
BTN4	FP 52 -CTTCTTCCCAAGGCTGAC-32	57°C / MboI	Jang <i>et al.</i> (2005)
	RP 52 -CTTACTGAGCTCTTCCAGG-32		
BTNZ	FP 52 -TCCCGAGAATGGGTTCTG -32	60°C / HaeIII	Jang <i>et al.</i> (2005)
	RP 52 -CACTGCCTGAGTTCACCTCAT-32		
BTII*	FP 52 -CCTGCTTATTTCCCTAGTCTC-32	57°C/ intron1	Primer designed
	RP 52 -CCACCCTAAGGTAGTCAATC-32		
BTI3*	FP 52 -AACTGGCTATAAAGCCCTAGA-32	57°C/ intron3	Primer designed
	RP 52 -ACTACACAAGGGAAGTGGGT-32		
BTI4*	FP 52 -AGATCTCACAGACATTCCAGA-32	62°C/ intron4	Primer designed
	RP 52 -TGCTGAACCAGAGGTAGAGTA-32		

*Primers were designed based on the published nucleotide sequence information of *Butyrophilin* gene of bovine. T^A, the optimized annealing temperature for each primer.

performance traits (Liefers *et al.* 2005, Brym *et al.* 2004, Winter *et al.* 2003 and Jang *et al.* 2005). It is believed that SNPs occurring within such genes may influence the milk production trait or at least be an effective DNA marker of a sub-region of the dairy animal genome.

In the present study, PCR-RFLP analysis of Murrah buffalo *Butyrophilin* gene revealed monomorphism indicating absence of variation at screened regions of gene. However, identification of BTI1 SNP (c.184C>T>G) in intron 1 of *Butyrophilin* gene and its statistical association with milk yield is a first report in Murrah Buffalo. The variance analysis revealed that the BTI1 SNP (c.184C>T>G) variant BTI1TT genotype was differing significantly with 305 days lactation milk yield of Murrah buffaloes. Similarly in bovine, *Butyrophilin* polymorphism in intron 1 had been reported to be associated with 305-days production traits ($P<0.05$) in Korean cattle (Jang *et al.* 2005). Bhattacharya *et al.* (2006) reported *Hae*III PCR-RFLP polymorphism in crossbred cattle and significant effects ($p<0.05$) of three genotypes, viz. AA, BB and AB on total milk solid, fat and SNF percentages, while showing non-significant impact on total protein %.

The PCR-SSCP followed by sequencing is found to be feasible approach to reveal DNA polymorphism at *Butyrophilin* gene locus in buffaloes. Whether this polymorphism could affect the expression of *Butyrophilin* gene remains further study. It is suggested to validate association of identified BTI1 SNP (c.184C>T>G) of Murrah Buffalo in large population with more records which might prove good candidate for marker assisted breeding for milk yield. The findings of the present research can be useful information resource for buffalo genetic improvement, conservation, management and breeding decisions.

SUMMARY

DNA polymorphism of *Butyrophilin* gene was analyzed using PCR-RFLP, SSCP and to find possible relationship (if any) of identified polymorphism with milk production traits in Murrah buffalo. Genomic DNA was isolated from 110 Murrah from Institute's herd. The PCR-RFLP polymorphism analysis of *Butyrophilin* gene using *Taq*I, *Mbo*I and *Hae*III restriction enzymes revealed single restriction pattern indicating absence of variation in buffalo. The PCR-SSCP analysis of intron 3 and 4 exhibited monomorphic SSCP patterns. However, intron 1 region of *Butyrophilin* revealed polymorphic BTI1SSCP with 3 patterns. The identified buffalo *butyrophilin* variants were found with following frequencies: A, 0.6; B, 0.31 and C, 0.09 in Murrah buffaloes. The direct DNA sequencing of 3 polymorphic SSCPs within Murrah buffalo *Butyrophilin* gene revealed BTI1 SNP (c.184C>T>G) confirming 3 variants, viz. A, B and C. The statistical analysis indicated BTI1TT SNP genotypes were significantly associated with 305 days lactation milk yield of Murrah buffaloes. The Murrah buffaloes with BTI1TT

SNP genotypes had 683.93 kg and 320.48 kg higher milk yield as compared to BTI1CT and BTI1CG SNP genotypes respectively. The identified association of BTI1TT SNP genotypes with milk yield after validation in large population with more records will be useful for designing selection and breeding strategies for genetic improvement of buffaloes for milk production.

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