Single-step genome-wide association studies (GWAS) and post-GWAS analyses to identify genomic regions and candidate genes for milk yield in Brazilian Girolando cattle

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ABSTRACT

Milk production is economically important to the Brazilian agribusiness, and the majority of the country's milk production derives from Girolando (Gir × Holstein) cows. This study aimed to identify quantitative trait loci (QTL) and candidate genes associated with 305-d milk yield (305MY) in Girolando cattle. In addition, we investigated the SNP-specific variances for Holstein and Gir breeds of origin within the sequence of candidate genes. A single-step genomic BLUP procedure was used to identify QTL associated with 305MY, and the most likely candidate genes were identified through follow-up analyses. Genomic breeding values specific for Holstein and Gir were estimated in the Girolando animals using a model that uses breed-specific partial relationship matrices, which were converted to breed of origin SNP effects. Differences between breed of origin were evaluated by comparing estimated SNP variances between breeds. From 10 genome regions explaining most additive genetic variance for 305MY in Girolando cattle, 7 candidate genes were identified on chromosomes 1, 4, 6, and 26. Within the sequence of these 7 candidate genes, Gir breed of origin SNP alleles showed the highest genetic variance. These results indicated QTL regions that could be further explored in genomic selection panels and which may also help in understanding the gene mechanisms involved in milk production in the Girolando breed.

Key words: breed of origin of alleles model, breed origin effect, gene network, tropical dairy cattle

INTRODUCTION

Milk production is economically important to Brazilian agribusiness. Most of the milk in Brazil is produced in grazing systems in challenging environments, and these circumstances should be considered in the breeding goal (Ferraz and de Felício, 2010). To meet these requirements, the Girolando breed was created by crossing Holstein and Gir animals, with the aim to sustainably produce milk in tropical and subtropical regions. Girolando cattle are present in the majority of Brazilian dairy farms and are responsible for 80% of the milk produced in the country (da Silva et al., 2018b).

The first crosses between Holstein (H) and Gir (G) in Brazil emerged in the 1940s. Years later, based on the increased importance of milk production, a program was implemented to create the Girolando breed to direct the crosses to generate offspring with higher genetic capacity for productivity traits, high fertility indexes, and heterosis. The Girolando's genetic composition ranges from 1/4 H:3/4 G to 7/8 H:1/8 G; however, the crossing schemes have been directed to focus the breed's genetic composition to 5/8 H:3/8 G. The Girolando progeny testing scheme started in 1997 and allowed dissemination of Girolando semen in Brazil based on reliable breeding values, which contributed greatly to genetic improvement in this breed (da Silva et al., 2018b).

In Brazil, Girolando is the fastest growing breed in semen production, and the average 305-d milk yield (305MY) increased 51.29% (3,599 to 5,445 kg) from 2000 to 2016. Annual genetic evaluations of Girolando animals and the recent inclusion of genomic information increase the efficiency of selection of young bulls and cows and contribute to further advancement in the Girolando breeding program (da Silva et al., 2017, 2018a).

In Girolando dairy cattle, researchers have been studying milk yield and milk composition traits

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(Canaza-Cayo et al., 2016), reproduction, tick and heat stress resistance traits, and the associations of these traits with specific gene polymorphisms (Canaza-Cayo et al., 2018; dos Santos et al., 2018; Otto et al., 2018). Genome-wide association studies have been used to identify SNP that are associated with QTL in dairy cattle (Silva et al., 2011; Meredith et al., 2012), and post-GWAS analyses, including gene networks and gene ontology (GO) enrichment analyses, have provided a better understanding of the molecular mechanisms involved in the evaluated trait and allow the identification of the most likely candidate genes (Soares et al., 2017; Marques et al., 2018). Nevertheless, GWAS for milk yield in the Girolando breed have not been yet reported.

The breed of origin of alleles (BOA) approach (Vandenplas et al., 2016) has been used in crossbred animals, and the results from this approach have been assigned to models that take breed-specific SNP effects into account, to improve genomic prediction and GWAS (Sevillano et al., 2017, 2018). According to Sevillano et al. (2018), the parental breed origin influences the estimated SNP effects and helps to describe the variance of SNP associated with performance in crossbred pigs.

The objectives of this study were to perform a GWAS to identify QTL associated with 305MY in Girolando cattle and to identify the most likely candidate genes through post-GWAS studies, including GO terms enrichment and gene-transcription factors networks. In addition, the breed of origin of SNP was investigated to test its effect in the specific variance of 305MY.

MATERIALS AND METHODS

Phenotypic Data

The phenotypic data consisted of 305MY records from Girolando cattle, obtained from herds supervised by the Brazilian Dairy Control Service or herds that collaborate with the Girolando Genetic Breeding Program Sires Progeny Test. The database used in this study included 166,628 lactations from 94,124 Girolando cows, edited for age at calving (547 to 9,095 d), calving season (dry or rainy), breed composition (determined by proportion of Holstein breed, 1/4, 3/8, 1/2, 5/8, 3/4, or 7/8), and contemporary group (determined by herd-year of calving). Data for cows older than 9,095 d of age at calving and for lactations suspended due to abnormal causes were eliminated, whereas size of herd and contemporary group, with only contemporary groups with at least 3 records and daughters from at least 2 sires per group, were considered for data inclusion. After data editing, the data file that was used for

analysis had an average parity of 2 (range 1 to 15) and an average contemporary group of 38 cows (range 3 to 1,420), with 4,335 levels. The pedigree file included 188,615 animals: 8,725 sires, 70,691 dams, including both purebred Holstein or Gir and crossbred Girolando cattle.

Genotyping and Quality Control

A total of 6,760 animals were genotyped using different SNP chip densities: 4,403 Girolando cattle genotyped with the Illumina Bovine SNP50 BeadChip v. 2 (50K; Illumina, San Diego, CA), and 1,256 Holstein, 624 Gir, and 477 Girolando cattle genotyped at high density with the Illumina Bovine HD BeadChip (777K). Genotypes generated with 20K, 50K, or 70K chips were imputed to the HD v. 2 panel using FImpute software (Sargolzaei et al., 2014) with the default parameter settings and using pedigree information (Oliveira Júnior et al., 2017). In total, 413K SNP were imputed. In the genotype quality control analysis, SNP with minor allele frequency ≤ 0.02 , maximum difference between observed and expected allele frequency for Hardy Weinberg Equilibrium of 0.15, GenCall score ≤ 0.70 , and call rate ≤ 0.98 were excluded from the data set. Samples with call rate < 0.90 were also excluded. After data trimming, 397,325 SNP and 6,760 samples remained for further analysis, including 1,256 Holstein, 624 Gir, and 4,880 crossbred Girolando cattle of different breed composition (1/4, 3/8, 1/2, 5/8, 3/4, or 7/8 Holstein). Among genotyped animals, 4,573 were females, of which 4,310 cows had at least 1 lactation record; and 2,187 were males, of which 1,035 bulls had daughters with phenotypes and 1,152 had no daughters or daughters without records.

Genome-Wide Association Analysis

The single-step GWAS was conducted using the BLUPF90 software family (Misztal et al., 2002). First, the variance components were estimated and genomic estimated breeding values (GEBV) were predicted with AIREMLF90 by applying the following single-trait animal model:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a} + \mathbf{W}\mathbf{p} + \mathbf{e},$$

in which $\mathbf{y}$ is the vector of phenotypic observations (305MY kg); $\boldsymbol{\beta}$ is the vector of fixed effects (contemporary group, calving season, and breed composition) and covariates (age at calving, including linear and quadratic components); $\mathbf{a}$ is the vector of random additive genetic effects; $\mathbf{p}$ is the vector of random permanent

environmental effects; $\mathbf{e}$ is the vector of residual effects; and $\mathbf{X}$, $\mathbf{Z}$, and $\mathbf{W}$ are the incidence matrices of β , $\mathbf{a}$, and $\mathbf{p}$, respectively. It was assumed that $\mathbf{a} \sim N(0, \mathbf{H}\sigma_a^2)$, $\mathbf{p} \sim N(0, \mathbf{I}\sigma_p^2)$, and $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$, where σ_a^2 , σ_p^2 , and σ_e^2 are the additive genetic, permanent environmental, and residual variances, respectively; $\mathbf{H}$ is a matrix combining genomic and pedigree information, as proposed by Aguilar et al. (2010), and $\mathbf{I}$ is the identity matrix.

The SNP effects were calculated using postGSf90 software (<http://nce.ads.uga.edu/>; Aguilar et al., 2010), and the GWAS results were reported as the percentage of genetic variance explained by overlapping windows of 0.5-megabase, in which the windows size was determined by a previous linkage disequilibrium study in an F₂ Girolando population (unpublished data). The percentage of additive genetic variance explained by the windows was calculated as described by Wang et al. (2014). A Manhattan plot showing the nonoverlapping windows that exhibit the highest additive genetic variance was created using the `mhtplot` function of R software (R version 3.3.1; R Foundation for Statistical Computing, Vienna, Austria).

Gene Search and Generation of Gene Networks

Within the top 10 windows explaining the highest percentage of additive genetic variance, putative candidate genes were identified based on initial and final coordinates of each selected window on the ARS-UCD1.2 assembly of the bovine genome, using the National Center for Biotechnology Information Map Viewer (<https://www.ncbi.nlm.nih.gov/genome/gdv/?org=bos-taurus>). The selection of the top 10 windows explaining the highest percentages of additive genetic variance is a methodology widely used in association studies to evaluate several traits in different animal species (Tiezzi et al., 2015; Irano et al., 2016; Sevillano et al., 2019).

The gene set was used to perform the biological processes enrichment analysis to identify the most likely candidate genes for 305MY. For that, a GO network highlighting biological roles and gene interactions for the 305MY trait was generated using the ClueGO application in Cytoscape (Bindea et al., 2009), based on a unilateral hypergeometric test and Bonferroni correction. After that, analyses of promoter sequences of candidate genes were performed. For these, promoter sequences (FASTA format) were collected as flanking sequences of 3,000 bp upstream and 300 bp downstream of gene transcription start sites in the bovine reference genome, as used by Soares et al. (2017). These data were sub-

mitted to the TFM-Explorer software (<http://bioinfo.lifl.fr/TFM/TFME/>), which uses weighting matrices from the JASPAR database (<http://jaspar.binf.ku.dk/>; Sandelin et al., 2004) to detect potential transcription factor (TF) binding sites, as described in Touzet and Varré (2007). The TF list was then analyzed in Cytoscape software (Shannon et al., 2003) using the Biological Networks Gene Ontology tool (BiNGO; Maere et al., 2005) to determine significantly overrepresented functional GO terms. Based on the overrepresented biological processes identified and literature review, we were able to select those TF most related to 305MY. The most likely candidate genes were then identified through the gene-TF network, which was built using the Network Analyzer tool in Cytoscape, to provide an overview of candidate genes and their predicted TF across the 305MY trait.

Estimation of SNP Allele Effects

Inference about the breed of origin of alleles allows inclusion of this information in models that take breed-specific SNP effects into account in crossbred performance (BOA model) and estimation of SNP-specific genetic variance by breed and across breeds. To accomplish this, SNP allele substitution effects were obtained through back-solving from breed-specific H and G GEBV in Girolando animals, which were estimated using the genomic BLUP model with breed-specific partial relationship matrices. A detailed description of this analysis can be found in the next sections. In short, deriving SNP allele effects included the following steps: (1) determination of BOA on BTA1, 4, 6, and 26; (2) generation of the breed-specific partial relationship matrices $\mathbf{G}^H$ and $\mathbf{G}^G$, and calculation of Girolando GEBV using the BOA model; (3) back-solving SNP allele effects for Girolando from GEBV; (4) calculation of SNP genetic variance.

Assignment of Breed of Origin of Alleles. The BOA approach (Vandenplas et al., 2016) was used to infer the breed of origin of alleles in the Girolando cattle population. This analysis consists of 3 steps. (1) Phasing the purebred and crossbred cattle genotypes with AlphaPhase 1.1 software (Hickey et al., 2011), in which haplotype phasing was performed using pedigree, 9 combinations of haplotype lengths (350:50, 250:100, 300:100, 350:100, 150:200, 200:200, 250:200, 300:200, and 350:200) used by Sevillano et al. (2016) and Vandenplas et al. (2016), and 2 more combinations of core and tail lengths (500:150, 600:150). The current study is the first to use the high-density SNP chip to apply the BOA approach, which allowed the inclusion of the 2 combinations of core and tail lengths (500:150,

600:150), adapted for the current study. All phasing analyses were run considering “Offset” and “NotOffset” modes, allowing each allele to be considered 22 times through different haplotypes of variable lengths. The “Offset” mode moves the start of each core to halfway along the first core, creating 50% overlapping between cores. (2) Determining the unique haplotypes for each pure breed. In this step, for assigning a breed of origin to a haplotype, at least 80% of its copies were required to be observed in a specific breed. If the percentage of haplotype copies identified in a single breed was less than 80%, the breed of origin of this haplotype was defined as “unknown.” The value of 80% for the relaxation factor parameter was chosen following the recommendation by Sevillano et al. (2016). (3) Assigning breed origin for each allele observed in Girolando animals based on the determined haplotypes. This approach was applied to BTA1, 4, 6, and 26, where the identified candidate genes for the 305MY trait were located. On average, 94.35% of the alleles of Girolando animals were assigned a breed of origin. After performing the BOA approach, SNP adjacent to and located in the candidate genes were selected and used to generate the breed-specific partial relationship matrices.

Genomic BLUP Model Including Breed-Specific Partial Relationship Matrices (BOA Model). To account for the breed-specific effect of alleles, a model with 2 breed-specific partial relationship matrices ($\mathbf{G}^H$ and $\mathbf{G}^G$) was fitted (BOA model; Sevillano et al., 2017):

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{g}^H + \mathbf{Z}\mathbf{g}^G + \mathbf{W}\mathbf{p} + \mathbf{e},$$

in which $\mathbf{y}$ is the vector of phenotypic observations (305MY kg); $\boldsymbol{\beta}$ is the vector of fixed effects (the same effects used in the single-step model), and $\mathbf{X}$ is the incidence matrix of fixed effects; $\mathbf{g}^H$ and $\mathbf{g}^G$ are the vectors of the additive genetic effect of purebred gametes in crossbred animals, and $\mathbf{Z}$ is the incidence matrix; $\mathbf{p}$ is the vector of random permanent environmental effects, and $\mathbf{W}$ is the incidence matrix; and $\mathbf{e}$ is the vector of residual effects. The variance of the permanent environmental effect and residual effect were as follows:

$$\text{Var}_{\mathbf{p}} = \sigma_p^2 \otimes \mathbf{I},$$

and

$$\text{Var}_{\mathbf{e}} = \sigma_e^2 \otimes \mathbf{I}.$$

The variance of additive genetic effect for breed H origin was

$$\text{Var}_{\mathbf{g}^H} = \sigma_{g^H}^2 \otimes \mathbf{G}^H,$$

where $\mathbf{g}^H$ is a random vector with additive effects from breed H gametes, $\sigma_{g^H}^2$ is the breed-specific additive genetic variance for breed H, and $\mathbf{G}^H$ is a breed-specific partial relationships matrix for breed H. The variance structure for breed G is defined similarly.

Both breed-specific partial relationship matrices, $\mathbf{G}^H$ and $\mathbf{G}^G$, were built using the breed of origin of selected alleles located in the candidate genes for 305MY in Girolando, using the first method from VanRaden (2008). The breed-specific partial relationship submatrices were defined (considering, e.g., the breed H origin) thus:

$$\mathbf{G}^H = (\mathbf{M}^H - \mathbf{C}^H \mathbf{D}^H)(\mathbf{M}^H - \mathbf{C}^H \mathbf{D}^H)' F^{-1},$$

where $\mathbf{M}^H$ is an $n \times m$ matrix containing H allele content for the Girolando animals, where n is the number of Girolando animals and m is the number of SNP. The element M_{ij}^H —that is, the H allele content for Girolando animal i on locus j —has values of 0, 1, or 2 if animal i has both alleles coming from the H breed of origin, and 0 or 1 if one allele comes from the H breed of origin. If none of its alleles on locus j were assigned to the H breed of origin, as when both alleles come from the G breed of origin, then the corresponding element in the centered matrix $(\mathbf{M}^H - \mathbf{C}^H \mathbf{D}^H)$ was set to zero, such that this element did not contribute to the computed H-specific partial relationships. $\mathbf{C}^H$ is an $n \times m$ matrix in which the element C_{ij}^H counts the total number of H alleles for Girolando animal i on locus j , and $\mathbf{D}^H$ is an $m \times m$ diagonal matrix with breed H-specific frequencies of the counted allele (p_j^H) on the diagonals,

where p_j^H was calculated across Girolando animals by counting the occurrences of alleles originating from breed H and coded as 1, divided by the total number of H alleles in the Girolando on locus j . The scaling factor (F) was defined as $F = \sum_j 2p_j^H(1 - p_j^H)$. The breed-specific partial relationship submatrix $\mathbf{G}^G$ is defined likewise. We used ASReml software (Gilmour et al., 2015) to simultaneously estimate the variance components and the additive genetic effects for breeds H and G, taking into account animals containing genotype and phenotype information only. Thus, the BOA model was applied to a Girolando population encompassing 4,310 phenotyped and genotyped animals.

Back-Solving SNP Allele Effects from GEBV. The SNP allele effects ($\hat{\alpha}_H$) were estimated based on

Figure 1. Manhattan plot of proportion of additive genetic variance (%) explained by nonoverlapping windows in the Girolando population evaluated for 305-d milk yield (kg). Each dot denotes a window.

the GEBV of breed H for Girolando ($\hat{a}_H$), as similarly performed by Wang et al. (2014), using

$$\hat{a}_H = \mathbf{W}^H \hat{\alpha}_H,$$

where $\mathbf{W}^H$ contains centered genotypes, which can be obtained respectively by

$$\mathbf{W}^H = (\mathbf{M}^H - \mathbf{C}^H \mathbf{D}^H)$$

and

$$\hat{\alpha}_H = \mathbf{W}^{H'} (\mathbf{W}^H \mathbf{W}^{H'})^{-1} \hat{a}_H = \mathbf{F}^{-1} \mathbf{W}^{H'} \mathbf{G}^{(H)-1} \hat{a}_H.$$

The SNP allele effects of breed G were calculated similarly to breed H.

SNP Genetic Variance. Each SNP j ($\sigma_{\text{SNP}_{H,j}}^2$) variance was estimated based on the SNP allele effects and allele frequencies of breed H for Girolando, as follows:

$$\sigma_{\text{SNP}_{H,j}}^2 = 2p_j^H (1 - p_j^H) (\hat{\alpha}_{H,j})^2,$$

where $\hat{\alpha}_{H,j}$ is the allele substitution effect for SNP j . The SNP genetic variances of breed G ($\sigma_{\text{SNP}_{G,j}}^2$) were calculated likewise.

RESULTS AND DISCUSSION

Genetic Parameters

In the current study, the heritability estimate for 305MY was 0.11 ± 0.005 , and the repeatability estimate was 0.33 ± 0.003 . In previous studies with Girolando cattle, heritability and repeatability estimates were higher than the estimates found in this study, with reported heritability values ranging from 0.15 to 0.35 (Ribeiro et al., 2017; Canaza-Cayo et al., 2018) and repeatability values ranging from 0.37 to 0.47 (Facó et al., 2008, 2009).

QTL Regions

Using the single-step GWAS approach, the top 10 identified genome regions associated with 305MY, in terms of percentage of genetic variance explained (%), were located on BTA1, 2, 4, 5, 6, 7, 16, and 26 (Figure 1), and jointly explained 4.75% of the additive genetic

variance (range 0.34 to 0.69). These small genetic variances show that the evaluated trait is highly polygenic, and the importance of each unique region or gene located on these regions to milk yield is small. In a previous study with Italian Holstein cattle performed by Fontanesi et al. (2014), a significant SNP associated with milk yield was found in the same region on BTA4 identified in the current study. Moreover, in a Holstein-Friesian cattle population, 3 significant SNP associated with the same trait were closely identified with QTL located on BTA6 and 26 of the current study (Meredith et al., 2012). The other significant SNP or QTL regions identified for 305MY in this study did not overlap with any identified QTL regions reported in the literature.

Candidate Genes

Putative candidate genes within the selected top 10 windows were identified based on the ARS-UCD1.2 assembly of the bovine genome. To better understand their functions, we grouped the genes according to their GO terms enrichment (Figure 2). In this GO network,

we identified the *LEP* and *CLOCK* genes, which are involved in regulation of hormone secretion, the *CASR* gene, which is associated with regulation of ATPase activity, and the *LRRC4* gene, which is involved in negative regulation of Janus kinase/signal transducers and activators of transcription (JAK/STAT cascade).

The *LEP* gene encodes leptin, a hormone secreted by adipocytes, which plays an essential role in the regulation of energy balance, fertility, and milk production (Silva et al., 2002; Liefers et al., 2005). In dairy cattle, leptin concentrations increase throughout pregnancy and are affected by polymorphisms in the leptin receptor gene (*LEPR*; Ingvarsen and Boisclair, 2001; Liefers et al., 2004). In previous studies, several SNP in the *LEP* gene showed significant association with 305-d milk yield (Banos et al., 2008; Clempson et al., 2011; Kiyici et al., 2018). Evidence of dominance effects on 305-d milk yield was found for a SNP in the *LEP* gene (Kiyici et al., 2018). Also, significant dominance effects on fat and protein contents were described for a SNP in the *LEP* gene by Suchocki et al. (2010). The *LEP* gene also modulates some *DGAT1* gene functions and

Figure 2. Functional networks showing gene interactions (triangle nodes) related to 305-d milk yield and their subnetworks related to regulation of hormone secretion, ATPase activity, and the JAK/STAT cascade (Janus kinase/signal transducers and activators of transcription) in the Girolando population. Node size denotes term enrichment significance, from the ClueGO Cytoscape plug-in (Bindea et al., 2009). The most enriched terms per group are shown in bold purple, green, and light green letters, whereas the sub-biological process terms are labeled in black.

suppresses the expression of *DGAT2* (Chen et al., 2002; Suzuki et al., 2005). Thus, the *LEP* gene is considered to be an important milk yield candidate gene in dairy cattle.

The clock circadian regulator (*CLOCK*) is one of the core circadian clock genes, which is regulated in the mammary, liver, and adipose tissue, and its expression is increased during the transition from pregnancy to lactation (Casey et al., 2009; Casey and Plaut, 2012). The *CLOCK* gene products make up core clock elements that generate circadian rhythms, which drive the rhythmic expression of numerous genes (Casey et al., 2009). During lactation, the cow's circadian system is responsible for initiating and sustaining lactation through regulation of hormone physiology and control of the metabolic demand needed to support milk synthesis (Casey and Plaut, 2012; Casey et al., 2014b). The decrease or disruption of *CLOCK* levels can result in impairment of milk production (Casey et al., 2016) and decrease of milk fat yield in dairy cattle (Casey et al., 2014a). This gene also plays an important role in the regulation of epithelial cell growth and differentiation in the mammary gland (Casey et al., 2016).

The calcium-sensing receptor (*CASR*) is a guanine nucleotide-binding protein-coupled cell surface receptor (GPCR; Brown et al., 1993) and is highly expressed in mouse mammary epithelial cells during lactation (VanHouten et al., 2004). The *CASR* gene promotes calcium transport into milk and coordinates the secretion of the parathyroid hormone-related protein (PTHrP) into milk and also into blood circulation, which is responsible for activation of bone resorption of maternal skeletal calcium stores (Wysolmerski, 2012; Mamillapalli et al., 2013; VanHouten and Wysolmerski, 2013). Furthermore, it has important functions in the maintenance of blood circulating calcium, adjusting milk calcium transport according to systemic availability of calcium and stimulating PTHrP secretion when necessary. Thus, *CASR* ensures a steady supply of calcium for milk production and protects the body against hypocalcemia, which may decrease overall milk production (VanHouten et al., 2004; VanHouten, 2005; Kim and Wysolmerski, 2016).

The leucine-rich repeats-containing 4 gene (*LRRC4*) is involved in the negative regulation of the JAK/STAT cascade (Wu et al., 2006). The STAT family members play important functions in different mammary developmental stages such as lactogenesis, mammary epithelial cell proliferation, differentiation, and apoptosis (Haricharan and Li, 2014). In the mouse mammary gland, the JAK/STAT signaling pathway is the main regulator of milk protein expression (Yang et al., 2000); however, according to Bionaz and Loor (2011), this

pathway plays a minor role in milk protein synthesis in dairy cattle. STAT5a is responsible for development of the mammary gland during pregnancy, lactation, and alveolar progenitor cell survival (Yamaji et al., 2009; Dong et al., 2010). In dairy cattle, SNP in STAT5a were associated with fertilization success, embryonic survival, and milk production traits, becoming a putative DNA marker for improving milk yield at 305 d and milk composition (Khatib et al., 2008; He et al., 2012; Raven et al., 2014). In the mammary involution stage, STAT5 is inactivated and STAT3 is activated. STAT3 is responsible for involution of the mammary gland by inducing apoptosis and, later, mammary epithelial cell clearing (Kritikou et al., 2003; Hughes et al., 2012). However, in bovines, the mammary involution stage is an important period to both cell death and epithelial cell renewal; therefore, for the most part, alveolar structure is maintained. Apoptosis, accompanied by an increase in cell proliferation, allows cell turnover to the next lactation (Capuco and Akers, 1999; Rowson et al., 2012).

The candidate gene set was also used to perform an analysis of regulatory sequences, and after identification of the TF most related to 305MY (Table 1), we generated a gene-TF interaction network, which allowed us to evaluate connected genes (Figure 3). E74-like factor 5 (ELF5) and STAT1 were the most enriched TF in the gene-TF network (Figure 3). ELF5 is a member of the ETS family of TF, found in the epithelial layer of different tissues (Zhou et al., 1998). This TF is expressed by mammary luminal progenitor cells, the expression of which is increased during pregnancy and lactation (Harris et al., 2006; Oakes et al., 2008; Bionaz and Loor, 2011). The ELF5 TF is a key regulator in secretory alveolar epithelium differentiation during mammary morphogenesis and is also important in lobuloalveolar development in the mammary gland (Harris et al., 2006). Expression of ELF5 is induced by progesterone, and working together, both ELF5 and progesterone have a greater effect on the differentiation of progenitor cells than they do separately (Lee et al., 2013). This TF also is a key member of the set of prolactin-regulated genes able to restore lobuloalveolar development and milk production in prolactin-deficient mammary epithelium in vivo (Harris et al., 2006). In virgin mammary epithelium of mice, forced expression of ELF5 was responsible for alveolar differentiation of mammary epithelium and precocious milk secretion during pregnancy, and its absence in the body resulted in failed alveolar morphogenesis and lactation (Oakes et al., 2008). In bovine and murine mammary glands, insulin plays a major role in milk protein synthesis via induction of ELF5 (Menzies et al., 2010; Bionaz and

Table 1. Enriched transcription factors (TF) associated with genes identified for milk yield in 305-d milk yield (kg) based on their gene ontology biological process and literature review

TF ¹	Biological process	Literature review ²
ELF5	Mammary gland epithelial cell differentiation	Key regulator in the secretory alveolar epithelium differentiation and in lobuloalveolar development in the mammary gland (Harris et al., 2006)
STAT1	JAK/STAT cascade ³	SNP in STAT1 were associated with milk production traits (Rychtářová et al., 2014; Cobanoglu et al., 2016)
STAT3	JAK/STAT cascade	Responsible for involution of mammary gland through inducing apoptosis and later mammary epithelial cell clearing (Kritikou et al., 2003; Hughes et al., 2012)

¹ELF5 = E74-like factor 5; STAT1 = signal transducer and activator of transcription family 1; STAT3 = signal transducer and activator of transcription family 3.

²The cited literature studies are just a sample of the vast available literature.

³JAK/STAT = Janus kinase/signal transducers and activators of transcription.

Loor, 2011; Sigl et al., 2014). Thus, the ELF5 TF plays an important role in milk and milk protein synthesis during lactation and mammary epithelium differentiation.

STAT1 is involved in all stages of mammary gland development and is expressed in the mature virgin mammary gland before pregnancy and after involution, during remodeling of the gland (Hughes and Watson, 2012; Haricharan and Li, 2014). Expression of STAT1 and STAT3 can be regulated by growth hormones,

cytokines, progesterone, and prolactin (Boutinaud and Jammes, 2004; Haricharan and Li, 2014; Leehy et al., 2018). STAT1 is also involved in milk production traits. Previous studies have described significant association between SNP in STAT1 with milk production, milk fat and protein yields, and percentages in different dairy cattle breeds (Cobanoglu et al., 2006; Rychtářová et al., 2014; Cobanoglu et al., 2016). A study of buffalo also found associations between STAT1 and protein percentage and 305-d milk yield, providing evidence that STAT1 is important to milk production traits in different species (Deng et al., 2016).

The STAT3 TF showed a low number of connections in the gene-TF network (Figure 3). STAT3 is a member of the STAT family and responsible for mammary gland involution after the lactation period (Chapman et al., 1999; Sargeant et al., 2014). In the first phase of involution, this TF is a key transcriptional regulator of genes associated with innate immunity, and it plays an important role in wound healing in the second phase (Hughes et al., 2012). During bovine mammary involution, the balance between STAT5 and STAT3 signaling is important in regulation of the transition from lactation to involution. At the onset of mammary gland involution, STAT5 is downregulated while STAT3 is upregulated (Singh et al., 2016, 2017). The negative relationship between STAT5 and STAT3 activation is important to preventing further STAT5 activation when milk production ceases (Granillo et al., 2007). According to recent studies, when involution is experimentally induced by abrupt cessation of milking, increased expression of STAT3 can be detected up to 72 h after milking, although it occurs at different rates among cows and quarters within the same mammary gland (Singh et al., 2016, 2017).

From the gene-TF network, additional candidate genes for 305MY trait were highlighted based on TF binding sites. The dedicator of cytokinesis 1 (*DOCK1*), also known as *DOCK180*, was one of the most enriched

Figure 3. Gene-transcription factor (TF) network: Genes located in the top 10 windows for 305-d milk yield (kg; green circle nodes) and their associated TF (yellow octagon nodes). Node size corresponds to network analyses (Cytoscape; Shannon et al., 2003), in which larger nodes denotes a higher edge density associated with the number of TF binding sites. Blue square nodes show the gene ontology (GO) biological processes related to TF.

genes in this network. This gene plays an important role in the initiation of mammary gland involution and epithelial phagocytes to mediate dead cell clearance in vitro and in vivo (Bagci et al., 2014). Previous studies have described impaired STAT3 activation at the onset of mammary involution in mutant mice containing inactivated DOCK1, which resulted in delays of the initiation and progression of morphological changes associated with involution and impairment of phagocytosis of apoptotic cells (Akakura et al., 2004; Bagci et al., 2014). Mammary gland involution is essential to ensure that the tissue conserves its ability to initiate another lactation cycle.

We also identified *SLC15A2* and *SND1* in the gene-TF network. Although not enriched in the network, these genes display important roles in milk production. Solute carrier family 15 member 2 (*SLC15A2*), also known as oligopeptide transporter 2 (*PEPT2*), is one of the main transporters for peptides (Kim et al., 1972). The *PEPT2* gene is responsible for independent transport of several different short-chain di- and tripeptides and a variety of pharmacologically important compounds (Daniel and Kottra, 2004). According to Groneberg et al. (2002), *PEPT2* mRNA and protein are expressed at high levels in lactating rat and human mammary gland epithelial cells collected from breast milk. Luminal expression of *PEPT2* provides an efficient pathway for reuptake of short-chain peptides derived from hydrolysis of milk proteins secreted into the lumen, contributing to a reduction in drug disposition in the milk (Groneberg et al., 2002). Later studies demonstrated that *PEPT2* mRNA and protein are also expressed in lactating and cultured bovine mammary gland epithelial cells. Coupled with lactogenic hormones (prolactin, hydrocortisone, and insulin), gene expression of *PEPT2* was significantly enhanced (Zhou et al., 2011; Cui et al., 2017). In the bovine mammary gland, reabsorption of peptides is very important for milk protein synthesis, in which *PEPT2* is essential to maintain peptide homeostasis and to reduce drug contamination in milk, especially during lactation (Zhou et al., 2011). Thus, the *PEPT2* gene might influence milk production in cattle (Yang et al., 2015; Cui et al., 2017).

Staphylococcal nuclease domain-containing 1 (*SND1*), also known as Tudor staphylococcal nuclease (*Tudor-SN*) or *p100*, encodes the SNDI protein, a multifunctional highly conserved protein with homologs found in the genomes of 21 diverse eukaryotes (Broadhurst et al., 2005). This protein was initially identified in HeLa cells, and its conservation throughout evolution suggests that SND1 is an essential transcriptional co-activator and shows important physiological functions (Tong et al., 1995). Expression of the *SND1* gene has been observed in different murine and bovine tissues

and can be upregulated in methionine-promoted milk synthesis and cell proliferation, as well as in response to lactogenic hormones during lactation (Broadhurst et al., 2005; Lu et al., 2012; Fashe et al., 2013). The *SND1* gene is responsible for the positive regulation of STAT5, mTOR, SREBP-1, and Cyclin D1 signaling pathways and affects milk synthesis and proliferation of bovine mammary epithelial cells (Ao et al., 2015), β -casein expression (Broadhurst and Wheeler, 2001), and the prolactin-induced transcription loop, cooperating with STAT5 in transcriptional activation (Paukku et al., 2003). In this way, *SND1* is a very important gene for milk synthesis and proliferation of bovine mammary epithelial cells (Lu et al., 2012; Ao et al., 2015).

SNP Genetic Variance

Post-GWAS analyses allowed identification of 7 candidate genes associated with 305MY, located on BTA1, 4, 6, and 26. After performing the BOA approach for these chromosomes, the SNP located in these genes were selected and their alleles used to estimate breed-specific effect of alleles. Within the 7 candidate genes, the alleles coming from the G breed of origin showed higher genetic variance than alleles coming from the H breed of origin (Figure 4). Allele frequencies, SNP-specific effects and SNP-specific variances by breed are detailed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17890>). Large variations for 305MY (kg) were observed in SNP-specific variances for both breeds, ranging from 0.01 to 18.85 ± 0.10 for G and 0.01 to 4.39 ± 0.03 for H (Figure 4). These results suggest that the SNP-specific variances in the candidate genes for 305MY in Girolando depend upon the breed of origin. The difference in SNP-specific genetic variance between alleles from the G and H breeds could result from genetic selection. Selection for one specific allele or trait in subsequent generations can result in fixation of alleles and substantial losses in genetic diversity, which can be intensified by use of genomic selection (Bulmer, 1971; Van Grevenhof et al., 2012; Engelsma et al., 2014). Because the Brazilian Dairy Gir Breeding Program started only recently, in 1984, the G breed did not experience an intensive selection process for milk production compared with the H breed, which may explain the higher genetic variability for 305MY in G as well as the higher SNP-specific variance of evaluated alleles coming from G.

CONCLUSIONS

This was the first study to evaluate the trait 305MY in a Girolando population using GWAS and post-GWAS analyses. From 10 regions, mostly explaining additive

Figure 4. SNP genetic variance for 305-d milk yield (kg) when alleles originate from Holstein (a) or Gir (b) breed. Each dot denotes a SNP.

genetic variance for 305MY in Girolando cattle, candidate genes were identified on the chromosomes 1, 4, 6, and 26. A total of 7 most likely candidate genes were identified based on the gene-TF networks and gene ontology enrichment analyses. These genes were found to be involved different stages of milk synthesis (*LEP*, *CLOCK*, *CASR*, *LRRC4*, *DOCK1*, *SLC15A2*, and *SND1*). Moreover, we noticed that alleles coming from the Gir breed of origin showed larger genetic variance than alleles coming from the Holstein breed of origin. These results suggest that Girolando SNP-specific genetic variance of alleles in the candidate genes for 305MY depend upon parental inherited origin. Our results could be further explored to validate QTL results for the Girolando 305MY trait.

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