POLYMORPHISMS IN THE MTRN1A GENE PROMOTER IN BUFFALOES IN THE AMAZON

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ABSTRACT

The work aimed genetically to characterize the Murrah, Mediterranean and mixed-breed populations based on polymorphisms in the melatonin 1A receptor gene promoter (MTRN1A) and associate the genotypes with milk production. The haplotype A (position -1511 T and -1482 presents ACAA) and haplotype B (position -1511 C and -1482 does not present ACAA) were found in all groups. The haplotype frequencies were in Murrah breed (A=0.66 and B=0.34), Mediterranean breed (A=0.73 and B=0.27), and mixed-breed (A=0.65 and B=0.35). The Murrah breed had the genotypes AA (0.44), AB (0.44) and BB (0.12), and the Mediterranean and mixed-breed buffaloes had the genotypes AA (0.52) and (0.46), AB (0.42) and (0.39), and BB (0.06) and (0.15), respectively. For the Murrah, Mediterranean, and mixed-breed buffaloes, respectively, the expected heterozygosity values were 0.45, 0.40, and 0.45, the inbreeding coefficients were 0.04, -0.03, and 0.18, and the Hardy-Weinberg probabilities were 0.92, 0.24, and 0.59, respectively. The genotypes evaluated did not have an effect on milk production; however, the single nucleotide polymorphisms (SNP) and the insertion/deletion polymorphism (INDEL) can be used in studies on genetic variability.

Keywords: *Bubalus bubalis*, buffaloes, SNP, INDEL, genotype, milk

INTRODUCTION

In Brazil, water buffalo (*Bubalus bubalis*) rearing has been developed on a large scale, with an annual increase of 3.5%. Brazil contains 1.37 million buffaloes, and the country's northern region holds 66.2% of the overall population, the northeastern region has 9.5%, the southeast has 12.7%, the south has 7.4%, 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, 2016). The production and marketing of buffalo milk and their dairy products are well differentiated and have grown increasingly to attract consumers seeking nutritional and functional quality (Araújo *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

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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 mechanisms that regulate milk synthesis are controlled by several genes that can be considered as candidates for genetic variation in economically important traits in livestock selection strategies. Additionally, it would be useful to identify polymorphisms in these candidate genes, including those associated with quantitative trait for marker-assisted selection, used single nucleotide polymorphism (SNP) insertion/deletion polymorphism (INDEL) markers for example, which can be applied in breeding programs in order to increase milk production (Naserkheil *et al*., 2019)

Among the various genes studied with reference to the production of milk in domestic animals, the melatonin 1A receptor gene (MTRN1A) is on chromosome 1 which is a fusion of *Bos Taurus* chromosome 1 and 27 (Miziara *et al*., 2007). Melatonin is produced by pineal gland at night in direct proportion to the period of darkness (Malpaux *et al*., 2001). The melatonin effect is carried out at hypothalamic level, by regulating of GnRH secretion (Malpaux *et al*., 1998). However the highest concentration of melatonin MTNR1A receptors has been evidenced in the Pars Tuberalis (PT), but this region seems to be particularly involved in the control of Prolactin secretion (Dardente, 2007; Dupré *et al*., 2008). Thus, polymorphisms found in MTRN1A can modulate the quantity of melatonin that affects the quantity of prolactin (Zetouni *et al*., 2014).

Therefore, the objective of this study was to identify polymorphisms in MTNR1A, and genetically characterize and associate polymorphisms with the production of milk in Murrah, Mediterranean, and mixed-breed herds in the Brazilian Amazonian region

MATERIALS AND METHODS

The total milk production was analyzed (the total of all the milk produced in each milking session within a 36 months period) of 69 buffaloes, 38 of them being Murrah breed, 18 Mediterranean and 13 mixed-breed of the Murrah with Mediterranean. A total of 5 mL of blood was collected from each animal and preserved in EDTA at 4°C to be analyzed in the laboratory.

Genomic DNA was extracted according to the protocol developed by Regitano and Coutinho (2001). Subsequently, the DNAs was quantified in 1.0% agarose gel and compared with increasing concentrations of DNA bacteriophage (50, 100 and 200 ng/µL). The purity of DNA was evaluated in the Biomate 3 spectrophotometer (Thermoscientific, USA) within the range A260 nm/A280 nm and samples with rates equal to or higher than 1.8 were selected.

The polymerase chain reactions (PCR) were conducted in a final volume of 20 µL to amplify a fragment of 162 pb. Two pairs of primers were designed using the Primer3 program (http://simgene.com/Primer3), forward 5'-TGCACACATCCTAGGACCTG-3' and reverse 5'-TCCAGGCCTTAAGGGTACAGTC-3'. The reactions contained 1X of Buffer 10X, 2 mM de $MgCl_2$, 1 mM of each dNTP, 2.5% of Bovine Serum Albumin (Invitrogen, Brazil), 10 nM of each primer (forward and reverse), 1U *T*aq DNA polymerase (Promega-Brazil) and 25 ng genomic DNA. The reactions were performed using a Veriti Termal Cycle (Apllied Biosystem-USA) with an initial denaturation temperature of 95°C for 5

minutes followed by 30 cycles with a denaturing temperature of 94°C for 45 seconds, an annealing temperature of 61°C for 1 minute, an extension temperature of 72°C for 2 minutes, followed by a final extension temperature of 72°C for 5 minutes.

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 minutes for denaturation, and refrigeration in ice for electrophoresis.

Subsequently, the samples were submitted to electrophoresis in 8% polyacrylamide gel (acrylamide:bisacrylamide at a proportion 29:1) with TBE buffer 1X for 8 h at 600V. Finally, the gels were blushed 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 (Applied Biosystems-USA) using a Big Dye kit of the same manufacturer (Applied Biosystems-USA). The sequences were edited using the programme Chromas Lite V.2.1.1 (Technelysium Pty Ltd 2013) and aligned by Clustal Omega programme (McWilliam *et al.*, 2013) with the reference sequences AY524665.1 of *Ovis aires* and CP027095.1 of *Bos mutus,* available in the Genbank in order to determine the SNPs.

The programme GENEPOP (Raymond and Rousset, 1995) was used to determine the haplotype and genotype frequencies, the heterozygosity observed and expected, the inbreeding coefficients (F_{IS}), the probabilities of the Hardy-Weinberg equilibrium and the estimates of the F statistic for population differentiation. The programme GenALEx (Peakall and Smouse, 2012) was used to calculate the Shannon index. The associations of the different genotypes with a role in milk production were tested via Analysis of Variance (ANOVA) using PROC GLM of SAS/ STAT 9.0 (SAS, 2004), obeying the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk}$$

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, γ_{ij} represents the effect of interactions between the genotype and race factors and ε_{ijk} represents experimental error. The level of significance established was 0.05.

RESULTS AND DISCUSSION

The PCR products were 162 bp long, and the PCR-SSCP technique was effective in finding polymorphisms in buffalo in the region promoter melatonin gene. Three different patterns of SSCP were observed and showed in Figure 1.

Each haplotype was sequenced and characterized as: haplotype A (position -1511 presents T and position -1482 presents an wild sequence of ACAA) and haplotype B (position -1511 presents C and position -1482 presents a ACAA deletion). However, Individuals presenting only haplotype A were considered genotype AA, and presenting only haplotype B were considered genotype BB, finally those that presented both haplotypes were considered AB.

All genotypes (AA, AB and BB) were observed in the Murrah breed, Mediterranean breed and Mixed-breed populations and allele haplotype A was the most frequent variant in both groups (0.66, 0.73 and 0.65, respectively). AA was

the most frequent genotype in the Mediterranean breed and in the Mixed-breed groups, while AA and AB genotypes were the most frequent in the Murrah breed.

Barbosa *et al.* (2017), investigated Murrah buffaloes for the presence of SNPs in the MTRN1A gene promoter region, and detected a polymorphic point at position -1511 and the indel region at position -1482, which was observed in our study. In adding, were observed that the SNP in -1511 position of T to C was counted in heterozygosity in all studied breeds, which did not observe for those authors.

The observed heterozygosities and 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 FIS 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).

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. Barbosa *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. 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).

Regarding associations between genotype and milk production, we found that the SNPs had no direct effect on variations in milk production. Zetouni *et al.* (2014) investigated buffaloes for the presence of polymorphisms in the exon 2 in the MTRN1A gene and three genotypes (CC, CT, and TT) were identified by PCR-RFLP, and there was a significant association with protein percentage in milk. When the mutations occur in the promoter region of the genes, alterations in gene expression can occur (Kininis and Kraus, 2008), which could result in different productive or reproductive roles in the buffaloes. Dubey *et al.* (2015) investigated the thyroglobulin promoter region of swamp and riverine buffaloes using a PCR-SSCP and sequencing technique and also analyzed the association of polymorphism with milk production characteristics and observed a significant effect on fat the percentage of buffalo belong to Mehsana and Nili Ravi dairy breeds.

Barbosa *et al.* (2017), study the promoter region of the melatonin receptor gene, and we found some SNPs were associated with reproductive characteristics such as calving interval and age at first calving. Association between seasonal reproductive activity and single nucleotide polymorphism (SNP), were found in works with Italian buffaloes (Carcangiu *et al*., 2011; Luridiana *et al*., 2012) and Indian Murrah buffalo (Gunwant *et al*., 2018).

In conclusion, buffalo MTRN1A gene was variable in the promoter region, the allele variant A was more representative, and the different genotypes were not associated with milk production. Therefore, these SNPs and the INDEL, can serve as population markers for studies on genetic variability in buffaloes.

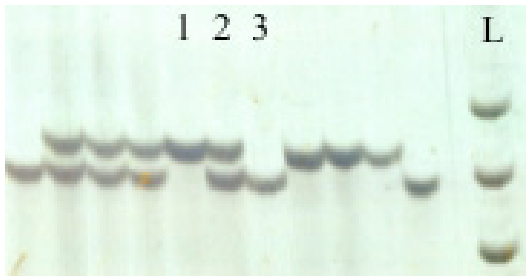

Figure 1. Silver-stained polyacrylamide gel showing the SSCP-derived genotypes of the MTRN1A gene. Number 1 genotype AA, number 2 genotype AB and number 3 genotype BB. The letter L indicate the ladder marker.

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

Breed	Genotypes	Allele	Ho_{ob}	He_{ob}	He_{exp}	SI	Fis	HWP
MU	AA (0.44)	A (0.66)	0.56	0.44	0.45	0.64	0.04	0.92
	AB (0.44)	B (0.34)						
	BB (0.12)							
ME	AA (0.52)	A (0.73)	0.58	0.42	0.40	0.59	-0.03	0.24
	AB (0.42)	B (0.27)						
	BB (0.06)							
MD	AA (0.46)	A (0.65)	0.61	0.38	0.45	0.64	0.18	0.59
	AB (0.39)	B (0.35)						
	BB (0.15)							

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

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

Genotype	Breed					
	Murrah		Mediterranean		Mixed-breed	
	Average	SD	Average	SD	Average	SD
AA	1913.69	419.43	1572.39	407.47	1652.76	404.62
AB	1459.46	495.45	1745.23	437.29	1695.55	617.12
BB	1577.04	184.85	1277.96	315.48	1375.00	251.19

SD, standart deviation

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