Genetic polymorphisms related to meat traits in purebred and crossbred Nelore cattle

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Abstract – The objective of this work was to estimate the allelic and genotypic frequencies of *CAST*/*Xmn*I, a calpastatin gene polymorphism, and CAPN530, a calpain 1 large subunit gene polymorphism, in different beef genetic groups (Nelore and Nelore x *Bos taurus*), and to investigate associations between these polymorphisms and carcass and meat traits. Three hundred animals – comprising 114 Nelore, 67 Angus x Nelore, 44 Rubia Gallega x Nelore, 41 Canchim, 19 Brangus three-way cross and 15 Braunvieh three-way cross – were genotyped by PCR-RFLP and phenotyped for rib-eye area (REA), back-fat thickness (BT), intramuscular fat (IF), shear force (SF) and myofibrillar fragmentation index (MFI). The occurrence of the two alleles of the *CAST*/*Xmn*I and CAPN530 single nucleotide polymorphisms (SNPs) in a *B. indicus* breed, which permitted association studies in purebred and crossbred Nelore cattle, was first shown in the present work. No relationship was found between the *CAST* or *CAPN1* SNPs and growth-related traits (REA) or fat deposition (BT and IF), since calpastatin and μ-calpain are not physiologically involved with these traits. Moreover, the association results between genotypes and aged meat tenderness (assessed by SF and MFI) showed that these markers are useless in assisted selection for purebred Nelore and their crosses with *B. taurus*.

Index terms: *Bos indicus*, calpain gene, calpastatin gene, meat tenderness, single nucleotide polymorphisms.

Polimorfismos genéticos relacionados às características da carne em bovinos Nelore puros e cruzados

Resumo – O presente trabalho objetivou estimar, em bovinos de corte de diferentes grupos genéticos (Nelore e Nelore x *Bos taurus*), as frequências alélicas e genotípicas dos polimorfismos *CAST*/*Xmn*I, do gene da calpastatina, e CAPN530, do gene da calpaína, bem como avaliar a ocorrência de associações entre esses polimorfismos e características da carcaça e da carne produzida. Trezentos animais – 114 Nelore, 67 Angus x Nelore, 44 Rubia Galega x Nelore, 41 Canchim, 19 tricross Brangus e 15 tricross Braunvieh – foram genotipados por PCR-RFLP e fenotipados para área de olho de lombo (AOL), cobertura de gordura subcutânea (CGS), gordura intramuscular (GI), força de cisalhamento (FC) e índice de fragmentação miofibrilar (IFM). A ocorrência dos dois alelos dos polimorfismos de nucleotídeo único (SNPs) *CAST*/*Xmn*I e CAPN530 em animais *B. indicus*, que viabilizou estudos de associação em gado Nelore puro e cruzado, foi demonstrada pela primeira vez neste trabalho. Nenhuma relação foi encontrada entre os SNPs dos genes *CAST* e *CAPN1* e as características de crescimento (AOL) ou deposição de gordura (CGS e GI), já que a calpastatina e a μ-calpaína não estão fisiologicamente envolvidas com essas características. Os resultados de associação entre os genótipos e a maciez da carne maturada (avaliada por FC e IFM) mostraram a pouca utilidade desses marcadores na seleção de bovinos Nelore puros ou cruzados com *B. taurus*.

Termos para indexação: *Bos indicus*, gene da calpaína, gene da calpastatina, maciez da carne, polimorfismos de nucleotídeo único.

Introduction

Tenderness is very relevant to beef consumers and is, therefore, a trait of interest for animal selection. The breakdown of myofibrillar proteins controlled by the μ-calpain enzyme and modulated by its inhibitor, calpastatin, is the main mechanism of post-mortem meat tenderization (Delgado et al., 2001). When calpastatin activity increases, it reduces μ-calpain activity, with

a negative effect on meat tenderness (Pringle et al., 1997). Therefore, genes encoding calpastatin (*CAST*) and μ-calpain (*CAPN1*) are considered important functional candidates for meat tenderness in livestock.

The *CAST* gene was mapped to chromosome BTA7 (Bishop et al., 1993), and the *CAPN1* gene, to the telomeric end of the BTA29 chromosome, the same position verified for meat tenderness QTL (Smith et al., 2000). Chung et al. (2001a) detected genetic variants at intron 6 of bovine *CAST*, which can be identified by PCR-RFLP using the *Xmn*I restriction enzyme. They also found some evidence indicating that genotyping this polymorphism could help identify animals with distinct calpastatin enzymatic activity. Two non-synonymous single nucleotide polymorphisms (SNPs) – CAPN316 (located in exon 9) and CAPN530 (located in exon 14) in the gene *CAPN1* have been associated with meat traits in *Bos taurus* beef breeds (Page et al., 2002, 2004; Corva et al., 2007). Rincon & Medrano (2006) developed a PCR-RFLP method for genotyping the CAPN530 polymorphism using the *Psy*I enzyme. However, this marker failed to show segregation in *B. indicus* cattle of the Brahman breed (Casas et al., 2005), discouraging association studies between this SNP and meat quality in other zebu-influenced breeds.

According to Casas et al. (2005), polymorphisms that segregate and associate with traits of interest in *B. taurus* may or may not segregate in *B. indicus*. Thus, it is necessary to develop and use other markers in candidate genes or chromosomal regions, to allow association studies in *B. indicus*. It is also important to consider major variations in allelic frequencies between breeds, even within subspecies. Furthermore, association results for molecular markers and production traits obtained in *B. taurus* populations are not directly applicable to *B. indicus* populations, because allelic substitution effects are specific to each population and its environment.

The objective of this work was to estimate the allelic and genotypic frequencies of the *CAST*/*Xmn*I and CAPN530 polymorphisms in different beef genetic groups (Nelore and Nelore x *B. taurus*), as well as to determine the occurrence of associations between these polymorphisms and carcass and meat traits.

Materials and Methods

Samples of Nelore (*B. indicus*, n = 114), Angus x Nelore (1/2 *B. taurus* + 1/2 *B. indicus*, n = 67), Canchim (5/8 *B. taurus* + 3/8 *B. indicus*, n = 41), Brangus three-way cross (9/16 *B. taurus* + 7/16 *B. indicus*, n = 19) and Braunvieh three-way cross (3/4 *B. taurus* + 1/4 *B. indicus*, n = 15) were analyzed. These animals, originating from commercial herds of seven farms, were bred in 2003, 2005, 2006 and 2007 in the feedlot of the Faculdade de Medicina Veterinária e Zootecnia of the Universidade Estadual Paulista, Botucatu, SP, Brazil, using an intensive system denominated "superprecoce", previously detailed by Curi et al. (2005). The remaining 44 samples representing the cross between Rubia Gallega sires (*B. taurus*) and Nelore dams (Rubia Gallega x Nelore: 1/2 *B. taurus* + 1/2 *B. indicus*), were raised in a semi-intensive system during 2006. The genetic composition of each group was described by Curi et al. (2009). The 300 animals used in the trial, 32 females and 268 males, were bred according to the Brazilian legislation for animal well-being (protocol nº 89/2006-CEEA issued by the Comitê de Ética em Experimentação Animal – CEEA, of the Faculdade de Medicina Veterinária e Zootecnia of the Universidade Estadual Paulista, Botucatu, SP, Brazil) and slaughtered at 15, 17 and 19 months of age.

After slaughter in collaborating abattoirs, the carcasses were identified and chilled for 24 hours. Two 2.54-cm thick samples of the *longissimus dorsi* muscle were then removed from an area between the 11th and 13th ribs of the left half of each carcass.

Samples collected between the 12th and 13th ribs provided rib-eye area (REA), back-fat thickness (BT) and shear force (SF) measurements. Samples collected between the 11th and 12th ribs were used to measure the myofibrillar fragmentation index (MFI) and intramuscular fat (IF, percentage of total lipids), as well as for the extraction of genomic DNA. The REA was measured by the quadrant-point method and the BT was determined with the aid of a ruler, both according to the USDA Quality Grade protocol (United States Department of Agriculture, 1997). After these initial measurements, carried out in the slaughterhouse, the samples of *longissimus dorsi* were deboned, vacuum wrapped and aged under controlled temperature (between 1 and 2°C) for 14 days, followed by freezing at -20°C. The other phenotypic traits, SF, MFI and IF, were determined in the laboratory following methods described by Wheeler et al. (1995), Culler et al. (1978) and Bligh & Dyer (1959) respectively.

Genomic DNA was extracted from meat samples (250 mg) by the non-phenolic method, with digestion with proteinase K and precipitation with NaCl and alcohol (Sambroock et al., 1989).

CAST and *CAPN1* genotyping was done by the PCR-RFLP method. To determine the A and B alleles of the *CAST* SNP, a fragment of approximately 2,000 base pairs located at intron 6 was amplified with forward 5' – AGC AGC CAC CAT CAG AGA AA – 3' and reverse 5' – TCA GCT GGT TCG GCA GAT – 3' primers and digested by the *Xmn*I restriction enzyme (Chung et al., 2001a). To identify the A and G alleles of CAPN530 polymorphism, a fragment of 341 base pairs located at exon 14 was amplified with forward 5' – CGT TTC TTC TCA GAG AAG AGC GCA GG G A – 3' and reverse 5' – CCT GCG CCA TTA CTA TCG ATC GCA AAG T – 3' primers and digested by the *Psy*I restriction enzyme (Rincon & Medrano, 2006). After digestion of the amplification products, the DNA fragments of *CAST* and *CAPN1* genes were separated in agarose gel (2 and 3% respectively).

The individual genotypes were determined by DNA fragment size analysis (measured in base pairs, bp), by comparison with a standard molecular weight of 100 bp. The allelic and genotypic frequencies were calculated for each polymorphism according to Weir (1996). The differences in allele frequency within and between the genetic groups studied was calculated using contingency tables (Curi & Moraes, 1981) adapted from Goodman (1965).

For the association studies, the traits of interest were analyzed using the general linear model (GLM) of the Statistical Analysis System (SAS Institute, 2004) program, and the least square means of the genotypes were compared by the Tukey's test. The Bonferroni correction was applied to analyses involving multiple comparisons. The linear model for adjustment of quantitative variables included the genotype and the contemporary group effects as follows: $Y_{ijk} = \mu + G_i + CG_j + e_{ijk}$, where Y_{ijk} is the trait of interest, μ is the general mean, G_i is the fixed effect of i^{th} genotype (i = 1, ..., 3), CG_j is the fixed effect of j^{th} contemporary group (j = 1, ..., 13), and e_{ijk} is the random error. The definition of contemporary groups included variations of genetic group, sex, age of slaughter, feedlot year and farm of origin. These variations could not be considered separately in the model, since there is an important confounding among them. The bull effect was not included in the model, since the number of genotyped offspring of individual bulls was very small. Thus, due to the large number of parents, the possibility of confounding between the genotype and bull effects on the evaluated traits was diluted. The interactions between SNPs and the studied genetic groups were not significant, and were not included in the final analysis.

Results and Discussion

The *CAST*/*Xmn*I polymorphism segregated in the six genetic groups studied with a significantly higher frequency of the A allele in all analyses (Table 1). The A allele frequency was significantly higher in the Angus x Nelore and Rubia Gallega x Nelore groups than in the Nelore and Canchim groups. Brangus and Braunvieh three-way crosses had an intermediate A allele frequency in comparison to the groups that differed statistically. Homozygous AA followed by heterozygous AB were the most frequent genotypes in all but the Canchim group, in which the homozygous BB was the second most frequent genotype.

No previous studies with *B. indicus* were found to adequately compare the results for allelic frequencies

Table 1. Allelic and genotypic frequencies for the *CAST*/*Xmn*I polymorphism in the different genetic groups and in the total animal sample[1].

Genetic group[2]	Allelic frequency		Genotypic frequency		
	A	B	AA	AB	BB
Nelore (114)	0.636Ba	0.364Ab	0.438	0.395	0.167
Angus x Nelore (67)	0.873Aa	0.127Bb	0.746	0.254	0.000
Rubia Gallega x Nelore (44)	0.795Aa	0.205Bb	0.659	0.273	0.068
Canchim (41)	0.621Ba	0.379Ab	0.512	0.219	0.269
Brangus three-way cross (19)	0.790ABa	0.210ABb	0.632	0.316	0.052
Braunvieh three-way cross (15)	0.800ABa	0.200ABb	0.600	0.400	0.000
Total (300)	0.732	0.268	0.577	0.310	0.113

[1]Means followed by equal letters, uppercase among genetic groups and lowercase within genetic groups, do not differ by the Goodman test, at 5% probability. [2]The number between parentheses indicates the number of animals in each genetic group.

of *CAST*/*Xmn*I. Nevertheless, the present findings and the frequencies of 0.75 and 0.25 for alleles A and B, described in *B. taurus* animals of the Angus breed by Chung et al. (2001a), suggest that the A allele frequency is higher than the B allele frequency in *B. taurus* and in *B. indicus*. Consequently, the absence of differences in *CAST*/*Xmn*I allele frequencies between *B. indicus* and *B. taurus* suggests a lack of association between this polymorphism and meat tenderness, a trait that differs between the subspecies (Wheeler et al., 1994).

Results for association analyses between *CAST*/*Xmn*I genotypes and studied traits (Table 2) were not significant ($p>0.05$). In spite of the *CAST*/*Xmn*I being a SNP in a non-coding region, it could be linked or in linkage disequilibrium with causative polymorphisms associated to phenotype differences. Also, no codifying RNA transcript from intronic regions are involved in a number of biological processes, such as controlling transcriptional and post-transcriptional levels of gene expression (Nakaya et al., 2007). Thus, intronic polymorphisms have gained importance as possible causative SNP and are not to be overlooked. However, as in Chung et al. (2001b), the results of the present trial show no association between the *CAST*/*Xmn*I polymorphism and meat tenderness based on shear force or myofibrillar fragmentation index. Even with a small number of animals genotyped, 47 *B. taurus* animals of the Angus breed, Chung et al. (2001b) found that genotypes had significant effect on calpastatin activity, although they could not explain the observed variation in meat tenderness. Chung et al. (2001b) and the present work showed greater meat tenderness in animals with two copies of the A allele, that were, however, not statistically significant. AA animals had lower shear force and higher myofibrillar fragmentation index when comparing minimum square means. These results may be explained by the low *CAST*/*Xmn*I effect or by the weak disequilibrium linkage between this polymorphism and the causative mutation. Considering the size of the effect, the association between *CAST*/*Xmn*I and the evaluated traits may have been significant with more animals in the sample. The weak linkage disequilibrium hypothesis showed that other polymorphisms described in the *CAST* gene were closely related to variation in meat tenderness, namely the SNP A2959G (access number AF159246), at region 3' UTR, and G/C (nucleotide 282 of access number AY008267), at intron 5, identified by Barendse (2004) and Schenkel et al. (2006) respectively. Curi et al. (2008) reported the genotyping of A2959G (AF159246) *SNP* of bovine *CAST* gene by PCR–RFLP technique for the first time. The accuracy of the method was confirmed through the direct sequencing of PCR products of nine individuals. The lack of connection between allelic forms of the *CAST* gene and growth related traits (such as REA) and fat (BT and IF) was expected since, in theory, calpastatin is not involved in their physiology. However, it may be possible to find linkage disequilibrium between this marker and functional polymorphisms in surrounding genes, as well as the possibility of pleiotropic effects. Chung et al. (2001b) found significant associations between *CAST* polymorphisms and percentage of kidney, pelvic and heart fat, and Schenkel et al. (2006) associated the gene with carcass fat yield.

The CAPN530 polymorphism segregated in all genetic groups studied (Table 3). The A allele frequency was lower than the G allele frequency in all analyses. There were no significant differences in allelic frequencies between genetic groups. The AA genotype was less frequent in all groups. Higher GG frequency was prevalent in all but one group, the Rubia Gallega x Nelore, in which the AG genotype was most prominent. In contrast with the results presented by Casas et al. (2005), which showed absence of the A allele in a *B. indicus* population of the Brahman breed, this study revealed a 21.9% frequency in Nelore *B. indicus*. Thus, for the first time, it was possible to study the association between this polymorphism and traits of economical interest in *B. indicus* bovines. The

Table 2. Least square means and standard errors of the rib-eye area (REA), back-fat thickness (BT), intramuscular fat (IF), shear force (SF) and myofibrillar fragmentation index (MFI) determined for genotypes of the *CAST*/*Xmn*I polymorphism.

Genotype[(1)]	REA (cm^2)	BT (mm)	IF (%)	SF ($kgf\ cm^{-2}$)	MFI
AA (163)	72.36±0.75	4.00±0.12	1.47±0.07	3.54±0.06	75.52±1.48
AB (91)	70.52±0.92	3.89±0.45	1.47±0.09A	3.75±0.08A	64.94±1.81
BB (30)	71.22±1.56	4.11±0.25	1.35±0.15	3.91±0.13	70.93±3.04

[(1)]The numbers between parentheses indicate the number of animals from each genotype.

results presented here and elsewhere (Page et al., 2004; White et al., 2005; Corva et al., 2007) suggest that the A allele frequency of CAPN530 is lower then the G frequency in both *B. taurus* and *B. indicus* animals, and that the A allele occurrence is not extremely different between breeds. In two different *B. taurus* populations – a commercial herd, Simmental x Angus, and a research herd, GPE Cycle 7 –, Page et al. (2004) showed frequencies of 37 and 28% for the A allele. White et al. (2005) found a 14% frequency in a study with animals that combined *B. taurus* and *B. indicus* influence. Finally, Corva et al. (2007) described frequencies between 2 and 18% in four genetic groups formed by the crosses of Angus, Hereford and Limousin breeds (all *B. taurus*). Thus, like for *CAST*/*Xmn*I, it appears there is not great difference between *B. taurus* e *B. indicus* for the allele frequencies of the CAPN530, and this may indicate a lack of association between polymorphism and meat tenderness.

The analyses also showed no association ($p>0.05$) between genotypes of CAPN530 and phenotypes (Table 4). The CAPN530 polymorphism in the *CAPN1* gene is a guanine (G allele) to adenine (A allele) substitution that causes a valine to isoleucine change at codon 530, domain III of the μ-calpain enzyme. The alteration between two apolar aminoacids is relatively conservative, although it may change protein stability and assemblage, thus affecting proteolytic activity (Page et al., 2002). Page et al. (2002, 2004) and Corva et al. (2007) found evidence of the association between polymorphism and meat tenderness. Interestingly, while Page et al. (2002, 2004) report a favorable effect of the G allele, Corva et al. (2007) dispute the superior meat tenderness – smaller shear force values – of the AG genotype in comparison with the GG genotype. Analogous to conclusions reported by White et al. (2005), the present work failed to show association between genotypes of CAPN530 and meat tenderness. In addition to the environment-genotype interaction, which may produce divergent results even with direct markers within a breed, this disagreement can be due to differences: in disequilibrium or in the linkage phase between markers and quantitative trait locus (QTL); in epistatic interactions affecting the candidate gene; and in the magnitude of the effect of the candidate gene over the phenotype (Curi et al., 2005). These differences often underpin comparisons amongst populations and breeds, and in bovines they normally concur to the diversity of two subspecies. Hence, the contrast between the findings of Page et al. (2002, 2004), the present results and those of White et al. (2005) may be understood, as the first works mentioned only used

Table 3. Allelic and genotypic frequencies for the CAPN530 polymorphism in the different genetic groups and in the total animal sample[1].

Genetic group[2]	Allelic frequency		Genotypic frequency		
	A	G	AA	AG	GG
Nelore (114)	0.219Ab	0.781Aa	0.070	0.298	0.632
Angus x Nelore (67)	0.343Ab	0.657Aa	0.134	0.418	0.448
Rubia Gallega x Nelore (44)	0.329Ab	0.671Aa	0.091	0.477	0.432
Canchim (41)	0.219Ab	0.781Aa	0.121	0.195	0.684
Brangus three-way cross (19)	0.263Ab	0.737Aa	0.105	0.316	0.579
Braunvieh three-way cross (15)	0.367Aa	0.633Aa	0.200	0.333	0.467
Total (300)	0.273	0.727	0.103	0.340	0.557

[1]Means followed by equal letters, uppercase among genetic groups and lowercase within genetic groups, do not differ by the Goodman test, at 5% probability. [2]The number between parentheses indicates the number of animals in each genetic group.

Table 4. Least square means and standard errors of the rib-eye area (REA), back-fat thickness (BT), intramuscular fat (IF), shear force (SF) and myofibrillar fragmentation index (MFI) determined for genotypes of the CAPN530 polymorphism.

Genotype[1]	REA (cm^2)	BT (mm)	IF (%)	SF ($kgf\ cm^{-2}$)	MFI
AA(30)	73.43±1.59	3.74±0.26A	1.31±0.16	3.74±0.14	69.17±3.14
AG (99)	70.42±0.93	4.12±0.15	1.58±0.09	3.65±0.08	71.84±1.82
GG (158)	71.91±0.72	3.94±0.12	1.42±0.07	3.65±0.06	74.07±1.41

[1]The number between parentheses indicates the number of animals from each genotype.

B. taurus animals, while the last two also included *B. indicus* animals. Overall, these results indicate that the CAPN530 SNP is not a functional marker for variation in beef tenderness. Also, in *B. indicus* populations it is not linked or in strong linkage disequilibrium with the causative polymorphism, which confirms that polymorphism is useless in marker-assisted selection for beef herds with a broad genetic background. However, a relatively novel *CAPN1* polymorphism, the CAPN4751 SNP, has shown great potential as a marker for meat tenderness selection in *B. taurus*, *B. indicus* and *B. indicus* x *B. taurus* beef herds (White et al., 2005; Van Eenennaam et al., 2007). As expected, no relation between allelic forms of the *CAPN1* gene and the growth and fat traits (REA, BT and IF) was found. On the other hand, with the possibility of linkage disequilibrium between the *CAPN1* and surrounding genes or of pleiotropic effects, positive associations between variants of the gene and carcass traits were described (Juszczuk-Kubiak et al., 2004; Casas et al., 2005; Cheong et al., 2008).

Conclusions

1. Despite novel demonstration of the occurrence of the two alleles of *CAS*T/*Xmn*I and CAPN30 polymorphisms in *Bos indicus* animals, there is no association between genotypes of these markers and meat tenderness.

2. The analyzed polymorphisms are useless in marker-assisted selection programs for Nelore and their crosses with *Bos taurus*.

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