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HSF1 and HSPA6 as functional candidate genes associated with heat tolerance in Angus cattle

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ABSTRACT - The purpose of this study was to access and characterize single nucleotide polymorphisms (SNP) located within the HSF1 and HSPA6 candidate genes for adaptability in Angus breed raised in subtropical climate. Samples of DNA from 20 animals representing extreme phenotypes for adaptability traits were obtained. Sequence variations in the candidate genes were described by sequencing target regions. We identified 12 SNP located in the HSF1 gene. Moreover, four of the six SNP found in the HSPA6 gene cause amino acid substitutions in protein-coding regions. We also identified a representative SNP (called tag SNP) in a region of the HSF1 gene with high linkage disequilibrium ($r^2 = 0.87$) that may represent 11 SNP located in this gene. Minor allele frequency observed for the SNP ranged from 0.10 to 0.50 and 0.02 to 0.21 for the HSF1 and the HSPA6 genes, respectively. Overall, almost all SNP analyzed showed significant deviation from the Hardy-Weinberg Equilibrium and half of the loci had heterozygosity greater than 0.50. The data suggest that there is sequence variability in these genes that could be exploited by breeding programs. There is genetic variation in HSF1 and HSPA6 genes in this populations of Angus breed, which is fundamental to obtain response to selection.

Key Words: Angus, molecular markers, thermoregulation

Introduction

According to the Instituto Brasileiro de Geografia e Estatística (IBGE, 2014), Brazil has the largest commercial cattle herd in the world with about 212 million animals, which places the country in the leading position as a beef exporter (ABIEC, 2014).

There is great concern about the thermal comfort of animals in Brazilian farming systems, due to the tropical climate of the country, characterized by high temperatures and solar radiation that can cause heat stress and affect the productive performance of the animals.

Heat stress provokes sorts of complex responses which are essential in the preservation of cell survival. In mammals, exposure to hypothermia or hyperthermia has been related to morphological and physiological alterations.

Bos indicus breeds are adapted to tropical environmental conditions, owing to their long-time adaptation with tropical climates in relation to taurine breeds; however, the meat from these animals have lower quality, particularly with regard to tenderness. The adverse effects of heat stress also affect the feed intake, milk production, growth rate, and reproduction which are lower in *B. indicus* than in *B. taurus* breeds (Gaughan et al., 2012).

To establish an economically viable farming system, it is necessary to look for breeds or individuals that have better genetic potential for adaptation to local environmental conditions and at the same time provide better quality beef.

The development of a breed of cattle that can tolerate heat stress and maintain productivity has been a long-term goal of researchers and cattle breeders. However, isolation of differences in thermoregulatory ability is challenging due to the many physical and genetic differences among bovines (Scharf et al., 2010).

Advances in molecular genetics have allowed the identification of genes and molecular markers related to adaptability, for example single nucleotide polymorphisms (SNP), that may be applicable in marker-assisted selection. Thus, to minimize problems related to heat stress, one

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strategy is to select animals with superior genetic potential for adaptation to tropical climates.

Some candidate genes have been indicated as associated with heat tolerance in cattle, including heat shock transcription factor 1 (HSF1) and heat shock 70kDa protein 6 (HSPA6) genes mapped to bovine chromosomes 14 and 3 (Zimin et al., 2009), respectively. These genes encode for heat shock proteins (HSP) that are involved in the reduction of cell damage caused by heat stress. The HSP are activated in response to stressors such as heat, physical strain, oxidative stress, among other factors. These proteins are released intracellularly and extracellularly in an inducible form in response to stress (Hecker and McGarvey, 2011).

Under stressful conditions, such as temperature rise, HSP levels are high, aiding the synthesis and maturation of new proteins that will replace those affected by stress. Heat shock proteins also provide a subsidy for cells to identify and facilitate the refolding of damaged proteins or to target them to a suitable proteolytic system, facilitating the elimination of proteins whose damage is not amenable to restoration. The increase of HSP in the damaged cells, besides helping in the repair of proteins, plays an important role in the maintenance of the viability, since it inhibits the apoptosis (Deb et al., 2014).

Among these proteins, the family HSP70 has a significant role in cell thermotolerance (Beckham et al., 2004) and animal survival (King et al., 2002). This family is the most conserved phylogenetically and of major importance for the folding of proteins in the cells. Therefore, it is known as the protein with the highest performance in response to cellular stress, being used in many studies as a stress indicator (Cole and Meyers, 2011; Monzo et al., 2012).

This study aimed to resequence the candidate genes HSF1 and HSPA6 to identify and characterize SNP related to adaptability in Angus cattle raised in a subtropical climate.

Material and Methods

The animals used in this study came from a farm under the ownership of Casa Branca Agropastoril Ltda., in the municipality of Silvianópolis, MG, Brazil. This region is at latitude 22°01'46" S, longitude 45°50'06" W, and altitude of 897 m and it is characterized by a wet subtropical climate with mean annual temperature of 19.9 °C.

Seventy-two Angus bulls of 15 months of age were raised in feedlot system, where they received water and commercial ration in covered trough and had access to shade.

The animals were herded into a cattle handling corral 12 h before the beginning of measurements of characteristics. In this period, the animals remained without access to shade, water, and food. They were taken to a covered trunk, one by one, to remain under the sun as long as possible before measurements.

Adaptability measures, respiratory rate (RR), and coat temperature (CT) were collected once a month for five consecutive months, from July to November 2012 at two different times: 7:00 and 13:00 h.

The respiratory rate was obtained by counting twice the number of breaths in the flank region in a period of 15 s and the average of these values was multiplied by four to obtain the number of breaths per minute. The coat temperature was measured in °C by a digital infrared thermometer targeted 20 cm below the vertebral column of the animal (Silva, 2000; Eustáquio Filho et al., 2011).

From a total of 72 animals, 20 presenting extreme phenotypes for adaptability traits such as RR and CT were selected for the target-resequencing and for identification of molecular markers.

The averages of the respiratory rate and the early afternoon coat temperatures were obtained to calculate an index, in which each trait corresponded to 0.50 of the index. This index was generated for each animal containing the two traits (RR and CT).

Animals were considered extreme "negative" if they were less tolerant to heat and "positive" if they were more tolerant to heat, based on the classification of the index obtained. Therefore, the ten highest values were classified as negative and the ten smallest values as extreme positive phenotypes. The phenotype information was used to perform a Fisher's exact test to determine if allele frequencies differed among extreme animals.

Genomic DNA was extracted from blood samples (5 mL) as described in Regitano and Coutinho (2001). Concentration of DNA was measured by spectrophotometry and the quality was verified by the 260:280 ratio. The DNA integrity was assessed by electrophoresis in agarose gel.

FASTA sequences of HSF1(Gene ID: 506235) and HSPA6 (Gene ID: 539835) genes were obtained from the public database National Center for Biotechnology Information (NCBI). Primers were designed for target regions using Primer 3 Plus software, available online (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Sequence variations in the candidate genes were described by sequencing target regions.

Forward (F) and reverse (R) primers were designed for the amplification of HSF1 and HSPA6 genes (Table 1), as well as their amplicon size (pb) and annealing temperatures (°C).

Reactions to amplify all target regions were carried out with final volume of 20 µL: 80ng of the DNA template, 0.165 µM of each primer, 1.5 mM of the reaction buffer with $MgCl_2$, 0.2 mM of each dNTP, and 0.650 unit of the Taq DNA polymerase. Polymerase chain reactions (PCR) were performed in a thermocycler model T100 (Bio-Rad) using a standard PCR program. The denaturation step of 95 ºC for 3 min was followed by 35 cycles at 94 ºC for 40 s, annealing temperature according to each primer pair for 30 s, and final extension of 72 ºC for 50 s. Finally, PCR products were visualized in agarose gel and then purified using a ExoSAP-IT enzyme following manufacturer's protocol.

The sequencing reactions were performed as described by Tizioto et al. (2013) using the ABI Prism BigDye Terminator v. 3.1 cycle sequencing kit. To avoid any potential contamination, sequencing products were purified using isopropanol and sodium acetate. The sequences were processed in an ABI Prism 3100 Avant.

The PhredPhrapConsed package (Ewing and Green, 1998) was used to identify SNP in the extreme-phenotype animals sequenced. The SNP locations were determined by mapping sequences to the FASTA files of the gene.

We predicted potential functional consequences of the variants by inspecting amino acid substitution and open reading frame changes with Gene Runner software (http://www.generunner.net).

Table 1 - Description of the primers used for amplification of HSF1 and HSPA6 genes in cattle

Primer	5´- 3´ sequence	Amplicon size (pb)	AT (C°)
	HSF1 gene		
Region1_F	TCCTTTTGGGGTCTTAGCCG		
Region1_R	AGGCACCTGGTAGAAAGCAG	845	57.5
Region2_F	CTGCGGAGCGAGGACATAAA		
Region2_R	CCAAGGCCACCTAATCCCAC	877	59.5
Region3_F	TCTGACCCCCTAAAGGCACA		
Region3_R	CGCACCTCTCGTCTACACTC	879	59.5
Region4_F	CTGTTCAGCCCCTCGGTTAC		
Region4_R	CCTGGCTCATCGGTCTGTTT	780	59.5
Region5_F	CTGTCTGTCCTACCACCCCA		
Region5_R	CATGGCTTGTCAGCATGGTC	795	57.5
	HSPA6 gene		
Region1_F	GGTCTCCCGCAACTGGATAAA		
Region1_R	GATTCTCAGGACGTTGAGCCC	703	57.7
Region2_F	TAATCACGGTGCCTGCCTAC		
Region2_R	GTAGTCATCACCCCACCAGC	808	59.5
Region3_F	GGCAGGGAGCTGAACAAAAG		
Region3_R	GTCTTGTACTTTGCGCCTGTC	651	57.7
Region4_F	GTGGAGAGGATGGTTCGTGAG		
Region4_R	AGGGTAAGATTCTCCTCCACTC	500	59.7

F - forward; R - Reverse; bp - base pairs; AT - annealing temperature.

Minor allele frequencies (MAF), identification of TagSNP, linkage disequilibrium (LD) extends and heterozygosity, and Hardy Weinberg equilibrium deviations were obtained using Haploview software (Barrett et al., 2005).

Fisher's exact test was applied to determine if allele frequencies differed among extreme phenotypes, by comparing genotype frequencies at each extreme using SAS software (Statistical Analysis System, version 9.2).

Results

The general averages estimated for RR and CT were 55.77 and 29.48, respectively. There was a significant difference between the morning and afternoon periods with values of 48.13 (RR, morning), 58.70 (RR, afternoon), and 28.08 (CT, morning), 31.05 (CT, afternoon).

Twelve SNP were identified in HSF1 gene; eight of these were mapped to coding regions, but they do not alter the amino acid sequence of the protein. The other markers identified were located in intron with uncharacterized function (Table 2).

From the SNP found in the exon of HSPA6 gene, only two (4 and 6) are synonymous mutations (Table 3). While SNP1 changes the amino acid alanine into serine, SNP2 changes histidine into glutamine, SNP3 replaces isoleucine by phenylalanine, and SNP5 modifies proline into alanine.

Among the markers identified, we found a TagSNP (SNP3) in the HSF1 gene (Figure 1). This polymorphism represents a set of SNP mapped physically close in the chromosome and, therefore, tend to have the same behavior in cell division. The haplotype formed by the SNP 6 and 11 showed a strong degree of LD with a maximum r^2 value of 0.87.

Table 2 - Description of single nucleotide polymorphisms (SNP) of HSF1 gene

SNP	Gene location (base pairs)	Gene region	5´- 3´ sequence	Consensus nucleotide	Mutant nucleotide
1	15.029	intron	CACCCCCAG	C	A
2	15.011	intron	AGAGGTAC	G	A
3	15.009	intron	AGGTACGTC	A	C
4	15.511	exon	GCGTCCTTC	C	T
5	15.954	intron	CACGTGGG	T	A
6	17.384	exon	GCAAGCAA	C	T
7	19.842	exon	CCGGCACTT	C	G
8	19.841	exon	CGGCACTTC	A	G
9	19.832	exon	GGGTCCACT	C	A
10	19.727	exon	TGGTCCGGA	C	T
11	19.728	exon	GTGGTCCGG	T	C
12	19.788	exon	CCCCCGGTG	C	T

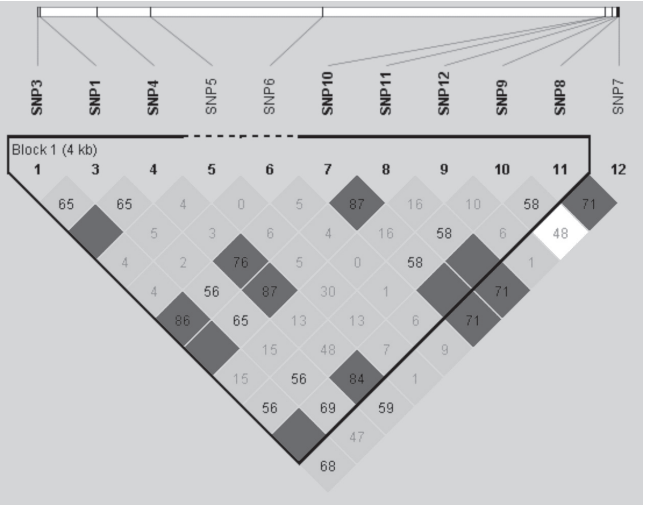
A - adenine; C - cytosine; G - guanine; T - thymine.

For the HSPA6, we found a weaker LD degree and it was not possible to construct haplotypes.

Single nucleotide polymorphisms 1, 2, 3, 4, 8, and 11 of HSF1 gene showed high degree of heterozygosity, with values above 0.70 (Table 4). In addition, markers 5, 6, and 9 of HSF1 gene and all SNP found in HSPA6 gene showed low level of heterozygosity, with values below 0.50 (Table 4).

In this study, only two types of genotypes for all SNP of both genes were found. For example, in SNP1 of the HSF1 gene, only GG and GT genotypes were found between the animals and the same for the other SNP in this gene and also in HSPA6 gene.

The MAF observed for SNP of HSF1 gene ranged from 0.10 to 0.50 and of HSPA6, from 0.02 to 0.21 (Table 4).



SNP - single nucleotide polymorphisms.
The black shading represents "strong linkage disequilibrium", gray shading evidence "genetic recombination", and white square represents "with no statistical evidence".

Figure 1 - Linkage disequilibrium pattern (r^2) among the SNP identified in the HFS1 gene.

Table 3 - Description of single nucleotide polymorphisms of HSPA6 gene

SNP	Gene location (bp)	Gene region	5′-3′ sequence	Consensus nucleotide	Mutant nucleotide	Amino acid substitution
1	1.564	exon	TCCGGAA	G	T	S instead of A
2	1.287	exon	GTCCACT	C	G	Q instead of H
3	1.195	exon	TTTATCTC	A	T	F instead of I
4	2.040	exon	CAGCTTC	C	T	*
5	2.002	exon	GTCCTCC	C	G	A instead of P
6	2.451	exon	CTGTGGT	T	A	*

A - adenine; C - cytosine; G - guanine; T - thymine; S - serine; A - alanine; Q - glutamine; H - histidine; F - phenylalanine; I - isoleucine; P - proline.
* Without amino acid change.

Discussion

The SNP (Table 2) can be silent mutations which changed the nucleotide, but not the amino acid in the protein. These mutations, also called even synonymous mutations, can affect the level of protein expression and other events of the protein formation process. According to Curi (2004), these mutations can promote alternative RNA processing and modify the open reading frame. Synonymous mutations are often overlooked in genetic analyses for disease-causing mutations unless they are directly associated with potential splicing defects. Several studies indicate that some SNP are associated with changes in protein expression and also protein folding and function. The effect of codon usage and mRNA structural changes in protein translation rates and how they can affect protein structure and function is beginning to be studied. Bartoszewski et al. (2016) stated that these synonymous mutations can alter the translational kinetics and protein folding and/or function and the mechanism of how this occurs is based on a model in which codon usage modulates the translational rate by introducing pauses caused by nonoptimal or rare codons or by introducing changes in the mRNA structure, which influences co-translational folding. Parmley et al. (2006) showed that silent mutations affected stability of the secondary structure of the mRNA in mammals.

Table 4 - Hardy Weinberg equilibrium (HWE) and heterozygosity (He) deviations, minor common allele frequencies (MAF), and P-values of Fisher's exact test performed for HSF1 and HSPA6 genes

SNP	HWE	He	MAF	P-value
		HSF1 gene		
1	0.0061	0.85	0.42	0.4945
2	0.0000	1.00	0.50	-
3	0.0245	0.92	0.46	0.6429
4	0.0034	0.93	0.46	0.6667
5	1.0000	0.06	0.03	0.6667
6	1.0000	0.06	0.03	0.6250
7	0.4218	0.61	0.30	0.2720
8	0.1103	0.81	0.38	0.4895
9	0.6678	0.20	0.26	0.4079
10	0.0660	0.53	0.37	0.3709
11	0.0264	0.76	0.40	0.4821
12	1.0000	0.61	0.10	0.2637
		HSPA6 gene		
1	0.8701	0.43	0.21	0.3427
2	1.0000	0.35	0.17	0.4196
3	1.0000	0.33	0.16	0.3394
4	1.0000	0.26	0.13	0.3251
5	1.0000	0.05	0.02	0.5263
6	1.0000	0.25	0.12	0.4308

Other authors also claim that, although SNP mapped to intergenic regions do not alter amino acid sequencing, they may be regulatory mutations playing an important role in the regulation and processing of mRNA (Zan et al., 2007).

However, the SNP in the coding region of HSP genes could affect peptide-binding kinetics or affinity of the HSP proteins and ATPase activity (Table 3), while nucleotide changes in the flanking regions (promoter and 5', 3' - untranslated region (UTR)), as already mentioned, might affect inducibility, degree of expression, or stability of HSP mRNA (Bernabucci et al., 2009).

According to Curi (2004), non-synonymous mutations can cause a dysfunction of the protein and, consequently, phenotypic variation. Thus, SNP found in this study may be important for formation, function, and expression of HSP proteins and future association studies using the SNP identified may be useful to develop strategies to implement marker-assisted selection for heat stress and tolerance in breeding programs of Angus breed.

In humans, HSP70 gene polymorphism in the coding region was positively associated with human longevity and survival advantage and was associated with an increased ability to respond to heat stress (Singh et al., 2006). Similarly, adaptation to different thermal conditions was associated with the specific SNP of HSP90 in ovine species (Salces-Ortiz et al. 2013).

Schwerin et al. (2003) reported the association of the SNP within HSP70 promoter to the shorter productive life in dairy cattle. Previous studies highlighted the role of polymorphism within HSP70 promoter region to the reproductive parameters including pregnancy rate, calf weaning weights, and fertility in dairy cattle (Banks et al., 2007; Starkey et al., 2007; Rosenkrans et al., 2010).

Li et al. (2011), while studying Holstein cows, found SNP associated with heat tolerance located at 900 pb and 4693 bp of HSF1 gene. Furthermore, Liu et al. (2010) and Adamowicz et al. (2005), also studying Holstein cows, found SNP in regulatory regions in other genes (HSP70A1A and ATPIA1, respectively) related to thermoregulation.

Li et al. (2010) also identified five novel SNP (one in CDS and four in 3′-UTR) and 11 different genotypes in HSP70.1 gene of Chinese Holstein cattle. Among these, three genotypes (AB, DD, and FF) showed higher potassium content in erythrocytes, higher milk fat and protein percentage, and higher milk yield, respectively. Thus, the authors suggested the probable associations of these genotypes with thermotolerance. Zhang et al. (2002) detected polymorphisms of the regulatory and coding regions of the HSP70 gene associated with different heat tolerance capabilities in broiler chickens. Huang et al. (2002) detected SNP in the 5′ flanking region of the HSP70.2 gene in boars and these SNP were found to be associated with semen quality traits including sperm motility, percentage of normal and abnormal sperms, sperm concentration, and semen volume. In pigs, a functional promoter and 3′-UTR variants of highly conserved inducible HSP70.2 gene significantly affected mRNA stability and cell response to stress (Schwerin et al. 2001, 2002).

The protein produced by HSP70A1A gene, a member of HSP70 family, is the main chaperone acting on a body subjected to heat stress. Homologous to this gene, HSPA6 may also act in severe conditions of heat stress. Mohanarao et al. (2013) observed an increase of 14.4 times in the expression of this gene when goats were exposed to high temperatures.

As observed, few reports have identified SNP within heat shock protein gene contributing to certain disease susceptibility and stress tolerance in *B. Taurus* and those found were related to dairy breeds. However, more studies should be reported on genetic variants and their characterization, as well as the associated with heat tolerance in Angus cattle.

Linkage disequilibrium refers to the non-random association of alleles of different loci (McKay et al., 2007). The higher the LD between SNP, the greater the chance of these SNP be inherited together during cell meiosis. Li et al. (2011), studying Holstein cattle in China, found two haplotypes in HSF1 gene, but with weak LD between polymorphisms ($r^2 = 0.10$). The LD differences between breeds and populations may influence the marker effects of this gene.

Strong LD was found in 0.2222 pairs of SNP (black shading, Figure 1). However, 0.7778 pairs of SNP (gray shading, Figure 1) may indicate genetic recombination. Interestingly, a few pairs of SNP with evidence of recombination (for example: SNP1 with SNP3 and SNP9 with SNP8) are neighbors. This fact can be explained by the occurrence of gene conversion.

Most of the SNP identified presented significant deviation from Hardy Weinberg equilibrium. This implies that there was a significant difference in genotype and allele frequencies within each gene locus, as well as the occurrence of inbreeding flow or genes from other populations (Table 4). It is important to consider that the sample size may be influencing these results as well as the sampling strategy of extreme phenotypes.

The equilibrium of a gene can be affected by factors such as mutation, migration, genetic drift, and selection because they promote changes in genotypic and allelic frequencies of a gene through generations. Li et al. (2011) also observed

deviations from equilibrium for both polymorphisms found in HSF1 gene.

Single nucleotide polymorphisms of HSF1 gene with high degree of heterozygosity (Table 4) can be considered a measure of genetic variability, which is fundamental to obtain response to selection. According to Ott (1992), marker heterozygosity above 0.70 is considered high polymorphism.

The markers with low level of heterozygosity, with values below 0.50, can be indicating moderate variability of genetic markers in the genes analyzed (Table 4).

Association studies commonly consider markers with MAF above 0.10. Thus, in HSPA6 gene, some SNP may not be appropriate for use in marker-assisted selection (Table 4).

Values of MAF, deviation from Hardy Weinberg equilibrium, and observation of only two genotypes must have been observed because of the strategy chosen of sampling phenotypic extreme animals. The lack of prior association between phenotypes and genotypes may be due to biased allelic frequency and to small size of sequenced animals.

Conclusions

There is genetic variation in HSF1 and HSPA6 genes in this populations of Angus breed, which is fundamental to obtain response to selection.

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