SINGLE STRAND CONFORMATION POLYMORPHISM WITHIN BUTYROPHILIN GENE AND ITS RELATIONSHIP WITH MILK YIELD IN INDIAN RIVERINE BUFFALOES

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ABSTRACT

Butyrophilin has been shown to be a mammary-specific gene and used as a genetic marker to investigate allelic substitution effects on milk production traits. The aim of the present study was to study genetic polymorphism of the butyrophilin gene using SSCP in the Murrah, Surti and Bhadawari breeds of riverine buffaloes (*Bubalus bubalis*). The association study was carried out to find possible relationships (if any) with milk production traits in Murrah buffalo. The SSCP analysis of the intron region of butyrophilin revealed polymorphic BTI1 SSCP in buffaloes. DNA sequencing of three polymorphic SSCPs within the Murrah buffalo butyrophilin gene revealed three variants.viz, A, B and C. The frequencies of identified buffalo butyrophilin variants were: A = 0.6, B = 0.31 and C = 0.09 in 55 Murrah buffaloes; in Surti A = 0.5, B = 0.3 and C = 0.2 while in Bhadawari A= 0.4, 0.5 and 0.1. The Murrah buffalo butyrophilin variant sequence (EU194868) was found in 96 % pairwise percent similarity with *Bos taurus* nucleotide sequence (AF005497). The statistical analysis using general linear model procedure (SYSTAT) for association study indicated BTI1 SSCP was significantly associated ($P \leq 0.05$) with 305-day lactation milk yield of Murrah buffaloes. The Murrah buffaloes with BTI1BB genotypes had 683.93 kg and 320.48 kg higher milk yield as compared to BTI1AA and BTI1CC genotypes, respectively. The association of BTI1 SSCP polymorphism with milk production traits will be useful for genetic improvement of buffaloes for milk production traits.

Keywords: riverine buffaloes, *Bubalus bubalis*, butyrophilin, DNA polymorphism, riverine PCR-SSCP, milk yield

INTRODUCTION

The buffalo is the most important farm animal species in Asia, especially in India, where it is extensively used for milk, meat, fuel and fertilizer (from manure) production , as well as for draught power (Borghese, 2005). India possesses more than half of the world buffalo population and many of the fine breeds are found in its different parts of

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the country. 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 the genetic basis of production traits and breeding strategies. The riverine buffalo contributes significantly to the Indian agriculture system and to the economy. Nearly 55% of milk production in India comes from buffaloes (FAO, 2007). There is therefore a needed to analyze and establish molecular markers for production traits in buffaloes. Currently, genetic marker research applied to animal breeding and production is focussed mainly on analysing mutations located within candidate genes and their association with economically important production traits (Kale and Yadav, 2011).

The Polymerase Chain reaction – single strand conformation polymorphism (PCR-SSCP) (Orita *et al.*, 1989) method is a powerful method for identifying sequence variation in amplified DNA fragments. Compared with the PCR-RFLP technique, PCR-SSCP has the advantage of being able to detect single base substitutions in other locations besides enzymatic restriction sites. Butyrophilin is the major glycoprotein of the 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 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).

Therefore the present work has been carried out to analyse butyrophilin gene polymorphism using PCR-SSCP in Murrah, Surti and Bhadawari breeds of riverine buffaloes (*Bubalus bubalis*). DNA sequencing was done for polymorphic SSCP variants obtained in Murrah buffaloes. The association study was carried out to find possible relationships (if any) with milk production traits in Murrah buffalo.

MATERIALS AND METHODS

The study was conducted on representatives of three important breeds of riverine buffaloes i.e. 55 Murrah, 10 Surti and 10 Bhadawari breed animals from Haryana and Punjab, Gujarat and Utter Pradesh and Madhya Pradesh states of India respectively. Blood samples (10 ml) were collected by jugular veinipuncture using vacuum tubes containing acid citrate dextrose solution (ACD) as an anticoagulant. Genomic DNA isolation was performed from blood using the phenol chloroform extraction protocol (Clamp *et al.*, 1993) with some modifications. The PCR primers (Table1) for PCR-SSCP analysis were designed using PRIMER3 software for the butyrophilin gene in buffalo on the basis of the cattle GenBank sequence.

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 10x PCR assay buffer containing 1.5 mmol $MgCl_2$, 200 µmol each of dNTPs, 0.75 U *Taq* DNA polymerase and 10 pmole of each primer. Amplification was carried out in a Biometra thermal cycler using PCR cycling conditions as (95°C for 5 minutes) and 34 cycles of 45 seconds at 95°C, annealing temperature (T^A°C) and 72°C consecutively, followed by a five minutes final extension at 72°C. In the process of PCR

amplification the annealing temperature (T^A) was optimised for each primer sequence (Table 1). 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 x TBE buffer using a 100 bp ladder as a marker for confirmation of the length of the PCR products. The agarose gels were stained with ethidium bromide (0.5 µg/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 butyrophilin gene PCR products were resolved by SSCP analysis. The various factors were tested for each fragment and optimized. viz, amount of PCR product acrylamide-bisacrylamide concentration, presence of glycerol, voltage, electrophoresis run time and at room temperature using ice chilled circulating water to electrophoresis assembly. The amplified PCR products were diluted in denaturing solution (95% formamide, 10 mmol NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mmol EDTA) and heat denatured at 95°C for ten minutes. After denaturation PCR products were immediately transferred to chilled ice pack and kept in -20°C for 10 minutes. The PCR products were resolved on a non-denaturing 12% acry1amide: bis-acrylamide (49:l) gel. The vertical gel electrophoresis was carried out in a Bio-Rad Protean® II Xi Cell electrophoreses unit using 1X TBE buffer at 10-12.5 volts/cm for 12 h at room temperature. Gels were silver-stained (Sambrook and Russell, 2001) and photographed using a digital camera for butyrophilin SSCP pattern analysis.

The PCR products representing different SSCP patterns in Murrah buffalo were directly sequenced using automated DNA sequencing service and analysed using various analytical tools for polymorphism detection. The DNA sequence polymorphism observed were used to genotype the Murrah, Surti and Bhadawari 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,

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

Y_{ij} : observation on j^{th} animal i^{th} genotype

μ : population mean

$_{gi}$: effect of i^{th} genotype (i=1, 2)

s_i : effect of i season

p_j : effect of j parity

h_k : effect of k year

e_{ijkl} : random error

RESULTS AND DISCUSSION

The butyrophilin gene was screened for SSCPs using primers BTI1, BTI3 and BTI4 (Table 1). The SSCP patterns for all the three primers were best resolved in 12% acrylamide-bisacrylamide with the same SSCP conditions *viz.* voltage (200 volts), temperature (25°C), time (12 h) with 10% glycerol. The butyrophilin gene fragments amplified using BTI1 yielded three polymorphic SSCP patterns *viz.* A, B and C in 12% non-denaturing PAGE (Figure 1) while fragments amplified using BTI3 and BTI4 primers did not exhibit polymorphic SSCP fragments. The frequencies of polymorphic SSCP patterns in BTI1 amplified fragments of buffalo were: A = 0.6, B = 0.31 and CC = 0.09 in 55 Murrah buffaloes; in Surti A = 0.5, B = 0.3 and C

= 0.2 while in Bhadawari A= 0.4, 0.5 and 0.1. This study revealed polymorphic SSCP in the intron 1 region of the butyrophilin gene of Murrah, Surti and Bhadawari breeds of buffaloes.

The PCR products generated using BTI1 primer representing three SSCP patterns (A, B and C) in Murrah buffalo were sequenced using automated DNA sequencing services (Bangalore Genei). The nucleotide sequences arising from this study were submitted in GenBank (EU194868, EU1997977 and EU199798). The polymorphic Murrah buffalo butyrophilin variant A sequence (EU194868) identified using BTI1 primer was compared with *Bos taurus* reference sequence (AF005497) using alignment tool (Geneious Software) which revealed eleven computational mutations. The Murrah buffalo butyrophilin variant sequence (EU194868) was found to have 96% pairwise percent similarity with *Bos taurus* nucleotide sequence (AF005497). The BTI1 SSCP polymorphism was used to genotype Murrah, Surti and Bhadawari populations of buffaloes in which BTI1 SSCP genotypes were in Hardy-Weinberg proportions (Table 2).

Association of butyrophilin allelic variants with milk production traits in Murrah buffaloes: The variance analysis indicated BTI1 SSCP was significantly associated ($P \leq 0.05$) with 305-day lactation milk yield of Murrah buffaloes. The Murrah buffaloes with BTI1BB genotypes had 683.93 kg and 320.48 kg higher milk yield as compared to BTI1AA and BTI1CC genotypes, respectively. The positive association of BTI1 SSCP polymorphism with milk production traits may be useful for improving milk performance in dairy buffaloes. However, it was observed that, BTI1 polymorphism genotypes did not significantly differ with fat and SNF percentage trait values of Murrah buffaloes. The least squares means for milk yield, fat and SNF percentage in Murrah buffaloes with different genotypes are given in Table 3.

Presently with improvements in selection and breeding technologies, selection pressure has been steadily growing. Among the tools at the disposal of modern animal breeders, marker assisted selection conceivably seems to be the most direct control, for it can hasten the rate of genetic gain of desirable traits in farm animals. Hence, in the present study, the sequence variations in buffalo butyrophilin gene were investigated by SSCP analysis of three amplified fragments. Three SSCP variants were detected in the BTI1 amplified fragment. The SSCP polymorphism was designated as BTI1 SSCP. The variance analysis indicated BTI1 SSCP was significantly associated ($P \leq 0.05$) with 305-day lactation milk yield of Murrah buffaloes. The Murrah buffaloes with BTI1BB genotypes had 683.93 kg and 320.48 kg higher milk yield as compared to BTI1AA and BTI1CC genotypes respectively. The statistical association of butyrophilin gene polymorphism revealed using SSCP followed by DNA sequencing is one of the first reports in Murrah buffaloes Similarly in previous studies, Jang *et al.* (2005) studied association of butyrophilin candidate genes with production traits in Korean dairy proven and young bulls and reported that BTN3 was associated with 305-day production traits ($p<0.05$). Bhattacharya *et al*. (2006) reported *Hae*III PCR-RFLP polymorphism in crossbred cattle and identified AA, BB and AB genotypes having significant effects ($p \leq 0.05$) on total milk solid, fat and SNF percentages.

CONCLUSION

In the present study, PCR-SSCP after direct sequencing was found to be feasible

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

Name of Primer	Primer Sequence		T^A / RE/ Gene region
BTI1	FP	5′-CCTGCTTATTTCCCTAGTCTC-3′	57°C/ intron1
	RP	5′-CCACCCTAAGGTTAGTCAATC-3′	
BTI3	FP	5′-AACTGGCTATAAAGCCCTAGA-3′	57°C/ intron3
	RP	5′-ACTACACAAGGGAACTGAGGT-3′	
BTI4	FP	5′-AGATCTCACAGACATTCCAGA-3′	62°C/ intron4
	RP	5′-TGCTGAACCAGAGGTAGAGTA-3′	

Table 2. The frequency of BTI1 SSCP genotypes and variants in Murrah, Surti and Bhadawari breeds of buffaloes.

Buffalo Breed	N	Number/ Frequency of genotypes						Variant Frequency		
		AA	BA	BB	CA	CB	CC	A	B	C
Murrah	55	33/0.6	0/0	17/0.31	0/0	0/0	5/0.09	0.6	0.31	0.09
Surti	10	5/0.5	0/0	3/0.3	0/0	0/0	2/0.2	0.5	0.30	0.2
Bhadawari	10	4/0.4	0/0	5/0.5	0/0	0/0	1/0.1	0.4	0.5	0.1

Table 3. Means and standard error (SE) of studied traits in reference to BTI1 polymorphism in Murrah buffalo.

Genotype	n	Milk Yield ± SE	FAT*±SE	SNF*±SE
BTI1 AA	33	2204.56 ± 223.75	0.297^{NS}±0.004	0.315^{NS}±0.001
BTI1 BB	17	2888.49^{S}± 295.83	0.296^{NS}±0.006	0.315^{NS}±0.002
BTI1 CC	05	2568.01± 415.78	0.309^{NS}±0.008	0.318^{NS} ±0.002

*are scale-transformed values; Within columns, means marked by the same superscript^{S} did differ significantly at P≤0.05.

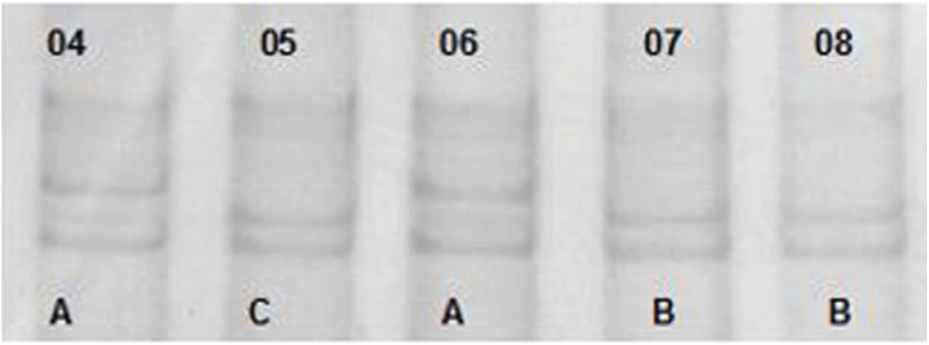

Figure 1. Single strand conformation polymorphism (BTI1) within butyrophilin gene in riverine buffaloes where,
A, B and C = SSCP patterns
04-08 = Animal numbers

technique to reveal nucleotide sequence variation. The variance analysis revealed that the BTI1 SSCP variant BTI1BB genotype was differing significantly with 305-day lactation milk yield of Murrah buffaloes. Validation of identified polymorphism with a larger dataset might be good candidates for marker assisted breeding for better milk production in dairy buffaloes. The observed nucleotide sequence variation in the butyrophilin gene and positive association with production traits can be useful information resource for buffalo genetic improvement, conservation, management and breeding decisions.

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