Two-stage genome-wide association study for the identification of causal variants underlying hoof disorders in cattle

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ABSTRACT

Feet and legs disorders influence dairy cattle breeding by their effect on animal welfare, economic losses due to lower production and fertility, costs of treatment, and problems with herd management. In our study, we estimated heritabilities and performed a 2-step GWAS for 3 traits describing hoof health: hoof health status defined by a veterinarian (HSV), hoof health status defined by a claw trimmer (HSC), and the total number of hoof disorders (NHD), scored in 1,998 Fleckvieh and 979 Braunvieh cows. The individuals were genotyped with a high-density (HD) panel consisting of 76,934 SNP. For significant genomic regions, the SNP information was enhanced by SNP imputed from the whole-genome sequence of Fleckvieh and Braunvieh bulls from the 1000 Bulls Genome project. The heritabilities were estimated to be 0.035 for HSV, 0.249 for HSC, and 0.279 for NHD. Based on the first-stage GWAS with SNP from the HD panel, 7 significant genomic regions on 6 chromosomes were defined: (1) 120 SNP spanning 15,522 bp on BTA1, including the *TOPBP1* gene; (2) 4,139 SNP spanning 1,426,046 bp on BTA7, including the *RIOK2* and *RGMB* genes; (3) 167 SNP spanning 167,352 bp on BTA13, including the *C13H20orf194* gene; (4) 2 regions on BTA14, one harboring 1,071 SNP spanning 380,024 bp, including *RRM2B* and *NCALD*, and the other comprising 632 SNP spanning 385,111 bp, including *STK3*; (5) 328 SNP on BTA15, spanning 235,567 bp between *FAM168A* and *PLEKHB1*; and (6) 1,549 SNP on BTA22, spanning 596,101 bp in the neighborhood of *PTPRG*. Then, we conducted a second-stage GWAS based on SNP from whole-genome sequences within the significant regions obtained in the first stage of the analysis. For HSV, the highest additive effect was estimated for 23 SNP located within a region on BTA15, close to *FAM168A*, corresponding to

a predicted gene sequence. For HSC, the highest additive effect was attributed to 44 SNP located within a region of BTA22 corresponding to 4 predicted gene sequences, with rs135082893 within a sequence encoding a microRNA. Another potential causal mutation for HSC was rs134142607 on BTA13, within the exon of *C13H20orf194*. For NHD, 33 SNP with the highest estimated effect were located on BTA7 within a region of a predicted gene positioned between *RIOK2* and *RGMB*. On BTA14, all significant SNP were located in introns of *STK3*, which is responsible for the “abnormal gait” phenotype in mice.

Key words: Braunvieh, hoof disorders, Fleckvieh, genome-wide association study (GWAS), imputation to sequence

INTRODUCTION

Feet and legs disorders influence dairy cattle breeding in various aspects. The most important is their effect on animal welfare, followed by economic losses due to lower production levels and problems with fertility, and finally by costs of treatment. Another negative effect related to these disorders is associated problems with herd management because of the incidence of involuntary culling of sick cows. Heritabilities of feet and legs disorders vary between 0.005 (Ring et al., 2018) and 0.34 (Swalve et al., 2008). Therefore, it is possible (and important) to identify genes that are responsible for variation in animals' susceptibility to feet and legs disorders. This will enable establishing genetic programs aimed at reducing the incidence of feet and legs disorders.

A key problem in the analysis of feet and legs disorders is the lack of routine recording of those traits in most countries and thus the shortage of a sufficient number of phenotypes to estimate genetic parameters and conduct GWAS. Some such studies, all relatively recent, exist (for a comprehensive review, see Herings-tad et al., 2018). Chapinal et al. (2013), Ødegård et al. (2013), van der Spek et al. (2013), Weber et al. (2013)

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Pérez-Cabal and Charfeddine (2015), Malchiodi et al. (2017) and Croué et al. (2017, 2019) estimated variance components for various traits related to feet and legs disorders. Buitenhuis et al. (2007), Swalve et al. (2014), and van der Spek et al. (2015) detected genomic regions responsible for variation in the disorders. In the latter context, the recent study of Wu et al. (2016) was especially valuable, because the authors used SNPs imputed from a 50K chip to whole-genome sequence (WGS) variants to estimate additive effects of the polymorphisms on the susceptibility to feet and legs disorders. However, despite a large sample size of 10,751 bulls representing 3 breeds and a very large number of SNPs (22,751,039), the study identified a small number of significant variants and found no overlap between the 3 analyzed breeds.

In our study, we followed Wu et al. (2016) in that we combined SNP chip and WGS information in a GWAS for hoof disorders to identify candidate genes harboring causal mutations in Braunvieh and Fleckvieh. However, to minimize the type I and type II error risk due to testing millions of single SNP–phenotype associations in a mid-sized sample, we used a 2-stage GWAS procedure.

MATERIALS AND METHODS

Data Set

The analyzed data set comprised records of hoof disorders scored on Austrian Braunvieh and Fleckvieh cows over several parities (between 1 and 13) within the frame of the Efficient Cow project (<http://en.zar.at/Projects/Efficient-Cow.html>). For each cow, 3 lameness-related traits collected until 100 DIM were considered: hoof health status defined by a veterinarian (HSV; binary), hoof health status defined by a claw trimmer (HSC; binary), and total number of hoof disorders (NHD; discrete values between 0 and 5). The incidence of hoof disorders as defined by the veterinarian varied between 3.55% in parity 1 and 7.82% in parities >4 (Figure 1). The incidence of hoof disorders defined by a claw trimmer was much higher and varied between 38.32% in parity 1 and 60.03% in parities >4 (Figure 2). In each parity, the largest fraction of cows were healthy with no hoof disorders, and the maximum number of 584 diagnoses was recorded for parities >4 (Figure 3). The number of cows with records varied between 2,977 for HSV (1,998 Fleckvieh and 979 Braunvieh) and 1,513 for HSC and NHD (1,064 Fleckvieh and 449 Braunvieh). Interestingly, after 100 DIM, no increase in disorder incidence was observed—prolonging trait recording until 300 DIM resulted in only a few additional diagnoses of hoof problems (Table 1). Therefore, only traits recorded until 100 DIM were ana-

Figure 1. The incidence of hoof health status defined by a veterinarian (HSV).

lyzed. The number of recorded parities per cow ranged between 1 and 8. Each cow was genotyped with the GeneSeek Genomic Profiler HD panel (Neogen Genomics, Lincoln, NE) consisting of 76,934 SNPs. The SNP preselection criteria comprised a minor allele frequency (MAF) of at least 0.01 and technical quality of genotyping expressed by a minimum call rate of 99%. After editing, 74,762 SNPs remained for further analysis, exhibiting an average MAF of 0.31 and an average call rate of 99.48%.

Variance Components

For NHD, variance components were estimated using the following linear mixed model:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}_u\mathbf{u} + \mathbf{Z}_v\mathbf{v} + \mathbf{Z}_p\mathbf{p} + \boldsymbol{\epsilon}, \quad [1]$$

where $\mathbf{y}$ is a vector of all records for NHD, including multiple parities; $\boldsymbol{\beta}$ is a vector of fixed effects comprising a general mean, breed, parity (from 1 to 4 and >4), calving year-season [composed of years 2012–2015 and season 1 (October–March) and 2 (April–September)]; percent of non-Holstein-Friesian genes (varying between 0 and 49%) and hoof status recording code (0 = unknown, 1 = officer, 2 = electronic via a vet, 3 = cow owner); $\mathbf{u}$ is a random additive polygenic effect of a cow; $\mathbf{p}$ is a random permanent environmental effect; $\mathbf{v}$ is a random veterinarian effect, and $\boldsymbol{\epsilon}$ is a vector of random residuals. It was assumed that $\mathbf{u} \sim N(0, \mathbf{A}\sigma_u^2)$,

Table 1. Descriptive statistics of traits

Trait ¹	Value ²						Mean	SD
	0	1	2	3	4	5		
HSV _{DIM100}	3,545	190					0.05	0.22
HSV _{DIM300}	3,547	190					0.05	0.22
HSC _{DIM100}	1,419	1,237					0.47	0.50
HSC _{DIM300}	1,421	1,274					0.47	0.50
NHD _{DIM100}	1,419	929	229	86	23	6	0.66	0.87
NHD _{DIM300}	1,421	930	229	86	23	6	0.66	0.86

¹HSV = hoof health status defined by a veterinarian; HSC = hoof health status defined by a claw trimmer; NHD = total number of hoof disorders; DIM100 = traits scored until 100 DIM; DIM300 = traits scored until 300 DIM.

²HSV and HSC were binary traits with values of 0 and 1; NHD had discrete values from 0 to 5.

$\mathbf{v} \sim N(0, \mathbf{I}_v \sigma_v^2)$, $\mathbf{p} \sim N(0, \mathbf{I}_p \sigma_p^2)$, and $\boldsymbol{\varepsilon} \sim N(0, \mathbf{I}_\varepsilon \sigma_\varepsilon^2)$, where $\mathbf{A}$ is an additive relationship matrix among individuals calculated based on pedigree; σ_u^2 , σ_v^2 , σ_p^2 , and σ_ε^2 are variances for additive polygenic, veterinarian, permanent environmental, and residual effects, respectively; $\mathbf{I}_v$, $\mathbf{I}_p$, and $\mathbf{I}_\varepsilon$ are identity matrices with dimension equal to vectors $\mathbf{v}$, $\mathbf{p}$, and $\boldsymbol{\varepsilon}$, respectively; and $\mathbf{X}$, $\mathbf{Z}_u$, $\mathbf{Z}_v$, and $\mathbf{Z}_p$ are design matrices for $\boldsymbol{\beta}$, u , v , and p , respectively.

For the binary traits HSV and HSC, variance components were estimated using the following logistic model:

$$\text{logit}(\mathbf{p}) = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}_u u + \mathbf{Z}_v v + \mathbf{Z}_p p + \boldsymbol{\varepsilon}, \quad [2]$$

with $\mathbf{p}$ representing the probability of hoof disease diagnosis and elements on the right-hand side of model

[2] being the same as in model [1] and depending on a trait v denoting a veterinarian or a claw trimmer effect. Effects of both models were estimated using the ASReml v3.0 software (Butler et al., 2009).

First-Stage GWAS with SNPs from the HD Panel

The additive effects of SNPs from the HD panel were estimated by applying the model for NHD:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{X}_g g + \mathbf{Z}_u u + \mathbf{Z}_v v + \mathbf{Z}_p p + \boldsymbol{\varepsilon}, \quad [3]$$

and for HSV and HSC:

$$\text{logit}(\mathbf{p}) = \mathbf{X}\boldsymbol{\beta} + \mathbf{X}_g g + \mathbf{Z}_u u + \mathbf{Z}_v v + \mathbf{Z}_p p + \boldsymbol{\varepsilon}, \quad [4]$$

Figure 2. The incidence of hoof health status defined by a claw trimmer (HSC).

Figure 3. The incidence of hoof disorders (NHD).

which complemented the right-hand side of equations [1] and [2] by $\mathbf{X}_g g$, where $\mathbf{X}_g$ is a design vector for SNP genotypes, parameterized as -1 , 0 , or 1 for a homozygous, heterozygous, and an alternative homozygous SNP genotype, respectively, and g represents an additive fixed SNP effect. The procedure was repeated for each SNP separately, with the variance components corresponding to random effects assumed as known and set to the estimates obtained in models [1] and [2]. To test the hypotheses ($H_0: g = 0$ vs. $H_1: g \neq 0$), the Wald test, $W = \hat{g}/\sigma_{\hat{g}}$, was used, where $\sigma_{\hat{g}}$ is the standard error of the estimated SNP effect $\hat{g}$. Under H_0 , this statistic followed the standard normal distribution. The multiple testing correction via Bonferroni's approach (Dunnett, 1955) does not account for the intercorrelation between tests, which here arises through linkage disequilibrium (LD) between SNPs, and therefore does not guarantee a predefined experiment-wise type I error rate. Therefore, 2 methods for estimating the effective number of tests (m_e^1 and m_e^2), accounting for the LD between SNPs, were applied. Let m be the number of tests performed for each SNP and $\lambda_i (i = 1, \dots, m)$ be the eigenvalues of a pairwise LD matrix between SNPs. The first estimator is given by

$$m_e^1 = \sum_{i=1}^m [I(\lambda_i \geq 1) + (\lambda_i - |\lambda_i|)],$$

where $I(\cdot)$ is an indicator function and $[\cdot]$ is a floor function (Li and Ji, 2005). The second estimator, given by

$$m_e^2 = m - \sum_{i=1}^m I(\lambda_i > 1)(\lambda_i - 1),$$

was proposed by Li et al. (2012); m_e^1 and m_e^2 were used for multiple testing correction of nominal P -values in place of the total number of tests (m) in the Bonferroni procedure. The main advantage of using m_e^1 and m_e^2 is that they account for the intercorrelation between tests and thus are lower than m , resulting in less conservative correction of the nominal significance.

Second-Stage GWAS with SNPs from WGS

Whole-genome DNA sequences of Braunvieh (50 individuals) and of Fleckvieh (30 individuals), available through the 1000 Bulls Genome Project (Hayes and Daetwyler, 2019), were used for the imputation of SNPs of phenotyped individuals within the significant genomic regions defined by the first-stage GWAS. Im-

putation was carried out separately for each breed using Beagle 4 software (Browning and Browning 2007). A significant genomic region was defined based on the location of the most significant SNP from the HD panel and positions of the closest downstream and upstream genes. It comprised SNPs from the HD panel and imputed SNPs from WGS with MAF > 0 (Table 2). Then, the additive effect of each SNP within the significant region was estimated using the same models as used for SNPs from the HD panel.

RESULTS

The lowest heritability of 0.035 was estimated for hoof health status defined by a veterinarian (HSV). For the remaining traits, similar heritabilities of 0.249 for hoof health status defined by a claw trimmer (HSC) and 0.279 for the total number of hoof disorders (NHD) were estimated (Table 3).

The estimated number of independent tests was lowest for m_e^1 (69,421) and 71,233 for m_e^2 , which resulted in reductions of, respectively, 7.1% and 4.7% compared with the number of tests in Bonferroni correction (74,762). Practically, these reductions did not affect the selection of significant SNPs in our GWAS models. Thus, the significance threshold after Bonferroni correction was set to 0.05. The GWAS detected 2 significant SNP for HSV located on BTA14 and BTA15; 3 significant SNP for HSC located on BTA1, BTA13, and BTA22; and 2 significant SNP for NHD located on BTA7 and BTA14 (Figures 4, 5, and 6, Table 4). Based on those locations, significant genomic regions were examined in detail using SNP imputed from WGS.

For HSV, the significant region defined on BTA14 spanned 380,024 bp between rs135667758 located in the intron of *RRM2B* and rs381281819 in the intron of *NCALD* (Figure 7, Table 2). Among the 1,071 SNP located within this region, the most significant group comprised 70 neighboring SNP spanning 11,049 bp. Because of complete LD between them, no new information was gained by WGS because the region contained the most significant SNP identified in the first-stage GWAS with the HD panel. All polymorphisms were in an intergenic region downstream of *NCALD*, which additionally corresponded to an intron of a computationally predicted gene (Table 5). The second significant region for HSV was at 235,567 bp on BTA15 between rs110476174 and rs385044070 (Figure 7, Table 2). Among the 328 SNP in this region, the highest effect (and thus significance) in the stage-2 GWAS was estimated for 23 SNP in complete LD, spanning 8,055 bp of an intergenic region between *FAM168A* and *PLEKHB1*, a sequence that corresponds to a computationally predicted gene (Table 5). As for BTA14, the BTA15 SNP from WGS

Table 2. Significant regions from the high-density (HD) panel

SNP ¹	Position ²	Annotation ²
HSV		
Significant region on BTA14		
rs135667758 (773 SNPs)	14:62,135,661	Intron of <i>RRM2B</i>
rs109154693 (295 SNPs)	14:62,359,983	Most significant SNP from the HD panel, intergenic
rs381281819	14:62,515,685	Intron of <i>NCALD</i>
Significant region on BTA15		
rs110476174 (295 SNPs)	15:52,890,666	Intron of <i>FAM168A</i>
rs136200469 (30 SNPs)	15:53,093,509	Most significant SNP from the HD panel, intergenic
rs385044070	15:53,126,233	Intron of <i>PLEKHB1</i>
HSC		
Significant region on BTA1		
rs211358724 (118 SNPs)	1:135,754,567	Intron of <i>TOPBP1</i>
rs43248859	1:135,770,089	Intron of <i>TOPBP1</i>
Significant region on BTA13		
rs109106381 (42 SNPs)	13:51,793,134	Intron of c13h20orf194
rs111006940 (122 SNPs)	13:51,842,153	Most significant SNP from the HD panel, intron of c13h20orf194
rs41698910	13: 51,960,486	Intron of c13h20orf194
Significant region on BTA22		
rs383295308 (281 SNPs)	22:39,357,403	Intron of <i>PTPRG</i>
rs110147660 (1,265 SNPs)	22:39,736,166	Most significant SNP from the HD panel, intergenic
rs134216087	22:39,500,891	Intergenic
NHD		
Significant region on BTA7		
rs109301309 (4,024 SNPs)	7:96,606,759	Exon of <i>RIOK2</i>
rs109798552 (112 SNPs)	7:97,930,272	Most significant SNP from the HD panel, intergenic
rs110192805	7:98,032,823	Exon of <i>RGMB</i>
Significant region on BTA14		
rs134709130 (469 SNPs)	14:65,398,603	Intergenic
rs110532594 (160 SNPs)	14:65,709,652	Most significant SNP from the HD panel, intron of <i>STK3</i>
rs110771871	14:65,783,714	Intron of <i>STK3c</i>

¹HSV = hoof health status defined by a veterinarian; HSC = hoof health status defined by a claw trimmer; NHD = total number of hoof disorders; the significance threshold after Bonferroni correction was set to 0.05. Counts in parentheses represent the number of SNPs located between SNPs marked by their rs code.

²Chr:position; corresponding to the ARS-UCD1.2 assembly accessed via the Ensembl database release 97 (<http://jul2019.archive.ensembl.org/index.html>).

Table 3. Estimated variance components ($\pm$ standard error)

Trait ¹	Variance component				
	Additive polygenic	Permanent environmental	Veterinarian/claw trimmer	Residual	Heritability
HSV	0.002 $\pm$ 0.001	0.002 $\pm$ 0.002	10 ⁻⁶ $\pm$ 10 ⁻⁶	0.044 $\pm$ 0.002	0.035 $\pm$ 0.02
HSC	0.075 $\pm$ 0.014	0.003 $\pm$ 0.014	0.061 $\pm$ 0.026	0.162 $\pm$ 0.010	0.249 $\pm$ 0.04
NHD	0.244 $\pm$ 0.030	10 ⁻⁷ $\pm$ 10 ⁻⁸	0.168 $\pm$ 0.072	0.460 $\pm$ 0.024	0.279 $\pm$ 0.03

¹HSV = hoof health status defined by a veterinarian; HSC = hoof health status defined by a claw trimmer; NHD = total number of hoof disorders.

Figure 4. P -values for hoof health status defined by a veterinarian (HSV). The lower horizontal line corresponds to the $-\log_{10}$ of the nominal type I error probability of 5% and the upper horizontal line corresponds to the genome-wise 5% significance threshold after Bonferroni correction.

did not improve the resolution in detecting association signals because 1 of the 23 SNP was already identified in the first-stage GWAS.

Interesting results were obtained for HSC (Figure 8), albeit not within the significant region on BTA1, where, because of high inter-SNP LD, the same effect was estimated for all 120 SNP across the 15,522-bp

region covering introns and exons of *TOPBP1* (Table 2). On BTA13, where the significant region of 167,352 bp between rs109106381 and rs41698910 included 167 SNP, one of the most significant SNP (rs134142607) was located within an exon of *C13H20orf194*. On BTA22, the significant region comprised 143,488 bp, with 1,549 SNP flanked by rs383295308 and rs134216087.

Figure 5. P -values for hoof health status defined by a claw trimmer (HSC). The lower horizontal line corresponds to the $-\log_{10}$ of the nominal type I error probability of 5% and the upper horizontal line corresponds to the genome-wise 5% significance threshold after Bonferroni correction.

Figure 6. P -values for the total number of hoof disorders (NHD). The lower horizontal line corresponds to the $-\log_{10}$ of the nominal type I error probability of 5% and the upper horizontal line corresponds to the genome-wise 5% significance threshold after Bonferroni correction.

High SNP effects were located between 13:39,665,432 and 13:39,697,735, a region that includes rs135082893 within an exon of a noncoding transcript sequence for the microRNA bta-mir-2369.

For NHD (Figure 9), 1 of 2 significant regions was detected on BTA7 between rs109301309 and rs110192805 (1,426,064 bp). The 34 most significant neighboring SNP were intergenic and contained the SNP that was the most significant in the first-stage GWAS. The genomic location of those SNP (7:97,891,749–7:97,941,381) is relatively close to and downstream of an exon of

RGMB gene (a distance of 91,238 bp) and an exon of a noncoding transcript of a long non-coding RNA (a distance of 65,299 bp). It also overlaps with a sequence corresponding to a computationally predicted gene. The second significant region was located on BTA14 between rs134709130 and rs110771871; it comprised 632 SNP spanning 385,111 bp downstream and within the *STK3* gene. The most significant part of the region corresponded to an intron of *STK3* and comprised 14 SNP, including the most significant SNP from the first-stage GWAS.

Table 4. Significant SNP from the high-density panel

SNP ¹	Position ²	Effect	Risk-increasing allele	P -value ³
HSV				
rs109154693	14:62,359,983	0.5793	C	0.0101
rs136200469	15:53,093,509	0.8224	A	<0.0001
HSC				
rs43247868	1:135,826,020	0.3459	A	<0.0001
rs111006940	13:51,842,153	0.3206	G	<0.0001
rs110147660	22:39,736,166	0.3912	C	0.0003
NHD				
rs109798552	7:97,930,272	0.1375	A	0.0005
rs110532594	14:65,709,652	0.1347	A	0.0005

¹HSV = hoof health status defined by a veterinarian; HSC = hoof health status defined by a claw trimmer; NHD = total number of hoof disorders. The significance threshold after Bonferroni correction was set to 0.05.

²Chr:position; corresponding to the ARS-UCD1.2 assembly accessed via the Ensembl database release 97 (<http://jul2019.archive.ensembl.org/index.html>).

³Corrected for multiple testing using the Bonferroni correction.

Figure 7. P -values and minor allele frequencies (MAF) for SNP within the regions significant for hoof health status defined by a veterinarian (HSV). The x-axis represents SNP positions expressed in base pairs, the y-axis represents $-\log_{10}(P)$, and the insets show the genomic context of the most significant SNPs on the ARS-UCD1.2 reference assembly (<http://jul2019.archive.ensembl.org/index.html>).

DISCUSSION

Heritabilities estimated in our study fell in the broad range of parameters reported previously and recently

summarized by Heringstad et al. (2018). However, significant SNP locations showed no overlap between our study and published GWAS (van der Spek et al., 2015; Wu et al., 2016; Naderi et al., 2018; Croué et al., 2019).

A similarly low concordance was observed by Wu et al. (2016), even within the same study and using different statistical models.

The relatively high heritabilities estimated in our study for HSC and NHD indicated good selection potential for reducing the susceptibility of hoof disorders as well as the possible involvement of candidate genes with medium to large effects. The low heritability of HSV indicated a very high environmental component, which was 1.7 times higher than for the similarly defined HSC. We hypothesize that the veterinary diagnosis is related to a much more severe hoof disorder stage, which involves the effect of many environmental factors, compared with a diagnosis made by claw trimmers on an earlier stage during routine hoof trimming, which often occurs at a subclinical stage of a disorder. Given the low heritability of this trait, no strong causal signals were expected.

One drawback of our study was the limited sample size, which influenced type I and type II error rates. This is also a possible explanation for the observed heterogeneity. In contrast, because hoof health traits are not subjected to routine recording in most countries, phenotyping is a limited factor in this and in other studies related to health traits. The training sample size available for imputation to WGS might have resulted in some imputation errors and did not permit good differentiation between closely linked SNPs in the second-stage GWAS. Within each significant region, groups of SNPs were in complete LD with the same estimated effects and thus significance. The lack of

recombination events between those SNPs in the training data set did not allow for statistical differentiation among them. However, we identified genomic regions with significant effects on hoof health status and thus potential candidate genes located within or close to these regions: *TOPBP1* (BTA1); *RGMB* (BTA7); *C13H20orf194* (BTA13); *NCALD*, *RRM2B*, and *STK3* (BTA14); *FAM168A* (BTA15); and *PTPRG* (BTA22). The most interesting findings indicate 2 possible causal mutations: rs135082893 within a sequence encoding a microRNA (*bta-mir-2369*) and rs134142607 within an exon of *C13H20orf194* and in the *STK3* gene, which is involved in a locomotion process as shown by its association with an “abnormal gait” phenotype in the mouse (<http://www.mousephenotype.org>).

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Table 5. The most significant stage-two SNPs selected within the significant regions defined in the first-stage genome-wide association study

Flanking SNP position (ID) ^{1,2}	No. of SNPs in the region	Region length (bp)	Genomic annotation	Effect	P-value
HSV					
14:62,352,133 (rs41724166)–62,363,182 (rs136907591)	70	11,049	Within a predicted gene sequence, downstream of <i>NCALD</i>	3.04	0.00240
15:53,090,834 (rs210116783)–53,098,889 (rs434361479)	23	8,055	Within a predicted gene sequence, upstream of <i>FAM168A</i>	4.30	0.00002
HSC					
13:51,826,644 (rs109930398)–51,870,091 (rs385239569)	15	35,189	Intron and exon of <i>C13H20orf194</i>	3.33	0.00086
22:39,665,432 (rs137006000)–39,697,630 (rs133309559)	44	32,198	Within predicted gene sequence, within noncoding <i>bta-mir-2369</i> upstream of <i>PTPRG</i>	4.03	0.00006
NHD					
7:97,891,749 (rs383780129)–97,941,381 (rs136660809)	33	49,632	Within a predicted gene sequence, upstream of <i>RIOK2</i> , downstream of <i>RGMB</i>	4.55	0.00001
14:65,701,029 (rs134461610)–65,742,648 (rs109459948)	14	41,619	Intron of <i>STK3</i>	3.27	0.00109

¹HSV = hoof health status defined by a veterinarian; HSC = hoof health status defined by a claw trimmer; NHD = total number of hoof disorders.

²Chr:position; corresponding to the ARS-UCD1.2 assembly accessed via the Ensembl database release 97 (<http://jul2019.archive.ensembl.org/index.html>).

Figure 8. P -values and minor allele frequencies (MAF) for SNPs within the regions significant for hoof health status defined by a claw trimmer (HSC). The x-axis represents SNP positions expressed in base pairs, the y-axis represents $-\log_{10}(P)$, and the insets show the genomic context of the most significant SNPs on the ARS-UCD1.2 reference assembly (<http://jul2019.archive.ensembl.org/index.html>).

Figure 9. P -values and minor allele frequencies (MAF) for SNPs within the regions significant for the total number of hoof disorders (NHD). The x-axis represents SNP positions expressed in base pairs, the y-axis represents $-\log_{10}(P)$, and the insets show the genomic context of the most significant SNPs on the ARS-UCD1.2 reference assembly (<http://jul2019.archive.ensembl.org/index.html>).

REFERENCES

- Browning, S. R., and B. L. Browning. 2007. Rapid and accurate haplotype phasing and missing data inference for whole genome association studies by use of localized haplotype clustering. *Am. J. Hum. Genet.* 81:1084–1097. <https://doi.org/10.1086/521987>.
- Buitenhuis, A. J., M. S. Lund, J. R. Thomasen, B. Thomsen, V. H. Nielsen, C. Bendixen, and B. Guldbrandtsen. 2007. Detection of quantitative trait loci affecting lameness and leg conformation traits in Danish Holstein cattle. *J. Dairy Sci.* 90:472–481. [https://doi.org/10.3168/jds.S0022-0302\(07\)72649-8](https://doi.org/10.3168/jds.S0022-0302(07)72649-8).
- Butler, D., B. R. Cullis, A. R. Gilmour, and B. J. Gogel. 2009. AS-Reml-R Reference Manual. Department of Primary Industries and Fisheries, Brisbane, QLD, Australia.
- Chapinal, N., A. Koeck, A. Sewalem, D. F. Kelton, S. Mason, G. Cramer, and F. Miglior. 2013. Genetic parameters for hoof lesions

- and their relationship with feet and leg traits in Canadian Holstein cows. *J. Dairy Sci.* 96:2596–2604. <https://doi.org/10.3168/jds.2012-6071>.
- Croué, I., F. Fikse, K. Johansson, E. Carlén, G. Thomas, H. Leclerc, and V. Ducrocq. 2017. Genetic evaluation of claw health traits accounting for potential preselection of cows to be trimmed. *J. Dairy Sci.* 100:8197–8204. <https://doi.org/10.3168/jds.2017-13002>.
- Croué, I., A. Michenet, H. Leclerc, and V. Ducrocq. 2019. Genomic analysis of claw lesions in Holstein cows: Opportunities for genomic selection, quantitative trait locus detection, and gene identification. *J. Dairy Sci.* 102:6306–6318. <https://doi.org/10.3168/jds.2018-15979>.
- Dunnett, C. W. 1955. A multiple comparisons procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* 50:1096–1121. <https://doi.org/10.1080/01621459.1955.10501294>.
- Hayes, B. J., and H. D. Daetwyler. 2019. 1000 Bull Genomes project to map simple and complex genetic traits in cattle: Applications and outcomes. *Annu. Rev. Anim. Biosci.* 7:89–102. <https://doi.org/10.1146/annurev-animal-020518-115024>.
- Heringstad, B., C. Egger-Danner, N. Charfeddine, J. E. Pryce, K. F. Stock, J. Kofler, A. M. Sogstad, M. Holzhauer, A. Fiedler, K. Müller, P. Nielsen, G. Thomas, N. Gengler, G. de Jong, C. Ødegård, F. Malchiodi, F. Miglior, M. Alsaaod, and J. B. Cole. 2018. Invited review: Genetics and claw health: Opportunities to enhance claw health by genetic selection. *J. Dairy Sci.* 101:4801–4821. <https://doi.org/10.3168/jds.2017-13531>.
- Li, J., and L. Ji. 2005. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity* 95:221–227. <https://doi.org/10.1038/sj.hdy.6800717>.
- Li, M. X., J. M. Yeung, S. S. Cherny, and P. C. Sham. 2012. Evaluating the effective numbers of independent tests and significant p-value thresholds in commercial genotyping arrays and public imputation reference datasets. *Hum. Genet.* 131:747–756. <https://doi.org/10.1007/s00439-011-1118-2>.
- Malchiodi, F., A. Koeck, S. Mason, A. M. Christen, D. F. Kelton, F. S. Schenkel, and F. Miglior. 2017. Genetic parameters for hoof health traits estimated with linear and threshold models using alternative cohorts. *J. Dairy Sci.* 100:2828–2836. <https://doi.org/10.3168/jds.2016-11558>.
- Naderi, S., M. Bohlouli, T. Yin, and S. König. 2018. Genomic breeding values, SNP effects and gene identification for disease traits in cow training sets. *Anim. Genet.* 49:178–192. <https://doi.org/10.1111/age.12661>.
- Ødegård, C., M. Svendsen, and B. Heringstad. 2013. Genetic analyses of claw health in Norwegian Red cows. *J. Dairy Sci.* 96:7274–7283. <https://doi.org/10.3168/jds.2012-6509>.
- Pérez-Cabal, M. A., and N. Charfeddine. 2015. Models for genetic evaluations of claw health traits in Spanish dairy cattle. *J. Dairy Sci.* 98:8186–8194. <https://doi.org/10.3168/jds.2015-9562>.
- Ring, S. C., A. J. Twomey, N. Byrne, M. M. Kelleher, T. Pabiou, M. L. Doherty, and D. P. Berry. 2018. Genetic selection for hoof health traits and cow mobility scores can accelerate the rate of genetic gain in producer-scored lameness in dairy cows. *J. Dairy Sci.* 101:10034–10047. <https://doi.org/10.3168/jds.2018-15009>.
- Swalve, H. H., H. Alkhoder, and R. Pijl. 2008. Estimates of breeding values for sires based on diagnoses recorded at hoof trimming: Relationships with EBV for conformation traits. *Interbull Bull.* 38:87–90.
- Swalve, H. H., C. Floren, M. Wensch-Dorendorf, K. Schöpke, R. Pijl, K. Wimmers, and B. Brenig. 2014. A study based on records taken at time of hoof trimming reveals a strong association between the IQ motif-containing GTPase-activating protein 1 (IQGAP1) gene and sole hemorrhage in Holstein cattle. *J. Dairy Sci.* 97:507–519. <https://doi.org/10.3168/jds.2013-6997>.
- van der Spek, D., J. A. M. van Arendonk, and H. Bovenhuis. 2015. Genome-wide association study for claw disorders and trimming status in dairy cattle. *J. Dairy Sci.* 98:1286–1295. <https://doi.org/10.3168/jds.2014-8302>.
- van der Spek, D., J. A. M. van Arendonk, A. A. A. Vallee, and H. Bovenhuis. 2013. Genetic parameters for claw disorders and the effect of preselecting cows for trimming. *J. Dairy Sci.* 96:6070–6078. <https://doi.org/10.3168/jds.2013-6833>.
- Weber, A., E. Stamer, W. Junge, and G. Thaller. 2013. Genetic parameters for lameness and claw and leg diseases in dairy cows. *J. Dairy Sci.* 96:3310–3318. <https://doi.org/10.3168/jds.2012-6261>.
- Wu, X., B. Guldbbrandtsen, M. S. Lund, and G. Sahana. 2016. Association analysis for feet and legs disorders with whole genome sequence variants in 3 dairy cattle breeds. *J. Dairy Sci.* 99:7221–7231. <https://doi.org/10.3168/jds.2015-10705>.

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