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Polymorphism analysis in genes associated with meat tenderness in Nelore cattle

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ABSTRACT

The aims of this study were to identify haplotyped loci associated with meat tenderness (WBSF) in candidate genes and to search for new polymorphisms in these regions that influence such trait in Nelore cattle. Fifty-two genes that had previously been associated with WBSF or that have biological functions that may influence tenderness were chosen for the haplotype association analysis in 1657 animals. Of the 52 tested candidate genes, two haplotyped loci located in *ASAP1* and *CAPN1* were significantly associated with WBSF. The effect of the significant haplotype alleles varied from −0.44 to 0.80 and −1.03 to 1.52 kg in *CAPN1* and *ASAP1*, respectively. Exonic regions near and within these loci were chosen for sequencing to search for new polymorphisms. Four SNPs were identified in *ASAP1* and seventeen in *CAPN1*. All SNPs detected in the sequenced regions in *ASAP1* showed no association with WBSF even though two of them (*rs714391435* and *rs109256712*) were in strong linkage disequilibrium (LD) with most SNPs that composed the significant haplotyped locus. Four SNPs located in *CAPN1* were found for the first time in this study. One of them (*rs1121961662*) showed significant association with WBSF and is also in strong LD with all SNPs within the significant haplotyped locus in *CAPN1* gene. The SNP *rs1121961662* could be used as a molecular marker for this QTL and may be included in low-density arrays to improve the selection of meat tenderness in *Bos indicus* animals.

1. Introduction

Brazil has been a major global producer and exporter of beef for over a decade (Anualpec, 2016) and the Brazilian herd composition is predominantly *Bos indicus*, with Nelore being the most prominent beef breed. *Bos indicus* breeds produce beef of lower tenderness than *Bos taurus* breeds (O'Connor et al., 1997; Bianchini et al., 2007) and meat tenderness is considered to be the most important trait influencing consumer satisfaction, impacting the profitability of the entire beef industry (Huffman et al., 1996; Boleman et al., 1997; Goodson et al., 2002). However, meat tenderness is a complex trait in which many genetic and non-genetic factors affecting it (Hocquette et al., 2012) and is expressed late in the animal's life when measured *post-mortem*. As a consequence, the genetic improvement of meat tenderness in beef cattle has been slow (Gutiérrez-Gil et al., 2008; Dunner et al., 2013). Therefore, the search for trait-associated SNP markers has become an important tool to identify quantitative trait loci (QTL), candidate genes and causal mutations that influence meat tenderness (Page et al., 2004; Van den Berg et al., 2013).

Candidate gene-association mapping approach can be effectively used to analyze genes with known function related to complex traits with higher statistical power when compared to GWA studies (Kwon and Goate, 2000; Patnala et al., 2013; Amos et al., 2011). Another strategy to increase the power for the identification of significant genomic regions is through haplotype-based association methods. Haplotypes are multi-allelic, reduce the number of tests and may capture more of the available linkage disequilibrium (LD) with QTL loci (Zhao et al., 2007; Cuyabano et al., 2014). The combination of these two approaches increases the chance of detecting regions that truly influence complex traits and may be explored more deeply to search for new polymorphisms that could be useful in selection programs. Therefore, the aims of this study were to identify haplotyped loci associated with meat tenderness in candidate genes and to search for new polymorphisms in these regions that influence such trait in Nelore

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cattle.

2. Material and methods

2.1. Animals

A total of 1657 Nelore bulls born between 2008 and 2011, raised on pasture and sourced from three different animal breeding programs were used in this study. The animals were finished in feedlots for approximately 90 days and then slaughtered in commercial slaughterhouses at a mean age of 729 ± 81 days. The animals belonged to 165 contemporary groups (CG) defined by the combination of farm and year of birth, management group as long-yearlings and month and year of slaughter. There were at least two animals in every CG.

2.2. Phenotype and genotype data

Briefly, to obtain the tenderness phenotypes, animals were slaughtered and the carcasses were identified and chilled for 24 to 48 hour *post-mortem*. Steaks of 2.54 cm thickness were collected from the *longissimus thoracis* muscle between the 12th and 13th ribs from the left half of the carcasses. The steaks were vacuum sealed and aged in a cold chamber for 150 h at 1 °C and then were frozen at −20 °C until they were later cooked in an oven to an internal temperature of 71 °C as proposed by Wheeler et al. (1995). After cooking, steaks were chilled overnight at 2 to 5 °C before coring. The Warner-Bratzler shear force (WBSF), a mechanical measurement of tenderness, was measured using a Salter shearing device (G-R Electric, Manhattan, KS). For precision, eight 1.27 mm meat cylinders were obtained from each sample and the average shear force was used in analysis. The mean of WBSF was 5.21 ± 1.38 kg with a minimum value of 1.56 kg and a maximum of 11.20 kg.

Tissues from the *longissimus thoracis* muscle were used to extract DNA using a DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Genotyping was performed by high-density bead array technology using the Illumina (San Diego, CA) BovineHD Infinium Assay® with an Illumina HiScan System®. The BovineHD BeadChip contains 777,962 SNP markers evenly distributed throughout the genome with a 3.43 kilobase (kb) average separation between the markers. The Illumina Genome Studio software was used to analyze the HiScan images to call genotypes. Forty-one samples for which the genotype call rate was < 90% were removed. The SNP markers with a call rate of < 95% or with a minor allele frequency < 5%, Hardy Weinberg equilibrium test statistic probability $< 10^{-5}$ or that were unmapped to autosomes or sex-linked were also excluded. After quality control a total of 1616 samples remained for analysis.

2.3. Candidate gene analysis

Fifty-two genes that had previously been associated with meat quality or that have biological functions that may influence tenderness were chosen for analysis and are described in Supplementary Table 1. The genomic coordinates for each gene based on the *Bos taurus* UMD3.1 reference assembly were expanded by 1500 bp upstream and downstream due to their potential for harboring regulatory elements and missing exons within the assembly (Whitacre et al., 2015). A total of 985 SNPs on the BovineHD assay were identified within these regions.

The software fastPHASE (Scheet and Stephens, 2006) was used for haplotype reconstruction and missing genotype estimation. The linkage disequilibrium (LD) calculation between SNPs and the haplotype block definition were performed using HaploView software (Barrett et al., 2005) based on estimates of D' (Gabriel et al., 2002).

Statistical analyses incorporating haplotype information were performed using the MIXED procedure of the SAS 9.3 (SAS Institute, 2011), for each haplotyped locus, as follows:

$$y_{ijk} = c_i + s_i + \sum_{l=1}^{n-1} b_l x_{li} + e_i$$

where, y is the WBSF phenotypes for animal i; c is the fixed effect of CG for animal i; s is the random effect of sire for animal i; b is the regression coefficient of the number of copies of the allele l; x is the number of copies of the allele l for animal i; e is the random error for animal i; and n is the haplotype allele number. The Bonferroni test was applied at an experiment-wise 10% ($P \leq 0.00028$) error rate to adjust for multiple testing.

2.4. Sequencing

Exons of each candidate gene located within, or adjacent to, the haplotyped loci that were significantly associated with WBSF were chosen for sequencing in 25 animals with the lowest degree of relatedness among the 1616 animals to identify polymorphic regions, since they are more likely to be genetically different from each other. After, the polymorphic regions were sequenced in 298 animals that belonging to the CGs from the initial 25 sequenced animals.

Primers were designed to amplify these regions (based on Gene ID: 281661 and 327705) using Primer3 software and their specificity was verified using OligoAnalyzer 3.1. The polymerase chain reaction amplification mixture contained 1.0 μL DNA (60 ng/μl), 1.5 μL of each primer (15 pM), 7.5 μL GoTaq Colorless Master Mix and 3.5 μL nuclease-free water in a final volume of 15 μL. A Master Cycler Gradient 5331 thermal cycler was used to perform the amplification using the conditions: denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing temperature dependent on each primer (Supplementary Table 2) for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Twenty-five unrelated animals were individually sequenced to identify variants in the amplicons produced by each primer pair. Sequencing was performed by the dideoxynucleotide chain termination reaction on an ABI 3730 XL sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). Polymorphisms within each amplicon were identified using the CodonCode Aligner program.

2.5. Polymorphism association analyses

In order to increase the number of individuals analyzed, genotypes called from the sequence data produced for the SNPs discovered in the sequenced genes were merged with the SNPs from the BovineHD assay and genotypes were imputed for all 1616 animals using BEAGLE v3.3.2 (Browning and Browning, 2007). HaploView software was used to perform the tests of Hardy-Weinberg equilibrium (HWE) for each SNP and to calculate the LD between SNPs. The same statistical analysis described for candidate genes analysis (above) was performed to the SNPs discovered in the sequenced genes. The SNP *rs714391435* were not included in the association analysis due to its very low MAF (0.02). The Bonferroni correction was applied at the 5% level to adjust for multiple testing ($P \leq 0.0025$).

3. Results and discussion

Two haplotyped loci were significantly associated with meat tenderness using the Bonferroni correction ($P \leq 0.00028$) and were located in the *ASAP1* and *CAPN1* genes (Table 1). The haplotyped locus in *ASAP1* was composed of 21 SNPs spanned 36,884 bp in intron 3 and the haplotyped locus in *CAPN1* spanned 3,128 bp with 3 SNPs between intron 13 and 19. The haplotype alleles, their frequencies and average effect are shown in Table 2. In *ASAP1*, the most favorable haplotype allele (GGGTACCAGCGGGCCAAAGTG) decreased 1.03 kg in WBSF and was observed at 7.6% frequency in this population. In *CAPN1*, the haplotype allele ATG showed the most favorable effect (−0.44 kg) on

Table 1
Description of the haplotyped loci significantly associated with meat tenderness after Bonferroni correction.

Gene	RefSNP	Chr	Pos	Region	Length
ASAP1	*rs110237617 - rs110329676 - rs110543908 - rs109736419 - rs109890073 - rs110270007 - rs136043279 - rs109331471 - rs110421714 - rs110868560 - rs109150109 - rs110631693 - rs110205824 - rs109516020 - rs109758436 - rs109254655 - rs110869257 - rs109958399 - rs134375889 - rs109522060 - rs41627745*	14	11,494,742–11,531,625	I 3	36,884
CAPN1	*rs17871058 - rs17872050 - rs17870847*	29	44,085,769–44,088,897	I 13–19	3,128

RefSNP, reference SNP ID number; Chr, chromosome; Pos, haplotype initial and final position in base pairs; I, intron; Length, haplotype length in base pairs.

Table 2
Frequency, average effect and respective standard error (SE) of the haplotyped loci alleles significantly associated with meat tenderness.

Genes	Alleles	Frequency	Average effect (SE)
ASAP1	TATCCTTCATAAATTGGGGCT	0.447	0.07 (0.30)
	GGTTCTTCATAAATTGGGGTG	0.339	−0.25 (0.32)
	GGGTACCAGCGGGCCAAAGTG	0.076	−1.03 (0.51)
	GGGTACCAGCGGGCCAAAATG	0.049	−0.40 (0.60)
	GGTCCTTCATAAACTAGGGCG	0.044	−0.08 (0.65)
	GGTTCTTCATAAACTAGGGTG	0.033	−0.13 (0.73)
	GGTTACCAGCGGGCCAAAGTG	0.012	1.52 (0.64)
CAPN1	GTG	0.591	−0.20 (0.14)
	GTA	0.185	−0.17 (0.16)
	ATG	0.121	−0.44 (0.23)
	GCA	0.103	0.80 (0.29)

Table 3
Description of the SNPs found in the candidate tenderness genes in Nelore cattle.

Gene	Chr	RefSNP	Position	Region	Consequence
ASAP1	14	*rs109552294*	11,558,261	Exon 4	Synonymous
		rs109256712	11,558,286	Intron 4	
		rs211332127	11,558,293	Intron 4	
		rs714391435	11,558,324	Intron 4	
CAPN1	29	*rs481432081*	44,085,260	Intron 12	Synonymous
		rs17871050	44,085,638	Exon 14	
		rs17871051	44,085,642	Exon 14	Non-synonymous
		rs1121961662	44,086,321	Intron 15	
		rs721496944	44,086,442	Intron 16	
		rs464736006	44,086,508	Intron 16	
		rs17872152	44,088,005	Intron 18	
		rs17872153	44,088,027	Intron 18	
		rs110896665	44,088,036	Intron 18	
		rs1131232536	44,088,078	Intron 18	
		rs447978510	44,088,087	Intron 18	
		rs1118716870	44,088,126	Intron 18	
		rs1122971334	44,088,158	Intron 18	
		rs715244311	44,088,332	Intron 19	
		rs110022482	44,089,241	Intron 20	
		rs110480144	44,089,263	Intron 20	
		rs719993085	44,089,483	Intron 21	

RefSNP, reference SNP ID number; Position, UMD3.1 coordinate of the SNP on the chromosome (base pair).

WBSF and was present in this population at 12.1% frequency.

The *ASAP1* gene (ArfGAP with SH3 domain, Ankyrin repeat and PH domain 1), also known as *DDEF1* (Gene ID: 327705), is located on chromosome 14 and encodes a signal transduction protein that enhances the potential of fibroblasts to differentiate into adipocytes (King et al., 1999). Cruz et al. (2012) reported that meat tenderness scores were significantly associated with adipocyte cell numbers and also proposed that increasing the numbers of adipocytes would lead to improved tenderness. Adipocytes are the cells that comprise adipose tissue found in subcutaneous, visceral, intermuscular and intramuscular (marbling) adipose depots (Hausman et al., 2014). Subcutaneous fat insulates the carcass and slows *post-mortem* chilling, which may improve tenderness by reducing the extent of cold-shortening and marbling improves meat tenderness by reducing tissue density and decreasing the strength of connective tissues (Fiems et al., 2000; Hausman et al., 2009). Tizioto et al. (2012), Tizioto et al. (2013) and Ramayo-Caldas et al. (2016) showed association of SNPs in *ASAP1* with meat tenderness in Nelore and French beef breeds.

The *CAPN1* gene (Gene ID: 281661) is located on chromosome 29 and encodes μ-calpain, the micromolar calcium activated neutral protein, which degrades myofibrillar proteins *post-mortem*. The calcium dependent proteolytic system has long been considered to be among the most important factors responsible for variation in *post-mortem* tenderization (Croall and DeMartino, 1991; Koohmaraie, 1994; Koohmaraie, 1996). A number of studies have shown significant associations between *CAPN1* and shear force, suggesting that variants in this gene may be responsible for variation in meat tenderness (Page et al., 2002; Casas et al., 2005; Casas et al., 2006; White et al., 2005; Curi et al., 2009).

For *ASAP1*, we sequenced only exon 4 due to its proximity to the significant haplotyped region and nine exons were located near or in the significant haplotyped region in *CAPN1* (Supplementary Table 2). The sequencing identified 21 SNPs, with 17 SNPs in *CAPN1* spanning part of intron 12 through part of intron 21 and 4 SNPs in *ASAP1*, including exon 4 and a part of intron 4 (Table 3). The SNP *rs109552294*, located in exon 4 of *ASAP1*, is a synonymous substitution and the remaining SNPs are in intron 4. Among the detected SNPs in *CAPN1*, the SNP *rs17871050* is a synonymous substitution while the SNP *rs17871051* produces a valine to isoleucine substitution (Val530Ile) in exon 14 of *CAPN1* and the remaining SNPs are located in introns. The sequences containing the polymorphisms were submitted to NCBI under accession numbers KT023263.1 and KT023264.1. The SNPs *rs1121961662*, *rs1131232536*, *rs1118716870* and *rs1122971334*, located in *CAPN1*, were identified for the first time in this study and were deposited into NCBI's dbSNP database.

In the intron 18 of *CAPN1*, where the SNPs *rs447978510*, *rs1118716870* and *rs1122971334* were located, there is also the microRNA 2407 (Gene ID: 100313430) located from 44,088,087 to 44,088,159 bp (73 nucleotides). MicroRNAs (miRNAs) act as post-transcriptional silencers leading to decreased gene expression and may affect their host gene (Bartel, 2004; Pillai et al., 2007). This miRNA may modulate *CAPN1* expression or may regulate genes that cooperate with *CAPN1* in its specific biological processes (Lutter et al., 2010; Bosia et al., 2012).

The LD between the SNPs that composed the significant haplotyped loci and the SNPs detected in the sequenced regions are shown as a heat map of the estimates of D' in Fig. 1. In *ASAP1*, the four SNPs detected in the sequenced regions (*rs109552294*, *rs109256712*, *rs211332127* and *rs714391435*) were in strong LD between them. However, only the SNPs *rs714391435* and *rs109256712* were in strong LD with most SNPs that composed the significant haplotyped locus. In *CAPN1*, all SNPs detected in the sequenced regions were in strong LD with the three SNPs within the significant haplotyped locus (*rs17871058*, *rs17872050* and *rs17870847*), but the SNPs *rs17871058* and *rs719993085*, *rs17870847* and *rs481732081* that showed low LD between them. The four SNPs identified for the first time in a bovine population (*rs1121961662*, *rs1131232536*, *rs1118716870* and *rs1122971334*) also were in strong LD with each other. According to Pritchard and Przeworski (2001) SNPs in strong LD tend to be inherited together more often than would be expected by chance.

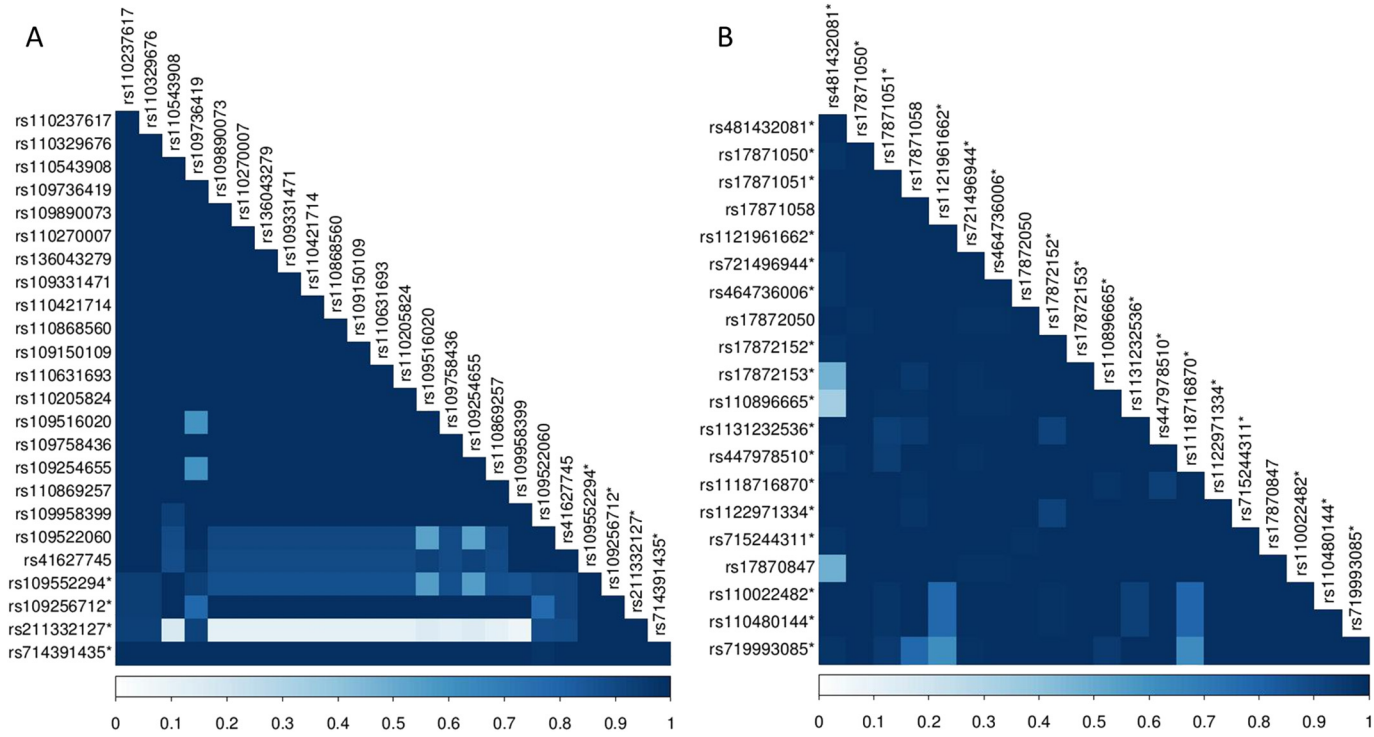

Fig. 1. Heat map of linkage disequilibrium (D') between the SNPs located in *ASAP1* (A) and *CAPN1* (B) genes. SNPs without the asterisks compose the significant haplotyped loci and SNPs with asterisks were discovered in the sequenced regions. Darker shading indicates regions of higher linkage disequilibrium.

Table 4
Allele frequency, effect and P-values for SNPs found in the tenderness candidate genes.

Gene	RefSNP	Allele frequency		Genotype Frequency			HWEpval	Effect	P-value
		A	**G**	**AA**	**AG**	**GG**			
ASAP1	*rs211332127*	0.36	0.64	0.1219	0.4696	0.4085	0.3815	0.017	0.2911
CAPN1	*rs481432081*	0.70	0.30	0.4937	0.4230	0.0833	0.5238	−0.007	0.5872
CAPN1	*rs17871051*	0.04	0.96	0.0006	0.0833	0.9161	0.3919	−0.147	0.4882
CAPN1	*rs17872152*	0.04	0.96	0.0006	0.0797	0.9197	0.4760	−0.129	0.5587
CAPN1	*rs17872153*	0.29	0.71	0.0760	0.4291	0.4949	0.1112	−0.016	0.5321
CAPN1	*rs1131232536*	0.11	0.89	0.0079	0.1955	0.7966	0.1914	0.117	0.2344
CAPN1	*rs110022482*	0.96	0.04	0.9155	0.0827	0.0018	1.0000	0.139	0.4472
CAPN1	*rs110480144*	0.96	0.04	0.9155	0.0827	0.0018	1.0000	−0.139	0.4472
		T	**C**	**TT**	**TC**	**CC**			
ASAP1	*rs109552294*	0.54	0.46	0.2842	0.5142	0.2016	0.1672	−0.017	0.3283
ASAP1	*rs714391435*	0.02	0.98	0.000	0.0453	0.9547	0.8491	–	–
CAPN1	*rs17871050*	0.19	0.81	0.0266	0.3204	0.6530	0.0231	−0.080	0.5088
CAPN1	*rs464736006*	0.81	0.18	0.6512	0.3223	0.0265	0.0231	0.073	0.5668
CAPN1	*rs447978510*	0.86	0.14	0.7435	0.2348	0.0217	0.4677	0.045	0.6147
CAPN1	*rs1118716870*	0.07	0.93	0.0036	0.1268	0.8696	0.7557	−0.168	0.0036*
CAPN1	*rs1122971334*	0.11	0.89	0.0079	0.1949	0.7972	0.2004	0.113	0.2567
CAPN1	*rs715244311*	0.19	0.81	0.0266	0.3198	0.6536	0.0311	−0.082	0.4983
CAPN1	*rs719993085*	0.14	0.86	0.0253	0.2342	0.7405	0.1149	−0.069	0.4598
		T	**G**	**TT**	**TG**	**GG**			
CAPN1	*rs110896665*	0.43	0.57	0.1762	0.5063	0.3175	0.1961	−0.041	0.8918
		G	**C**	**GG**	**GC**	**CC**			
ASAP1	*rs109256712*	0.49	0.51	0.2299	0.5184	0.2517	0.1450	0.004	0.7274
CAPN1	*rs1121961662*	0.93	0.07	0.8714	0.1255	0.0031	0.5310	0.187	0.0023**
		A	**C**	**AA**	**AC**	**CC**			
CAPN1	*rs721496944*	0.19	0.81	0.0278	0.3205	0.6517	0.0498	−0.077	0.5316

RefSNP, reference SNP ID number; HWEpval, P-values for the tests of Hardy-Weinberg equilibrium; effect, allelic substitution effect.
** P-value after Bonferroni correction at 5% (P ≤ 0.0025)
* P-value after Bonferroni correction at 10% (P ≤ 0.005).

The allele and genotype frequencies, P-values for HWE, allelic substitution effect and the result of the association analysis of the SNPs detected in the sequenced regions are presented in Table 4. All SNPs detected in the sequenced regions were in HWE (P ≥ 0.001). Few SNPs in *ASAP1* have been shown to affect WBSF in Nelore (Tizioto et al., 2012 and Tizioto et al., 2013) and French beef breeds (Ramayo-Caldas et al., 2016). The associations of the four SNPs found in *ASAP1* in this study with WBSF have not previously been investigated in beef cattle;

however, they showed low effect and no evidence of association with WBSF in the population studied.

Several studies have reported SNPs located in exons and introns of *CAPN1* affecting WBSF in *Bos taurus*, *Bos indicus* and crossbred populations (Casas et al., 2006; Curi et al., 2009; Page et al., 2002; McClure et al., 2012). However, studies investigating the associations of the SNPs found in *CAPN1* in this study were not found in the literature, except for the SNP *rs17871051*, which has previously been reported to be associated with WBSF in beef cattle (Page et al., 2002; Page et al., 2004; Lee et al., 2014; McClure et al., 2012). Only the SNP *rs1121961662*, located in intron 15 of *CAPN1*, showed significant association with WBSF, after Bonferroni correction at 5% ($P \leq 0.0025$), and the greatest allelic substitution effect (0.185 kg). SNPs located in intron may be in strong LD with the causal mutation or may be regulatory elements influencing the expression of the genes that host them (Cooper, 2010). This SNP (*rs1121961662*) is in strong LD with the SNP *rs17871051* which showed no association with WBSF in the present study and this lack of association could be due to the low frequency of the A allele (0.04). Pinto et al. (2010) also reported low frequency of the A allele and no association with WBSF of the SNP *rs17871051* in another Nelore cattle population. Casas et al. (2005) reported that the G allele seemed to be fixed in Brahman (*Bos indicus*). Therefore, the SNP *rs1121961662* could be used as a molecular marker for this QTL and included in low-density arrays to improve the selection of meat tenderness in *Bos indicus* breeds.

4. Conclusions

Haplotypes capturing the linkage disequilibrium between markers allowed the identification of two significant regions in *ASAP1* and *CAPN1* influencing meat tenderness in Nelore cattle. One polymorphism (*rs1121961662*), detected for the first time in a bovine population, was associated with meat tenderness and was in strong linkage disequilibrium with the significant haplotyped locus in *CAPN1* gene. Future investigation to better understand how these genes are influencing meat tenderness and to validate the SNP *rs1121961662* could be helpful to improve such trait in Nelore cattle.

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Authors' contributions

CUB conceived the study, analyzed and interpreted the results and, wrote the manuscript. RE, DAG, DGMG and AFBM helped conceive the study. JFT, JED and LGA assisted with preparation of the manuscript and aided with interpretation of results; TB and HNO assisted with analyses, preparation of the manuscript and interpretation of results. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical clearence

All animal procedures were approved by the São Paulo State University (Unesp), School of Agricultural and Veterinary Science Ethical Committee (Approval No. 18.340/16).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mgene.2018.08.002.

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