1. Introduction

Brazilian bovines breeds have shown low indices of productivity, which may be related to climate, nutrition or genetic inheritance. The increasing productive performance of bovines is a common goal for many cattle breeding programs worldwide. In order to promote a better adaptation of high yield bovine breeds to adverse environments, Brazilian breeding programs have generated new breeds from zebu and taurine crosses, such as, for example, the Brazilian breeds Girolando (Gir × Holstein), Brangus (Nelore × Angus) and Simbrasil (Guzera × Simental). These breeds have shown better adaptation to the hot and humid climate, boosting dairy cattle productivity (Bicalho et al., 2006).

We have decided to investigate the variations of specific gene polymorphisms in the Girolando breed due to some extrinsic and intrinsic factors linked to the environment and to the genetic background, respectively. Javanmard et al. (2005) have explained that both factors play a decisive role in the metabolism of the Girolando breed in terms of milk synthesis. Amongst these factors, it is worth highlighting the feeding (Restle et al., 2005), type of management (Cerdôtes et al., 2004; Khatib et al., 2006; Oliveira and Nogueira, 2006; López et al., 2007) and genotype (Smith et al., 2000; Komisarek et al., 2004; Grisart et al., 2004; Khatib et al., 2006).

Selection of specific genotypes or alleles from candidate genes in bovines that possess economically favorable traits are advantageous (Williams, 2005) and among them, the diacylglycerol O-acyltransferase 1 (DGAT1) and leptin (LEP); however, they have not been simultaneously investigated nor have been evaluated in the Brazilian Girolando breed (Gir × Holstein, backcrossed to Holstein). Our aim was to determine the influence of fat-related genes, DGAT1 and LEP, and their polymorphisms on performance traits of milk production in the Girolando breed. Results indicated that the K allele of the DGAT1 gene showed a significant association with total and average daily milk production with additive effect. The LEP gene showed that the A allele and its homozygote are highly prevalent and almost fixed in this population and may have been favorably selected during backcrossing for the origin of this breed. The important impact of the K allele of the DGAT1 gene on milk production corroborates the initiative of performing marker-assisted selections with this gene in breeding programs of the Girolando breed.
2002, 2005) and immunological properties of the herd (Fruhbreck et al., 1998; Lord et al., 1998). In bovines, the LEP gene is found on chromosome 4, which consists of 3 exons and 2 introns, although only 2 exons express the protein (Stone et al., 1996). Lindersson et al. (1998) discovered a QTL for the production of milk near the LEP gene, at a distance of 82.8 centimorgans (cM). Various polymorphisms in the LEP gene, which influence milk production, reproduction and physiology of nutrients’ ingestion such as three SNPs in exon 2 (Arg/Cy) and exon 3 (Ala/Val, Glu/Arg), other polymorphisms located in the promoter region and nine SNPs in the intron 2, were also found in the bovine genome (Javanmard et al., 1998; Lord et al., 1998). In bovines, the LEP gene is found on chromosome 4, which consists of 3 exons and 2 introns, although only 2 exons express the protein (Stone et al., 1996). Lindersson et al. (1998) discovered a QTL for the production of milk near the LEP gene, at a distance of 82.8 centimorgans (cM). Various polymorphisms in the LEP gene, which influence milk production, reproduction and physiology of nutrients’ ingestion such as three SNPs in exon 2 (Arg/Cy) and exon 3 (Ala/Val, Glu/Arg), other polymorphisms located in the promoter region and nine SNPs in the intron 2, were also found in the bovine genome (Javanmard et al., 2010, Veerkamp et al., 2000, Alfonso (2005) and Williams (2005) indicated the LEP as a candidate gene for marker-assisted selection, due to it is important physiological role, and its putative association with the productive performance of dairy cattle, which was demonstrated elsewhere (Akers, 2006; Liefers et al., 2005).

This investigation aimed determining the influence of polymorphisms of the two metabolic fat-related genes DGAT1 and LEP in the milk production and its components in the Girolando breed.

2. Materials and methods

2.1. Sample collection

Blood samples were collected from 349 females Girolando breed (3/8 Gir + 5/8 Holstein). All the animals under analysis were milked twice a day. Due to differential frequencies of lactation, the lactation order greater than seven (8–11) were considered as seventh order, as registered by the Brazilian Association of Girolando Breeders (ABGC), the official dairy control program monitored by the Dairy Control Service of the Association (SCL). Feeding regimes were grouped into 1 = pasture with a regular mineralized salt supplement; 2 = pasture with greater mineralized supplementation and/or confined animals. For the total production, the linear effect of the lactation length (in days) as co-variable was also considered. Information about the daily and annual production of milk, period of lactation of the cow, and birth intervals were provided by the SCL.

2.2. DNA extraction and genotyping

Lymphocyte cells were isolated from PBMC samples by washing them with 0.9% saline solution and precipitating them at 2500 rpm for 10 min. The lymphocyte cell pellet was washed three times with a red blood cell lysis buffer (5 mM Sucrose, 10 mM Tris–HCl pH 7.5, 5 mM MgCl2, 1% Triton X-100) and once with nuclear lysis buffer (10 mM Tris–HCl, 2 mM EDTA, pH 8.0, 400 mM NaCl). The resulting pellet was incubated with a cellular lysis solution containing protease K (10 mg/mL) overnight at 37 °C. Lastly, DNA was extracted from the cells using phenol–chloroform (Sambrook and Russell, 2001).

Genotyping of both DGAT1 and LEP was performed by conventional PCR–RFLP, Polymerase Chain Reaction–restriction fragment length polymorphism as described elsewhere (Liefers et al., 2002) and by PCR–SSCP (PCR – Single Strand Conformation Polymorphisms) as described by Orita et al. (1989). The PCR–SSCP electrophoretic patterns were compared with previous PCR–RFLP profiles for both genes, from which genotypes and their frequencies were calculated. The PCR–SSCP was also used to verify if novel mutations were present in the same genomic region.

For the DGAT1 genotyping, a 411-bp fragment located in the exon 8, which includes the K232A polymorphism, was used for PCR–RFLP and PCR–SSCP analyses. The sequences of primers consisted of: sense 5′-GCACCATCTCTTCTCAG-3′ and antisense 3′-GGAACCGTCTTCGATAG-3′ (Liefers et al., 2002). A 20 μL reaction mix contained 1 μL of genomic DNA, 0.5 U of Taq DNA polymerase (Platinum, Invitrogen), 10 pmol of each primer, 200 μM of dNTPs, 1, 5 mM of MgCl2, 1X reaction buffer and water. The PCR reaction included an initial denaturation of 94 °C for 4 min, followed by 10 cycles of 60 s at 94 °C, 60 s at 66 °C (1 °C cycle−1) and 1 min at 72 °C, and a further 25 cycles 1 min at 94 °C, 2 min at 56 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C.

For the LEP genotyping, a PCR product of 400 bp, located in the intron 2 between exons 2 and 3, was used for PCR–RFLP and PCR–SSCP analyses. The sequences of primers were: sense 5′-TGGACTGCG TTGTATTTTCTTCT-3′ and antisense 5′-GTCCCCGTTTCTGGCTACC TAACT-3′ near the location of the polymorphism (Liefers et al., 2002). A 20 μL reaction mix contained 1 μL of genomic DNA, 0.5 U of Taq DNA polymerase (Platinum, Invitrogen), 10 pmol of each primer, 200 μM of dNTPs, 1, 5 mM of MgCl2, 1X reaction buffer and water. The PCR reaction included an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C, 55 °C and 72 °C (1 min each) and finished with a final extension of 15 min at 75 °C.

Two microlitres of each PCR product was added to 18 μL of Low Ionic Strength – LIS solution (10% sacarose, 0.01% Bromphenol Blue and 0.01% Xylene cyanol) homogenized and heated at 95 °C for 12 min and immediately transferred to an ice bath and loaded onto a 12% polyacrylamide gel (49:1; acrylamide: bis-acrylamide). Electrophoresis was carried out at room temperature, 15 mA (150 W, close to 1000 V) in 1X TBE buffer during 16 h. The PAGE was silver stained and dried, according to the Popsescu (1993) adapted for a porous transparent cellophane paper fixation, performed at room temperature for 12 h stretched on the surface of a glass plate.

2.3. Statistical analysis

The allelic and genotypic frequencies were calculated according to Weir (1996). Hardy–Weinberg equilibrium (HWE) for each gene. The total milk production, daily milk production, lactation length and birth intervals were associated to the gene polymorphisms, using the following mixed model:

\[
y_{ijk(l)mnop} = \mu + R_i + G_j + \delta_{ijk(l)} + S_{mnop(l)} + A_n + M_l + O_p + \beta(I_{jk(l)mnop} - \Gamma) + e_{ijk(l)mnop}
\]

where: \(y_{ijk(l)mnop}\) = the breeding value; \(\mu\) = general mean; \(R_i\) = fixed effects of the \(i\)th herd, \(i = 1, 2, \ldots, 18\); \(G_j\) = fixed effects of the \(j\)th genotype; \(j = 1, 2, 3\); \(\delta_{ijk(l)}\) = random effect of the \(l\)th cow (with \(l = 1, 2, \ldots, 350\) to total milk yield; \(I_{jk(l)} = 1, 2, \ldots, 352\) for daily milk yield and lactation length within \(k\)th bull (com \(k = 1, 2, \ldots, 136\), in the \(j\)th genotype and \(l\)th herd; assuming that \(\delta_{ijk(l)} \sim N(0, \sigma^2_\delta)\), which \(\sigma^2_\delta\) is the variance and covariance matrices, considering residues’ independence; \(S_{mnop(l)}\) = fixed effect of the \(m\)th feeding regimes within the \(l\)th herd; \(m = 1, 2, 3\); \(A_n\) = fixed effect of the \(n\)th month of birth; \(n = 1, 2, \ldots, 13\) for the total production of milk and \(n = 1, 2, \ldots, 14\) for the daily milk yield and lactation length; \(M_l\) = fixed effect of the \(l\)th month of birth; \(l = 1, 2, \ldots, 12\); \(O_p\) = fixed effect of the \(p\)th birth order; \(p = 1, 2, \ldots, 7\); \(\beta\) = linear coefficient (co-variable) associated with lactation length (for the total production of milk); \(I_{jk(l)mnop}\) = lactation length (for the total production of milk); \(I_{l(l)mnop}\) = lactation length (for the total production of milk); \(e_{ijk(l)mnop}\) = random effect associated with \(l\)th cow within \(k\)th bull, in the \(j\)th genotype and \(l\)th herd in the \(p\)th birth order; assuming \(\epsilon_{ijk(l)mnop} \sim N(0, \sigma^2_\epsilon)\), which \(\sigma^2_\epsilon\) is the variance and covariance matrices, considering residues’ dependence.

The data were analyzed through a mixed model, adapted by Buchanan et al. (2003), using the statistics software SAS version 9.1.3 (System and SAS release, 2005). This model also included the fixed effects of the year and the month in which lactation began, order of lactation, feeding regimes on different cattle ranches and the genotype. Furthermore, the random effects of the bull (father of the
cow), cow on the ranch and residue were taken into consideration. Simultaneously, the structure of variable components, due to repeated measures on the same animal, was also taken into account. For the total production, the linear effect of the lactation length (in Figure 1. PCR–SSCP genotyping for genes DGAT1 (A) and LEP (B) performed in a 12% polyacrylamide (49:1, acrylamide:bis) gel electrophoresis carried out with 15 mA in 1X TBE buffer during 16 h at room temperature, followed by silver-staining. The genotypes are indicated at bottom of the gel.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Alleles</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DGAT1</strong></td>
<td>A</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>0.46</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>LEP</strong></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 1
Estimated allelic and genotypic frequencies for the DGAT1 and LEP genes in Girolando cattle breed.

Table 2
Additive and dominance effects of DGAT1 and LEP alleles on four productive performance traits in the dairy cattle Girolando breed.

<table>
<thead>
<tr>
<th>Gene effects</th>
<th>Total milk production</th>
<th>Daily milk production</th>
<th>Birth intervals</th>
<th>Lactation length</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DGAT1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>213</td>
<td>0.05 *</td>
<td>212</td>
<td>0.05 *</td>
</tr>
<tr>
<td>Dominant</td>
<td>239</td>
<td>0.41</td>
<td>227</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>LEP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>175</td>
<td>0.63</td>
<td>162</td>
<td>0.55</td>
</tr>
<tr>
<td>Dominant</td>
<td>169</td>
<td>0.23</td>
<td>155</td>
<td>0.19</td>
</tr>
</tbody>
</table>

DF = degrees of freedom; *P > F (probability for the F test) = P < 0.05.

Figure 2. Regression analyses for allelic substitution for the DGAT1 gene polymorphisms (AA, KA, KK) considering the mean minimum squares of the total milk production and the average daily milk production in the Girolando breed.

days) as co-variable was also considered. Mean comparisons for genotypes and genes, DGAT1 (AA, KA and KK) and LEP (AA, AB and BB), were performed by using pairwise comparisons through the Student's t-test, with a 5% probability of significance. Regression analysis was performed to investigate the effect of allelic substitution on performance traits. Allelic and gene frequencies were plotted against each other to analyze their distribution across the population.

3. Results

The PCR-SSCP technique was successful in substituting the PCR-RFLP analysis, and has easily discriminated all the genotypes for both DGAT1 and LEP genes. The DNA banding pattern shown in the modified PAGE electrophoresis presented very specific profiles (Fig. 1). Genotypes were identified based on cross-matched profiles presented by their counterpart analysis with PCR–RFLP, which evidenced the same banding patterns described by Liefers et al. (2002, data not shown). No mutations or differential band profiles were observed in PCR–SSCP; therefore, the polymorphisms are the only ones within the genomic regions investigated. However, our banding patterns for A and K alleles did not match with profiles described elsewhere (Ripoli et al., 2006), probably because of the many different parameters used, such as: electrophoresis voltage, time, temperature, gel concentration, acrylamide:bis-acrylamide ratio, and the low-ionic strength buffer used for the ampiclon denaturation (LIS–SSCP), which may have influenced in the conformational pattern of each single stranded DNA molecule.

The allelic and genotypic frequencies for the DGAT1 and LEP genes are presented in Table 1. The DGAT1 gene frequency had a greater prevalence of the K allele (0.54), with KK and KA genotypes predominating in the population, with frequencies of 0.27 and 0.54, respectively. However, the LEP gene polymorphisms in this breed was more restrict and due to the higher frequency of the A allele (0.87), with very high frequencies for the AA (0.75) and AB (0.24) genotypes. The genotypic frequencies of both genes (DGAT1 and LEP) are in agreement with the Hardy–Weinberg equilibrium for the population under study (p > 0.05).

A significant additive effect was observed for DGAT1 alleles, specifically for the total milk production and average daily milk production (p < 0.05) (Table 2). It has also been shown that the effect of A → K allele substitution promotes an increase of 106.46 kg in the total milk production and 0.365 kg in the daily milk production average (Fig. 2). The LEP alleles and genotypes did not present any significant impact in any of the four traits investigated (p > 0.05).

Mean comparisons among genotypes for all four productive performance traits of the Girolando breed are presented in Table 3. Mean values for the DGAT1 genotypes were significantly different for the total milk production and daily milk production average (p < 0.05).

In order to investigate possible interactions between genes, we have performed a simple combined analysis of frequencies, and although individual genotypic frequencies for both genes follow the Hardy–Weinberg equilibrium, the combined genotypic frequencies (Table 4) has shown a partial disequilibrium for specific genotypic interactions, specifically the AA (DGAT1) × BB (LEP) that had no observations. Due to the very low frequencies of BB genotypes, the sample size did not present statistical power to test genotypic interactions; however, it is interesting to emphasize that the most favorable DGAT1 genotypes (KA and KK) combined with the AA genotype of LEP presented a frequency of 0.62.

4. Discussion and conclusions

The values found of DGAT1 gene were those that had been expected, as the Girolando breed results from the crossbreeding involving tropically adapted taurine and zebu breeds, with the latter presenting a high frequency of the K allele (Winter et al., 2002). In the Holstein breed, a large spectrum of variations in the allelic frequency of the DGAT1 gene in populations selected for dairy production can be found, with the frequency of the K varying from 0.35 to 0.7, as reported by Grisart et al. (2001) and Spelman (2002). Komisarek et al. (2004) found a frequency of 0.83 and 0.71 for the K and A alleles respectively in the Jersey breed. European breeds have high frequencies of the K allele, despite being traditionally bred for meat production, and as such, this allele is considered an ancestral part of the haplotype of DGAT1. On the other hand, a low frequency of the K allele is found in populations of the zebu breeds (Winter et al., 2002). In Indian Holstein Bulls the results indicated allelic frequency of K allele (0.59) was higher compared to A allele (0.41) for DGAT1 (Patel et al., 2009).

In relation to the frequency of alleles of the LEP gene similar results were related by Liefers et al. (2002) where only one homozygote animal for the B allele was found and Ripoli et al. (2011) where the frequencies among bovine breeds were 0.900, 0.100, and 0.00 for AA, AB and BB, respectively, (p > 0.21). Furthermore, in the work of Liefers et al. (2002), cows with the genotype Sau3IA-AB produced 1.32 kg/d more milk and consumed 0.73 kg/d more food compared to the SauAI-AA genotype, suggesting that the RFLP-B allele could obtain a greater production of milk without having a negative effect on the balance of energy and fertility of the animals.

This result stems from the exclusively additive effect of the DGAT1 gene in the studied population. A number of studies have associated the K allele with high production of fat in the milk of dairy cattle, a tendency that is supported by the high frequency of this allele in individuals from the Holstein breed selected for this end (Komisarek et al., 2004). This behavior of the K variant of the polymorphism of the DGAT1 gene, was also observed in other breeds of dairy cattle, such as: the Holstein–Friesian (Grisart...
et al., 2001; Spelman, 2002; Thaller et al., 2003), in the Jersey and Ayrshire, (Spelman, 2002) in the Fleckvieh (Thaller et al., 2003). Grisart et al. (2004) have shown that the allele of the DGAT1 gene which encodes lysine in the diacylglycerol transferase synthesized 1.5-fold more triacylglycerides than the variant for alanine, which may explain the phenotypic effects shown here. The results of the LEP gene corroborate the findings of Zwiernicki et al. (2002) and Madeja et al. (2004) which also failed to find a significant relationship between the alleles of the leptin gene and components of dairy production. However, controversy remains, as other studies, using different polymorphisms, have demonstrated the impact of the LEP gene on the increase of milk production (Veerkamp et al., 2000; Liefers et al., 2002; Buchanan et al., 2003). The additive effect of DGAT1 K allele and its significant impact on milk production, plus the greater frequency of the specific genotypic interaction KK/KA (DGAT1) x AA (LEP) may suggest that both genes are under selection in this Girolando breed, but the favorable LEP allele (A) has been selected and fixed in a faster rate, which may explain in part the missing genotype (BB) and the small frequency of the heterozygote (AB). The Brazilian Girolando breed is originated from zebu and taurine backcrosses aiming the adaptation to the environment with higher milk yields; therefore, the importance of the LEP gene cannot be discarded, especially because of the higher frequency of its most productive genotype (AA) in combination with KK and KA genotypes of the DGAT1 gene.

Although the LEP gene polymorphisms did not present any significant impact on milk production and associated traits, it was shown that the A allele and its homozygote are prevalent and almost fixed in this population and may have been favorably selected during backcrossing to originate this Brazilian breed.

In brief, we have shown the important impact of the K allele of the DGAT1 gene on total milk production and on daily milk production average with an additive effect, which corroborates the initiative of marker-assisted selections in breeding programs of the Girolando breed.

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References


