Fine Mapping of Genetic Loci on *Bos taurus* autosome 18 associated with Functional Traits and Conformation Traits in German Holstein cattle

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Gutachter:

Prof. Dr. Reinhard Schröder
Institut für Biowissenschaften, Abteilung Genetik, Universität Rostock

PD Dr. Christa Kühn
Forschungsbereich Molekularbiologie, Leibniz-Institut für Nutztierbiologie, Dummerstorf

Prof. Dr. Georg Thaller

Institut für Tierzucht und Tierhaltung, Christian Albrechts-Universität zu Kiel

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1. Introduction

The domestication of cattle was a process that started around 8,000 to 11,000 years ago (Beja-Pereira et al., 2006; Burt, 2009). Since then the intraspecies evolution of cattle was influenced by adaptation and selection for differing human demands for milk, meat, leather and hides, as well as for the usability of cattle as draft animals. At present, more than 800 cattle breeds have been established based on breed related phenotypes, and cattle have become one of the most important farm animals in the world (Food and Agriculture Organization of the United States, 2009). In the 18th century, cattle breeding became organized when selective breeding was established and the first dairy cattle breeding cooperatives were founded (Hodgson, 1956; Rumler, 1981; Michaels, 2001). Nowadays, cattle are preferentially raised as livestock for dairy products, beef and hides, and as draft animals, but they are also considered as model organisms for basic studies of reproduction, metabolism and disease resistance.

Cattle breeding is aimed at the improvement of the economic efficiency of milk and beef production in respect to economical, social and natural challenges (Groen et al., 1997). Economically important traits are production traits, traits affecting the output of an animal (milk and beef) and functional traits, traits affecting the working expenses, such as disease resistance, reproduction, nutrient transformation and milkability, in other words traits that affect housing and feed diet costs, replacement costs and costs for veterinary treatments. Trait characteristics are influenced by environmental factors, such as farm management, housing and nutritional standards, and most of the economical important traits are affected by more than one genetic locus (quantitative, polygenic or complex traits). Each genetic locus that contributes to the variation within a quantitative trait is referred to as quantitative trait locus (**QTL**).

In the 20th century, the main focus in dairy cattle breeding was a higher productivity and the improvement of the economic efficiency to increase profitability and optimize the usage of

resources, in respect to reduce costs for consumers (Oltenacu and Algers, 2005). This goal was achieved by improving farm management and by selection particularly aimed at high milk yield, which resulted in a sharp increase in average milk yield over the last 50 years. In Germany, for example, the average milk yield of dairy cows increased from 2,643 kg/cow in 1951 (Arbeitsgemeinschaft Deutscher Rinderzüchter e.V., 1953) to 7,879 kg/cow in 2008 (Arbeitsgemeinschaft Deutscher Rinderzüchter e.V., 2009). The same trend of improved milk yield was observed in other countries, and well organized farms already have achieved an average milk yield of 10,000 kg/cow (Oltenacu and Algers, 2005). However, the selection on high production and the increase in milk yield was accompanied by undesirable effects on health, reproduction and longevity (Essl, 1998; Rauw et al., 1998; Oltenacu and Algers, 2005), and many studies reported unfavorable correlations between milk production traits and functional traits (Simianer et al., 1991; Lucy, 2001; Holtsmark et al., 2008). Furthermore, the consumers' concern in animal health and animal welfare, as well as the consumers' desire for low prices increased the economic pressure on dairy farms (Kalm, 2002; Williams, 2005). In dairy cattle breeding, health related traits like udder health, and reproduction traits such as the rate of stillbirth and calving ease, are traits with a substantial impact on costs for veterinary treatments and costs for replacements. Implementing these traits in new breeding schemes may have favorable effects on the economy of milk production. Especially in Europe, where low milk prices and the milk quota system additionally increased the interest in a reduction of costs for milk production, the improvement of health and reproduction traits parallel to a steady increase in productivity will be the main challenges in dairy cattle breeding in future.

1.1 Molecular tools in genetic selection

Several developments in molecular genetics have resulted in an advance in dairy cattle breeding: (i) the investigation of the bovine genome sequence (Van Tassell et al., 2008; The Bovine Genome Sequencing and Analysis Consortium et al., 2009; Zimin et al., 2009) resulted in the detection of a high number of DNA variations that are suitable for genetic analyses (Werner et al., 2004; The Bovine HapMap Consortium et al., 2009); (ii) the development of high throughput genotyping methods (Ziegle et al., 1992; Mansfield et al., 1997; Oliphant et al., 2002; Matsuzaki et al., 2004; Matukumalli et al., 2009) made genotyping of large numbers of animals cost effective; (iii) the possibility to investigate the transcriptome (Suchyta et al., 2003) and proteome of cattle (O'Donnell et al., 2004) advanced the annotation of the bovine genome (Sonstegard et al., 2002); and (iv) the establishment of respective databases made species specific data accessible to the scientific community (Fadiel et al., 2005). These developments resulted in the identification of chromosomal regions and genes affecting production, health and reproduction traits (Dennis et al., 1989; Shuster et al., 1992; Schwenger et al., 1993; Spelman et al., 1996; Berg et al., 1997; Grisart et al., 2002; Kühn et al., 2003; Rincon and Medrano, 2003; Blott et al., 2003; Schrooten et al., 2004), and the implementation of gained molecular genetic information in new breeding schemes will complement selection previously based on phenotype and pedigree information.

Two approaches are mainly used to identify chromosomal regions and genes affecting a specific trait: The candidate gene approach and the genetic mapping approach. The aim of both approaches is the identification of DNA variations that control trait specific phenotype variations. DNA variations that are verified to be associated with effects on a trait specific phenotype can be used as molecular genetic markers to select animals carrying alleles associated with favorable effects on the target trait. For this purpose the marker assisted

selection (**MAS**) is used, which, in comparison to conventional selection strategies, implements molecular genetic information in addition to phenotype and pedigree information.

1.1.1 The genetic mapping approach

The basic idea of mapping is to identify chromosomal regions, genes and polymorphisms affecting a target trait by tracking marker inheritance in families segregating for the target trait (Geldermann, 1975). For that purpose, marker information is set in correlation to phenotypes, which are quantified in the offspring within a resource population, and genetic linkage is analyzed between molecular genetic markers and target traits. If a parent is heterozygous for a QTL and for a molecular marker that is in linkage with the QTL, it is highly probable that a significant difference in the phenotype of the target trait may occur between animals that inherited different QTL alleles and that this difference is also in accordance to different inherited molecular marker alleles (Geldermann, 1975), which can be traced by molecular genetic method. The aim of the genetic mapping approach is the identification of quantitative trait loci and of genes and the causative mutation within these genes, which are contributing to the phenotypic variation of a target trait.

Family Designs

Two family designs are proposed for genetic mapping analyses in cattle, the daughter design and the granddaughter design (Weller et al., 1990). Using a daughter design, sires that are assumed to be segregating for a target trait and their daughters are genotyped at specific marker loci, and phenotypes are quantified in the daughters. Thereafter, marker inheritance can be traced, and genetic linkage between molecular marker and phenotype can be estimated. The statistical power of the daughter design to identify chromosomal regions affecting a target trait is dependent on the QTL effect, on the heritability of the trait and on the number of sires' daughters. More daughters will provide more statistical power but will also increase

costs for genotyping. Using a granddaughter design, grandsires that are assumed to be segregating for the target trait and their sons are genotyped and phenotypes of their granddaughters/daughters are quantified. The phenotypes of the granddaughters/daughters can be used to estimate the genetic merit of the genotyped sons, and marker inheritance can be traced between grandsire and sire. Thereby, the genetic linkage can be estimated between molecular marker and genetic merit of the sons. The statistical power of the granddaughter design depends on the number of genotyped sons and on the number of phenotyped daughters of genotyped sons. The more phenotyped daughters are available for a genotyped son, the more precise the genetic merit of the son can be estimated. The benefit in using granddaughter designs is that less genotyping is necessary to achieve the same statistical power of an equivalent daughter design. Because of the reliability of phenotypic estimates (breeding values) for genotyped sires that are based on many observations in their daughters, the accuracy of phenotypic estimates is more accurate than direct phenotypes quantified for genotyped daughters in the daughter design (Weller et al., 1990; Georges et al., 1995). Depending on the QTL effect and on the heritability of the investigated trait, two to five times more animals have to be genotyped using a daughter design in comparison to a granddaughter design (Weller et al., 1990).

However, both family designs, the daughter design and the granddaughter design, are well applicable in the commercial dairy cattle population. The use of proven sires in dairy cattle breeding results in a high number of offspring for a single proven sire (hundreds to thousands of daughters per sire) and the intensive recording of economical important traits in the dairy cattle population provide a reliable source of phenotypic data. To investigate more complex traits that are not recorded routinely and require additional phenotyping and additional analyses (e.g. feed intake, disease resistance or metabolism), usually experimental resource populations are raised. For this purpose, prevalently crosses between two breeds are used, because of phenotypic differences that are commonly higher between breeds (e.g. dairy cattle,

beef cattle) than differences within a breed (Ron and Weller, 2007). This approach is very expensive for dairy cattle due to the high costs for raising and maintenance as well as due to the long generation intervals of cattle. In respect to the number of genotyped animals, backcrosses or F2 population achieve the highest statistical power.

Recently, the development of high throughput genotyping methods (Mansfield et al., 1997; Oliphant et al., 2002; Matsuzaki et al., 2004; Matukumalli et al., 2009) and the identification of a high number of molecular markers that are distributed uniformly over the whole genome (Itoh et al., 2005; Van Tassell et al., 2008) enabled fast and cost effective genotyping of a high number of markers covering the whole genome. These developments made genome wide association studies or whole genome association studies possible (Kolbehdari et al., 2008; Feugang et al., 2009). Due to the use of single marker regression models that are applied to estimate the association of a single marker with a specific trait, the whole genome association studies are usually independent of families.

Molecular Markers

The first DNA markers used to analyze linkage between molecular genetic markers and economical important traits were restriction fragment length polymorphisms (**RFLP**) and DNA fingerprints (Georges et al., 1990). Due to the development of new molecular technologies, such as the PCR technology (Saiki et al., 1988), a large number of new DNA variations were identified, that were suitable for genetic analyses (Fries et al., 1990; Vaiman et al., 1997; The Bovine HapMap Consortium et al., 2009). Microsatellite markers, for example, were one of the first DNA markers that found broad application in livestock genetics (Rohrer et al., 1994; Crawford et al., 1994; Cheng et al., 1995; Vaiman et al., 1996). Microsatellite loci consist of simple sequence repeats of mono-, di-, tri- or tetranucleotides, accordingly they are also referred to as short tandem repeats (**STR**) or simple sequence repeats (**SSR**) (Ellegren, 2004). The number of di-, tri- or tetranucleotide repeats typically

varies between five and twenty. Microsatellites are one of the most variable types of DNA sequences in the genome, and due to the development of the PCR technique and due to the development of high throughput genotyping methods, microsatellite analyses and genotyping became cost effective and applicable (Ziegle et al., 1992; Mansfield et al., 1997). In respect to the abundance and to the high heterozygosity, microsatellite markers were important for the construction of the first bovine whole genome genetic linkage maps (Barendse et al., 1994; Bishop et al., 1994; Kappes et al., 1997; Barendse et al., 1997; Thomsen et al., 2000; Ihara et al., 2004) and for the first whole genome analyses to detect QTL in cattle (Georges et al., 1995; Ashwell and Van Tassell, 1999; Schrooten et al., 2000; Kühn et al., 2003). Today microsatellites are also important markers in parentage testing and in forensics (Bowling et al., 1997; Pajnic et al., 2001; van Asch et al., 2009; van de Goor et al., 2009).

The size of a chromosomal region that is identified to harbor a QTL is dependent on the QTL effect, on the heritability of the trait, on the mapping strategy applied, on the number of animals investigated, on the information content of DNA markers and on the physical distance between DNA markers on a respective chromosome. By increasing the marker density in a respective chromosomal region, it is possible to identify chromosomal regions that harbor a causative gene or polymorphisms, more precisely. Increasing knowledge of the bovine genome sequence and new developments in human genetics (Wang et al., 1998) indicated single nucleotide polymorphisms (**SNP**) as suitable markers for fine mapping previously identified QTL regions. SNP are uniformly and densely distributed throughout the whole genome and are more abundant than microsatellite marker. Additionally, the development of chip based genotyping methods enabled cost effective genotyping of a large number of SNP in a single analysis (Oliphant et al., 2002; Matsuzaki et al., 2004; Matukumalli et al., 2009). Bovine SNP were mainly detected by comparative sequencing of individuals from different breeds (The Bovine HapMap Consortium et al., 2009), different populations (Van Tassell et al., 2008) or by comparative sequencing of candidate genes. Up to

date more than two million SNP have been detected in cattle (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/), and the new developed BovineSNP50 genotyping Beadchip (Matukumalli et al., 2009) harbors 54,000 SNP that can be analyzed in a single assay.

Mapping strategies

One of the first steps to identify genes or polymorphisms affecting the phenotypic variation of a target trait is to perform a whole genome scan to identify chromosomal regions that are in linkage to the target trait. This primary analysis is also referred to as whole genome linkage analyses and provides first information on a chromosomal region containing a QTL. To perform a whole genome scan, informative molecular markers that are evenly distributed over the whole genome (commonly one marker every 10 to 20 cM) have to be available for genotyping, and an appropriate mapping population has to be selected in respect to the traits of interests (Weller et al., 1990). The size of the identified chromosomal regions in linkage analyses is dependent on the distance between two marker loci (marker interval) and on the number of recombination events that occurred between two closely linked marker loci. To narrow down the position of a QTL that was identified in a whole genome scan, the marker density within the QTL region has to be increased. Nowadays, additional markers for fine mapping a known QTL region are commonly obtained by selecting additional markers from published linkage maps (Cattle Genome Mapping Project: http://www.marc.usda.gov/genome/cattle/cattle.html) or by investigating sequence databases like GenBank (Benson et al., 2008), the NCBI Trace Archives (NCBI Trace Archives: http://www.ncbi.nlm.nih.gov/Traces/home/) or the NCBI SNP database (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/) for further polymorphisms within the target region, that are suitable for genetic analyses. Additionally, it is also possible to identify new polymorphisms by comparative sequencing of functional candidate genes that are located

within the previously identified chromosomal regions, or by positional cloning (Grisart et al., 2002).

So far several statistical methods have been developed for mapping QTL including linear regression methods, maximum likelihood analyses, squared difference regression methods, residual maximum likelihood analyses based on mixed models and Bayesian analyses (Hoeschele et al., 1997). In 2000 Meuwissen and Goddard described a method for fine mapping QTL using linkage disequilibria with closely linked marker loci. This method considers ancient recombination events between two closely linked marker loci to identify the minimum haplotype associated with the QTL. Linkage mapping methods only consider recombination events that occur in the genotyped animals, comprising two or three generations. Hence, the number of recombination events between two closely linked markers is low, unless a large number of animals are genotyped. The linkage disequilibrium (**LD**) method developed by Meuwissen and Goddard (2000) utilizes the assumption that identical marker haplotypes are more likely to be identical-by-decent (**IBD**) and derive from a common ancestor. Based on this assumption, marker haplotypes that have identical marker alleles in a region surrounding the QTL are also more likely to show similar haplotype effects and thus carry identical QTL alleles. Marker haplotype effects are dependent on the position of the QTL in relation to the marker haplotype and by considering covariances between marker haplotype effects the QTL can be positioned (Meuwissen and Goddard, 2000). The advantage of LD methods is that the position of a QTL can be mapped more precisely in comparison to LA. Hence, the number of genes within identified chromosomal regions is decreased (Andersson and Georges, 2004).

Farnir et al. (2000) discovered that an extensive LD exists in dairy cattle and that LD observed between nonsyntenic loci, and loci that are up to 10 cM apart on the same chromosome might generate false-positive results, if only LD is considered. By incorporating linkage information from linkage analysis (**LA**) this problem can be avoided. Since then

combined linkage and linkage disequilibrium (**LALD**) mapping methods have been developed and several studies have performed LALD mapping to refine position of previously known QTL (Olsen et al., 2008; Kim et al., 2009; Nilsen et al., 2009). Meuwissen et al. (2002) have shown that combined LALD mapping is viable to narrow down the position of a QTL to 1 cM and less. A prerequisite for LA, LD and hence for LALD mapping methods is the knowledge of the marker order in the respective chromosomal region under investigation. Due to the use of linkage information between marker loci in LA or the use of marker haplotypes in some LD analyses the wrong marker order can cause fatal errors.

1.1.2 The candidate gene approach

A candidate gene is a gene which is assumed to have a significant effect on the phenotypic variation of a target trait. The candidate gene approach is used to identify such genes and the causative mutation within these genes that are responsible for phenotypic variation. Two distinct strategies are considered. On the one hand it is possible to identify candidate genes by investigating known physiological pathways affecting a target trait (functional candidate gene approach) and on the other hand it is possible to identify candidate genes by investigating the function of genes that are located within a chromosomal region containing a QTL (positional candidate gene approach). Both strategies utilize the knowledge accumulated by the scientific community.

A great challenge in investigating a QTL region for functional candidate genes in cattle and in using databases storing information on physiological pathways and gene functions is that the databases are commonly attributed to human and mice, and that the genome annotation of cattle is still poor compared to the annotations of human and mouse genomes. To overcome these issues and to utilize the more complete annotation of human and mouse genomes, usually comparative maps between human, mouse and cattle are established (Goldammer et al., 2002; Wind et al., 2005).

Ron and Weller (2007) propose four criteria that can be applied to select candidate genes out off a known QTL region. These criteria can also be applied to select functional candidate genes without considering information about QTL regions. The first criterion proposed is the known physiological involvement of a candidate gene in the phenotype of interest. If the physiology of a specific trait is known, it is possible to identify the biochemical pathways and genes that are involved by investigating literature databases such as PubMed (PubMed: http://www.ncbi.nlm.nih.gov/pubmed/), ISI Web of Knowledge (ISI Web of Knowledge: http://isiwebofknowledge.com/) or iHOP (iHOP: http://www.ihop-net.org/UniPub/iHOP/), or by investigating databases that store information about biological pathways and gene functions such as KEGG (KEGG: Kyoto Encyclopedia of Genes and Genomes: http://www.genome.jp/kegg/). Fadiel et al. (2005) have summarized information about databases providing general and species specific information, that are available to the scientific community. The second criterion purposed by Ron and Weller (2007) is to consider known effects of gene knock-out experiments, mutations or transgenics in other species like mouse or rat. Gene knock-outs or mutations that are known to be associated with effects on the phenotype of interest can be considered as prime candidate genes. A reliable resource to investigate mutations, knock-outs and transgenics is, for example, the international database resource for the laboratory mouse (Mouse Genome Informatics: http://www.informatics.jax.org/), which provides integrated genetic, genomic, and biological data for the laboratory mouse. The last two criteria suggested by Ron and Weller (2007) are related to the gene expression, whether it is expressed in tissues related to the trait or during developmental stages related to the phenotype. For that purpose, the Gene Expression Omnibus (Gene Expression Omnibus GEO: http://www.ncbi.nlm.nih.gov/projects/geo/) available at NCBI or the Gene Portal Hub (BioGPS: http://biogps.gnf.org/?referer=symatlas#goto=welcome) can be used to investigate tissue and development specific gene expression.

A more powerful approach considering gene expression is the investigation of gene expression profiles of animals with extreme phenotypes (Feugang et al., 2009) or of animals that are known to have inherited different QTL alleles of a known QTL (Griesbeck-Zilch et al., 2008; Griesbeck-Zilch et al., 2009). In this approach, genes that are differentially expressed between animals with extreme phenotypes or between animals that inherited different QTL alleles can be used to identify affected biological pathways and key regulators of differentially expressed genes, which then can be considered as candidate genes for further analyses.

By applying the criteria purposed by Ron and Weller (2007) a list of genes that meet at least some of the criteria can be generated, and the genes with the highest functional relevance can be selected for further analyses. These genes then can be further analysed including structural analyses and mutation screening, to identify polymorphisms that may contribute to the phenotypic variation of the target trait. To accelerate the search for candidate genes, bioinformatic tools such as Suspects (Adie et al., 2005; SUSPECTS Candidate Gene Search: http://www.genetics.med.ed.ac.uk/suspects/) have been developed. These tools automate and simplify the first steps of the candidate gene approach, basically database queries, but are commonly attributed to the human genome.

An example for the identification of a candidate gene by the use of a positional candidate gene approach is the Diacylglycerol O-acyltransferase 1 (***DGAT1***) gene. *DGAT1* was selected as candidate gene within a region harbouring a QTL for milk fat production (Riquet et al., 1999). It was known to be involved in fat metabolism (Cases et al., 1998) and additional information from knock-out experiments in mice indicated that mice lacking the *DGAT1* gene do not lactate (Smith et al., 2000). Grisart et al. (2002) identified a non-conservative *K232A* substitution in the *DGAT1* gene by positional cloning, and association studies revealed that this polymorphism is associated with major effects on milk fat content and other milk characteristics (Grisart et al., 2002). These studies showed that it is possible to identify genes

affecting a target trait by investigating the function and expression of genes, which are located within a previously identified QTL region.

1.2 Marker assisted selection and genomic selection

Marker assisted selection (**MAS**) is used to implement gained molecular genetic information for a specific trait in selection and to complement selection based on phenotype and pedigree information. Three types of molecular genetic markers are considered for MAS: direct markers that represent the causal mutation affecting a target trait; indirect markers that are in population wide LD to the causal mutation; and indirect markers that are in population wide linkage equilibrium to the causal mutation (Dekkers, 2004). The highest potential genetic gain related to MAS is expected for traits with a low heritability, that are difficult to record (feed intake, product quality) or that are not recorded at all (disease resistance). Traits that are expressed in only one sex or that are recorded late in life may additionally benefit from MAS (Lande and Thompson, 1990; Dekkers, 2004).

Commonly selection is based on phenotypic information and on estimated breeding values (**EBV**), which are calculated based on phenotype and pedigree information. A breeding value represents the sum of gene effects of an animal on a specific trait and is used to evaluate the genetic merit in relation to the population mean. The identification of genes and polymorphism affecting trait specific phenotypes, facilitates the implementation of molecular genetic information in breeding programs. For monogenic traits, the application of MAS is straightforward. By identifying the underlying gene and mutation affecting the trait, individuals carrying the unfavorable allele can be excluded from breeding. In cattle, for example, the development of a genetic test for bovine leukocyte adhesion deficiency (Shuster et al., 1992), a monogenic disorder, resulted in the rapid elimination of carriers in U.S. Holstein bulls (Dekkers, 2004). For quantitative traits or indirect markers the application of

MAS is more complex. Prior to the inclusion of a marker, the marker characteristics have to be evaluated in the target population. The allele frequency of the marker has to be determined, the association between marker and target trait has to be confirmed, the phenotypic effect has to be calculated and correlated effects on other traits have to be verified (Dekkers, 2004). Additionally, for indirect markers that are in linkage with the target trait, the linkage phase between marker locus and causal mutation has to be verified for each family and each generation. However, direct markers and to a lesser extend indirect markers that are in LD to the causative mutation can be used for population-wide selection without considering the family background. In general, marker information can be included in selection in terms of molecular scores that represent the molecular genetic information based on the absence or presence of marker alleles or on estimated marker or QTL effects. Benefits from marker assisted selection are assumed (i) for the accuracy of selection, because of marker genotypes that could provide information additional to the phenotypic information; (ii) for the decrease of generation intervals, because of the possibility to select at earlier stages of life based on marker information; and (iii) for the evaluation of selection candidates, because of genotyping important marker loci in a large number of animals will allow a pre-selection of candidates for later selection independent of trait recording. A summary of markers that are applied in MAS in farm animals is given by Dekkers (2004). So far routine MAS programs have been implemented for the German and French Holstein Population (Bennewitz et al., 2003; Guillaume et al., 2008).

Genomic selection is an extrapolation of MAS and is still in the beginnings and still developing. With the advent of SNP and chip based genotyping methods (Matukumalli et al., 2009) the marker coverage of the whole genome will soon achieve a density to assume that every QTL is in linkage disequilibrium to at least one marker locus (Goddard and Hayes, 2007). Hence, estimated effects for marker genotypes that are in LD to the QTL will potentially be sufficient to explain the total genetic variation for a specific trait. The sum of

estimated QTL/marker effects can be used to calculate a genomic EBV, which then represents the genetic merit of an animal. The accuracy of the genomic EBV is estimated to be between 0.75 and 0.85 (Schaeffer, 2006), and it is assumed that an increasing number of available markers and an increasing number of phenotyped animals, genotyped at a high number of marker loci, will further advance genomic selection and the accuracy of statistical methods used for genomic prediction (Goddard and Hayes, 2007; VanRaden, 2008).

Genomic selection will change cattle breeding in the future. Genotyping will become more and more important and may become routine in addition to the conventional trait recording. Furthermore, the possibility to estimate the genetic merit of an animal, already early in life (at birth), will greatly reduce the generation intervals, which will affect the costs for maintenance of proven bulls and may lead to a change in breeding program designs (Schaeffer, 2006). However, the statistical methods to estimate genomic EBV are still in development and the existence of epistatic QTL effects and interaction of QTL is not accounted for, so far. Hence, the identification of the mutations that affect phenotypic variation will still be important to increase the accuracy of genomic EBV, especially for QTL with small, dominant or epistatic effects.

1.3 Udder health and udder conformation traits

Udder health has become an important aspect in dairy cattle breeding. Mastitis or the inflammation of the mammary gland effects milk production, milk quality, animal welfare and is a major cause for premature culling (Seegers et al., 2003). Effects on decreases in reproduction that are related to mastitis have been discussed also (Ahmadzadeh et al., 2009). Hence, costs for veterinary treatments, discarded milk, replacements and animal welfare are unfavorably affected by mastitis. Mastitis has the highest economical impact of all productive diseases (Kossaibati and Esslemont, 1997) and mastitis incidence is reported to be 44.1 cases in 100 cow years, considering clinical mastitis cases (Fourichon et al., 2001). The economical impact of mastitis is mainly attributed to the productive losses and to the control costs comprising discarded milk, veterinarian's time and herdsmen's time as well as drug costs. The total costs for a mild mastitis case are assumed to be € 226.13 and can increase to € 2360.57 for fatal clinical mastitis cases (Esslemont and Kossaibati, 2002). Annual cost per cow are estimated to be around $ 95 considering clinical mastitis cases (Hultgren and Svensson, 2009) and costs increase to € 272.63 if mild and clinical mastitis cases are considered (Esslemont and Kossaibati, 2002).

1.3.1 Intramammary infection and somatic cell count

The common route of pathogens into the mammary gland is the invasion through the teat channel (Sutra and Poutrel, 1994; Hogan and Smith, 2003; Rainard and Riollet, 2006). A hematogenous infection through the blood is uncommon but may also occur. After traversing the teat channel pathogens can multiply in the milk or mammary gland and may establish an intramammary infection. The characteristics of an infection depend on the pathogen and can vary between pathogen strains (Hogan and Smith, 2003; Bannerman, 2009).

Milk from a healthy udder contains in average 50,000 somatic cells per milliliter, and macrophages are the predominant cell type among other cells like B and T lymphocytes,

polymorphonuclear neutrophil leukocytes (**PMN**) and epithelial cells (Gruet et al., 2001). During infection bacteria or bacterial products like lipopolysaccharides (**LPS**) trigger the recruitment of PMN from the blood via macrophage and mammary epithelial cell (**MEC**) signaling (Rainard and Riollet, 2006). PMN are the first cells recruited into the milk and the amount of PMN as well as the swiftness of the recruitment process is essential for the defense of the mammary gland and for bacterial killing (Li et al., 2002; Rainard and Riollet, 2006). The amount of PMN and the rapidity of the recruitment depend on pathogens involved and on the cows' predisposition. The number of PMN increases rapidly when the infection advances and in clinical mastitis cases the somatic cell count (**SCC**) (the number of somatic cells) can increase to 10^7 cells per milliliter milk. For sub-clinical mastitis cases the threshold of 200,000 cells per milliliter is set to distinguish between healthy quarters (SCC < 200,000) and quarters with sub-clinical mastitis (Dohoo and Leslie, 1991). Hence, the number of somatic cells in the milk from infected udders is increased and this is why the somatic cell count is considered as substitute trait for mastitis incidence in genetic evaluations (Schutz, 1994). The main reasons to apply SCC in genetic evaluations are the availability of somatic cell count records and the moderate to high correlation between SCC and clinical mastitis as well as between SCC and sub-clinical mastitis (Rupp and Boichard, 2003; Hinrichs et al., 2005). In some Nordic countries like Denmark, Finland, Sweden and Norway, mastitis data is recorded by veterinarians and is collected by a national dairy cow recording system (Valde et al., 2004). However, in many countries including Germany, mastitis data is not recorded and collected, but the somatic cell count, which is used as a quality measure for milk and as an indicator trait for udder health, is routinely recorded for sire evaluations (Vereinigte Informationssysteme Tierhaltung w.V.; Description of genetic evaluation: http://www.vit.de/index.php?id=zw-milch-zws-beschreibung&L=1). For genetic evaluations usually the somatic cell score (**SCS**) is used. The SCS is the $\log_2$ transformed SCC:

$$SCS = \log_2 (\text{somatic cell count} / 100{,}000) + 3$$

and in statistical analyses the advantages over SCC are the normal distribution and a uniform variance among samples (Schutz, 1994).

1.3.2 Clinical and sub-clinical mastitis

Mastitis can occur as clinical mastitis, commonly caused by environmental pathogens and as sub-clinical mastitis commonly caused by contagious pathogens (Gruet et al., 2001). Contagious pathogens like *Staphylococcus aureus* can establish a persistent infection in the mammary gland. After an acute phase with clinical signs the infection usually results in a persistent sub-clinical infection, which shows no apparent signs of local inflammation and is typically manifested as an increase in the somatic cell count. No visible changes in the appearance of the milk or the udder occur but the milk composition is altered, bacteria can be detected in the milk and the milk production is reduced (Sutra and Poutrel, 1994; Harmon, 1994). If the pathogen can persist for a longer time period in the mammary gland, the infection may become chronic and may persist for a lifetime. Clinical cases of contagious infections may also occur but are less common.

Environmental pathogens like *Escherichia coli* invade the mammary gland through the teat channel and multiply rapidly in the milk or colostrum. The majority of cases cause a clinical mastitis. Sub-clinical infections caused by environmental pathogens are less common. Clinical infections trigger dramatic changes in the milk composition and the appearance of the milk alters. Clinical mastitis is often accompanied by swelling, heat or pain in the udder, by an elevated rectal temperature, by lethargy or anorexia and in fatal cases it can cause the death of the cow (Harmon, 1994; Hogan and Smith, 2003). In comparison to sub-clinical infections the SCC is commonly increased to a higher level during clinical infection.

1.3.3 Mastitis pathogens

Mastitis pathogens can be subdivided in contagious pathogens, which are mainly detected in infected udders and on the skin of the udder and teat, and in environmental pathogens, which

are mainly detected in the surroundings of the cow, within the bedding, manure and soil. The major contagious pathogens are *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus agalacticae* and the major environmental pathogens are coliforms like *Escherichia*, *Klebsiella* and *Enterobacter*. The most prevalent environmental pathogens are *Escherichia coli* and *Streptococcus uberis* (Harmon, 1994; Zadoks and Fitzpatrick, 2009). Coagulase-negative staphylococci (**CNS**) like *Staphylococcus simulans* and *Staphylococcus chromogenes* have been reported as minor pathogens in bovine mastitis (Harmon, 1994) but in the last years they are one of the most common isolates from sub-clinical mastitis cases in many countries (Pyörälä and Taponen, 2009). Further minor pathogens which usually cause sub-clinical mastitis are spirochetes, mycobacteria, nocardia, mycoplasmas, yeast and funghi (Watts, 1988).

1.3.4 Risk factors, QTL mapping and candidate genes for udder health and related traits

Bovine mastitis is a complex trait and many factors contribute to the health status of the udder. Factors affecting the risk to acquire an intramammary infection, are on the one hand hygiene management, housing and feeding; factors that are attributed to farm management (Schukken et al., 1991; Barkema et al., 1999; Svensson et al., 2006) and on the other hand cow characteristics like parity, stage of lactation and the genetic predisposition to mastitis; factors that are attributed to the cow itself (Hogan and Smith, 2003; Valde et al., 2004; Sordillo, 2005; Fox, 2009).

Risk factors attributed to parity and stage of lactation

A critical period for mastitis is the time around transition, particularly the first two weeks prior to calving and the first weeks after calving. In the first weeks of lactation the cow is unable to meet the demands for high milk production and enters a period of negative energy balance. Body reserves have to be mobilized to counter energy deficits and host defense

mechanisms are unfavorable affected (Collard et al., 2000; Pyörälä, 2008; Mehrzad et al., 2009). Within this time period the risk for an intramammary infection is highest (Hogan and Smith, 2003; Valde et al., 2004). The majority of clinical mastitis cases that occur in the first two weeks after calving are attributed to infections that were acquired during the first two weeks of the dry period or the two weeks prior to calving (Hogan and Smith, 2003; Fox, 2009). Risk factors affecting mastitis susceptibility at these time points are typically attributed to morphological, physiological and immunological changes during involution and colostrogenesis (Nickerson, 1989).

For multiparous cows, additionally an increased risk concerning the severity of mastitis is reported (Smith et al., 1985; Hogan and Smith, 2003; Pyörälä, 2008). Mehrzad et al. (2009) investigated phagocytic and bactericidal activity of PMN in primiparous and multiparous cows. They reported that phagocytic and bactericidal activity is reduced in PMN of multiparous cows, which maybe one factor contributing to the increased severity of mastitis in older cows.

Risk factors attributed to udder and teat morphology

Udder and teat morphology also affect the risk of mastitis (Rupp and Boichard, 2003). A loosely attached and deep udder is closer to the floor and has more contact with the rear legs. Hence, the possibility to become dirty and acquire small injuries on the udder skin is increased. The dirtiness of the udder contributes to the environmental pathogen burden and small injuries on the udder skin increase the risk of contagious pathogens colonizing on the udder skin (Sutra and Poutrel, 1994; Barkema et al., 1999). Hence, the risk for intramammary infections is increased for loosely attached and deep udders. Several studies have investigated udder conformation traits and showed that a high and tightly attached udder has favorable effects on udder health (Rogers et al., 1991; Rupp and Boichard, 2003; Kadarmideen, 2004; Nemocova et al., 2007).

The teat canal is the barrier that separates the environment from the interior of the mammary gland. Between milking and during the dry period the teat canal is closed by the teat sphincter muscle and is lined by keratin (Nickerson, 1989; Pyörälä, 2008). During the dry period the accumulation of keratin can completely seal the teat canal. Keratin contains long-chain fatty acids, cationic proteins and xanthine oxidase, which are reported to have microbicidal activity against mastitis pathogens. Removal of the keratin is linked with an increased mastitis incidence (Capuco et al., 1992). Hence, a high risk factor for mastitis is the time at milking and after milking, when the teat canal is open and the keratin plug is removed. After milking it takes two hours until the teat sphincter has contracted and the teat canal is closed again. Milking hygiene and cow hygiene play an important role at this time point. Several studies have investigated the influence of the teat shape on udder health and reported correlation between udder health traits and teat diameter, teat end callosity and milk leakage (Chrystal et al., 1999; Neijenhuis et al., 2001; Klaas et al., 2005). Cows that have an increased teat diameter and leak milk between milking are at high risk for mastitis, which may be related to an incomplete sealing of the teat channel between milking that is caused by a weak teat sphincter. In addition, the teat shape has an important impact on the risk of mastitis that is attributed to the use of automated milking machines. Automated milking machines can induce mechanical and circulatory impairments in teat tissues, which may result in an increased colonization of contagious pathogens on the teat skin. An improper teat shape is more prone to teat lesions during automated milking, further increasing the risk of mastitis (Rainard and Riollet, 2006).

Risk factors attributed to the innate and adaptive immune system

The genetic predisposition to mastitis does not only include udder morphology, teat shape and energy metabolism, it also includes facets of the innate and adaptive immune system responsible for detection, localization, killing and clearance of pathogens. Defense

mechanisms attributed to the innate or adaptive immune system include (i) cellular defenses comprising neutrophils, macrophages, B- and T- Lymphocytes, natural killer cells, mast cells and other immunocytes; (ii) humoral defenses including the complement system, cytokines, secreted antibodies and bacteriostatic or bactericidal proteins like lysozyme and (iii) morphological defenses comprising udder and teat morphology. The coordinated interaction of the innate and adaptive immune system is essential for a successful elimination of pathogens, and dysfunctions in detection, signaling or killing of pathogens may increase the risk of mastitis. Distinct pathways possibly affecting susceptibility to mastitis have been discussed in many reviews (Detilleux, 2002; Sordillo, 2005; Rainard and Riollet, 2006; Bannerman, 2009) and a small number of genes have been identified that are related with mastitis traits in cattle so far (Ogorevc et al., 2009).

QTL mapping and candidate genes for mastitis

Considering the diversity of pathogens which can cause mastitis and considering the complexness of the immune system in respect to specific host-pathogen interactions, the number of genetic loci that may contribute to the genetic predisposition to mastitis has to be very high. This is also reflected by the number of QTL detected for mastitis related traits. QTL for SCC, SCS or mastitis incidence were found on nearly all chromosomes. Khatkar et al. (2004) and Hu et al. (Hu et al., 2007; Hu and Reecy, 2007) have reviewed literature on dairy cattle QTL including QTL for SCC, SCS and mastitis incidence and have developed online databases storing QTL information (Combined QTL Map of Dairy Cattle Breeds: http://www.vetsci.usyd.edu.au/reprogen/QTL_Map/; AnimalQTLdb: http://www.animalgenome.org/QTLdb/). So far 71 QTL for mastitis related traits were detected in cattle. Interestingly, some QTL detected in whole genome scans were located in chromosomal regions also known to harbour genes attributed to the innate and adaptive immune system. The beta-defensins, for example, are located in a cluster on *Bos taurus*

autosome 27 (**BTA27**). Beta-defensins are small cationic peptides that exert antimicrobial activity (White et al., 1995). Lingual antimicrobial peptide (**LAP**), a member of the beta-defensin family, is expressed in mammary epithelial cells and expression is induced in response to mastitis (Swanson et al., 2004). In the same region, where the beta defensin cluster is located on BTA27, Kühn et al.(2003) have detected a QTL for SCC and Klungland et al. (2001) a QTL for clinical mastitis. A similar situation is found on BTA23, where the *major histocompatibility complex class II* (**MHC class II**) genes are located (Brinkmeyer-Langford et al., 2009). MHC class II molecules play an important role in antigen presentation and QTL for mastitis traits were repeatedly reported within a region on BTA23, where the MHC class II genes are located (Reinsch et al., 1998; Heyen et al., 1999; Boichard et al., 2003; Ashwell et al., 2004; Holmberg and Andersson-Eklund, 2004).

Genes affecting SCC or mastitis incidence and hence contribute to the risk of mastitis have also been identified but most of the studies have not been confirmed in independent studies, and for most of the candidate genes no functional proof is given so far. The most frequently reported genes that are assumed to be associated with increased SCC or mastitis incidence are the *major histocompatibility complex class II, DRB3* (***BoLA-DRB3***) (do Nascimento et al., 2006; Rupp et al., 2007), *toll-like receptor 4* (***TLR4***) (Sharma et al., 2006; Wang et al., 2007) and *Interleukin 8 receptor alpha* (***IL8RA***) (Rambeaud and Pighetti, 2007; Leyva-Baca et al., 2008). These genes are related to immune functions, more precisely to pathogen detection (*TLR4*), neutrophil recruiting (*IL8RA*) and antigen presentation (*BoLA-DRB3*). Further genes that are assumed to be associated with mastitis traits are *Interleukin 8 receptor beta* (*IL8RB*) (Youngerman et al., 2004), *fez family zinc finger 2* (***FEZF2***) (Sugimoto et al., 2006), *fibroblast growth factor 2* (***FGF2***) (Wang et al., 2008), *nucleotide-binding oligomerization domain containing 2* (***NOD2***) (Pant et al., 2007), *chemokine (C-C motif) receptor 2* (***CCR2***) (Leyva et al., 2007) and *cytochrome P450, subfamily XI B, polypeptide 1* (***CYP11B1***) (Kaupe et al., 2007). As mentioned above most of these studies have not been confirmed in

independent studies or independent cattle populations so far and no functional proof is given for most of these candidate genes. For *IL8RA*, for example, the association to SCS could not be confirmed in an independent cattle population (Goertz et al., 2009), indicating population specific linkage disequilibrium between the causal mutation and the SNP within *IL8RA* that was reported to be associated with SCS (Rambeaud and Pighetti, 2007; Leyva-Baca et al., 2008).

1.4 Calving ease and Stillbirth

Calving ease and stillbirth are both related to the complex process of birth. In animal breeding, calving ease is defined as the difficulty of calving, and in the German selection programs calving ease is scored in three categories. Category one includes easy and normal calvings, category two includes difficult calvings and category three includes difficult calvings requiring veterinarian assistance. Stillbirth, however, is defined as a classical "All-or None" trait and is considered as a calving where the calf was born dead or died within the first 48 hours (Vereinigte Informationssysteme Tierhaltung w.V.; Description of genetic evaluation: http://www.vit.de/index.php?id=zw-milch-zws-beschreibung&L=1). Calving ease and stillbirth are evaluated for direct effects, which are attributed to effects on the calf and for maternal effects that are related to effects on the dam. Calving ease and stillbirth are correlated traits and approximately 50 % of stillborn calves had difficulties at calving (Berglund et al., 2003; Steinbock et al., 2003).

1.4.1 Economical impact

The economical impact of calving traits is attributed to increased farm management costs and production losses. The costs of stillbirth are predominantly assigned to the replacement of the calf and Meyer et al. (2001) have estimated the cost for replacements caused by stillbirth to be more than $125.3 million per year in the United States. An increase of cost by $75.9 million was observed between 1985 and 1996.

For calving ease Dematawena and Berger (1997) estimated the costs to be \$0.00, \$50.45, \$96.48, \$159.82 and \$379.61 for unproblematic calvings (1) to extreme difficult calvings (5) They did not consider costs for herdsmen and veterinarians and costs for cow culling in their study. Hence, the additional time for herdsmen and veterinarians spend to watch and assist a calving were not included. The production losses assigned to calving ease and stillbirth are predominantly decreases in milk, fat and protein yield as well as an increase in the number of days from calving to conception (days open) and an increase in number of services needed for conception (Thompson et al., 1983; Dematawena and Berger, 1997; Meyer et al., 2001). Both, days open and the number of services needed for conception, affect the productive life of the cow and additionally decrease milk, fat and protein yields.

1.4.2 Genetic parameters, incidence and trends

In the German Holstein population the genetic correlation between maternal and direct effects on calving traits is assumed to be slightly negative (r_g = - 0.10), and the heritability is estimated to be h^2 = 0.05 for maternal and direct effects on calving ease as well as for maternal and direct effects on stillbirth (Vereinigte Informationssysteme Tierhaltung w.V.; Description of genetic evaluation: http://www.vit.de/index.php?id=zw-milch-zws-beschreibung&L=1). The genetic correlation between calving ease and stillbirth is moderate to high (r_g = 0.8 for direct effects and r_g = 0.74 for maternal effects). The low heritability and the slightly negative correlation between direct effects and maternal effects on calving traits is one reason, why selection to improve calving performance is hampered.

Incidence of stillbirth has continuously increased in the last decades. For Holsteins in the United States, Meyer et al. (2001) reported an increase in stillbirth from 9.5 % in 1985 to 13.2 % in 1996 for primiparous cows and an increase from 5.0 % to 6.6 % for multiparous cows. Accordingly, Steinbock et al. (2003) have found an increase from 6 % in 1986 to almost 9 % in 1996 in first calvings of Swedish Holstein cows. An increase in stillbirth for multiparous

cows as well as an increase in calving ease for primiparous and multiparous cows was not reported for Swedish Holstein cattle in their study.

Steinbock et al. (2003) also investigated correlations between parities. Genetic correlation between first and second parity for stillbirth were 0.45 and 0.48 for direct and maternal effects and correlation between first and second parity for calving ease direct and maternal effects were 0.61 and 0.71, respectively. Further studies have confirmed a correlation different to 1.0 between first and later parities, indicating that the genetic background of first and second parity calving traits are probably not identical (Meyer et al., 2000; Steinbock et al., 2003; Wiggans et al., 2008). Only 20 to 50 % of the genetic variation of calving traits are assumed to be common to first and later parity calvings (Steinbock et al., 2003).

1.4.3 Risk factors

Calving is a complex process, and as for mastitis many factors contribute to the risk of calving difficulties and stillbirth. One factor is the breed. It is known that between breeds differences in the risk of calving difficulties and stillbirth exist. Stillbirth rate in Holsteins, for example, is approximately twice as high as in Swedish Red and White, Danish Red and Danish Jersey (Steinbock et al., 2003; Hansen et al., 2004; Heringstad et al., 2007). Furthermore, Hansen et al.(2004) have shown that an increased use of selected Holstein-Frisian sires from the United States had an unfavorable effect on direct and maternal genetic effects for stillbirth in the original Danish Black and White breed. These changes are assumed to be related to the increased proportion of Holstein-Frisian genes deriving from Holstein-Frisian sires from the United States.

Factors affecting the risk of calving ease and stillbirth, common for all breeds, are, besides congenital defects of unknown cause, on the one hand genetic factors predominantly attributed to weight and size of the calf and the dam and on the other hand non-genetic factor including farm management, environment and physiological stages of the dam. Risk factors

attributed to the environment include year, time of year at calving (Meyer et al., 2001; Johanson and Berger, 2003; Steinbock et al., 2003) and herd (Steinbock et al., 2003), which may be related to temperature, humidity, housing and food availability. Risk factors attributed to the calf and/or the dam are dam parity (Meyer et al., 2001; Johanson and Berger, 2003; Steinbock et al., 2003), sex of the calf and calf birth weight (Johanson and Berger, 2003), calf size, incompatibility between calf and pelvic opening of the dam (Thompson et al., 1983; Johnson et al., 1988; Mee, 2008) as well as the gestation length (Meyer et al., 2000; Johanson and Berger, 2003) and the birth of twins (Ettema and Santos, 2004). The two most important factors affecting the risk of stillbirth and calving ease, particularly for heifers, is the calf birth weight and the pelvic size of the dam. Both are assumed to be useful to predict stillbirth and dystocia (Johanson and Berger, 2003) and are related to the incompatibility between calf and pelvic opening of the dam. However, the predominant cause for dystocia in multiparous cows is the fetal malposition, which is affected by the number of fetuses, parity and calf sire breed, (Mee, 2008) as well as to the incompatibility between calf and pelvic opening of the dam.

1.4.4 QTL mapping for calving ease, stillbirth and related traits

So far, most QTL for calving traits have been detected in whole genome scans and genes associated with stillbirth or calving ease have not been reported. Khatkar et al. (2004) have reviewed QTL mapping studies in cattle and have summarized information about QTL for calving traits that have been reported before 2004. More recent studies have also identified QTL for calving traits and traits that have a possible impact on calving performance: Kneeland et al. (2004), for example, reported QTL for birth weight on BTA2, BTA6, BTA14, BTA19, BTA21 and BTA23 in a commercial beef cattle line. Ashwell et al. (2005) detected QTL for calving ease on BTA8, BTA9, BTA17, BTA23, BTA24 and BTA27 in US Holstein cattle and Kolbehdari et al. (2008) identified QTL for maternal calving ease on BTA7, BTA9, BTA23, BTA24, BTA28, BTA 29 and for direct calving ease on BTA4, BTA6, BTA8,

BTA11, BTA18, BTA21, BTA23 in Canadian Holstein. Interestingly, some of the regions reported to harbor QTL for calving traits are assumed to affect more than one trait associated with calving, reflecting the complex interaction of factors influencing calving performance. One example is a SNP (ss86324977) reported by Cole et al. (2009), which is reported to affect body depth, rump width, stature, strength, daughter calving ease and sire calving ease in North American Holstein bulls. For MAS especially chromosomal regions that have favorable correlations between effects on different traits are of interest. QTL with opposite effects on maternal and direct calving traits, for example, are not useful for MAS, as improvement in one trait would have negative effects on the other trait. Thomasen et al. (2008) and Cole et al. (2009) both have investigated effects of QTL to identify regions with favorable effects on maternal and direct calving traits. They reported that QTL in the middle to telomeric region on BTA18, showed a favorable correlation between maternal and direct effects on calving traits, indicating this region to be suitable for MAS.

1.5 The experimental objectives

In 2003 Kühn et al. have performed a whole-genome scan to detect quantitative trait loci for functional traits in the German Holstein population. They used a granddaughter design comprising 16 families and 872 sons to investigate calving traits, reproduction traits, udder health related traits and functional herd life. Kühn et al. (2003) identified 5 chromosomes harboring more than one QTL for functional traits. For *Bos taurus* autosome 18 (**BTA18**) a genome-wide significant QTL for somatic cell count (**SCC**), which is an index trait for udder health, was identified and putative QTL affecting calving traits, reproduction traits and functional herd life were detected. Functional herd life is considered to be a characteristic independent from milk production, that is attributed to the genetic vitality, robustness, fertility and health of a cow (Vereinigte Informationssysteme Tierhaltung w.V.; Description of genetic evaluation: http://www.vit.de/index.php?id=zw-milch-zws-beschreibung&L=1). Breeding values for functional herd life represent an approximation for longevity in relation to the population mean and reflects the productive lifespan of an animal. The detection of QTL for udder health (somatic cell count) in addition to the detection of QTL for reproduction and calving traits as well as for functional herd life indicated that genetic loci located on BTA18 might have a substantial impact on the economic efficiency of dairy cattle breeding in the German Holstein population.

Subsequent studies investigating udder health traits on BTA18 in the German Holstein population (Brink, 2003; Xu et al., 2006) confirmed the findings of Kühn et al. (2003), and Brink (2003) found first indications that more than one QTL for somatic cell count could be located in the middle to telomeric region on BTA18. These findings were confirmed by Xu et al. (2006) in a study investigating 51 half-sib families comprising 2768 sires. Studies in other cattle populations also confirmed QTL for udder health related traits on BTA18 (Holmberg and Andersson-Eklund, 2004; Schulman et al., 2004; Lund et al., 2007). QTL for calving

traits on BTA18 that were previously reported by Kühn et al. (2003), have also been confirmed in independent studies (Kolbehdari et al., 2008; Thomasen et al., 2008; Cole et al., 2009), and studies reporting QTL for conformation traits on the same chromosome (Ashwell et al., 2005; Schnabel et al., 2005; Kolbehdari et al., 2008; Cole et al., 2009) indicated that conformation might be a functional background of QTL for calving performance and udder health on BTA18. To gain insights in the functional background of QTL for calving performance and udder health on BTA18 in the German Holstein population the following objectives were deduced for this study:

I. Selection and genotyping of new markers in the middle to telomeric region on BTA18 to increase the marker density for fine mapping QTL;
II. Evaluation and verification of the marker order on BTA18;
III. Selection and analyses of candidate genes for predisposition to mastitis;
IV. Mapping and fine mapping of QTL for somatic cell score, calving ease, stillbirth and for conformation traits that are related with calving performance and udder health;
V. Identification of molecular markers in linkage disequilibrium to calving and udder health related traits that are suitable for MAS.

2. Results and Publications

In the following two paragraphs the linkage map construction and candidate gene selection is described in more details, providing additional results that were not included in the publications.

2.1 Linkage Map construction

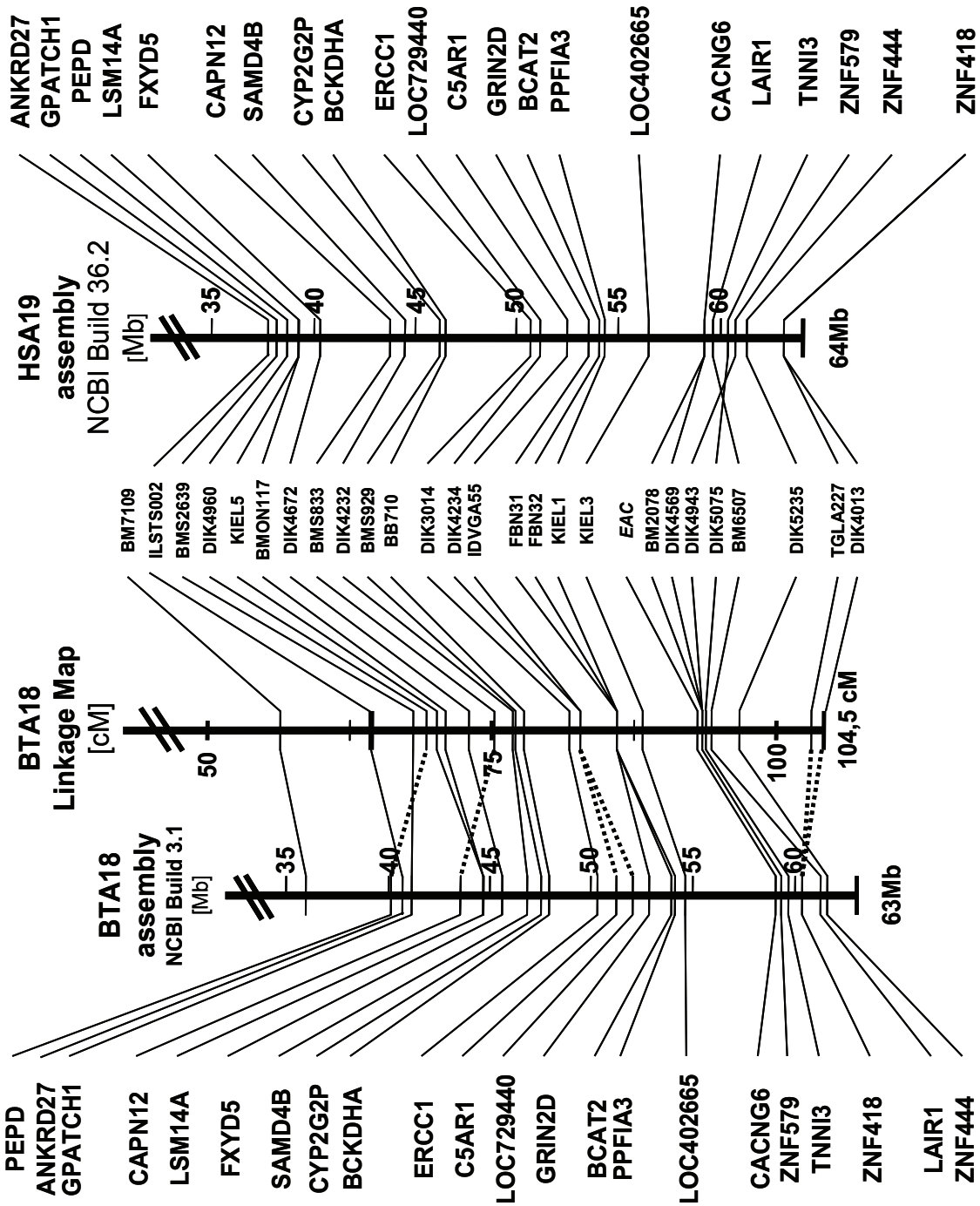
In our studies a total of 38 markers covering BTA18 were used to calculate the final linkage map and 28 of these markers were included in fine mapping. Previous studies investigating BTA18 in the German Holstein population used seven (Kühn et al., 2003), fifteen (Brink, 2003) and five markers (Xu et al., 2006) covering BTA18. Genotyping data was available from previous studies for 16 markers, whereas 22 of the 38 markers were newly selected and genotyped. Ten of the 16 markers that were genotyped in earlier studies had to be excluded from fine mapping, because of missing genotyping data for one or more families. This was done to avoid technical difficulties in LD and combined LALD analyses caused by markers not genotyped in all families. The 22 new markers were selected based on previously reported QTL-regions for somatic cell count on BTA18 (Brink, 2003; Kühn et al., 2003; Xu et al., 2006) and were located in the middle to telomeric region on BTA18 covering approximately 30 Mb. Fifteen microsatellite markers were selected from the bovine linkage map of the United States Department of Agriculture's Meat Animal Research Center (Cattle Genome Mapping Project: http://www.marc.usda.gov/genome/cattle/cattle.html) and seven SNP were selected from a total of 39 polymorphisms that were previously detected in candidate genes for SCS (Brand et al., 2009b). A summary of all markers used to calculate the final linkage map and their position on BTA18 is given in table 1. The final linkage map had a total length of 102 cM.

Table 1: List of markers on BTA18 including marker position in the final linkage map. Polymorphism information content is given only for markers considered for fine-mapping.

Marker Name	Position on BTA18 (cM)	Polymorphism Information Content
IDVGA31	0	---
TGLA357	13.7	---
ABS13	18.6	---
HAUT14	43.3	---
CDH1_c.2102C>T	52	0.375
BM7109	55.7	0.669
ILSTS002	63.6	0.614
BMS2639	67.5	0.693
DIK4960	68.4	0.656
KIEL5	69.7	---
BMON117	69.7	0.713
DIK4672	69.7	0.161
HAMP_c.86+430G>A	69.7	0.044
HAMP_c.366+109G>A	69.7	0.375
BMS833	73.8	0.354
DIK4232	75.6	0.643
BMS929	75.9	---
BB710	76.3	0.589
PVRL2_c.-1268G>C	79	0.312
PVRL2_c.392G>A	79	0.342
DIK3014	80.1	0.468
DIK4234	80.7	0.712
CALM3_c.3+1678C>T	81.2	0.375
CALM3_c.3+1795C>T	81.2	0.258
IDVGA55	81.2	---
FBN31	83.7	---
FBN32	83.7	0.484
KIEL1	83.7	---
KIEL3	85.9	---
EAC	91.2	0.840
BM2078	91.4	0.703
DIK4569	91.4	0.730
DIK4943	91.4	0.279
DIK5075	91.9	0.168
BM6507	92.5	0.617
DIK5235	94.7	0.659
TGLA227	100.8	0.770
DIK4013	102	0.570

The methods considered for mapping QTL comprised LD and combined LALD analyses. By increasing the marker density to an average marker interval of less than 2 cM, the fine mapping of previously reported QTL regions in the middle to telomeric region on BTA18 was enabled. The marker order was very important to avoid difficulties in LA, LD or combined LALD mapping based on marker haplotypes. Different methods were applied to verify the refined marker order used in the studies presented here. First, published linkage maps (Ihara et al., 2004; Snelling et al., 2005) and published radiation hybrid maps (Itoh et al., 2005) were investigated and compared to own linkage mapping results. Second, the bovine sequence assemblies Build2.1 and Build3.1 as well as the human sequence assembly build 36.2, available at NCBI (NCBI Map Viewer: http://www.ncbi.nlm.nih.gov/mapview/), were used to compare linkage maps with published sequence maps. This was done to utilize the advanced assembly and annotation of the human genome for the evaluation of the marker order as well as for candidate gene selection. *Homo sapiens* autosome 19 (**HSA19**) is known to be homologous to the telomeric region on BTA18 (Brunner et al., 2003), and the NCBI Blast tool (NCBI BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to build up a comparative map between BTA18 and HSA19. Map positions were assigned by aligning gene loci on BTA18, which were located next to microsatellite marker loci on BTA18, with the homologous genes in the human genome. The comparative map BTA18/HSA19, constructed for candidate gene selection and evaluation of the marker order, is shown in Figure 1. Several discrepancies could be detected between bovine sequence assemblies Build2.1 and Buid3.1 (data not shown) as well as between bovine sequence assemblies and published and own linkage maps (Figure 1). SNP were not included in the comparative map shown in Figure 1, because they were incorporated into the linkage map after candidate gene selection.

Figure 1: Comparative Map BTA18/HSA19. Left and right columns show gene symbols in the order to the position of the gene loci in the bovine sequence assembly Build3.1 (left) and in the order to the position of the gene loci in the human sequence assembly build 36.2 (right). Dashed lines indicate discrepancies between the refined marker order used for our linkage map and the bovine sequence assembly Build3.1. Only the middle to telomeric region of BTA18 considered for fine mapping is shown.



Finally, radiation hybrid (**RH**) mapping was performed to ascertain the marker order in a critical region on BTA18. This was necessary because the marker position could not be assigned with certainty by linkage mapping due to a low number of recombination events between these markers loci. Additionally, some of these markers were not included in published linkage maps and alignments of the marker loci to the bovine as well as to the human genome were inconclusive. For this purpose, a 12 000 rad whole genome radiation hybrid panel (Rexroad et al., 2000), comprising a set of 180 bovine-hamster hybrid cell lines, was used to analyze the physical distances between closely linked marker loci by RH-mapping.

Bovine-hamster hybrid cell lines are established by fusion of irradiated bovine fibroblasts with hamster host cells, which results in hybrid cells containing an intact hamster genome and, based on the irradiation dose, a number of chromosomal fragments representing a subset of the bovine genome. Using PCR-techniques the presence or absence of marker loci can be verified in a set of different bovine-hamster hybrid cell lines, which in total represent the whole bovine genome. Marker specific co-retention patterns can be analyzed between tested marker loci and are used to calculate physical distances between them. For RH-mapping distances between markers are calculated in centiRay (**cR**) and one cR is equivalent to a 1 % chance that a chromosome break occurred between two marker loci. The resolution of a radiation hybrid panel is attributed to the irradiation dose and a higher dose results in smaller chromosomal fragments, which allows a higher resolution between closely linked marker loci. The 12 000 rad whole genome radiation hybrid panel developed by Rexroad et al. (2000) and the software RHMAP (Boehnke et al., 1996) were used in these studies to ascertain the marker position for seven markers. A LOD-score criterion of 8 was applied to identify linkage groups (Figure 2). A list of primers used to detect marker loci by PCR is given in table 2.

Figure 2: Graphical illustration of two linkage groups identified by RH-mapping of seven markers on BTA18. Marker positions are given in centiRay (cR).

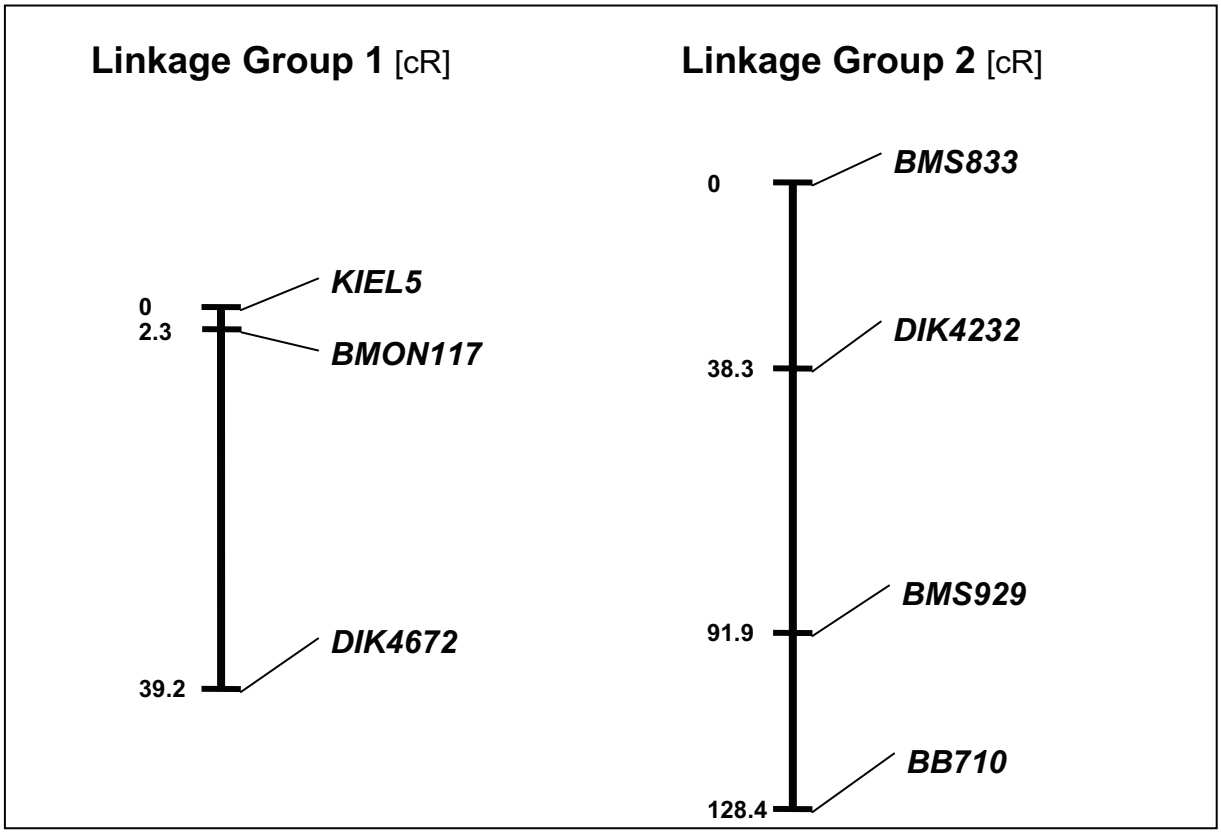


Table 2: List of primers used for radiation hybrid mapping

Marker	Orientation	Sequence	Annealing Temperatures
Kiel5	forward	GGTGGTTGTGGTAAGAGGTG	62°C
	reverse	GACAGAGGCTTTACTTGGTTTC	
BMS929	forward	CATTGTATTCACTTGGCTGGTTC	60°C
	reverse	CTTGGACAGGACGGGCTTC	
BMS833	forward	AGTAAGGGGGTTCACAAGTA	56°C
	reverse	GCACGGGGCATGGGTTCA	
BMON117	forward	GGGAGTGGAAGATGAGGCTG	65°C
	reverse	GAGAAGAAAAGAGGGAGGGAGG	
DIK4672	forward	ATGCCCATCGAAATGGATAA	58°C
	reverse	ACGTTGCTGCAAATGACACT	
BB710	forward	CTCCATGCCAATCAATCAAG	58°C
	reverse	CTCCCTTCAGAAACAGATGC	
DIK4232	forward	TTGTGAGGTAAAGGGACATGA	55°C
	reverse	GCCAGATTTGCCAACTGTTT	

After evaluation of the marker order, candidate genes were selected and analyzed. Thereafter, seven candidate gene SNP were selected and incorporated into the linkage map, based on own linkage mapping results and sequence information provided by NCBI GenBank (NCBI GenBank: http://www.ncbi.nlm.nih.gov/Genbank/). The refined marker order used to calculate the final linkage map is in accordance with previously published linkage- and RH-maps (Ihara et al., 2004; Snelling et al., 2005; Itoh et al., 2005) as well as with the newest bovine sequence assembly Btau4.0 (The Bovine Genome Sequencing and Analysis Consortium et al., 2009) and the whole genome assembly published by Zimin et al. (2009). Discrepancies that occurred to the bovine sequence assemblies Build2.1 and Build3.1 were overcome in the new bovine sequence assemblies. A complete list of markers included in fine mapping and their position in published linkage and RH-maps as well as in the bovine sequence assemblies Build3.1 and Btau4.0 is available online as supplemental table in (Brand et al., 2009b) or on CD in the BMC_Genetics_Sup directory (Appendix 8.3).

2.2 Candidate gene selection

Candidate genes for SCS were selected prior to fine mapping QTL for SCS. They were selected based on the positional information of QTL for SCS derived from the studies performed by Kühn et al. (2003), Brink (2003) and Xu et al. (2006) as well as on own initial mapping results. Nearly 500 genes were located within the previously reported QTL-regions on BTA18, including members of large gene families such as the leukocyte immunoglobulin-like receptor family, the killer cell immunoglobulin-like receptor family, the pregnancy specific beta-1-glycoprotein family and the carcinoembryonic antigen-related cell adhesion molecule family. Additionally, more than 100 genes for zinc finger proteins were located in the telomeric region on BTA18. The high number of tandem duplications in the telomeric region on BTA18 (The Bovine Genome Sequencing and Analysis Consortium et al., 2009) and the high number of gene families might be one reason, why the bovine sequence assembly of BTA18 was and partly still is difficult.

To select candidate genes based on gene function and biochemical pathways, different databases were investigated and different bioinformatic tools were used. The comparative map of BTA18/HSA19 was used to select the respective regions on HSA19, which were homologous to the regions on BTA18, spanning previously reported QTL for SCC and SCS. Based on the human genome annotation, the Entrez gene database (Maglott et al., 2007), OMIM database (Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/omim) and ihop database (iHOP: http://www.ihop-net.org/UniPub/iHOP/) were used to preselect genes based on gene function. Additionally the bioinformatic tools Suspects (Adie et al., 2005; SUSPECTS Candidate Gene Search: http://www.genetics.med.ed.ac.uk/suspects/) and GeneScoreLite were used. GeneScoreLite is similar to Suspects and was developed by the Technische Universität München (Osman M. and Fries R.) within the FUGATO M.A.S.net project (BMBF project FUGATO M.A.S.net:

FKZ 0313390A). Both tools use chromosomal positions and keywords to perform a ranking of genes of the selected chromosomal region. Suspects exploits databases including OMIM (Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/omim), GAD (Genetic Association Database: http://geneticassociationdb.nih.gov/), HGMD (Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/ac/index.php) and GO (Gene Ontology Database: http://www.geneontology.org/), whereas GeneScoreLite exploits literature available at PubMed (PubMed: http://www.ncbi.nlm.nih.gov/pubmed/). Selection criteria applied were on the one hand pathways associated with udder and teat morphology, including mammary gland development and connective tissue, and on the other hand pathways associated with innate and adaptive immune system, especially pathogen recognition and pathogen adherence as well as initiation of immune response. By combining results from own literature research and from the ranking lists derived from Suspects and GeneScoreLite, a number of genes were preselected that were examined in depth by investigating literature. *Calmodulin 3* (phosphorylase kinase, delta) (*CALM3*), *Hepcidin antimicrobial peptide* (*HAMP*), *cadherin 1, type 1, E-Cadherin* (ephitelial) (*CDH1*), and *poliovirus receptor-related 2* (herpesvirus entry mediator B) (*PVRL2*) were selected as candidate genes, due to their involvement in smooth muscle contraction (*CALM3*) (Walsh, 1994; Pfitzer, 2001), their antifungal and antibacterial activity (*HAMP*) (Park et al., 2001) and their function in tissue and organ development (*CDH1, PVRL2*) (Ivanov et al., 2001; Niessen, 2007; Ebnet, 2008; Takai et al., 2008). For all four candidate genes gene expression was investigated. On the one hand it was possible to use initial unpublished results derived from gene expression analyses of healthy cows with different predisposition to mastitis (Hartman A., Schwerin M., FBN Dummerstorf, personal communication) and on the other hand gene expression databases like the Gene Expression Omnibus (Gene Expression Omnibus GEO: http://www.ncbi.nlm.nih.gov/projects/geo/) available at NCBI or The Gene Portal Hub (BioGPS: http://biogps.gnf.org/?referer=symatlas#goto=welcome) were investigated. In

addition, the expression of selected candidate genes in udder parenchyma of lactating and non lactating cows as well as in liver tissue was verified (Figure 3). For this purpose, RNA was isolated from mammary gland tissue of lactating and non-lactating cows as well as from liver tissue of a non-lactating cow, and cDNA was synthesized according to Weikard et al (2005). Tissue specific cDNA was used to verify gene expression by PCR. Primers used for PCR are given in Table 3. Gene expression could be confirmed for all four selected candidate genes in all tissues except for *HAMP*. *HAMP* expression could be verified in liver tissue only. This may be related to the facts that we investigated apparently healthy cows and that *HAMP* is predominantly expressed in the liver (Park et al., 2001) and is induced *TLR4* dependent in myeloid cells (Peyssonnaux et al., 2006). In this context, it would be interesting to investigate *HAMP* gene expression in mammary gland tissue and milk from cows suffering from mastitis, to investigate a possible function of *HAMP* in udder infections. Expression of *CDH1*, *PVRL2* and *CALM3* could be confirmed in all tissue as expected.

Figure 3: Expression of candidate genes in genomic DNA (**1)**, parenchyma of lactating **(2)** and non lactating cows **(3)** as well as in liver tissue **(4) M**: Marker; **nC**: negative Control. **A**: *HAMP*; **B**: *PVRL2*; **C**: *CALM3*; **D**: *CDH1*.

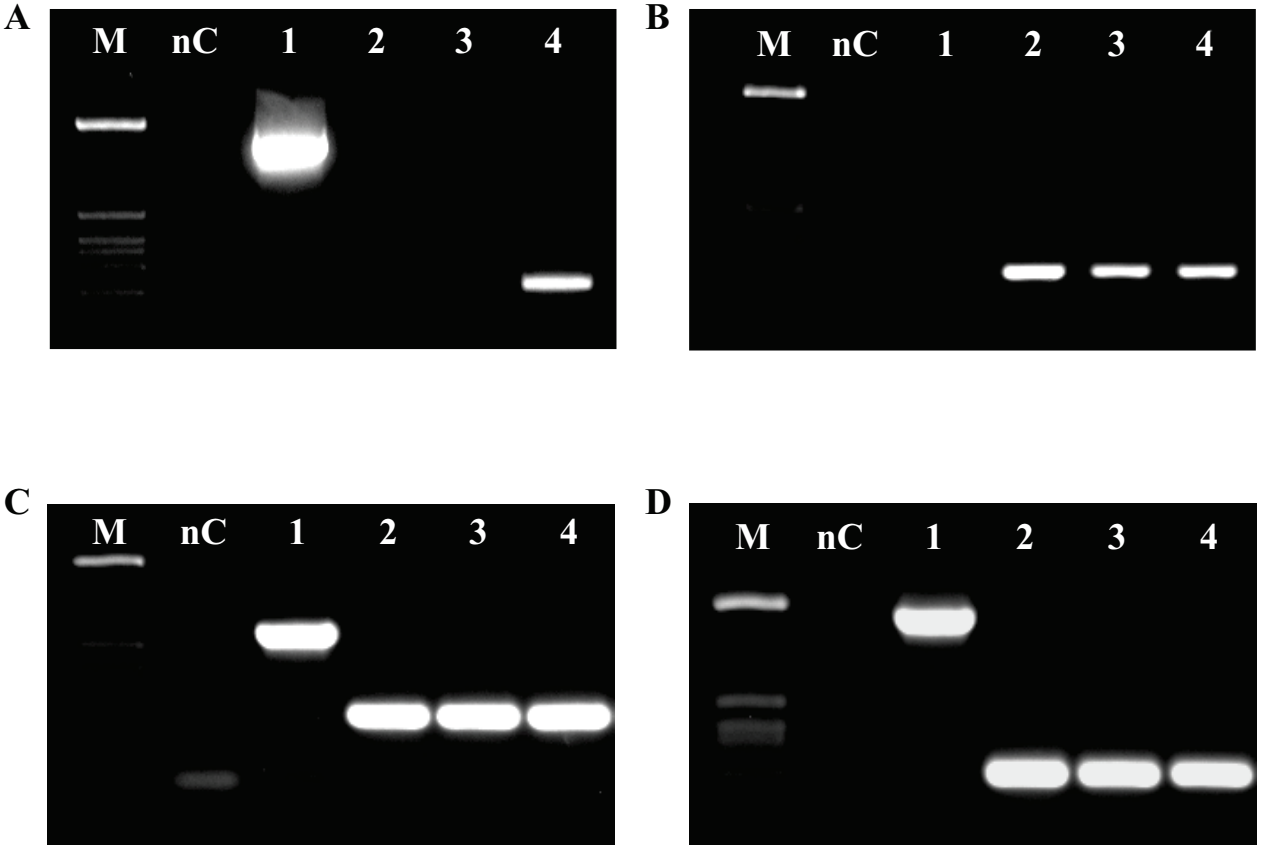


Table 3: List of primers used for expression analyses

Gene	Orientation	Sequence	Annealing Temperatures
HAMP	forward	CAGACGGCACAATGGCAC	55°C
	reverse	GGGCAGCAGGAATAAATAAG	
PVRL2	forward	GGCTGTCCTTCGTCAATAC	50°C
	reverse	CAGATGACCTTGACACCGT	
CALM3	forward	GAACCCCACTGAAGCCGAGC	60°C
	reverse	TGGCCGTCCCCGTCAATGT	
CDH1	forward	CATAGACAACCAGAACAAAGAC	58°C
	reverse	CCCTATGTAACTGGCTCAAG	

2.3 Publications

2.3.1 Refined Positioning of a Quantitative Trait Locus Affecting Somatic Cell Score on Chromosome 18 in the German Holstein using Linkage Disequilibrium

Christine Baes, Bodo Brand, Manfred Mayer, Christa Kühn, Zengting Liu, Friedrich Reinhardt, Norbert Reinsch

J. Dairy Sci. (2009), **92**:4046-4054

INTERPRETIVE SUMMARY

Genetic selection for udder health is widely based on the indicator trait somatic cell score, which is highly correlated with clinical mastitis and has a moderate heritability. In this study a combined linkage and linkage disequilibrium analysis was used to fine map a previously reported quantitative trait locus affecting somatic cell score in the German Holstein population. The quantitative trait locus in the marker interval identified was estimated to be responsible for between 5.89 % and 13.86 % of the genetic variation in somatic cell score.

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Refined positioning of a quantitative trait locus affecting somatic cell score on chromosome 18 in the German Holstein using linkage disequilibrium

C. Baes,*[1] B. Brand,*[1] M. Mayer,* C. Kühn,* Z. Liu,† F. Reinhardt,† and N. Reinsch*[2]
*Forschungsinstitut für die Biologie Landwirtschaftlicher Nutztiere, 18196 Dummerstorf, Germany
†Vereinigte Informationssysteme Tierhaltung w. V., 27283 Verden, Germany

2.3.2 Identification of a two-marker-haplotype on *Bos taurus* autosome 18 associated with somatic cell score in German Holstein cattle

Bodo Brand, Christine Baes, Manfred Mayer, Norbert Reinsch, Christa Kühn

BMC Genetics (2009), **10**:50

INTERPRETIVE SUMMARY

Udder health has a substantial impact on the economy of milk production and the welfare of dairy cows. The somatic cell score is highly correlated with clinical mastitis and is implemented in routine sire evaluations as an indicator trait for udder health. In the German Holstein population quantitative trait loci for somatic cell score have previously been reported on *Bos taurus* autosome 18. In this study a refined analysis of the telomeric region on chromosome 18 using a combined linkage and linkage disequilibrium approach is described. Two putative QTL for somatic cell score were identified and a supplementary analysis of maternal two-marker-haplotypes indicated one specific haplotype to be in linkage disequilibrium with a locus affecting somatic cell score in German Holstein cattle.

BMC Genetics

Research article

Identification of a two-marker-haplotype on Bos taurus autosome 18 associated with somatic cell score in German Holstein cattle

Bodo Brand†[1], Christine Baes†[2], Manfred Mayer[2], Norbert Reinsch[2] and Christa Kühn*[1]

Address: [1]Research Unit Molecular Biology, Research Institute for the Biology of Farm Animals, 18196 Dummerstorf, Germany and [2]Research Unit Genetics and Biometry, Research Institute for the Biology of Farm Animals, 18196 Dummerstorf, Germany

Email: Bodo Brand - brand@fbn-dummerstorf.de; Christine Baes - baes@uni-hohenheim.de; Manfred Mayer - mmayer@fbn-dummerstorf.de; Norbert Reinsch - reinsch@fbn-dummerstorf.de; Christa Kühn* - kuehn@fbn-dummerstorf.de

* Corresponding author †Equal contributors

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Abstract

Background: The somatic cell score (SCS) is implemented in routine sire evaluations in many countries as an indicator trait for udder health. Somatic cell score is highly correlated with clinical mastitis, and in the German Holstein population quantitative trait loci (QTL) for SCS have been repeatedly mapped on *Bos taurus* autosome 18 (BTA18). In the present study, we report a refined analysis of previously detected QTL regions on BTA18 with the aim of identifying marker and marker haplotypes in linkage disequilibrium with SCS. A combined linkage and linkage disequilibrium approach was implemented, and association analyses of marker genotypes and maternally inherited two-marker-haplotypes were conducted to identify marker and haplotypes in linkage disequilibrium with a locus affecting SCS in the German Holstein population.

Results: We detected a genome-wide significant QTL within marker interval 9 (*HAMP_c.366+109G>A* - *BMS833*) in the middle to telomeric region on BTA18 and a second putative QTL in marker interval 12-13 (*BB710* - *PVRL2_c.392G>A*). Association analyses with genotypes of markers flanking the most likely QTL positions revealed the microsatellite marker *BMS833* (interval 9) to be associated with a locus affecting SCS within the families investigated. A further analysis of maternally inherited two-marker haplotypes and effects of maternally inherited two-marker-interval gametes indicated haplotype *249-G* in marker interval 12-13 (*BB710* - *PVRL2_c.392G>A*) to be associated with SCS in the German Holstein population.

Conclusion: Our results confirmed previous QTL mapping results for SCS and support the hypothesis that more than one locus presumably affects udder health in the middle to telomeric region of BTA18. However, a subsequent investigation of the reported QTL regions is necessary to verify the two-QTL hypothesis and confirm the association of two-marker-haplotype *249-G* in marker interval 12-13 (*BB710* - *PVRL2_c.392G>A*) with SCS. For this purpose, higher marker density and multiple-trait and multiple-QTL models are required to narrow down the position of the causal mutation or mutations affecting SCS in German Holstein cattle.

Background

Udder health, somatic cell score and subclinical and clinical mastitis remain major challenges for the economy of milk production in respect to milk production efficiency and animal health and welfare. Several studies have attempted to identify chromosomal regions, genes and polymorphisms that influence udder health in order to improve breeding strategies. SCS has been used as an indicator of udder health and is implemented in routine sire evaluations in many countries [1]. SCS has a low to medium heritability (h^2 = 0.15; [1]) and a strong correlation to mastitis in the German Holstein population (r_g = 0.84; [2]). However, selection on low SCS as well as on decreased mastitis incidence is hampered by three aspects: first the low heritability of SCS and liability to mastitis, second the difficulties in recording mastitis related data and third by potential population-wide antagonisms between milk production traits (milk, fat and protein yield) and udder health [1,2]. Recently, marker assisted selection (MAS) has been determined as a promising tool to improve current selection strategies based on phenotypic data [3]. MAS implements genetic marker information of confirmed QTL regions to identify individuals with favourable genetic background concerning the trait of interest. Thus, the confirmation and fine mapping of known QTL regions and the estimation of QTL effects will advance the use of MAS.

For clinical mastitis (CM) or SCS, QTL have been detected on nearly all autosomes [4] and several studies repeatedly detected QTL for SCS or CM in the telomeric region of BTA18 [5-12]. In addition, Kühn et al. [13] have shown in a proof-of-principle approach that information of 5 markers located in the telomeric region of BTA18 indeed enabled successful MAS, which identified halfsib heifers prior to first calving that exhibited significant differences in SCS after parturition.

The intention of this study was to further analyse the middle to telomeric region of BTA18 with the aim of identifying markers and marker haplotypes in linkage disequilibrium (LD) with SCS in German Holstein cattle to improve MAS for udder health. Therefore, we increased the marker density in the telomeric region on BTA18 and selected four functional candidate genes within the QTL regions reported by [5,7,11]. Polymorphisms detected within these candidate genes were used as additional markers for fine mapping previously identified QTL regions and to analyse effects of candidate gene polymorphisms on SCS in the German Holstein. In the present study, we detected a genome-wide significant QTL in the middle to telomeric region on BTA18. Furthermore, we analysed effects of maternally inherited marker haplotypes and identified a two-marker-haplotype associated with SCS in German Holstein cattle.

Methods

Selection of candidate genes

Based on the positional information derived from the previously mentioned QTL regions identified by [5,7,11] and preliminary results from microarray experiments, an intensive literature research of genes bearing potential function in innate immune defence, immune defence, mammary gland development or udder morphology was performed. To enable the investigation of positional candidate genes within the telomeric region on BTA18, a comparative map for BTA18 and *Homo sapiens* autosome 19 (HSA19) was constructed to take advantage of the more advanced gene annotation of the human genome (Additional file 1: marker table). Four candidate genes were selected: *calmodulin 3* (phosphorylase kinase, delta) (*CALM3*), *Hepcidin antimicrobial peptide* (*HAMP*), *cadherin 1, type 1, E-Cadherin* (ephitelial) (*CDH1*), and *poliovirus receptor-related 2* (herpesvirus entry mediator B) (*PVRL2*). Calmodulin 3 is a ubiquitously expressed Ca^{2+}-binding protein that is involved in many Ca^{2+} modulated signal pathways. It was selected due to its influence on smooth muscle contraction [14,15] and the potential effects on milk leaking and milk flow, which is a trait with a substantial genetic correlation to SCS (r_g = 0.4; [1]). *HAMP* was selected as an immunological candidate gene because of its antifungal and antibacterial activity [16], the toll-like receptor-4 dependent induction by bacterial pathogens in myeloid cells [17] and to a lesser extent due to its function as a key regulator of iron metabolism [18]. For *CDH1* and *PVRL2* initial results from own microarray experiments indicated their differential expression in clinically unaffected heifers with different predisposition to udder infection. Additionally, both genes are involved in cell-cell junctions that have a strong impact on tissue development. E-Cadherin, a Ca^{2+}-dependent cell-cell adhesion molecule, is involved in tissue and organ development as part of the cadherin-catenin-complex [19,20], and poliovirus receptor-related 2, a Ca^{2+}-independent immunoglobulin-like cell adhesion molecule, is involved in the organisation of intercellular junctions as part of the nectin-afadin-complex [20-22]. Furthermore E-Cadherin can serve as a receptor for pathogens and is involved in their internalization [23,24].

Screening for polymorphisms

Polymorphisms within the candidate genes were detected by comparative sequencing of genomic DNA from heifers selected for QTL alleles associated with high or low SCS. The heifers originate from most likely QTL heterozygous sires selected from the German Holstein population based on marker information regarding a confirmed QTL for SCS on BTA18. The selection strategy for the heifers and their phenotypes are described by Kühn et al. [13] in detail. For sequencing, we selected three pairs of halfsibs, where one daughter inherited the SCS increasing paternal

chromosomal region (q) and the other inherited the SCS decreasing paternal chromosomal region (Q). In addition, two animals were screened for variants which originated from a genetically divergent Charolais × Holstein F_2 cross background.

For each gene except *CDH1* the entire gene (large introns excluded), 800 to 1500 bp of the promoter region and up to 500 bp downstream of the transcripts were investigated. For *CDH1*, only the genomic sequence spanning exon 13 to 15 was analysed. Primer information and the genomic position for each primer are given in additional file 2: primer table sequencing. Sequencing was performed by amplification of genomic DNA and subsequent sequence analysis with the DYEnamic ET Terminator Cycle Sequencing reaction and the MegaBACE™1000 DNA Analysis System (GE Healthcare, Munich, Germany). For evaluation of polymorphisms BioEdit 7.0.5.2 [25] was used. Essentially, polymerase chain reaction (PCR) primers were used for sequencing. Additional sequencing primers were used only for longer PCR fragments or PCR fragments that were difficult to sequence.

Families

The pedigree material used for genotyping included a total of 1,054 animals originating from six paternal halfsib families. Some of the animals are a subset of the granddaughter designs previously described by [7,11]. Numbers of sons per grandsire ranged from 60 to 353, with an average family size of 175 sons. The German genetic evaluation center (VIT) in Verden, Germany provided additional pedigree information including non-genotyped ancestors of genotyped animals (7,627 animals).

Phenotypes

The phenotype information for SCS was provided by the VIT as daughter yield deviations (DYD) for the first lactation (Table 1). SCS is the $\log_2$ transformed somatic cell count ($\log_2$ (somatic cell count/100000) + 3). DYD for SCS were calculated based on a random regression test day model [26]. The reliabilities associated with the DYD were expressed as the number of effective daughter contributions (EDC) as described by [26]. DYD and EDC for genotyped animals were obtained from the official release of the April 2008 routine genetic evaluation.

Marker Set

The marker set included a total of 28 markers covering the telomeric region of BTA18 from *CDH1* to *DIK4013*. Six of the 28 markers were already genotyped within previous QTL mapping studies [5,7,11] and include an erythrocyte antigen marker. The other 22 markers were newly selected and genotyped. Fifteen new microsatellite and seven new single nucleotide polymorphism (SNP) markers were chosen based on the information of the putative QTL positions reported by [5,7,11]. The fifteen new microsatellite markers were selected using the bovine linkage map of the United States Department of Agriculture's Meat Animal Research Center (MARC USDA) [27]. SNP markers were selected from all detected polymorphisms within candidate genes based on allele frequencies, position (coding- or non-coding region), effect of the SNP (synonymous or nonsynonymous) and whether they are in linkage disequilibrium to each other.

Genotyping

Microsatellite markers were genotyped by PCR or Multiplex-PCR with fluorescence labelled primers followed by a fragment length analysis using the MegaBACE™1000 DNA Analysis System and MegaBACE Fragment Profiler Version 1.2 software (GE Healthcare, Munich, Germany) (Additional file 3: primer table genotyping). The genotyping methods used for detection of SNP were PCR-restriction fragment length polymorphism (RFLP) assays for *CDH1_c.2102C>T* [NCBI dbSNP: rs41862198], *HAMP_c.366+109G>A* [NCBI dbSNP: ss141026745], *PVRL2_c.-1268G>C* [NCBI dbSNP: ss141026747], *PVRL2_c.392G>A* [NCBI dbSNP: rs41884977], *CALM3_c.3+1795C>T* [NCBI dbSNP: ss141026788] and a multiplex pyrosequencing assay for *HAMP_c.86+430G>A* [NCBI dbSNP: ss141026740] and *CALM3_c.3+1678C>T* [NCBI dbSNP: ss141026787] (Table 2). The enzymes used for detection of RFLPs were identified with the NEBcutter V2.0 webtool [28,29] and PCR primers were designed with primer analysis software Oligo 4.1 (National Bioscience Inc., Plymouth, MN, USA). For the RFLP assays, a PCR specific for each SNP was used to amplify genomic DNA, and PCR products were subsequently incubated for 8 h with SNP specific restriction enzymes. For visualization of the RFLP a 2.5% agarose gel was used.

Table 1: Descriptive statistics for daughter yield deviations

Trait	Number of Phenotypes	Mean	Standard Deviation	Minimum	Maximum
SCS	1058	- 0.1024	0.383	-1.211	1.072

Arithmetic mean and standard deviation as well as lowest and highest daughter yield deviation for SCS considering all animals are given. SCS is the $\log_2$ transformed somatic cell count ($\log_2$ (somatic cell count/100000) + 3).

Table 2: Primer and enzymes used for genotyping of SNP

SNP	Method		Primer	Enzyme	Alleles
CDH1_c.2102C>T	RFLP	forward	5'-CAT AGA CAA CCA GAA CAA AGA C	*Eco*57I	A/G
		reverse	5'-TGG ACC TCT GGG GAG ACT G	(Fermentas Life Sciences)	
HAMP_c.366+109G>A	RFLP	forward	5'-AGA CAC CCA CTT TCC CAT C	*Csp*6I	A/G
		reverse	5'-AGC TCC ACA GTC TCT TCT C	(Fermentas Life Sciences)	
PVRL2_c.-1268G>C	RFLP	forward	5'-AAT GCC AGT CAA TCA CAG TCT C	*Hinc*II	G/C
		reverse	5'-GGA TTC TAC ACC CGC TGC TC	(Fermentas Life Sciences)	
PVRL2_c.392G>A	RFLP	forward	5'-TTC CTC AAA CTG TCT TAT CTG G	*Hin*6I	A/G
		reverse	5'-GTG TAG TTG CCC TCG TCC TC	(Fermentas Life Sciences)	
CALM3_c.3+1795C>T	RFLP	forward	5'-TTG AGA GAA AAC CAG CAG AC	*Bse*YI	C/T
		reverse	5'-CCA GGC AGC AGT GTT AGA	(New England Biolabs Inc.)	
CALM3_c.3+1678C>T	pyro-sequencing	forward	5'-GAG CCC TCC CTG AGT GCT TC		C/T
		reverse	5'-AGC GGC TGC CTG TTC TCC		
		sequencing	5'-AGG ATG GCT GCA CAC		
HAMP_c.86+430G>A	pyro-sequencing	forward	5'-AAA AGA TGG TGG GAG AGT AAT GG		A/G
		reverse	5'-CCT CTG CAC TTG CCT GTA AGA CTT		
		sequencing	5'-CCA AAT AGG TCA AAT AAC A		

Summary of methods and primers used for genotyping SNP. Forward primers for pyrosequencing were biotyinlated at the 5'-end for purification of single strand DNA.

Primer design for pyrosequencing was performed with Pyrosequencing™ Assay Design Software (Biotage AB, Uppsala, Sweden). A SNP specific PCR was used for amplification of genomic DNA and the products of both PCR were merged and analysed with the PSQ™HS 96A pyrosequencing system (Biotage AB, Uppsala, Sweden) in a multiplex run.

Linkage Map

The genetic linkage map was calculated based on a refined marker order using CRIMAP software [30]. The marker order on BTA18 used for the calculation was evaluated in two steps. First, information from published linkage maps [31,32], RH-maps [33], and human and bovine sequence-assemblies [34] were merged and compared to own linkage-mapping results. Second, in a region including the markers *BMON117*, *DIK4672*, *BMS833*, *DIK4232* and *BB710*, no unequivocal marker order was obtained. Thus, the marker order in this region was verified by RH-mapping using the 12000 rad whole-genome radiation hybrid panel [35] and RH-MAP3.0 software [36]. For some marker groups, a recombination rate of zero was calculated. To avoid technical difficulties arising in the calculation of transmitting probabilities the marker spacing was set to small values greater than zero (Additional file 1: marker table).

QTL Mapping

A combined linkage and linkage disequilibrium analysis (LALD) was performed using the software system TIGER [37]. TIGER is a UNIX script linking several individual Fortran programmes to perform combined linkage and linkage disequilibrium analysis. Six steps are implemented in the script. First, allele frequencies and transmitting probabilities for each putative QTL position are calculated using BIGMAP [38]. The putative QTL positions were considered as the midpoint of each marker interval, resulting in a total of 27 putative QTL positions. Second, the identical by descent (IBD) sub-matrices for each putative QTL position are computed based on the gene dropping procedure described by [39-41] and third, the IBD sub-matrices are tested for positive definiteness and inverted. The software program COBRA [42] computes a condensed gametic relationship matrix and its inverse at each putative QTL position for the calculation of gametic effects. Transmitting probabilities and IBD sub-matrices are used for the set up of the condensed gametic relationship matrix. Finally, an LALD analysis is performed analysing every putative QTL position with restricted maximum likelihood (REML) methods applied in ASReml [43]. A detailed description of the QTL mapping procedure and the model applied in ASReml is given by [12].

Analyses were conducted using a likelihood ratio test, where the REML of the full model was compared with the REML of the model missing the QTL effect. Chromosome-wide and genome-wide significance thresholds were determined as restricted log likelihood ratio (RLRT) equivalents of logarithm of odds (LOD) units [44] where a LOD > 2 indicates chromosome-wide (RLRT = 9.2) and a LOD > 3 indicates genome-wide significance (RLRT = 13.8) [45]. Confidence intervals were estimated using the LOD drop-off method described by [44].

Association analysis

To investigate the association of candidate gene polymorphisms and markers flanking interval 9 and interval 12-13

with SCS, a mixed model including a random polygenic effect and the fixed effect of marker genotypes was applied in ASReml:

$$y_{ij} = \mu + MG_i + a_j + e_{ij} \quad (1)$$

where **y** is a vector of phenotypic observations (DYD) for sires, μ is the overall mean, $\mathbf{MG}_i$ is the fixed effect of the marker genotype **i**, $\mathbf{a}_j$ is the random polygenic effect of animal **j** and $\mathbf{e}_{ij}$ is the random residual. The polygenic effect that accounts for the family structure of the population was estimated using an extended pedigree of non-genotyped ancestors of genotyped animals including a total of 7,627 animals. To account for multiple testing, a 5% experiment-wise significance threshold was obtained by Bonferroni correction of the nominal p-value assuming a 5% Type 1 error ($p_{exp} = 0.0057$).

Analysis of maternally inherited two-marker haplotypes and two-marker-interval gametes

Due to the limited number of sires, paternally inherited chromosomes could have a strong impact on genotype effects estimated in their offspring. To exclude these effects, maternally inherited two-marker-intervals including flanking markers of most likely QTL position were investigated. The most probable linkage phases of genotyped sires were calculated to determine the maternally inherited haplotypes using BIGMAP. The TIGER software system was then applied to estimate maternally inherited gametic effects for the putative QTL positions in interval 9 and interval 12 based on IBD sub-matrices. Finally, the estimated gametic effects of maternally inherited two-marker-interval gametes were analysed and plotted using SAS software (SAS Institute, Cary, NC, USA).

To verify differences in estimated two-marker-interval gamete effects, we performed an association analysis including a fixed maternally inherited two-marker-haplotype effect independent of the IBD sub-matrices. For this purpose, the same model was used as for marker genotypes (1), except that the fixed genotype effect was replaced by the fixed maternally inherited two-marker-haplotype effect. Two-marker-haplotypes with the highest and lowest mean for IBD gametic effects were tested against the total of all other haplotypes within respective intervals. For interval 12-13 additionally the most frequent two-marker-haplotype was investigated.

Results

Screening for polymorphisms

Sequence analyses were based on the provisional reference sequences obtained from NCBI [46]. For *CALM3* [GenBank: NM_001046249; GeneID: 520277], the entire gene (10728 bp) was resequenced including ~1.3 kb upstream from the transcription start (assumedly promoter region). A total of eleven polymorphisms were detected (Additional file 4: polymorphisms): ten SNP and one 12 bp deletion within the assumed promoter region.

For *HAMP* [GenBank: NM_001114508; GeneID: 512301], in silico analyses revealed that this gene is not annotated in Btau4.0 but has been annotated in NCBI Build3.1. The sequence of *HAMP* is still located on BTA18 in Btau4.0 [GenBank: NC_007316.3: 476114 bp-477570 bp]. The whole gene (2777 bp) was resequenced, and three SNP were detected (Additional file 4: polymorphisms), one SNP within intron 1 and two SNP within the first 110 nucleotides downstream of *HAMP* transcript.

For *CDH1*, only the coding sequence was provided as provisional mRNA reference sequence [GenBank: NM_001002763; GeneID: 282637]. Due to discrepancies between the mRNA reference sequence and the genome assembly sequence, only the genomic reference sequence was used for sequence comparisons. The genomic sequence including exon 13 to 15 (1673 bp) was investigated and six SNP were detected (Additional file 4: polymorphisms) two of them were located in exon 13 and cause an amino acid substitution.

For *PVRL2* [GenBank: NM_001075210.1; GeneID: 505580], the whole gene, excluding 13181 bp of intron 2 and 670 bp of intron 4, was resequenced (11766 bp). A total of 17 SNP and two length polymorphisms were identified (Additional file 4: polymorphisms). The examination of the promoter region (1 kb upstream of transcription start) of *PVRL2* revealed a gap of 367 bp with missing sequence information in the genome assembly Btau4.0. By a BLAST search [47] a whole genome shotgun sequence (WGS)-Trace [Trace Archive: 1632871820] that overlaps the gap was identified within the NCBI Trace Archives [48]. This WGS-Trace was incorporated in the reference sequence and sequence information was confirmed by resequencing. Coordinates of polymorphisms upstream of the translation initiation codon ATG are indicated according to the updated sequence [GenBank: FJ829796].

Markers and Map

The genetic marker map covered 50 cM of the telomeric region of BTA18 (Additional file 1: marker table). Marker intervals ranged from 0.05 cM to 7.9 cM with an average marker interval of 1.85 cM. The number of alleles for each marker ranged from two for SNP to 34 for the erythrocyte antigen marker. The marker order is in good agreement with previously published linkage- and RH-maps [31-33] and no discrepancies to the bovine sequence assembly Btau4.0 [49] were observed (Additional file 1: marker table).

QTL Mapping

The restricted Log Likelihood Ratio profile of the combined LALD analysis is shown in Figure 1. Two peaks exceeding the genome-wide significance level can be observed. The maximum of the first peak is located in marker interval 9 at position 71.775 cM with *HAMP_c.366+109G>A* and *BMS833* as flanking markers. The maximum of the second peak is located in marker interval 12 at position 77.6 cM with the flanking markers *BB710* and *PVRL2_c.-1268G>C*. The LOD drop-off method was used to estimate confidence intervals for each of the peaks. An approximate 96% confidence interval included marker intervals 3 to 9 for the first peak and the marker intervals 2 to 15 for the second peak.

Association analysis

An association analysis between the candidate gene polymorphisms and SCS was performed to evaluate the influence of the selected candidate genes on the variation in SCS in German Holstein cattle. The markers *BMS833* and *BB710* were also included in the association analysis, as they are flanking markers of the maxima observed in the LALD test statistic. The association analyses (Table 3) indicated a significant effect of the *BMS833* genotype (p = 0.004) on SCS within the 5% experiment-wise significance threshold (p_{exp} = 0.0057). *PVRL2_c.392G>A* (p = 0.017) and *CALM3_c.3+1678C>T* (p = 0.055) approached nominal significance, but were not significant at the 5% experiment-wise significance level. For the genotypes of SNP markers *CDH1_c.2102C>T*, *HAMP_c.366+109G>A*, *HAMP_c.86+430G>A*, *PVRL2_c.-1268G>C* and *CALM3_c.3+1795C>T* no significant effects on SCS were observed.

Analysis of maternally inherited two-marker haplotypes and two-marker-interval gametes

To exclude any specific sire gamete effects that occur in a paternal halfsib design, maternally inherited gametic effects for two-marker-interval gametes were analysed and plotted for the putative QTL positions in interval 9 and interval 12-13. In interval 9 (*HAMP_c.366+109G>A* - *BMS833*), six maternally inherited two-marker-allele combinations occurred. Two-marker-interval gametes including *BMS833* alleles *115* and *119* were excluded, because they both occurred only once (Table 4; Figure 2A). Gametes carrying the *BMS833* allele *117* in interval 9 have a mean estimated maternally inherited gametic effect of 0.022 (± 0.003) and 0.028 (± 0.003), respectively, whereas gametes carrying allele *113* of marker *BMS833* have mean effects of - 0.0006 (± 0.0014) and - 0.0059 (± 0.0022), respectively. Investigating the gametic effect of each flanking marker of interval 9 alone (Figure 2B), revealed that the main differences in gametic effects in the two-marker-interval results from the discrimination by microsatellite marker alleles. The difference in the mean effects for two-marker-interval gametes carrying the two

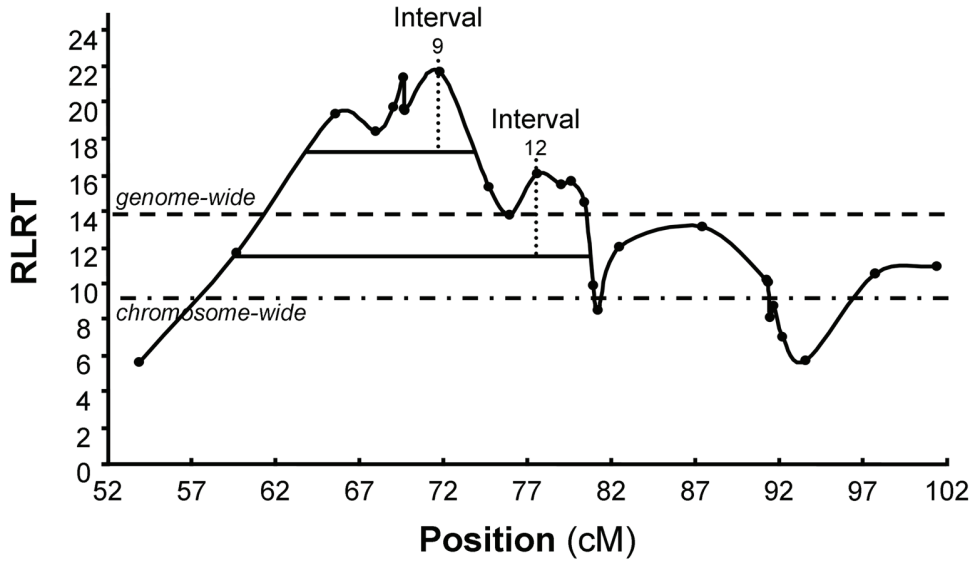

Figure 1
LALD Profile. Restricted Log Likelihood Ratio (RLRT) profile of a combined linkage and linkage disequilibrium analysis testing for a putative QTL affecting SCS on BTA18. The profile is plotted for putative QTL positions located at the midpoint of each marker interval. QTL positions are indicated by black dots. Thick black lines indicate confidence interval for the first maximum at interval 9 (upper line) and the second maximum at interval 12 (lower line). Dashed lines indicate genome- and chromosome-wide significance thresholds.

Table 3: Association analyses of marker genotypes with SCS in six German Holstein halfsib families

Marker/SNP	Position in cM	Nominal p-value
CDH1_c.2102C>T	52	0.121
HAMP_c.86+430G>A	69.7	0.132
HAMP_c.366+109G>A	69.75	0.416
BMS833	73.8	0.004*
BB710	76.3	0.216
PVRL2_c.-1268G>C	78.9	0.325
PVRL2_c.392G>A	79.1	0.017
CALM3_c.3+1678C>T	81.15	0.055
CALM3_c.3+1795C>T	81.25	0.223

Summary of results from association analyses for marker genotypes based on a mixed model (1) including a fixed genotype effect and a random polygenic effect. The experiment-wise 5% significance level is $p_{exp} = 0.0057$.

alleles of *HAMP_c.366+109G>A* is 0.009, whereas the difference between gametes carrying alleles *113* and *117* of marker *BMS833* is 0.0268.

For interval 12-13 (*BB710 - PVRL2_c.392G>A*) we selected *PVRL2_c.392G>A* (interval 13) as a flanking marker. Both polymorphisms, *PVRL2_c.-1268G>C* (interval 12) and *PVRL2_c.392G>A* (interval 13) are located in close vicinity within the *PVRL2* gene and showed a high linkage disequilibrium ($r^2 = 0.68$). In interval 12-13 (*BB710 - PVRL2_c.392G>A*), two-marker-interval gametes with twelve different two-marker-allele combinations were observed. Two-marker-interval gametes carrying the two *BB710* alleles *243* and *245* were excluded, because they both only occurred once. In addition, the frequencies for allele combinations *253-G* and *257-G* were smaller than 1% and therefore gametes carrying these two allele combinations are not included in Figure 3 (Table 4). Analogous to the situation in interval 9, the SNP alleles of *PVRL2_c.392G>A* seem to have only a small influence on discriminating estimated two-marker-interval gamete effects (Figure 3B), because the difference in the mean effects discriminated by the alleles is 0.0078. For microsatellite marker alleles (Figure 3A), two-marker-interval gametes carrying the allele *249* have mean effects of 0.0378 (± 0.0018) and 0.0176 (± 0.0009) and gametes carrying allele *253* have mean effects of - 0.0150 (± 0.0021) and - 0.0253 (± 0.0012). The biggest differences in the mean estimated gametic effects was observed for the two two-marker-interval gametes *249-G* (0.0378 (± 0.0018)) and *253-A* (-0.0253 (± 0.0012)). Thus, the difference in the mean maternally inherited gametic effects equals 0.0631, which is equivalent to 0.16 phenotypic standard deviations.

To validate the differences observed between maternally inherited two-marker-interval gametes, we performed a direct association analysis for maternally inherited two-marker-haplotypes without considering IBD coefficients in interval 9 (*HAMP_c.366+109G>A - BMS833*) and interval 12-13 (*BB710 - PVRL2_c.392G>A*) (Table 5). Only the two-marker-haplotypes with the highest and lowest mean for IBD gametic effects were tested against the total of all other haplotypes. For interval 12-13, we also tested *255-A*, because it was the most frequent haplotype (frequency at 40%) in the data set. In interval 9, no significant association of the maternally inherited haplotypes *G-113* and *G-117* were observed, whereas haplotype *249-G* (p = 0.027) in interval 12-13 seems to be associated with SCS in the German Holstein population.

Table 4: Estimated effects on SCS for maternally inherited two-marker-gametes in interval 9 and interval 12-13

Interval	Haplotype	Number of Animals	Haplotype Frequencies	Mean of Estimates	Standard Error Mean
9	*A - 113*	258	0.2986	-0.0006	0.0014
9	*A - 117*	198	0.2292	0.0217	0.003
9	*G - 113*	339	0.3924	-0.0059	0.0022
9	*G - 117*	69	0.0799	0.0276	0.003
12 - 13	*249 - G*	38	0.0409	0.0378	0.0018
12 - 13	*249 - A*	262	0.2820	0.0176	0.0009
12 - 13	*251 - G*	78	0.0840	-0.0012	0.0007
12 - 13	*251 - A*	32	0.0344	0.0002	0.0024
12 - 13	*253 - G*	3	0.0032	-0.0150	0.0021
12 - 13	*253 - A*	44	0.0474	-0.0253	0.0012
12 - 13	*255 - G*	53	0.0571	0.0122	0.0009
12 - 13	*255 - A*	375	0.4037	-0.0043	0.0009
12 - 13	*257 - G*	4	0.0043	-0.0019	0.0022
12 - 13	*257 - A*	40	0.0431	0.0144	0.0022

Summary of means for gametic effects estimated for maternally inherited two-marker-interval gametes considering IBD coefficients. Haplotype frequencies were determined by direct counting.

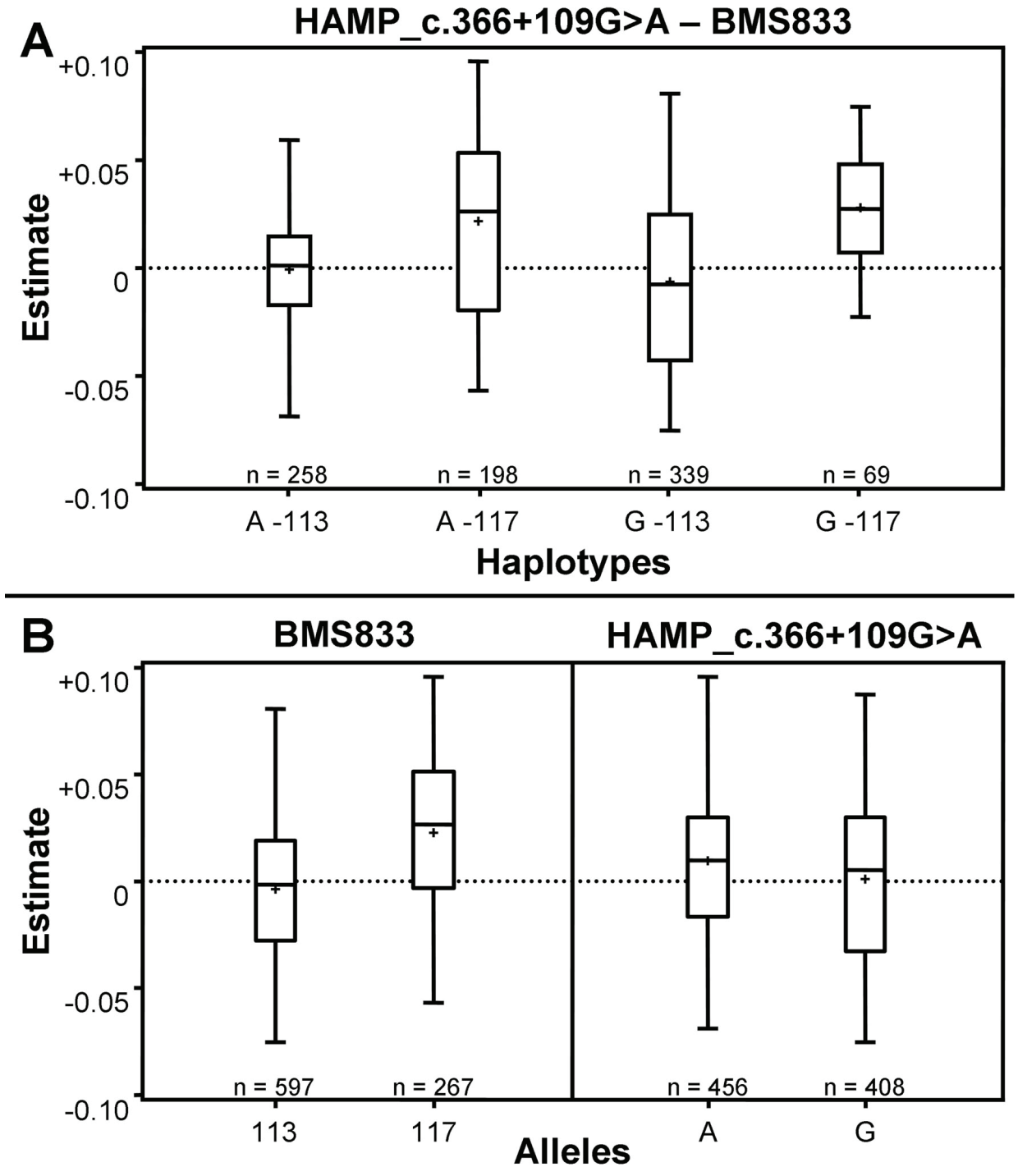

Figure 2
Box-whisker plot for estimated effects of maternally inherited two-marker-interval gametes in interval 9. Two-locus-interval (A) and single locus (B) gametes of the two-marker-interval flanking the putative QTL in interval 9 are written on the X-axis. The boxes contain 50% of all values, where (+) represents the mean, and the horizontal line within the box (-) represents the median. The first and third quartiles are represented by the lower and upper edge of the box and the whiskers extend to the highest and lowest values.

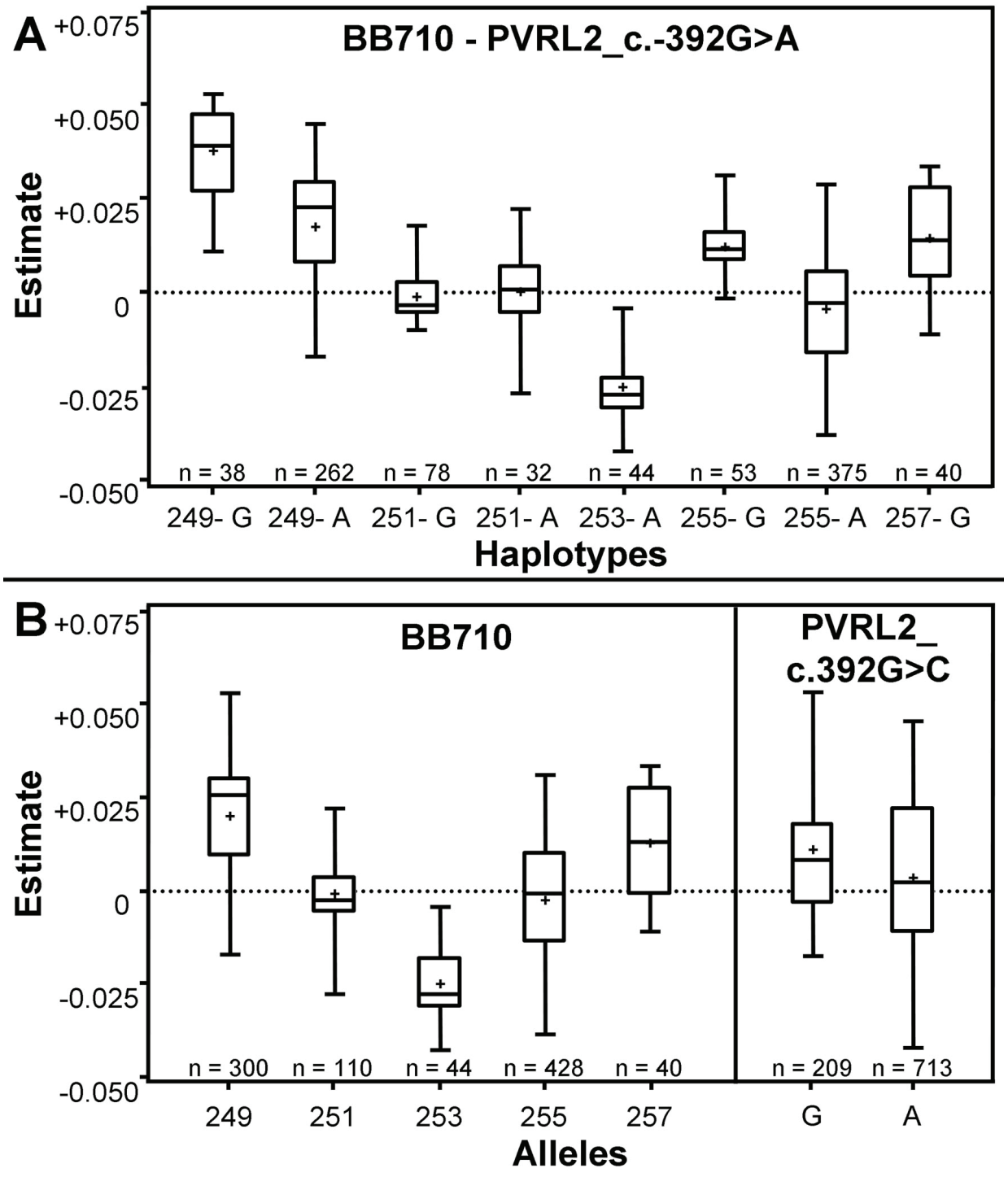

Figure 3
Box-whisker plot for estimated effects of maternally inherited two-marker-interval gametes in interval 12-13. Two-locus-interval (A) and single locus (B) gametes of the two-marker-interval *BB710* (interval 12) and *PVRL2_c.392G>A* (interval 13) are written on the X-axis. Estimates are for the putative QTL position in interval 12. The boxes contain 50% of all values where (+) represents the mean and the horizontal line within the box (-) represents the median. The first and third quartiles are represented by the lower and upper edge of the box and the whiskers extend to the highest and lowest values.

Table 5: Association analyses of maternally inherited two-marker-haplotypes with SCS

Interval	Haplotype	Haplotype Frequencies	Nominal p-value	Effect Estimates
9	*G - 113*	0.3924	0.555	-0.016
9	*G - 117*	0.0799	0.422	0.038
12 - 13	*249 - G*	0.0409	0.027*	0.142
12 - 13	*253 - A*	0.0474	0.207	-0.073
12 - 13	*255 - A*	0.4037	0.057	-0.048

Summary of results from an association analyses for maternally inherited two-marker-haplotypes based on a model (1) including a fixed two-marker-haplotype effect and a random polygenic effect. Individual two-marker-haplotypes were compared to all other maternally inherited haplotypes in the data set. Haplotype frequencies were determined by direct counting.

Discussion

In an approach to identify marker and marker haplotypes affecting SCS in the German Holstein population, we analysed association of marker genotypes and maternally inherited two-marker-intervals flanking putative QTL positions detected in an LALD analysis for SCS on BTA18, and showed that the two-marker-haplotype *249-G* of interval 12-13 (*BB710 - PVRL2_c.392G>A*) is in LD with SCS in the German Holstein population.

Initially, we detected a genome-wide significant QTL for SCS on BTA18 (Figure 1). The maxima of the QTL test statistic were in interval 9 (*HAMP_c.366+109G>A - BMS833*) and in interval 12 (*BB710 - PVRL2_c.-1268G>C*). In the same region telomeric on BTA18, several studies reported QTL for SCS. Kühn et al. [7], Brink [5] and Xu et al. [11] all identified a QTL for SCS in the German Holstein population at the telomeric end of BTA18 near marker *TGLA227*. Schulman et al. [10] reported a QTL for SCS as well as for mastitis at the telomeric end of BTA18 in Finnish Ayrshire cattle, and Ashwell et al. [50] localized a QTL for SCS in the middle to telomeric region on BTA18 at marker BM2078 in US Holstein, whereas Lund et al. [8] reported a QTL for SCS in Finnish Ayrshire, Swedish Red and White, and Danish Red with the maximum of the test statistic in the middle of BTA18 between the markers ILSTS002 and BMS2639. Interestingly Lund et al. [8] as well as Xu et al. [11] indicated that there might be more than one QTL for SCS on BTA18, but in both studies no significant evidence could be provided for a second QTL. Due to the lower marker density in previous studies and different approaches to detect QTL, a direct comparison between our results and previously mentioned studies is impeded. Nevertheless, the confidence intervals of the maxima observed in our LALD analysis did not include marker *TGLA227* at the telomeric end of BTA18, and the QTL position reported by Lund et al. [8] as well as the assumption of a second QTL by Xu et al. [11] further in the middle of BTA18, indicated that we identified a second QTL for SCS in German Holstein cattle and possibly discovered a third QTL on BTA18 in our studies. Further indications for a third QTL on BTA18 in German Holstein cattle arise from association and haplotype analyses but no formal proof for a third QTL is given in our studies.

Subsequent to QTL mapping, we performed an association analysis of marker genotypes to verify the association of candidate gene polymorphisms and flanking markers of the most likely QTL positions observed in LALD analysis with SCS. Our results indicated that the microsatellite marker *BMS833* (p = 0.004) is associated with SCS and *PVRL2_c.392G>A* (p = 0.017) and *CALM3_c.3+1678C>T* (p = 0.055) showed a respective tendency of association. Corresponding to the results of our LALD analysis, these results confirmed the position of one QTL for SCS in interval 9, as *BMS833* is one of the flanking markers of this interval. *PVRL2_c.392G>A* and *CALM3_c.3+1678C>T* are flanking markers of the intervals 13 (*PVRL2_c.-1268G>C - PVRL2_c.392G>A*), 14 (*PVRL2_c.392G>A* - DIK3014) and intervals 16 (DIK4234 - *CALM3_c.3+1678C>T*) and 17 (*CALM3_c.3+1678C>T* - *CALM3_c.3+1795C>T*). The weak association observed for these markers is also in accordance to our LALD analysis, because interval 13 and 14 are within the genome-wide significance threshold in LALD analysis. Additionally, the second maximum observed in our LALD analysis in interval 12 is in an immediately adjacent interval and the putative QTL position in interval 12 is approximately 1 MB upstream of *PVRL2_c.392G>A*. For *CALM3_c.3+1678C>T*, only interval 16 (*DIK4234 - CALM3_c.3+1678C>T*) is within the chromosome-wide significance level in our LALD test statistic confirming the weaker association with SCS observed. To further test the results of our LALD analysis and the association of *BMS833* genotypes with SCS on a population wide level, we investigated maternally inherited two-marker-intervals for interval 9 (*HAMP_c.366+109G>A - BMS833*) as well as for interval 12-13 (*BB710 - PVRL2_c.392G>A*). First we analysed effects of maternally inherited two-marker-interval gametes estimated based on IBD coefficients and second we performed a direct association analysis for maternally inherited two-marker-haplotypes of interval 9 and interval 12-13 without considering IBD coefficients.

For interval 9 (*HAMP_c.366+109G>A - BMS833*), maternally inherited two-marker-interval gametes showed differences in estimated effects for SCS (Figure 2). The variance of estimated gametic effects was higher for two-marker-interval gametes in interval 9 compared to two-marker-interval gametes in interval 12-13 and single marker analyses revealed that the microsatellite marker alleles are the main force in discriminating the effects of maternally inherited two-marker-interval gametes (Figure 2, Figure 3). Association analysis for maternally inherited two-marker-haplotypes of interval 9 showed that none of the maternally inherited two-marker-haplotypes in interval 9 (*HAMP_c.366+109G>A - BMS833*) are in LD with SCS in the German Holstein population. However, association analysis of *BMS833* genotypes showed an association with SCS in our half-sib design indicating that the association is due to linkage but not linkage disequilibrium of the BMS833 locus with the causal mutation affecting SCS.

For interval 12-13 (*BB710 - PVRL2_c.392G>A*), the biggest difference in gametic effects estimated for maternally inherited two-marker-interval gametes was observed between *249-G* (0.0378 (± 0.0018)) and *253-A* (- 0.0253 (± 0.0012)), where a positive mean of estimates indicates an unfavourable effect on SCS (high number of cells) and a negative mean of estimates indicates a favourable effect on SCS (low number of cells). Association analyses for the maternally inherited two-marker-haplotypes of interval 12-13 showed that *249-G* ($p = 0.027$) is associated with SCS at the nominal 5% significance level in the German Holstein population. The weak association of *PVRL2_c.392G>A* genotype with SCS within the families we investigated and the association of maternally inherited two-marker-haplotype *249-G* with SCS indicates that *PVRL2_c.392G>A* is not the causal mutation affecting SCS in German Holstein cattle, but the causal mutation has to be located near or within marker interval 12-13 (*BB710 - PVRL2_c.392G>A*). Combining results obtained for markers *BMS833* and *PVRL2_c.392G>A* it still remains unclear whether there are two mutations (two QTL), one located near marker *BMS833* (interval 9) and one near or within interval 12-13, or only one mutation, presumably in interval 12-13, affecting SCS in the middle part of BTA18.

The candidate gene polymorphisms we investigated were not the causal mutations affecting SCS in German Holstein cattle. However, the results of association analyses for single marker genotypes and maternally inherited two-marker-haplotypes indicated that *HAMP* and *PVRL2* were selected within the region harbouring at least one QTL for SCS. Particularly *PVRL2* still remains interesting. On the one hand, the association of the *PVRL2_c.392G>A* genotype and that of the *249-G* haplotype suggests that another polymorphism within *PVRL2* is the causal mutation affecting SCS in the German Holstein population. On the other hand, *PVRL2* was selected as a gene with possible impact on mammary gland development or udder morphology and several studies have detected QTL for udder conformation on BTA18 [51,52]. Therefore, it is also possible that *PVRL2* does not directly affect SCS but affects udder conformation traits like udder depth or fore udder attachment that are correlated with SCS [1].

Conclusion

In summary, our results suggest that the chromosomal region including interval 9 (*HAMP_c.366+109G>A - BMS833*) and interval 12-13 (*BB710 - PVRL2_c.392G>A*), in the middle to telomeric region on BTA18 has a strong impact on SCS in the German Holstein population. The analyses of maternally inherited two-marker-interval gamete effects and the association of the maternally inherited two-marker-haplotype *249-G* of interval 12-13 (*BB710 - PVRL2_c.392G>A*) with SCS indicates that micosatellite marker *BB710* could be a suitable candidate-marker for MAS, but association of microsatellite marker *BB710* with SCS has to be verified. To confirm the association of the two-marker-haplotype *249-G* with SCS and approve the hypothesis of two QTL in this region a further investigation is necessary. Thus, a mapping of udder conformation traits including a multiple-trait and multiple-QTL model might be useful to verify the existence of two QTL, and whether they are both directly affecting SCS or one is affecting a correlated trait. Likewise, a higher marker density within this region has to be achieved and families segregating for different *BB710* alleles have to be identified. Hence, it might be useful to cover the region including interval 9 and interval 12-13 with equally distributed SNP to narrow down the position of the casual mutation or mutations affecting SCS in the German Holstein population by a further fine mapping approach.

Authors' contributions

BB carried out the genotyping work, the polymorphism screening, the linkage map construction, participated in the statistical analyses and drafted the manuscript. CB facilitated the statistical analyses by the development of software packages, participated in the statistical analyses and helped drafting the manuscript. MM participated in the development of software packages and the statistical analyses. NR participated in design and coordination of the study. CK devised the design of the study, coordinated the study, and participated in the statistical analyses and in drafting the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

***Marker table**. Summary of marker information including marker name, intervals, number of alleles, polymorphism information content, position of markers in own linkage map and in published linkage and RH maps and position in the bovine whole genome assemblies NCBI Build3.1 [GenBank: CM000194.3] and Btau4.0 [GenBank: NC_007316.3] as well as comparative position in the human whole genome assembly HSA36.3. Markers of intervals with marker spacing set to small values greater than zero are indicated by *. For some markers no accession number was available, therefore references for sequence information are given [5,53]. The polymorphism information content was calculated using the software PowerMarker v3.25 [54]. Marker positions are in order to positions of 5'-nucleotides of upstream primers for microsatellite markers or the direct position of the SNP. In Build3.1 several discrepancies in the sequence assembly were discovered. Discrepancies to the refined marker order are highlighted in yellow. Comparative positions in HSA36.3 were assigned by BLAST search of marker sequences in the bovine whole genome assembly Btau4.0 to identify the nearest gene locus on BTA18 and locating this gene locus in HSA36.3 using NCBI Map Viewer [34].*

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Additional file 2

***Primer table sequencing**. Summary of primers used for sequencing, including primer sequence, position in Btau4.0 and polymorphisms detected in PCR fragments.*

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Additional file 3

***Primer table genotyping**. Summary of primers used for genotyping microsatellites, including primer sequence and position in Btau4.0.*

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Additional file 4

***Polymorphisms**. Summary of polymorphisms identified by comparative sequencing including polymorphism name, accession number and sequence information for 200 nucleotides surrounding the polymorphism.*

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2.3.3 Quantitative Trait Loci Mapping of Calving and Conformation traits on *Bos taurus* autosome 18 in the German Holstein Population

B. Brand, C. Baes, M. Mayer, N. Reinsch, T. Seidenspinner, G. Thaller and Ch. Kühn

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INTERPRETIVE SUMMARY

Functional herd life in dairy cattle is substantially affected by udder health and calving performance. In an attempt to reveal the functional background of previously reported quantitative trait loci for calving traits and udder health on bovine chromosome 18, linkage, linkage disequilibrium and combined linkage and linkage disequilibrium analyses were performed. Quantitative trait loci affecting calving performance and conformation traits were identified, and further analyses indicated that variation in conformation traits might be a functional background for quantitative trait loci affecting calving traits and udder health traits on chromosome 18 in the German Holstein population.

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Quantitative trait loci mapping of calving and conformation traits on *Bos taurus* autosome 18 in the German Holstein population

B. Brand,* C. Baes,†‡ M. Mayer,† N. Reinsch,† T. Seidenspinner,§ G. Thaller,§ and Ch. Kühn*[1]
*Research Unit Molecular Biology and
†Research Unit Genetics and Biometry, Research Institute for the Biology of Farm Animals (FBN), D-18196 Dummerstorf, Germany
‡Department of Animal Husbandry and Animal Breeding, University Hohenheim, D-70593 Stuttgart, Germany
§Institute for Animal Breeding and Husbandry, Christian-Albrechts-University, D-24098 Kiel, Germany

3. Discussion

3.1 Udder health and udder type traits

To complement our investigation on udder health and to investigate the molecular background of QTL for somatic cell count, that were previously reported on BTA18, we included the somatic cell score and udder health related udder type traits in our analyses. The mapping approach comprised separate and combined linkage- and linkage disequilibrium analyzes that were used to identify and fine map QTL on BTA18. The studies presented here (Baes et al., 2009; Brand et al., 2009a; Brand et al., 2009b) are the first studies that applied LD and combined LALD mapping methods to investigate SCS and udder type traits on BTA18 in the German Holstein population. Summarizing results, we were able to detect a QTL for SCS responsible for between 5.89 and 13.86 % of the genetic variation in somatic cell score (Baes et al., 2009). The position of the QTL was within a region that was repeatedly reported to contain QTL for SCS (Ashwell et al., 1997; Brink, 2003; Kühn et al., 2003; Lund et al., 2007) and clinical mastitis (Holmberg and Andersson-Eklund, 2004; Schulman et al., 2004). Furthermore, in our analyses, a second peak in the QTL tests statistic that exceeded the genome-wide significance threshold indicated that there might be a second QTL for SCS in the middle to telomeric region on BTA18. Within this region, the maternally inherited haplotype *BB710* - *PVRL2_c.392G>A* showed a significant association with SCS (Brand et al., 2009b).

In the German Holstein population, previous studies reported QTL for SCS near marker *TGLA227* (Brink, 2003; Kühn et al., 2003; Xu et al., 2006). *TGLA227* is located at the telomeric end of BTA18, at 100.8 cM in our linkage map. The QTL for SCS at marker *TGLA227* could not be confirmed in our analyses. In our analyses, the maximum of the LALD statistic was in marker interval *HAMP_c.366+109G>A* - *BMS833* (71.775 cM), approximately 30 cM upstream of marker *TGLA227*, and the haplotype *BB710* -

PVRL2_c.392G>A (77.7 cM) that was shown to be associated with SCS, was located approximately 20 cM upstream of marker *TGLA227*. Because the confidence interval of the QTL for SCS did not include marker *TGLA227* in our LALD analyses, we assumed that we have identified a second QTL for SCS on BTA18 in the families we investigated. This assumption is supported by the studies of Xu et al. (2006) and Brink (2003) as well as by the studies of Lund et al. (2007). Lund et al. (2007) detected a QTL for SCS in three Nordic dairy cattle breeds at marker *BMS2639* that is positioned at 67.5 cM in our linkage map, approximately 4 cM upstream of *HAMP_c.366+109G>A - BMS833*. They additionally found indication of a second QTL for SCS at the telomeric end of BTA18, where Xu et al. (2006) and Brink (2003) both detected a QTL for SCS at marker *TGLA227.* Additionally, Xu et al. (2006) and Brink (2003) both found indications for a second QTL further upstream on BTA18. Xu et al. (2006) assumed the position of a second putative QTL at marker *BM2078* that is positioned at 91.3 cM in our linkage map, and Brink (2003) assumed the position of a second putative QTL near marker *BMS833* at 73.8 cM, corresponding to our linkage map. The second QTL assumed by Xu et al. (2006) is approximately 20 cM downstream of marker interval *HAMP_c.366+109G>A - BMS833,* and the position of the second QTL assumed by Brink (2003) is within the maximum of our LALD QTL test statistic for SCS. However, in all three studies no formal, significant evidence for a second QTL for SCS was found. Similar in our analyses, no significant evidence for a second QTL for SCS was found, when applying a 2-QTL LALD model to our dataset. But considering that Xu et al. (2006), Brink (2003) and Lund et al. (2007) reported divergent positions of QTL maxima and that in all three studies an indication for a second QTL for SCS on BTA18 was found, it is highly probable that there is more than one QTL in the middle to telomeric region on BTA18 affecting SCS.

Interestingly, the position of the second QTL suggested by Xu et al. (2006) at marker *BM2078* is within the maxima of the QTL test statistics for udder composite index, udder depth and fore udder attachment that were detected in our LALD analyzes (Brand et al.,

2009a). The maximum for udder composite index and for fore udder attachment were located in marker interval 20 (*EAC-BM2078*) and the maximum of the QTL test statistic for front teat placement was in marker interval 21 (*BM2078-DIK4569*). Udder depth and fore udder are assumed to affect SCS and clinical mastitis. The genetic correlation with SCS as well as with clinical mastitis is reported to range from – 0.19 to – 0.70 (Rupp and Boichard, 2003), indicating that a high and tightly attached udder has favourable effects on udder health. Hence, it is possible that the second putative QTL for SCS assumed by Xu et al. (2006) is attributed to indirect effects of udder type traits affecting the susceptibility to mastitis. Likewise, it is possible that the second putative QTL for SCS detected in our analyses at marker interval *BB710* - *PVRL2_c.392G>A* is attributed to indirect effects of udder type traits affecting SCS. Marker interval *BB710* - *PVRL2_c.392G>A* is located approximately 12 cM upstream of marker *BM2078*, and *PVRL2* was selected as a candidate gene for SCS because of the possible involvement in mammary gland development and udder morphology as part of the nectin-afadin-complex (Niessen, 2007; Ebnet, 2008; Takai et al., 2008).

To get a more complete picture of udder health traits and udder type trait segregating on BTA18 and to identify the causal mutations on BTA18 affecting SCS and udder type traits, further analyses are necessary. On the one hand methods that are suitable to detect multiple QTL and are capable to analyse multiple traits should be applied to quantify correlated effects of QTL for SCS and QTL for udder type traits on BTA18. On the other hand the marker density in the middle to telomeric region has to be increased and more families have to be analysed, to increase the power to detect multiple QTL. In our analyses six half-sib families were investigated and a total of 1,054 animals were genotyped at 28 marker loci in the middle to telomeric region on BTA18. By increasing the marker density to 1 cM and less, especially in the region between marker interval *HAMP_c.366+109G>A* - *BMS833* and marker interval *BB710* - *PVRL2_c.392G>A*, and by investigating more families, the resolution of the QTL mapping process could be increased. Furthermore, it might be that the investigated families

are to some extend segregating for different QTL for SCS on BTA18, and that QTL in repulsion phase additionally decrease the power to detect multiple QTL. Hence, more families have to be analysed to identify families segregating for different QTL for SCS on BTA18 for further analyses.

In summary, our results confirmed that the middle to telomeric region on BTA18 has a strong impact on udder health and udder health related conformation traits and that genetic loci on BTA18 may also contribute to the genetic variance of functional herd life in the German Holstein population. Furthermore, the association of maternally inherited two-marker-haplotype *BB710 - PVRL2_c.392G>A* with the causal mutation affecting SCS provided evidence that the two-marker-haplotype *BB710 - PVRL2_c.392G>A* is a candidate marker that could be used for population-wide marker assisted selection (MAS) for SCS. In a proof-of-principle approach Kühn et al. (2008) have shown that the middle to telomeric region on BTA18 indeed is suitable for MAS on SCS. They used 5 indirect linkage equilibrium markers covering nearly 30 cM in the telomeric region on BTA18, to determine QTL heterozygous sires for selection and showed that a selection of halfsib heifers for low SCS is possible already early in life. Before implementation of haplotype *BB710 - PVRL2_c.392G>A* in MAS, the marker has to be further validated in animals comprising multiple independent families.

3.2 Calving and body type traits

To identify genetic loci that are associated with effects on calving performance in the German Holstein population, calving traits comprising calving ease and stillbirth were investigated. Calving traits were analyzed for maternal and direct effects separately, because they have a divergent physiological background. Maternal effects (m) on calving traits are attributed to effects on the dam, e.g. size of the dam and proportion of the dam's pelvis, and direct effects (p) on calving traits are attributed to effects on the calf, e.g. size and birth weight of the calf. In our analyses maternal effects and direct effects on calving traits were further separated in first- (p1) and further-parity (p2) calvings for direct effects, and in first- (m1), second- (m2) and third-parity (m3) calvings for maternal effects. This was done, because it is assumed that first and further parity calving traits are biologically distinct traits (Wiggans et al., 2008) and that about 20 to 50 % of the genetic variation of calving traits are common to first and later parity calvings only (Steinbock et al., 2003). Furthermore, Seidenspinner et al. (2009) have shown, that the use of parity specific calving traits is beneficial for QTL mapping studies. They compared results of QTL analyses considering parity-specific phenotypes with results obtained from QTL analyses considering across parity phenotypes for calving traits and reported that the results differed markedly between first and later parity as well as across parity analyses. Accordingly, these results also indicated that first and later parity calving traits are affected by distinct physiological pathways and are biologically distinct traits. In addition to the calving traits and to complement our investigation on calving traits in the German Holstein population, we also investigated body type traits that are assumed to affect calving performance. Body type traits considered for our analyses were stature, body depth, chest width, rump angle, and rump width, which are traits that are predominantly related to the size and anatomical proportions of the calf and the dam.

The study presented here (Brand et al., 2009a) is the first study that used phenotypic measures for first and later parity calving traits and applied LD and combined LALD methods to map QTL for calving traits as well as for body type trait on BTA18 in the German Holstein population. Applying LALD methods, we were able to identify a genome-wide significant QTL for calving ease p2 and chromosome-wide significant QTL for body depth, rump angle and stillbirth p2 in the middle to telomeric region on BTA18. QTL affecting stature, chest width or rump width, traits that are assumed to affect calving performance and that are related to the size and to the anatomical proportions of the calf and the dam, were not detected in the families we investigated. Additional analysis of maternally inherited marker alleles, that were performed to exclude any specific sire effect that could occur in a paternal halfsib design, indicated, that maternally inherited alleles of microsatellite markers *DIK4234* and *DIK3014* are associated with stillbirth m3, stillbirth p2, calving ease p2 and body depth, respectively. Hence, QTL for maternal and direct effects on calving traits initially reported by Kühn et al. (2003) could be confirmed. QTL affecting stature, chest width or rump width, that were previously reported by Cole et al. (2009), could not be confirmed in the families we investigated.

Cole et al. (2009) investigated genetic effects on dairy traits in 5,360 North American Holstein bulls. They analyzed 38,416 SNP covering the whole genome and identified a single SNP (ss86324977) on BTA18 with large effects on body depth, rump width, stature, strength, daughter calving ease and sire calving ease. Additionally, they identified BTA18 to have large effects on longevity and total merit, confirming our hypothesis that genetic loci on BTA18 might have a substantial impact on economically important traits. Similar results were reported by Kolbehdari et al.(2008). They performed a whole-genome-scan to map QTL for conformation and functional traits in Canadian Holstein cattle and identified two SNP on BTA18 that are associated with effects on over all conformation, overall rump and mammary system (rs41636734) as well as with direct effects on calving ease (rs41636749), respectively.

The SNP identified by Cole et al. (2009) and Kolbehdari et al.(2008) were located approximately in the middle between marker interval 15 (80.4 cM) and marker interval 25 (93.6 cM) corresponding to our linkage map. In interval 15 (*DIK3014-DIK4234*) we identified the maternally inherited marker alleles of the flanking markers to be associated with effects on stillbirth m3, stillbirth p2, calving ease p2 and body depth, respectively, and in interval 24 (*DIK5075-BM6507*) and interval 25 (*BM6507-DIK5235*) we detected QTL for rump angle, calving ease p2 and stillbirth p2 in our LALD analyzes. These results indicated that there might be two loci on BTA18 affecting calving traits. However, it might be that the position of QTL for calving traits, that is approximately 10 cM apart from the position of markers that are assumed to be in LD to the causal mutation affecting calving traits, is attributed to the different methods applied and to the size of marker intervals in this region. In LALD analyses, LA and LD information including haplotype identical by descent probabilities are merged, whereas in association analyses, only LD between alleles at the marker and the putative causal mutation affecting the target trait is tested. Taken together it remains unclear if there is more than one QTL affecting calving traits in the middle to telomeric region on BTA18, but the SNP reported by Cole et al. (2009) , that is assumed to affect calving traits and body type traits as well as the results from Kolbehdari et al. (2008) and our analyses indicate, that there might be a single locus affecting body conformation traits and calving traits on BTA18.

Thomasen et al. (2008) investigated calving traits for first parity in Danish Holstein and identified QTL affecting maternal and direct effects on stillbirth, direct effects on calf size, and direct effects on calving difficulties in the middle to telomeric region on BTA18. They also analyzed the correlation of QTL effects and reported a positive correlation of QTL effects for maternal effects on stillbirth, direct effects on calving ease and calf size. In our analyses the effects of maternally inherited *DIK4234* alleles on stillbirth m3, calving ease p2 and body depth showed also the same direction of effects, also indicating a positive

correlation. These results highlight the telomeric region on BTA18 for marker assisted selection for maternal stillbirth, because the positive correlation with direct effects on calving traits is in contrast to the genome-wide negative correlation between maternal and direct effects on calving traits reported for the German Holstein population (Vereinigte Informationssysteme Tierhaltung w.V.; Description of genetic evaluation: http://www.vit.de/index.php?id=zw-milch-zws-beschreibung&L=1). Thomasen et al. (2008) suggested a underlying mechanism for the correlated QTL effects on direct effects on calving ease, maternal effects on stillbirth and direct effects on calf size that is attributed to the birth weight or size of the calf. Likewise, Cole et al. (2009) suggested a possible underlying mechanism for the correlated effects on stillbirth, calving ease and conformation traits in their studies, which is related to the birth weight or size of the calf. In our analyses we could not find indication for QTL affecting stature, chest width or rump width but the concurrent association of maternally inherited marker alleles with body depth and maternal effects on calving ease for marker *DIK4234* also indicated that variation in body conformation might be the cause for divergent performance regarding calf delivery associated with this marker. Thus, our results support the hypothesis of a locus with favorably correlated effects on maternal and direct effects on calving traits. Whether these correlated effects are attributed to direct effects resulting in a disproportion in size of calf and dam or whether this is attributed to yet unrecognized physiological effects remains open.

To identify the causal mutations affecting calving and conformation traits and to identify the underlying physiological pathway affecting calving traits on BTA18, further analyses are needed. For this purpose, the marker density in the telomeric region on BTA18 has to be increased and a multiple trait model that is capable to identify multiple QTL should be applied to achieve a higher resolution of the map and to investigate correlated effects on multiple traits. Additionally the number of families should be increased and traits affecting calf size and birth weight should be included in further analyses, to become a more complete

picture of calving traits and traits affecting calving performance on BTA18 in the German Holstein population.

In summary, the studies presented here confirmed that the middle to telomeric region on BTA18 has a strong impact on calving performance. The microsatellite marker *DIK4234* was identified as a candidate marker for MAS for maternal stillbirth and the indicated positive correlation of maternal and direct effects on calving traits highlighted this marker for MAS, because the favourable correlation of maternal and direct effects on calving traits is in contrast to the genome wide negative correlation of maternal and direct effects on calving traits in the German Holstein population. Furthermore, the knowledge of congruence of QTL regarding calving and conformation traits and the favorable correlation of QTL effects on calving traits and conformation traits will aid future selection of candidate genes, because these observations imply a respective functional background of QTL for calving traits in the German Holstein population.

4. Summary

Functional herd life and the economic efficiency of milk production are substantially affected by udder health and calving performance. The experimental work presented here was targeted at the characterization and analysis of the molecular background underlying genetic variation of udder health and calving traits on the bovine chromosome 18. In the German Holstein population previous studies reported the telomeric region on BTA18 to harbor QTL affecting functional traits. Based on these studies, molecular tools that are used to identify trait associated gene loci had to be improved. By increasing the marker density within the previously reported QTL-regions and by establishing a comparative map of BTA18/HSA19 the fine-mapping of the middle to telomeric region on BTA18 and the selection of positional candidate genes for the somatic cell score based on the human genome annotation was enabled.

Linkage- and linkage-disequilibrium analyses were performed separately to initially identify QTL affecting udder health, calving traits and conformation traits that are assumed to affect udder health and calving performance on BTA18. QTL identified in separate LA- and LD-analyses were further analyzed by applying combined linkage- and linkage disequilibrium methods. Genome-wide significant QTL were identified for somatic cell score, direct effects on calving ease, fore udder attachment, udder depth as well as for udder composite index. Chromosome-wide significant QTL were detected for direct effects on stillbirth, rump angle, body depth and front teat placement. For maternal effects on stillbirth a suggestive QTL was detected. Hence, previously known QTL for udder health, calving traits and conformation traits could be confirmed and, furthermore, new QTL for conformation traits could be detected in the German Holstein population. The coincidence of QTL for udder health and conformation traits in the telomeric region on BTA18 indicated that udder type traits like udder depth and fore udder might be a functional background underlying QTL affecting udder

health on BTA18. Similar for calving traits, overlapping QTL for calving traits and QTL for body depth suggested a common physiological mechanism which affects body depth as well as calving ease and stillbirth. This hypothesis was corroborated by association studies. Maternally inherited *DIK4234* alleles could be identified to be associated with maternal effects on stillbirth, body depth and direct effects on calving ease. Furthermore, the allele associated with a lower stillbirth rate was also associated with a decline in body depth and fewer problems at calving. Whether the potential interrelation is due to direct effects resulting in a disproportion in size of calf and dam or due to other physiological mechanisms, remains open.

For somatic cell score the maternally inherited two marker haplotype *BB710* - *PVRL2_c.392G>A* was identified to be associated with effects on somatic cell score. *Poliovirus receptor-related 2 (PVRL2)* was selected as a candidate gene for SCS because of the possible involvement in mammary gland development and udder morphology as part of the nectin-afadin-complex.

In summary, haplotype *BB710* - *PVRL2_c.392G>A* and microsatellite marker *DIK4234* were identified as two candidate markers for MAS for udder health and maternal effects on stillbirth. Further investigations are necessary to validate marker effects and characterize correlated effects on other traits.

5. Zusammenfassung

Eutergesundheit und Kalbmerkmale haben einen wesentlichen Einfluss auf die funktionale Nutzungsdauer und die wirtschaftliche Effizienz der Milchproduktion. Diese Arbeit war darauf ausgerichtet, auf dem Rinderchromosom 18 (**BTA18**) molekulare Grundlagen der genetischen Variation von Eutergesundheit und Kalbemerkmalen zu untersuchen und zu charakterisieren. Ausgangspunkt der Arbeit waren vorausgegangene Studien in der Deutschen Holsteinpopulation, die bereits quantitative trait loci (**QTL**) für funktionale Merkmale in der telomeren Region auf BTA18 identifizieren konnten. Darauf aufbauend mussten molekulare Werkzeuge zur Identifizierung von merkmalsassoziierten Genorten weiterentwickelt werden. Dazu gehörte, dass die Markerdichte in den aus vorausgegangenen Studien bekannten QTL-Regionen erhöht und eine komparative Genkarte BTA18/HSA19 erstellt wurde. Auf diese Weise konnte die Feinkartierung in der mittleren bis telomeren Region von BTA18 ermöglicht und positionelle Kandidatengene für die somatische Zellzahl mit Hilfe der komparativen Genkarte aus dem humanen Genom abgeleitet werden.

Es wurden separate Kopplungs- und Kopplungsungleichgewichts-Analysen durchgeführt, um QTL für Eutergesundheit, Kalbemerkmale und für Exterieurmerkmale, die Einfluss auf Eutergesundheit und Kalbemerkmale haben, auf BTA18 zu identifizieren. Merkmale, für die ein signifikanter QTL in der Kopplungs-Kartierung oder Hinweise auf einen QTL in der Kopplungsungleichgewichts-Kartierung detektiert wurde, konnten mittels kombinierter Kopplungs- und Kopplungsungleichgewichts-Kartierung feinkartiert werden. In der kombinierten Kopplungs- und Kopplungsungleichgewichts-Kartierung konnten genomweit signifikante QTL für die somatische Zellzahl, direkte Effekte auf Kalbeverlauf, für die Vordereuteraufhängung, die Eutertiefe, sowie für den Merkmalskomplex Euter gefunden werden. Chromosomweit signifikante QTL wurden für direkte Effekte auf Totgeburten und für Beckenneigung, Körpertiefe und die Strichplatzierung vorne identifiziert. Für maternale

Effekte auf Totgeburten konnte ein Hinweis auf einen möglichen QTL gefunden werden. Somit konnten bereits bekannte QTL für Eutergesundheit, Kalbemerkmale und Exterieurmerkmale auf BTA18 bestätigt, sowie weitere QTL für Exterieurmerkmale in der Deutschen Holsteinpopulation neu identifiziert werden. Die Clusterung von QTL für Eutergesundheit und Exterieurmerkmale in der telomeren Region von BTA18, deutete darauf hin, dass Eutermerkmale wie zum Beispiel Eutertiefe und Vordereuteraufhängung ein funktionaler Hintergrund von QTL für Eutergesundheit auf BTA18 sein könnten. Ähnlich bei Kalbemerkmalen, hier deutete eine Überlappung von QTL für Kalbemerkmale mit dem QTL für Körpertiefe auf einen gemeinsamen physiologischen Mechanismus hin, der sowohl die Körpertiefe als auch den Kalbeverlauf und die Totgeburtenrate beeinflussen könnte. Diese Hypothese wurde in weiterführenden Assoziationsstudien bekräftigt. Es konnte gezeigt werden, dass maternal ererbte Allele des Markers *DIK4234* mit Effekten auf Totgeburten maternaler Effekt, Kalbeverlauf direkter Effekt und Körpertiefe assoziiert sind und dass das Allel, das mit einer geringeren Totgeburtenrate assoziiert ist, auch mit einer geringeren Körpertiefe der Tiere und mit weniger Problemen im Kalbeverlauf assoziiert ist. Ob dabei ein möglicher Zusammenhang auf physiologische Mechanismen zurückzuführen ist, welche die Größe des Kalbes und der Mutterkuh direkt beeinflussen, oder ob andere Mechanismen eine mögliche Ursache sein könnten, bleibt noch ungeklärt.

Für die somatische Zellzahl konnte der maternal ererbte zwei-Marker-Haplotyp *BB710* - *PVRL2_c.392G>A* als merkmalsassoziiert nachgewiesen werden. *Poliovirus receptor-related 2* (*PVRL2)* wurde als Kandidatengen für die somatische Zellzahl wegen des möglichen Einflusses auf die Euterentwicklung und Eutermorphologie als Komponente des Nektin-Afadin-Komplexes ausgewählt.

Zusammenfassend lässt sich herausstellen, dass mit dem Haplotyp *BB710* - *PVRL2_c.392G>A* und dem Mikrosatelliten-Marker *DIK4234* zwei Kandidaten für die Markergestützte Selektion auf Eutergesundheit und maternale Effekte auf Totgeburten

identifiziert wurden. Weiterführende Untersuchungen zur Validierung der Marker und zur weiteren Charakterisierung von korrelierten Marker-Effekten auf weitere Merkmale müssen noch durchgeführt werden.

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NCBI BLAST. http://blast.ncbi.nlm.nih.gov/Blast.cgi

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NCBI Trace Archives. http://www.ncbi.nlm.nih.gov/Traces/home/

AnimalQTLdb. http://www.animalgenome.org/QTLdb/

BioGPS. http://biogps.gnf.org/?referer=symatlas#goto=welcome

Combined QTL Map of Dairy Cattle Breeds. http://www.vetsci.usyd.edu.au/reprogen/QTL_Map/

Vereinigte Informationssysteme Tierhaltung w.V. Description of genetic evaluation. http://www.vit.de/index.php?id=zw-milch-zws-beschreibung&L=1

Gene Expression Omnibus GEO. http://www.ncbi.nlm.nih.gov/projects/geo/

Gene Ontology Database. http://www.geneontology.org/

Genetic Association Database. http://geneticassociationdb.nih.gov/

Human Gene Mutation Database. http://www.hgmd.cf.ac.uk/ac/index.php

iHOP. http://www.ihop-net.org/UniPub/iHOP/

ISI Web of Knowledge. http://isiwebofknowledge.com/

KEGG: Kyoto Encyclopedia of Genes and Genomes. http://www.genome.jp/kegg/

Mouse Genome Informatics. http://www.informatics.jax.org/

NCBI dbSNP. http://www.ncbi.nlm.nih.gov/projects/SNP/

NCBI GenBank. http://www.ncbi.nlm.nih.gov/Genbank/

Online Mendelian Inheritance in Man (OMIM). http://www.ncbi.nlm.nih.gov/omim

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7. Cumulative list of publications

7.1 Publications

Baes, C., B. Brand, M. Mayer, C. Kuhn, Z. Liu, F. Reinhardt, and N. Reinsch. 2009. Refined positioning of a quantitative trait locus affecting somatic cell score on chromosome 18 in the German Holstein using linkage disequilibrium. J. Dairy Sci. 92:4046-4054.

Brand, B., C. Baes, M. Mayer, N. Reinsch, and C. Kuehn. 2009. Identification of a two-marker-haplotype on Bos taurus autosome 18 associated with somatic cell score in German Holstein cattle. BMC Genet. 10:50.

Brand, B., C. Baes, M. Mayer, N. Reinsch, T. Seidenspinner, G. Thaller, and C. Kühn. 2009. Quantitative trait loci mapping of calving and conformation traits on Bos taurus autosome 18 in the German Holstein Population. J. Dairy Sci.: doi:10.3168/jds.2009-2553.

7.2 Lectures and poster presentations

Brand, B., C. Baes, M. Mayer, N. Reinsch, T. Seidenspinner, G. Thaller, M. Schwerin, and C. Kühn. 2009. Feinkartierung von Kalbemerkmalen und Exterieurmerkmalen auf dem Rinderchromosom 18. Vortragstagung der DGfZ und GfT am 16./17. September 2009 in Gießen

Brand, B.; Schwerin, M.; Kühn, Ch., 2008. Bovine chromosome 18: Mapping of quantitative trait loci for somatic cell score and udder conformation traits. XXXI. Conference of the International Society for Animal Genetics., s 2141.

Brand, B.; Weikard, R.; Schwerin, M.; Kühn, C., 2007. Abwehrvermögen gegen Mastitis: QTL-Kartierung und Kandidatengenanalyse für Mastitisempfänglichkeit auf dem Rinderchromosom 18. Vortragstagung der DGfZ und GfT vom 26/27 September 2007 in Stuttgart-Hohenheim.

8. Appendix

8.1 Abbreviations

$	dollar
%	percent
°C	degree Celcius
€	euro
al.	alteri
bp	base pair
BTA	*Bos taurus* autosome
CALM3	calmodulin 3 (phosphorylase kinase, delta)
CDH1	cadherin 1, type 1, E-Cadherin (ephitelial)
cM	centiMorgan
CNS	coagulase-negative staphylococci
cR	centiRay
DGAT1	diacylglycerol O-acyltransferase 1
DNA	deoxyribonucleic acid
DYD	daughter yield deviation
EBV	estimated breeding value
EDC	effective daughter contribution
FBN	Leibniz Institute for farm animal science
h^2	heritability
HAMP	hepcidin antimicrobial peptide
HSA	*Homo sapiens* autosome
IBD	identical-by-decend
kg	kilogram

LA	linkage analysis
LALD	linkage and linkage disequilibrium
LD	linkage disequilibrium
log_2	logarithmus dualis
LPS	lipopolysaccharides
MAS	marker assisted selection
Mb	mega bases
MEC	mammary epithelial cells
MHC	major histocompatibility complex
ml	milliliter
NCBI	National Center for Biotechnology Information
nt	nucleotide
PCR	polymerase chain reaction
PMN	polymorphonuclear neutrophil leukocytes
PVRL2	poliovirus receptor-related 2 (herpesvirus entry mediator B)
QTL	quantitative trait loci
rad	radiation absorbed dose
REML	restricted (residual) maximum likelihood
RFLP	restriction fragment length polymorphisms
r_g	genetic correlation
RH	radiation hybrid
RNA	ribonucleic acid
SCC	somatic cell count
SCS	somatic cell score
SNP	single nucleotide polymorphism
SSR	simple sequence repeats

STR	simple tandem repeats
UK	United Kingdom
US	United States

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8.3 CD

Content:

1. PhD-Thesis: (BodoBrand_PHD2010.pdf)

2. Supplemental Data: (BMC_Genetics_Sup directory)

Brand, B., C. Baes, M. Mayer, N. Reinsch, and C. Kuehn. 2009. Identification of a two-marker-haplotype on Bos taurus autosome 18 associated with somatic cell score in German Holstein cattle. BMC Genet. 10:50.

8.4 Erklärung zum eigenen Anteil an den Veröffentlichungen

Hiermit erkläre ich, dass ich im Rahmen dieser Arbeit 22 der 28 für die Feinkartierung genutzten Marker ausgewählt und an 960 Tieren aus 6 Halbgeschwister Familien der Deutschen Holsteinpopulation genotypisiert habe. Die Markerabfolge auf BTA18 wurde von mir evaluiert und die auf dieser Markerabfolge beruhende genetische Kopplungskarte erstellt. Dabei wurde unter anderem eine RH-Kartierung für 7 Marker durchgeführt, deren Position mittels Kopplungskartierung nicht aufgeklärt werden konnte. Die Erstellung der komparativen Karte BTA18/HSA19 mittels Sequenzvergleichen, sowie die Auswahl von Kandidatengen, die Resequenzierung der Kandidatengene und die Identifizierung von Polymorphismen in den Kandidatengenen wurden ebenfalls von mir durchgeführt. Die statistische Auswertung der Daten wurde basierend auf den von Frau Baes entwickelten Softwareprogrammen durchgeführt. Dabei wurden von mir die Kartierung von Kalbemerkmalen und Exterieurmerkmalen und die Assoziationsstudien für alle Merkmale durchgeführt. Die Manuskripte für die Publikationen mit den Titeln: “Identification of a two-marker-haplotype on *Bos taurus* autosome 18 associated with somatic cell score in German Holstein cattle” und “Quantitative Trait Loci Mapping of Calving and Conformation traits on *Bos taurus* autosome 18 in the German Holstein Population” wurden von mir verfasst.

Rostock,

8.5 Selbstständigskeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Quellen und Hilfsmitteln dazu verwendet habe und die den benutzten Werken wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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