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Association between *IGF2* and *CYP21* gene polymorphisms and characteristics of economic interest in Nellore cattle

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ABSTRACT. We analyzed two single nucleotide polymorphisms (SNPs) of the *IGF2* and *CYP21* genes in Nellore cattle participating in the Brazilian Animal Breeding Program. The SNPs were found in exon 6 of the *IGF2* (insulin-like growth factor 2) gene (RFLP/*Mbo*II) as well as in the promoter region of the *CYP21* (steroid 21-hydroxylase) gene (RFLP/*Hpa*II) of these animals. The TC heterozygotes were significantly more frequent than CC and TT homozygotes in the RFLP/*Mbo*II polymorphism. The T allele was significantly more frequent than the C allele in RFLP/*Hpa*II polymorphism. This population was found

to be in Hardy-Weinberg equilibrium for these SNPs. Association of these polymorphisms with expected progeny differences of reproductive and productive traits was investigated, but proved to be significant only for DP550 (expected progeny differenced for weight at 365 days - *IGF2* - RFLP/*Mbo*II) and DP450 (expected progeny differenced for weight at 450 days - *CYP21* - RFLP/*Hpa*II). This is the first study on the occurrence of these two polymorphisms in this Zebu breed of cattle. A total of 147 Nellore animals participating in the Breeding Program of the Nellore Breed (PMGRN) under the management of the National Association of Breeders and Researchers (ANCP) in the city of Ribeirão Preto were analyzed.

Key words: Genotype-phenotype association; SNPs; *IGF2*; *CYP21*; Cattle

INTRODUCTION

The beef cattle industry is one of the most important economic activities of Brazilian agribusiness and Zebu breeds and their cross-breeds represent a large percentage of the total cattle population. Breeders are always aware of new knowledge and the application of new technologies to increase the profitability of their production systems and competitiveness in order to maintain their market positions. The breeding program is one of the most important technologies used in the production process, which consider genetics the main pillar or instrument when it comes to the selection of genetically superior animals that can pass their desired traits to their offspring. This breed is used extensively in the country due to its great potential for production, rusticity and adaptation to various environmental conditions (ANUALPEC, 2009).

Currently, research in molecular biology has led to the generation of techniques and knowledge that assist and complement the traditional system of genetic improvement, intensifying research on the occurrence of different types of molecular markers in the bovine genome, in order to provide more information to assist studies on the quantitative characteristics of zootechnical interest (Regitano, 2005; Garcia, 2006). The direct application of molecular techniques has allowed the detection of SNPs (single nucleotide polymorphisms), a type of polymorphism that is characteristically abundant in the bovine genome, as evidenced in researches of genotype-phenotype association to observe genotypic variations related to phenotypic characteristics (Andréa et al., 2007; de Souza et al., 2007; Carrijo et al., 2008; Caetano, 2009; Curi et al., 2009).

Reflecting the growing demand for breeding stock, which is mainly responsible for most of the genetic advancements achieved in cattle populations, the country seeks to invest in the identification of genetically superior animals originating from the various ongoing genetic improvement programs of Zebu breeds in order to provide the market with animals able to transmit precocity, higher reproductive efficiency and rapid weight gain, among other desired characteristics. These periodical genetic evaluations make it possible to group animals with a genetic profile more suitable to each production system in the different regions of the country in order to ensure sustainable development (Freitas, 2004; Regitano, 2005; Lôbo et al., 2010).

The association of phenotypic characteristics and the use of marker-assisted selection provide an appropriate standard of selection for the breeders, because measurement of the parameters is performed in younger animals, allowing proper management of their genetic

potential (Goddard and Hayes, 2007). According to Meuwissen et al. (2001), studies on production and fertility aspects have contributed to establishing the connection between specific breeds that are improved, such as Holstein cattle for milk production and Zebu cattle (*Bos Taurus* and *Bos indicus*) for their adaptation to the tropics, and tolerance to various climatic conditions and environments.

Recently, molecular genetics has shown great advances, mainly due to the development of methods for the analysis of genetic material using a large number of samples, with consequent cost reduction (Garrick and Golden, 2009; Suekawa et al., 2010). Research groups in various countries (e.g., Netherlands, New Zealand, USA, and Australia) have conducted studies on cattle using this technology.

Specific regions of the *IGF2* and *CYP21* genes were analyzed in order to assist breeding programs by providing additional information. The restriction fragment length polymorphism (RFLP)/*Mbo*II polymorphism is located in exon 6 of the *IGF2* gene (insulin-like growth factor 2), located on bovine chromosome 29, which plays an important and essential role in cell proliferation and differentiation for growth and embryonic development in mammals (Flisikowski et al., 2005). The RFLP/*Hpa*II polymorphism is in the Bov-A2 element (considered to be a short interspersed nucleotide element, SINE) present in the promoter region of the *CYP21* gene (steroid 21-hydroxylase) on bovine chromosome 23. In humans, this gene is involved in the synthesis of steroid hormones, and its mutation is associated with congenital adrenal hyperplasia (Damiani et al., 2000). Thus, the aim of the present study was to identify two SNPs in Nellore cattle and connect aspects of the genotype that may be associated with some expected progeny differences for reproductive traits and growth in these animals.

MATERIAL AND METHODS

Genomic DNA was isolated from semen samples of 147 Nellore animals participating in the Breeding Program of the Nellore Breed (Programa de Melhoramento Genético da Raça Nelore - PMGRN) under the management of the ANCP (National Association of Breeders and Researchers) in the city of Ribeirão Preto. From a subgroup of 105 young bulls that was part of a larger group of 147 genotyped animals for the two polymorphisms studied here, we obtained the respective productive and reproductive records of the offspring of these bulls, and for each locus the probability of inheritance of the less frequent paternal allele was calculated. Also, the contemporary offspring from non-genotyped bulls were included in the analysis, and the probability of inheritance of the less frequent allele was then calculated from the allelic frequencies estimated for the total population.

DNA extraction was performed according to the protocol developed by Olerup and Zetterquist (1992), with modifications. The samples were genotyped by PCR-RFLP with primers specific for each polymorphic region (*IGF2* - RFLP/*Mbo*II: forward 5'GCCTCTC GCTGTCCTCTC3' and reverse 5'CAGCCCGTCCTCCCTAAAG3'; *CYP21* - RFLP/*Hpa*II: forward 5'CCCACCGAGTCCTGCCAC3' and reverse 5'GAGGGGGCAGTTGAAGGAC3'). The genotypic and allelic frequencies of the population were calculated and Hardy-Weinberg equilibrium was assessed by the chi-square (χ^2) test, considering a significance level of 5%. The statistical analysis was aimed to determine a possible association of the reported genotypes with the phenotypic traits of nine productive and reproductive characteristics of each animal registered at PMGRN. The methodology used to estimate the average effects of genes was based on

mixed models (BLUP - best linear unbiased prediction), usually used in the genetic evaluation of animals in the Breeding Program of the Nellore Breed - PMGRN (Lôbo et al., 2010).

The definition of contemporaneous groups included year and month of birth, sex, herd, and feed management. The assessed characteristics were: weight at 120, 240, 365, 450, and 550 days of age, scrotal circumference at 365, 455 and 550 days of age, and age at first birth. The BLUP method involves the solution of equations for each contemporaneous group and an equation for each animal, using data on the relationships among the animals to form an array. This method is generally used to calculate expected progeny differences and requires estimates of genetic parameters, variance and covariance of genetic and environmental characteristics.

The analysis model includes as random effects, the animal direct genetic effects, and maternal, genetic and permanent environment effects, and as fixed effects, the contemporary group and age class of dam, and also the random error. The maternal genetic effect was only included for analysis of the weights at 120 and 240 days and the permanent maternal environment for the weights at 120, 240 and 365 days and the scrotal circumference at 365 days. Besides these, we included as covariate the effect of the probability that the animal had received from his sire and the less frequent allele of each locus studied. The means and sample size for the traits analyzed according to the polymorphisms studied are presented in Table 1. In statistical analysis, we used the production records of the animals sired by the genotyped bulls and other animals that shared the contemporaneous group with at least one of the male sons of these bulls. In the case of non-genotyped bulls, the probability of the animal receiving the less frequent allele of the father was calculated from the allele frequencies estimated. The way the loci and several characteristics were tested and the correction for multiple comparisons were conducted using the Bonferroni method (Bland and Altman, 1995). The genetic parameters necessary for carrying out the same analysis were the same used in genetic evaluation by PMGRN/ANCP (Lôbo et al., 2010).

Table 1. Means and numbers of animals (in parentheses) for the traits analyzed, according to animals' genotype for *IGF2* RFLP/*Mbo*II and *CYP21* RFLP/*Hpa*II polymorphism.

Trait	N amount	*IGF2* - RFLP/*Mbo*II				*CYP21* - RFLP/*Hpa*II			
		Overall average	CC	TT	TC	TT	CC	CT	
P120[a]	199,207	126.5	127.29 (6,039)	131.2 (1,896)	127.9 (15,715)	127.9 (23,230)	129.9 (74)	132.7 (346)	
P240[a]	152,859	183.9	184.03 (4,448)	192.1 (1,454)	186.2 (12,630)	186.1 (18,181)	179.0 (27)	191.6 (324)	
P365[a]	132,444	236.0	350.73 (3,452)	241.6 (1,122)	410.1 (10,647)	240.0 (14,913)	214.7 (19)	235.0 (289)	
P450[a]	111,205	272.8	270.99 (2,893)	279.6 (947)	279.2 (9,205)	277.6 (12,751)	245.8 (19)	270.4 (275)	
P550[a]	61,908	317.7	314.34 (1,945)	323.9 (544)	322.3 (5,810)	321.0 (8,119)	325.4 (7)	300.4 (173)	
PE365[b]	45,973	20.1	19.68 (1,053)	20.6 (391)	20.2 (3,871)	20.2 (5,229)	20.0 (4)	20.4 (82)	
PE455[b]	45,539	23.0	22.09 (1,115)	23.6 (368)	23.1 (4,053)	22.9 (5,425)	23.3 (7)	22.7 (104)	
PE550[b]	22,763	26.3	25.09 (711)	26.9 (153)	26.2 (2,316)	26.0 (3,108)	27.1 (4)	25.1 (68)	
IPP[c]	60,409	35.9	36.0 (1,519)	34.8 (301)	35.8 (3,779)	35.8 (5,534)	34.2 (65)	- -	

[a]Pounds; [b]cm; [c]month.

RESULTS AND DISCUSSION

The allelic and genotypic frequencies of the *IGF2* RFLP/*Mbo*II and *CYP21* RFLP/*Hpa*II polymorphisms were estimated by the genotyping of 147 animals. The results obtained show that the genotypic frequencies for the *IGF2* - RFLP/*Mbo*II were 25% homozygote (CC and TT) and 50% heterozygote (CT) genotypes (Table 2). This indicates equilibrium in allele frequency. The genotyping of the *CYP21* - RFLP/*Hpa*II polymorphism demonstrated a higher incidence of the T allele, explaining the predominant frequency of the TT genotype (Table 2).

Table 2. Allelic and genotypic frequencies for *IGF2* RFLP/*Mbo*II and *CYP21* RFLP/*Hpa*II polymorphisms and chi-square (χ^2) values for Hardy-Weinberg equilibrium test.

	IGF2 RFLP/*Mbo*II			*CYP21* RFLP/*Hpa*II		
Allelic frequency	C	T		T	C	
	0.5034	0.4965		0.9557	0.0442	
Genotypic frequency	CC	TT	TC	TT	CC	CT
	0.2517	0.2449	0.5034	0.9183	0.0068	0.0748
χ^2*		0.0069			1.9339	

*χ^2 = 1 degree of freedom (P > 0.05).

The average effect of the replacement of the less frequent allele, as well as the probability, the standard error, and the results of *t*- and Bonferroni tests estimated for each trait for the association of *IGF2* - RFLP/*Mbo*II polymorphisms are shown in Table 3. The analysis with reproductive and productive traits did not have a significant impact on the traits analyzed, except for weight at 550 days, where each animal that received the C allele tended to weigh on average 8.04 kg more than its contemporary.

Table 3. Average allelic effect, standard error, *t*-test, nominal and corrected by Bonferroni method P values for the T allele of *IGF2* RFLP/*Mbo*II polymorphism.

Trait	Average effect	Standard error	P value	Bonferroni
PE365[a]	0.0150	0.1595	0.4626	1.00
PE455[a]	0.1998	0.2193	0.1812	1.00
PE550[a]	0.2655	0.3391	0.2168	1.00
IPP[b]	-0.1317	0.4075	0.3733	1.00
P120[c]	0.8737	0.8239	0.1445	1.00
P240[c]	1.0407	1.3248	0.2161	1.00
P365[c]	4.0209	1.5532	0.0048	0.09
P450[c]	4.1152	1.7448	0.0092	0.17
P550[c]	8.0494	2.7076	0.0015	0.03*

*Significant test (P < 0.05); [a]cm; [b]month; [c]pounds.

As shown in Table 4, we did not find a significant association of the *CYP21* - RFLP/*Hpa*II polymorphism with the phenotypic data of the same population of young bulls previously assessed for *IGF2* polymorphism. The effect of the replacement (T/C) was positive for weight at 455 days. The results of the Bonferroni test were found to be significant (5%), and each animal that received the C allele tended to weigh on average 10.2 kg less than its contemporary.

Table 4. Average allelic effect, standard error, *t*-test, nominal and corrected by Bonferroni method P values for the T allele of *CYP21* RFLP/*Hpa*II polymorphism.

Trait	Average effect (kg)	Standard error	*t*	P < *t*	Bonferroni
PE365[a]	-0.5176	0.3503	1.4776	0.0698	1.00
PE455[a]	-1.0509	0.4621	2.2744	0.0115	0.21
PE550[a]	-0.3461	0.6939	0.4988	0.3090	1.00
IPP[b]	0.3675	0.9651	0.3808	0.3517	1.00
P120[c]	-1.2296	1.7803	0.6907	0.2449	1.00
P240[c]	-5.1653	2.8369	1.8208	0.0343	0.62
P365[c]	-7.1020	3.4171	2.0784	0.0188	0.34
P450[c]	-10.2908	3.7347	2.7555	0.0029	0.05*
P550[c]	-5.4879	6.0385	0.9088	0.1817	1.00

*Significant test (P < 0.05); [a]cm; [b]month; [c]pounds.

Several studies have investigated allelic profiles by SNP genotyping in cattle populations (Damiani et al., 2000; Flisikowski et al., 2005; de Souza et al., 2007; Andréa et al., 2007; Biase et al., 2007; Curi et al., 2009; Bagnicka et al., 2010; Souza et al., 2010). Up to 2009, around 500 bulls from 123 farms participating in the Genetic Improvement Program of the Nellore Breed in Brazil have been evaluated for the future multiplication of "positive animals" in commercial herds by artificial insemination, and of this total number, 25% were evaluated in the present study.

The analysis of the association of the *IGF2* - RFLP/*Mbo*II polymorphism with the reproductive and productive traits did not show a significant effect on the majority of traits analyzed, except for weight at 550 days, as can be observed in Table 2. The SNP RFLP/*Mbo*II analyzed in this study is present in exon 6 of the *IGF2* gene, which is considered a regulatory region due to its location in the 5'UTR; these regions can be targeted by regulatory proteins with a substantial number of mRNA structural motifs, which have been identified in transcriptional and post-transcription regulation of gene expression (Martins da Silva et al., 2008). The *IGF2* gene encodes a fetal mitogenic protein (IGF-II) structurally related to insulin, and has been the most extensively studied imprinted mammalian gene owing to its pivotal role in the regulation of embryonic development and relationship to disease (Berkowicz et al., 2010). The effect of the T/C allele replacement for *IGF2* was considered to be statistically significant (3%) in Bonferroni test results. According to the average allelic effect, the animals that received the T allele (less frequent in the population) from the sire tended to have greater weight than their contemporary at this stage of life. In an association study of two SNPs located in the bovine *IGF2* gene (exons 2 and 10) with milk production traits in dairy cattle, Bagnicka et al. (2010) identified significant differences ($P < 0.001$) for the CT/GT haplotype. However, the same authors, in the analysis of the CT/GG haplotypes, verified that they seemed inferior for milk traits (daily yield, fat and protein content), and lower daily milk yield and lactose were associated with the genotype CT/GT, while the highest milk yield was associated with the genotypes TT/GG and CT/GG.

Blott et al. (2003) reported that a polymorphism in the growth factor receptor gene, located on chromosome 20, had an influence on milk production and composition in a dairy cattle population. Qun Zhao (2002) studied an SNP in intron 8 of the *IGF2* gene (RFLP/*Aci*I) in beef cattle and detected significant associations with growth and carcass traits. Previous studies have shown that *IGF2* is an important regulator of body size, where in humans, a polymorphism in the 3'UTR (RFLP/*Apa*I) associated with measurement of weight in middle-aged men has been reported (O'Dell and Day, 1998). DeChiara et al. (1990) showed that *IGF2* gene knockout mice had significant fetal growth retardation, especially in the early stages of pregnancy, confirming that the *IGF2* gene affects growth rates and body composition. Goodall and Schmutz (2003) identified two SNPs, one in exon 2 and the other in intron 8, and in 2007, the same authors reported the association of RFLP/*Bsr*I SNPs with loin-eye area and percentage of carcass fat in beef cattle (Goodall and Schmutz, 2007). In this study, the association of these SNPs with average effect of the reproductive and productive traits and the frequency of the less frequent allele showed a significant impact (3% for the Bonferroni test) for weight at 550 days, where each animal that received the T allele (less frequent in the population) from the sire tended to have greater weight than its contemporary at this stage of life, indicating a probably role of the *IGF2* gene.

In the same population studied, we did not find a significant association of the *CYP21*

- RFLP/*Hpa*II polymorphism with the phenotypic data for the majority of the traits analyzed (Table 4), but the effect of the replacement (T/C) was positive only for weight at 455 days, and the results of the Bonferroni test were found to be significant (5%), showing lower frequencies of the C allele in the population in this progeny test population. The fact that the sample used in this study was composed of animals under progeny test and the lower frequencies of the C allele in the population may have influenced these results.

The RFLP/*Hpa*II polymorphism present in the Bov-A2 element (promoter region of the *CYP21* gene) is involved in the synthesis of steroid hormones in humans, and its mutation is associated with congenital adrenal hyperplasia (Damiani et al., 2000). This disease is caused by defects of any of the six enzymes that are required for the synthesis of cortisol and aldosterone from cholesterol in the adrenal cortex (Lee, 2001). In a study with Nellore heifers, Andréa et al. (2007) did not find a significant association between *CYP21* gene polymorphisms with weight at 450 days and between prolactin gene polymorphisms with age at first calving. In pigs, Moe et al. (2009) did not find a significant association between SNPs at the *CYP21* and the estimated steroid levels of these animals.

Therefore, further studies are needed before these SNPs can be used for marker-assisted selection in larger populations. It is also important to determine whether the gene regions play a role in the development of the traits. It is important to mention that the animals assessed were part of a special group (animals pre-selected for the test).

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