

Identification of SNPs and changes in protein coding of genes associated with beef quality in Nellore cattle

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ABSTRACT. We investigated single nucleotide polymorphisms of four genes (calpain (*CAPN-9* and *CAPN-14*), calpastatin – *CAST*, diacylglycerol acyltransferase – *DGAT*, and leptin - *LEP*) that are related to beef quality, and we examined changes in the synthesis of proteins that they encode in Nellore cattle. Samples from 95 adult males of commercial origin were analyzed. Genomic DNA was extracted from the *longissimus dorsi* (sirloin) muscle tissue, identified with PCR-single-stranded conformation polymorphism analysis. For each different pattern identified, the products were sent for sequencing and analyzed using sequence scanner software. The data were analyzed by determining the absolute and relative frequencies of the polymorphisms identified in each gene. For the *CAPN-9* gene, sequencing showed five polymorphisms (G/A, T/A, T/C, T/C, and A/G), of which two involved amino acid substitutions (c.5861G>A and c.5498A>G). Sequencing of the *CAPN-14* gene revealed four polymorphisms (A/C, G/A, T/C, and C/G), with two involving amino acid substitutions (c.11054 T>C

and c.11161C>G). Sequencing of the *CAST-5* gene revealed five polymorphisms (C/T, T/C, C/A, C/A, and G/T), four of which involved amino acid substitutions (c.29919C>T, c.29963A>C, c.29978C>A, and c.30019G>T). Sequencing of the *DGAT* gene revealed six polymorphisms (T/A, G/A, A/T, G/C, A/G, and G/A), four of which involved amino acid substitutions (c.11730A>T, c.11809G>C, c.11858A>G, and c.11927G>A). Sequencing of the *LEP* gene revealed three polymorphisms (C/T, C/T and T/C), with one involving an amino acid substitution (c.14962T>G). These genes had a large number of polymorphisms resulting in amino acid differences in Nellore Cattle. These polymorphisms involving amino acid changes may promote functional changes in beef characteristics of Nellore cattle given that these genes are associated with beef quality parameters.

Key words: Leptin; Calpastatin; Calpain; Diacylglycerol Acyltransferase

INTRODUCTION

Molecular biology techniques have developed rapidly in recent years; they allow the determination of the frequencies and population genetic parameters of genes that are associated with economically relevant characteristics in cattle. In recent years, studies have shown that performance, fattening, carcass and meat quality traits in many cattle breeds are associated with polymorphisms and with genotypic and allelic frequencies of several genes (*LEP*, *CAST*, *CAPN1*, *GHR*, *FABP4*, *DGAT1*, *CSN3*, *LGB*, *MYF5*, *TG*, *IGF1*, *IGF1R*, *LGB*, *ANK1* and *OLR1*) (Thaller et al., 2003; Curi et al., 2005; Borges et al., 2014; Ardicli et al., 2017; Ardicli et al., 2019a; Ardicli et al., 2019b; Carvalho et al., 2019; Tyulebaev et al., 2019). These traits related to beef quality are very important since they involve economic issues along the beef production chain (farm to fork). Brazil, is the most important country for beef export in the world and the main breed used is Nellore. This breed and hybrids with this breed constitutes most of the zebu animals in the Brazilian beef herd. It is used as purebred or crossbreeding with European breeds in order to achieve greater performance and better carcass parameters and meat quality (Dani et al., 2008). We examined some genes (*LEP* - leptin; *DGAT* - diacylglycerol acyltransferase; *CAPN* - calpain; and *CAST* - calpastatin) that are associated with these traits.

The study of the *LEP* gene is of great importance due to its physiological relevance, involving traits of economic interest in cattle, such as carcass fat deposition and tenderness, drip loss, total lipids and fatty acids profile in beef (Fortes et al., 2009; Orrù et al., 2011; Pinto et al., 2011). Another gene used was diacylglycerol acyltransferase (*DGAT*), which is associated with fat composition, lipid profile, subcutaneous fat deposition, intramuscular fat, ribeye area and cooking loss in cattle (Winter et al., 2002; Conte et al., 2010; Borges et al., 2014). For the calpain (*CAPN*) and calpastatin (*CAST*) genes, SNPs are related to carcass and meat quality traits, especially the tenderization process (Casas et al., 2005; Schenkel et al., 2006; Curi et al., 2010; Bolormaa et al., 2011; Lara et al., 2012; Desgarnes-Alcalá et al., 2017; Sun et al., 2018) and beef color (Pinto et al., 2011). Thus these associations demonstrate the importance of understanding the genetic variation and occurrence of mutations of these genes in Nellore cattle.

We evaluated SNPs in genes that have been associated with meat quality traits and carcass parameters in cattle (*CAPN* -9; *CAPN* -14; *CAST* -5; *DGAT* and *LEP*) to assess changes in the proteins that they encode in Nellore cattle.

MATERIAL AND METHODS

Animals and experimental site

The experiment was conducted with samples from animals slaughtered from the feedlot of the Frialto group, located 15 km from the city of Sinop, MT, Brazil. To perform this study, we used 95 male Nellore animals between 13 and 36 months of age, unrelated and belonging to the contemporary group considering the management in the feedlot for 88 days. The animals were fed with complete diet offered *ad libitum* and had an average initial weight of 386.19 ± 4.48 kg and a weight at slaughter of 527.82 ± 17.96 kg. At the end of the period of time in the feedlot, the animals were slaughtered, under humane conditions, in the slaughterhouse of the Frialto group after a 12-hour fast. After 24h freezing, a sample from *longissimus dorsi* (sirloin) was collected and kept frozen for DNA analyses. This study was approved by the Ethics Committee on Animal Use (CEUA) of Federal University of Lavras (UFLA) and registered under number 040/12.

SNP prospecting

Extraction and amplification of genomic DNA

Genomic DNA was extracted from samples of the *longissimus dorsi* (sirloin) muscle following CTAB protocol for DNA extraction (Cationichexadecyl trimethyl ammonium bromide) described by Stefanova et al. (2013). The concentration and purity of the extracted DNA were quantified in a NanoDrop ND-1000 Spectrophotometer UV/Vis (absorbance at 260 nm (A260) and 280 nm (A280)). The samples were diluted with sterile water to obtain the desired final concentration of 10 ng DNA/ μ L. Next, gel electrophoresis was performed to assess DNA integrity and thus confirm the high quality of the extracted genomic DNA.

For the DNA analysis, primers were designed for the genes evaluated (calpain - *CAPN*-9 (exon 9), calpain - *CAPN*-14 (exon 14), calpastatin - *CAST*-5 (exon 5), diacylglycerol acyltransferase - *DGAT* (exons 16 and 17) and leptin - *LEP* (exon 2)) using software tools available online (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) (Table 1).

First, genetic sequences homologous to the selected genes were analyzed with Basic Local Alignment Search Tool (BLAST) software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/>). Next, Oligo Perfect and Oligo Analyzer were used to design primer pairs for each gene.

Conventional polymerase chain reaction (PCR) was performed with 50 ng of genomic DNA in a final volume of 25 μ l (1 \times reaction buffer, 200 μ M dNTPs, 1.25 U of Taq DNA polymerase, 2.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 μ M of each primer and H₂O).

Table 1. Primer sequences used for the amplification (35 cycles) of fragments of the *CAPN-9*, *CAPN-14*, *CAST-5*, *DGAT* and *LEP* genes in meat samples from Nellore cattle.

Gene	Forward primer (5'-3') Reverse primer (5'-3')	Fragment length (bp)	Annealing temperature
<i>CAPN-9</i>	F=5'GGACTCAGGCTTGAACAGAGG3' R=5'GCATGAAGTCTCGGAAGGA3'	500	60°C
<i>CAPN-14</i>	F=5'GTAGAAAGCCCTCCCTGTC3' R=5'TCAGAGCCTCACTCTCCTCA3'	593	61°C
<i>CAST-5</i>	F=5'GGATTATTATCAACCAGACACCAAC3' R=5'CAATACCTGCTGATGCCACA3'	393	61°C
<i>DGAT</i>	F=5'TCTTCCACGAGGTCAAGTGC3' R=5'GGCAAAGCAGTCCAACACC3'	427	61°C
<i>LEP</i>	F=5'CTCTAGGGAAAAGGCGGAGTC3' R=5'CAGCCAGAAGCTCAGGTTTC3'	500	60°C

PCR was performed in a thermocycler (Mastercycler Eppendorf, USA) with the following thermal cycling profile: 5 minutes for initial denaturation at 95°C, 30 seconds for denaturation at 95°C, 30 seconds for primer annealing (60°C, 61°C, 61°C, 61°C, 61°C and 60°C for *CAPN-9*, *CAPN-14*, *CAST-5*, *DGAT* and *LEP*, respectively), and 1 minute for extension at 72°C. The final extension was 2 minutes at 72°C. The PCR products were visualized by electrophoresis (TAE buffer) in a 1% agarose gel containing 200 ng/mL ethidium bromide.

The amplified products for the different primers were subsequently subjected to electrophoresis (TBE buffer (45 mM Tris-borate, pH 8.0, and 1 mM EDTA)) in a 1.0% agarose gel containing 0.8 µg/mL ethidium bromide, and the resulting electrophoretic profiles were visualized in the gel and recorded using a photodocumentation system (Spectroline Ultraviolet Transilluminator).

Polyacrylamide gel electrophoresis/mutation screening

The screening of mutations of the *CAPN-9*, *CAPN-14*, *CAST-5*, *DGAT* and *LEP* genes was performed by the SSCP (single-stranded conformation polymorphism) comparative method, for which 1 µL of each PCR product was added to and diluted in 10 µL of denaturing buffer (98% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). After denaturation at 95 °C for 5 minutes, the samples were immediately placed on ice and then loaded on a 12.5% acrylamide:bisacrylamide (37.5:1) gel. The samples were subjected to polyacrylamide gel electrophoresis in a horizontal gel (35×15) at 20°C in 0.5× TBE buffer for 6 hours at 180 V, 150 mA and 100 W. The gels were stained based on the procedure reported by Byun et al. (2009).

Sequencing and data analysis

The samples obtained from the PCR-SSCP analysis were sequenced by capillary electrophoresis in an ABI 3130 sequencer using POP7 polymer and BigDye v3.1 according to the capillary method described by Sanger, Nicklen and Coulson (1977). The data generated were studied and analyzed using the free Sequence Scanner Software (Applied Biosystems®). MEGA X was used to analyze the sequencing chromatograms; the

computational tools in software allowed the alignment of the nucleotide sequences and prediction of the corresponding amino acids. A similarity search was performed using the computational tool BLAST and sequences stored in GenBank®. The NCBI (National Center for Biotechnology Information) Orf Finder tool was used to transform the nucleotide sequences into proteins, and the Omega Clustal tool was then used to align the protein sequences.

Data analysis

The data were analyzed by determining the relative frequency of the polymorphisms identified in each gene of each genetic group. Allele frequency was calculated by direct counting and was defined as the proportion of the different alleles of a gene in the total diploid population, according to the formula of Ramalho et al. (2012).

RESULTS

In this study, the PCR products of the evaluated genes (*CAPN-9*, *CAPN-14*, *CAST-5*, *DGAT* and *LEP*) were subjected to gel electrophoresis, separating the DNA fragments according to size, i.e., DNA of the same length formed a single band in the gel, with the same band sizes presented for each specific gene. Therefore, the isolated bands were used directly for PCR-SSCP analysis.

The PCR-SSCP technique revealed the presence of three genotypes (AC, BC and AA) and three alleles (A, B and C) for the *CAPN-9* gene (Figure 1A); eight different genotypes (AG, EF, AC, BB, CE, DG, AH and BI) with nine different alleles (A, G, E, F, C, B, D, H and I) for the *CAPN-14* gene (Figure 1B); and four different genotypes (ABC, AC, ABCD and EE) with five different alleles (A, B, C, D and E) for the *CAST-5* gene (Figure 1C).

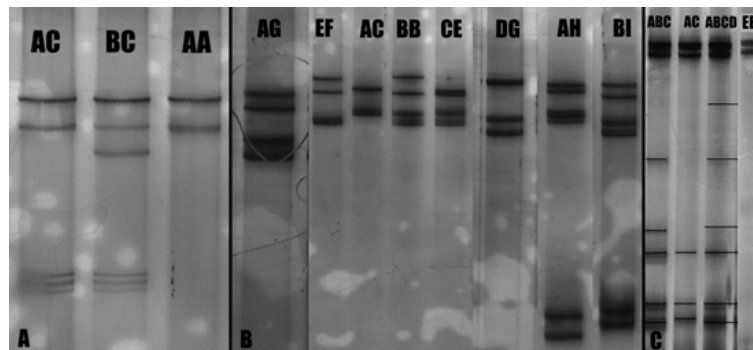


Figure 1. The PCR-SSCP technique (12.5% polyacrylamide gel) revealed (A) three different genotypes (AC, BC and AA) with three different alleles (A, B, C) for the bovine *CAPN-9* gene SNPs; (B) eight different genotypes (AG, EF, AC, BB, CE, DG, AH and BI) with nine different alleles (A, G, E, F, C, B, D, H and I) for the bovine *CAPN-14* gene SNPs; and (C) four different genotypes (ABC, AC, ABCD and EE) with five different alleles (A, B, C, D and E) for the bovine *CAST-5* gene.

For the *DGAT* gene, four different genotypes (AE, AD, BB and AC) with five different alleles (A, B, C, D and E) were identified (Figure 2A), and for the *LEP* gene, five

different genotypes (AA, AB, BB, BC, ABC) with three different alleles (A, B and C) were identified (Figure 2B).

Based on the genotype frequency analysis of the *CAPN-9* gene, the AC genotype (55%) was predominant in the Nellore population studied, with genotypes AA (39%) and BC (6%) having the lowest occurrence. For this gene, the allele frequencies observed were A, C, and B were 66.65%, 30.50% and 2.85%, respectively.

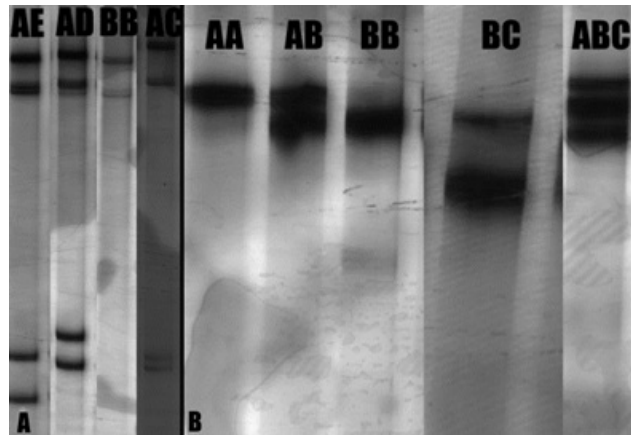


Figure 2. The PCR-SSCP technique (12.5% polyacrylamide gel) revealed (A) four different genotypes (AE, AD, BB and AC) with five different alleles (A, B, C, D and E) for the bovine *DGAT* gene SNPs; and (B) five different genotypes (AA, AB, BB, BC, ABC) with three different alleles (A, B and C) for the bovine *LEP* gene SNPs.

The data obtained from the sequencing of each genotype of the *CAPN-9* gene revealed the presence of five base substitution polymorphisms (G/A; T/A; T/C; T/C; and A/G) (Figure 3A). Analyzing the five SNPs detected in the sequencing of the *CAPN-9* gene, two polymorphisms (c.5861G>A and c.5498A>G) resulted in the substitution of the amino acid arginine for lysine and the amino acid proline for serine (Figure 3B).

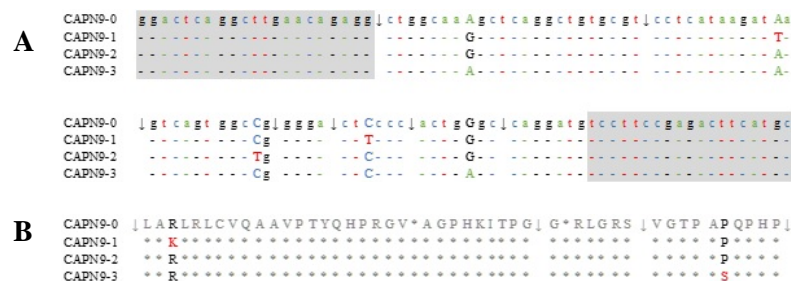


Figure 3. (A) Alignment of the nucleotide sequences, using tools from the MEGA X site, of the bovine *CAPN-9* gene with the sequence deposited in GenBank (accession number: AH009246.3). The nucleotides in exon 9 are indicated with lowercase letters. The SNPs are represented by uppercase letters. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image. The shaded regions indicate the primer binding regions. Hyphens indicate sequence identity. *CAPN9-0* (GenBank reference sequence: AH009246.3) and *CAPN9-1*, 2 and 3 (sequences evaluated in the present study). (B) Alignment of the amino acids of the bovine *CAPN-9* gene amplification products using Clustal W2. The uppercase letters are the amino acids. Asterisks (*) indicate identical residues. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image.

For the *CAPN-14* gene, the genotype frequencies were as follows: AG (16%); EF (11%), AC (14%), CE (15%), DG (4%), AH (7%), BI (18%) and BB (15%); and the allele frequencies were as follows: A (18.80%), B (18.80%), C (16.00%), D (2.20%), E (14.35%), F (6.10%), G (10, 50%), H (2.75%) and I (10.50%).

The data obtained from the sequencing of each genotype of the *CAPN-14* gene revealed the presence of four base substitution polymorphisms (A/C; G/A; T/C; and C/G) (Figure 4A) with two polymorphisms (c.11054 T>C and c.11161C>G) that resulted in the substitution of the amino acid proline for serine and of the amino acid aspartate for glutamic acid, respectively (Figure 4B).

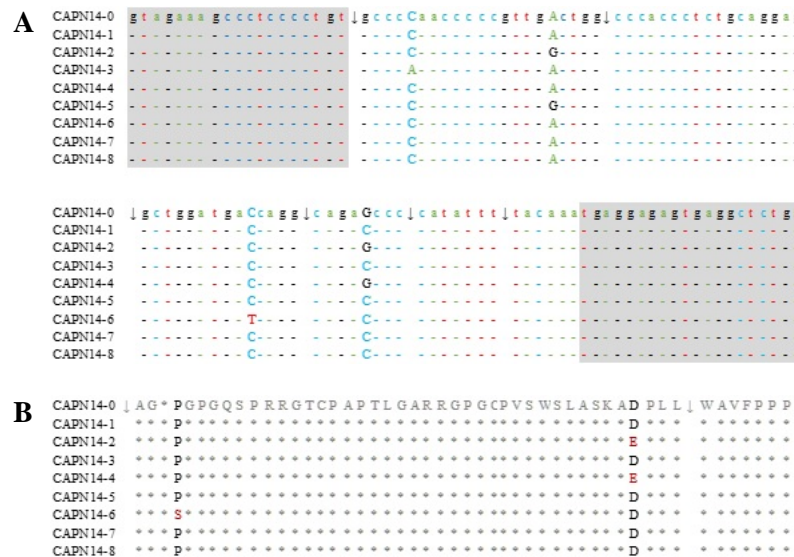


Figure 4. (A) Alignment of the nucleotide sequences, using tools from the MEGA X site, of the bovine *CAPN-14* gene with the sequence deposited in GenBank (accession number: AH009246.3). The nucleotides in exon 14 are indicated with lowercase letters. The SNPs are represented by uppercase letters. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image. The shaded regions indicate the primer binding regions. Hyphens indicate sequence identity. *CAPN14-0* (GenBank reference sequence: AH009246.3) and *CAPN14-1*, 2, 3, 4, 5, 6, 7 and 8 (sequences evaluated in the present study). (B) Alignment of the amino acids of the bovine *CAPN-14* gene amplification products using Clustal W2. The uppercase letters are the amino acids. Asterisks (*) indicate identical residues. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image.

The following results were observed for the genotype frequencies of the *CAST-5* gene in the studied Nelore population: AC (53%), ABCD (9%), ABC (21%) and EE (17%). The highest allele frequencies for the *CAST-5* gene were observed for the A (37.72%) and C (37.72%) alleles, followed by B (13.63%), E (6.82%) and D (4.10%) alleles.

The data obtained from the sequencing of each genotype of the *CAST-5* gene revealed the presence of five base substitution polymorphisms (C/T; T/C; C/A; C/A; and G/T) (Figure 5A). For the five SNPs detected in the *CAST-5* gene, four polymorphisms (c.29919C>T; c.29963A>C; c.29978C>A; and c.30019G>T) resulted in the substitution of the amino acid threonine for proline, proline for threonine, valine for isoleucine and glycine for valine, respectively (Figure 5B).



Figure 5. (A) Alignment of the nucleotide sequences, using tools from the MEGA X site, of the bovine *CAST-5* gene with the sequence deposited in GenBank (accession number: AH014526.2). The nucleotides in exon 5 are indicated with lowercase letters. The SNPs are represented by uppercase letters. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image. The shaded regions indicate the primer binding regions. Hyphens indicate sequence identity. *CAST5-0* (GenBank reference sequence: AH009246.3) and *CAST5-1, 2, 3* and *4* (sequences evaluated in the present study). (B) Alignment of amino acids from bovine *CAST-5* gene amplification products using Clustal W2. The uppercase letters are the amino acids. Asterisks (*) indicate identical residues. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image.

For the *DGAT* gene, the genotype frequency analysis revealed the following: AE (12%), AD (21%), BB (58%) and AC (9%). The allele frequencies for the *DGAT* gene were A (29%), B (40%), C (6%), D (15%) and E (8%). Based on the sequencing of each genotype of the *DGAT* gene, there were six base change polymorphisms (T/A; G/A; A/T; G/C; A/G; and G/A) (Figure 6A). For the six SNPs detected, four polymorphisms were found (c.11730A>T; c.11809G>C; c.11858A>G; and c.11927G>A) that resulted in the substitution of the amino acid leucine for histidine, arginine for glycine, arginine for histidine and arginine for proline, respectively (Figure 6B).

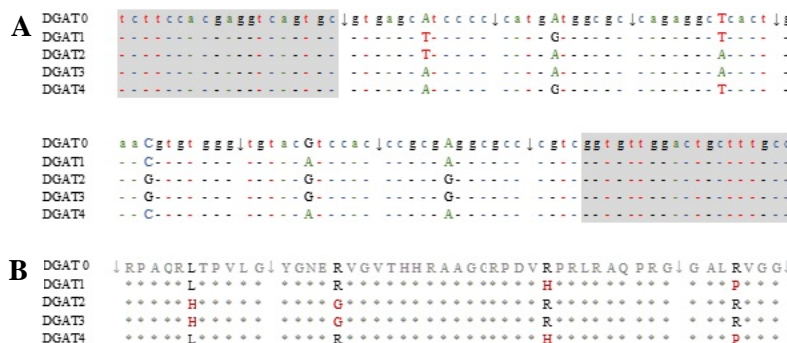


Figure 6. (A) Alignment of the nucleotide sequences, using MEGA X tools, of the bovine *DGAT* gene with the sequence deposited in GenBank (accession number: AJ318490.1). The nucleotides in exons 16 and 17 are indicated with lowercase letters. The SNPs are represented by uppercase letters. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image. The shaded regions indicate the primer binding regions. Hyphens indicate sequence identity. *DGAT-0* (GenBank reference sequence: AJ318490.1) and *DGAT-1, 2, 3, 4* (sequences evaluated in the present study). (B) Alignment of the amino acids of the bovine *DGAT* gene amplification products using ClustalW2. The uppercase letters are the amino acids. Asterisks (*) indicate identical residues. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image.

For the *LEP* gene, the genotype frequencies were AA (12%), AB (36%), BB (36%), BC (10%) and ABC (6%), and the allele frequencies were A (33.33%), B (56.42%) and C (10.25%).

Based on the sequencing of each *LEP* gene genotype, there were three base substitution polymorphisms (C/T, C/T and T/C) (Figure 7A); however, only one polymorphism (c.14962T>G) resulted in an amino acid substitution, i.e., arginine to tryptophan (Figure 7B).

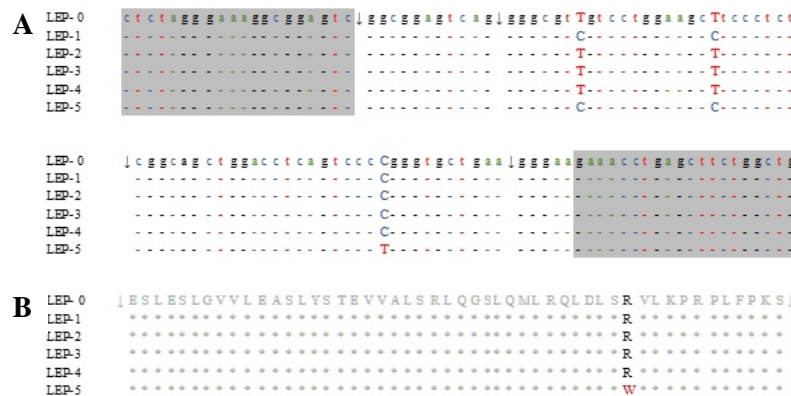


Figure 7. (A) Alignment of the nucleotide sequences, using MEGA X tools, of the bovine *LEP* gene with the sequence deposited in GenBank (accession number: JQ711179.1). The nucleotides in exon 2 are indicated with lowercase letters. The SNPs are represented by uppercase letters. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image. The shaded regions indicate the primer binding regions. Hyphens indicate sequence identity. LEP-0 (GenBank reference sequence: JQ711179.1) and LEP-1, 2, 3, 4, 5, 6, 7 and 8 (sequences evaluated in the present study). (B) Alignment of the amino acids of the bovine *LEP* gene amplification products using Clustal W2. The uppercase letters are the amino acids. Asterisks (*) indicate identical residues. The arrows indicate sequenced regions that did not show SNPs and were excluded for better visualization of the image.

DISCUSSION

This study investigated the occurrence of SNPs and the variation in the number of genotypes and alleles for the *CAPN-9*, *CAPN-14* and *CAST-5* genes among other studies in the literature.

For the *CAPN* (exon 14) and *CAPN* (exon 9) genes, Chung et al. (2014) evaluated the genetic variants using the PCR-SSCP technique; they found three different band patterns (GG, GA and AA and CC, CG and GG, respectively) for each gene that was associated with meat tenderness in Hanwoo cattle. Sun et al. (2018) evaluated the relationship between SNPs of the *CAST* gene (exon 9) using the PCR-SSCP technique and meat quality traits in Chinese cattle and three distinct patterns (TT, CT and CC) were identified that were associated with parameters related to meat tenderness and marbling score. Similarly, Pinto et al. (2011) working with Nellore Cattle, found three genotypes (TT, CT and CC) for CAPN1 (Intron 17) that were associated to beef redness and yellowness. Thus, the results of the present study indicate that there are greater variability in the SNPs for these genes in the Nellore cattle breed and depending on the region of the gene was studied and the type of mutation, consequently will have different results on meat quality.

For the *CAPN-9* gene, three genotypes were identified, with one being predominant. Similar results were found by Rosa et al. (2020), who conducted a study in Indonesia with 29 Aceh cattle and by Desgarenes-Alcalá et al. (2017) in 331 dual-purpose cattle in the Papaloapan region of Mexico using the PCR-RFLP technique to evaluate polymorphisms in the *CAPN* gene (exon 9). A study with cattle in China, Xin et al. (2011) used the PCR-SSCP technique to evaluate the relationship between *CAPN* gene polymorphisms (exon 17) and observed three genotypes. And, Pinto et al. (2011) for *CAPN1* (intron 17) for SNP *CAPN4751* to Nellore Cattle in Brazil found similar to those found in the present study, differing only in the genotype frequencies, thus indicating the similarity polymorphism of this gene in different cattle breeds.

The highest allele frequencies for the *CAPN-9* gene were observed for alleles A, C and B, in decreasing order. In previous studies two alleles (C and G) were identified for the *CAPN-9* gene in Aceh cattle in Indonesia (Desgarenes-Alcalá et al. (2017) and Rosa et al. (2020)); for *CAPN1* in Nellore cattle in Brazil two alleles (C and T) were found by Pinto et al. (2011); and only two alleles were found by Xin et al. (2011) for cattle evaluated in China for the *CAPN* gene (exon 17). In the animals of the present study, despite the presence of a greater number of alleles, there was a predominance of two alleles (A and C) in the Nellore cattle population studied that represented 97.15%. This lower variation in the number of alleles within this gene in the animals in the present study and in other breeds of beef cattle may be linked to their purpose because these breeds are selected especially for meat production, with a lower occurrence of crosses and heterosis with other breeds, potentially leading to the low variation in allele frequency.

Mutations in the *CAPN-9* gene are related to the cytosine/guanine (C/G) substitution, which produces a change from the amino acid glycine to alanine (Casas et al., 2005). *CAPN* (exon 9) in cattle is located at the telomeric end of chromosome 29, a region where quantitative trait loci related to meat tenderness, growth and feed efficiency are located (Casas et al., 2003; Pinto et al., 2011). Ardicli et al. (2019a) conducted a study with 108 Angus, Angus × Hereford × Nellore, Brahman, Hereford, Limousine and Charolais cattle using the PCR-RFLP technique to determine genotype/allele frequencies and *CAPN* gene polymorphisms (exon 9) and found the g.5709 C>G polymorphism, which results in the substitution of guanine for cytosine (C/G) and an amino acid substitution, i.e., alanine for glycine. In the present study, polymorphisms were also identified that resulted in the substitution of the amino acid arginine for lysine and the amino acid proline for serine. Arginine and lysine have similar characteristics (both have a hydrophobic R group); therefore, a substitution of one for the other is conservative, without compromising the enzymatic function of calpain (Buchanan et al., 2002). Conversely, proline and serine are amino acids with different characteristics (hydrophobic and hydrophilic R group, respectively); a substitution for one or the other would be nonconservative, likely altering the function of calpain in domain II and causing functional changes in proteolytic activity of calpain, with consequent variations in postmortem myofibrillar protein degradation (Curi et al., 2010). The substitution of the C allele in this genetic marker is associated with meat quality, especially tenderness (Casas et al., 2005; Miquel et al., 2009). Therefore, determination of the genotype and allele distributions corresponding to the *CAPN* gene (exon 9) can provide economic benefits in farm-raised and imported cattle (Ardicli et al., 2019b).

The analyses performed for the genotype frequencies of the *CAPN-14* gene show that the BI genotype was predominant in the Nellore population studied, with the DG and AH genotypes having the lowest occurrence. The highest allele frequencies for the *CAPN-14* gene were observed for the A and B alleles, in that order. In a study by Desgarenes-Alcalá et al. (2017) to determine the frequencies of alleles of the *CAPN* gene (exon 14) associated with the meat quality of Aceh cattle, the RFLP technique identified three genotypes, AA, GG and AG, and two alleles (G and A). However, Lara et al. (2012), when conducting a study to identify the variability in *CAPN* (exon 14) in 727 cattle of European and zebu breeds and their crosses (*Bos taurus taurus* x *Bos taurus indicus*), identified new SNPs for meat tenderness and six genotypes using the PCR-SSCP technique (XX, XY, YY, XZ, YZ and ZZ).

Regarding the *CAPN-14* gene, four SNPs were identified. In the present study, polymorphisms were also found that resulted in the substitution of the amino acids proline for serine and aspartate for glutamic acid. Proline and serine have different characteristics (hydrophobic and hydrophilic groups, respectively); a substitution for one or the other would be nonconservative and, thus, more likely to alter the function of calpain in domain III, which binds Ca^+ and is involved in the process of enzyme dissociation. Aspartate and glutamic acid have similar characteristics; a substitution from one to the other would be a conservative substitution, without compromising the enzymatic function of calpain (Buchanan et al., 2002). However, Pinto et al. (2010) evaluated the occurrence of SNPs in the *CAPN* gene (exon 14) in 638 Nellore cattle found a polymorphism that resulted in the substitution of adenine for guanine (A>G) and amino acid changes, i.e. isoleucine for valine, which was related to a reduction in shear force at 14 and 21 days of meat aging. Similar results were found by Carvalho et al. (2017) for SNPs in the *CAPN* gene (exon 14) in 605 Nellore cattle, in which a reduction in shear force at 21 days of aging was observed; and by Pinto et al. (2011) studying the SNP CPAN4751 polymorphism of the Gene *CAPN* 1 (Intron 17), where this mutation represent the substitution of cytosine for thymine (C>T) and association with an increase in redness and yellowness of beef at 7, 14 and 21 days was reported. Thus, although it is suggested that the amino acid change identified in the present study may not result in a calpain activity changes, the base pair changes in the genes *CAPN* are reported and was associated to change of meat characteristics.

For the *CAST-5* gene, four genotypes were identified in the studied Nellore population, with a predominance of two alleles (A and C). On the other hand, occurrence of different genotypes of the *CAST* gene in cattle has been reported are frequent identified three genotypes and two alleles like as reporter by Desgarenes-Alcalá et al. (2017) for the *CAST* gene using the PCR-RFLP technique in crossbreeding cattle in Mexico; and by Tyulebaev et al. (2019), in 84 Simmental bulls; and by Schenkel et al. (2006) for 628 cattle from various breeds (Angus, Limousin, Charolais, Simmental and Others breed). And this author reporter association of these genotypes and alleles for meat quality traits such as meat tenderness, *Longissimus dorsi* area, lean yield and increase of fat yield. Thus, these results showed that Nellore Cattle has a greater number of genotype than found in other breeds and they could be associated with other traits of meat quality besides tenderness.

For the *CAST-5* gene, five base substitution polymorphisms were identified. Sun et al. (2018), who evaluated the relationships between *CAST* genotypes (exon 9) and meat quality traits in 132 Chinese Simmental cattle, found a SNP in the *CAST596* gene (T>C) that resulted in the substitution of thymine for cytosine, causing the substitution of the

amino acid arginine for cysteine. However, these authors did not observe a significant association with some of the evaluated traits, such as marbling score and shear force. Lee et al. (2019) evaluated the association of three SNPs in the *CAST* gene (exon 7) with meat tenderness in two muscle cuts (*longissimus thoracis* and *semimembranosus*) from Hanwoo steers and observed the following SNPs: G>A, G>A and G>C; these SNPs were significantly related to increased shear force in both muscles. Also is reported association of shear force of *longissimus* muscle in Australian cattle with c.28i2A>G polymorphism of gene *CAST* (CAST2832) by Bolormaa et al. (2011). In the Nellore cattle in the present study, a greater number of polymorphisms was identified in the exon 5 region of the calpastatin gene, as observed by other authors in other regions of the *CAST* gene (exon 9 and exon 7). Exon 5 of calpastatin is located in domain L and, despite having no inhibitory capacity, plays a key role in regulating the inhibitory efficiency of calpastatin (Tullio et al., 2014). Thus, the occurrence of mutations in these exons can modify the regulatory function of the gene (Avern et al., 2001), altering the inhibitory efficiency of calpastatin in the meat of Nellore cattle and consequently influencing the tenderization process of these animals in the *post mortem* period.

Calvo et al. (2014), who, using the PCR-RFLP technique, evaluated mutations in the *CAST* gene (exon 7) and tested the association of these changes with meat tenderness in 196 Parda de Montaña and Pirenaica cattle in Spain, identified a polymorphism that caused the substitution of adenine with guanine (A>G), producing a substitution of the amino acid threonine with alanine, which was related to high shear force values. Similar results were also found by Enriquez-Valencia et al. (2017), who estimated the allele and genotype frequencies for the *CAST* gene (exon 7) in different genetic groups of 114 beef cattle produced in Brazil (Nellore and its crosses with *Bos taurus*); they identified the substitution of adenine for guanine (A>G), producing a substitution of the amino acid threonine with alanine, which was related to low shear force values and a low myofibrillar fragmentation index. Thus, the *CAST-5* polymorphisms that resulted in the substitution of the amino acids threonine for proline, proline for threonine, valine for isoleucine and glycine for valine were also identified. However, the amino acids threonine and proline have different characteristics (hydrophilic and hydrophobic groups, respectively); a substitution for one to the other would be nonconservative, potentially affecting the regions of interaction between the L-domain of calpastatin and calpain and generating a more stable union between these proteins, thus affecting the tenderization process (Curi et al., 2010; Calvo et al., 2014). The amino acids valine, isoleucine and glycine have similar characteristics; a substitution of one for any other would be conservative, without compromising the enzymatic function of calpastatin (Buchaman et al., 2002).

For the population of cattle studied, in the *DGAT* gene, four genotypes with five different alleles were identified, with the BB genotype having the highest prevalence in the studied Nellore population; alleles B and A had the highest allele frequencies. Venkatachalapathy et al. (2013) investigated the occurrence of SNPs in the *DGAT* gene (exon 16) using the PCR-SSCP technique in cattle in India and observed two distinct patterns (CC and CT). The association of SNPs *DGAT* gene and performance traits of a commercial cattle herd, was identified by PCR-RFLP technique, where three genotypes (AA, KA and KK) and two alleles (A and K) for 296 Holstein-Friesian bulls was found by Ardicli et al. (2019b); while in 390 Holstein cows and 160 buffaloes in Romania and was related effects on the fat percentage and fatty acid profiles for three genotypes (KK, KA and

AA) and two alleles (A and K) (Tabaran et al., 2015). Urtnowski et al. (2011) identified three different genotypes (AA, GA and GG) and two alleles (A and G) using the RFLP and also find association between polymorphisms in the *DGAT* gene (exon 8) and meat quality in 156 Polish Holstein young bulls.

The sequencing of each genotype of the *DGAT* gene revealed the presence of six base substitution polymorphisms, with four promoting changes in amino acid synthesis. Urtnowski et al. (2011), who studied the association between polymorphisms in the *DGAT* gene and meat production and quality traits in 156 Polish Holstein young bulls, found that one of the mutations, an AA>GC substitution, caused an amino acid substitution, i.e., lysine to alanine (K232A). This substitution affects the intramuscular fat content and changes the physicochemical characteristics of meat (Winter et al., 2002; Thaller et al., 2003). Similar, analyzing the *DGAT1* gene polymorphism in Nellore Cattle Borges et al. (2014) found five alleles (4R, 5R, 6R, 7R and 8R) where the 5R allele was associated with increased intramuscular fat percentage, while the 6R allele had a negative effect on cooking losses and the 7R allele a positive effect on ribeye area. Yuan et al. (2013) found four base substitutions in exon 17 (C>T; T>G; C>T; and C>T); the SNPs C>T and T>G caused a substitution of the amino acids threonine for alanine and valine to glycine, respectively. According to these authors, these SNPs identified in *DGAT* were significantly associated with carcass fat quality traits in Chinese commercial cattle, concluding that the substitution of these amino acids (alanine and glycine) was desirable because they are associated with better meat quality and carcass traits, such as shear force, marbling, and meat and fat color.

Furthermore in the present study, leucine for histidine, arginine for glycine, arginine for histidine and arginine for proline substitution also occurred. The substitution of arginine for histidine probably would not modify the enzymatic function of diacylglycerol acyltransferase because these two residues have similar characteristics (Buchanan et al., 2002). However, the amino acid substitutions involving arginine and glycine and arginine and proline could affect the regions where the enzyme diacylglycerol acyltransferase and its cell receptors interact. These substitutions could affect satiety signaling related to the amount of body energy deposited in the form of fat (Salman et al., 2007). So, the polymorphisms found with the occurrence of amino acids changes in the present study could promote alterations in relation to the intramuscular fat composition of the meat of Nellore Cattle or modified another beef parameters.

For the *LEP* gene, five genotypes with three different alleles were identified; the AB and BB genotypes and the B allele were the most prevalent. Similar to the results of the present study, in the *LEP* gene (exon 3) using the PCR-SSCP technique, Dubey et al. (2008) found variation in Sahiwal cattle in India with four distinct genotypes (AA, BB, CC and DD); while Papaleo Mazzucco et al. (2016) in Angus and Hereford cattle, detected three genotypes (CC, CT and TT) and two alleles (C and T). Using PCR-RFLP technique to evaluate the association of SNPs of the *LEP* gene with performance traits of commercial cattle was identified three genotypes (CC, CT, and TT) and two alleles (C and T) in 296 purebred Holstein-Friesian bulls by Ardicli et al. (2019b) and in 81 Simental bulls by Ardicli et al. (2017). Similar results were also reported in the leptin gene (exon 2) in Japanese cattle by Kawaguichi et al. (2020) and Pinto et al. (2011) in Nellore Cattle, where three genotypes and two alleles was reported. This difference between the number of genotypes cited in the literature may be related to the region of the gene evaluated. These authors of the cited studies investigated exon 3 and another SNPs of the leptin gene, while

in the present study, exon 2 was investigated. The exon 2 region of the leptin gene has an important physiological role associated with the manifestation of traits such as carcass fat deposition, milk production, consumption capacity, and feed conversion (Salman et al., 2007).

Nkrumah et al. (2005), who evaluated, using the PCR-RFLP technique, *LEP* gene polymorphisms and associations with fat content in the carcass of 150 bulls, identified six SNPs in exon 2 that resulted in the substitution of cytosine for thymine and thus arginine for cysteine, a substitution that was associated with the production of meat with greater marbling. In Simmental crossbred steers, in exon 2 of leptin, a polymorphism (T>C) was identified that replaced cysteine with arginine, increasing the ribeye area and fat thickness (Tian et al. 2013). Similarly, Giblin et al. (2010) conducted a study with 848 Dutch-Friesian bulls in Ireland to quantify the associations between SNPs of the *LEP* gene and performance traits. These authors observed a polymorphism at the position of amino acid 80 in exon 3, with the substitution of alanine for valine, with increased milk fat production in the animals with the C allele. Furthermore, a polymorphism in exon 2 of leptin was associated with greater fat deposition in Angus and Hereford carcasses, as shown by Buchanan et al. (2002). Pinto et al. (2011) studying the leptin Gene SNP T945M (C>T) that results in a substitution of the amino acid threonine by methionine, found the association of it to drip loss at the 7th day of postmortem aging beef in Nellore cattle. Otherwise, Orrù et al. (2010) studying the *LEP* gene polymorphism for 103 Simmental bulls for exon 2 found amino acid changes from Tyrosine to Phenylalanine (SNP g.1127A>T) and change Arginine to Cysteine (SNP g.1180C>T) and both did not showing relation to fatty acids composition of the muscle. However, in the exon 3 of leptin gen the snps with change amino acid like as Alanine for Valine (g.3100C>T); asparagine to serine (g.3157A>G) and the snp g.978C>T in the intron of *LEP* gene (without amino acid change) was associated to change of fatty acids composition. Then only SNP g.3257C>T in the *LEP* exon 3 affected the total lipid content in muscle. The animals in the present study, the occurrence of polymorphisms in this region of the gene (exon 2 of the leptin gene) that result in the substitution of the amino acid arginine for tryptophan, which are amino acids with different characteristics, may cause changes in leptin action and function and carcass fat deposition traits or effects on drip loss in the meat.

In the present study are found some SNPS with results an amino acids changes for the genes studied. Although these modification could be associated to beef quality characteristics this results should not consider isolate because of according some studies have showing that occurrence of SNPs in different genes can be associated to same characteristic of meat quality in cattle (Bolormaa et al., 2011; Orrù et al., 2011; Tyulebaev et al., 2019).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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