

Polymorphisms in candidate genes and their association with carcass traits and meat quality in Nellore cattle

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Abstract – The objective of this work was to estimate the allele polymorphism frequencies of genes in Nellore cattle and associate them with meat quality and carcass traits. Six hundred males were genotyped for the following polymorphisms: DGAT1 (VNTR with 18 nucleotides at the promoter region); ANK1, a new polymorphism, identified and mapped here at the gene regulatory region NW_001494427.3; TCAP (AY428575.1:g.346G>A); and MYOG (NW_001501985:g.511G>C). In the association study, phenotype data of hot carcass weight, ribeye area, backfat thickness, percentage of intramuscular fat, shear force, myofibrillar fragmentation index, meat color (L*, a*, b*), and cooking losses were used. Allele B from the *ANK1* gene was associated with greater redness (a*). Alleles 5R, 6R, and 7R from the *DGAT1* VNTR gene were associated with increased intramuscular fat, reduced cooking losses and increased ribeye area, respectively. The single nucleotide polymorphism (SNP) of the *TCAP* gene was not polymorphic, and MYOG alleles were not associated with any of the evaluated characteristics. These results indicate that *ANK1* and *DGAT1* genes can be used in the selection of Nellore cattle for carcass and meat quality.

Index terms: *Bos indicus*, ankyrin, assisted selection, beef cattle, myogenin, titin.

Polimorfismos em genes-candidatos e suas associações com características de carcaça e qualidade da carne em bovinos Nelore

Resumo – O objetivo deste trabalho foi estimar as frequências de polimorfismos alélicos em genes de bovinos Nelore e associá-los às características de carcaça e qualidade da carne. Seiscentos machos foram genotipados quanto aos seguintes polimorfismos: DGAT1 (VNTR com 18 nucleotídeos na região promotora); ANK1, novo polimorfismo, identificado no presente estudo e mapeado na região gênica regulatória NW_001494427.3; TCAP (AY428575.1:g.346G>A); e MYOG (NW_001501985:g.511G>C). No estudo de associação, foram utilizados os dados fenotípicos de massa da carcaça quente, área de olho do lombo, espessura de gordura subcutânea, percentagem de gordura intramuscular, força de cisalhamento, índice de fragmentação miofibrilar, coloração da carne (L*, a*, b*) e perdas por cocção. O alelo B do gene *ANK1* foi associado ao aumento da coloração vermelha (a*) da carne. No gene *DGAT1*, os alelos 5R, 6R e 7R foram associados ao aumento de gordura intramuscular, à redução das perdas por cocção e ao aumento da área de olho de lombo, respectivamente. O SNP (polimorfismo de nucleotídeo único) do gene *TCAP* não apresentou polimorfismo, e os alelos do gene *MYOG* não foram associados a nenhuma das características avaliadas. Os resultados indicam que os genes *ANK1* e *DGAT1* podem ser utilizados na seleção de animais Nelore quanto à qualidade de carne e carcaça.

Termos para indexação: *Bos indicus*, anquirina, seleção assistida, bovino de corte, miogenina, titina.

Introduction

Visual, sensory, and nutritional attributes of meat, as well as food safety and animal welfare define meat quality. These features, desired and valued by consumers, influence consumer choice at the time of

purchase and should be the target of breeding programs in beef cattle.

Meat quality, like most economically important traits in livestock, is complex, polygenic and controlled by an unknown number of quantitative trait loci (QTL) (Grisart et al., 2002). The candidate gene approach

can be used when the gene is located in a region that hosts a QTL or when there is prior information on gene effect on a trait (Te Pas & Soumillion, 2001).

The *DGATI* gene encodes the protein acyl CoA:diacylglycerol acyltransferase (DGAT) which catalyses the final step in triacylglycerol synthesis (Cases et al., 1998; Grisart et al., 2002). This enzyme may also be involved in the synthesis and storage of lipids (Cases et al., 1998). Two markers of this gene have been associated with variation in milk and meat fat content: lysine to alanine substitution at position 232 (K232A) and a variable number of tandem repetitions (VNTR) at the promoter region. The VNTR of the *DGATI* gene comprises repetitions of an 18-nucleotide sequence, that is rich in guanine and cytosine, and is a target for transcription factor Sp1 binding (Fürbass et al., 2006). Due to the high content of C and G, this fragment has also the potential to set CpG islands, which are important epigenetic modifications for transcriptional regulation (Fan & Zhang, 2009).

Titin is a huge protein that links the Z line to myosin in the sarcomere (Warriss, 2000). It is broken down during the postmortem period (Warriss, 2000) and is associated with tenderization 24-48 hours after slaughter (Hopkins & Taylor, 2004). The titin-cap (*TCAP*) gene encodes telethonin, a protein that interacts with titin at the Z line (Cheong et al., 2007). Associations between single nucleotide polymorphisms (SNPs) of the *TCAP* gene and marbling in *Bos taurus* showed that the guanine to adenine substitution reduced the marbling score (Cheong et al., 2007).

The *MYOD* family consists of a gene group (*MYOD1* or *MYF3*; *MYOG*, myogenin or *MYF4*; *MYF5*; and *MYF6* or herculin) involved in regulating muscular hypertrophy and hyperplasia (Houba & Te Pas, 2004; Bhuiyan et al., 2009). *MYOG* is expressed before birth, during the differentiation/fusion of myoblasts, into multinucleated myofibrils (Houba & Te Pas, 2004). In the postnatal period, it can be associated with repair to damage in muscle fibers and hypertrophic growth (Te Pas & Soumillion, 2001). In pigs, this gene has been associated with an increase in the number and cross-sectional area of muscle fibers (Kim et al., 2009), birth and carcass weight, and growth rate (Te Pas et al., 1999). However, in *B. taurus* cattle, the effect was evident only on the water-holding capacity and tenderness of meat (Ujan et al., 2011).

Ankyrins are proteins involved in interactions between the plasmatic membrane and cytoskeleton (Bagnato et al., 2003). *ANK1*, *ANK2*, and *ANK3* encode ankyrins expressed in specific tissues (Rubtsov & Lopina, 2000). Alternative splicing of *ANK1* produces ankyrin R that links sarcoplasmic reticulum to the M and Z lines of sarcomere in muscle fibers (Rubtsov & Lopina, 2000; Bagnato et al., 2003). This ankyrin has a domain that is sensitive to proteolysis, and may be the target of calpain, which could affect meat tenderness (Rubtsov & Lopina, 2000).

Regarding the four mentioned genes, there is little or no information concerning the frequency of polymorphisms in *B. indicus* cattle. Due to differences in ancestry between *B. taurus* and *B. indicus*, it is important to determine whether markers identified in taurine can be used in Zebu cattle (Casas et al., 2005). These results can be used to implement marker-assisted selection, from which it is possible to predict animals which could provide better performance and accelerate genetic gain in the herd.

The objective of this work was to estimate the allele polymorphism frequencies of genes in Nellore cattle and associate them with meat quality and carcass traits.

Materials and Methods

Six hundred Nellore bulls (*B. indicus*), with a mean age of 24 months, that were feedlot for 90 to 100 days were used. Animals were part of the Delta G Connection breeding program which provided data management, performance records, and animal's pedigree. Slaughter occurred in a commercial abattoir, between 2010 and 2011 in different locations, where carcasses were weighed while hot (HCW), identified and cooled for a period of 24 to 48 hours. Next, samples were taken from the *longissimus dorsi* muscle, between the 9th and 13th ribs of the left half of carcasses.

Samples collected between the 12th and 13th ribs were used to measure the ribeye area, backfat thickness, shear force, cooking losses (losses), and meat color (L*, lightness; a*, redness; b*, yellowness), using *CIELab* system of the Chroma Meter CR-400 (Konica Minolta Sensing, Inc., Tokyo, Japan). The remaining meat was used to analyze the myofibrillar fragmentation index, percentage of intramuscular fat, and DNA extraction. Ribeye area was determined by the quadrant method and backfat thickness with a caliper (in mm); both

measurements were performed in accordance with USDA standard methods (United States Department of Agriculture, 2000). Cooking losses were determined by the weight difference between raw and cooked meat. Other phenotypic measurements (shear force, myofibrillar fragmentation index, intramuscular fat, L *, a *, b *) were determined according to methods described by Wheeler et al. (1995), Culler et al. (1978), Bligh & Dyer (1959) and Renner (1982), respectively.

DNA was extracted from meat in the Laboratory of Molecular Genetics, Faculdade de Ciências Agrárias e Veterinárias (Unesp), Jaboticabal, SP, Brazil, using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA), according to the protocol for purification of total DNA from animal tissues provided by the manufacturer.

PCR reactions were performed using the enzymes Taq DNA polymerase (Invitrogen, Grand Island, NY, USA) for *MYOG* and *TCAP*, and GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) for *DGATI* and *ANKI*, in accordance with the manufacturers' recommendations. The nucleotide sequence of the four pairs of primers (Bioneer, Daejeon, Korea) was obtained from the literature (Table 1). Amplification was performed in a MyCycler thermocycler (Biorad, Hercules, CA, USA) with an initial denaturation at 95°C for 5 min, followed by 35 to 40 cycles at 95°C for 1 min (Table 1), 30 to 45 s at a specific annealing temperature for each polymorphism, 72°C for 1 min, and a final extension step at 72°C for 5 min. Fragment sizes of each polymorphism resulting from PCR are described in Table 1.

Except for *DGATI*, which was genotyped only by PCR products length analysis (VNTR allele size discrimination), the resulting PCR fragments of *ANKI*, *MYOG*, and *TCAP* were digested with the restriction

enzymes (New England Biolabs, Ipswich, MA, USA) *MspI*, *NgoMIV*, *BtsCI*, respectively (PCR-RFLP technique). The incubation time and temperature of each enzyme were: 37°C for 12 hours, for *MspI*; 37°C for 16 hours, for *NgoMIV*; and 50°C for 12 hours, for *BtsCI*. Digestion products were dyed with ethidium bromide and were subjected to electrophoresis in 2% agarose gel for *MYOG*, and 3% for *ANKI* and *TCAP*. The PCR fragments of *DGATI* were separated in high-resolution 4% agarose gel. A standard molecular weight of 100 bp was added to each gel, to indicate the size of the produced fragments. Individual genotypes for each polymorphism were determined by size analysis of the identified fragments in gel.

From the identified genotypes in gel, allelic and genotypic frequencies for each polymorphism were calculated. Analyses were performed using the GLM procedure of the SAS software. The correction of hypothesis tests for multiple comparisons was done by the Bonferroni method. The allele substitution effect of polymorphisms on the traits of interest was determined by regression of the number of allele copies. The used model was: $Y_{ijk} = \mu + CG_i + b_1 \times n_{jk} + b_2 \times AS_j + \epsilon_{ijk}$, in which: Y_{ijk} is the trait of interest; μ is the mean; CG_i is the fixed effect of the i^{th} contemporary group ($i = 1, \dots, 45$); b_1 represents the regression coefficient for the covariate allele numbers; n_{jk} are the allele numbers of the genotype of the j^{th} animal; b_2 is the regression coefficient for the covariate age at slaughter; AS_j is the age at slaughter of j^{th} animal; and ϵ_{ijk} is the random error.

To avoid dependencies on the polymorphisms of *MYOG* and *ANKI*, regression was performed only on B and C alleles, respectively. For the VNTR of *DGATI*, with multiple alleles and very low frequency of 4R and 8R alleles, dependency was avoided by

Table 1. Forward (F) and reverse (R) primers and their references, annealing temperatures and time (ATT), number of cycles, and fragment size (FS) used to amplify *TCAP*, *MYOG*, *ANKI*, and *DGATI* genes in cattle.

Gene (access number)	Primer sequence (5' to 3')	Reference	ATT	N° of cycles	FS (bp)
<i>TCAP</i> (AY428575.1)	F: GGGAGTGAGCAGTCATCATGGC R: AGAGGCAGCACCCGCTGGT	Cheong et al. (2007)	60°C/45s	36	517
<i>MYOG</i> (NW_001501985.2)	F: CTGAGAGAGAAGCGCACACT R: CTGGCTCAACTAGACCGACT	Bhuiyan et al. (2009)	56°C/30s	38	680
<i>ANKI</i> (NW_001494427.3)	F: GGTGTTTTGCTTCTCCAGC R: CTGAGTGACTGAGCACTGTAATGA	Aslan et al. (2010)	52°C/30s	35	701
<i>DGATI</i> (AJ318490.1)	F: TCAGGATCCAGAGGTACCAG R: CAATGAGAAGGCACGGACTGTGAA	Gautier et al. (2007)	58°C/30s	40	210, 228, 246, 264, and 282

considering the mean effects of 4R and 8R together as zero. In the *DGATI* analysis, the total reduction in the sum of squares caused by the effect of three alleles in the model was computed to obtain the nominal p-value for the locus.

The contemporary group was formed considering management (year of birth, and animal management at birth, weaning, and yearling) and slaughter groups. The term slaughter group means all bovine slaughtered on the same day and in the same abattoir. Groups with less than three animals were excluded from the analysis. Bull effect was not included in the model because the offspring number of the same bull was very small, so the possibility of bias the allele effect with the bull effect was minimized.

CpG islands of *DGATI* were identified using the MethPrimer program (Urogene, 2013). Criteria used were: size greater than 100 base pair (bp); GC content greater than 50%; and observed/expected GC ratio greater than 0.6. Sequences with four to eight repetitions of VNTR were evaluated.

Results and Discussion

The contemporary group formation reduced the number of measurements of all evaluated traits; and about 100 animals depended on these traits (Table 2). For *TCAP*, the *BtsCI* enzyme cleaved fragments of 177, 154, 128, and 58 bp, for allele A, and fragments of 305, 154, and 58 bp, for allele G. Digestion with the *NgoMIV* enzyme for *MYOG* resulted in two fragments of 476 and 204 bp for allele C and an undigested fragment of 680 bp for allele G.

Table 2. Descriptive statistics for meat quality traits.

Trait	Number of measurements	Mean	Coefficient of variation
Hot carcass weight (kg)	509	268.40	8.0
Cooking losses (%)	516	23.62	14.7
Ribeye area (cm ²)	515	65.62	11.8
Backfat thickness (mm)	515	5.20	45.5
Shear force (kg)	515	4.77	27.1
L*: lightness	516	33.24	17.1
a*: redness	514	18.34	21.4
b*: yellowness	514	10.13	30.6
Myofibrillar fragmentation index	503	24.81	35.2
Intramuscular fat (%)	501	0.757	37.5

For *ANK1*, SNP number four (rs135054140) was expected to be found, as described by Aslan et al. (2010). However, the PCR-RFLP revealed a new SNP, before the one identified by these authors (Figure 1). When the *MspI* enzyme produced fragments of 436 and 265 bp, the allele was named A; in the other allele, named B, three fragments were seen, one with 436 bp and two others with unknown size. Based on the knowledge of nucleotide sequences and on the enzyme recognition site (5' C[▼]CGG 3'), it is possible to infer that the fragment lengths are most likely 112 and 153 bp, 121 and 144 bp, or 151 and 114 bp. However, the exact location of this polymorphism has yet to be confirmed by sequencing.

DGATI gene polymorphism was characterized by the presence of five alleles (4R, 5R, 6R, 7R and 8R) with 210, 228, 246, 264 and 282 bp, respectively. According to Gautier et al. (2007), 228 bp alleles of this gene have five repetitions of the VNTR sequence with 18 nucleotides. Thus, alleles with four to eight repetitions were verified. The observed allele frequencies were: 0.039, 4R allele; 0.431, 5R allele; 0.455, 6R allele; 0.073, 7R allele; and 0.004, 8R allele. As previously explained, for the purposes of statistical analysis, the effect of less frequent alleles 4R and 8R were set at zero.

Allelic and genotypic frequency estimates showed that the *TCAP* gene was not polymorphic (frequency <1%) for mutation g.346G>A in *B. indicus* cattle (Table 3). Therefore, no association study was performed for this gene.

The replacement of one random allele by alleles 5R, 6R, and 7R of *DGATI* influenced the percentage of intramuscular fat (increased), cooking losses (decreased), and ribeye area (increased), respectively. A to B substitution in *ANK1* was associated with greater redness. No association was found between G to C substitution on any of the evaluated traits in *MYOG* (Table 4).

The 5R allele of *DGATI* increased the percentage of intramuscular fat. This effect was expected, since the gene encodes the DGAT enzyme (Cases et al., 1998; Grisart et al., 2002), and VNTR may be involved in the transcription of this gene (Kühn et al., 2004). However, the results do not yet indicate how many VNTR repetitions would favorably increase the synthesis of triglycerides. Other studies have determined positive associations for seven (Kühn et al., 2004) and six repetitions (Curi et al., 2011).

The VNTR of *DGATI* even seems to be involved in gene transcription regulation through the constitution of CpG islands. This is a genomic region with size larger than 200 bp, C+G content greater than 50%, and an observed/expected CpG ratio greater than 0.6 (Fan & Zhang, 2009). Gene promoters are usually associated with CpG islands, a fact that is related to the strong correlation between them and transcription initiation (Deaton & Bird, 2011). The results of CpG island identification in the *DGATI* varied according to the number of VNTR repetitions. With four and five repetitions (equal to alleles 4R and 5R, respectively), no CpG island was identified. However, with six to eight repetitions, the program recognized the presence of a CpG island. The region becomes a CpG island after a certain number of polymorphism repetitions is achieved, and this could influence the regulation of *DGATI* and the observed phenotype. Whereas 5R allele is associated with percentage of intramuscular fat and it is in the transition point to a CpG island setting, the CpG island could be the result of downregulated gene expression. However, more studies with methylation and gene expression are required to confirm this hypothesis.

It is possible that *DGATI* is related to fat deposition in meat and milk, but through different mechanisms. While the lysine allele of K232A polymorphism of *DGATI* was strongly associated with increased fat content in milk (Grisart et al., 2002; Sanders et al., 2006; Gautier et al., 2007), the same effect was not determined for marbling and fat thickness (Casas et al., 2005; Souza et al., 2010). However, the VNTR of *DGATI* gene, which was associated with intramuscular fat (in this study) and with backfat thickness (Curi et al., 2011), showed little (Gautier et al., 2007) or no effect (Sanders et al., 2006) on milk fat. These findings indicate that the K232A polymorphism is associated with fat in milk, and the VNTR with fat in meat. Further evidence of this hypothesis is the fact that the lysine allele of polymorphism K232A is almost fixed in Zebu cattle population (Souza et al., 2010), even though it has high fat content in milk and low marbling compared with taurine.

Selecting cattle for increased intramuscular fat content can improve juiciness, flavor and tenderness of meat. Fat accounts for 3–10% of the variation in these traits (Nishimura et al., 1999). The influence on juiciness and flavor directly improves meat flavor after

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GGTGTTTTGCTTCTCCAGCTGTATTGACGCTACTGGGTTCTGTCCTGCCTGAGAAAAGTCTCTCCAGCC
TCTCTCCTGGCCCCCTTGGCCACCCTCTGCACTGAGGCTGGTCCAGGGGCCCCGATGGGCAGCCATCTGG
CCTCAGCTTTTCCGCAAGATCCAAGCGGGCCCTTCTGTGAGGCTACAGCTGTGGTAAAAACCACCACCAA
GGGCCGCTCCGTGACACTCTGTTCCACAGCAGCTGGAAACCCCAAGCTGAGGGGCCSGGCCATCCCCA
GCAGACCAGCACTGAGGCGGTGGGGAGGGCAAACAGGTTCCAGGGCACCTTTAGGCTAAAAATACCC
ACTATAAGTATCATCAAACCAGCAATGTTAATAAAGGAAATCAGTCCTGATTGTTTCATTGGAAGGACT
GATGCTGCAGATGAAGCTCCAATCCTTGGCCACCTGATGTGAAGAACTGATTCATTGGAAAAGACCCT
GATGCTGGGAAAGACTGAAGGCAGGAGGA GAAGGGGACGACAAAGGATGAGATGGTTGGATGGCATC
ACCAACTCAGTGGACATGAGTCTGAGTAAACTCTGGGAGTTGGGGATGGACAGGGAGGCCTGGCGTAC
TGTGGTCCATGGGGTCACAAAGAGTCGGACATGACTTAGTGACTGAACAACAAGAATGA TCATTACAG
TGCTCAGTCACTCAG
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Figure 1. Partial nucleotide sequence in a regulatory region of the *ANK1* gene (gi: 355475919). Based on the fragment size produced by PCR-RFLP technique, the new polymorphism can be located at position -534 (replacement of G to A in bold line), -544 (replacement of G to A in dashed line), or -574 (replacement of C to G in dotted line), related to the transcription start site described by Aslan et al. (2010). “S” in bold represents the SNP4 (rs135054140) described by these authors. PCR primer locations are highlighted in gray.

cooking and the saliva flow in the mouth (Warriss, 2000). Softness improvement would be indirect through disjunction and disorganization of intramuscular connective tissue, which allows of separation of myofibril connections (Nishimura et al., 1999; Warriss, 2000). However, according to Nishimura et al. (1999), more than 8% of intramuscular fat is necessary to affect tenderness.

The 6R allele of *DGATI* had a negative effect on cooking losses, while 7R had a positive effect on ribeye area. In principle, these findings are not directly explained by the physiological mechanisms related to *DGATI*, which is involved in the synthesis of triacylglycerol. However, these polymorphisms may be in linkage disequilibrium with another gene that influences these traits. Although there are no reports on the association of the polymorphisms K232A (Casas et al., 2005; Souza et al., 2010) and VNTR (Curi et al., 2011) of *DGATI* with ribeye area or cooking losses, these two associations are beneficial for meat technology and livestock. Loss reduction due to cooking prevents meat dryness, while the increase of the ribeye area, which is a measure of muscularity, can raise the yield of meat cuts.

The B allele of *ANKI* was associated with increased meat redness. This association was not expected, due to the biological function of the gene product. This

effect may be related to linkage disequilibrium of this polymorphism with some polymorphism from another gene on the same chromosome. Previous studies have linked SNPs in the *ANKI* promoter region to tenderness and intramuscular fat in *B. taurus* (Aslan et al., 2010). In pigs, this gene was associated with drip loss and intramuscular fat (Aslan et al., 2012), but this is the first report of an association with meat color in cattle.

Meat color is one of the aspects which most influence consumer choice (Nam et al., 2009), and a strong red color is considered more desirable. It is related to a higher proportion of type I muscle fibers (Gentry et al., 2004), which have oxidative metabolism and a greater concentration of myoglobin than fibers of glycolytic metabolism, type II. Type I fibers are also positively correlated with greater acceptance of meat color (Nam et al., 2009). However, variation in the values of redness, even if significant, cannot have a direct influence on product acceptability (Nam et al., 2009) because the result could be outside the range of human perception, and choice is a multifaceted process involving numerous subjective elements.

In general, the current work results highlight the importance of developing appropriate molecular markers for the selection of *B. indicus*.

Conclusion

The genes *DGATI* and *ANKI* can be used in the selection of Nellore cattle regarding carcass and meat quality, after validation studies with independent populations.

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Table 3. Allele and genotype frequencies of the *ANKI*, *MYOG*, and *TCAP* genes.

Allele	Allele frequency		Genotype frequency		
<i>ANKI</i>	0.43 (A)	0.57 (B)	0.20 (AA)	0.47 (AB)	0.33 (BB)
<i>MYOG</i>	0.54 (C)	0.46 (G)	0.30 (CC)	0.47 (GC)	0.23 (GG)
<i>TCAP</i>	0.004(A)	0.996 (G)	0.0 (AA)	0.008 (AG)	0.992 (GG)

Table 4. Allele substitution effect (\pm standard error) of alleles 5R, 6R and 7R of the *DGATI* gene, B allele of the *ANKI* gene, and C allele of the *MYOG* gene on the percentage of intramuscular fat, ribeye area, cooking losses, and redness of meat (a).

Gene	Intramuscular fat (%)	Ribeye area	Cooking losses	a
<i>DGATI</i>				
Allele 5R	0.078 \pm 0.04*	0.534 \pm 1.00	-0.892 \pm 0.50	0.172 \pm 0.51
Allele 6R	0.071 \pm 0.04	1.317 \pm 1.00	-0.989 \pm 0.50*	0.264 \pm 0.51
Allele 7R	0.051 \pm 0.05	2.711 \pm 1.22*	-0.279 \pm 0.61	-0.358 \pm 0.62
<i>ANKI</i>	-0.016 \pm 0.02	-0.116 \pm 0.48	0.293 \pm 0.23	0.516 \pm 0.24*
<i>MYOG</i>	-0.006 \pm 0.02	-0.585 \pm 0.46	0.108 \pm 0.22	0.335 \pm 0.23

*Significant at 5% probability.

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