



ANIMAL SCIENCE

CSN1S1 and CSN3 gene variants in female Murrah buffaloes in the Brazilian Amazon

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Abstract: The characteristics of milk are controlled by several genes, with emphasis on the four genes from casein, CSN1S1; CSN1S2; CSN2 and CSN3, which are responsible encoding of fractions the milk protein. The study of genetic variants in these genes, seek to investigate alleles, insertions or deletions, that can directly reflect on productive characteristics, indicating differences in milk quality, composition and yield. The CSN1S1 and CSN3 genes were analyzed in lactating Murrah buffaloes using nucleotide sequencing. An SNP was found in the amplified fragment of the CSN1S1 gene, located in nucleotide number 2,123 of the promoter region in position nt-258 (A/G). As for the CSN3 gene, two SNPs of exon number 4 were identified in codons 33 (ACC/ATC) and 34 (ACC/ACT) of the analyzed fragment. This study contributes to important associations between genetic variants and the desired characteristics of milk and its derivatives in future studies, because the variants found may be associated with the quality of milk, enabling genetic selection to be assisted by molecular markers, indicating a major advance that makes it possible to select animals early.

Key words: Amazon, *Bubalus bubalis*, milky quality, single nucleotide polymorphism.

INTRODUCTION

Firly introduced in Amazon in 19th century, the buffalo (*Bubalus bubalis*) adaptation to Amazon environment was exceptional, and the acceptance of its beef and milk lead to a fascinating herd increased in the last decades (Sheikh et al. 2006). However, few attentions are paid to genetics characteristics of herds to increase products quality (Sheikh et al. 2006).

The characteristics of milk are controlled by several genes, in particular, the four casein genes, CSN1S1, CSN1S2, CSN2, and CSN3, which are responsible for encoding the four fractions of the protein α -casein - α -casein s1, α -casein s2, β -casein and κ -casein (El Nahas et al. 2013).

The CSN1S1 gene is located on chromosome 7 and is formed by 19 exons (NCBI- Gene ID

102396531-). Most studies that investigated the polymorphism of this gene involved the exons 17 and 19 (El Nahas et al. 2013). However, the studies have only found polymorphisms in the promoter region of this gene, as this region is fundamental for the transcription of the gene (Alves & Souza 2013). The CSN3 gene is located on chromosome 7 across five exons (NCBI- Gene ID 102395364). This study aimed to investigate the occurrence of variants in the CSN1S1 and CSN3 genes in buffalo females of the Murrah racial breed.

MATERIALS AND METHODS

The experimental procedures were authorized by the Ethics Committee on the Use of Animals

in Experiments (CEUA) from the Federal Rural University of the Amazon (16/2015, process 23084.04411/2015-81). The experiment was performed using 39 buffalo females of the Murrah racial breed belonging to the dairy herd of one commercial farm, in the Brazilian Amazon. The farm adopts a sustainable semi-intensive breeding system with less animals to improve quality and area use, consequently the sample in this study is reduced. We collected of each animal a tail broom hair with approximately 40 hair strands, containing their respective hair follicles, and packed in individual microtubes (1.5 mL).

DNA extraction was performed using the Wizard® Promega commercial kit (Promega Bio Sciences, LLC. San Luis Obispo, USA). To determine the CSN1S1 alleles that are located in the promoter region –in the 5' untranslated region (UTR) and exon 1– fragments of 313 bp were amplified. To determine the alleles of the CSN3 gene located between exon 4 and intron 4, fragments of 348 bp were amplified by PCR using the primers designed by Freitas (2015) (CSN1S1) and Fonseca et al. (2013) (CSN3).

Amplification reactions were performed in a thermocycler SimpliAmp™ Thermal Cycler (Applied Biosystems, A24811). Each 25 µL of the polymerase chain reaction (PCR) was prepared using the reagents described in Table I.

To perform PCR, a temperature gradient test was used to identify the ideal annealing temperature for the primers. The conditions for the amplification of both genes consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min.

To verify the products of the amplification reaction, each sample, stained with bromophenol blue BlueJuice™, was subjected to electrophoresis using 1% agarose gel stained with 3 µL of SYBR Safe™ DNA Gel Stain (Invitrogen,

Table I. Quantities of each reagent used in the preparation of the PCR mix, to the CSN1S1 and CSN3 genes.

Reagents	Quantities (µl)
Buffer ((NH ₂) ₂ SO ₄) (Qiagen)	2.0
Mg ⁺ Cl (Qiagen)	1.0
dNTP	2.0
Taq DNA polymerase (Qiagen)	0.2
Primer Forward	0.5
Primer Reverse	0.5
Ultrapure water	17.8
DNA genomic 100 ng	1.0

Thermo Fisher Scientific, USA). Samples that showed amplifications were purified, using the Exo/SAP Go purification kit (Boca Scientific Inc., USA), following the manufacturer's recommendations. Each reaction was prepared in a final volume of 15 µL using the commercial kit BigDye® Terminator Cycle Sequencing v3.1 (version 3.1, Applied Biosystems, USA), according to the manufacturer's instructions. The amplifications were performed in a Veriti® Thermal cycler (Applied Biosystems, Thermo Fisher Scientific, USA). For sequencing, a new step of purifying the samples was performed using absolute and 70% ethanol.

Each sample was sequenced and separated by capillary electrophoresis in an automatic sequencer model ABI-3130® Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA). The electropherograms generated from the sequencing were edited and analyzed using the BioEdit software (version 7.2.1). Then, the sequences were edited to remove low quality sections through the basic local alignment research tool (BLAST) at 100% compatibility level and aligned using the ClustalW tool. The generated sequences for each PCR product were aligned with the forward or reverse primer as

well as with the reference sequence for each gene, where the Gene ID of CSN1S1 and CSN3 are 102396531 and 102395364, respectively.

RESULTS AND DISCUSSION

Genetic polymorphisms have been studied in casein genes. The fragments analyzed in this study show a strong association with important characteristics of milk in cattle (Alipanah et al. 2005, Heck et al. 2009), making studies that confirm this association also in buffaloes necessary, allowing in the future that these variants can be used in selection programs assisted by molecular markers.

The *CSN1S1* gene amplified has fragment 313 bp in size and is located upstream (towards 5') of the transcriptional start site of exon 1, comprising 273 and 40 nucleotides in the promoter and exon 1, respectively.

The analysis of the fragments that correspond to the 5' UTR promoter region and part of exon 1 in buffaloes were not mentioned in the literature as such fragments are not included in a coding region (Cosenza et al. 2015). However, it is known that the 5' UTR region is important for gene transcription, since transcription begins in the promoter region, and consequently, the correct recognition of the promoter region becomes critical when investigating gene expression regulation (Alves & Souza 2013). The buffalo nucleotide sequences

of the 39 samples were found to be identical, except for nucleotide number - 2.123, in which one SNP was found to be located of the 5' UTR promoter region in the *CSN1S1* gene (-2123 A > G). The observed SNP allowed the identification of three distinct genotypes of AA, GG, and AG, with only one individual showing a homozygous GG pattern.

Regarding the analysis of the genotype frequencies of the polymorphism found in the *CSN1S1* gene of the females studied (Table II), we were able to observe a higher percentage of the AA genotype, followed by the heterozygous genotype AG and GG, respectively. The expected genotypic frequency was different from the observed frequencies, and observed heterozygosity was greater than the expected heterozygosity (0.38 vs. 0.34), thus suggesting an excess of heterozygotes in relation to the Hardy-Weinberg equilibrium model. Although the population analyzed is a small sample, these results suggesting an endogamy absence (Barros et al. 2011)

The *CSN3* gene amplified fragment consist of 350 bp located between exon and intron 4, comprising 238 and 112 nucleotides in the exon and intron, respectively. Two variants were identified, located in nucleotides 377 and 381 of exon 4 (T>C), allowing the identification of three distinct genotypes of CC, TT, and CT. The two variants (nucleotides 377 and 381 located in general position in relation to the complete

Table II. The observed, expected, and allelic genotypic frequencies of the casein *CSN1S1* and *CSN3* genes. 'N', 'Reg', and 'Gen' refer to the nucleotide number, gene region, and genotype, respectively. 'F' indicates frequency, where 'FGo', 'FGe', and 'Fa' refer to the observed, expected, and allelic genotypic frequencies, respectively.

Gene	Reg	Genotype frequencies									Allele frequency			
		Gen	FGo	FGe	Gen	FGo	FGe	Gen	FGo	FGe	Allele	Fa	Allele	Fa
CSN1S1	5' UTR	AA	0,59	0,61	AG	0,38	0,05	GG	0,03	0,34	A	0,78	G	0,22
CSN3	exon 4	CC	0,53	0,54	CT	0,41	0,07	TT	0,06	0,39	C	0,74	T	0,26
CSN3	exon 4	CC	0,53	0,54	CT	0,41	0,07	TT	0,06	0,39	C	0,74	T	0,26

Reg = gene region; Gen = genotype; FGo = frequency genotypic observed; FGe = frequency genotypic expected; Fa = frequency genotypic allelic.

gene) were present at codons 33 (ACC>ATC) and 34 (ACC>ACT) in the amplified fragment. Such variants correspond to codons 135 and 136 of the mature peptide, which, once translated, result in the amino acids threonine and isoleucine (Figure 1).

Variant B (T allele) that results in the amino acids isoleucine and threonine in codons 135 and 136 of the mature κ -casein peptide, was associated with a higher percentage of fat, protein, and casein in bovine milk, thus, significantly influencing the production of cheeses, due to the superior coagulation power of the rennet contained in them, compared to variant A (C allele) (Heck et al. 2009). In this context, it is possible to suggest the development of experiments that prove that this truth extends to buffaloes, enabling this variant to be used in selection programs assisted by molecular markers.

Regarding the analysis of the genotypic frequencies in the CSN3 gene (Table I), it was possible to observe a higher percentage of the CC genotype, followed of the heterozygous genotype

CT, and TT, respectively. The expected genotypic frequency, demonstrated that the possibility the population may be evolving, since the genotypic frequencies were altered. In addition, as the observed heterozygosity was greater than the expected heterozygosity, this suggested an excess of heterozygotes in relation to the Hardy-Weinberg equilibrium model. In this context, it is possible to infer that there was an absence of inbreeding crosses in the studied population, due to the number of heterozygous individuals observed, although the studied population is small.

CONCLUSION

We identified variants in the CSN1S1 gene and differences of the amino acids in CSN3 gene of the mature peptide of Murrah buffalo. Therefore, we suggest that further studies be carried out with the variants found, using a larger population and confirming the association between polymorphisms and productive characteristics

Alelo C

91 ACA CCT ACC ACC GAA GCA ATA GAG AAC ACT GTA GCT ACT CTA GAA 135
31 T P T T Q A I Q N T V A T L Q 45

Thr / Thr

Alelo T

91 ACA CCT ATC ACT GAA GCA ATA GAG AAC ACT GTA GCT ACT CTA GAA 135
31 T P I T Q A I Q N T V A T L Q 45

Ile / Thr

Figure 1. Nucleotide and amino acids sequences of the DNA segment amplified by PCR of the female Murrah buffalo breed. Nucleotides, from nt-91 to nt-135 (nucleotides 377 and 381 located in general position in relation to the complete gene), and amino acids, from 31 to 45 of the alignment are illustrated. The C allele is shown in codons 33 threonine (Thr) (ACC) and 34 Thr (ACC), while the T allele is shown in codons 33 isoleucine (Ile) (ATC) and 34 Thr (ACT).

of milk in buffaloes to increase buffalo products quality in semi-intensive farms.

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Author contributions

NBCM and MDNR designed the experiment and carried out the research trial. NBCM completed the statistical analyses. NBCM, SLMG, RSRP, ALS, MR, RM, RPM, IGH and MDNR revised the scientific content and drafted the manuscript. NBCM & MDNR assisted with the experimental design and research trial, while all authors provided editorial suggestions and approved the final manuscript.

