Genome-wide association study using haplotype libraries and repeated-measures model to identify candidate genomic regions for stillbirth in Holstein cattle

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ABSTRACT

Reduced fertility is one of the main causes of economic losses on dairy farms, resulting in economic losses estimated at \$938 per stillbirth case in Holstein herds. The identification of genomic regions associated with stillbirth could help to develop better management and breeding strategies aimed to reduce the frequency of undesirable gestation outcomes. Here, 10,570 cows and 50,541 birth records were used to perform a haplotype-based GWAS. A total of 41 significantly associated pseudo-SNPs (haplotypes within haplotype blocks converted to a binary classification) were identified after Bonferroni adjustment for multiple tests. A total of 117 positional candidate genes were annotated within or close (in a 200-kb interval) to significant pseudo-SNPs (haplotype blocks). The guilt-by-association functional prioritization identified 31 potential functional candidate genes for reproductive performance out of the 117 positional candidate genes annotated. These genes play crucial roles in biological processes associated with pregnancy persistence, fetus development, immune response, among others. These results helped us to better understand the genetic basis of stillbirth in dairy cattle and may be useful for the prediction of stillbirth in Holstein cattle, helping to reduce the related economic losses caused by this phenotype.

Key words: functional candidate gene, genome-wide association study, haplotype, Holstein, stillbirth

INTRODUCTION

Reduced fertility is one of the main causes of economic losses on dairy farms. Stillbirth is defined as calf death just before or during parturition, or within

48 h after birth (Berglund et al., 2003). The financial losses are estimated to be \$938 per stillbirth case in Holstein herds (Mahnani et al., 2018). The frequency of stillbirth events is an indicator of welfare shortfalls in dairy herds and must be controlled in the herds. Usually, genetic studies investigate stillbirth using only the first birth record due to the higher heritability (h^2) of first parities ($h^2 = 0.11$) compared with later parities ($h^2 = 0.01$; Guarini et al., 2018). However, the same cow may show different outcomes in different gestations. Therefore, evaluation of a single time point may provide a biased indication of the genetic causes of stillbirth. Consequently, the use of statistical models that consider all the birth records of the cows is an interesting alternative to study the genetic causes of stillbirth events. The identification of genomic regions associated with stillbirth could help to develop better management and breeding strategies aimed to reduce the frequency of this undesirable gestation outcome. The use of haplotype libraries for GWAS can increase detection power of candidate regions due to an increased genetic variation captured by haplotypes and the finer mapping of the candidate regions (Barendse, 2011; Wu et al., 2014; Chen et al., 2018; Id-Lahoucine et al., 2019; Feitosa et al., 2021). In livestock species, the haplotype and repeated record approaches are not widely applied due to several factors, such as the complexity to estimate haplotype blocks, low SNP panel density, and the common focus on first-parity cows.

Therefore, the objectives of this work were (1) to identify candidate genomic regions associated with stillbirth using a haplotype library and a repeated measures model and (2) to prioritize potential functional candidate genes associated with biological processes associated with stillbirth in dairy cattle. The combination of both approaches, the use of repeated measures and haplotype-based association studies, is hypothesized to help identify candidate regions for stillbirth in the cattle genome, providing a better understanding of the biological basis of reproduction in dairy cattle.

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MATERIALS AND METHODS

Phenotypic and Genotypic Data

The phenotypic and genotypic information of 61,264 cows were provided by Lactanet. A total of 136,282 stillbirth records from Holstein cows collected between April 1997 and June 2019 were analyzed. The stillbirth events were defined as a calf born dead or a calf that died in the first 48 h after calving. All the animals were imputed and phased for the 50,925 SNP markers composing the BovineSNP50K BeadChip (Illumina Inc.) array using the FImpute software version 2.2 (Sargolzaei et al., 2011). The original SNP panels used to genotype the animals before imputation and the number of animals per panel are shown in Supplemental Table S1 (<https://data.mendeley.com/datasets/nnp5hdy9cv/1>; Fonseca et al., 2021). All phenotypic and genotypic data used in this study were obtained from pre-existing databases. Therefore, no animal care committee approval was necessary for the purposes of this study.

Haplotype Block Detection and Pseudo-SNP Encoding

The haplotype blocks were detected using the Haploview software version 4.2 (Barrett et al., 2005). The command line option from Haploview was used, and the haplotype blocks were called using the Gabriel blocks algorithm (Gabriel et al., 2002). Only autosomal markers with Hardy-Weinberg equilibrium test probability threshold $> 10^{-6}$, minor allelic frequency > 0.01 , and maximum distance between markers equal to 2 kb were kept for the analyses. In addition, only haplotypes with a frequency higher than 0.01 were retained for further analyses.

The haplotype blocks were mapped against the phased genotypes, and the 2 individual haplotypes in a haplotype block were extracted for each individual. Subsequently, the number of copies of each haplotype within each haplotype block was counted for each individual, creating a pseudo-SNP matrix (haplotypes within haplotypic blocks converted to a binary classification). The pseudo-SNP matrix was then used in the GWAS, and the association of each pseudo-SNP with the stillbirth events was tested as described below.

Haplotype-Based GWAS Using Repeated Measures for Stillbirth

The software ASReml 4.1 (Gilmour et al., 2015) was used to identify the pseudo-SNPs associated with the stillbirth events. For this analysis, only cows with 3 or

more records were retained (i.e., 10,570 cows and 50,541 records). The statistical model used was as follows:

$$y_{ijklmnopq} = \mu + Cs_i + Csex_j + Ca_k + Psnpl_l + HYS_m + Cadd_n + SS_o + Pe_p + e_{ijklmnopq}$$

where $y_{ijklmnopq}$ is the q th phenotypic observation of the n th cow; μ is the overall mean, Cs_i is the effect of i th calf size, $Csex_j$ is the effect of the j th calf sex, Ca_k is the effect of the k th cow age class (<36 , $36-50$, $50-63$, $63-75$, ≥ 75 mo), and $Psnpl_l$ is the allele substitution effect of the l th pseudo-SNP effect, which were all treated as fixed effects; HYS_m is the random effect of the m th herd-year-season, $Cadd_n$ is the random additive genetic effect of the n th cow, and SS_o is the random additive genetic effect of the o th service sire. Additionally, Pe_p is the random permanent environment effect of the p th cow with records, and $e_{ijklmnopq}$ is the random residual effect associated with the q th phenotypic observation. The cow age classes were defined as described by Jamrozik et al. (2016). The additive relationship matrix based on pedigree (21,278 animals, from which 11,436 were parents, going back 1 generation) was used for modeling the covariance among additive polygenic animal effects. The significantly associated pseudo-SNPs were identified using Bonferroni adjustment for multiple tests at a 5% genome-wide significance level.

Functional Candidate Genes Search

The positional candidate genes (genes annotated within and in a 200-kb interval, 100 kb downstream and upstream from the start and end haplotypes coordinates) were annotated using the R package GALLO (Fonseca et al., 2020a). The cattle QTL annotated were annotated using the haplotype coordinates, without additional intervals due to the extensive length of some QTL, from the Animal QTLdb database (<https://www.animalgenome.org/cgi-bin/QTLdb/index>; Hu et al., 2019). The QTL enrichment analysis was performed using GALLO as described by Fonseca et al. (2020a). A guilt-by-association approach was performed to identify potential functional candidate genes (Fonseca et al., 2020b; Martins de Carvalho et al., 2020; Sweett et al., 2020; Lam et al., 2021). Briefly, a trained list containing genes associated with stillbirth and pregnancy persistence was obtained from GUILDify (Guney et al., 2014), using the following list of keywords: stillbirth, pregnancy, and pregnancy loss. In the current study, the top 100 genes with the highest guild scores were selected to compose the trained list. Subsequently, TopGene suit (Chen et al., 2009) was used to perform a functional annotation from both trained list and posi-

tional candidate gene list annotated from the significant haplotypes. A multivariate analysis was performed to associate the functional profile of each positional candidate gene with the functional profile of the trained list. The functional profile was created using information from gene ontology terms for the 3 main categories of molecular function, biological process, and cellular component; human and mouse phenotypes; metabolic pathways; Pubmed publications; and diseases from ToppGene database (Chen et al., 2009; Cánovas et al., 2012; Fonseca et al., 2018). An overall P -value was calculated using a statistical meta-analysis. Subsequently, the genes with a significant P -value, after a 5% false discovery rate adjustment for multiple tests, were selected as prioritized genes. The complete description of the meta-analysis to calculate the final P -value is available in Chen et al. (2009).

RESULTS

Phenotypic Data Distribution

The data set used for the GWAS was composed of 50,541 birth records from 10,570 Holstein cows (cows with 3 or more birth records). A frequency of 11.47% stillbirth cases (5,796 cases) was identified among the 50,541 birth records. An average ($\pm$ SD) of 4.78 ± 1.31 records per individual, with a maximum of 13 records, was observed in this data set. The mean of stillbirth events per individual was $0.55 (\pm 0.59)$, with a minimum number of stillbirth events of 0 and a maximum of 5. The number of animals by number stillbirth events was as follows: 5,285 animals with 0 events, 4,808 animals with 1 event, 445 animals with 2 events, 31 animals with 3 events, and 1 animal with 5 events.

Haplotypes Significantly Associated with Stillbirth

An average of 60.8 haplotype blocks were identified per chromosome. The chromosomes BTA1 and BTA28 had the largest and smallest number of haplotype blocks with 145 and 16, respectively (Supplemental Table S2, <https://data.mendeley.com/datasets/nnp5hdy9cv/1>; Fonseca et al., 2021). The distribution of number of markers and haplotype length (in kb) are shown in Figure 1. The maximum average number of markers per haplotype block was identified in BTA8 (4.9) and the maximum average length was identified in BTA5 (198.9 kb). A total of 41 significantly associated pseudo-SNPs were identified after Bonferroni correction (Figure 2 and Supplemental Table S3, <https://data.mendeley.com/datasets/nnp5hdy9cv/1>; Fonseca et al., 2021). A total of 117 genes were annotated within the significant haplotypes (Supplemental Table S4, <https://data.mendeley.com/datasets/nnp5hdy9cv/1>; Fonseca et al., 2021).

[.mendeley.com/datasets/nnp5hdy9cv/1](https://data.mendeley.com/datasets/nnp5hdy9cv/1); Fonseca et al., 2021).

Functional Annotation of Candidate Regions

There were 31 genes, out of 117 genes annotated within the significant haplotypes, significantly associated with the functional profile of the trained list in the guilt-by-association analysis. A total of 20 unique pseudo-SNPs were mapped within the coordinates of these 31 functional candidate genes. The functional profile of the trained list and the prioritization results can be found in Supplemental Tables S5 and S6 (<https://data.mendeley.com/datasets/nnp5hdy9cv/1>; Fonseca et al., 2021), respectively. The trained list was composed of genes directly associated with the regulation of fertility and embryonic development, substantiating the group of genes chosen to compose the trained list. The list of prioritized genes and each one of the haplotypes mapped within the coordinates of the functional candidate genes are presented in Table 1.

Enrichment Analysis for Quantitative Trait Loci Within the Candidate Genomic Regions

The QTL annotation analysis using the list of 41 significantly associated pseudo-SNPs (Supplemental Table S7, <https://data.mendeley.com/datasets/nnp5hdy9cv/1>; Fonseca et al., 2021) indicated that the majority of the QTL annotated in these regions are mainly related to reproductive processes, accounting for 33.5% of all QTL classes (Figure 3). Among them, “calving ease,” “interval to the first estrus after calving,” and “interval from first to last insemination” are the most frequently annotated traits within those candidate regions. Additionally, “stillbirth” and “stillbirth (maternal)” QTL were also previously reported within the candidate regions identified in the present study.

The list of QTL annotated within the significantly associated haplotypes was also used to perform an enrichment analysis to test if candidate genomic coordinates were associated with specific traits more than expected by chance. The enrichment analysis was performed for each trait annotated using the Animal QTLdb database and the R package GALLO for each chromosome individually, helping to reduce the specialization bias observed on some chromosomes such as the BTA14 for milk production (Bennewitz et al., 2004). The QTL enrichment analysis indicated a total of 45 enriched QTL (Supplemental Table S8, <https://data.mendeley.com/datasets/nnp5hdy9cv/1>; Fonseca et al., 2021), from which 13 were reproduction-related QTL (Figure 4). The BTA5 and BTA14 were those with the largest number of reproduction-related QTL identified as

Figure 1. Violin plots showing the haplotype block distribution for number of markers in the block (a) and its length in kb (b) per chromosome (CHR). The shapes around the boxplots represent the kernel density for the data distribution, and the dots represent records above the 1.5 interquartile range threshold.

Figure 2. Manhattan plot for the GWAS using haplotype libraries for stillbirth. The y-axis presents the nonadjusted P -values, and the x-axis shows the chromosomes. The dashed horizontal line delimits the Bonferroni adjustment for multiple tests threshold.

Table 1. Functional profile of prioritized candidate genes mapped within and close to (within a 200-kb interval) haplotype coordinates significantly (Bonferroni adjustment for multiple tests at a 5% genome-wide significance level) associated with stillbirth events

Gene	Haplotype	Wald F-statistic	Adjusted P-value	Fertility-related function or evidence	Reference
<i>HBS1L</i>	9:72923043-73175040	44.66	3.23 ⁻⁷	Controls fetal and maternal hemoglobin levels, higher levels of which are associated with higher risk of stillbirth	Blankson et al., 1993; Stephaansson et al., 2000; Bhattarai et al., 2019 Vassalli et al., 1991
<i>PLG</i>	9:96487958-96605772	41.43	1.68 ⁻⁶	Regulates plasminogen levels, responsible for degradation of intravascular thrombi and extracellular matrices	
<i>MECOM</i>	1:98318674-98615181	33.28	1.10 ⁻⁴	Mutations on <i>MECOM</i> cause thrombocytopenia, abnormal low levels of platelets, and aplastic anemia, which are associated with worse pregnancy outcomes	Parnas et al., 2006
<i>GRM1</i>	9:83383631-83534942	33.18	1.13 ⁻⁴	Protective potential against periventricular leukomalacia in premature infants	Jantzie et al., 2010
<i>PEX3</i>	9:80738394-81029512	31.29	3.00 ⁻⁴	Mutations on <i>PEX3</i> are associated with the development of Zellweger syndrome, which are associated with stillbirth	Wapner and Lewis, 2002; Lee et al., 2020
<i>ORC5</i>	4:45313027-45597060	31.09	3.31 ⁻⁴	Identified as differentially expressed in preeclampsia cases during a meta-analysis	Sitras et al., 2015
<i>KDM4C</i>	8:37579398-38343034	29.06	9.45 ⁻⁴	Crucial for preimplantation development and potential interaction with hormone signaling and hypoxia	Sankar et al., 2017; Krieg et al., 2018
<i>GLDC</i>	8:37579398-38343034	29.06	9.45 ⁻⁴	Mutations in <i>GLDC</i> are associated with partially penetrant neural tube defects	Narisawa et al., 2012
<i>SYNJ2</i>	9:94536565-94552504	27.94	1.68 ⁻³	Association with number of piglets born alive	Jiang et al., 2020
<i>IFT57</i>	1:52880869-53135919	27.27	2.41 ⁻³	Mutations associated with orofaciogigital and skeletal dysplasia, and differentially expressed pre- and postpuberty in the uterus of <i>Bos indicus</i> heifers	Thevenon et al., 2016; Fortes et al., 2018
<i>AHI1</i>	9:73314138-73624020	26.49	3.56 ⁻³	Mutations in <i>AHI1</i> cause Joubert syndrome, associated with neurological disorder related to cerebellar hypoplasia	Valente et al., 2006
<i>CACNA1C</i>	5:108312689-108749971	24.98	7.76 ⁻³	<i>CACNA1C</i> mutations cause LQTS type 8 (Timothy syndrome), one of the leading causes of sudden cardiac death in early life	Fukuyama et al., 2014; Strand et al., 2020
<i>PCMTD1</i>	14:20893054-21022129	24.05	1.25 ⁻²	Expressed in the endometrial tissue of high- and low-fertility heifers and associated with puberty age in both male and female cattle	Fortes et al., 2013; Killeen et al., 2014; Mota et al., 2017
<i>DISC1</i>	28:4545373-4689264	23.88	1.36 ⁻²	Variants on <i>DISC1</i> are an important factor for schizophrenia-related behavioral (SRB) phenotypes, and maternal immune activation during gestation interacts with <i>DISC1</i> . In addition, women with SRB have an increased risk of worse pregnancy outcomes.	Jablensky et al., 2005; Lipina et al., 2013
<i>NDUFB10</i>	25:1488094-1735703	23.31	1.84 ⁻²	Differentially expressed in the endometrial tissue of heifers with low circulating progesterone concentration	Forde et al., 2012
<i>RPS2</i>	25:1488094-1735703	23.31	1.84 ⁻²	Differentially expressed between the endometrial tissue of pregnant and nonpregnant ewes	Zhao et al., 2015
<i>GFER</i>	25:1488094-1735703	23.31	1.84 ⁻²	Preserves mouse embryonic stem cell mitochondrial morphology and function	Todd et al., 2010
<i>SLC9A3R2</i>	25:1488094-1735703	23.31	1.84 ⁻²	Hypomethylated in cord blood DNA of hypertensive disorders of pregnancy samples	Kazmi et al., 2019
<i>TSC2</i>	25:1488094-1735703	23.31	1.84 ⁻²	Mutations in <i>TSC2</i> result in tuberous sclerosis associated with missed abortion and fetal abnormalities	Zhao et al., 2020
<i>SNAI2</i>	14:19654205-19945845	23.05	2.11 ⁻²	Overexpression of <i>SNAI2</i> in mice results in a 20% incidence of sudden death, cardiomegaly, and cardiac failure	Pérez-Mancera et al., 2006
<i>RAB35</i>	17:62414020-62627915	23.02	2.14 ⁻²	Depletion of <i>RAB35</i> affects spindle formation and actin-based spindle migration during oocyte meiosis in mice	Zhang et al., 2019
<i>GCN1</i>	17:62414020-62627915	23.02	2.14 ⁻²	Regulates cell cycle and proliferation during embryonic development. Additionally, it is crucial for <i>GCN2</i> activation, which in knockout mice results in an increase of neonatal and prenatal mortalities.	Zhang et al., 2002; Yamazaki et al., 2020

Continued

Table 1 (Continued). Functional profile of prioritized candidate genes mapped within and close to (within a 200-kb interval) haplotype coordinates significantly (Bonferroni adjustment for multiple tests at a 5% genome-wide significance level) associated with stillbirth events

Gene	Haplotype	Wald F-statistic	Adjusted P-value	Fertility-related function or evidence	Reference
<i>RPLP0</i>	17:62414020-62627915	23.02	2.14 ⁻²	Upregulated in resorbed bovine embryos and dominantly expressed in the mammary vasculature during lactation	El-Sayed et al., 2006; Lee et al., 2010
<i>PXN</i>	17:62414020-62627915	23.02	2.14 ⁻²	Plays an important role in the implantation stage through its disassembly, resulting in the loss of uterine epithelial cells. Additionally, it is associated with perinatal pulmonary arterial remodeling.	Diagne et al., 2003; Kaneko et al., 2008
<i>SIRT4</i>	17:62414020-62627915	23.02	2.14 ⁻²	Member of sirtuin family (<i>SIRT1</i> , <i>SIRT2</i> , and <i>SIRT3</i>), previously associated with term labor, with potential roles in the regulation of pregnancy and parturition	Lappas et al., 2011; Lim et al., 2016
<i>CYP7A1</i>	14:24396306-24825146	22.48	2.84 ⁻²	Controls bile acid production, which is increased during pregnancy, necessary for lipids and lipid-soluble vitamin absorption. However, hypercholeolemia can lead to intrahepatic cholestasis of pregnancy, which increases the risk of fetal distress, spontaneous preterm delivery, and stillbirth.	Glantz et al., 2004; Moscovitz et al., 2016
<i>SDCBP</i> <i>ACADM</i>	14:24396306-24825146 3:69084257-69175016	22.48 22.36	2.84 ⁻² 3.01 ⁻²	Upregulated in the myometrium of pregnant women Mutations on <i>ACADM</i> are associated with medium-chain acyl-CoA dehydrogenase deficiency, which is associated with increased morbidity, mortality, and sudden infant death	Rehman et al., 2003 McConkie-Rosell and Iaifolla, 1993; Maier et al., 2005
<i>MLLT3</i>	8:23670246-23814719	22.23	3.22 ⁻²	Differentially expressed in the bovine endometrium during the luteal and implantation stage, and differentially expressed pre- and postpuberty in the uterus of <i>Bos indicus</i> heifers	Fortes et al., 2018; Musavi et al., 2018
<i>TNFRSF13C</i>	5:112885057-112970228	21.86	3.93 ⁻²	Plays important immunoregulatory roles in the human placenta	Hunt et al., 2010

enriched (5 and 3, respectively; Table 2). Additionally, the relationship between the enriched fertility-related QTL, the haplotypes significantly associated with stillbirth, and the genes mapped within the haplotypes is shown in the Figure 5.

DISCUSSION

The top 5 significant functional candidate genes (*HBS1L*, *PLG*, *MECOM*, *GRM1*, and *PEX3*) mapped within the haplotype blocks related to pseudo-SNPs associated with stillbirth events play important roles related to fertility and the reproduction system. Among them, the *HBS1L* gene (HBS1 like translational GTPase) mapped within the coordinates of the most significant haplotype in BTA9 (BTA9:72923043-73175040). The *HBS1L* gene is one of the main regulators of the fetal hemoglobin levels (Thein et al., 2007; Stadhouders et al., 2014). During pregnancy, fetal hemoglobin can leak into the maternal circulation, causing a spontaneous free oxygen radical accumulation, which is associated with preeclampsia in humans (Hansson et al., 2015). In addition, increased maternal hemoglobin levels are associated with higher risk of stillbirth (Lu et al., 1991; Blankson et al., 1993; Stephansson et al., 2000; Bhat-tarai et al., 2019).

The second most significant haplotype (BTA9: 96487958-96605772), also located on BTA9, mapped within the coordinates of the *PLG* gene (plasminogen). The plasminogen activator system plays a crucial role in the degradation of intravascular thrombi and extracellular matrices (Vassalli et al., 1991). The PAI-1 (plasminogen activators inhibitors 1) are the main inhibitors of these system and are responsible for inhibiting the trophoblast invasion during the pregnancy (Chakraborty et al., 2002). Mutations on *PAI-1* are associated with female fertility diseases and worse pregnancy outcome, with recurrent pregnancy losses (Ye et al., 2017).

The *MECOM* gene (MDS1 and EVI1 complex locus) is mapped in the genomic region of the third most significant haplotype located on BTA1 (BTA1: 98318674-98615181) and is crucial for the long-term hematopoietic stem cell maintenance (Zhang et al., 2011). Mutations on the *MECOM* gene cause thrombocytopenia, abnormal low levels of platelets, and aplastic anemia (Nielsen et al., 2012; Niihori et al., 2015). The development of severe cases of thrombocytopenia during the pregnancy are associated with adverse perinatal outcomes, such as intrauterine growth restrictions and stillbirth in humans (Parnas et al., 2006). Additionally, *MECOM* seems to be essential for the distal segment and renal progenitor formation, which controls nephron multiciliate cells in zebrafish; consequently, this gene

plays a crucial role in the embryo development (Li et al., 2014).

The haplotype block mapped on BTA9: 83383631–83534942 harbors the *GRM1* gene (glutamate metabotropic receptor 1), for which the gene product is called mGluR1. The expression of mGluR1 is predominantly postsynaptically in neuros of several structures of the central nervous system, such as hippocampus, hypothalamus, amygdala, among others (Shigemoto et al., 1992). The activation of the receptors for mGluR1, and another member of this family, mGluR5, results in a protective potential against periventricular leukomalacia in premature infants (Jantzie et al., 2010), which is a common abnormality found in brains of stillborns (Grafe and Kinney, 2002).

Another significantly associated pseudo-SNP is located in the haplotype block mapped on BTA9: 80738394–81029512. This haplotype block harbors the *PEX3* gene (peroxisomal biogenesis factor 3), which is responsible for peroxisomal membrane biogenesis (Matsui et al., 2013). Mutations on *PEX3* are associated with the development of Zellweger syndrome, which is characterized by the deficiency to import peroxisomal matrix proteins (Baes et al., 1997). Interestingly, the occurrence of Zellweger syndrome is associated with stillbirth in humans (Wapner and Lewis, 2002).

Additionally, on top of the most significant genes described above, the other 26 functional candidate genes were also associated with relevant biological processes or phenotypes related to fertility. For example, the genes *PCMTD1*, *NDUFB10*, *RPS2*, *SDCDB*, and *MLLT3* are differentially expressed in the endometrium of heifers in different conditions, such as in pre- and postpubertal stages (Rehman et al., 2003; Forde et al., 2012; Fortes et al., 2013, 2018; Killeen et al., 2014; Zhao et al., 2015; Mota et al., 2017; Musavi et al., 2018). In addition, *GLDC*, *IFT57*, *AHI1*, *CACNA1C*, *GFER*, *RAB35*, and *PXN* were previously associated with the regulation of the embryonic development (Diagne et al., 2003; Valente et al., 2006; Kaneko et al., 2008; Todd et al., 2010; Narisawa et al., 2012; Fukuyama et al., 2014; Thevenon et al., 2016; Zhang et al., 2019; Strand et al., 2020). Interestingly, among the functional candidate genes, *KDM4C*, *SYNJ2*, *DISC1*, *SLC9A3R2*, *TSC2*, *SIRT4*, *CYP7A1*, and *ACADM* are associated with abortion, bad pregnancy outcomes, and early live death in different species (McConkie-Rosell and Iafolia, 1993; Glantz et al., 2004; Jablensky et al., 2005; Maier et al., 2005; Lappas et al., 2011; Lipina et al., 2013; Moscovitz et al., 2016; Lim et al., 2016; Sankar et al., 2017; Krieg et al., 2018; Kazmi et al., 2019; Jiang et al., 2020; Zhao et al., 2021).

It is important to highlight the relevance of BTA9 on the results obtained here. The smallest *P*-values were identified on this chromosome, as well as the largest number of functionally prioritized candidate genes (*HBS1L*, *PLG*, *GRM1*, *PEX3*, *SYNJ2*, and *AHI1*). Additionally, the QTL enrichment analysis reinforced the involvement of the genomic regions on BTA9 associated with stillbirth with fertility-related traits through an enrichment for conception rate and daughter pregnancy rate. The enrichment for other fertility-related QTL (interval to first estrus after calving, calving ease, age at puberty, pregnancy rate, and stillbirth) on genomic regions associated with stillbirth obtained in the present study reinforced the putative association of these candidate regions with fertility in dairy cattle.

Figure 3. The QTL annotation within positional candidate haplotype blocks for stillbirth. (a) Percentage of each QTL class annotated within the 41 significantly (5% false discovery rate adjustment for multiple tests) associated pseudo-SNPs. (b) Contribution of each fertility-related trait for the total percentage of reproduction QTL (33.5%).

Figure 4. Bubble plot showing the enrichment results for the QTL annotated within the significant haplotypes after Bonferroni adjustment for multiple tests. The darker the color of the circle, the smaller the P -value; area of the circle corresponds to the number of QTL annotated. The x-axis shows the richness factor, which is a ratio between the number of each QTL class annotated and the total number of that QTL class in the QTL database. CHR = chromosome.

The inclusion of other -omics technologies can be helpful to improve the detection power of genetic components associated complex traits, such as stillbirth, due to a larger proportion of the biological processes involved in the regulation and development of these

phenotypes (Cánovas et al., 2014, 2017). Stillbirth is a complex trait with genetic and environmental components. In Canadian Holsteins, the heritability for this trait is quite low, ranging from 0.11 in first parities to 0.01 in later parities (Guarini et al., 2018). The data

Table 2. Enrichment analysis for QTL annotated within significant (5% false discovery rate adjustment for multiple tests) haplotypes after Bonferroni adjustment for multiple tests

QTL	CHR ¹	Number of QTL	FDR ²
Interval to first estrus after calving	25	37	1.28^{-11}
Calving ease	25	48	5.18^{-10}
Age at puberty	14	7	0.0002
Calving ease (maternal)	8	4	0.0023
Conception rate	9	6	0.0026
Interval to first estrus after calving	14	4	0.0028
Stillbirth (maternal)	14	2	0.0090
Pregnancy rate	20	1	0.0157
Age at puberty	7	1	0.0173
Calving ease (maternal)	5	3	0.0257
Stillbirth	5	3	0.0324
Daughter pregnancy rate	9	2	0.0385
Daughter pregnancy rate	5	3	0.0432

¹CHR = chromosome.

²FDR = false discovery rate.

Figure 5. Chord plot showing the relationship between the prioritized functional candidate genes (left-hand side) and enriched fertility-related QTL (right-hand side). On the left-hand side, following gene names, in parentheses, are the coordinates of the significant haplotypes mapped to the genes. Darker colors in the chords connecting the genes and QTL represent smaller *P*-values associated with the haplotype in the GWAS for stillbirth.

set analyzed in the current study was obtained from the same population from which the abovementioned heritabilities were calculated. Due to this low heritability, usually only the records from the first parity are used for genetic evaluations. A satisfactory detection power was obtained with the model applied in the current study using multiple birth records from the same animal, which can be a more complete and helpful approach for GWAS and animal selection.

CONCLUSIONS

The prediction of stillbirth cases using genomic data can be helpful for the cattle industry due to the

economic losses associated with this trait. The use of haplotype libraries combined with a repeated-records model in the GWAS showed to be an interesting alternative to use all available data to detect genomic regions associated with stillbirth in the cattle genome. In addition, the haplotypes significantly associated with stillbirth cases identified by the GWAS were annotated for positional functional candidate genes for reproductive performance. These genes play crucial roles in biological processes associated with pregnancy persistence, fetus development, and immune response, among others. The results reported here helped us to better understand the genetic basis of stillbirth in dairy cattle and may be useful for prediction of stillbirth in the Holstein cattle.

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