Polymorphisms in the leptin gene promoter in Brazilian beef herds

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ABSTRACT. Brazil is the world's largest producer of beef cattle; however, the quality of its herds needs to be improved. The use of molecular markers as auxiliary tools in selecting animals for reproduction with high pattern for beef production would significantly improve the quality of the final beef product in Brazil. The leptin gene has been demonstrated to be an excellent candidate gene for bovine breeding. The objective of this study was to sequence and compare the leptin gene promoter of Brazil's important cattle breeds in order to identify

polymorphisms in it. Blood samples of the Nellore, Guzerat, Tabapuã, and Senepol breeds were collected for genomic DNA extraction. The genomic DNA was used as a template for polymerase chain reaction (PCR) to amplify a 1575-bp fragment, which in turn was sequenced, aligned, and compared between animals of different breeds. Twenty-three single nucleotide polymorphic sites, including transitions and transversions, were detected at positions -1457, -1452, -1446, -1397, -1392, -1361, -1238, -963,-901, -578, -516, -483, -478, -470, -432, -430, -292, -282, -272, -211, -202, -170, and -147. Additionally, two insertion sites at positions -680 and -416 and two deletion sites at positions -1255 and -1059 were detected. As the promoter region of the leptin gene has been demonstrated to vary among breeds, these variations must be tested for their use as potential molecular markers for artificial selection of animals for enhanced beef production in different systems of bovine production in Brazil.

Key words: Molecular markers; Animal breeding; SNP; Gene promoter; Candidate gene

INTRODUCTION

Brazil is considered the largest producer of beef in the world. However, it faces several challenges in improving the quality of the final product. Among the numerous challenges, two in particular should be emphasized: 1) the selection of animals with high reproductive and productive patterns and 2) management techniques for bovine production in variable grass systems, i.e., grass production with optimal adaptability and high nutritional value and adaptation of animals to different climatic conditions and sanitary control (Ferraz and Felício, 2010).

Fontanesi et al. (2014) have emphasized the use of molecular markers as a tool for assisting the selection of animals with excellent phenotypes to determine herd quality. The two main characteristics of the beef cattle are 'precocity', i.e., the accelerated growth and pubertal maturity that possibly leads to a shortened generational interval (Andrea et al., 2011) and 'flavor', i.e., the tenderness and juiciness of meat. These two traits are the most important requirements of the beef cattle market (Hunt et al., 2014).

Leptin is considered a multifunctional hormone that not only acts in the homeostasis of body weight, but also thermogenesis, angiogenesis, hematopoiesis, osteogenesis, chondrogenesis, both neurological and immunological functions, and blood pressure (Bouloumié et al., 1998; Fantuzzi and Faggioni, 2000; Mantzoros, 2000; Sagawa et al., 2002). A study on the reproductive performances of cows using molecular markers of the leptin gene concluded that this gene plays an important role in bovine pregnancy (Almeida et al., 2003) and weight gain performance (Almeida et al., 2007), and is also associated with the deposition of fat and tenderness of meat (Fortes et al., 2009).

The majority of research on genetic polymorphisms of the bovine leptin gene has been related to its exon and intron sequences and their relationship with the protein structure of leptin hormone. However, it has been reported that the polymorphisms found in the leptin gene promoter regulate the expression of leptin gene in fatty tissues and are associated with differences in serum leptin concentration, growth rate, body weight, food intake, feeding

behavior, carcass measurements and merit, and production and composition of milk in beef cattle (Nkrumah et al., 2005). Therefore, the objectives of this study was to sequence a 1575-bp fragment of the leptin gene promoter in the Nellore, Tabapuã, Guzerat, and Senepol breeds, and compare these sequences to find out which breeds stand out for their high relevance in beef production in Brazil.

MATERIAL AND METHODS

This study was evaluated and approved by the Ethics Committee for the Use of Animals in Experiments (CEUA) of Universidade Federal Rural da Amazônia (UFRA), Pará, Brazil, under the protocol numbers UFRA 02384.007128/2013-40 and CEUA 024/2016. All procedures were performed in accordance with the regulations of the National Council for Animal Experimentation Control, Brazil.

Sampling and laboratory procedures

A total of 180 animals were used in this study. Of these, 40 animals, each of the Nellore and Guzerat breeds, were obtained from the farmers of Imperatriz, State of Maranhão; 70 animals of the Tabapuã breed from Paragominas, State of Pará; and 30 animals of the Senepol breed from Uberlândia, State of Minas Gerais in Brazil. Five milliliters of blood from the jugular vein of each animal was collected in a tube containing EDTA.

Blood samples were hemolyzed with bi-distilled water and centrifuged for 5 min at 2000 *g* in order to separate the leukocytes, which were used for the genomic DNA extraction using phenol-chloroform-isoamyl alcohol method (Sambrook et al., 1989).

Polymerase chain reactions (PCR) were performed in order to amplify the 1575-bp fragments of the leptin gene promoter from the genomic DNA of different animals. A primer pair was designed on the basis of the GenBank reference sequence of *Bos taurus* leptin promoter (GenBank accession No. AJ571671) using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/). The primer sequences were: forward 5'-AGGCGGAGAGGAGGAAAGAT-3' and reverse 5'-CCTCTTATAGCCGCCGAAG-3'. PCR was performed in a reaction volume of 25 µL comprising the following reagents: 10X PCR buffer, 1 mM $MgCl_2$, 1.25 mM each dNTP, 10 nM each primer, 20% betaine, 2 U Platinum High-Fidelity *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 50 to 100 ng genomic DNA. The following reaction conditions were used: initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min; annealing at 53°C for Nellore and Tabapuã DNA, 55°C for Senepol DNA, and 58°C for Guzerat DNA for 45 s; and extension at 72°C for 1 min 30 s. Final extension was carried out at 72°C for 10 min.

The PCR products were visualized on 1.5% agarose gel stained with GelRed™ Nucleic Acid Stain (Biotium, Fremont, CA, USA). The samples with high pattern were purified by Illustra™ ExoProStar™ one-step enzyme (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Subsequently, all purified samples were sequenced using BigDye Direct Sanger Sequencing Kit (Invitrogen) by an automatic DNA sequencer ABI 3500xl (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

The resulting DNA sequences were edited and aligned with the GenBank reference

sequence AJ571671 using the BioEdit program (Hall, 1999; http://www.mbio.ncsu.edu/bioedit/bioedit.html). All detected polymorphisms, insertions, and deletions were compared among different breeds. The allele frequencies were determined by direct counting.

RESULTS

After several attempts to amplify the 1575-bp fragment from all blood samples collected, isolated fragments of the leptin gene promoter were obtained from 40 Nellore, 70 Tabapuã, 40 Guzerat, and 29 Senepol animals. Of these, 39 animals were successfully sequenced: 12 Nellore, 10 Tabapuã, 11 Guzerat, and 6 Senepol. Subsequently, the sequence alignment of the DNA sequence of these animals with the GenBank reference sequence AJ571671 led to the identification of 23 single nucleotide polymorphisms (SNPs) in the region between positions -1442 and -133 of the leptin gene promoter in the bovine breeds. Of these SNPs, 14 were transition and 8 were transversion types. An SNP at position -170 might be a transition or transversion (Table 1). Varying numbers of SNPs were found in each breed: 13 Guzerat, 1 Nellore, 15 Senepol, and 9 Tabapuã.

Table 1. SNPs detected in the leptin gene promoter of the four beef bovine breeds from Brazil, their allele frequencies, and comparison with the reference sequence of *Bos taurus* leptin gene promoter (GenBank accession No. AJ571671).

Positions	AJ571671	Guzerat	Nellore	Senepol	Tabapuã
-1457	A	G(0.27)	A	G(0.33)	G(0.17)
-1452	A	G(0.55)	A	G(0.33)	G(0.17)
-1446	T	T	T	C(0.33)	T
-1397	A	A	A	T(0.17)	A
-1392	G	G	G	A(0.33)	A(0.17)
-1361	G	G	G	A(0.17)	G
-1251	T	T	T	T	T
-1238	G	G	G	C(0.33)	C(0.17)
-963	C	C	C	T(0.33)	T(0.17)
-901	A	A	A	T(0.33)	A
-578	C	G(0.45)	C	G(0.33)	C
-516	T	T	T	T	A(0.17)
-483	G	A(0.36)	G	G	G
-478	C	T(0.41)	C	C	C
-470	A	T(0.09)	A	A	A
-432	A	G(0.09)	A	A	A
-430	G	C(0.09)	G	G	G
-292	T	C(0.55)	T	C(0.33)	T
-282	G	T(0.55)	G	G	G
-272	G	G	G	A(0.33)	A(0.17)
-211	A	G(0.55)	A	A	A
-202	G	A(0.36)	A(1.00)	A(0.17)	A(0.33)
-201	C	C	C	C	C
-170	C	C	C	A(0.17) T(0.17)	C
-147	C	T(0.55)	C	T(0.33)	T(0.17)

Both deletions and insertions were found at some sites. An insertion at position -680 was found only in the Tabapuã breed, while the insertion at position -415 was found in the Guzerat, Senepol, and Tabapuã breeds; however none were observed in the Nellore breed. Deletions were observed at position -1255 in both Senepol and Tabapuã breeds, while a deletion at position -1059 was found in all breeds (Table 2).

Table 2. Insertions and deletions detected in the leptin gene promoter of the four beef bovine breeds from Brazil, their allele frequencies, and comparison with the reference sequence of *Bos taurus* leptin gene promoter (GenBank accession No. AJ571671).

Positions	AJ571671	Guzerat	Nellore	Senepol	Tabapuã
Insertions					
-680	CTA				CTTA (0.17)
-416	GGT	GGGT (0.50)		GGGT (0.33)	GGGT (0.17)
Deletions					
-1255	AG			AG (0.33)	AG (0.17)
-1059	TGT	TGT (0.83)	TGT(1.00)	TGT (0.67)	TGT (0.83)

DISCUSSION

The bovine leptin gene promoter demonstrated a high degree of polymorphism. This has also been noted by Liefers et al. (2005) and Nkrumah et al. (2005). A total of 25 polymorphic sites were detected in the 1600-bp long fragment of the leptin gene. On an average, this corresponded to one SNP per 52 bp. Liefers et al. (2005) analyzed a 1600-bp fragment of the leptin gene promoter in a Holstein-Friesian mixed breed and found 20 polymorphic sites, with an average of one SNP per 80 bp. Furthermore, Nkrumah et al. (2005) detected SNPs at positions -207 (C→T), -528 (C→T), and -1759 (C→G) in the leptin gene promoter using the GenBank reference sequence AB070368.1. None of these polymorphisms were found in our study, or in that of Liefers et al. (2005).

The SNP A1457G, previously identified in the Holstein breed by Liefers et al. (2005), was present among all Nellore samples in our study. However, the mean frequency among the others breeds was consistent with the findings of da Silva et al. (2012). On the other hand, the SNP at position -1457 showed no significant association with both growth and carcass traits in Nellore cattle (Silva et al., 2014). Although the Nellore breed shared almost 100% of its sequence with the sequence described by Taniguchi et al. (2002), some novel polymorphisms were observed in a mixed herd of the Holstein-Friesian breed studied by Liefers et al. (2005). Insertions at both positions -680 and -416 were characteristic of the Tabapuã breed, while insertion at only position -416 was detected in the Guzerat and Senepol breeds. Besides these insertions, a GenBank reference sequence AB070368.1 shows an insertion of cytosine (C) at position -498, which was not observed in our sequences, as well as in the sequences of Taniguchi et al. (2002) and Liefers et al. (2005). The Senepol and Tabapuã breeds presented the same deletion of two base pairs (AG) at position -1255. Likewise, Liefers et al. (2005) observed these insertions in a mixed-breed herd of Holstein-Friesian. However, the four breeds in our study have demonstrated a deletion of three base pairs (GTG) at position -1057, in consistency with the sequences of Taniguchi et al. (2002) and Liefers et al. (2005).

Most of the polymorphisms described in this study are highly relevant for leptin expression and may be associated with the characters of reproduction and production, which are the important characteristics of several bovine breeds of beef cattle in Brazil. Therefore, it is important to further analyze these leptin gene promoter polymorphisms as potential molecular markers for assisted artificial selection. The detection of such polymorphisms would likely be helpful in the development of breeds with enhanced production characteristics in different beef production systems in Brazil.

Conflicts of interest

The authors declare no conflict of interest.

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