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DGAT1 K232A polymorphism in Brazilian cattle breeds

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ABSTRACT. Recent reports identified *DGAT1* (EC 2.3.1.20) harboring a lysine to alanine substitution (*K232A*) as a candidate gene with a strong effect on milk production traits. Our objective was to estimate the frequency of the *DGAT1 K232A* polymorphism in the main Zebu and Taurine breeds in Brazil as well as in Zebu x Taurine crossbreds as a potential QTL for marker-assisted selection. Samples of 331 animals from the main Brazilian breeds, Nellore, Guzerat, Red Sindhi, Gyr, Holstein, and Gyr x Holstein F1 were genotyped for *DGAT1 K232A* polymorphism (A and K alleles) using the PCR-RFLP technique. The highest frequency of the A allele was found in the Holstein sample (73%) followed by Gyr x Holstein F1 (39%). Gyr and Red Sindhi showed low frequencies of A alleles (4 and 2.5%, respectively). The A allele was not found in the Nellore and Guzerat samples. Our results could be used to guide association studies between this locus and milk traits in these breeds.

Key words: *DGAT1*, PCR-RFLP, Candidate genes, Zebu, Cattle

INTRODUCTION

Candidate gene approaches provide tools for identifying and mapping genes affecting quantitative traits. A candidate gene can be defined as a gene with biological effects on the physiology of a trait of interest (functional) or as a gene closely linked to a functional gene (positional). Polymorphisms within selected candidate genes can be tested for their association with quantitative traits to better understand their effects and can be used in marker-assisted selection (MAS) programs (Wu et al., 2005). MAS together with traditional selection methods can be most effective for complex traits, improving accuracy, reducing generation interval and accelerating genetic progress (Drogemuller et al., 2001).

Candidate genes associated with milk proteins, somatotropic axis and lymphocyte antigens are the genes most studied in cattle (Kemenes et al., 1999). There is strong evidence that a polymorphism of the growth hormone receptor gene located on BTA 20 is associated with a major effect on milk yield and composition (Blott et al., 2003). Tsiaras et al. (2005) reported a polymorphism of the Kappa-casein gene affecting milk and fat yield in Holstein cows. Machado et al. (2005) found an association of BoLA DRB3 alleles *16 and *29 with milk production in Gyr cattle.

The current bovine linkage map is highly saturated with markers (Ihara et al., 2004). This made it possible to develop genome scan approaches aimed at detecting quantitative trait loci (QTLs) and also to isolate candidate genes in the QTL regions. Many genome scans found QTLs on chromosomes BTA 06, 14, 20, and 26 with major effects on milk production traits (Coppieters et al., 1998; Ashwell et al., 2004; Chen et al., 2006; Gautier et al., 2006).

Fine-mapping efforts located a QTL in a 3-cM marker interval on the proximal end of BTA 14 (Farnir et al., 2002). In this region, a strong candidate gene called *DGAT1* was found affecting milk traits (Winter et al., 2002; Grisart et al., 2002). *DGAT1* encodes the enzyme acyl-CoA:diacylglycerol-acyltransferase that plays a fundamental role in the metabolism of cellular diacylglycerol in physiological processes, such as intestinal fat absorption, lipoprotein assembly, adipose tissue formation, and lactation, involved with the metabolism of triacylglycerol in higher eukaryotes (Cases et al., 1998).

Further studies found a non-conservative substitution of lysine by alanine (*DGAT1 K232A*) as a consequence of an adenine/adenine to guanine/cytosine substitution at position 10433 and 10434 of exon VIII in European bovine populations (Spelman et al., 2002; Thaller et al., 2003; Weller et al., 2003). After sequencing this region in *Bos taurus taurus*, *Bos taurus indicus*, *Bos grunniens*, and *Bubalus bubalus*, Winter et al. (2002) observed that the alanine-encoding haplotype is present only in *Bos taurus taurus*, indicating that the lysine-encoding variant is likely the ancestral state of *DGAT1*. A significant decrease in protein and milk yields, and increase in fat yield were associated with the lysine substitution (K allele). The alanine variant (A allele) was associated with an increase in protein and milk yields and decrease in fat yield (Spelman et al., 2002; Thaller et al., 2003; Weller et al., 2003).

In spite of having the largest effect on milk production traits, *K232A* is not the only polymorphism affecting milk production at the end of BTA 14 (Bennewitz et al., 2004). Variable number of tandem repeat polymorphisms in the promoter region of the *DGAT1* gene also affect milk production traits, probably due to variation at the transcriptional level of *DGAT1* gene (Kühn et al., 2004).

Dairy herds in Brazil are mainly composed of Zebu breeds (*Bos taurus indicus*) and their crossbreds. Despite having a reduced milk yield performance in relation to Taurine breeds, Zebu breeds are well adapted to tropical environments and are resistant to endo- and ectoparasites in these environments (Byford et al., 1976). Thus, breeding programs focusing on the improvement of Zebu cattle milk production in addition to its robustness are of great interest for the Brazilian milk industry.

To our knowledge, few studies have been published on *DGAT1* in Zebu breeds (Pappas et al., 2004; Casas et al., 2005; Kaupe et al., 2005). The objective of the present study was to determine the frequency of the *DGAT1 K232A* polymorphism in the main Brazilian breeds, Nellore, Guzerat, Red Sindhi, Gyr, Holstein, and Gyr x Holstein F1, as a potential marker for MAS in these breeds.

MATERIAL AND METHODS

Animals

Samples were obtained from the Embrapa Dairy Cattle DNA Bank consisting of 331 animals of the Brazilian breeds Nellore, Guzerat, Red Sindhi, Gyr, Holstein, and Gyr x Holstein F1 (Table 1). Semen samples of Gyr, Nellore, Guzerat, and Holstein breeds were collected from artificial insemination companies that commercialize the major genetic contributors for the current population of these breeds in Brazil. Samples of Red Sindhi breed were collected from female animals because of the reduced and concentrated population size of this breed in a few herds of Northeastern Brazil. Samples from Gyr x Holstein F1 were collected from female animals located in the main milk production regions in Brazil.

Table 1. Characterization of samples.

Cattle breed	Species	N[1]	Sex
Gyr	*Bos taurus indicus*	53	Male
Guzerat	*Bos taurus indicus*	53	Male
Nellore	*Bos taurus indicus*	62	Male
Red Sindhi	*Bos taurus indicus*	60	Female
Holstein	*Bos taurus taurus*	50	Male
Gyr x Holstein F1	*Bos taurus indicus* x *taurus*	53	Female
Total		331	

[1]Number of animals sampled.

DNA genotyping

DNA extraction was based on the phenol-chloroform procedure described by Sambrook et al. (2001). A fragment of 411 bp of the *DGAT1* gene containing the *K232A* substitution was amplified for each sample. PCR reactions were performed in a total volume of 20 µL using 60 ng genomic DNA as template, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5%

DMSO, 0.8 U *Taq* polymerase, and 0.5 µM of each primer. Primer sequences were: forward - 5'-GCACCATCCTCTTCCTCAAG-3' and reverse - 5'-GGAAGCGCTTTCGGATG-3'. The PCR profile included an initial denaturation step at 94°C for 4 min, 10 cycles of 94°C (60 s), 66°C (↓ 1°C/cycle for 60 s) and 72°C (60 s) followed by 25 cycles of 94°C (60 s), 56°C (120 s) and 72°C (60 s), and a final extension step of 10 min at 72°C.

The PCR performance was verified by electrophoresis of an aliquot (10 µL) of the amplified product in 1.5% agarose gels stained with ethidium bromide for 30 min and photographed under UV light using a digital camera apparatus (EagleEye II, Stratagene Co.). The *K232A* polymorphism in the *DGAT1* gene was detected by the PCR-RFLP technique. For that, 10 µL amplified DNA was digested with 2 U of *Cfr*I restriction enzyme (MBI Fermentas) for 6 h at 37°C followed by a denaturation step of 20 min at 65°C. PCR and restriction reactions were performed in a 9700 PCR System (Applied Biosystems).

Digested products were visualized in 5% native polyacrylamide gels (height: 25 cm) in 1X TBE (400V for 100 min) stained with silver nitrate. Allele sizes were estimated by comparison to a 100-bp ladder. An undigested fragment of 411 bp indicated the K allele, and two fragments of 203 and 208 bp indicated the A allele. For each genotyping set, a sample previously known as alanine homozygous (AA) was added to avoid miss-identification of alleles caused by a partial digestion of the restriction enzyme (positive control).

Statistical analysis

Allelic and genotypic frequencies were calculated using the free computational software TFPGA, version 1.3 (Miller, 1997), available on-line from http://www.marksgeneticsoftware.net.

The observed heterozygosity was obtained as follows:

$$H(direct) = \sum_{i} \sum_{j \neq i} \frac{N_{lij}}{N} \qquad \text{(Equation 1)}$$

where N_{lij} is the number of heterozygous individuals in the *l* locus; *N* is the number of individuals analyzed.

The Hardy-Weinberg expected heterozygosity was obtained from the estimated allele frequencies:

$$H(HW) = 1 - \sum_{i=1}^{n} {p_{li}}^2 \qquad \text{(Equation 2)}$$

where p_{li} is the frequency of the *i* allele at the *l* locus; *n* is the number of alleles at the *l* locus.

Hardy-Weinberg heterozygosity and equilibrium were evaluated by the exact probability test (Haldane, 1954) under the assumption of a chi-square (χ^2) distribution using the free software TFPGA, version 1.3 (Miller, 1997). Comparisons between the genotypic frequencies of breeds were conducted using Fisher's exact test available on the SAS® procedures (SAS, 2002). A significance level of 0.05 was assumed for the tests.

RESULTS AND DISCUSSION

The addition of DMSO to the PCR reactions allowed an equal amplification of both alleles. The genotyping of the *DGAT1 K232A* was successfully performed by PCR-RFLP technique for all samples showing a clear separation of three different genotypes (Figure 1). The positive controls included in the genotyping sets were important to assure that the digestion step worked in all samples.

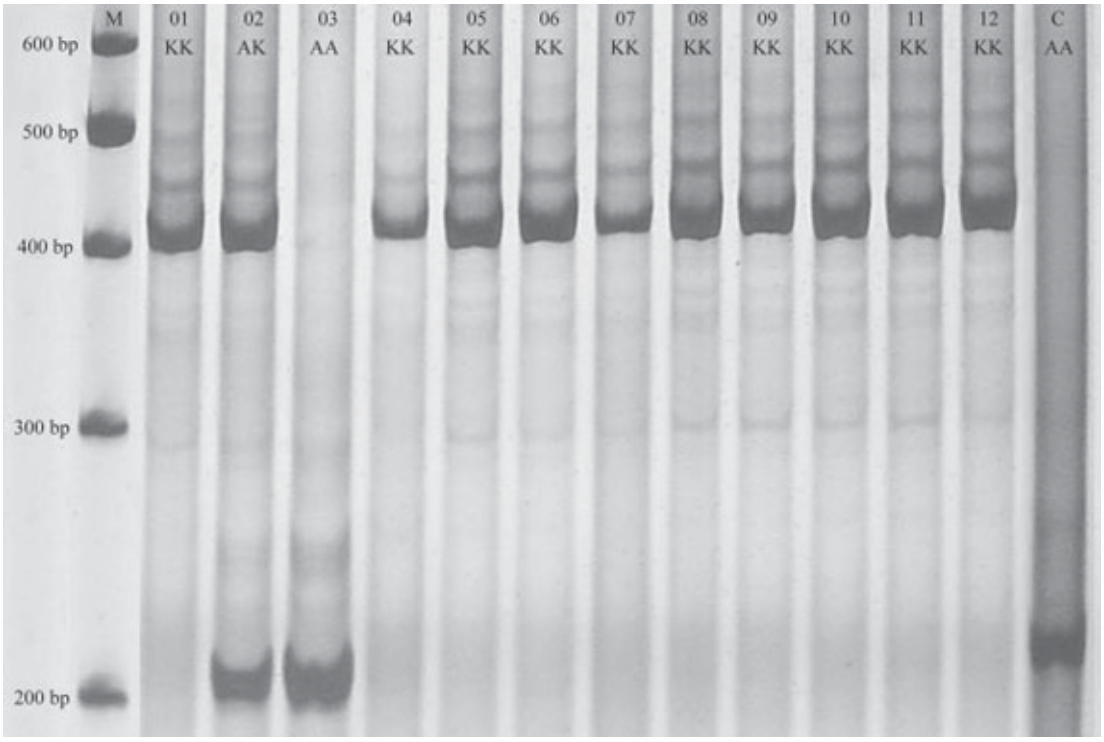

Figure 1. Polyacrylamide gel showing the genotypes (KK, AK and AA) of *DGAT1 K232A* polymorphisms of bovine animals. *Lanes*: M, 100-bp DNA ladder; *lanes 1* to *12*, bovine samples and *C*, positive control (AA genotype).

Genotypic and allelic frequencies are shown in Table 2. The exact probability of Haldane (1954) found a deviation from the Hardy-Weinberg equilibrium of the *DGAT1 K232A* genotypic frequencies for Holstein and Gyr x Holstein F1. This could be related to the sampling of the genotyped sires, but the intense use of these sires in commercial herds must also be taken into account since it could reflect the actual allelic and genotypic frequencies for this locus. This disequilibrium can be a result of indirect selection for this locus from the selection for milk production. There were no significant differences among Zebu breeds for allelic and genotypic frequencies for this locus. Otherwise, a substantial difference was found between Zebu breeds and Holstein, for which A allele and AA genotype were found at a high frequency, as well as between Zebu breeds and Gyr x Holstein F1.

The Holstein breed showed the highest frequency for the A allele (73%) among all genotyped breeds. This value was higher than the frequency obtained for the A allele in New

Table 2. *K232A DGAT1* genotypic and allelic frequencies (%) including observed and expected heterozygosity.

Breed	Genotypic frequencies (%)				Allelic frequencies (%)		Heterozygosity (%)	
	KK	AK	AA	EP[1]	K	A	Observed	Expected
Gyr	94.0	4.0	2.0	0.06	96.0	4.0	4.0	7.0
Guzerat	100.0	0.0	0.0	-	100.0	0.0	0.0	0.0
Nellore	100.0	0.0	0.0	-	100.0	0.0	0.0	0.0
Red Sindhi	95.0	5.0	0.0	1.00	97.5	2.5	5.0	5.0
Holstein	14.0	26.0	60.0	0.03*	27.0	73.0	26.0	39.0
Gyr x Holstein F1	30.0	62.0	8.0	0.04*	61.0	39.0	61.0	48.0

[1]Exact probability for Hardy-Weinberg equilibrium testing (Haldane, 1954).
*Significant for EP (<0.05).

Zealand Holstein (40%) and German Holstein (42%) populations. This frequency, however, is lower than the ones found in New Zealand Jersey (88%) and German Brown Swiss (98%), both taurine dairy breeds (Spelman et al., 2002; Kaupe et al., 2004).

The large use of imported semen from the United States in the Brazilian Holstein population (Costa et al., 2001) suggests a similarity between the allelic frequencies of Holstein populations in these countries. The high frequency of A allele found in the Holstein population in our study could be explained by a possible high frequency of this allele in the US gene pool that could be the result of an intensive indirect selection for milk production. This could also be explained by a high frequency of the A allele in the semen sample imported from US which has a great impact in the Brazilian gene pool.

The Gyr x Holstein F1 crossbred showed a frequency of 39% for the A allele. This value was similar to the ones found in purebred New Zealand (40%) and German Holstein (42%) (Spelman et al., 2002; Kaupe et al., 2004). The highest observed heterozygosity (61%) as well as expected heterozygosity (48%) was found in the Gyr x Holstein F1 crossbred and could be explained by differences in gene frequencies among Gyr and Holstein purebred parents and by the high frequency of homozygous AA genotype in the Brazilian Holstein population. Characterization of the *DGAT1 K232A* polymorphism in this crossbred population is of great interest for the Brazilian milk industry, since the majority of the Brazilian dairy cattle is composed of Gyr x Holstein F1 crossbred animals (Facó et al., 2005).

All animals examined in the Nellore population were homozygous for the K allele. Similar results were reported by Kaupe et al. (2004) who found a frequency of 1% for the A allele. Pappas et al. (2004) evaluated the *DGAT1 K232A* polymorphism in Nellore and Nellore crossbreds and found a frequency of 45% for the A allele in the Nellore x Angus crossbred, 44% for the Nellore x Canchim crossbred and 0% for the purebred Nellore, concluding that the A allele in these crossbred populations originated from the taurine germplasm.

In the Guzerat population, all animals were also homozygous for the K allele. No other report has been found in the literature for this particular breed which has been used in Brazil for both meat and dairy purposes. Therefore, we suggest that additional genotyping must be performed on a large sample of the Guzerat population to confirm the null frequency of the A allele before studies on association of this allele with milk traits can be carried out in this breed.

A frequency of 2% was found for the A allele in the Red Sindhi breed. This result suggests that MAS for the A allele could be carried out, since the Red Sindhi breed is commonly used for dairy purposes in Northeastern Brazil. Furthermore, although our results indicate that this locus is under Hardy-Weinberg equilibrium, Faria et al. (2001) pointed out risks of extinction for this breed in Brazil due to the high inbreeding level and low-effective population size.

There are two populations for the Gyr breed in Brazil: the Dairy Gyr and the Standard Gyr. Long-term progeny tests have been carried out to select proven bulls for milk production generating the Dairy Gyr population (Martinez et al., 2005). As a result, Dairy Gyr have been intensively used as purebred and crossbred (Gyr x Holstein) for dairy purposes in Brazil. In the Dairy Gyr population, we found a frequency of 4% for the A allele and only one animal was homozygous for the A allele. Pappas et al. (2004) also found a frequency of 4% for the A allele in Dairy Gyr. These findings would be of great interest for MAS of proven bulls. The presence of the A allele in the Brazilian Dairy Gyr population also raises the hypothesis of the introgression of Taurine genes from Holstein cattle in this breed at the foundation of this population, since the A allele showed a high frequency in the Brazilian Holstein breed.

CONCLUSIONS

Brazilian Holstein and Gyr x Holstein F1 showed a very high frequency of the A allele. In Nellore and Guzerat breeds, the frequency of the A allele was null. For Gyr and Red Sindhi breeds a low frequency of A allele was found. Our results could be used to guide association studies between this locus and milk traits, e.g., milk production, fat and protein yield and fat and protein content in these breeds.

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