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Polymorphisms in the *DGAT1* gene in buffaloes (*Bubalus bubalis*) in the Amazon

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ABSTRACT. Water buffaloes (*Bubalus bubalis*) are quite well adapted to climatic conditions in the Amazon, and in this biome, they are noted for the considerable amount of meat and milk they produce and how hard they are able to work. Because of a lack of research dedicated to improving the rearing of buffaloes in the Amazon, the objective of this study was to genetically characterize the Murrah and Mediterranean breeds, as well as a mixed-breed population, based on polymorphisms in the diacylglycerol *O*-acyltransferase 1 gene (*DGAT1*), and associate the genotypes with milk production. By using the polymerase chain reaction-single-strand conformation polymorphism technique, the alleles A (0.79), B (0.20), and D (0.01) were found in the Murrah breed. In the Mediterranean and mixed-breed buffaloes, we found

alleles A (0.69) and (0.77) and B (0.31) and (0.23), respectively. The Murrah breed had the genotypes AA (0.63), AB (0.29), BB (0.05), and AD (0.03), and the Mediterranean and mixed-breed buffaloes had the genotypes AA (0.44) and (0.61), AB (0.50) and (0.31), and BB (0.06) and (0.08), respectively. For the Murrah, Mediterranean, and mixed-breed buffaloes, respectively, the expected heterozygosity values were 0.34, 0.43, and 0.35, the inbreeding coefficients were 0.78, -0.15, and 0.17, and the Hardy-Weinberg probabilities were 0.70, 0.67, and 0.52. The genotypes evaluated did not have an effect on milk production; however, the single nucleotide polymorphisms can be used in studies on genetic variability.

Key words: SNP; Buffalo; Milk; Genotype; PCR-SSCP

INTRODUCTION

In Brazil, water buffalo (*Bubalus bubalis*) rearing has been developed on a large scale, with an annual increase of 4.3%. Brazil contains 1.3 million buffaloes, and the country's northern region holds 64.1% of the overall population, the northeastern region has 9.7%, the southeast has 11.5%, the south has 9.0%, and the center-west has 5.8%. The northern state of Pará is the main buffalo-rearing state with 38.5% of the national total, followed by Amapá state with 18.1% (IBGE, 2011). The production and commercialization of buffalo milk and its derivatives are quite varied (Dias et al., 2012). The production and consumption of buffalo milk is increasing, because of an increase in demand for foods derived from it, such as milk and cheese. Therefore, the components of buffalo milk, such as fats and total solids, make buffalo milk higher quality than cow milk (Rosales and Batalha, 2013).

The use of molecular markers that are associated with zootechnical data significantly contributes to animal production, avoids threats to the breeds during breeding control, and increases the yield of these animals in Brazil (Mariante et al., 2011). Single nucleotide polymorphism (SNP) markers are amongst the most varied molecular markers, and are characterized by a change in a unique nitrogenous base, allowing for the detection of two nucleotides in the same position and demonstrating codominant inheritance (Vignal et al., 2002).

Among the various genes studied with reference to the production of milk in domestic animals, diacylglycerol *O*-acyltransferase 1 (*DGAT1*) controls the rate of triglyceride synthesis via adipocytes (Yen et al., 2008). *DGAT1* in buffaloes is approximately 8.3 kb long, contains 17 exons, and is located on the 14th chromosome (Yuan et al., 2007). SNP-type polymorphisms were found by Raut et al. (2012) at the extension of exon 7 to exon 9 in *DGAT1* in buffaloes, and they concluded that it would be possible to associate them with milk yield. Another study showed that minisatellite markers in *DGAT1* are associated with milk constituents in buffaloes (Cardoso et al., 2015). Tăbăran et al. (2015) reported the influence of polymorphisms in *DGAT1* on the percentage and profile of milk fat in Romanian Holstein cattle; therefore, they should be used in marker-assisted selection (Hill et al., 2016). Lacorte et al. (2006) used polymerase chain reaction (PCR)-restriction fragment length polymorphism to reveal that SNPs in *DGAT1* exhibited a high frequency of the A allele in Holstein and Gyr x Holstein F_1 cattle. However, the A allele has not been found in the Nellore and Guzerat breeds, and is only found at a low frequency in the Gyr and Red Sindhi breeds. These findings demonstrate that cattle have low

genetic variability. Genetic polymorphisms only occur in some cattle breeds, and in buffaloes, they are conserved on the K-allele sequence. Therefore, the objective of this study was to identify polymorphisms in *DGAT1*, and genetically characterize and associate polymorphisms with the production of milk in Murrah, Mediterranean, and mixed-breed herds in the Brazilian Amazonian region.

MATERIAL AND METHODS

The milk production in one control day (the total milk produced in each milking session within a 24-h period) of 83 buffaloes (38 Murrah, 32 Mediterranean, and 13 mixed-breed) was analyzed. The mixed-breed cattle were between 1/2, 1/4, 7/8, 15/16, 17/32, 25/32, and 33/64 of Murrah and Mediterranean. All of the cattle were taken from the PROMEBULL project, which was developed in Campo Experimental de Terra Alta in Pará at the Embrapa Amazônia Oriental (1°05'37.75"S and 47°54'56.76"W), and from Unidade de Pesquisa Dr. Felisberto Camargo in Belém, Pará (1°26'28.29"S and 48°24'20.58"W). A total of 5 mL of blood was collected from each animal and preserved in EDTA at 4°C until analysis.

Genomic DNA was extracted according to the protocol developed by Regitano and Coutinho (2001). Subsequently, the DNA was quantified on 1.0% agarose gel and compared with increasing concentrations of DNA bacteriophage (50, 100, and 200 ng/µL; Invitrogen). The purity of the DNA was evaluated using a BioMate™ 3 spectrophotometer (Thermo Scientific, USA) within the range A_{260}-A_{280} nm, and samples with rates equal to or higher than 1.8 were selected.

The PCRs were conducted in a final volume of 20 µL to amplify a 412-bp fragment, which included intron 7, exon 8, and intron 8. The primers used were forward 5'-GCACCATCCTCTTCCTCAAG-3' and reverse 5'-GGAAGCGCTTTCGGATG-3' (Winter et al., 2002). The reactions contained 1X buffer (10X), 2 mM $MgCl_2$, 1 mM each dNTP, 2.5% bovine serum albumin (Invitrogen), 10 nM each primer (forward and reverse), 1 U *Taq* DNA polymerase (Promega, Brazil), and 25 ng genomic DNA. The reactions were performed using a Veriti™ Thermal Cycler (Applied Biosystems, USA), with an initial denaturation temperature of 95°C for 5 min followed by 35 cycles with a denaturing temperature of 94°C for 45 s, an annealing temperature of 57°C for 1 min and 30 s, an extension temperature of 72°C for 2 min, followed by a final extension temperature of 72°C for 5 min.

Polymorphisms were detected using the single-strand conformation polymorphism (SSCP) technique, with 2 µL PCR products mixed with 6 µL loading buffer (0.05% xylene-cyanol, 0.05% bromophenol blue, 0.5 mM EDTA, pH 8.0, and 98% formamide) followed by a heating session at 95°C for 10 min for denaturation, and refrigeration in ice for electrophoresis. Subsequently, the samples were subjected to electrophoresis on 8% polyacrylamide gel (acrylamide:bisacrylamide at a proportion of 29:1) with 1X TBE buffer for 8 h at 600 V. The gels were then stained with silver nitrate.

Samples that exhibited different migration patterns were purified using a QIAquick® PCR Purification Kit (Quiagen, USA), and were then sequenced in a 3130 Genetic Analyzer (Applied Biosystems) using a BigDye® Kit (Applied Biosystems). The sequences were edited using Chromas Lite V.2.1.1 (Technelysium Pty Ltd., Australia) and aligned by Clustal Omega (McWilliam et al., 2013) with the GenBank reference sequences FJ014704 and DQ182702, in order to determine the SNPs.

GENEPOP (Raymond and Rousset, 1995) was used to determine allele and genotype frequencies, the observed and expected heterozygosities, inbreeding coefficients

(F_{IS}), Hardy-Weinberg equilibrium probabilities, and F-statistic estimates for population differentiation. GenAlEx (Peakall and Smouse, 2012) was used to calculate the Shannon index. Associations between the different genotypes and milk production were investigated by conducting analysis of variance using PROC GLM in SAS/STAT® 9.0 (SAS Institute Inc., 2004) with the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk} \quad \text{(Equation 1)}$$

where Y_{ijk} represents the production of milk, μ represents the average of all observations, α_i represents the effect of the genotype factor, β_j represents the effect of the breed factor, y_{ij} represents the effect of interactions between the genotype and breed factors, and ε_{ijk} represents the experimental error. The level of significance was set at 0.05.

RESULTS

The PCR products were 412 bp long, and the PCR-SSCP technique was effective in finding polymorphisms in buffalo *DGAT1*. Four patterns of bands were found for this gene, which were sequenced and compared with those described by Raut et al. (2012). The allele variant A produced cytosine (C) at position 75, C at position 135, C at position 256, and guanine (G) at position 277. The allele variant B produced C at position 75, G at position 136, thymine (T) at position 256, and G at position 277. The allele variant D produced C at position 75, C at position 135, C at position 256, and T at position 277. A and B allele variants were observed in this study, as well as a new allele variant, variant D (Figure 1).

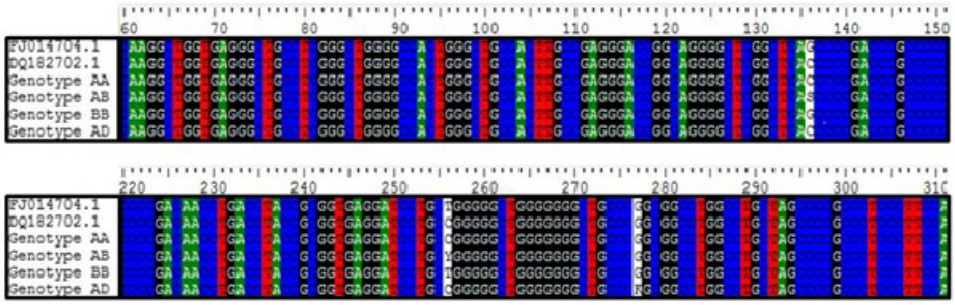

Figure 1. Alignment of nucleotide sequences, showing evidence for the presence of single nucleotide polymorphisms at positions 136, 256, and 277.

Four genotypes were observed in the Murrah breed and three were observed in the Mediterranean and mixed-breed groups (Table 1). Allele variant A was the most frequent variant in the Murrah, Mediterranean, and mixed-breed groups (0.79, 0.69, and 0.77, respectively) (Table 1). AA was the most frequent genotype in the Murrah and mixed-breed groups, while AB was the most frequent in the Mediterranean breed (Table 1).

The observed heterozygosities were lower than 0.50 in the Murrah and mixed-breed groups and equal to 0.50 in the Mediterranean group, while the expected heterozygosities were lower than 0.50 in all of the groups studied. The Shannon index was lower than 1.00 in all of

the groups, and the F_{IS} values indicated endogamy in the Murrah and mixed-breed groups but not in the Mediterranean group. The Hardy-Weinberg equilibrium did not differ significantly ($P > 0.05$) from the proportions expected in any of the breed groups (Table 1).

Table 1. Genotypic, allelic, and genetic characteristics of the populations.

Breed	Genotype	Allele	H_{Oob}	H_{Eob}	H_{Eexp}	SI	F_{IS}	HWP
MU	AA (0.63)	A (0.79)	0.68	0.32	0.34	0.56	0.78	0.70
	AB (0.29)	B (0.20)						
	BB (0.05)	D (0.01)						
	AD (0.03)							
MD	AA (0.44)	A (0.69)	0.50	0.50	0.43	0.62	-0.15	0.67
	AB (0.50)	B (0.31)						
	BB (0.06)							
ME	AA (0.61)	A (0.77)	0.69	0.31	0.35	0.54	0.17	0.52
	AB (0.31)	B (0.23)						
	BB (0.08)							

MU, Murrah; MD, Mediterranean; ME, mixed; H_{Oob}, observed homozygosity; H_{Eob}, observed heterozygosity; H_{Eexp}, expected heterozygosity; SI, Shannon index; F_{IS}, inbreeding coefficient; HWP, Hardy-Weinberg probability. Frequencies are in parentheses.

Table 2 presents the results for milk production. There were no significant associations between genotype and milk production in any of the breed groups studied ($P > 0.05$).

Table 2. Milk production performance (kg) of each genotype within the breed groups.

Genotype	Breed					
	Murrah		Mediterranean		Mixed	
	Average	SD	Average	SD	Average	SD
AA	4.55	1.72	4.78	1.89	4.08	2.32
AB	4.28	0.77	3.77	1.43	4.69	1.63
BB	4.61	0.33	4.66	2.31	1.95	0.00
AD	3.76	0.00				

SD, standard deviation.

DISCUSSION

The sequence obtained included intron 7, exon 8, and intron 8 of *DGAT1* (Yuan et al., 2007), and all of the variations were detected in introns 7 and 8, as described by Raut et al. (2012). However, a novel variant was detected (D), which has a different SNP configuration according to Raut et al. (2012). The allele variant A was the most frequent in all of the breed groups, and, consequently, the genotype AA was found at a high frequency in the Murrah and mixed-breed groups. Very similar results were obtained by Raut et al. (2012) when studying Murrah and Pandharpuri buffaloes.

Shi et al. (2012) investigated Murrah buffaloes for the presence of SNPs between introns 7 and 8 of *DGAT1*, and detected a polymorphic point at position 256, which was observed in our study. The same workers compared the complete 412-bp sequence with that in cows, and observed differences in positions 111, 112, and 338, with T, C, and C in cows and G, A, and G in buffaloes, respectively. We also found G, A, and G in these positions in the three breed groups studied.

Heydarian et al. (2014) investigated 278 bp between introns 7 and 8 in four breeds of buffalo (Bhadawari, Mehsana, Murrah, and Surti) that are native to five provinces of Iran. Their

sequence corresponds with that found between positions 60 and 337 in our study. However, the authors did not observe polymorphisms in positions 75, 136, or 277, and only observed a SNP at position 256. The C allele at position 256 was the most frequent in all of the breeds, and the CC genotype was the most frequent in two of the breeds; in the other two breeds, the genotype frequencies were equal (Heydarian et al., 2014).

In the present study, the different variants and allele frequencies resulted in low genetic variability in Murrah, Mediterranean and mixed-breed, despite there being no deviation from the Hardy-Weinberg equilibrium, but this low variability indicates that inbreeding has occurred. Machado et al. (2016) also observed inbreeding (based on a SNP found on an intron of the melatonin receptor gene) in two Amazonian populations of buffaloes that are characterized by their systems of rearing (Terra Firme and Varzea); the authors concluded that both populations had low to moderate genetic variability.

Regarding associations between genotype and milk production, we found that the SNPs had no direct effect on variations in milk production. Some studies have reported that SNPs in *DGAT1* are significantly associated with milk yield, high milk fat yield, high fat, and high protein in dairy cows and buffaloes (Lacorte et al., 2006; Tantia et al., 2006; Hill et al., 2016). However, the SNPs found in this study were in an intron region, which is not important for amino acid chains on proteins, although some polymorphisms found in the intron regions of enzyme genes can affect enzyme functionality, as reported by Buraczynska et al. (2004) for the endothelial synthase nitric oxide gene and by Sachse et al. (1999) for the cytochrome isoenzyme P450 gene (*CYP1A2*).

In conclusion, buffalo *DGAT1* was variable in the region between introns 7 and 8, with a new allele variant (D) found, but the allele variant A was more representative, and the different genotypes were not associated with milk production. Therefore, these SNPs can serve as population markers for studies on genetic variability in buffaloes.

Conflicts of interest

The authors declare no conflict of interest.

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