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Short Communication

Identification of novel single nucleotide polymorphisms in the DGAT1 gene of buffaloes by PCR-SSCP

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Abstract

Diacylglycerol O-acyltransferase 1 (DGAT1) is a microsomal enzyme that catalyzes the final step of triglyceride synthesis. The DGAT1 gene is a strong functional candidate for determining milk fat content in cattle. In this work, we used PCR-SSCP (polymerase chain reaction-single-strand conformation polymorphism) and DNA sequencing to examine polymorphism in the region spanning exon 7 to exon 9 of the DGAT1 gene in Murrah and Pandharpuri buffaloes. Three alleles (A, B and C) and four novel single-nucleotide polymorphisms were identified in the buffalo DGAT1 gene. The frequencies of the alleles differed between the two buffalo breeds, with allele C being present in Murrah but not in Pandharpuri buffalo. The allele variation detected in this work may influence DGAT1 expression and function. The results described here could be useful in examining the association between the DGAT1 gene and milk traits in buffalo.

Key words: buffalo, DGAT1, PCR-SSCP, polymorphism, SNP.

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Diacylglycerol O-acyltransferase 1 (DGAT1) is one of the key enzymes in controlling the rate of triglyceride synthesis in adipocytes. Since the DGAT1 gene maps to the quantitative trait loci (QTL) for milk fat percentage in the centromeric region of chromosome 14 (BTA14), this gene has been studied as a candidate for association with the milk fat content in cattle.Mutation analysis in cattle has revealed 19 polymorphic sites within the DGAT1 gene (Winter *et al.*, 2002). Among these polymorphisms the GC/AA exchange at positions 10433/10434 (GenBank accession no. AJ318490) in exon 8 that results in a *K232A* amino acid exchange was found to affect the milk fat content (Spelman *et al.*, 2002; Boichard *et al.*, 2003) and has been extensively studied in several cattle populations (Lacorte *et al.*, 2006). The influence of DGAT1 *K232A* on milk fat content differs among populations (Spelman *et al.*, 2002; Thaller *et al.*, 2003), suggesting that further variations in the genomic region of DGAT1 may be involved. In this context, polymorphism in the promoter region has been reported to have functional relevance for DGAT1 transcription (Kühn *et al.*, 2004).

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Buffaloes are the major milk-producing domestic animals in India, with a milk fat content ranging from 7% to 11% among different breeds. DGAT1*K* is reported to be fixed in five Indian breeds of buffalo (Tantia *et al.*, 2006). Since the regulation of DGAT1 expression in adipocytes occurs largely at the transcriptional and post-transcriptional levels (Yu *et al.*, 2002) it is possible that mutations in the introns and untranslated regions may also influence DGAT1 expression and, consequently, the milk fat content. The aim of this study was to examine the polymorphism in introns 7 and 8 of the DGAT1 gene in Murrah and Pandharpuri buffaloes using polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), a rapid, precise technique that allows the identification of single-nucleotide polymorphisms (SNPs).

Forty-four buffaloes (22 Murrah and 22 Pandharpuri) reared in different agroclimatic areas were used in this study. Murrah is the major buffalo breed on northern India whereas Pandharpuri is from western India. High milk yields and breed-specific traits were the main selection cri-

teria in both cases. Unrelated animals reared in the districts of Rohtak, Hisar, Jind and Bhiwani in the case of Murrah and in the districts of Solapur, Sangli and Kohlapur in the case of Padharpuri buffaloes were sampled and only healthy animals with the characteristic traits of each breed were studied.

Ten millilitres of venous blood was collected from each animal into tubes containing 500 µL of 2.7% EDTA as anticoagulant. The samples were transported to the laboratory on ice and stored at -20 °C until isolation of genomic DNA. Genomic DNA was isolated from 5 mL of blood by the phenol-chloroform extraction method (Sambrook and Russel, 2001). The quality and purity of the isolated DNA were determined by agarose gel electrophoresis and spectrophotometry, respectively. The DNA samples that showed a single band on agarose gel and had an A_{260nm}/A_{280nm} ratio of ~1.8 were used for further analysis. DNA was quantified by comparison with lambda DNA of known concentration in 0.8% agarose gels.

A region of the buffalo DGAT1 gene spanning parts of exon 7 and exon 9 (including intron 7, exon 8 and intron 8 entirely) was amplified by PCR using forward (5'-GCACCATCCTCTTCCTCAAG-3') and reverse (5'-GGAAGCGCTTTCGGATG-3') primers (Winter *et al.*, 2002). Although this region of the buffalo gene has already been amplified and sequenced by others (Tantia *et al.*, 2006), in the present study we used PCR-SSCP to examine polymorphism in the exon 7 to exon 9 region of the gene. The PCR mixture (final volume: 25 µL) contained 10 pM of each primer, 100 µM of each dNTP, 1.5 mM $MgCl_2$, 100 ng of genomic DNA as template and 1 U of *Taq* DNA polymerase (Fermentas) in PCR buffer. The samples were denatured at 94 °C for 5 min and then subjected to 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The length of the amplified fragment was 411 bp in both breeds.

Four microliters of each PCR product was mixed with 14 µL of formamide loading buffer (0.05% xylene-cyanole, 0.05% bromophenol blue, 5.5 mM EDTA, pH 8.0, in formamide) and forward and reverse primer solution (final concentration: 150 nM), denatured at 95 °C for 5 min and snap-chilled on ice for at least 10 min. Samples were then loaded onto a non-denaturing 15% polyacrylamide gel (acrylamide:bis-acrylamide ratio of 49:1) and electrophoresis was done at 10 °C in 0.5X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 20 h at 200 V. The bands were silver stained (Bassam *et al.*, 1991). Two PCR products corresponding to each of the different PCR-SSCP alleles/patterns identified were purified using PCR product purification kits (Qiagen) and sequenced by an automated dye terminator cycle sequencing method in an ABI PRISM 377 DNA sequencer. The sequences were analyzed using DNASTAR Lasergene 7.0 sequence analysis software to identify SNPs in the different PCR-SSCP alleles.

SSCP detects single-base sequence changes by abnormal electrophoretic migration of one or both single strands in a non-denaturing polyacrylamide gel (Gruszczynska *et al.*, 2005). Four PCR-SSCP patterns were observed in Murrah buffaloes while only two patterns were seen in Pandharpuri buffaloes (Figure 1). Since single-stranded DNA (ssDNA) usually yields two bands for homozygotes and four for heterozygotes the patterns were denominated as genotypes AA, AB, BB and AC in Murrah and as AA and AB in Pandharpuri. The lower band in the AC genotype sample could not be separated. Similar results have also been reported for a heterozygous genotype of the kappa-casein bovine gene for which only three PCR-SSCP bands were confidently identified (Barroso *et al.*, 1998).

The addition of forward and reverse primers (final concentration of each primer: 150 nM) to the gel loading mixture greatly improved the resolution and reproducibility of the SSCP bands. This finding confirmed the idea that primer addition during SSCP analysis leads to hybridization of the primer with ssDNA, reduces DNA reannealing and affects the mobility and sensitivity of the SSCP bands (Zhu *et al.*, 2006). However, despite this manoeuver, an additional band was observed in all of the samples with allele A, *i.e.*, genotypes AA, AB and AC. Since this band was not seen in the BB genotype and since sequencing of the PCR products of the AA genotype revealed no discrepancy between the sequences of different samples or the forward and reverse sequences, we concluded that the additional band was an alternative form of the allele A DNA sequence. This conclusion agrees with the demonstration that strands with the same sequence may yield different stable confor-

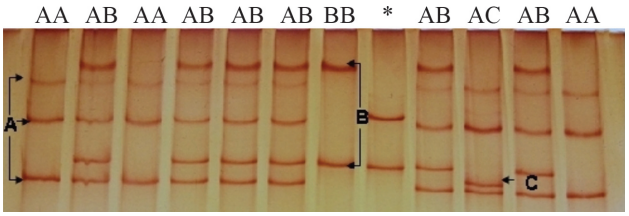

Figure 1 - Silver-stained polyacrylamide gel showing the PCR-SSCP patterns of alleles of the DGAT1 gene amplified from genomic DNA of Murrah and Pandharpuri buffalo. The SSCP-derived genotypes are indicated at the top of each lane. Bands A, B and C in lanes AA, BB and AC, respectively, indicate the alleles identified in this work. *PCR-SSCP pattern of the DGAT1 gene fragment amplified from sheep (control).

Table 1 - Nucleotide substitutions in the PCR-SSCP alleles of the buffalo DGAT1 gene.

Allele (Accession number)	Nucleotide positions*			
	75	136	256	277
A (DQ886485)	C	C	C	G
B (FJ014704/FJ014706)	C	G	T	G
C (FJ014705)	A	C	C	T

*Based on the sequence in GenBank, accession number FJ014704.

Table 2 - Genotype and allele frequencies based on PCR-SSCP analysis of the DGAT1 gene in Murrah and Pandharpuri buffaloes.

Breed	Genotype frequency				Allele frequency		
	AA	AB	BB	AC	A	B	C
Murrah (n = 22)	0.27	0.55	0.09	0.09	0.59	0.36	0.05
Pandharpuri (n = 22)	0.45	0.55	-	-	0.73	0.27	-

mations known as isoconformers (Ripoli *et al.*, 2006; Chessa *et al.*, 2010).

Samples with different PCR-SSCP patterns were sequenced to identify the underlying SNP. The sequences have been deposited in GenBank under accession numbers FJ014704 (allele B in Murrah), FJ014705 (allele C in Murrah) and FJ014706 (allele B in Pandharpuri). Polymorphism was seen as a substitution at four positions (Table 1). Seven SNPs have been reported based on PCR-SSCP studies of the entire DGAT1 gene in Chinese buffalo (Yuan *et al.*, 2007) and 19 SNPs (DQ886485) were reported based on sequencing studies in Indian buffalo (Mishra *et al.*, 2007), but none have been reported for the region studied here. The sequence of allele A identified here was identical to that reported for the DGAT1 gene in Indian buffalo (DQ886485). Alleles B and C were novel and differed from allele A at two positions each, whereas allele B differed from C at four positions; the sequences of all three alleles differed from those of Chinese buffalo (AY999090). Three PCR-SSCP alleles have been reported in the DGAT1 gene fragment spanning the region studied here, with two of the alleles being different confirmations of the same sequence (Ripoli *et al.*, 2006). In contrast, the sequences of the three alleles identified here differed from each other.

The GC/AA substitution in exon 8 of the DGAT1 gene in cattle has been associated with milk fat content (Winter *et al.*, 2002; Weller *et al.*, 2003; Grisart *et al.*, 2004). Exon 8 however, was found to be conserved in the three PCR-SSCP alleles identified here. This finding agrees with reports that DGAT1*K*, which is associated with a high milk fat content, is fixed in Indian cattle and buffalo breeds (Tantia *et al.*, 2006). Table 2 shows that the allele and genotype frequencies varied between the two breeds studied here (Table 2). The presence of allele C only in heterozygous individuals and only in Murrah buffaloes indicated that this may be a mutation specific to the Murrah breed or a rare allele in Pandharpuri buffaloes (Falconer and Mackay, 1996).

The SNPs identified here occurred in introns 7 and 8 of the DGAT1 gene. Polymorphisms in introns affect the functionality of genes for enzymes such as endothelial nitric oxide synthase (Buraczynska *et al.*, 2004) and the cytochrome P450 isoenzyme CYP1A2 (Sachse *et al.*, 1999). Since the DGAT1 gene is also an enzyme gene, the SNPs identified here may influence the functionality of this gene, *i.e.*, its effect on milk fat content, which varies markedly among Indian buffalo breeds. In this regard, a phylogeographic study (Berrebi *et al.*, 2005) of large populations of different buffalo breeds would be useful in determining the general distribution of the alleles identified in this study.

In conclusion, the buffalo DGAT1 gene shows considerable genetic variation and contains several novel SNPs. The variation in the distribution of several alleles between Pandharpuri and Murrah buffalo, which also differ markedly in their milk fat content, indicates a need for studies on the association between these SNPs and milk fat content. The findings described here should be useful in determining the role that DGAT1 plays in regulating milk fat synthesis and in improving the quality of buffalo milk.

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