Using imputed whole-genome sequence variants to uncover candidate mutations and genes affecting milking speed and temperament in Holstein cattle

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ABSTRACT

Milking speed (MS) and temperament (MT) are 2 workability traits of great importance in dairy cattle production and breeding. This is mainly due to an increased intensification of the worldwide production systems and greater adoption of precision technologies with less human-cattle interaction. Both MS and MT are heritable traits and thus, genomic selection is a promising tool to expedite their genetic progress. However, the genetic architecture and biological mechanisms underlying the phenotypic expression of these traits remain underexplored. In this study, we investigated the association of >5.7 million imputed whole-genome sequence variants with MT and MS in 4,381 and 4,219 North American Holstein cattle, respectively. The statistical analyses were performed using a mixed linear model fitting a polygenic effect. We detected 40 and 35 significant SNPs independently associated with MT and MS, respectively, which were distributed across 26 chromosomes. Eight candidate genes (*GRI-N3A*, *KCNJ3*, *BOSTAUV1R417*, *BOSTAUV1R419*, *MAP2K5*, *KCTD3*, *GAP43*, and *LSAMP*) were suggested to play an important role in MT as they are involved in biologically relevant pathways, such as glutamatergic synapse, vomeronasal receptor and oxytocin signaling. Within their coding and upstream sequences, we used an independent data set to further detect or validate significantly differentiated SNP between cattle breeds with known differences in MT. There were fewer candidate genes potentially implicated in MS, but immunity-

related genes (e.g., *BOLA-NC1* and *LOC512672*), also identified in other populations, were validated in this study. The significant SNP and novel candidate genes identified contribute to a better understanding of the biological mechanisms underlying both traits in dairy cattle. This information will also be useful for the optimization of prediction of genomic breeding values by giving greater weights to SNP located in the genomic regions identified.

Key words: behavior, docility, genome-wide association study, GWAS, milking rate

INTRODUCTION

Dairy cattle breeding programs have been refined over time to include novel traits of increasing interest in the worldwide dairy cattle industry (Chesnais et al., 2016; Miglior et al., 2017; Chang et al., 2020). In this context, milking temperament (MT) and milking speed (MS) are 2 key workability traits that are directly associated with milk production efficiency. This is mainly due to an increased number of cows per farm and reduced human-cattle interactions, greater intensification of production systems, and larger adoption of precision technologies [e.g., automated milking systems (AMS); Carlström et al., 2013; Brito et al., 2020]. Milking temperament reflects behavioral responses to human or equipment during the milking process and can be subjectively scored on a linear scale of 1–5 points from very nervous to very calm animals (Sewalem et al., 2010; Szentléleki et al., 2015). Milking speed is mainly associated with the required milking time in relation to milk yield. Milking speed has been subjectively scored based on different scaling systems [e.g., from 1–8 points in the United States (Wiggans et al., 2007); or from 1–5 points in Canada and France (Sewalem et al., 2010);

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Marete et al., 2018)]. In addition to subjective scores, the use of precision technologies, such as AMS, is a promising alternative to quantitatively evaluate both MS and MT (Brito et al., 2020; Chang et al., 2020). In addition to production efficiency, MS has been genetically linked with udder and overall health conditions (Sewalem et al., 2010; Jardim et al., 2018), highlighting an important genetic relationship to be considered when designing selection indices.

Both MT and MS are low to moderately heritable, with estimates ranging from 0.07 to 0.47 for MT, and from 0.10 to 0.37 for MS, respectively (Schmutz et al., 2001; Rensing and Ruten, 2005; Wiggans et al., 2007; Sewalem et al., 2011; Haskell et al., 2014; Marete et al., 2018; Chang et al., 2020). Official genetic evaluations for MT and MS have been released for more than 2 decades in some countries (e.g., Canada; Chesnais et al., 2016), but only in recent years these traits have been included in other dairy cattle breeding programs around the world (Boichard and Brochard, 2012; Byrne et al., 2016).

Single nucleotide polymorphism arrays have been the predominant genotyping platform used for GWAS purposes (Visscher et al., 2017; Ma et al., 2019), especially because they are cost-effective for most livestock species. However, the detection power using SNP chip genotyping methods is affected by the extent of linkage disequilibrium (**LD**) between the genotyped SNP and the unknown causal mutations affecting the target traits (Druet et al., 2014). An alternative to identify the potential causal mutations consists on thoroughly genotyping all genome-wide SNP by resequencing the entire genome [whole-genome sequence (**WGS**)]. Generation of WGS data is still expensive to be performed in large scale, but genotype imputation from SNP chip panel to WGS has been shown to work well (Larmer et al., 2017). Therefore, genotype imputation based on LD patterns has been widely used for generating large-scale WGS data sets (Halperin and Stephan, 2009; Bouwman et al., 2018; Twomey et al., 2019). In dairy cattle, initiatives, such as the 1,000 Bull Genomes Project (www.1000bullgenomes.com; Hayes and Daetwyler, 2019), have created databases of WGS for key ancestors, which can be used as reference populations for imputing genotypes from medium- and high-density SNP panels to WGS variants. For instance, WGS variants imputed based on the 1,000 Bull Genomes Project database have been used in GWAS for udder index and MS in Nordic Holstein cattle (Jardim et al., 2018) and endo-parasite phenotypes in dairy and beef cattle (Twomey et al., 2019).

Canada's Ten Thousand Cows Genome Project was designed to genotype thousands of nonelite cows with

the least possible amount of preferential treatment, coming from herds with known history of exemplary phenotype recording. Therefore, the main objectives of this study were to perform a GWAS for MT and MS using imputed WGS variants from North American Holstein cattle to identify prospective candidate mutations, genes, and biological pathways associated with MS and MT in dairy cattle.

MATERIALS AND METHODS

Ethics Statement

No Animal Care Committee approval was necessary for the purposes of this study, as all information required was obtained from pre-existing databases.

Animals and Phenotypes

The pedigree, phenotypic, and genomic data sets were provided by the Canadian Dairy Network, a member of Lactanet (Guelph, ON, Canada). A total of 4,381 and 4,219 animals with phenotypic records (deregressed breeding values) for MT and MS traits, respectively, were used in this study (Table 1). Within the first 6 mo of lactation, each first-parity cow was evaluated for MT and MS score by the Dairy Herd Improvement (Guelph, ON, Canada) field staff at the time of milking. Scores were given using a 5-point linear scale for both traits (MT: 1 = very nervous, 2 = nervous, 3 = average, 4 = calm, and 5 = very calm; MS: 1 = very slow, 2 = slow, 3 = average, 4 = fast, and 5 = very fast; Sewalem et al., 2011). On the phenotypic scale, the most desirable values are 3 or 4 for MS and 3, 4, or 5 for MT. The scores for each cow were given by the herd owner (or cow handler or milker). This is a subjective measurement, but it is consistent within each herd. The herd owner (or cow handler or milker) received a clear explanation about the trait definitions and the scoring system. Official genetic evaluations for MS and MT and their associated reliabilities were provided by Canadian Dairy Network based on their routine genetic evaluation models (www.cdn.ca). The deregressed EBV (**dEBV**) were computed following VanRaden et al. (2009), and only dEBV with accuracy greater than 28 were kept for further analyses. The dEBV (instead of raw phenotypic records) were used for the GWAS to enable incorporation of information available for non-genotyped (and therefore, no WGS available) animals and because dEBV are more accurate than individual phenotypic records (Oliveira et al., 2018). This has been done in several other GWAS studies (e.g., Abo-Ismael et al., 2017).

Table 1. Summary of the phenotypes (deregressed estimated breeding values, dEBV) for milking temperament (MT) and milking speed (MS) in North American Holstein cattle

Traits	Sample size	dEBV				Reliability	
		Mean	Min	Max	CV%	Mean	SD
MT	4,381	111.42	−1,828.3	2,027.3	9.18	29.9	4.2
MS	4,219	105.70	−1,464.8	1,663.8	7.78	30.0	4.1

Genotyping, Imputation, and Quality Control

Imputed WGS data containing 29,548,077 SNP were available for 9,131 Holstein cows. Genotype imputation was performed in 2 steps: (1) imputation from medium density panels (**MD**, 9,131 cows and 56,955 or 60,914 SNP; Illumina, San Diego, CA) to the BovineHD panel [**HD**, 311,725 SNP after a preliminary quality control (**QC**); Illumina, San Diego, CA]; and (2) imputation from HD to WGS. The reference population for step 1 had 2,397 animals (from the same herds and Holstein population), whereas for step 2, there were 1,147 animals with WGS data (from the 1,000 Bull Genomes Project, which also included North American Holstein animals; Hayes and Daetwyler, 2019). Prior to the stepwise imputation, SNP with call rate lower than 0.95, extreme departure from Hardy-Weinberg equilibrium ($P < 10^{-8}$), and excess of heterozygosity greater than 0.15 were removed from the data set during the QC. Only SNP present in both MD and HD were retained in the MD data set. After QC, 38,955 SNP remained in the MD data set for performing the imputation analyses. A total of 297,114 SNP (from the initial 311,725 SNP) remained in the HD panel data set after QC. Subsequently, imputation from HD to WGS was performed for each of the 29 cattle autosomal chromosomes. Genotype imputation was performed using the FImpute software (Sargolzaei et al., 2014).

For the imputed WGS SNP, an additional QC was performed by requiring the individual and genotype missing rates to be lower than 0.1, minor allele frequency (**MAF**) higher than 0.01, and no extreme deviation from Hardy-Weinberg equilibrium ($P < 10^{-8}$). A total of 5,771,890 and 5,808,944 high-confidence WGS SNP remained for the GWAS analyses of MT and MS, respectively. All QC were conducted using the PLINK software (Purcell et al., 2007).

Association Analyses and Statistical Model

Association analyses were performed using the GCTA package (Yang et al., 2011) and fitting a mixed linear model (**MLM**), including a polygenic effect. Therefore,

SNP effects were estimated using the following statistical model:

$$\mathbf{y} = \mathbf{1}\mu + \mathbf{X}\mathbf{b} + \mathbf{Z}\mathbf{u} + \mathbf{e},$$

where $\mathbf{y}$ is the vector of phenotypes (dEBV) for MS or MT; $\mathbf{1}$ is a vector of ones; μ is the overall mean; $\mathbf{b}$ is the fixed effect of the SNP tested for association, $\mathbf{X}$ is a vector containing the genotype score for the tested SNP; $\mathbf{u}$ is the vector of the remaining polygenic effect with $\mathbf{u} \sim N(0, \mathbf{G}\sigma_u^2)$, where $\mathbf{G}$ is the genomic-based relationship matrix (**GRM**), and σ_u^2 is the additive genetic variance of polygenic effects; $\mathbf{Z}$ is the incidence matrix for $\mathbf{u}$; and $\mathbf{e}$ is a vector of residual effects with $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$, where $\mathbf{I}$ is an identity matrix and σ_e^2 is the residual variance.

To avoid double-fitting candidate SNP as both fixed effect and random effect in the polygenic effect (Yang et al., 2014) and also reduce computational costs, GRM were alternatively constructed by randomly sampling 50,000 SNP from all chromosomes, except the one in which the analyzed SNP were located (i.e., total of 29 GRM). After GWAS analyses, all SNP were ranked based on their P -values and clumped according to their LD pattern ($r^2 > 0.9$), which has been suggested as a preferable method compared with the pruning strategy (Privé et al., 2018). The genomic inflation factor (λ) was calculated as the ratio of the median of the observed distribution of χ^2 statistic to the expected median,

$$\hat{\lambda} = \frac{\text{median}(\chi^2)}{0.4549},$$

for which 95% CI of λ value was further derived.

Multiple Testing Correction and Significance Testing

As a very large number of variants were tested, the Bonferroni adjustment procedure would be highly

conservative (Johnson et al., 2010). Thus, to avoid the occurrence of excessive false-negative results, we alternatively calculated the threshold of significant testing as 0.05 divided by the number of independent chromosome segments (M_e) at chromosome-wise levels (Li et al., 2015). The M_e is a function of effective population size (N_e) and chromosome length [L , in centi-Morgans (cM)], and can be calculated as (Goddard et al., 2011):

$$M_e = \frac{2N_e L}{\log(N_e L)}.$$

One cM was considered to be equivalent to 1 Mb (Wang et al., 2016) and N_e equal to 115 (Stachowicz et al., 2011). As a result, SNP were considered as statistically significant if their $-\log_{10} P$ -value was higher than the chromosome-wide threshold, which ranged from 4.36 to 4.87 across chromosomes (average $\pm$ SD = 4.61 $\pm$ 0.14).

Functional Analyses

The SNP coordinates were based on the ARS-UCD1.2 assembly of the cattle reference genome (GCA_002263795.2). The annotation information was retrieved from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and used for detecting the genes located within $\pm$ 100 kb from the significant SNP, regardless of their LD pattern, but at a distance where, on average, high LD is expected in North American Holsteins (Bohmanova et al., 2010). Functional enrichment analyses of all candidate genes were conducted using the DAVID tool (Huang et al., 2009).

In addition to within-breed genetic variability (such as in Holstein cattle), there are clear genetic differences in MT between taurine (*Bos taurus taurus*) and indicine (*Bos taurus indicus*) cattle breeds (Haskell et al., 2014). Therefore, to further validate the genomic regions identified, all SNP within coding regions and 1 kb upstream regions of the most likely candidate genes found to be significantly associated with MT in Holstein cattle based on their functional implications were further investigated using 25 worldwide cattle breeds (data available from the Bovine Genome Variation Database; Chen et al., 2019). These breeds were classified into taurine (104 animals from 13 breeds) and indicine (43 animals from 12 breeds) groups (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18897>). All SNP with the MAF lower than 5% in the taurine group were subjected to statistical testing for intergroup differences using the Fisher exact test (Bonferroni-adjusted P -value <0.05). Allele frequency differences

for the statistically significant SNP were schematically illustrated using the sequence logos (Wagih, 2017).

RESULTS

WGS Data Sets

After QC and LD-based clumping (Privé et al., 2018), a total of 1,952,421 and 1,968,045 informative SNP were kept for MT and MS, respectively. All SNP for MT were distributed across the 29 autosomes, in which the mean ($\pm$ SD) and median pairwise distances ranged from 933 $\pm$ 2,680 bp and 314 bp (BTA23), to 1,594 $\pm$ 3,340 bp and 568 bp (BTA13), respectively. Similar SNP distribution was observed for MS with overall mean ($\pm$ SD) and median pairwise distances of 1,262 $\pm$ 3,008 bp and 402 bp, respectively. Furthermore, about 2.2, 38.7, and 59.1% of all these remaining SNP were located within exonic, intronic, and intergenic regions, respectively. The MAF distribution was: 42.9% of SNP with MAF lower than 0.05, 13.0% between 0.05 and 0.10, and 44.1% of SNP with MAF greater than 0.10 for MT. Similar values were observed for MS (42.7, 13.1, and 44.2%, respectively).

GWAS for Milking Temperament

A total of 40 SNP, with an average ($\pm$ SD) MAF of 0.123 $\pm$ 0.145, located on 19 chromosomes were significantly associated with MT (Figure 1). Among them, the 3 highest $-\log_{10} P$ -values (>6) were observed on BTA9, BTA23, and BTA10, respectively, and BTA1, BTA3, BTA6, BTA8, BTA9, BTA10, BTA12, BTA16, BTA22, and BTA27 had more than one significant SNP. For instance, there were 3 significant SNP closely located together within a 4.4-kb region on BTA16, 5 SNP within a 0.33-Mb region on BTA27, and 3 SNP within a 0.36-Mb region on BTA9.

A total of 50 protein-coding and 20 lncRNA genes were detected within $\pm$ 100 kb from the 28 significant SNP; whereas no adjacent genes were found for the remaining 12 SNP reported for MT (Table 2). Among the 28 significant SNP, 18 were located within intronic regions, and 10 within intergenic regions. More than 5 candidate genes were clustered and simultaneously associated with 1 or 2 significant SNP on BTA3, BTA9, BTA18, BTA21, and BTA22. The 2 significant SNP on BTA1 were located in the last intron and 5' upstream region of the limbic system associated membrane protein (*LSAMP*) gene, respectively. One significant SNP was located within the third intron of the glutamate ionotropic receptor NMDA type subunit 3A (*GRIN3A*) gene on BTA8. Two significant SNP on BTA9 were located within the eighth and sixth introns of the TBC1

domain family member 32 (*TBC1D32*) gene. On BTA16, 3 significant SNP were located together within the 5' upstream region of potassium channel tetramerization domain containing 3 (*KCTD3*) and 3' downstream region of the Usherin (*USH2A*) genes. Nine and 7 candidate genes were associated with one intergenic SNP on BTA18 and 2 intronic SNP on BTA22, respectively. Three significant SNP were located together within the fourth intron of the zinc finger matrin-type 4 (*ZMAT4*) gene located on BTA27. Potential population stratification has been accounted for, as shown by the quantile-quantile (QQ) plot (Figure 2A). The estimated genome inflation factor was equal to 1.061, with a narrow 95% confidence interval of 1.046 to 1.075.

GWAS for Milking Speed

A total of 35 SNP with an average ($\pm$ SD) MAF of 0.151 ± 0.154 , were detected to be significantly associated with MS and distributed across 19 chromosomes (Figure 3). There were multiple significant SNP simultaneously observed on BTA4, BTA6, BTA8, BTA11, BTA14, BTA20, BTA23, BTA24, and BTA27. On BTA14, 4 significant SNP were closely positioned within a 4.5-Mb region. Two significant SNP were closely located within a 7.4-kb region on BTA8 and 2.7-kb region on BTA23, respectively. Although there were 5 significant SNP on BTA11, all of them were far from each other (at least 1 Mb apart).

A total of 75 candidate genes were detected for MS, consisting of 62 protein-coding and 13 lncRNA, which were located adjacently to 29 significant SNP (Table 3). Among all significant SNP, 1, 20 and 8 were located in exon, intron, and intergenic regions, respectively. On BTA5, 5 candidate genes (i.e., *LOC512464*, *LOC112441543*, *HIGD1C*, *METTL7A*, and *TM-PRSS12*) were associated with a significant SNP that was located within the third exon of the HIG1 hypoxia inducible domain family member 1C (*HIGD1C*) gene. More than 5 candidate genes were observed to be associated with one significant SNP on BTA6, BTA8, and BTA10. Two significant SNP on BTA8 were located within the first exon and first intron of a protein-coding gene (*LOC781261*), respectively, and 6 additional candidate genes were observed (i.e., *EPHX2*, *GULO*, *HTD2*, *APTX*, *DNAJA1*, and *SMU1*). The most significant SNP on BTA11 was observed in the second intron of neurexin-1 (*NRXN1*) gene. On BTA14 3 adjacent SNP were found in the fifth intron of the nuclear receptor coactivator 2 (*NCOA2*) gene. There was a gene-rich region on BTA23, in which a total of 12 candidate genes were detected to be close to 2 significant SNP that were located within the second and third introns of the E3 ubiquitin-protein ligase TRIM39 (*LOC615278*) gene. Similar to MT, there is no evidence of uncorrected population stratification according to the QQ plot and genome inflation factor of 1.015 (95% CI of 1.001–1.029, Figure 2B).

Figure 1. Manhattan plot of the GWAS results for milking temperament based on imputed whole-genome sequence data. Statistically significant SNP are denoted by triangles.

Functional Analyses of Candidate Genes for Milking Temperament

The functionally enriched categories of candidate genes are shown in Table 4 and Supplemental Table S2 (<https://doi.org/10.3168/jds.2020-18897>). The candidate genes associated with MT were significantly enriched into 5 functional categories ($P < 0.05$), including the glutamatergic synapse pathway (bta04724), InterPro entry of vomeronasal receptor, type 1 (IPR004072), GO terms of pheromone receptor activity

(GO:0016503) and heart development (GO:0007507), and oxytocin signaling pathway (bta04921). Three genes of potassium voltage-gated channel subfamily J member 3 (*KCNJ3*), protein phosphatase 3 regulatory subunit B, β (*PPP3R2*), and *GRIN3A* were involved in the glutamatergic synapse pathway. Two genes of *BOSTAUV1R419* and *BOSTAUV1R417* were enriched for the vomeronasal receptor activity. Three genes of the ribosomal protein S6 kinase A2 (*RPS6KA2*), mitogen-activated protein kinase kinase 5 (*MAP2K5*), and *TBC1D32* were enriched in the GO term of heart

Table 2. Description of SNP and candidate genes significantly associated with milking temperament in North American Holstein cattle¹

Chr	Position (bp)	P-value	MAF	SNP effects	Candidate genes (within ± 100 kb)	Location
BTA1	60,491,479	1.1797E-05	0.159	128.9	<i>GAP43</i> and <i>LSAMP</i>	Intron
	61,279,697	8.8392E-06	0.294	106.6		Intergenic
BTA2	41,433,774	9.5752E-06	0.104	156.5	<i>KCNJ3</i>	Intergenic
BTA3	12,962,750	1.4497E-05	0.011	446.5	<i>FCRL5</i> , <i>LOC100849046</i> , <i>LOC112445937*</i> , <i>LOC107132121*</i> , <i>LOC101904923*</i> , <i>LOC112445938*</i> , <i>LOC784007</i> , and <i>LOC112445939*</i>	Intron
	26,543,998	1.3290E-06	0.013	464.2	<i>CD2</i> , <i>LOC112445966*</i> , and <i>IGSF3</i>	Intergenic
	61,388,404	1.5201E-05	0.026	282.1	—	NA
BTA6	32,808,304	1.3681E-05	0.031	-280.1	—	NA
	38,881,648	7.0356E-06	0.155	135.5	—	NA
BTA8	89,513,243	6.7747E-06	0.025	-313.4	<i>NXNL2</i>	Intergenic
	91,408,357	1.2190E-05	0.056	-198.8	<i>GRIN3A</i> and <i>PPP3R2</i>	Intron
BTA9	4,614,751	1.4705E-05	0.027	-294.5	—	NA
	29,540,351	5.1005E-06	0.120	-147.4	<i>LOC101905771*</i> and <i>LOC112448126*</i>	Intron
	29,866,887	1.2670E-05	0.120	148.8	<i>TBC1D32</i>	Intron
	29,898,522	1.4876E-05	0.118	148.2		Intron
	62,471,145	9.7777E-06	0.031	270.3	<i>RARS2</i> , <i>SLC35A1</i> , <i>CFAP206</i> , <i>LOC112448057*</i> , and <i>C9H6orf163</i>	Intron
	101,306,064	3.0359E-07	0.380	-110.7	<i>PRR18</i> , <i>SFT2D1</i> , <i>LOC101903545*</i> , <i>MPC1</i> , <i>LOC107132798*</i> , <i>LOC101903629*</i> , and <i>RPS6KA2</i>	Intron
BTA10	14,638,815	1.6677E-05	0.035	-261.1	<i>MAP2K5</i> , <i>LOC112448375*</i> , <i>LOC112448376*</i> and <i>SKOR1</i>	Intron
	20,196,716	9.0145E-07	0.010	532.6	<i>NEO1</i> , <i>HCN4</i> and <i>REC114</i>	Intron
BTA11	59,587,479	5.4882E-06	0.018	-365.4	<i>LRRTM4</i> , and <i>LOC112448801*</i>	Intergenic
BTA12	80,284,325	2.2104E-05	0.053	204.9	—	NA
	80,304,993	2.1666E-05	0.071	185.7	—	NA
BTA14	49,856,629	1.6748E-05	0.010	455.6	—	NA
BTA16	18,812,423	4.7750E-06	0.019	-366.8	<i>KCTD3</i> and <i>USH2A</i>	Intergenic
	18,815,839	8.6765E-06	0.015	-406.2		Intergenic
	18,816,814	4.4486E-06	0.013	-448.0		Intergenic
BTA17	56,150,431	2.1443E-05	0.016	-378.0	<i>HSPB8</i> and <i>SRRM4</i>	Intergenic
BTA18	58,270,112	2.7762E-06	0.047	-231.1	<i>BOSTAUV1R417</i> , <i>BOSTAUV1R418</i> , <i>BOSTAUV1R419</i> , <i>LOC112442216</i> , <i>LOC112442217</i> , <i>LOC101910094</i> , <i>LOC101903564*</i> , <i>LOC787057</i> , and <i>LOC112442214</i>	Intron
BTA21	30,325,061	2.7060E-05	0.057	-195.8	<i>OTUD7A</i> , <i>LOC530472</i> , <i>LOC101907006*</i> , <i>ADAMTS7</i> , <i>TBC1D2B</i> and <i>LOC112443141*</i>	Intron
BTA22	59,471,127	1.8203E-06	0.054	-224.4	<i>EEFSEC</i> , <i>LOC107131703*</i> , <i>RUVBL1</i> , <i>SEC61A1</i> , <i>LOC112443432*</i> , <i>KBTBD12</i> , and <i>MGLL</i>	Intron
	59,619,868	2.5847E-05	0.018	-346.9	—	Intron
BTA23	2,338,177	3.3135E-07	0.049	-256.5	—	NA
BTA24	19,206,273	2.7365E-05	0.039	-235.4	—	NA
BTA27	35,413,178	2.8713E-05	0.308	-100.6	—	NA
	35,420,916	3.9903E-05	0.438	-92.5	—	NA
	35,737,738	2.6732E-05	0.407	91.8	<i>ZMAT4</i>	Intron
	35,737,851	1.2819E-05	0.419	95.7		Intron
	35,739,454	4.1478E-05	0.422	90.4		Intron
	39,668,668	3.1432E-05	0.231	-106.6	—	NA
	44,198,674	1.9565E-05	0.438	-92.4	<i>ZNF385D</i>	Intron
BTA29	23,753,023	4.4578E-06	0.055	-219.8	<i>NELL1</i>	Intron

¹Chr = chromosome; MAF = minor allele frequency; * = lncRNA genes.

Figure 2. Quantile-quantile plots of P -values and genomic inflation factors (λ) for milking temperament (A) and milking speed (B) in North American Holstein cattle. Red lines represent theoretical distribution of P -values under the null hypothesis of no association, from which departures would indicate deviations of null hypothesis.

development. Among these genes that were enriched for the above functions, 3 genes (i.e., *MAP2K5*, *KCNJ3* and *PPP3R2*) were involved in the oxytocin signaling pathway. Using a less stringent threshold of $P < 0.1$, 5 functional categories have been additionally suggested, including the photoreceptor cell maintenance (GO term), MAPK signaling pathway, ion channel (UP_KEYWORDS), Rab-GTPase-TBC domain (INTERPRO), and long-term potentiation pathway

(Supplemental Table S2, <https://doi.org/10.3168/jds.2020-18897>).

Within both coding region and upstream sequences of candidate genes that most likely have functional implications, 31 SNP with statistically significant differences of allele frequencies between the taurine and indicine breeds were further detected based on an independent data set. Among the 3 genes involved in glutamatergic synapse pathway, there was one upstream SNP and 4

Figure 3. Manhattan plot of the GWAS results for milking speed based on imputed whole-genome sequence data. Statistically significant SNP are denoted by triangles.

synonymous SNP in *GRIN3A* and 3 upstream SNP in *KCNJ3* (Figure 4A and B). Four and one SNP were detected for the *BOSTAUV1R417* and *BOSTAUV1R419* genes, respectively; and among them one nonsynonymous mutation (*p*.Trp112Leu at *c*.335G>T) was present in *BOSTAUV1R417* (Figure 4C). However, only 3 upstream SNP were found in the *MAP2K5* gene in the oxytocin signaling pathway (Figure 4D). There were 6, 4, and 5 significant SNP detected in the *KCTD3*, *GAP43*, and *LSAMP* genes, respectively, which were not functionally enriched, but supported by strong association (Figures 4E and F). Furthermore, the *GAP43* gene had one nonsynonymous mutation of *p*.Pro172Ser at *c*.514C > T.

Functional Analyses of Candidate Genes for Milking Speed

A total of 17 candidate genes were significantly enriched in 8 functional categories ($P < 0.05$, Table 4 and Supplemental Table S2, <https://doi.org/10.3168/jds.2020-18897>). There were 5 genes located on BTA10 (i.e., *LOC521749*, *LOC788778*, *LOC788790*, *LOC516467*, and *LOC504344*), which were involved in 2 GO terms: detection of chemical stimulus involved in sensory perception (GO:0050907), and transmembrane signaling receptor activity (GO:0004888). Three other GO terms, including the positive regulation of mitochondrial membrane potential (GO:0010918), positive

Table 3. Description of SNP and candidate genes significantly associated with milking speed in North American Holstein cattle¹

Chr	Position (bp)	P-value	MAF	SNP effects	Candidate genes (within ± 100 kb)	Location
BTA4	18,630,065	3.2270E-06	0.010	405.2	—	NA
	58,610,485	1.3116E-05	0.343	80.3	—	NA
BTA5	28,975,642	1.6849E-05	0.080	-135.9	<i>LOC512464</i> , <i>LOC112441543</i> , <i>HIGD1C</i> , <i>METTL7A</i> , and <i>TMPRSS12</i>	Intron
BTA6	1,623,443	1.6472E-05	0.011	-377.5	—	NA
	110,828,142	8.5310E-06	0.209	100.2	<i>CC2D2A</i> , <i>FBXL5</i> , <i>LOC104968939*</i> , <i>LOC100849050</i> , and <i>BST1</i>	Intergenic
BTA8	59,424,073	1.5306E-05	0.091	132.0	<i>VCP</i> , <i>FANCG</i> , <i>PIGO</i> , <i>STOML2</i> , <i>FAM214B</i> , and <i>UNC13B</i>	Intergenic
	74,722,735	1.2250E-05	0.025	249.2	<i>EPHX2</i> , <i>GULO</i> , <i>HTD2</i> , <i>LOC781261</i> , <i>APTX</i> , <i>DNAJA1</i> , and <i>SMU1</i>	Intron
	74,730,092	7.3227E-06	0.029	240.4	—	Exon
BTA9	2,526,481	1.7919E-06	0.190	-108.3	—	NA
BTA10	28,155,181	1.8039E-05	0.025	243.1	<i>LOC788797</i> , <i>LOC788790</i> , <i>LOC788778</i> , <i>LOC504344</i> , <i>LOC788740</i> , <i>LOC521749</i> , and <i>LOC516467</i>	Intergenic
BTA11	33,416,170	5.1299E-06	0.036	-213.7	<i>NRXN1</i>	Intron
	55,348,499	1.5597E-05	0.011	-364.7	<i>CTNNA2</i>	Intron
	76,490,540	1.7029E-05	0.145	-112.0	—	NA
	77,563,811	1.6850E-05	0.031	-227.4	<i>LOC112448818*</i>	Intron
BTA13	86,130,607	1.1258E-05	0.079	-141.6	<i>LOC112448828*</i> , <i>LPIN1</i> , and <i>NTSR2</i>	Intron
	2,738,460	1.8323E-05	0.017	299.7	<i>PAK5</i>	Intron
BTA14	29,408,225	9.6177E-06	0.037	-208.9	<i>CYP7B1</i>	Intron
	33,747,936	1.7425E-05	0.358	-80.0	<i>PRDM14</i> and <i>NCOA2</i>	Intergenic
	33,891,204	5.4418E-06	0.403	-79.8	—	Intron
	33,892,181	2.6174E-06	0.431	-85.7	—	Intron
BTA16	64,722,850	1.7814E-05	0.046	-186.0	<i>NCF2</i> , <i>ARPC5</i> , <i>RGL1</i> , and <i>APOBEC4</i>	Intron
BTA18	32,867,187	1.1727E-05	0.079	142.7	<i>CDH11</i>	Intergenic
BTA19	60,394,610	2.9330E-05	0.400	-73.9	—	NA
BTA20	836,751	1.7188E-05	0.094	-136.6	<i>SLIT3</i> and <i>LOC107131552*</i>	Intron
	62,244,291	1.7709E-05	0.116	-114.6	<i>CTNND2</i> , <i>LOC112443009*</i> , and <i>LOC112443010*</i>	Intron
BTA22	67,686,024	2.7311E-06	0.452	-82.1	<i>ICE1</i>	Intron
	6,287,984	2.3214E-05	0.012	-350.3	<i>OSBPL10</i>	Intron
BTA23	28,460,633	2.5912E-05	0.414	-76.3	<i>PPP1R10</i> , <i>ATAT1</i> , <i>MRPS18B</i> , <i>ABCF1</i> , <i>PRR3</i> , <i>GNL1</i> , <i>RPP21</i> , <i>LOC615278</i> , <i>LOC512672</i> , <i>LOC104975665*</i> , <i>LOC104975667*</i> , and <i>BOLA-NC1</i>	Intron
	28,463,360	1.9085E-05	0.380	-81.2	—	Intron
BTA24	10,100,503	1.9220E-05	0.068	-147.3	<i>CDH19</i>	Intergenic
	14,505,480	1.3665E-05	0.028	225.1	<i>LOC112444187*</i>	Intergenic
BTA25	39,791,709	2.0311E-05	0.330	80.3	<i>SDK1</i> and <i>LOC112444323*</i>	Intron
BTA26	46,250,006	4.2956E-06	0.215	102.0	<i>C26H10orf90</i>	Intergenic
BTA27	14,713,849	3.9453E-05	0.061	-156.8	<i>STOX2</i> , <i>LOC101904332*</i> , <i>ENPP6</i> , <i>LOC104976048*</i> , and <i>LOC112444584*</i>	Intron
	40,306,840	3.4765E-05	0.021	-259.6	<i>RARB</i>	Intron

¹Chr = chromosome; * = lncRNA genes; MAF = minor allele frequency.

regulation of protein complex assembly (GO:0031334), and cellular response to DNA damage stimulus (GO:0006974), have been associated with 5 genes of the stomatin-like 2 (*STOML2*), transitional endoplasmic reticulum ATPase (*VCP*), interactor of little elongation complex ELL subunit 1 (*ICE1*), fanconi anemia complementation group G (*FANCG*), and G protein nucleolar 1 (*GNL1*). There were 10 additional functional categories, such as the GO term of cellular response to hormone stimulus, that have been associated with the candidate genes of MS with $P < 0.1$ (Supplemental Table S2, <https://doi.org/10.3168/jds.2020-18897>).

DISCUSSION

Although GWAS have been extensively performed for traditional traits in dairy cattle breeding, there is still a lack of research for workability traits. In this context, Hiendleder et al. (2003) first reported 3 QTL using microsatellite markers and proposed tyrosinase (*TYR*) as a putative candidate gene on BTA29 for MT. By genotyping 1,536 genome-wide SNP markers in Canadian Holstein bulls, 10 significant SNPs associated with MT were found on 7 chromosomes (Kolbehdari et al., 2008). A total of 406 and 601 SNP were significantly associated with MT and MS, respectively, in North American Holsteins using the BovineSNP50 chip (Abo-Ismael et al., 2014). For MS, Marete et al. (2018) identified 971 significant SNP distributed across 22

chromosomes in French Holstein cattle. Among these significant SNP, the authors observed the strongest association on BTA14 with a putative candidate gene of zinc finger and AT-hook domain containing (*ZFAT*). In Nordic Holstein cattle, a large number of significant SNPs associated with MS were detected on 8 chromosomes (BTA4, BTA5, BTA6, BTA9, BTA12, BTA13, BTA19, and BTA20), with the most significant association on BTA19 (Jardim et al., 2018). Additionally, other significant SNPs and QTL associated with MS in dairy cattle have also been previously reported in the literature (Schrooten et al., 2000; Hiendleder et al., 2003; Kolbehdari et al., 2008; Guo et al., 2012; Abo-Ismael et al., 2014; Kramer et al., 2014).

Although MS can be quantitatively measured in AMS by dividing milking yield by milking time (Siewert et al., 2018; Vosman et al., 2018), as far as we know, these quantitative records have not been employed in GWAS yet. Quantitatively scoring MT is more challenging compared with MS, however, precision technologies such as activity-monitors, video-imaging, and even traits derived from AMS are being actively developed (Brito et al., 2020; Chang et al., 2020). Wethal et al. (2020) reported a stronger genetic correlation for farmer-assessed MS than for MT between AMS and traditional milking systems. We expect that the detection power of GWAS based on quantitative measurements will be improved. However, this requires the availability of a large number of animals with phenotypic measure-

Table 4. Significantly enriched functions of candidate genes for milking temperament and milking speed in North American Holstein cattle

Category	Term	Genes	P-value
Milking temperament			
KEGG_PATHWAY	bta04724:Glutamatergic synapse	<i>GRIN3A</i> , <i>KCNJ3</i> , and <i>PPP3R2</i>	0.013
INTERPRO	IPR004072:Vomeronasal receptor, type 1	<i>BOSTAUV1R419</i> and <i>BOSTAUV1R417</i>	0.017
GOTERM_MF_DIRECT	GO:0016503~pheromone receptor activity	<i>BOSTAUV1R419</i> and <i>BOSTAUV1R417</i>	0.017
GOTERM_BP_DIRECT	GO:0007507~heart development	<i>RPS6KA2</i> , <i>MAP2K5</i> and <i>TBC1D32</i>	0.020
KEGG_PATHWAY	bta04921:Oxytocin signaling pathway	<i>MAP2K5</i> , <i>KCNJ3</i> and <i>PPP3R2</i>	0.021
Milking speed			
GOTERM_BP_DIRECT	GO:0050907~detection of chemical stimulus involved in sensory perception	<i>LOC521749</i> , <i>LOC788778</i> , <i>LOC788790</i> , <i>LOC516467</i> , and <i>LOC504344</i>	0.001
GOTERM_MF_DIRECT	GO:0004888~transmembrane signaling receptor activity	<i>LOC521749</i> , <i>LOC788778</i> , <i>LOC788790</i> , <i>LOC516467</i> and <i>LOC504344</i>	0.003
GOTERM_BP_DIRECT	GO:0010918~positive regulation of mitochondrial membrane potential	<i>STOML2</i> and <i>VCP</i>	0.009
UP_KEYWORDS	Cell membrane	<i>LOC521749</i> , <i>CDH11</i> , <i>LOC788778</i> , <i>ENPP6</i> , <i>STOML2</i> , <i>LOC788790</i> , <i>NRXN1</i> , <i>LOC516467</i> , <i>LOC504344</i> and <i>CDH19</i>	0.030
INTERPRO	IPR002591:Type I phosphodiesterase/nucleotide pyrophosphatase/phosphate transferase	<i>ENPP6</i> and <i>PIGO</i>	0.032
INTERPRO	IPR001039:MHC class I, α chain, $\alpha 1/\alpha 2$	<i>BOLA-NC1</i> and <i>LOC512672</i>	0.048
GOTERM_BP_DIRECT	GO:0031334~positive regulation of protein complex assembly	<i>ICE1</i> and <i>VCP</i>	0.049
GOTERM_BP_DIRECT	GO:0006974~cellular response to DNA damage stimulus	<i>FANCG</i> , <i>GNL1</i> and <i>VCP</i>	0.049

ments, which is increasing as precision technologies are becoming more common worldwide.

GWAS of Workability Traits Using WGS Variants

Although the genetic parameters of workability traits in dairy cattle have been studied for more than 3 decades (Agyemang et al., 1982; Erf et al., 1992), their underlying genetic architecture has remained less known compared with other economically important traits in dairy cattle. Both MT and MS were first subjected to GWAS in Dutch Holstein cattle in 2000 using a few hundred microsatellite markers (Schrooten et al., 2000), in which only 3 candidate genomic regions for MS were reported. Later, the 50k and 770k SNP panels were used for scanning candidate genes and QTL as-

sociated with MT and MS in North American Holstein (Abo-Ismael et al., 2014), Brown Swiss (Kramer et al., 2014), and French Holstein (Marete et al., 2018) cattle. However, the detection power and mapping precision of causal mutations are relatively low when using low-density markers. This can be considerably improved by using WGS variants from a large number of individuals (Wu et al., 2017). In this study, we used imputed WGS variants to perform GWAS for MT and MS in over 4,000 North American Holstein cows. After QC, these WGS variants were distributed across all autosomes with mean pairwise distance of ~1.3 kb, which facilitates the detection and fine-mapping of candidate mutations and genes. A similar study recently used imputed WGS variants for GWAS of udder index and MS in Nordic Holstein cattle (Jardim et al., 2018).

Figure 4. Functionally implicated genes and SNP with differentially distributed allele frequencies between taurine and indicine breeds associated with milking temperament, based on an independent data set. The association results for each gene are zoomed out on the bottom of panel with y-axis showing log-transformed P -values, in which red point(s) represent the statistically significant SNP. The gene structure and orientation (+, forward; -, reverse) are shown on the middle of panel, and exons and introns are denoted by the blue boxes and solid lines, respectively. Above the gene structure, sequence logos indicate the statistically significant differences of allele frequency between taurine (*Tau*) and indicine (*Ind*) breeds. The height of nucleotides is scaled and measured in bits. SNP positions are named by referring to coding sequence with nonsynonymous mutations in bold. A total of 8 genes are presented in panels A–F, with their respective gene names labeled under the structure diagram.

In GWAS, potential confounding factors, such as population stratification and extrinsic bias of genotyping approaches, must be properly accounted for to avoid false positive associations (Sul et al., 2018). Therefore, we calculated the individual's genetic relationships from a random subset of SNP and included them into MLM that were implemented using the GCTA package (Yang et al., 2011). In this regard, the potential confounding factors have been well controlled for both MT and MS traits, according to the estimated genomic inflation factors and QQ plots. Appropriate accounting for confounding factors can be observed when the genomic inflation factor is lower than 1.1 (Himes et al., 2009; Yang et al., 2011), which is the case in the present study. Another issue in GWAS is the adjustment of multiple testing for controlling Type I and Type II errors. In this study, we used a less stringent strategy by dividing the estimated number of independent chromosome segments, which is associated with the effective population size and chromosome length. This strategy has also been employed in other GWAS studies using imputed WGS variants, such as de Vlaming et al. (2017), van den Berg et al. (2019), and Sanchez et al. (2020).

Candidate Genes for Milking Temperament

Although MT is usually defined as a behavioral response to handling and constrain during milking, it can also indicate the overall cow temperament or personality. In this context, a recent study reported that behavioral traits are well consistent during early and late life stages in cattle (Neave et al., 2020). The moderate genetic contributions to individual differences on behavioral characteristics have been widely reported in the literature (Schmutz et al., 2001; Sewalem et al., 2011; Haskell et al., 2014; Chang et al., 2020). Due to the feasibility of genomic selection and its anticipated economic benefits, some efforts have been made for mapping QTL associated with MT in dairy cattle over the past 2 decades. There are significant genetic markers associated with behavior-related traits in both dairy and beef cattle located in almost all autosomal chromosomes. However, very few genomic regions have been confidently identified in multiple publications, except for BTA29 (Glenske et al., 2011). Significant SNP were also detected in this study over 19 chromosomes using imputed WGS data, but none of them overlapped with the reported QTL in the literature or records available in the Animal QTLdb (Hu et al., 2019). This indicates that MT has a polygenic inheritance mode. Furthermore, the differences observed across studies can be a result of population heterogeneity or indicate a relatively high ratio of false positives among the reported

results (Visscher et al., 2017). Among 29 autosomes, significant markers located in BTA17 and BTA27 were reported for the first time in this study.

Individual temperament is regulated by neurophysiological processes. In humans, several candidate genes have been proposed to be related to behavior (Anholt and Mackay, 2012; Veroude et al., 2016). In this study, we identified 8 novel candidate genes that are likely associated with MT in Holstein cattle, including *GRI-N3A*, *KCNJ3*, *BOSTAUV1R417*, *BOSTAUV1R419*, *MAP2K5*, *CTD3*, *GAP43*, and *LSAMP*. The *GRIN3A* and *KCNJ3* genes encode the 3A subunit of *N*-methyl-*D*-aspartate glutamate receptors and a member of G protein-gated inwardly rectifying potassium channels, which are located in the postsynaptic and presynaptic membrane, respectively. Therefore, *GRIN3A* and *KCNJ3* play fundamental roles in the glutamatergic synapse regulation and are involved in human cognitive disorders (Yamada et al., 2012; Takata et al., 2013; Volk et al., 2015). Kukekova et al. (2018), recently identified a significant association between the *KCNJ3* and *GRIN2B* (which is a coordinate member for *GRIN3A*) genes with tame and aggressive behaviors in red fox (*Vulpes vulpes*). We also observed 2 vomeronasal type-1 receptor (V1R) genes (*BOSTAUV1R417* and *BOSTAUV1R419*), which suggest that the chemosensory vomeronasal organ would participate in the expression of MT in cattle (Ibarra-Soria et al., 2014). The reduced aggressive behavior was observed by the depletion of a cluster of V1R genes in mice (Del Punta et al., 2002). The oxytocin signaling pathway, which has been widely involved in animal and human reproductive and social behavior (Grinevich et al., 2016; Jurek and Neumann, 2018), was identified and included the *MAP2K5* gene. One SNP in the microRNA target site of *MAP2K5* has been associated with anxiety and depressive disorders in human (Jensen et al., 2014). The *KCTD3* gene and other members of the potassium channel tetramerization domain family have been found in human neurodevelopmental and neuropsychiatric disorders (Teng et al., 2019). The 2 adjacent genes, *GAP43* and *LSAMP*, identified on BTA1 play important roles in regulating neuronal development, for which the altered or depleted expression were significantly associated with emotional and social behavior (Shen et al., 2012; Philips et al., 2015). In summary, we speculate that these candidate genes would influence MT in Holstein cattle by participating in molecular regulation of signaling transduction and neural response.

In addition to the novel candidate genes mentioned above, the 2 most likely candidate genes (*TYR* on BTA29: 6,326,410–6,435,488 bp, and dopamine receptor D4, *DRD4*, on BTA29: 50,667,785–50,673,523 bp), which were formerly reported to be associated with

temperament traits in cattle (Hiendler et al., 2003; Garza-Brenner et al., 2017), were not detected in this study. Only one significant SNP on BTA29 was detected, which is distantly located from the 2 candidate genes. Meanwhile, the novel candidate gene, neural EGFL-like 1 (*NELL1*), located on BTA29, has no known functional implication with respect to temperament according to the functional enrichment analysis, as well as literature evidence (Li et al., 2018). The genetic variation in temperament traits have been reported not only within but also across breeds (and subspecies), and taurine breeds tend to be relatively more docile than indicine breeds (Haskell et al., 2014). Therefore, coding and upstream SNP whose allele frequencies are significantly differentiated between taurine and indicine breeds were further systematically revealed in this study. These candidate SNP should be further investigated using resequencing of these regions in a large number of animals with divergent breeding values for temperament traits. Animal age is a factor that can influence MT. However, records from later parities were not available in this population. It is suggested that future studies investigate the genomic background of MT in later parities, but care should be taken with regards to possible preferential treatment or culling of cows with undesirable temperament, as this could bias the results.

Candidate Genes for Milking Speed

Although elevated MS can save milking time and increase economic efficiency, one main concern is the unfavorable genetic correlations of MS with both susceptibility to clinical mastitis and somatic cell score (Martin et al., 2018). Therefore, the accurate identification of causal mutations and genes is a feasible approach to genetically improve MS without leading to the deleterious consequences on health conditions. Using SNP chip panels and imputed WGS variants, few GWAS analyses have been performed for MS in dairy cattle. These GWAS have identified significant markers on all autosomes, except BTA15. Among them, BTA6 (Guo et al., 2012), BTA14 (Marete et al., 2018), and BTA19 (Jardim et al., 2018) had the strongest associations. In this study, SNP significantly associated with MS were identified across 19 chromosomes and did not overlap with any known QTL for MS trait. The most significant associations found in this study were observed on BTA9, BTA14, and BTA20. These findings suggest that the associations reported for MS in dairy cattle are not well replicable among different studies even at the chromosome level. This might be due to multiple factors, such as population heterogeneity, polygenic architecture of the trait, analytical

limitations, trait measurement differences, and others (Visscher et al., 2017).

In comparison to MT, the underlying molecular mechanism of MS and related candidate genes are less known. The most well-known biological process is the oxytocin-mediated milk-ejection reflex that stimulates milk ejection from alveoli into cisternal cavities and hence promotes the rapid and complete milk removal (Bruckmaier and Blum, 1996). As a neuroendocrine response to the teat stimulation, milk-ejection reflex can be disrupted by the reduced release of oxytocin from pituitary into the blood circulation or local blockade of oxytocin effects on mammary gland cells (Bruckmaier, 2005). Many candidate genes have been proposed to be associated with MS in dairy cattle, such as the *ZFAT*, potassium voltage-gated channel subfamily B member 2 (*KCNB2*), and mitogen-activated protein kinase 10 (*MAPK10*) (Guo et al., 2012; Jardim et al., 2018; Marete et al., 2018). However, few of them are known to be functionally involved in the oxytocin-related biological processes. In this study, no clear biological insight among the detected candidate genes was obtained for MS. One of the possible explanations is that the mechanical and biological determinants of MS in dairy cattle are not well known yet, which therefore complicate functional dissection for the identified candidate genes. Similar to former studies (Guo et al., 2012; Marete et al., 2018), few genes were detected in this study, which are associated with immune responses, including nonclassical MHC class I antigen (*BOLA-NC1*) and major histocompatibility complex class I (*LOC512672*). This provides further support on the link between MS and udder health.

Implications and Limitations of the Results

In this study, significant SNP and candidate genes associated with MT and MS in dairy cattle were comprehensively revealed, which would have 2 possible implications. First, these significant SNP and genomic regions could be incorporated into genomic prediction models by giving greater weights to SNP located in these genomic regions, by using the weighted single-step GBLUP (Zhang et al., 2016) or Bayesian (van den Berg et al., 2020) approaches. The prior information-based differential weightings of SNPs have been suggested to be helpful for improving the accuracy of genomic breeding values (Fragomeni et al., 2017; Teissier et al., 2018). Second, these genomic regions should be preferably included in functional studies on the molecular regulation mechanism for these traits. For example, less is known about the functional mechanism of the *KCNJ3* gene in influencing individual behavioral mechanisms, although

its genetic association has been consistently found in humans (Yamada et al., 2012), red fox (Kukekova et al., 2018), and dairy cattle (in this study). Furthermore, the variants identified here could be included in commercial SNP chip panels to improve the accuracy of genomic predictions for MS and MT.

The WGS imputation accuracy can affect the statistical power of GWAS, and it is mainly affected by the MAF and genetic relatedness between imputed (or target) animals and the reference population (Larmer et al., 2017). To reduce unfavorable influences of the poorly imputed variants and animals on subsequent GWAS results, robust QC, such as removing rare variants with a MAF lower than 1% and individuals with genotype missing rate greater than 10% was performed here. However, it is also expected that some low-frequency variants could be associated with MT and MS if true WGS variants had been used. Based on results of this study and former publications as discussed above, it is possible to speculate that both MT and MS are highly polygenic traits without obvious major genes in dairy cattle. With the availability of high-throughput precision technologies, it is likely that both MS and MT can be assessed in a more object manner, which could facilitate the detection of important genomic regions in future studies.

CONCLUSIONS

Milking temperament and milking speed in Holstein cattle were shown to have a clear polygenic inheritance mode, with several significant SNPs located across 19 chromosomes for each trait. A total of 8 candidate genes (*GRIN3A*, *KCNJ3*, *BOSTAUV1R417*, *BOSTAUV1R419*, *MAP2K5*, *KCTD3*, *GAP43*, and *LSAMP*) were suggested to have clear functional implications in MT. Important biological pathways were identified for MT (e.g., glutamatergic synapse pathway and oxytocin signaling pathway) and MS (e.g., cellular response to hormone stimulus and immune response). Various candidate genes identified were previously reported to be associated with behavioral traits in humans and other species. These findings contribute to a better understanding of the biological mechanisms underlying phenotypic expression of MT and MS in dairy cattle and will be useful in the optimization of genomic prediction of breeding values for these 2 traits.

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