INTRODUCTION

The candidate gene approach is one of the most important approaches to search for genetic markers associated to production traits and in analyzing polymorphism of structural and protein coding genes. Currently, genetic markers research applied to animal breeding and production is focused mainly on analysing mutations located within candidate genes and their association with economically important production traits (Oikonomou et al. 2011). There are several validated DNA polymorphisms in various loci related with milk production traits in dairy cattle (Chamberlain et al. 2012). Diacylglycerol transferase1 (DGAT1) was the first identified gene encoding a protein with DGAT activity (Cases et al. 1998) in which a missense mutation (Lys32→Ala) has been shown to be significantly associated with variation in milk fat percentage in cattle (Winter et al. 2002). DGAT-like activity has also been shown in other enzymes encoded by other genes and led to the detection of diacylglycerol transferase2 (DGAT2), monoacyl glycerol transferases 2 (MOGAT2), leptin and butyrophilin genes were analysed using PCR-SSCP in Murrah buffalo. The single strand conformation polymorphism (SSCP) analysis of amplified gene fragment in exon 5 of MOGAT2, exon 3 of leptin and intron 1 of butyrophilin gene revealed different patterns. A, B and C showed the following frequencies for each candidate gene in 53 (A=0.49, B=0.36 and C=0.15), 65 (A=0.38 and B=0.62) and 55 samples (A=0.6, B=0.31 and C=0.09) from Murrah buffaloes, respectively. The strand conformation polymorphism (SSCP) analysis followed by DNA sequencing revealed one single nucleotide polymorphism (SNP) that was (c.193T>C) in MOGAT2, one single nucleotide polymorphism (SNP) (c.25 T>C) in leptin and one single nucleotide polymorphism (SNP) (c.184C>T>G) in butyrophilin gene confirmed BTI1 SSCP. The statistical analysis using general linear model procedure for association study, indicated that Murrah buffalo monoacyl glycerol transferases 2 (MOGAT2) (c.193T>C) single nucleotide polymorphism (SNP) and (c.25 T>C) in leptin genotypes were not significantly different (P>0.01) in Murrah buffalo for milk production traits milk yield, fat percentage and single nucleotide polymorphism (SNP) percentage. However, the statistical analysis for association study indicated BTI1 SSCP was significantly (P≤0.05) associated with 305-days lactation milk yield. 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 butyrophilin single strand conformation polymorphism (SSCP) polymorphism with milk yield will be a useful tool for future selection and breeding strategies and genetic improvement of buffaloes for milk yield.

KEY WORDS butyrophilin, DGAT2 / MOGAT2, leptin, milk yield, Murrah buffalo, PCR-SSCP.
transfereases 1 (MOGAT1) and monoacyl glycerol transfereases 2 (MOGAT2) which are the members of the same family (Winter et al. 2003). Leptin is a 16-kDa protein secreted predominantly from white adipose tissue and it performs important roles in controlling the body weight, feed intake, immune function and reproduction in dairy cattle (Block et al. 2001). Nevertheless, Liefers et al. (2005) reported the relationship between the two extreme genotype combinations (polymorphisms located at a 135 bp region of the leptin promoter) showing and increase in milk production on dairy cattle. Butyrophilin has been shown to be a mammary-specific gene and it is normally used as a genetic marker to investigate allelic substitution effects on several quantitative milk production traits (Franke et al. 1981). Recently, the association of butyrophilin gene polymorphism with milk yield, protein yield and SNF yield was reported in Korean dairy proven and young bulls (Jang et al. 2005).

India is a rich country in buffalo genetic resource having 97 million of buffaloes in 2003 which accounts for 59.5% to total world buffalo’s population. Buffalo is the most important farm animal species in Asia, especially in India, where it is extensively used for milk, meat, fuel and fertilizer production (from manure) as well as for draught power (Borghese, 2005). The buffaloes contribute 55.6% to the total world buffalo’s population. Buffalo is the most important farm animal species in Asia, especially in India, where it is extensively used for milk, meat, fuel and fertilizer production (from manure) as well as for draught power (Borghese, 2005). The buffaloes contribute 55.6% to the total world buffalo’s population. Buffalo is the most important farm animal species in Asia, especially in India, where it is extensively used for milk, meat, fuel and fertilizer production (from manure) as well as for draught power (Borghese, 2005). The buffaloes contribute 55.6% to the total world buffalo’s population.

**MATERIALS AND METHODS**

**Sample collection**
The study group included a total 173 well maintained Murrah buffaloes at NDRI, Karnal herd whose breeding tracts are the Haryana and Punjab states of India. The SSCP analysis of candidate genes (DGAT2/MOGAT2, leptin and butyrophilin genes) was carried out using vacuum tubes containing acid citrate dextrose solution (ACD) as anticoagulant.

**DNA isolation and primers**
Genomic DNA isolation was carried out from blood using the phenol chloroform extraction protocol (Clamp et al. 1993) with some modifications like addition of sodium acetate (3 M) to the aqueous phase looking for the final concentration of 0.33 M and thorough mixing. 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 working aliquots were maintained at -20 °C. The PCR primers (Table 1) designed for DGAT2 / MOGAT2, leptin and butyrophilin genes analysis in buffaloes were used on the basis of cattle GenBank sequence encompassing polymorphic restriction site with PRIMER3 software (Rozen and Skaletsky, 1998). There is a close evolutionary relationship between cattle and buffaloes that hence the bovine genome is a useful source of markers for the buffalo genome mapping (Gallagher and Womack, 1992). Therefore, the designing of primers based on cattle genome works for some of the buffalo genomic regions.

**Polymerase chain reaction (PCR) amplification**
The PCR was carried out on about 100 ng of genomic DNA in a 25 μL reaction volume. The reaction mixture consisted of 2.5 μL of 10 × PCR assay buffer containing 1.5 mM MgCl2, 200 μM each of dNTPS, 0.75 units Taq DNA polymerase and 10 pmol of each primer. Amplification was carried out in a Biometra thermal cycler using the following PCR cycling conditions: 95 °C for 5 min and 34 cycles of 45 seconds at 95 °C, T° A °C and 72 °C consecutively, followed by a five minute final extension at 72 °C. 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 × 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 μg/mL). The amplified products (5 μL) were detected on 2% agarose gel using 1 μL of loading dye as a stop dye; electrophoresis was performed and visualized using UV light after ethidium bromide staining.

**Strand conformation polymorphism (SSCP) analysis**
The DGAT2 / MOGAT2, leptin and butyrophilin gene PCR products were resolved by SSCP analysis (Orita et al. 1989).
Various factors were tested for each fragment in order to optimize factors such as the amount of PCR product (4-10 μl), denaturing solution, acrylamide concentration (8 to 14%), percentage cross linking (2%), presence or absence (10%) glycerol, voltage (60 to 400 volts), running time (12 to 30 hours) and temperature (10-15 °C and room temperature) (by indicating the final optimized conditions used on this experiment in the results section). 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 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 20% acrylamide: bis-acrylamide (49:1) gel. The vertical gel electrophoresis was carried out in a Bio-Rad Protean® II Xi electrophoresis unit using 1X TBE buffer at 10-12.5 V/cm for 24 hours at room temperature. Gels were silver-stained (Sambrook and Russell, 2000) and photographed using digital camera for SSCP pattern analysis.

**DNA sequencing**

The PCR products representing different SSCP patterns were analyzed using florescent sequencing technique from automated DNA sequencing service (Bangalore Genei). The chromatograms were used to verify the nucleotide sequence on the basis of peaks. The nucleotide sequence alignments were carried out using alignment tools from Geneious software, Clustalw and BLAST to reveal single base variations. These allelic variants nucleotide sequences were analysed and submitted in NCBI GenBank. The observed DNA sequence polymorphisms observed were used to genotype Murrah buffalo population.

**Statistical analysis**

The frequency of polymorphic allelic variants, genotypes and their accordance with Hardy-Weinberg law was assessed by POPGENE 1.31 software. The association between polymorphic allelic variants of candidate genes and milk production traits were analysed using GLM procedure (SYSTAT). The following model was used:

\[
Y_{ijkl}= \mu + g_i + s_j + p_k + h_l + \epsilon_{ijkl}
\]

Where:

- \(Y_{ijkl}\): observation on the \(m\)th animal of \(i\)th genotype, \(l\)th season, \(k\)th parity, and \(h\)th year.
- \(\mu\): population mean.
- \(g_i\): effect of \(i\)th genotype (\(i=1, 2\) and 3).
- \(s_j\): effect of \(j\)th season.
- \(p_k\): effect of \(k\)th parity.
- \(h_l\): effect of \(l\)th year.
- \(\epsilon_{ijkl}\): random error associated with the measurement on the \(m\)th animal of the \(i\)th genotype, \(l\)th season, \(k\)th parity and \(h\)th year, assuming \(\epsilon_{ijkl} \sim \text{NII}(0, \sigma^2_e)\).

**RESULTS AND DISCUSSION**

The DGAT2 gene fragments amplified using primers: DGAT2-1141, DGAT2-1561 and DGAT2-1861 (Table 1) did not exhibit SSCP polymorphisms indicating monomorphic nature of intron 4 region of buffalo DGAT2 gene. The SSCP analysis of the amplified gene fragment of exon 5 of the MOGAT2 gene resulted in three different patterns; A, B and C (Figure 1) with the following frequencies (A=0.49, B=0.36 and C=0.15).

**Table 1** Details of primer sequences used for Murrah buffalo DGAT2 / MOGAT2, leptin and butyrophilin gene PCR-SSCP analysis

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Primer sequence</th>
<th>Annealing temperature / region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DGAT2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DGAT2-1141</td>
<td>FP: 5′-TTGACACGCTGACAGAAGGTA-3′</td>
<td>54 °C / intron 4</td>
</tr>
<tr>
<td>DGAT2-1561</td>
<td>FP: 5′-GGTGACAAAAATGCAACACTT-3′</td>
<td>61 °C / intron 4</td>
</tr>
<tr>
<td>DGAT2-1861</td>
<td>FP: 5′-TACTTCTACCGCCCTGGTGG-3′</td>
<td>61 °C / intron 4</td>
</tr>
<tr>
<td><strong>MOGAT2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOGAT2</td>
<td>FP: 5′-TTTGTCTTTATGGCCCTACCGG-3′</td>
<td>65 °C / exon 5</td>
</tr>
<tr>
<td><strong>Leptin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEPE3</td>
<td>FP: 5′-CAATGACCTGGAGAACCTC-3′</td>
<td>62 °C / exon 3</td>
</tr>
<tr>
<td><strong>Butyrophilin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTI1</td>
<td>FP: 5′-CCTGCTTATTTCCCTAGTCTC-3′</td>
<td>57 °C / intron 1</td>
</tr>
<tr>
<td>BTI3</td>
<td>FP: 5′-ACTGCTATAAACGCCTAGA-3′</td>
<td>57 °C / intron 3</td>
</tr>
<tr>
<td>BTI4</td>
<td>FP: 5′-AGATCTCAGACATCCCAGAAGGTA-3′</td>
<td>62 °C / intron 4</td>
</tr>
</tbody>
</table>

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The SSCP patterns for LEPE3 primer were better resolved in 12% acrylamide-bisacrylamide with other conditions kept similar: voltage (200 volts), temperature (25 °C), time (8 h) with 10% glycerol.

The SSCP analysis of amplified exon 3 fragment of the leptin gene using primer LEPE3 resulted in two different patterns: A and B (Figure 1) with the following frequencies in 65 Murrah buffaloes (A=0.38 and B=0.62). The Butyrophilin SSCP patterns were better resolved in 12% acrylamide-bisacrylamide with SSCP conditions: voltage (200 volts), temperature (25 °C), time (12 h) with 10% glycerol. The frequency of polymorphic SSCP patterns in BTI1 (Figure 1) amplified fragments was found with the following frequencies: A= 0.6, B= 0.31 and C= 0.09 in 55 Murrah buffaloes. The gene sequences arising out of this, i.e. EU239373 and EU239374 were submitted to GenBank. Leptin gene sequence analysis revealed one SNP (T-C substitution) in exon 3 at 25th nucleotide position denoted as (c.25 T>C) and nucleotide sequences arising out of this were submitted to GenBank. The ANOVA results indicated non-significant (P>0.01) effects of different Murrah buffalo MOGAT2 genotypes:

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>Variant genotype</th>
<th>N</th>
<th>Milk yield±SE</th>
<th>FAT±SE</th>
<th>SNF±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOGAT2</td>
<td>c.193T &gt; C TT</td>
<td>26</td>
<td>1775.81±241.06</td>
<td>0.297±0.005</td>
<td>0.315±0.001</td>
</tr>
<tr>
<td></td>
<td>c.193T &gt; C TC</td>
<td>27</td>
<td>1678.21±264.85</td>
<td>0.297±0.005</td>
<td>0.314±0.001</td>
</tr>
<tr>
<td>Leptin</td>
<td>c.25T &gt; C CC</td>
<td>40</td>
<td>2052.87±290.38</td>
<td>0.297±0.004</td>
<td>0.315±0.001</td>
</tr>
<tr>
<td></td>
<td>c.25T &gt; C CT</td>
<td>25</td>
<td>2421.37±280.95</td>
<td>0.294±0.004</td>
<td>0.315±0.001</td>
</tr>
<tr>
<td>Butyrophilin</td>
<td>BTI1 AA</td>
<td>33</td>
<td>2204.56±223.75</td>
<td>0.297±0.004</td>
<td>0.315±0.001</td>
</tr>
<tr>
<td></td>
<td>BTI1 BB</td>
<td>17</td>
<td>2888.49±295.83</td>
<td>0.296±0.006</td>
<td>0.315±0.002</td>
</tr>
<tr>
<td></td>
<td>BTI1 CC</td>
<td>05</td>
<td>2568.01±415.78</td>
<td>0.309±0.008</td>
<td>0.318±0.002</td>
</tr>
</tbody>
</table>

The least squares mean values of the milk production traits studied in Murrah buffaloes differing in their MOGAT2 (c.193T>C) genotypes are given in Table 2. In a bovine MOGAT2 gene polymorphism study Winter et al. (2003) found 15 SNPs outside exons and two silent exon SNPs and a standard chi-square test did not reveal a significant association of allele frequencies with breeding values for milk fat content in any of the three analyzed dairy breeds. Similarly, results of ANOVA in leptin gene indicated non significant (P>0.01) effect of allelic variants (c.25T>C) on milk yield, fat percentage and SNF percentage. The least square mean values of milk production traits not differing in their leptin (c.25T>C) genotypes are given in Table 2. In this line, other researchers reported only SNPs in bubaline Leptin gene including the 5’ region (Vallinoto et al. 2004). The butyrophilin gene after variance analysis indicated that, BTI1 SSCP was significantly (P<0.05) associated with 305-days 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.

The means within the same column with at least one common letter, do not have significant difference (P>0.05). NS: non significant and SE: standard error.

SNF: solids not fat.
Furthermore, Jang et al. (2005) reported in cattle that butyrophilin polymorphism (BTN3) was associated with 305 days production traits (P<0.05).

CONCLUSION

The present study revealed that PCR-SSCP and DNA sequencing are one of the most effective molecular biological techniques to detect DNA sequence variation at candidate gene loci in buffaloes. The possible reasons for non-significant effect of DGAT2 / MOGAT2 and leptin allelic variants might be due to the fact that sample size was small, absence of some genotypes, high standard error and uneven distribution of data. The studies concerning associations between DGAT2 / MOGAT2 and leptin gene polymorphism and production traits of riverine buffaloes are, however, fairly scarce. As these two candidate genes have genetic and functional similarity with identified markers is necessary to characterize them completely in buffalo genome. However, in butyrophilin gene, the variance analysis revealed that the BTI1 SSCP variant BTI1BB genotype was differing significantly with 305-days lactation milk yield of Murrah buffaloes. Therefore, validation of the identified polymorphism with a larger dataset might be a good candidate for marker assisted breeding in order to get better milk production in dairy buffaloes. The observed nucleotide sequence variation in the butyrophilin gene and positive association with production trait can be also a useful information resource for buffalo genetic improvement, conservation, management and breeding decisions.

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