TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Tierzucht

Genome-wide analysis of complex traits in cattle

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. A. Tellier

Prüfer der Dissertation:
1. Univ.-Prof. Dr. H.-R. Fries
2. Univ.-Prof. Dr. Ch.-C. Schön
3. Assoc. Prof. Dr. B. Hayes, La Trobe University Melbourne / Australien (nur schriftliche Beurteilung)
   Univ.-Prof. Dr. Th. A. Meitinger (nur mündliche Prüfung)

Die Dissertation wurde am 21.08.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum für Ernährung, Landnutzung und Umwelt am 28.01.2013 angenommen.

Publications arising from this thesis

Peer reviewed

Pausch H, Flisikowski K, Jung S, Emmerling R, Edel C, Götz K-U, Fries R (2011): Genome-Wide Association Study Identifies Two Major Loci Affecting Calving Ease and Growth-Related Traits in Cattle. *Genetics*, 187:289 –297.

Pausch H, Jung S, Edel C, Emmerling R, Krogmeier D, Götz K-U, Fries R (2012): Genome-wide association study uncovers four QTL predisposing to supernumerary teats in cattle. *Animal Genetics*. Article first published online: 15 Mar 2012 DOI:10.1111/j.1365-2052.2012.02340.x

Pausch H, Wang X, Jung S, Krogmeier D, Edel C, Emmerling R, Götz K-U, Fries R (2012): Identification of QTL for UV-protective Eye Area Pigmentation in Cattle by Progeny Phenotyping and Genome-wide Association Analysis. *PLoS ONE* 7: e36346.

Pausch H, Aigner B, Emmerling R, Edel C, Götz KU, Fries R (2012): Imputation of high-density genotypes in the FV population. Submitted to *Genetics Selection Evolution*

Fries R, **Pausch H** (2011): Individual genome sequencing of livestock animals – en route to genomic selection 2.0. *Züchtungskunde* 83:371-381

Jansen S, Aigner B, **Pausch H**, Wysocki M, Eck S, Benet-Pagès A, Wieland T, Strom T, Meitinger T, Fries R (2012): Assessment of the genomic variation in the Fleckvieh breed by re-sequencing at low to medium coverage. In preparation

Venhoranta H*, **Pausch H***, Wysocki M*, Szczerbal I*, Taponen J, Uimari P, Lohi H, Fries R, Switonski M, Andersson M (2012): The genetic background of the predominantly left-sided gonadal hypoplasia in Finnish PSK and Swedish Highland cattle. In preparation (*joint first authorship)

Wang X, Wurmser C, **Pausch H**, Jung S, Reinhardt F, Tetens J, Thaller G, Fries R (2012): Identification and dissection of four major QTL affecting milk fat content in the German-Holstein-Friesian population. *PLoS ONE* 7: e40711

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Pausch H, Edel C, Emmerling R, Götz KU, Fries R (2011): Imputation von hoch-dichter SNP Information beim Fleckvieh. Tagungsband der DGFZ/GfT-Gemeinschaftstagung 2011

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Fries R, **Pausch H**, Jansen S, Aigner B, Wysocki M (2012): Assessment of the genomic variation in a cattle population based on low-coverage re-sequencing. Book of Abstracts of the 63rd EAAP Annual Meeting, Bratislava

Gollnick NS, Jung S, Langenmayer MC, Majzoub M, Hermanns W, **Pausch H**, Fries R (2010): Schwere Hauterkrankungen bei Kälbern der Rasse Deutsches Fleckvieh (DFV): Ist eine neue Variante der Bovinen Hereditären Zink Defizienz die Ursache? 8. Buiatriktagung der Klinik für Wiederkäuer der LMU, München, Deutschland.

Publications arising from this thesis

Gollnick NS, Jung S, Langenmayer MC, Majzoub M, Hermanns W, **Pausch H**, Fries R (2011): Investigating a genetic variant of the bovine hereditary zinc deficiency syndrome in the German Fleckvieh population. 6. European Congress of Bovine Health Management, Liège, Belgien

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1st Chapter

General Introduction

The genetic architecture of quantitative traits

Most economically important traits in livestock species exhibit a complex genetic architecture with a small number of loci with major effects and a large number of loci with infinitesimal effects [1], [2],[3],[4]. Genomic regions affecting quantitative phenotypes are denoted as '*quantitative trait loci*' (QTL) [5]. The proportion of genetic variation explained by a QTL quantifies its effect size and its importance for the considered trait [6]. Assuming a diallelic locus and pure additivity allows to assess the trait variation explained by a QTL as $2pq\alpha^2/\sigma_a^2$ [7], where p and q are the frequencies of the QTL alleles, α is the allelic substitution effect and σ_a^2 is the additive genetic variation of the respective trait. The size of QTL effects is breed specific as allele frequencies differ among breeds [8],[9]. Furthermore, QTL effects are not persistent over time as genes with favourable effects underly both natural and artificial selection [10],[11]. The frequency of favourable alleles (with initial frequency > 0.5) increases considerably under selection resulting in a decrease of the QTL variation [10].

Mapping genomic regions underlying quantitative traits

The main objective of QTL mapping in livestock populations was the identification of suitable markers to improve economically relevant traits *via* marker-assisted selection (MAS) [12]. QTL mapping experiments in cattle populations were successfully performed using progeny-derived phenotypes for artificial insemination bulls which were genotyped for a small number of polymorphic microsatellites (*i.e.* granddaughter designs (*e.g.* [13])). This approach allowed for the identification of numerous QTL for various phenotypes [12]. However, the limited number of markers applied, resulted in large confidence intervals and imprecise mapping of QTL-regions (*e.g.* [14],[15]), rendering the fine-mapping and the identification of the underlying gene(s) (and polymorphism(s)) a difficult task. Furthermore, the identified markers were of limited suitability for

MAS, as they were (in most cases) in linkage disequilibrium (LD) with the underlying quantitative trait nucleotide (QTN) only. Addressing the QTN directly rather than using anonymous marker in LD with the QTN, is more efficient and persistent for MAS [16],[17].

High-throughput genotyping

The first assembly of the bovine genome permitted comprehensive insights into the genomic variation between as well as within cattle breeds [18]. The availability of the bovine reference sequence allowed primarily for the systematic assessment of a large number of single nucleotide polymorphisms (SNPs) by whole genome re-sequencing of individual animals [19],[20],[21],[22]. Thus detected SNPs enable to compile high-throughput genotyping arrays interrogating a large number of polymorphic sites simultaneously [23],[24]. Currently, the most dense bovine genotyping array interrogates genotypes for 777,962 SNPs per individual. However, genotyping of cattle populations is mostly performed with a medium-dense genotyping array interrogating 54,602 SNPs only. Genotype imputation allows to combine different datasets and to infer missing genotypes *in silico* [25]. The haplotype diversity of the entire population can be assessed by genotyping a substantial number of representative individuals with high-density SNP panels [26]. Missing genotypes of individuals genotyped at a lower density can be imputed accurately based on high-density haplotype information.

Implementation of genotype information in breeding programs

The availability of high-density SNP panels resulted in major modifications of cattle breeding programs. While traditional breeding schemes relied on progeny phenotyping and subsequent sire evaluation, the genetic value of individuals can now be accurately inferred based on genotype information only (*i.e. genomic selection* [27]). The implementation of genotype information not

only increases genetic gain but also dramatically reduces breeding costs of traditional breeding schemes [28]. However, as the current implementation of genomic selection relies on linkage disequilibrium between markers and causal variants, the accuracy of genomic breeding values declines considerably over generations [29]. The most efficient and sustainable genomic breeding program is supposed to utilize whole-genome sequence information and concomitantly causal variants directly [30],[6]. So far, the number of validated QTNs is very limited and their identification still renders a difficult task [31],[32].

Quantitative trait loci mapping with high-density genotypes

Genome-wide association studies (GWAS) based on densely spaced SNPs offer a powerful approach to the identification of genomic regions underlying phenotypic variation (*e.g.* [33],[34]). Fine-mapping and molecular-genetic dissection of significantly associated regions permit insights into the genetic mechanism(s) causing phenotypic variation and to pinpoint potential causal variants. However, strong linkage of potentially functional adjacent SNPs often precludes to finally proof the causality of individual variants [35]. The identification of the underlying QTN is further complicated by the fact that several adjacent markers with small effects might form a composite QTL [36].

Genome-wide association studies in humans

Since the first GWAS in 2005 [37], hundreds of variants have been identified to contribute to the genetic variation of complex traits in humans (*e.g.* [38],[39]). However, the identified QTNs account for a minor part of the heritability only [40]. A major part of the '*missing heritability*' [41] is most likely attributable to numerous undetected loci with small effects [42]. Large-scale GWAS and

high-density SNP panels are indispensable to detect small effect loci [43],[3]. However, even studies with hundreds of thousands of individuals and millions of SNPs have limited power to identify QTL accounting for a small part of the genetic variation [44].

Genome-wide association studies in cattle populations

In cattle, GWAS have been successfully applied to identify genomic regions underlying both mendelian (*e.g.* [45],[46],[47]) and quantitative traits (*e.g.* [4],[48]). GWAS of quantitative traits in livestock populations are often performed by using progeny-derived phenotypes such as breeding values or daughter yield deviations for the genotyped animals. Progeny-derived phenotypes are high-heritability traits as they are assessed based on a large number of records. The availability of high-heritability phenotypes facilitates QTL mapping considerably and compensates for the comparatively small sample sizes in livestock GWAS [3].

Population stratification in genome-wide association studies

An important prerequisite for unbiased QTL mapping is homogeneity of the mapping population. However, in typical livestock populations a limited number of founder animals explains a large proportion of the genetic diversity of breeding populations [49]. The resulting small effective population size causes substantial long-range linkage disequilibrium [3],[50]. Furthermore, the wide-spread use of genetically superior artificial insemination bulls results in large half-sib families. Allele frequencies between half-sib families might differ considerably and thereby confound QTL mapping by causing spurious associations [51]. Comparing the observed with the expected test statistics under the assumption of no association allows to assess the extent of spurious associations [52]. Confounding due to population stratification was primarily observed in case-control designs when allele frequencies differed between groups due to ancestry [53],[54]. Confounding also causes

spurious associations in genome-wide analysis of quantitative traits when subgroups of individuals share the same ancestry. Family-based designs (*e.g.* the transmission disequilibrium test, TDT [55]) are robust to population substructure. However, obtaining phenotype information from relatives is notoriously difficult for large-scale association studies precluding the application of family-based designs [56]. Furthermore, association studies are more powerful than family-based linkage studies especially for the identification of small effect variants [57],[58].

Accounting for population stratification

Several approaches are common to account for non-random population structure in GWAS (see [59] for a review). Initially, Yu et al. proposed a mixed-model based approach to account for the covariance among related individuals [60]. Therefore, the pairwise relationship is calculated based on pedigree and marker information, respectively. While the pedigree relationship is an expectation of the proportion of shared genes only, the genomic relationship reflects the realized proportion of common genes among individuals [61]. Especially in studies with incomplete pedigree information, the genomic relationship allows for a better resolution of cryptic relatedness among samples [62], [63]. While numerous approaches are available to build the genomic relationship based on SNP genotypes (*e.g.* [63],[64],[65]), VanRaden's approach [66] and derivatives thereof [67],[68] are most common for livestock populations. Sophisticatedly implemented algorithms allow for the efficient computation of large-scale mixed-model based GWAS (*e.g.* [69],[70],[71],[72]).

Another widely used approach to account for sample structure, albeit rather in human than in livestock GWAS, is accounting for the top axes of variation as inferred by principal components analysis (PCA) [73]. To this end, an eigenvector decomposition of a genetic covariance matrix built

based upon SNP genotypes is performed. The GWAS is subsequently carried out by applying the most informative axes of variation as covariates in a linear regression model [74]. However, the decision about the appropriate number of employed axes is controversial [75].

Outline of the Thesis

In **Chapter 2** of the present thesis a GWAS for calving and growth-related traits was performed in the German Fleckvieh (FV) population. The GWAS was facilitated by using breeding values as high-heritability phenotypes. Two major QTL were identified on bovine chromosomes 14 and 21 after careful correction for population stratification applying a principal components analysis (PCA) based approach. The underlying genomic regions are known to affect growth-related traits in mammals and most likely affect both fetal and postnatal growth in cattle.

Two different approaches to account for population stratification and the resulting inflation of false positive association signals were compared using real world data in **Chapter 3**. In order to identify genomic regions predisposing to the development of supernumerary teats in cattle, a GWAS was performed using a PCA based and a mixed model based approach, respectively. The GWAS with the PCA and mixed model based approach identified three and four QTL, respectively. The mixed model based approach outperformed the PCA based approach.

The successful mapping of twelve QTL regions underlying a special aspect of skin pigmentation in cattle is reported in **Chapter 4**. A high-heritability trait was assessed by phenotyping a large number of progeny groups of artificial insemination bulls. The successful imputation of high-density genotype information increased the power of the GWAS and enabled the mapping of QTL that account for a minor part of the genetic variation only. The results indicate that applying dense SNP panels clearly reduces the '*missing heritability*'.

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2nd Chapter

Genome-wide association study identifies two major loci affecting calving ease and growth related traits in cattle

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published in *Genetics* 187(1): 289–297

Abstract

Identifying quantitative trait loci (QTL) underlying complex, low heritability traits is notoriously difficult. Prototypical for such traits, calving ease is an important breeding objective of cattle (*Bos taurus*) improving programs. To identify QTL underlying calving ease, we performed a genome-wide association study using estimated breeding values (EBVs) as highly heritable phenotypes for paternal calving ease (pCE) and related traits. The massively structured study population consisted of 1800 bulls of the German Fleckvieh (FV) breed. Two pCE – associated regions on bovine chromosomes (BTA) 14 and 21 ($P = 5.72 \times 10^{-15}$ and $P = 2.27 \times 10^{-8}$, respectively) were identified using principal components analysis to correct for population stratification. The two most significantly associated SNPs explain 10% of the EBV variation. Since marker alleles with negative effect on pCE have positive effects on growth-related traits, the QTL may exert their effects on the birthing process through fetal growth traits. The QTL region on BTA14 corresponds to a human chromosome (HSA) region that is associated with growth characteristics. The HSA region corresponding to the BTA21 pCE QTL is maternally imprinted and involved in the Prader-Willi and Angelman syndromes. Re-sequencing of positional candidate genes on BTA14 revealed a highly significantly ($P = 1.96 \times 10^{-14}$) associated polymorphism ablating a polyadenylation signal of the gene encoding ribosomal protein S20 (*RPS20*). Our study demonstrates the leverage potential of EBVs in unravelling the genetic architecture of lowly heritable traits.

Introduction

The recent availability of genome-wide SNP panels in cattle and other livestock species enables the mapping of quantitative trait loci (QTL) as well as the prediction of an animal's genetic merit without relying on phenotypic information [1]. However, the complex genetic architecture of agriculturally important traits renders the systematic identification and characterization of

individual QTL a difficult task. The proportion of trait variance explained by an average QTL is very small. First mapping results in cattle seem to validate the classical quantitative genetic model of a large number of loci of small additive effects ([2],[3],[4]) and agree with findings from mapping QTL in the human genome [5]. Besides the relative contribution of a QTL to the trait variation, the heritability of the trait is a major determinant of the mapping power [1]. The heritability of calving traits, *i. e.* traits that describe the birthing process (dystocia in the case of difficulties) and the perinatal viability (stillbirth) of the calf as affected by the birthing process, are low, ranging from 0.04 to 0.15 ([6],[7],[8]). Calving traits are of considerable economic importance due to veterinary treatment costs, calf loss and lower production of cows affected by dystocia. Estimated breeding values (EBVs) for calving traits are used as selection criteria in attempts to reduce calving problems both in dairy and beef breeds (e.g. [9],[10]). Calving traits are complex since they are influenced by a sire-effect through the size of the calf as well as dam-effects consisting mainly of the pelvic dimensions. Routine progeny testing results in highly reliable EBVs for calving traits and thereby boosts the heritability to levels that make them amenable to QTL mapping even with medium-sized samples. An important prerequisite for unbiased QTL mapping based on linkage disequilibrium (LD) is homogeneity of the mapping population [11]. The heavy use of genetically superior bulls, facilitated by artificial insemination, and introgression lead to massively stratified populations. We attempted to correct for population stratification by principal components analysis (PCA) - based approaches that have been successfully in human genome-wide QTL mapping [12]. Here we report the mapping of two loci affecting very low heritability calving traits in a heavily structured dual purpose (dairy, beef) cattle population. The mapping approach was facilitated by the use of EBVs and consequent correction of population stratification.

Material and Methods

Animals and phenotypes

Bulls of the dual purpose breed FV (n = 1829) were genotyped using the Illumina BovineSNP 50K Bead chip® comprising 54,001 single nucleotide polymorphisms (SNPs). Phenotypes in the form of EBVs for beef (daily gain (DG)) and conformation traits (body size (BS)) as well as functionality traits such as paternal calving ease (pCE) and paternal stillbirth incidence (pSB) were obtained from the Bavarian State Research Center for Agriculture (www.lfl.bayern.de/bazi-rind) (November 2009 version, Supporting Table 1). Breeding value estimation was based on best linear unbiased prediction (BLUP) animal model. The calving process is described by a score ranging from 1 (unassisted delivery) to 4 (surgical delivery, fetotomy). Stillbirth is recorded as categorical trait (alive or not 48 hours post partum). Paternal and maternal effects on calving ease and stillbirth incidence are estimated multivariately for the first versus later parities. Parity specific EBVs are combined to produce paternal and maternal EBVs, respectively.

Genotypes and quality control

Of 1829 genotyped FV animals, six were excluded from further analyses due to genotype call rates below 90%. The remaining samples exhibited an average genotyping rate of 99.14%. 549 SNPs were omitted because their chromosome position was not known. 728 SNPs were discarded because genotyping failed in more than 10% of animals, 8480 SNPs were excluded due to a minor allele frequency smaller than 1% and 810 SNPs showed a significant ($P < 1 \times 10^{-3}$) deviation from the Hardy-Weinberg-equilibrium, indicating genotyping errors, and were thus not considered for further analyses. Pairwise identity by descent (IBD) was calculated based on identity by state (IBS) information derived from the remaining 43,863 SNPs using the method-of-moments approach implemented in *PLINK* [13]. IBD relationship of each pair of animals was compared with the

corresponding pedigree relationship calculated using the *PyPedal* package [14]. Comparison of the marker with the pedigree relationship revealed several inconsistencies, most likely resulting from mislabeling of DNA samples and false relationships. Unresolved inconsistencies led to the exclusion of 23 animals (Supporting Figure 1). The final set consisted of 1800 animals.

Single marker analysis

Single marker analysis was first carried out without considering population stratification. The EBVs were regressed on the number of copies of one of the alleles as implemented by the *PLINK --assoc* option. Quantile – quantile plots of the expected *vs.* the observed P values were inspected for an inflation of small P values indicating false positive association signals due to a structured population. The genome-wide inflation factor was computed according to Devlin and Roeder [11].

We next applied a PCA - based approach, implemented in the *EIGENSOFT 3.0* package [12], for eliminating false positive association signals due to ancestry differences and resulting population stratification. One SNP of a pair in LD with $r^2 > 0.25$ was excluded using the *PLINK --indep-pairwise* option (500 SNP window, shifted at 50 SNP intervals). A *smartpca* version of the *EIGENSOFT 3.0* package (compiled from source code with modifications for the bovine chromosome complement) was ran on the pruned data set consisting of 20,000 autosomal SNPs with the following option: the value of each marker is replaced with the residuals from a multivariate regression without intercept on the 5 preceding markers to further reduce redundancies due to LD. Eigenvalues (λ) and eigenvectors were calculated for all axes of variation. Correlation of ancestry adjusted EBVs and genotypes was calculated using the previously obtained eigenvectors with a *smarteigenstrat* version of *EIGENSOFT 3.0* compiled for the bovine chromosome complement. The resulting test statistic is equal to $(N - K - 1)$ times the squared correlation and χ^2 – distributed, where N is the number of samples and K the number of axes with an eigenvalue that

amounts to at least 70% of the mean eigenvalue (Jolliffe's criterion, [15]) used to adjust for ancestry [12]. Quantile – quantile plots were inspected and the genomic inflation factors were calculated (see above) to judge the extent of false positive signals. SNPs were considered as significantly associated for P values below the 5% Bonferroni-corrected type I error threshold for 43,863 independent tests. Allele substitution effects were estimated for each significant marker in a linear regression model implemented in *R* (www.r-project.org) with axes of variation with $\lambda \geq 0.7$ as covariables.

Haplotype analysis

Haplotypes for each chromosome region with significant association signals were reconstructed using default parameters in *fastPHASE* [16] and inspected by means of bifurcation plots obtained with *sweep* [17] to visualize recombination events and to define the length of haplotypes. The resulting haplotypes were analyzed for association in a multilinear regression model implemented in *R* (see above).

Estimating the power of the genome-wide association study

According to Goddard and Hayes [1] the correlation (*r*) between marker and trait, $r_{t \cdot m}$, is equal to $r_{m \cdot q} \cdot r_{q \cdot g} \cdot r_{g \cdot t}$, with *m* representing the marker genotype, *q* the QTL, *g* the genotypic value and *t* the phenotypic value (EBV) of an animal. $r^2_{m \cdot q}$ measures the LD between marker and trait, $r^2_{q \cdot g}$ the variance explained by the QTL and $r^2_{g \cdot t}$ the reliability of the EBV. Using this equation and the formula for the standard error of the correlation coefficient, the number of animals (*N*) required for identifying a QTL can be calculated as follows:

$$N = \left(\frac{1 - r_{t \cdot m}^2}{r_{t \cdot m} \frac{1}{z_{(1-\alpha)}}} \right)^2$$

where z is the normal score and α the Bonferroni-corrected type I error rate for 43,863 independent tests. Assuming a reliability of the EBV of 0.9, a LD between marker and QTL of $r^2 = 0.35$ and the QTL to explain 4% of the genetic variance, the required number of animals amounts to about 1700. Thus the power of our study with $N = 1800$ should allow to identify QTL explaining at least 4% of the genetic variance using EBVs of high reliability.

Annotation and polymorphism analysis of candidate genes

The *GENOMETHREADER* software tool [18] was used to predict the genomic structure and localization of the candidate genes based on the University of Maryland *UMD3.1* assembly of the bovine genome sequence [19] and the Dana-Farber Cancer Institute bovine gene index release 12.0 [20] together with the annotated RNA sequences of the *UMD3.1* assembly [19]. The *GENOMETHREADER* output was viewed and edited using the *Apollo* sequence annotation editor [21]. The exons and the promoter regions of the candidate genes were PCR-amplified (the primers are listed in Supporting Table 2) and re-sequenced in 12 FV bulls with specific genotypes for the SNP with the most significant signal for the pCE EBV (*BTA14 – ARS-BFGL-NGS-104268*), *i.e.* in one bull with GG and in 11 bulls with AG genotypes.

Genotyping of candidate gene polymorphisms

Genotypes of selected SNPs were determined by TaqMan® genotyping assays (Applied Biosystems Applera, Darmstadt, Germany). DNA samples were available for 810 FV animals only. Candidate gene polymorphisms were genotyped in these animals, and the genotypes of the remaining 990 animals of the study population were inferred using the EM algorithm implemented in *fastPHASE*.

Results

Single marker analysis

In a first attempt to identify QTL for pCE, we applied a linear regression model that did not account for the covariance of related animals. This model yielded 1146 autosomal SNPs exceeding the significance threshold and a genome-wide inflation factor of 4.75. However, an apparent association signal was observed on chromosome 14 (P: 1.64 x 10^{-55}; Supporting Figure 2). The inflation of significant association signals most likely results from relatedness of animals leading to massively structured population. The 1800 bulls within our study descend from 234 sires and 328 maternal grand sires. The paternal half sib families and the maternal grand sire families encompass up to 81 and 137 members, respectively. This is manifested by an average coefficient of relationship of 0.047 and distinct clusters of related animals (Supporting Figure 3A and 3B). Recent introgression of HF into FV can be uncovered by PCA . A 50% HF sire was broadly used within the FV population in the early 1980s to improve milk performance and udder quality of cows. Of 1800 FV bulls within the study population, 1050 exhibit HF ancestry *via* two of his sons (both 25% HF), as can be visualized by contrasting the top two axes of variation of the PCA (Supporting Figure 3C). Thus, HF admixture and the paternal and maternal sire families lead to a massively structured study population and concomitant inflation of significant association signals. Therefore, the association study was repeated, now correcting for population stratification using a PCA-based approach implemented in the *EIGENSOFT 3.0* package. The correction was based on 773 axes of variation that met the Jolliffe's criterion. In addition to the highly significant association with the pCE EBV on chromosome 14, that was already observed in the analysis without correction for stratification, the PCA - based analysis now also revealed significant association on chromosome 21 (Figure 1A). The Q - Q plot (Figure 1B) and an inflation factor of 0.97 document that the PCA-based analysis successfully eliminated association artifacts resulting from population stratification.

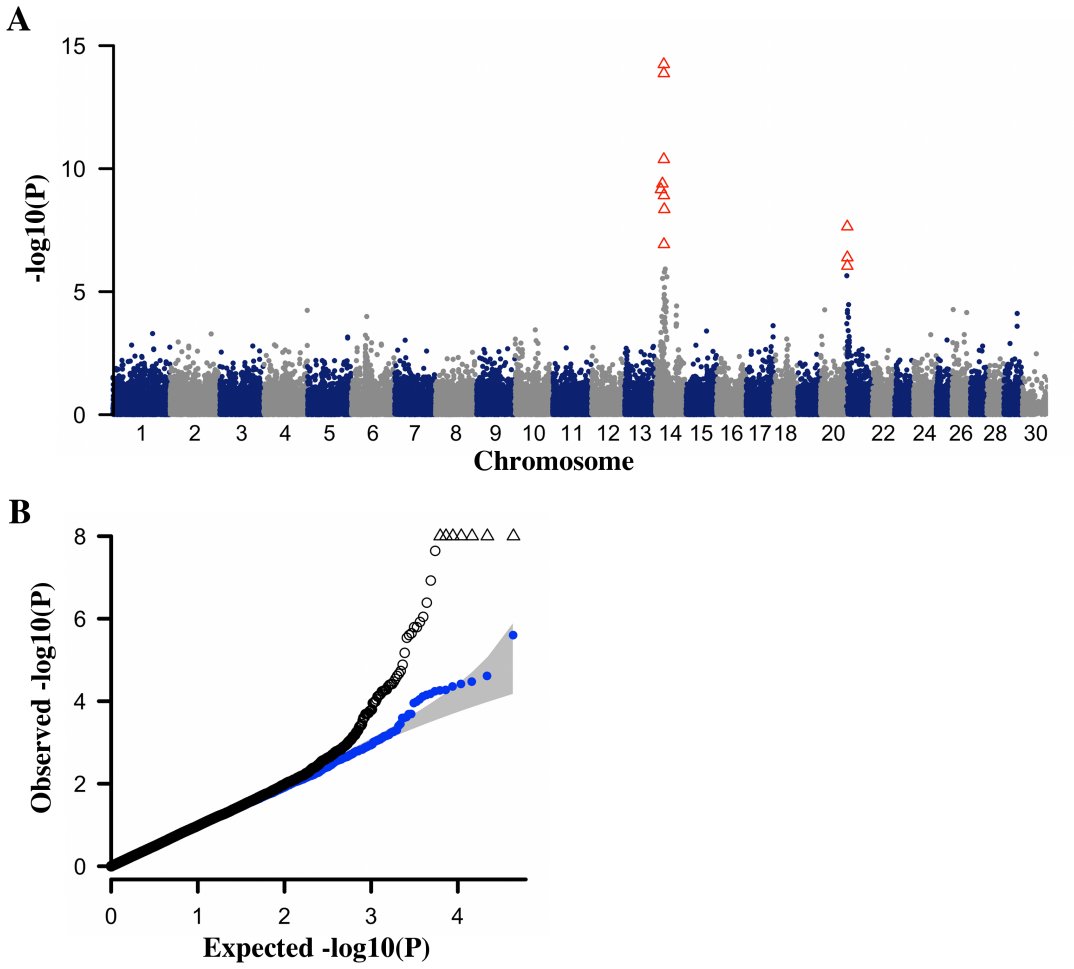

Figure 1: Association of 43,863 SNPs with the estimated breeding value (EBV) for paternal calving ease (pCE) in the Fleckvieh breed. (A) Manhattan plot. Red triangles represent SNPs with P < 1.14 x 10-6 (Bonferroni corrected significance level). (B) Quantile-quantile plot. The shaded area represents the 95% concentration band under the null hypothesis of no association. The open black dots represent the P values of the entire study, open triangles represent SNPs with P < 1 x 10-8. The filled blue dots indicate the P values excluding those from the associated regions on chromosome 14 and 21.

Eight SNPs on chromosome 14 and three SNPs on chromosome 21 meet the Bonferroni-corrected significance threshold (Table 1). Of the eight significant SNPs on chromosome 14, six lie within a 1.4 Mb interval (from 24.06 Mb to 25.4 Mb). Two significant SNPs outside this interval are in LD (r^2 = 0.48 and 0.68) with the most significantly associated SNP on chromosome 14. Three

significantly associated SNPs in high LD define a second pCE QTL region on chromosome 21 (2.15 Mb to 2.39 Mb). While the minor allele of the significant SNPs on chromosome 14 has a negative effect on the pCE EBV, it is the major allele of the significant SNPs on chromosome 21 that lowers the pCE EBV. The most significant SNP on chromosome 14 exhibit an allele substitution effect of -7.01, corresponding to 58% of the standard deviation of the EBV. The substitution effect of the major allele of the most significant marker on chromosome 21 is -2.93, *i.e.* 24% of the standard deviation of the EBV (Figure 2A). pCE is highly correlated with the paternal stillbirth incidence (pSB) as well as with growth related EBVs such as for DG and BS (Supporting Table 3). Consequently, association signals can also be observed for these EBVs, particularly on chromosome 14 (Table 1, Supporting Figure 4). The QTL alleles that lower the pCE and pSB EBVs have a positive effect on the growth related EBVs. There are several chromosome regions showing suggestive association ($P < 1 \times 10^{-3}$, Supporting Table 4), most prominently a second region on chromosome 14 with 5 SNPs located between 58.3 and 59.3 Mb.

Haplotype analysis

Haplotype analysis was carried out for the associated regions on chromosome 14 and 21 in an attempt to delineate the chromosomal segment carrying the pCE QTL. On chromosome 14, the allele that lowers the pCE EBV could be pinpointed to a specific haplotype that spans 1.58 Mb (starting at 23.82 Mb) and encompasses 23 SNPs (Table 2). This haplotype version occurs in a frequency of 10% in the study population. Its negative effect on the pCE EBV ($P = 1.56 \times 10^{-16}$) is more prominent than of any of the associated SNPs (-0.66 σ_A vs. -0.62 σ_A, Figure 2B). This is a strong indication for the causal variant lowering the pCE EBV to exclusively reside on this haplotype version.

On chromosome 21, the associated SNPs are contained within a haplotype spanning 0.6 Mb (starting at 1.78 Mb). The most frequent haplotype version occurs in a frequency of 66% and has a negative effect on the pCE EBV ($P = 3.15 \times 10^{-7}$). However it explains less of the genetic variance than the most significant SNP does (-0.18 σ_A vs. -0.24 σ_A).

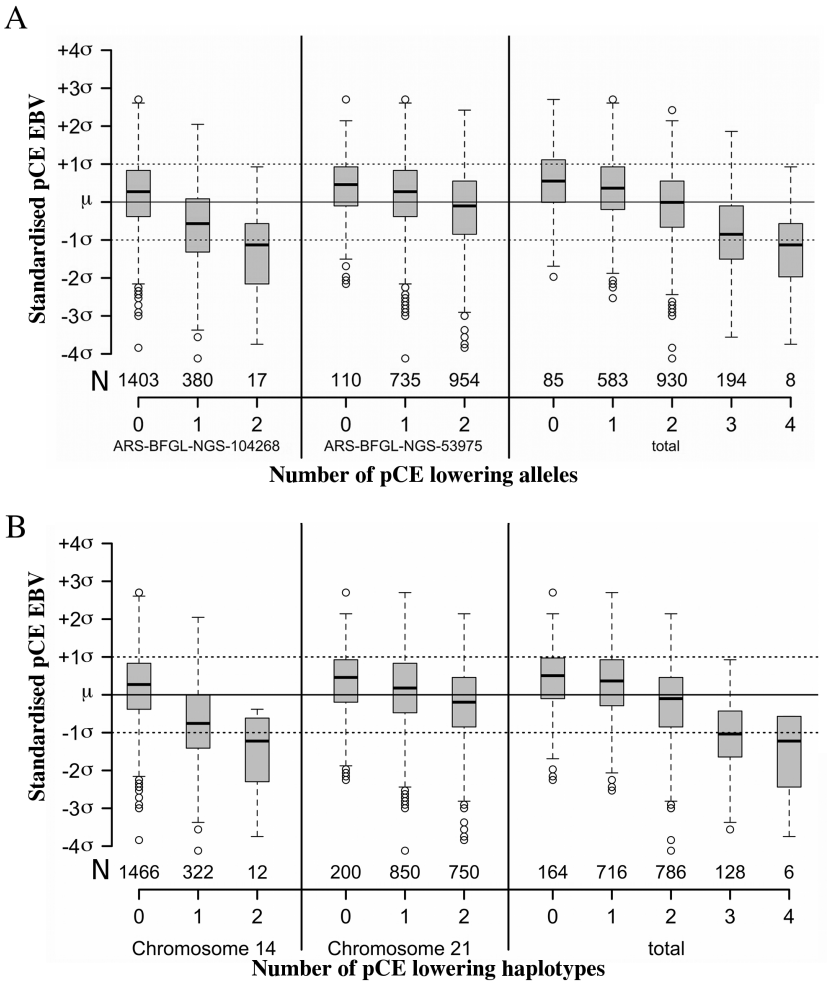

Figure 2: Effect of the most significantly associated markers on the estimated breeding value (EBV) for paternal calving ease (pCE) in the Fleckvieh breed. The boxplots show the effects of the most significantly associated SNPs (A) and haplotypes (B) on chromosome 14 and 21 separately and combined. The solid line represents the population mean, the dotted lines indicate one standard deviation of the EBV.

Table 1: SNPs showing significant association with paternal calving ease (pCE), paternal stillbirth incidence (pSB), daily gain (DG) and body size (BS) EBVs in 1800 Fleckvieh animals

SNP	Chromosome	Minor allele (minor allele frequency)	Physical position (bp)	EBV	Eigenstrat statistic	P value	α
ARS-BFGL-NGS-104268	14	A (0.12)	24,057,354	pCE	60.99	5.72×10^{-15}	-0.58
				pSB	47.12	6.69×10^{-12}	-0.54
BTA-91250-no-rs	14	A (0.10)	24,145,838	pCE	59.32	1.34×10^{-14}	-0.62
				pSB	47.51	5.47×10^{-12}	0.58
				BS	26.89	2.15×10^{-7}	0.45
BTB-01417924	14	G (0.13)	24,182,406	pCE	43.54	4.15×10^{-11}	-0.46
				pSB	33.79	6.13×10^{-9}	-0.42
Hapmap59686-rs29020689	14	A (0.14)	24,365,162	pCE	39.07	4.08×10^{-10}	-0.40
				pSB	38.27	6.18×10^{-10}	-0.42
ARS-BFGL-NGS-28867	14	G (0.10)	20,323,857	pCE	38.03	6.96×10^{-10}	-0.50
				pSB	33.78	6.17×10^{-9}	-0.49
				DG	25.50	4.42×10^{-7}	0.41
UA-IFASA-7112	14	G (0.09)	16,109,986	pCE	36.94	1.22×10^{-9}	-0.51
				pSB	32.09	1.47×10^{-8}	-0.49
Hapmap46735-BTA-86653	14	G (0.20)	25,401,722	pCE	34.40	4.48×10^{-9}	-0.36
				pSB	28.89	7.65×10^{-8}	-0.34
ARS-BFGL-NGS-53975	21	G (0.27)	2,151,256	pCE	31.25	2.27×10^{-8}	0.24
BTB-01532239	14	A (0.28)	24,437,778	pCE	28.05	1.19×10^{-7}	-0.26
				pSB	26.94	2.10×10^{-7}	-0.27
ARS-BFGL-NGS-114372	21	C (0.22)	2,381,941	pCE	25.65	4.09×10^{-7}	0.24
Hapmap52072-rs29018920	21	A (0.22)	2,333,804	pCE	24.12	9.01×10^{-7}	0.23

Eleven SNPs meet the genome-wide significance level of $P < 1.14 \times 10^{-6}$. SNPs are arranged in the order of increasing P values for the association with the paternal calving ease EBV. The P value for each trait x genotype combination is obtained by a principal components analysis - based approach to account for population stratification. The allelic substitution effect (α) is given for the minor allele in additive genetic standard deviations of the EBV. Physical positions are based on the UMD3.1 assembly of the bovine genome sequence.

Table 2: SNPs within the haplotype associated with the estimated breeding value (EBV) for paternal calving ease (pCE) on bovine chromosome 14

SNP	Physical position (bp)	Haplotype allele	Minor allele (allele frequency)	Eigenstrat statistic	P value	α
BTB-01953819	23,817,572	A	G (0.26)	0.37	0.54	0.03
Hapmap45796-BTA-25271	23,853,811	T	A (0.07)	5.39	0.02	-0.18
ss250608741 *	23,884,989	G	A (0.09)	1.06	0.3	0.06
ARS-BFGL-BAC-8052	23,893,220	G	A (0.01)	6.72	9.55×10^{-3}	-0.45
ARS-BFGL-NGS-97821	23,946,436	G	A (0.1)	0.98	0.32	0.07
ARS-BFGL-NGS-104268	24,057,354	A	A (0.12)	61.00	5.71×10^{-15}	-0.58
BTA-91250-no-rs	24,145,838	A	A (0.1)	59.32	1.34×10^{-14}	-0.62
BTB-01417924	24,182,406	G	G (0.13)	43.54	4.15×10^{-11}	-0.46
ARS-BFGL-NGS-110427	24,326,513	A	G (0.11)	0.02	0.89	-0.01
Hapmap59686-rs29020689	24,365,162	A	A (0.14)	36.94	1.22×10^{-9}	-0.40
ARS-BFGL-NGS-102351	24,407,125	G	G (0.25)	18.34	1.85×10^{-5}	-0.21
BTB-01532239	24,437,778	A	A (0.28)	28.04	1.19×10^{-7}	-0.26
BTB-01530788	24,524,205	A	G (0.34)	8.65	3.27×10^{-3}	0.12
BTB-01530836	24,573,257	G	A (0.35)	4.30	0.04	0.07
BTB-00557585	24,607,527	A	G (0.35)	4.75	0.04	0.08
BTB-00557532	24,643,266	A	G (0.35)	4.53	0.03	0.07
ss250608762 *	24,759,177	G	T (0.01)	1.00	0.32	-0.14
Hapmap40120-BTA-34288	24,787,245	C	A (0.09)	0.28	0.6	-0.05
ss250608721 *	24,954,981	A	A (0.16)	58.57	1.96×10^{-14}	-0.47
ss250608720 *	24,955,318	T	C (0.32)	3.56	0.06	0.06
Hapmap41234-BTA-34285	25,107,556	G	A (0.04)	13.89	1.94×10^{-4}	-0.42
BTB-02056709	25,175,950	A	G (0.18)	2.55	0.11	-0.08
BTB-00559128	25,215,027	A	G (0.21)	0.01	0.92	0.00
BTB-00557354	25,254,540	G	A (0.12)	1.63	0.2	0.09
Hapmap46986-BTA-34282	25,307,116	A	G (0.46)	9.62	1.93×10^{-3}	0.13
BTB-01779799	25,351,733	G	A (0.44)	19.00	1.30×10^{-5}	0.19
Hapmap46735-BTA-86653	25,401,722	G	G (0.2)	34.40	4.48×10^{-9}	-0.36

23 SNPs belong to the BovineSNP50 Bead chip collection and 4 additional SNPs designated by * result from re-sequencing. The P values were obtained by using a principal components analysis - based approach.. Genotypes for SNPs resulting from re-sequencing were determined in 810 animals and imputed for the remaining 990 animals of the study population. The allelic substitution effect (α) is given for the minor allele in additive genetic standard deviations of the pCE EBV. SNPs are arranged according to their physical position (UMD3.1 assembly of the bovine genome sequence).

Identification and analysis of candidate genes

The assessment of the transcriptional content of the pCE EBV associated regions was based on the *UMD3.1* assembly and annotation of the bovine genome [19]. The 23.82 – 25.40 Mb interval on chromosome 14 encompasses 13 transcripts / genes (Figure 3A). The associated region on bovine chromosome 14 is conserved in human chromosome 8q21 which has been shown to be associated with adult height [22]. Since adult stature is positively correlated with fetal size and fetal size is an important determinant of the birthing process, we considered *PLAG1, MOS, CHCHD7, RDHE2* (alias: *SDR16C5*)*, RPS20, LYN, TGS1, PENK,* as proposed by Gudbjartsson [22] as positional and functional candidate genes for the pCE QTL in cattle. Of this list, *PLAG1*, *TGS1, RPS20* and *LYN* together with *SOX17*, another gene in the critical region that we considered a functional candidate, were re-sequenced in a panel of 12 animals of our study population. Totally, we screened 30.3 kb resulting in the detection of 48 polymorphisms (Supporting Table 5). We decided to genotype four putatively functional SNPs, located in *SOX17* (*ss250608762*), *RPS20* (*ss250608720*, *ss250608721*) and *TGS1* (*ss250608741*), in 810 animals and analyzed the association with the pCE EBV in the complete study population using genotype imputation (Figure 3B & 3C). Only *ss250608721* produced a highly significant signal ($P = 1.96 \times 10^{-14}$). The polymorphism affects a polyadenylation signal of a cistron encompassing the genes for the ribosomal protein S20 (*RPS20*, a ribosomal component) and the small nucleolar RNA U54 (*SNORD54*, a ribosomal RNA modifying RNA) (Figure 4).

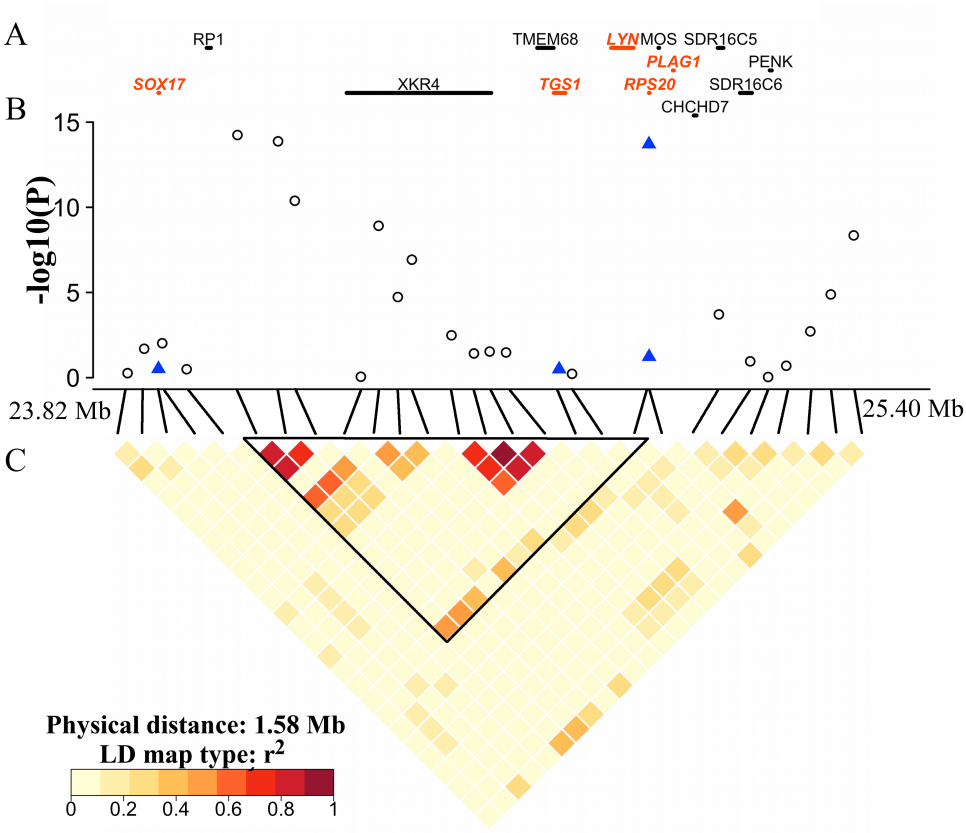

Figure 3: Detailed view of the region on chromosome 14 delineated by the haplotype associated with the estimated breeding value (EBV) for paternal calving ease (pCE). (A) Map of genes contained in this region. Red symbols indicate genes re-sequenced in the present study. (B) P values of 27 SNPs from analysis of association with the pCE EBV. The open black dots indicate results from genotyping of the entire study population, the blue triangles represent P values resulting from imputation based on 810 genotyped animals. (C) Heatmap of the pairwise linkage disequilibrium (r^2). The triangle delineates a linkage disequilibrium block containing the most significantly associated SNPs, including the potentially functional ss250608721 variant in RPS20.

The association signals on chromosome 21 result from the most proximal region on the chromosome (Supporting Figure 5). The region contains, among other transcripts, those encoding *SNURF-SNRPN* and *UBE3A*. These two transcripts are encoded in the human chromosome interval 15q11-15q13 that is subject to imprinting. The lack of a functional paternal copy of 15q11-15q13

causes the Prader-Willi syndrome, while the lack of a functional maternal copy of *UBE3A* is implicated in the Angelman syndrome [23]. The *SNURF-SNRPN* mRNA is derived from a single large transcriptional unit of which more than 70 snoRNAs of the C/D box type are processed [24]. Preliminary BLAST-analyses indicate the presence of a snoRNA cluster in the proximal region of bovine chromosome 21. However, a systematic annotation has not been attempted. The lack of detailed knowledge of the genomic organization, the imprinting status and transcriptional content of the associated region on chromosome 21 precluded the analysis of candidate genes, although a functional implication of the region in fetal growth and thus pCE seems obvious when considering that fetal growth retardation is symptomatic for the Prader-Willi syndrome.

ACTCTGAG AATAACTACG AGGAAAACTC CTTGTGGTGA AGGTTCTAAG
ACTTGGGATC GATTCCAAAT GAGGATCCAC AAGCGACTCA TTGACCTGCA
CAGTCCTTCT GAAATTGTCA AGCAGATCAC TTCCATCAGT ATTGAGCCGG
GAGTCGAGGT GGAAGTCACC ATTGCTGATG CCTAAATCAA CCTTTTT**AAT**
AAATCGATAA TCAGTTGTTA AATTTTGTTG ACTTTTATTT ATAATACTGA
CAAGTCTTAG GAATAGAGTT GTGTTATGGA ATTGATTCCA CCTTTTAAGT
★ **ss250608721 [A/G]**
AGAATAATCA GA**ATTTAAA**TG GATAGCCTGT GGTGGTTGAC CCTGTTTCCA
TTGTAAGCTA GTTTACACGT GACAGCATTT GAAAATGGAA TTGTGTTAAC
TGCCACATGA GATCTTGCTA TTGATGAATA TTGGGGTTTT TGAACATGTG
TGTGGGTTTA ACTTTGGAAC TGTCAGCAGT TTCTGGGATG ATTCTAATGT
TACCAGCTGT GGTTAGAAAC CTAGTGTGGT AAAAGGCAAG GAACTCAAAA
TCTAGGTCTT AATATCTGAC CCTGCCATTT GTCATAACTC CTTTTAAACT
TGGCAAATTT CAGTAAGTTG TGGAAACTTT TATAGATACT GGTGCCTTAA
TTTTAGATAA GAAAATAC**AA TAAA**ATATAA AACAATCCTA GTACTTAGGT
TATATCTATA GAATGTGTTT TATATATACT CCTTGGAATC ACTTTTCACT
TTATGTATAT AAGTCATTCC GTACGAAGCT AAGTTAAGCA TTTATATGTT

Figure 4: Predicted 3' UTR of cattle RPS20. The grey-shaded sequence designates the predicted exon 4, while the predicted polyadenylation (poly(A)) sites are denoted by underscore. The star locates the candidate quantitative trait nucleotide position, ablating a poly(A) site.

Discussion

Our genome-wide association study based on a dense SNP marker map provides strong evidence for two QTL on chromosome 14 and 21, respectively, that together explain at least 10% of the variation of the pCE EBV in the German FV breed. The two QTL also explain a substantial fraction of the pSB EBV as well as of EBVs of postnatal growth such as DG and BS. Stillbirth can be considered as the dichotomic manifestation of the calving ease score, as dystocia is a major cause of perinatal mortality. The correlation of pCE with growth-related traits and the coincident QTL point to fetal growth and the resulting birth weight as major determinant for the ease of delivery ([25],[26]). Thus, the two QTL might primarily affect fetal growth. One could expect that they would explain a larger fraction of the genetic variation of birth weight, a trait that is not routinely measured in dairy cattle. Improving postnatal growth along with lactation traits is a major breeding objective of the FV breed. This dual purpose selection is likely to act on the two QTL identified in our study. Animals known to carry favorable alleles for the chromosome 14 and 21 QTL could now be more stringently selected with regard to beef traits. However, the identification of QTL that either affect prenatal or postnatal growth but not both would facilitate to efficiently improve postnatal beef performance without antagonistically compromising calving ease. In any case, conventional selection schemes seem to allow favorable selection responses for calving ease and postnatal growth despite the genetic antagonism ([27],[28],[29]).

A key factor for successfully mapping of a QTL for a complex trait with very low heritability such as pCE was the use of reliably estimated breeding values for calving traits. If one assumes a heritability of 0.08, a LD between marker SNPs and QTL of $r^2 = 0.35$ and 4% of the genetic variation explained by the QTL, one would require approximately 20,000 individuals for the successful identification of a QTL (see Material and Methods). Using EBVs with a reliability of

90%, *i.e.* a *quasi* heritability of 0.9, requires less than 1800 animals to detect association. Breeding values are routinely estimated for many traits and are thus indispensable for dissecting complex trait variation in livestock species.

Another key factor for successfully mapping the two QTL was careful correction for extensive relationship among the study animals. The adjustment along 773 axes of variation allowed to account for major as well as for more subtle relationships that can possibly not be revealed by pedigree analyses. The association signal on chromosome 21 became apparent only when population structure was corrected for. Thus, PCA based elimination of false positive association signals might enable the detection of QTL with a smaller impact on the trait variation that would otherwise be "buried" in the false positive signals. Suggestive signals ($P < 1 \times 10^{-3}$, Supporting Table 4) are thus more likely to represent real QTL.

Our findings about two highly significant QTL for pCE as well as about additional suggestive QTL are supported by several previous studies on calving ease and growth trait QTL, based on microsatellite marker analyses. Kneeland et al. [30] identified three regions on chromosome 14 to affect birth weight in a composite breed. The proximal region from 26.0 to 26.7 cM most likely corresponds to the highly significant QTL region identified in our study, the more distal region between 36.2 and 46.2 cM may corroborate a suggestive QTL region resulting from our study. Davis et al. [31] also identified a QTL affecting birth weight at 42 cM. Koshkoih et al. [32] provide additional evidence for two birth weight QTL on chromosome 14 at 26 and 50 cM, respectively, in a cross of Limousin and Jersey animals. Maltecca et al. [33] recently identified a birth weight QTL at 19 cM on chromosome 14 in a Jersey – Holstein cross. There are also reports on QTL for postnatally measured growth traits in Wagyu ([34],[35]) and a Jersey – Limousin cross [36], indirectly supporting our suggestive evidence for a secondary pCE QTL on chromosome 14. Casas

et al. [37] and Davis et al. [31] identified a QTL for birth weight in the very proximal region of chromosome 21 in crosses of Brahman with Hereford and Charolais, respectively, providing supportive evidence for the pCE QTL identified in the present study.

There is also support in the literature for suggestive QTL on other chromosomes: Olsen et al. [38] and Holmberg and Andersson-Ecklund [39] identified in a Swedish and Norwegian dairy cattle population, respectively, a dystocia / stillbirth QTL at 36 – 37 cM on chromosome 6. We observe a suggestive pCE QTL at about 40 Mb on chromosome 6. Gutierrez-Gil et al. [40] identified a fetal growth / birth weight QTL in the same region based on a Charolais – Holstein cross. Eberlein et al. [41] provide evidence for the gene (*NCAPG*) encoding the Non-SMC Condensin I Complex, Subunit G to encompass this QTL, also based on a Charolais – Holstein cross. However, a prominent calving ease QTL in the Holstein breed on chromosome 18 [2] could not be detected in the present study or is not segregating in the Fleckvieh breed.

A preliminary candidate gene analysis identified a highly significantly pCE-associated SNP in a cistron encoding a ribosomal protein (*RPS20*) and a internally nested small nucleolar RNA (*SNORD54*). The SNP affects a polyadenylation site. Alternative polyadenylation at tandem poly(A) sites yield transcripts with different 3' UTR sequences providing the potential of differential regulation of mRNA expression by RNA binding proteins and / or miRNAs ([42],[43]). The marker allele causing the gain of an upstream polyadenylation signal is associated with a lower pCE EBV, *i.e.* a higher incidence of calving difficulties. This is hypothetically compatible with a shorter and more highly expressed mRNA encoding ribosomal components, leading to a higher ribosome assembly rate and concomitantly stronger fetal growth. Thus we consider the polymorphism as a candidate quantitative trait nucleotide (QTN) position. Interestingly, the pCE QTL on BTA21 is also in a chromosome region encoding factors involved in ribosomal assembly, specifically small

nucleolar RNAs (snoRNAs). It is therefore possible that both QTL affect ribosomal biogenesis. Mutation disturbing ribosome assemblies are often associated with abnormal fetal growth ([44], [45]).

Acknowledgements

This study is part of the project FUGATO-plus GenoTrack and was financially supported by the German Ministry of Education and Research, BMBF (grants 0315134A and 0315134D), the Förderverein Biotechnologieforschung e.V. (FBF), Bonn, and Lohmann Tierzucht GmbH, Cuxhaven.

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Supporting information for the

2nd Chapter

Supporting Table 1: Characteristics of the considered estimated breeding values (EBVs) of 1800 Fleckvieh bulls

EBV	Mean (μ)	Standard deviation (σ)	Mean reliability (r^2)
Daily gain (**DG**)	101.07	11.68	0.92
Paternal calving ease (**pCE**)	101.09	10.07	0.92
Paternal stillbirth incidence (**pSB**)	100.55	9.39	0.83
Body size (**BS**)	100.86	9.97	0.90

Supporting Table 2: Primers used for the re-sequencing of bovine *LYN*, *PLAG1*, *RPS20*, *SOX17* and *TGS1* genes

Gene	Primer_id	Region	Direction	Sequence
LYN	6964	PROM	forward	GGCCAGTACTTTGCATGTGA
	6965	EX1	reverse	ATTACGCAGCCATGTTTTGA
	6966	EX2	forward	GCTCTGCAGGACTGTTCCTC
	6967	EX2	reverse	GATGGAGAGATGGACGGATG
	6968	EX3	forward	GAACAGGGAAGGTGAACGAA
	6969	EX3	reverse	GGCAGCACAGATGGATAAGG
	6970	EX4	forward	CCCATGGTATGCAGGATCTTA
	6971	EX5	reverse	TCACTTGGCTGTAAAGCTGAAA
	6972	EX6	forward	AGGGCCATGTTGTTTATCCA
	6973	EX6	reverse	ATGGACTGTAGCCCACCAAG
	7010	EX6	forward	CCCCATAATGCCAATCTTGT
	7011	EX6	reverse	TGATCCTGCAACTTTATCCAAA
	6974	EX7	forward	CTTGGCGAGTTGGAAATGAT
	6975	EX7	reverse	CTGGAAGAGGGCATGACAAC
	6976	EX8	forward	CCAGGGAAGTCCCTAAAGGT
	6977	EX8	reverse	TTCTCCAGGCAAGAATACCG
	7006	EX8	forward	TCCCTTCTTTTCCCTCCCTA
	7007	EX8	reverse	CGAGCCTGCTGTTGATAGTCT
	6978	EX9	forward	GTCAAAGGGGACAGGTCAGA
	6979	EX9	reverse	GGGGTAGACAGGGAAGGAAA
	6980	EX10	forward	GAAAAGCTGGGACAATGACG
	6981	EX10	reverse	TGCCTGTTGTAATCGCTTTG
	6982	EX11	forward	TCCTTCTCCAGGGGATCTTT
	6983	EX11	reverse	GAGGAGCCCTGTGTCTTGTC

	7012	EX11	forward	CCGCAAAGAAGGAAAGTGTG
	7013	EX11	reverse	GACAAGAAGGCGGAGAAGTG
	6984	EX12	forward	CTTGGGGCTAGGTCTTCAGT
	6985	3'UTR	reverse	TCTGCGACTCACTGAAATGG
PLAG1	6986	PROM	forward	TTCTCTGGGCCTCTCACTTT
	6987	EX1	reverse	AGCTTCTCCGATGACAGGTT
	6988	EX2	forward	GGATCTCAGGGGATCTGTGA
	6989	EX2	reverse	GCGGAAAGAGGTGGATACAA
	6990	EX3	forward	GGTCTGCGGTGTTTAGGTGT
	6991	EX3	reverse	GGAGGAGTTCGTCCTTGATG
	6992	EX3	forward	GCACATGAAGAAGAGCCACA
	6993	EX3	reverse	CCGTGGGACTCTACTGGAAA
	6994	EX3	forward	AGGAGGAGGCACACTCTTCA
	6995	3'UTR	reverse	CAGCAAACATTTGAGCCAGA
RPS20	6996	PROM	forward	TGCAGATGACACCACCCTTA
	6997	PROM	reverse	CGGAGTTCACCCAAACTCAT
	7016	PROM	forward	AGATGGGCATACCAGACCAC
	7017	PROM	reverse	GGCCAAGTAATGTCTCTGCTTT
	6998	EX1	forward	ACCTCATGCGAAGAGCTGAC
	7014	EX1	reverse	CCTTACGCCTTCCTCTTTGA
	6999	EX2	forward	CCTGGAGGCATCTCATAAGC
	7015	EX2	reverse	AACACGGCACACACCAAGT
	7000	EX3	forward	CAGGGAATGGGCTTATGAGA
	7001	EX3	reverse	GCCAAAGCTCCAGATGTTTC
	7002	EX4	forward	CCGGTTGCTTTTAAACATGG
	7003	3'UTR	reverse	TGAGTTCCTTGCCTTTTACCA
SOX17	7138	PROM	forward	GTTGGCTGATGTTTGGTGTG
	7139	PROM	reverse	CAGGTCCCAAGTTTCAGCTC
	7413	PROM	forward	CCAAGCATCGAAACACAAAA
	7414	PROM	reverse	GGTGTCTCTCCACCCCCTAC
	7415	PROM	forward	TCCATCCTATGCATCCTGTG
	7416	PROM	reverse	TGGCCAAAAAGTGGTTGTAG
	7417	PROM	forward	TGAATCTCAGAGACCCAGGAA
	7418	PROM	reverse	TTCGAGAGGCCTTCTTTGTG
	7419	PROM	forward	GGGCAAGGTCCTTAACGTCT
	7420	PROM	reverse	ACTCAACCTGGAGCTGAGGA
	7140	EX1	forward	TTTTCTTAGGGGCAGGTGTC
	7141	EX1	reverse	ACTCACCCAGCATCTTGCTC
	7532	EX1	forward	TGAGCTGAAACTTGGGACCT

	7533	EX1	reverse	CTCGCCCTTCATCTTCATGT
	7534	EX1	forward	GTACGCCAGTGACGAGCAGA
	7535	EX1	reverse	GCCGCTTGGAGAGTAGGAGA
	7142	EX2	forward	CCCCAGCCTTCAACCTTT
	7143	EX2	reverse	CGGGGCGTAGCTGTAAGG
	7144	EX2	forward	CCCTGGGCCTTACAGCTAC
	7145	EX2	reverse	TCCTTGGGGAGGTGTGTAAC
	7146	3'UTR	forward	AACTATCCCGACGTGTGAGC
	7147	3'UTR	reverse	GGGTCACCTGAAATGCATAAG
	7421	3'UTR	forward	AGGGGAAGCCCTCAAATAAA
	7422	3'UTR	reverse	TGCCCATTGTAAATCACCTG
	7423	3'UTR	forward	ATCACTGTCCTGCCCTGTCA
	7424	3'UTR	reverse	CCATTGCCTTCTCCGATAGT
	7425	3'UTR	forward	CATTTGATGTGCAAACCTTCA
	7426	3'UTR	reverse	TATGGCAACAGCATGCAGAA
	7427	3'UTR	forward	TCTCTGGTGGTCCAGTGGTT
	7428	3'UTR	reverse	TATGCTTCCCAACGAACCTT
TGS1	6884	PROM	forward	CCGTAAGACCAGACGCACAG
	6885	EX1	reverse	CCCCTTTTTCGTAAGCATCA
	6886	EX2	forward	TCAATCCTTGTTAGAACCCTGT
	6887	EX2	reverse	AGGCCAGACTGTGGATGTTC
	6888	EX3	forward	TGCACACCTTTACTTTGAGCA
	6889	EX3	reverse	AATCCTCACGCACGAGACAT
	6890	EX4	forward	AGTCCATACGGTCGCAGAGT
	6891	EX4	reverse	TGTGAGGCATCAAAAGTCCA
	6892	EX4	forward	CATGCAGATCAGACCCTGTG
	6893	EX4	reverse	TGTATCCGACTCCTAGCAACC
	6894	EX5	forward	GGTCTGCCATGCAGTTCTTT
	6895	EX5	reverse	CTTCTTGACCCAGGAATGGA
	6896	EX6	forward	TCCCAAACACTGCTAGGTAAT
	6897	EX6	reverse	CAATGAAATTACATGTGGCTAGA
	6898	EX7	forward	TGCAGTCCTCTGCATGTTTA
	6899	EX7	reverse	GGCCTCCAGGATGGTACTTA
	6900	EX8	forward	GCAGCTTGTCAGGTCAAAAA
	6901	EX8	reverse	CAGAACACGCAGCCTACAGA
	6902	EX9	forward	TCTGTAGGCTGCGTGTTCTG
	6903	EX9	reverse	AAATGCTGCAAAGGACATGA
	6904	EX10	forward	GAAAATTGGGACTGGGGATA
	6905	EX10	reverse	AAACACAACAGTACCCAAAGTG

6906	EX11	forward	CTGCTCAGAAGATGCAGTCG
6907	EX11	reverse	CCAGGAACAGGTTCTGAGGA
6908	EX12	forward	AGGAACCTGGAGGGCTAGAG
6909	EX12	reverse	GCTATGTCAGGTGTGCAGGA
6910	EX13	forward	TGAACATTTGAGATGCCTCATT
6911	3'UTR	reverse	GCCAAAGCCATGTTTTGTTT

Supporting Table 3: Correlation between the estimated breeding values EBVs for daily gain (DG), paternal calving ease (pCE), paternal stillbirth incidence (pSB) and body size (BS) of 1800 animals

	pCE	pSB	BS
DG	-0.21	-0.18	0.39
pCE		0.86	-0.36
pSB			-0.23

Supporting Table 4: SNPs showing suggestive associations ($1.14 \times 10^{-6} < P < 1 \times 10^{-3}$) with the estimated breeding value (EBV) for paternal calving ease (pCE)

SNP	Chromosome	Minor allele and MAF	Physical position (BP)	Trait	Eigenstrat statistic	P value	α
ARS-BFGL-NGS-93455	1	A (0.24)	109,649,036	pCE	12.10	5.10×10^{-4}	0.17
BTA-49059-no-rs	2	A (0.02)	112,990,834	pCE	12.04	5.22×10^{-4}	0.47
ARS-BFGL-NGS-19373	4	G (0.4)	119,924,805	pCE	16.18	5.75×10^{-5}	0.18
ARS-BFGL-NGS-32612	5	A (0.42)	110,671,789	pCE	11.49	7.00×10^{-4}	0.15
ARS-BFGL-NGS-13748	5	A (0.42)	110,704,158	pCE	11.32	7.66×10^{-4}	0.15
Hapmap26308-BTC-057761	6	G (0.22)	38,576,012	pCE	11.81	5.91×10^{-4}	0.18
BTB-00251059	6	G (0.06)	42,190,501	pCE	15.09	1.02×10^{-4}	0.35
Hapmap47224-BTA-24614	6	G (0.44)	43,303,952	pCE	11.29	7.81×10^{-4}	-0.15
Hapmap23217-BTA-152007	7	A (0.42)	28,940,286	pCE	10.94	9.40×10^{-4}	-0.17
ARS-BFGL-NGS-104767	10	A (0.17)	1,361,856	pCE	11.14	8.45×10^{-4}	-0.21
BTA-70225-no-rs	10	G (0.39)	56,285,758	pCE	12.75	3.57×10^{-4}	-0.16
ARS-BFGL-NGS-55539	10	A (0.31)	58,488,593	pCE	10.86	9.82×10^{-4}	0.18
BTB-01518485	14	G (0.14)	58,203,661	pCE	13.40	2.52×10^{-4}	0.26

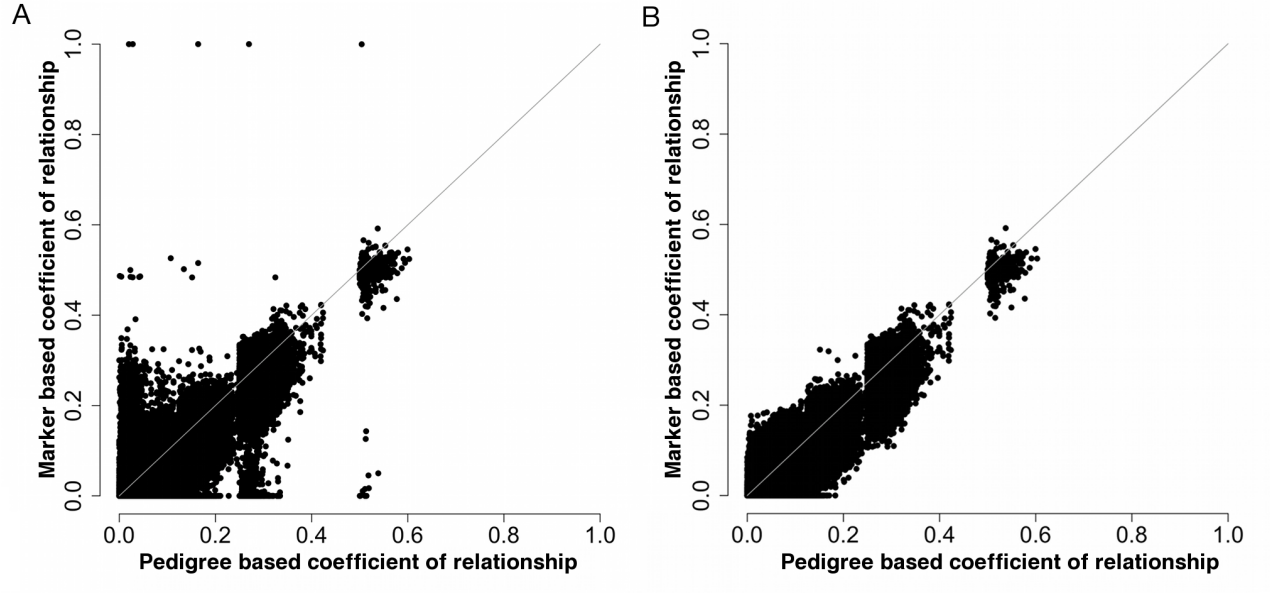
BTB-01518486	14	A (0.1)	58,262,807	pCE	15.33	9.03×10^{-5}	0.28
BTB-01289984	14	G (0.2)	58,491,253	pCE	13.79	2.04×10^{-4}	0.18
BTB-00574555	14	A (0.1)	59,225,067	pCE	13.78	2.05×10^{-4}	0.28
UA-IFASA-7897	14	A (0.12)	59,280,392	pCE	16.96	3.81×10^{-5}	0.29
ARS-BFGL-NGS-27017	15	A (0.18)	57,333,896	pCE	12.54	3.97×10^{-4}	0.20
ARS-BFGL-NGS-94657	17	G (0.47)	74,234,279	pCE	13.48	2.42×10^{-4}	-0.13
UA-IFASA-6850	17	G (0.34)	74,256,192	pCE	11.51	6.91×10^{-4}	-0.13
Hapmap51998-BTA-43053	18	G (0.27)	36,985,552	pCE	11.15	8.39×10^{-4}	-0.17
BTB-01393816	20	A (0.21)	2,754,521	pCE	11.62	6.52×10^{-4}	-0.19
Hapmap40409-BTA-26097	20	G (0.27)	11,576,011	pCE	16.28	5.47×10^{-5}	0.21
ARS-BFGL-NGS-42400	24	A (0.47)	47,413,118	pCE	11.90	5.61×10^{-4}	0.15
BTB-01710538	25	G (0.1)	29,635,262	pCE	10.98	9.23×10^{-4}	0.23
BTB-00920322	26	C (0.47)	3,930,593	pCE	16.33	5.33×10^{-5}	-0.14
ARS-BFGL-NGS-16336	26	A (0.35)	34,398,368	pCE	11.92	5.54×10^{-4}	-0.59
Hapmap42269-BTA-61597	26	A (0.29)	41,041,883	pCE	15.80	7.06×10^{-5}	-0.42
UA-IFASA-6120	29	G (0.27)	37,014,709	pCE	13.38	2.54 x 10-4	-0.19
ARS-BFGL-NGS-104213	29	G (0.02)	37,152,168	pCE	15.64	7.68 x 10-5	-0.59

Supporting Table 5: Characterization of the *PLAG1*, *RPS20*, *LYN*, *SOX17* and *TGS1* polymorphisms. The * indicates SNPs that were genotyped in 810 animals of the study population.

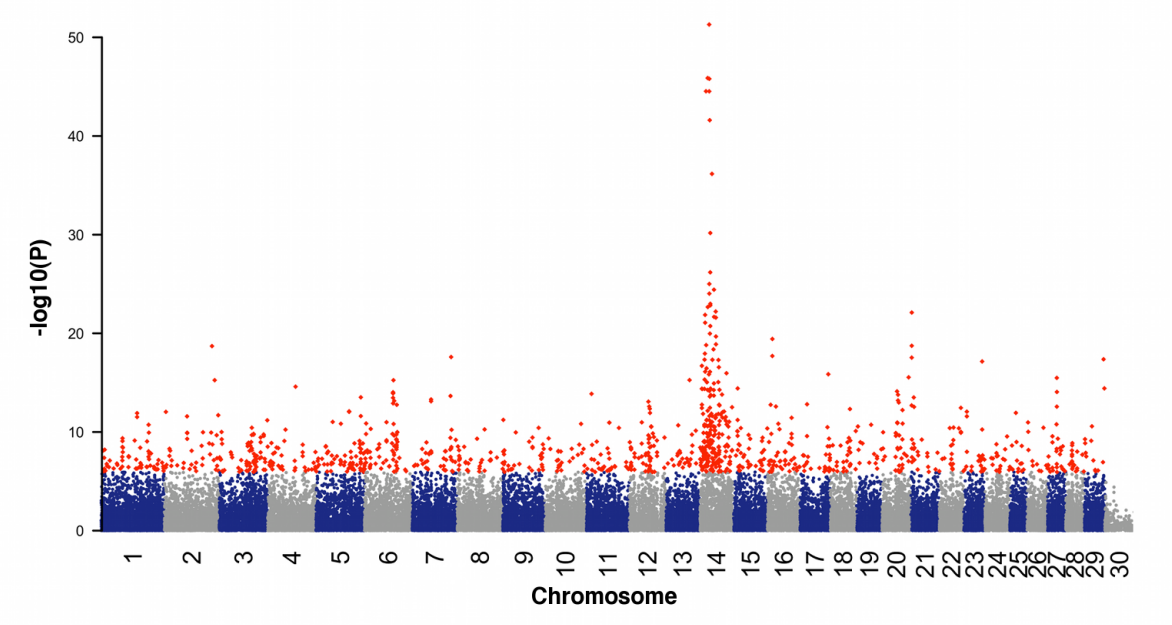
Gene	SNP_ID	Localization	SNP	AminoAcid
PLAG1	ss250608717	INT2	AT	
	ss250608718	PROM	CT	
RPS20	ss250608719	EX3	CT	G42
	ss250608720*	INT3	CT	
	ss250608721*	3'UTR	AG	
	ss250608722	3'UTR	AG	
	ss250608723	PROM	AC	
	ss250608724	PROM	AG	
	ss250608725	PROM	AG	
	ss250608726	PROM	CG	
LYN	ss250608727	INT11	CG	
	ss250608728	INT11	CT	

	ss250608729	EX12	CT	T454
	ss250608730	EX12	AG	T489
	ss250608731	3'UTR	CT	
	ss250608732	INT10	CT	
	ss250608733	INT4	CT	
	ss250608734	INT5	AG	
	ss250608735	INT1	AG	
	ss250608736	INT2	GT	
	ss250608737	INT11	AG	
	ss250608738	EX6	CT	G177
	ss250608739	EX6	CT	S205
SOX17	ss250608740	3'END	AG	
	ss250608741*	EX1	AG	A50T
TGS1	ss250608742	EX3	CT	S90
	ss250608743	INT3	CT	
	ss250608744	INT3	AT	
	ss250608745	EX4	CT	S297T
	ss250608746	INT4	CT	
	ss250608747	INT2	AG	
	ss250608748	INT5	CT	
	ss250608749	INT6	AG	
	ss250608750	INT6	AG	
	ss250608751	INT6	CT	
	ss250608752	3'UTR	CG	
	ss250608753	INT11	GT	
	ss250608754	INT12	INS T	
	ss250608755	INT10	CG	
	ss250608756	EX9	DEL GAA	K626-
	ss250608757	INT10	GT	
	ss250608758	INT7	CT	
	ss250608759	EX11	AT	I733
	ss250608760	EX11	CT	V758
	ss250608761	EX8	AG	P561
	ss250608762*	EX8	GT	P594S
	ss250608763	INT8	CT	
	ss250608764	PROM	CG	

Supporting Figure 1: Pairwise pedigree *vs.* IBD relationship for 1823 Fleckvieh bulls before (A) and after (B) the exclusion of 23 animals with inconsistencies.

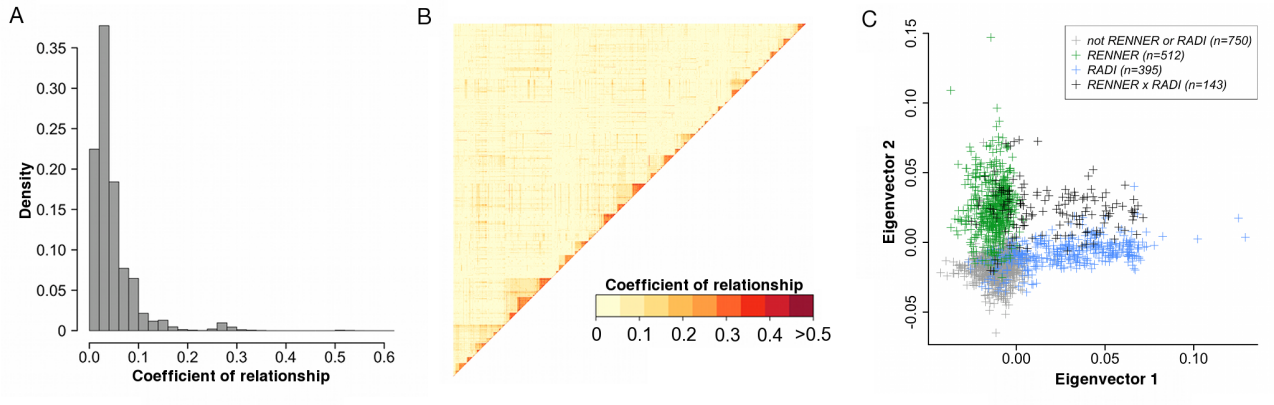


Supporting Figure 2: Manhattan plot for association of 43863 SNPs with the estimated breeding value (EBV) for paternal calving ease (pCE) without considering population stratification.



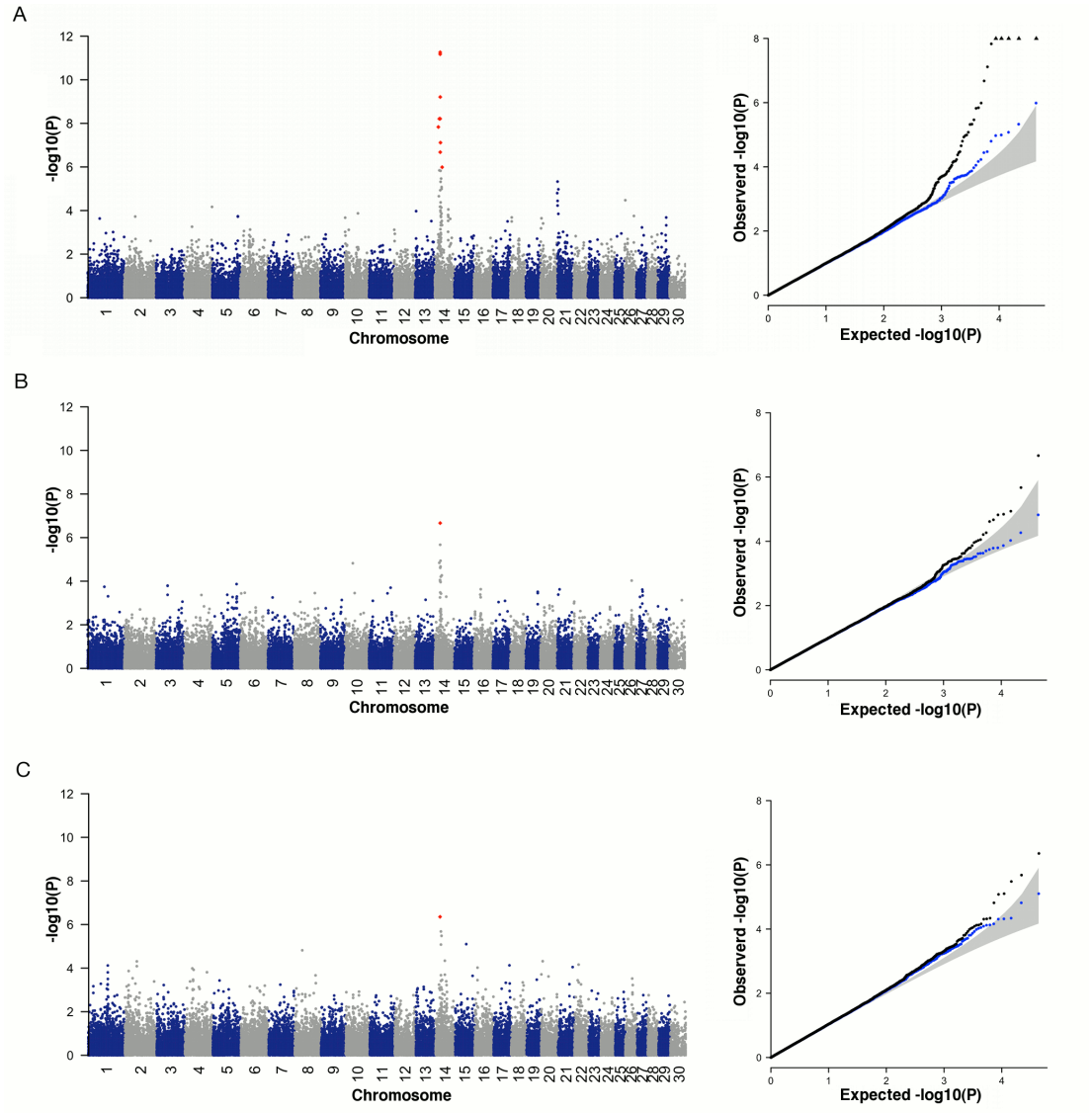
The red dots represent SNPs with $P < 1.14 \times 10^{-6}$ (Bonferroni corrected significance level).

Supporting Figure 3: Population stratification within the study population.



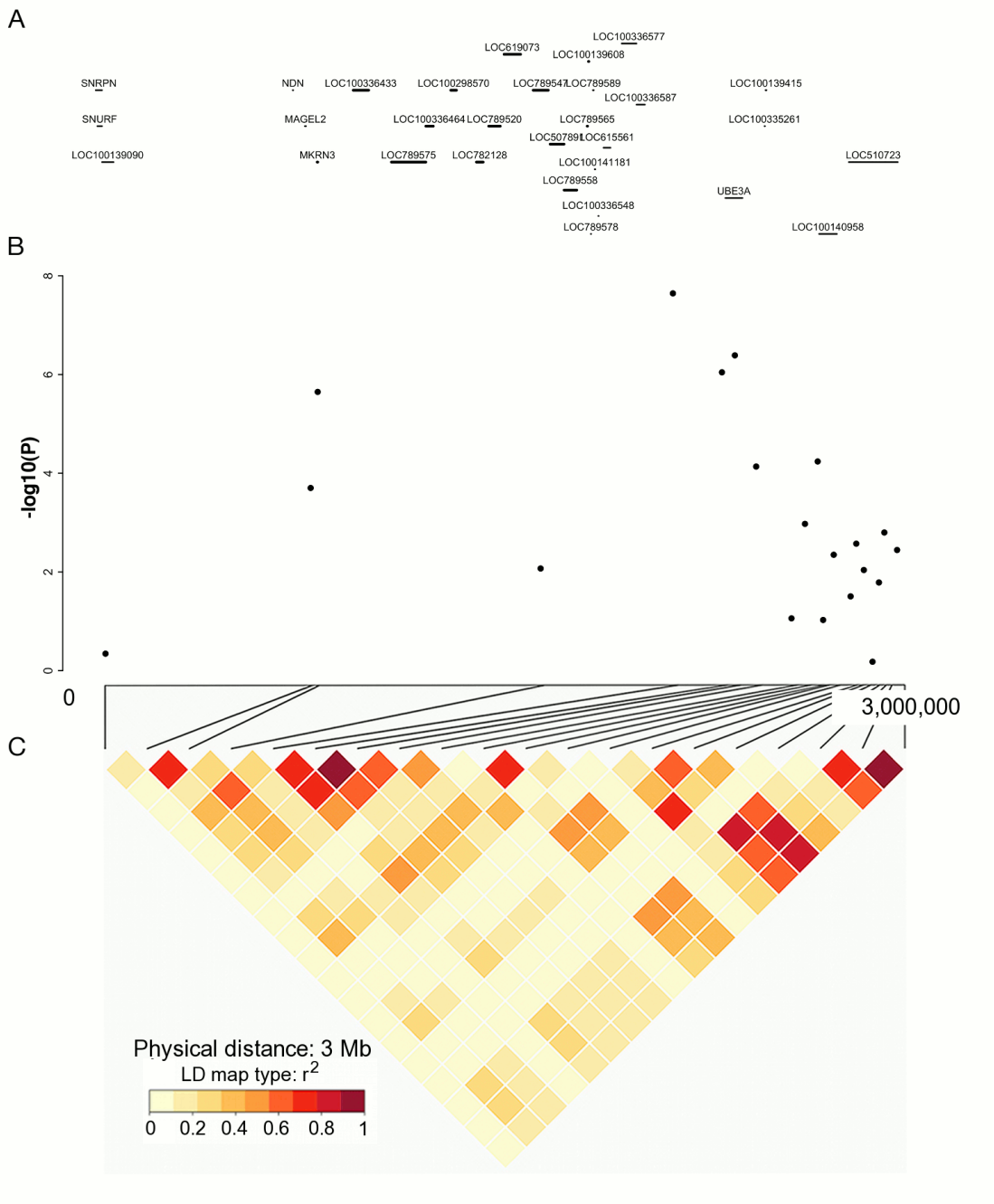
(A) Distribution of the coefficients of relationship of 1800 Fleckvieh animals. (B) Heatmap of the coefficients of relationship presents cluster of related individuals. (C) Plot of the first two eigenvectors visualizing recent introgression of Holstein-Friesian into the Fleckvieh breed (through *RENNER* or *RADI*, both 25% HF) and resulting stratification.

Supporting Figure 4: Manhattan plots and corresponding Quantile-quantile plots for association of 43863 SNPs with the expected breeding value for paternal stillbirth incidence (A), body size (B) and daily gain (C) after correction for population stratification.



The red dots represent SNPs with $P < 1.14 \times 10^{-6}$ (Bonferroni corrected significance level). The 95% concentration band under the null hypothesis of no association is indicated by the shaded area in the QQ plots. The black symbols represent the P values of the entire study (triangles represent SNPs with $P < 1 \times 10^{-8}$). The blue dots indicate the P values excluding those from the associated regions on chromosome 14.

Supporting Figure 5: Detailed view of the region on chromosome 21 delineated by the haplotype associated with the estimated breeding value (EBV) for paternal calving ease (pCE).



(A) Map of genes contained in this region. (B) P values of 20 SNPs from analysis of association with the pCE – EBV in 1800 Fleckvieh animals. (C) Heatmap of the pairwise linkage disequilibrium (r^2).

3rd Chapter

Genome-wide association study uncovers four QTL predisposing to supernumerary teats in cattle

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published in *Animal Genetics* 43(6): 689–695

Summary

Supernumerary teats (hyperthelia, SNTs) are a common abnormality of the bovine udder with a medium to high heritability and a postulated oligogenic or polygenic inheritance pattern. SNTs not only negatively affect machine milking ability but also act as a reservoir for bacteria. A genome-wide association study was carried out in order to identify genes involved in the development of SNTs in the dual-purpose Fleckvieh breed. 2467 progeny-tested bulls were genotyped at 43,698 SNPs and daughter yield deviations (DYDs) for "udder clearness" (UC) were used as high-heritability phenotypes. Massive structuring of the study population was accounted for by principal components analysis-based and mixed model-based approaches. Four loci on BTA5, BTA6, BTA11 and BTA17 were significantly associated with the UC DYD. Three associated regions contain genes of the highly conserved *Wnt* signalling pathway. The four QTL totally account for 10.7% of the variance of the UC DYD while the major fraction of the DYD variance is attributable to chromosomes with no identified QTL. Our results support both an oligogenic and a polygenic inheritance pattern of SNTs in cattle. The identified candidate genes permit insights in the genetic architecture of teat malformations in cattle and provide clues to unravel the molecular mechanisms of mammary gland alterations in cattle and other species.

Introduction

Supernumerary teats (hyperthelia, SNTs) represent a common abnormality of the bovine udder. SNTs negatively affect machine milking ability, especially if SNTs are connected to lactating mammary glands ([1],[2]). SNTs may be positioned at the rear udder (caudal), between the normal front and rear teats (intercalary) or as appendix of normal teats (ramal). Caudal SNTs are the most frequent abnormality [3]. More than 40% of the cows of the Fleckvieh (FV) breed exhibit SNTs [2]. Other breeds are affected to a lesser degree, e.g., 31% of Brown Swiss cows and 15% of Holstein

Friesian cows [4]. While the occurrence of supernumerary nipples / teats in humans and other species have been attributed to different modes of monogenic inheritance ([5],[6],[7],[8]), inheritance of SNTs in cattle seems to be oligo- or polygenic with a heritability between 15 and 60% ([9],[2]). There are no clues to external environmental effects on the development of SNTs. However, SNTs emerge more frequent in animals born of cows of the second and later parities [2], indicating a possible intrauterine hormonal effect of lactation. Reduction of the occurrence of SNTs is an important breeding goal in many breeding programs and highly reliably breeding values are estimated to this end. In this study, we performed a genome-wide association study based on a panel of 54001 single nucleotide polymorphisms (SNPs) and daughter yield deviations (DYDs) for the incidence of SNTs or so called “udder clearness” (UC) in an attempt to identify loci predisposing to the development of SNTs in the FV breed.

Material and Methods

Animals and phenotypes

2545 progeny-tested bulls of the dual purpose Fleckvieh (FV) breed were genotyped with the Illumina BovineSNP 50K Bead chip® interrogating 54,001 SNPs. The bulls descend from 316 different sires and 403 maternal grand-sires. The paternal half-sib families and maternal grand-sire families encompass up to 94 and 185 members with an average of eight and six members, respectively. Phenotypes in the form of daughter yield deviations ([10],[11],[12]) (DYDs) for “udder clearness” (UC) with an average reliability of 0.82 were obtained from the Bavarian State Research Centre for Agriculture (http://www.lfl-bayern.de, August 2011 version). UC is routinely assessed during the examination of first-crop daughters of test bulls by a score ranging from 1 to 9. The absence of supernumerary teats (SNTs) is recorded with 9, whereas the presence of SNTs, depending on the location and quantity, is scored as 1 to 8.

Genotypes and quality control

Of 2545 genotyped animals, six were removed from the dataset because genotyping failed for more than 10% of the SNPs. The chromosomal position (according to the University of Maryland UMD3.1 assembly of the bovine genome sequence [13] was determined for 53,452 SNPs. 549 SNPs with unknown chromosomal position and 8470 SNPs with minor allele frequencies < 0.01 were excluded. 761 SNPs were missing in more than 10% of the animals and 911 SNPs deviated significantly from the Hardy-Weinberg equilibrium ($P < 0.001$). The final dataset comprised 2539 animals and 43,698 SNPs. DYDs for UC were available for 2467 animals.

Genome-wide association study

Two different approaches were applied to account for population stratification and the resulting inflation of false positive association signals. Initially, a principal components analysis (PCA)–based approach as implemented in *Eigenstrat* [14] (ES) was applied including 671 axis of variation with an eigenvalue (λ) > $\bar{\lambda}$ (Kaiser's criterion) to account for sample structure. The axis of variation were inferred using *smartpca* [14] and a reduced dataset, pruned for closely linked SNPs ($r^2 > 0.2$) [15]. As a second approach to correct for population stratification, EMMAX *[16]* was applied to fit the model $y = \mu + Xb + Zu + e$, where y is a vector of daughter yield deviations (DYDs) for UC, μ is the overall mean, b is a vector of SNP effects, X is a design matrix of SNP genotypes, u is a vector of additive polygenic effects, Z is a incidence matrix relating u to individuals and e is a vector of random residual deviates ~ $N(0, I\sigma_e^2)$. u is assumed to be normal distributed with ($0, \sigma_g^2 G$), where σ_g^2 is the additive genetic variance and G is the genomic relationship matrix (GRM) of the 2467 animals obtained as proposed by VanRaden [17]. The

genomic inflation factor was calculated according to Devlin and Roeder [18] and SNPs with P < 1.14 x 10^{-6} (Bonferroni-corrected significance level) in either of the models were considered as significantly associated.

Partitioning of the genetic variance onto particular chromosomes

In order to partitioning the DYD variance onto different chromosomes and QTL, a GRM was built (see above) for the 30 chromosomes and four QTL separately. The GRM for each of the four identified QTL was built based on SNPs within a 5 Mb interval surrounding the most significantly associated SNP. All other SNPs except those within the 5 Mb interval were used to build the GRM for the chromosome harbouring the QTL. We applied *GCTA* [19] to fit the model $y=\sum_{i=1}^{34} g_i+e$, where y is a vector of DYDs for UC, g is a vector of genetic effects attributed to the ith chromosome / QTL, and e is a vector of random residual deviates. g_i is assumed to be normal distributed with $N(0,G_i\sigma_{gi}^2)$, where G_i is the GRM built using SNPs of the ith chromosome / QTL. Variance components were estimated with the effects of all chromosomes / QTL fitted simultaneously. The proportion of variance attributable to the ith chromosome / QTL was calculated as $\sigma_i^2/(\sum_{j=1}^{34}\sigma_j^2+\sigma_e^2)$.

Results

Association study

Five SNPs delimitating three QTL regions on BTA5, BTA11 and BTA17 met the genome-wide threshold of significance of $P < 1.14 \times 10^{-6}$ after PCA-based correction of population structure (Figure 1A). The successful elimination of false positive association signals is evidenced by a genomic inflation factor of 1.01. The characteristics of the significantly associated SNPs are listed in Table 1.

The mixed model-based approach yielded an inflation factor of 0.99 and six significantly associated SNPs within four QTL regions on BTA5, BTA6, BTA11 and BTA17, among them *ARS-BFGL-NGS-10494* on BTA6, which did not meet the threshold of significance with the PCA-based model (Table 1)(Figure 1B).

Table 1: Significantly associated SNPs with the daughter yield deviation for "udder clearness" in 2467 Fleckvieh animals

Chromosome	SNP-id	Physical position (Basepairs)	SNT-predisposing allele and allele frequency	P (PCA – based model)	P (Mixed model)	Neighboring genes
5	ARS-BFGL-NGS-26008	163,482	A (0.65)	**4.78×10^{-7}**	**2.38×10^{-7}**	*LOC783893, LOC783966, LOC782348*
	BTB-01498763	186,792	A (0.65)	**6.52×10^{-7}**	**3.69×10^{-7}**	
6	ARS-BFGL-NGS-10494	18,961,422	G (0.59)	1.41×10^{-5}	**1.89×10^{-8}**	*LEF1, DKK2*
11	BTA-16600-no-rs	11,612,663	A (0.20)	**1.67×10^{-7}**	**7.72×10^{-11}**	*EXOC6B*
	BTB-02007301	11,754,597	A (0.18)	**1.90×10^{-7}**	**1.96×10^{-11}**	
17	Hapmap49912-BTA-21169	62,783,598	A (0.56)	**7.10×10^{-10}**	**8.57×10^{-10}**	*TBX3, TBX5, RBM19*

Six SNPs located on four different chromosomes meet the genome-wide significance threshold of $P < 1.14 \times 10^{-6}$. The P values are given for the PCA-based as well as for the mixed model-based approach to account for population stratification. The bold typed letters indicate genome-wide significance. The SNPs are arranged according to their physical position, based on the UMD3.1 assembly of the bovine genome sequence.

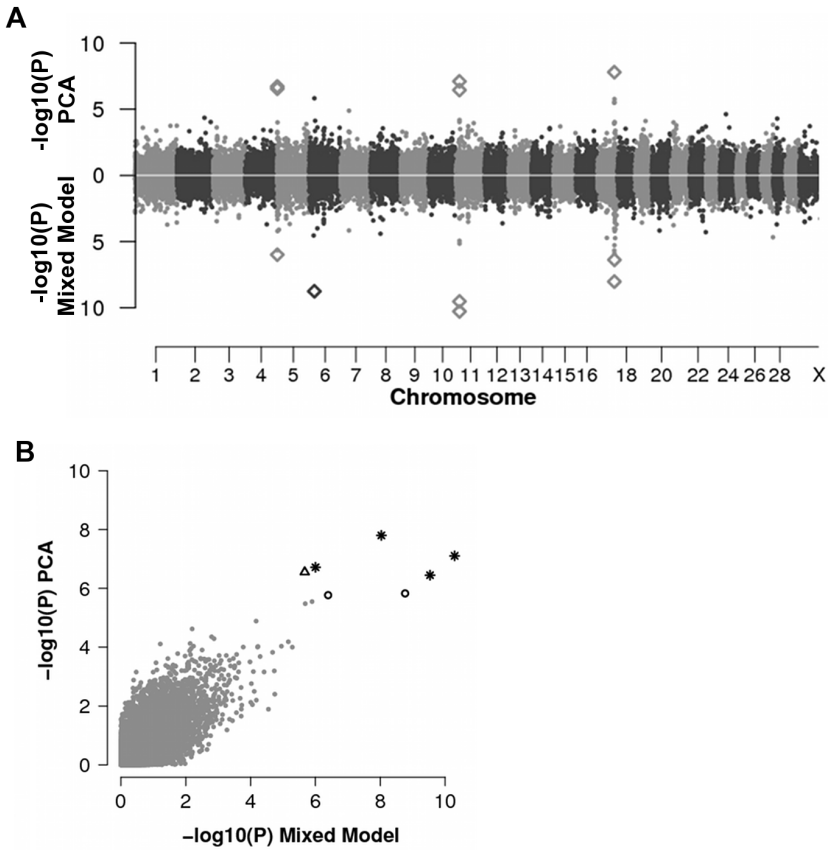

Figure 1: Association of 43,698 SNPs with the daughter yield deviation for "udder clearness" in the Fleckvieh breed. A) The results for the principal components analysis (PCA)-based and for the mixed model-based approach to account for population stratification are shown above and below the horizontal line, respectively. Open symbols represent SNPs with $P < 1.14 \times 10\text{-}6$. B) Correlation between the -log10(P) - values resulting from the analysis with two different approaches. Stars represent significantly associated SNPs after correction for population stratification with both models, open circles represent SNPs that are significantly associated with the mixed model- based approach, only.

Identification of functional genes in the associated regions

QTL that were significantly associated in at least one of the applied models were considered for the identification of genes known to be involved in mammary gland development. The gene content of the four associated regions was analysed based on the University of Maryland UMD3.1 annotation

[13]. The BTA5 region contains a cluster of sequences (*LOC783893*, *LOC783966*) similar to genes encoding ankyrin repeat domain containing proteins that are known to be involved in *Wnt* signalling [20], as well as *frizzled-3* (*LOC782348*), a gene encoding a transmembrane receptor required for the activation of *Wnt* signalling [21]. The associated region on BTA6 contains the lymphoid enhancer binding factor 1 encoding gene (*LEF1*) and the dickkopf homolog 2 encoding gene (*DKK2*). *LEF1* mediated *Wnt* signalling is required for the morphogenesis of the mammary gland during embryogenesis [22], while *DKK2* acts as an inhibitor of the canonical *Wnt* signalling [23]. Two members of the T-Box transcription factor gene family, *TBX3* and *TBX5* and the RNA binding motif protein 19 encoding gene *RBM19* are in the associated region on BTA17. *TBX3* is regulated as downstream target of *Wnt* signalling [24]. Two associated SNPs on BTA11 are located in introns of the exocyst complex component 6B encoding gene *EXOC6B*. The protein encoded by *EXOC6B* belongs to the network for the generation of the apical surface and lumen during gland formation [25].

Assessing the impact of the four QTL

Alleles lowering the DYD for UC can be considered as alleles that are predisposing to SNTs. We determined the SNTs-predisposing allele for the most significantly associated SNP for each of the four identified QTL regions. Figure 2 presents the frequency distribution of animals with an increasing number of SNTs-predisposing alleles (from 0 to 8). The DYD for UC decreases nearly linearly from 0.73 (0 alleles) to -0.22 (7 alleles) with an increasing number of predisposing alleles. Animals with 8 SNTs-predisposing alleles were not considered due to the low number of observations (n=4). Fitting a linear regression model yielded an average substitution effect of -0.14 DYD points for each SNTs-predisposing allele, corresponding to -0.19σ of the UC DYD.

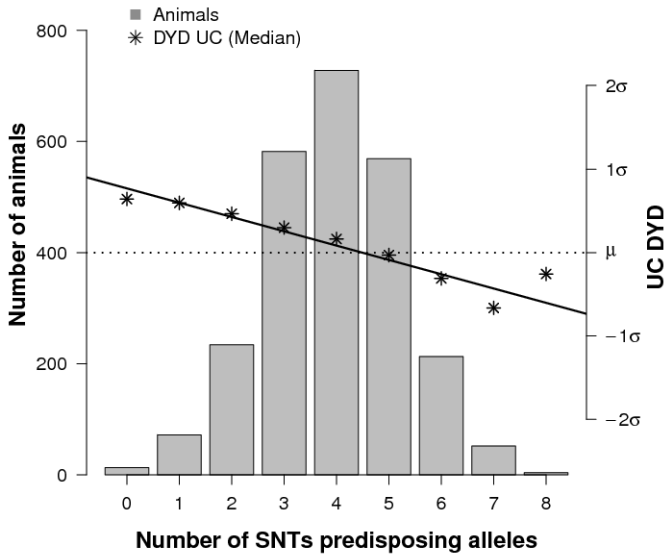

Figure 2: The combined impact of the four identified QTL on BTA5, BTA6, BTA11 and BTA17 on the daughter yield deviation for "udder clearness" in the Fleckvieh breed. 2467 Fleckvieh animals are grouped according to the number of alleles that predispose to supernumerary teats (SNTs). Black symbols represent the median daughter yield deviation (DYD) for "udder clearness" (UC) for each group. The black line is a linear regression line through these points, whereas the black dotted line represents the population mean. The grey bars indicate the number of animals with an increasing number of the SNTs-predisposing alleles.

Proportion of variance explained by each chromosome

Genomic relationship matrices for the 2467 animals were built for each chromosome / QTL separately in order to partitioning the genetic variance onto different chromosomes. The proportion of the DYD variance attributable to a particular chromosome / QTL was then estimated with the effects of all chromosomes / QTL fitted simultaneously. Totally, the 43,698 SNPs account for 52.27% of the DYD variance. The contribution of particular chromosomes varies strongly (Figure 3). A major fraction of the DYD variance is attributable to BTA5 (6.90%), BTA11 (4.94%), BTA6 (4.83%) and BTA17 (3.94%) the four chromosomes harbouring significantly associated SNPs for the UC DYD. Interestingly, a large fraction of the DYD variance results from BTA6, although

association analysis with PCA-based correction of population structure did not reveal significantly associated SNPs on this chromosome. The major fraction of the DYD variance on BTA6, BTA11 and BTA17 is attributed to the identified QTL, whereas the QTL on BTA5 accounts only for a minor fraction of the chromosomal variance.

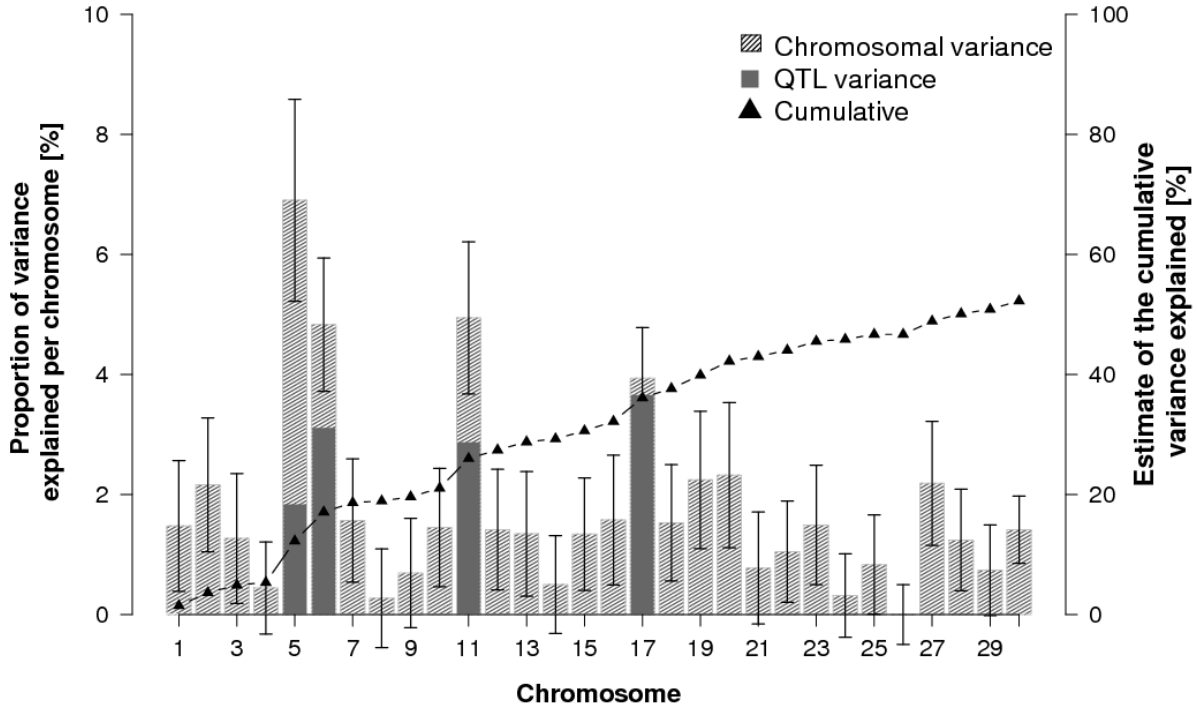

Figure 3: Partitioning of the DYD variance onto 30 chromosomes and four identified QTL. The grey shaded bars indicate the fraction of DYD variance attributed to a particular chromosome and the corresponding standard error. The dark grey bars represent the fraction of DYD variance attributed to each of the four identified QTL regions. The black triangles represent the cumulative proportion of DYD variance explained.

Discussion

We carried out an association study with a medium-sized sample of 2467 progeny-tested Fleckvieh bulls and a dense set of 43,698 genome-wide distributed SNPs. This enabled the detection of four QTL predisposing to supernumerary teats in cows. We accounted for the massively structured study

population by using a mixed model-based and a PCA-based approach. Both approaches have already been successfully applied to identifying QTL in highly structured cattle populations (e.g. [26],[27]). The PCA–based approach allowed for the identification of three QTL on BTA5, BTA11 and BTA17 for UC. The mixed model-based approach uncovered an additional QTL on BTA6. However, the P value of the corresponding SNP derived from the PCA-based approach is only slightly above the threshold of significance. Different treatment of the effective genomic relationship among the animals seems to be the major reason for the different P values. A subset of 671 principal components was extracted to represent the relatedness between animals with the PCA-based model, while the entire SNP information was incorporated in the genomic relationship matrix for the mixed model. The mixed model-based approach leads to a better sensitivity at maximum specificity as manifested by a unity inflation factor.

The four identified QTL can be considered as the major determinants for the development of SNTs in the FV breed since the four QTL account for 10.71% of the DYD variance. However, the largest proportion of the DYD variance is attributable to chromosomes with no identified QTL. BTA20, e.g., with no identified QTL, explains nearly 2.4% of the DYD variance. Thus the genetic architecture of the UC DYD is characterized by a large number of genes with small effects and only few genes with major effects, as it is typical for many complex quantitative traits [28]. These findings agree with an oligogenic / polygenic inheritance pattern for SNTs as proposed by Brka [2]. However, one must be aware of the complexity of the phenotype in the present study. Breeding value estimation for UC is carried out based on scoring the locations and shapes of SNTs. More specific phenotypes with regard to the positions of SNTs, *i.e.* caudal, intercalary or ramal, might allow for the detection of distinct QTL for different types of SNTs. It is well known, that distinct signalling pathways are essential for the development of specific mammary placodes [29].

The candidate genes identified in the QTL regions indicate the highly conserved *Wnt / ß-catenin* signalling pathway as the major determinant for the development of SNTs in cattle. *Wnt* signalling not only initiates the development of the embryonic mammary gland [30], but also induces mammary placode formation *via TBX3* expression [31]. Mutations resulting in a loss of function of *TBX3* have been shown to cause the ulnar mammary syndrome (UMS) in humans and mice, for which both supernumerary as well as aplastic nipples and mammary glands are characteristic ([32], [33],[34]). In cattle, the genomic region on chromosome 17 encompassing *TBX3* is associated with absent teats in Japanese Black cattle [35], supporting our findings of a QTL for mammary gland morphology on BTA17.

The appearance of SNTs ranges from rudimentary buds to fully developed teats. Discontinuous *Wnt* signalling *via LEF1* leads to underdeveloped mammary placodes [36], resulting in rudimentary teats and teat malformations. The prevention of malformations of the mammary gland such as inverted teats is a major objective of swine breeding. Polymorphisms within the coding region of *LEF1* have already been shown to be associated with the occurrence of inverted teats in swine [37]. The candidate genes resulting from the present study may therefore provide clues for identifying molecular alterations leading to teat abnormalities even in other species than cattle.

DKK2, a second functional candidate in the associated region on BTA6, is in close vicinity to *LEF1*. Possibly, both genes *DKK2* and *LEF1* are involved in the development of SNTs in cattle. *DKK2* acts as an antagonist of the *Wnt* signalling pathway and locally down-regulates *Wnt* signalling during normal eye development, whereas a lack of *DKK2* expression results in an increased ectopic activity of the *Wnt* signalling [38]. An increased activity of the *Wnt* signalling pathway results in the formation of ectopic placode-like structures [39]. An analogous mechanism was reported for *frizzled 3*, a functional candidate in the BTA5 QTL-region. *frizzled 3* acts as a transmembrane

receptor for *Wnt* proteins and activates the canonical *Wnt* signalling pathway [40]. Over-expression of *frizzled-3* was shown to result in ectopic eye development [41]. Similar interactions of *DKK2* and *frizzled 3* with *Wnt* signalling during embryonic mammary gland development are plausible, as SNTs can be considered as ectopic features of the mammary gland.

EXOC6B, located in the QTL region of BTA11 is another functional candidate gene for teat morphology and function. Its product is an exocyst component and may therefore affect lumen formation ([25],[42]) in supernumerary teats. Variants of this gene may predispose to lactating SNTs, which are particularly undesirable.

As primiparous cows are not lactating during pregnancy, the hormonal environment of the fetus differs for the first *vs*. later parities. Pregnancy and lactation result in distinct hormone release patterns in mammals, implicating differential expression of genes within the *Wnt* signalling pathway [43]. Our results provide evidence for *Wnt* signalling being involved in abnormal teat development. The higher incidence of SNTs in cows born from later parities [2] may therefore be explained by a stronger response to lactational hormones of *Wnt* signalling in pertinently predisposed animals.

Acknowledgements

This study is part of FUGATO-plus GenoTrack and was financially supported by the German Ministry of Education and Research, BMBF (grants 0315134A and 0315134D), the Förderverein Biotechnologieforschung e.V. (FBF), Bonn, and Lohmann Tierzucht GmbH, Cuxhaven.

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4th Chapter

Identification of QTL for UV-protective eye area pigmentation in cattle by progeny phenotyping and genome-wide association analysis

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published in *PLoS ONE* 7(5): e36346

Abstract

Pigmentation patterns allow for the differentiation of cattle breeds. A dominantly inherited white head is characteristic for animals of the Fleckvieh (FV) breed. However, a minority of the FV animals exhibits peculiar pigmentation surrounding the eyes (ambilateral circumocular pigmentation, ACOP). In areas where animals are exposed to increased solar ultraviolet radiation, ACOP is associated with a reduced susceptibility to bovine ocular squamous cell carcinoma (BOSCC, eye cancer). Eye cancer is the most prevalent malignant tumour affecting cattle. Selection for animals with ACOP rapidly reduces the incidence of BOSCC. To identify quantitative trait loci (QTL) underlying ACOP, we performed a genome-wide association study using 658,385 single nucleotide polymorphisms (SNPs). The study population consisted of 3579 bulls of the FV breed with a total of 320,186 progeny with phenotypes for ACOP. The proportion of progeny with ACOP was used as a quantitative trait with high heritability (h^2=0.79). A variance components based approach to account for population stratification uncovered twelve QTL regions on seven chromosomes. The identified QTL point to *MCM6*, *PAX3*, *ERBB3*, *KITLG*, *LEF1*, *DKK2*, *KIT*, *CRIM1*, *ATRN*, *GSDMC*, *MITF* and *NBEAL2* as underlying genes for eye area pigmentation in cattle. The twelve QTL regions explain 44.96% of the phenotypic variance of the proportion of daughters with ACOP. The chromosomes harbouring significantly associated SNPs account for 54.13% of the phenotypic variance, while another 19.51% of the phenotypic variance is attributable to chromosomes without identified QTL. Thus, the missing heritability amounts to 7% only. Our results support a polygenic inheritance pattern of ACOP in cattle and provide the basis for efficient genomic selection of animals that are less susceptible to serious eye diseases.

Introduction

High-density SNP panels offer a new approach to deciphering the genetic architecture of complex traits [1] [2]. Large scale genome-wide association studies (GWAS) have identified hundreds of variants contributing to the genetic variation of quantitative traits in humans, *e.g.* [3] [4]. Comprehensive GWAS have also been applied successfully to identify quantitative trait loci (QTL) for important traits in livestock species, frequently facilitated by using breeding values (or daughter yield deviations) *e.g.* [5],[2],[6]. Breeding values are highly heritable phenotypes as they are estimated on the basis of a large number of progeny records. Utilizing breeding values as phenotypes not only compensates smaller sample size in livestock GWAS [7] but also enables QTL mapping for traits which are recorded in the progeny.

Phenotypes for skin and coat pigmentation are readily accessible and accurately recordable traits with medium to high heritabilities ([8], [9]). Skin and coat pigmentation traits have been studied and characterized extensively in humans (see [10] for a review), in laboratory animals [11] and in domestic animals (see [12] for a review). Variations of skin and coat colours naturally arose in the course of adaption to altering environmental conditions, *e.g.* reaction to thermal stress [13] and increasing exposure to ultraviolet (UV) radiation [14].

Excessive exposure to UV radiation and a lack of ambilateral circumocular pigmentation (ACOP) are two predisposing factors to bovine ocular squamous cell carcinoma (BOSCC, eye cancer) [15] [16] [17]. Eye cancer is the most prevalent malignant tumour affecting cattle and causes substantial economic losses [16]. Breeds with white heads, *e.g.* Fleckvieh and Hereford, are particularly susceptible to BOSCC [18]. While the incidence of BOSCC in pertinently exposed Simmental (*i.e.* Fleckvieh) cattle is up to 53% [19], investigations concerning the prevalence of BOSCC in German herds have not been performed. Although there is evidence for a genetic predisposition to BOSCC,

the heritability for BOSCC is low [20]. The susceptibility to BOSCC is considerably reduced in animals with ACOP, a highly heritable trait which can be easily identified [21]. Since the heritability for eye-area pigmentaion is higher than for the susceptibility to BOSCC ([20],[21]), selection for ACOP is expected to rapidly decrease the number of affected animals [22] [17]. Furthermore, eye irritation and subsequent infection with bovine infectious keratoconjunctivitis (BIK, pinkeye) is more frequent in cattle without ACOP [23]. Selection for ACOP reduces the incidence of BIK and thus enhances animal welfare in areas with increased solar radiation. However, as excessive exposure to UV radiation is not a major challenge for German cattle, selection for ACOP does not take place in the German FV population.

The aim of the present study was to gain insights into the genetic architecture of a special aspect of skin pigmentation and to provide the basis for more efficient selection for animals that are less susceptible to serious eye diseases. Recording the pigmentation status in large progeny groups of artificial insemination bulls provided a highly heritable phenotype for a genome-wide association study. Using densely spaced SNPs, the association study identified twelve QTL regions.

Material and Methods

Animals and phenotypes

The proportion of daughters with ACOP (**Figure 1**) was assessed for 3579 progeny tested bulls of the Fleckvieh (FV) breed. Eye-area pigmentation is routinely recorded during the examination of first-crop daughters of test bulls as a categorical trait. However, phenotypes for ACOP are not recorded routinely for male animals. Phenotypic records for 320,186 FV cows were provided from the Bavarian State Research Center for Agriculture (http://www.lfl.bayern.de). The number of daughters per sire ranged from 20 to 3949 with a median of 59 daughters. An approximately normally distributed phenotype was obtained by square root transformation of the proportion of

daughters with ACOP (**Figure S1**). Genetic parameters were estimated using the random effect model $y = g + e$ [24], where y is the square root transformed proportion of daughters with ACOP, g is a vector of random genetic effects and e is a vector of random residual deviates ($e \sim N(0, I\,\sigma_e^2)$) . g is normally distributed with ($0, \sigma_g^2 A$), where σ_g^2 is the genetic variance and A is the numerator relationship matrix among the 3579 bulls tracing back pedigree information to 1920.

Figure 1. An animal of the dual-purpose Fleckvieh breed with ambilateral circumocular pigmentation. A white head is characteristic for animals of the Fleckvieh (FV) breed. However, in some half-sib families animals with pigmented skin around the eyes prevail. The pigmentation is restricted to the circumocular area and is not connected to the body pigmentation. Although there is variation regarding both the dimension and shape of ambilateral circumocular pigmentation (ACOP) in FV cattle, ACOP is routinely assessed as categorical trait only. The figure was kindly supplied by BAYERN-GENETIK GmbH (http://www.fleckvieh.de).

Genotypes and quality control

Genotyping was performed with three different genotyping arrays. 3387 FV bulls were genotyped with the Illumina BovineSNP 50K Bead chip® interrogating 54,001 (version 1, 54K*v1*) and 54,609 (version 2, 54K*v2*) SNPs, respectively. Additionally, 810 FV bulls were genotyped with the Illumina

BovineHD Bead chip® interrogating 777,962 SNPs (777K). 521 bulls of the 777K data set were also genotyped with the 54Kv1 genotyping array **(Table S1-S3).** The chromosomal position of the SNPs was determined according to the University of Maryland UMD3.1 assembly of the bovine genome sequence [25]. Quality control was performed for the three datasets separately using *PLINK* [26]. Animals with more than 10% missing genotypes were not considered for further analyses. Those SNPs with unknown, Y-chromosomal or mitochondrial position or if genotyping failed in more than 10% of the animals were excluded. Additionally, SNPs with a minor allele frequency (MAF) < 0.5% and SNPs showing significant (P < 0.0001) deviation from the Hardy-Weinberg Equilibrium were omitted for subsequent analysis. The genomic relationship was calculated as proposed by VanRaden [27] and compared with the pedigree relationship. Animals showing major discrepancies of the pedigree and genomic relationship were omitted. A detailed overview of the number of SNPs and animals not passing the quality criteria is given in **Table S1**.

In silico genotyping

A subset of 38,820 SNPs was interrogated with all three genotyping arrays. However, genotypes for 488, 394 and 611,702 SNPs were exclusively interrogated with the 54K*v1*, 54K*v2* and 777K array, respectively **(Table S2 & S3)**. The datasets were combined and missing genotypes were inferred using *findhapV2* [28]. After genotype imputation, the resulting dataset comprised 3643 animals and 658,385 SNPs with an average genotyping rate of 99.68% per individual. Progeny records were available for 3579 individuals only.

Evaluation of imputation accuracy

Imputation accuracy was evaluated within 802 animals of the high-density dataset to assess the quality of the imputed genotypes. We randomly selected 400 animals as reference population with

full genotype information. Genotypes were masked for the remaining 402 animals for all SNPs except for 40,062 SNPs interrogated by the 54K*v1* Bead Chip. Genotypes for the 613,232 masked SNPs were subsequently inferred using *findhapV2* and compared with the true genotypes. The number of SNPs used for the evaluation of the imputation accuracy is given for each chromosome in **Table S4**. In total, 99.52% of the genotypes could be inferred with an average genotypic concordance of 95.78%. The allele frequency was the major determinant for imputation accuracy (**Figure S2**).

Genome-wide association study

A genome-wide association study was performed using a variance components based approach to account for population stratification and to eliminate the resulting inflation of false positive associations. We used *EMMAX* [29] to fit the model $Y = Xb + u + \epsilon$, where Y is the square root transformed proportion of daughters with ACOP, b is the SNP effect, X is a design matrix of SNP genotypes, u is the additive genetic effect with ($0, \sigma_a^2 G$), where σ_a^2 is the additive genetic variance and G is the genomic relationship matrix (GRM) among the 3579 animals with phenotype information built based on 658,385 SNPs following VanRaden's approach (see above). SNPs were considered as significantly associated on a genome-wide level for P values below 7.59 x 10^{-8} (Bonferroni-corrected type I error threshold for 658,385 independent tests).

Estimating the power of the genome-wide association study

The required sample size (N) for a GWAS to identify a QTL explaining a given fraction (q^2) of the trait variance can be estimated as $N = (z_{(1-\alpha/2)} + z_{(1-\beta)})^2 / q^2$, where α is the significance level, z is the normal score and $(1-\beta)$ is the power to detect association [30]. Considering 3579 animals and

the Bonferroni-corrected significance threshold for an average number of 22.000 tests per chromosome, the power to detect a QTL accounting for at least 1% of the trait variance is approximately 90% in the present study.

Partitioning of the genetic variance

In order to estimate the proportion of phenotypic variance attributed to a particular chromosome, a GRM was built (see above) for each of the 30 chromosomes separately. We used *GCTA* [31] to fit the model $y=\sum_{i=1}^{30} g_i+e$, where y is a vector of the square root transformed proportion of daughters with ACOP, g is a vector of genetic effects attributed to the ith chromosome, and e is a vector of random residual deviates. g_i is assumed to be normally distributed with $N(0,G_i\sigma^2_{gi})$, where G_i is the GRM built based on SNPs on the ith chromosome. Variance components were estimated with the effects of all chromosomes fitted simultaneously and the proportion of phenotypic variance attributable to the ith chromosome was calculated as $\sigma^2_{g_i}/(\sum_{j=1}^{30}\sigma^2_{g_j}+\sigma^2_e)$. To estimate the proportion of phenotypic variance explained by each of the twelve identified QTL regions, SNPs within a 5 Mb interval centred on the most significantly associated SNP were considered for building the GRM for each QTL region. All SNPs except those within the 5 Mb interval were used to build the GRM for the chromosome harbouring the QTL. Variance components were estimated with the effects of all 30 chromosomes and twelve QTL regions fitted simultaneously (see above).

Results

The proportion of daughters with ambilateral circumocular pigmentation (ACOP) (**Figure 1**) was obtained by phenotyping a median number of 59 daughters for 3579 bulls. It ranged from 0 to 69.1% with an average of 22.6% of the daughters per genotyped sire. After square root transformation, 66.67%, 95.87% and 99.89% of the values were within one, two and three standard deviations, respectively (**Figure S1)**. The resulting trait is a highly heritable progeny-derived phenotype for the bulls. Using the numerator relationship matrix among the 3579 animals built based on in-depth pedigree information in a random effect model, the heritability was estimated to be 0.79 (± 0.04).

Association study

The genome-wide association study based on a variance components based approach to account for population stratification identified twelve QTL regions on seven chromosomes (**Figure 2**). Among them, eight met the Bonferroni-corrected threshold for genome-wide significance, four were significantly associated on a chromosome-wide scale. A detailed overview of the characteristics of the identified QTL regions is given in **Table 1** and **Figure S3**. An additional analysis conditional on the most significantly associated SNP indicates the presence of a second independent QTL on BTA6 and BTA22, respectively (**Table 2, Figure S4 & S5**).

Identification of functional genes within the associated regions

The gene content of the associated regions was analysed based on the University of Maryland UMD3.1 assembly of the bovine genome [25]. Strong association was observed in close vicinity to *KIT* (BTA6), *KITLG, ERBB3* (BTA5) and *MITF* (BTA22), four genes which play central roles in the migration of melanoblast cells and melanocyte development [32][33][34]. Two QTL on BTA2 point

to *PAX3* and *MCM6* as candidate genes for ACOP in cattle. *PAX3* is a transcription factor known to be involved in melanogenesis [35]. *MCM6* is up-regulated during pheomelanogenesis [36]. A QTL on BTA6, although only associated on a chromosome-wide level, is located between *DKK2* and *LEF1*. *DKK2* plays an essential role during eye development [37]. *LEF1* interacts with *MITF* via Wnt signalling [38]. The QTL on BTA11 is in close vicinity to *CRIM1*, which is up-regulated in developing ocular tissues [39]. *ATRN*, a candidate gene for the BTA13 QTL, was shown to influence coat colour in mice [40]. On BTA14, association of a region containing *GSDMC* (alias *MLZE*) was observed. *MLZE* is up-regulated in growing metastatic melanomas and is supposed to be important for melanoma progression [41]. A QTL on BTA22 identifies *NBEAL2* as candidate gene for ACOP. *NBEAL2* shows homology to *LYST*, which is responsible for pigmentation defects in humans and mice [42] [43]. We found no gene in immediate vicinity of the third BTA22 QTL.

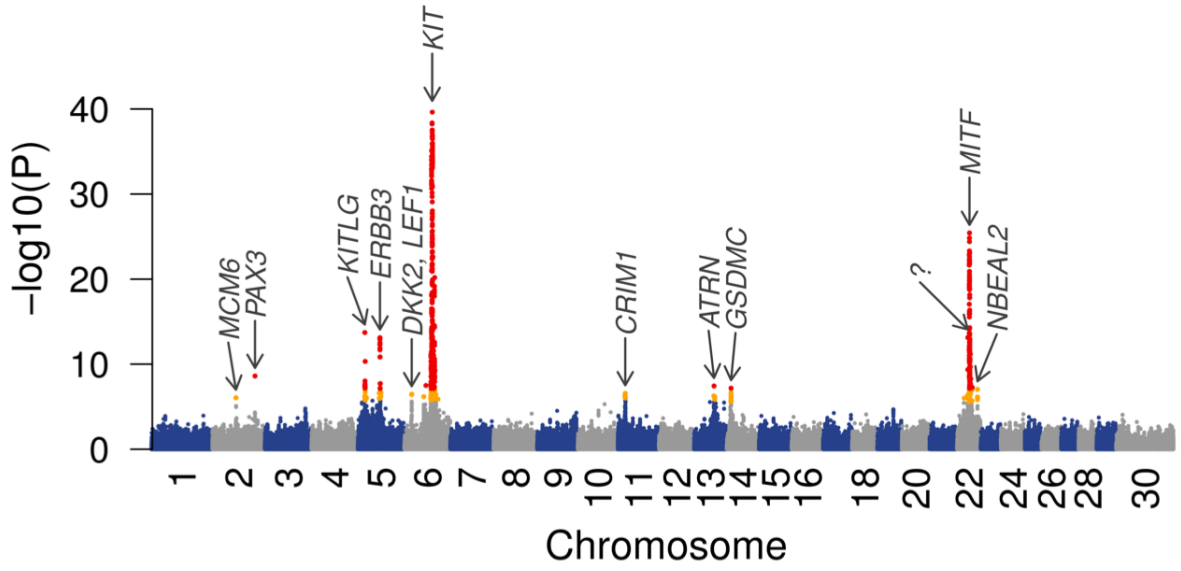

Figure 2. Manhattan plot of association of 658,385 SNPs with the proportion of daughters with ambilateral circumocular pigmentation in 3579 bulls of the Fleckvieh breed. The chromosomes are separated with alternating colours. Orange and red dots indicate chromosome-wide and genome-wide (P < 7.59 x 10-8) significantly associated SNPs, respectively. The vertical axis is truncated for P values below -log10(10-41). Twelve identified QTL regions are indicated with arrows and gene identifiers.

Table 1: The most significantly associated SNP for each of the twelve identified QTL regions for ambilateral circumocular pigmentation in the Fleckvieh breed

Chromosome	SNP-id	Physical Position (bp)	Minor allele frequency	P	Candidate gene
2	BovineHD0200017704	61,628,137	0.38	9.01×10^{-7}	*MCM6*
	UA-IFASA-5029	111,206,088	0.01	2.49×10^{-9}	*PAX3*
5	BovineHD0500005310	18,206,817	0.13	1.90×10^{-14}	*KITLG*
	BovineHD0500016261	57,554,914	0.32	7.59×10^{-14}	*ERBB3*
6	BovineHD0600005244	18,975,451	0.43	3.51×10^{-7}	*DKK2, LEF1*
	BTB-00263209	72,382,208	0.13	2.46×10^{-73}	*KIT*
11	BTB-00753516	19,344,832	0.32	2.72×10^{-7}	*CRIM1*
13	BovineHD1300014790	51,984,994	0.23	3.72×10^{-8}	*ATRN*
14	Hapmap22917-BTC-068800	12,075,830	0.33	6.75×10^{-8}	*GSDMC*
22	BovineHD2200008080	27,931,961	0.02	7.32×10^{-14}	-
	BovineHD2200009208	32,245,023	0.17	3.75×10^{-26}	*MITF*
	BovineHD2200015054	53,016,253	0.2	9.18×10^{-8}	*NBEAL2*

The SNPs are arranged according to their physical position based on the UMD3.1 assembly of the bovine genome. The P values were obtained by using a variance components based approach to account for population stratification.

Table 2: The most significantly associated SNPs on chromosomes 6 and 22 after analysis conditional on the top SNP

Chromosome	SNP-id	Physical Position (bp)	Minor allele frequency	P	Candidate gene
6	BovineHD0600020013	72,025,871	0.07	1.11×10^{-11}	*KIT*
22	BovineHD4100015611	32,787,124	0.03	6.11×10^{-17}	*MITF*

The SNPs are arranged according to their physical position based on the UMD3.1 assembly of the bovine genome. The P values are obtained by using a variance components based approach to account for population stratification conditional on the most significantly associated SNP for BTA6 (BTB-00263209) and BTA22 (BovineHD2200009208), respectively.

Allelic effects of significantly associated SNPs

Alleles raising the proportion of daughters with ACOP were determined for the most significantly associated SNP for each QTL (**Table 1 & Table 2**). **Figure 3** shows the frequency distribution of animals with an increasing number of alleles (from 1 to 16) predisposing to an increased number of progeny with ACOP. The proportion increases nearly linearly with an increasing number of predisposing alleles. Bulls with at least 15 predisposing alleles had > 50% progeny with ACOP while the fraction is <20% for sires with less than seven predisposing alleles.

Variance explained by all SNPs

The genomic relationship matrix, which was also applied for the genome-wide association study, was fitted in a mixed linear model to estimate the proportion of phenotypic variation accounted for by all 658,385 SNPs. To quantify the benefit of a high-density SNP map, the genomic relationship based on only 43,029 SNPs, which were genotyped in all animals, was estimated additionally. The very dense SNP map explains 73.64% of the phenotypic variation (*i.e.*93.22% of the heritability) while the medium-dense map explains 69.97% (*i.e.* 88.56% of the heritability) (**Figure S6**).

Partitioning of the genetic variation

We next built genomic relationship matrices among the 3579 animals for each chromosome separately in order to partitioning the phenotypic variation onto different chromosomes. The proportion of phenotypic variation attributable to a particular chromosome was then estimated with the effects of all chromosomes fitted simultaneously. The contribution of particular chromosomes varies strongly (**Figure 4)**. A major fraction of the phenotypic variation is attributable to BTA5 (10.68%), BTA6 (18.29%) and BTA22 (12.53%), three chromosomes harbouring at least two identified QTL for ACOP. Totally, the seven chromosomes with identified QTL account for 54.13%

of the phenotypic variation. This fraction decreases to 50.82% when the genomic relationship matrices built based on SNPs of the medium-density datasets were fitted (F**igure S6**). There was no significant association between chromosome length (in Mb units) and the proportion of phenotypic variance explained (P=0.22, r^2=0.06) (**Figure S7**).

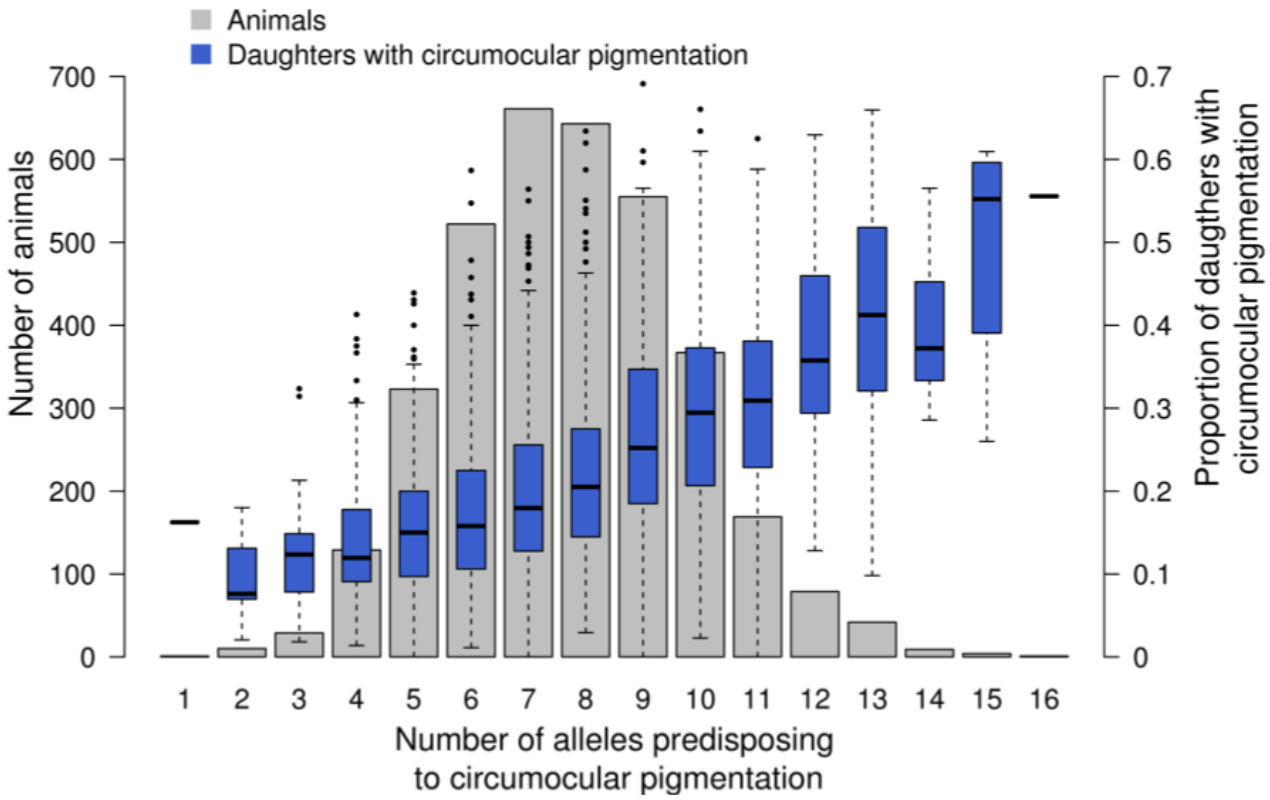

Figure 3. The effect of 14 significantly associated SNPs on the proportion of daughters with ambilateral circumocular pigmentation. 3579 Fleckvieh animals are grouped according to the number of alleles that predispose to ambilateral circumocular pigmentation (ACOP). The blue boxplots represent the proportion of daughters with ACOP for each group. The grey bars indicate the number of sires with an increasing number of predisposing alleles.

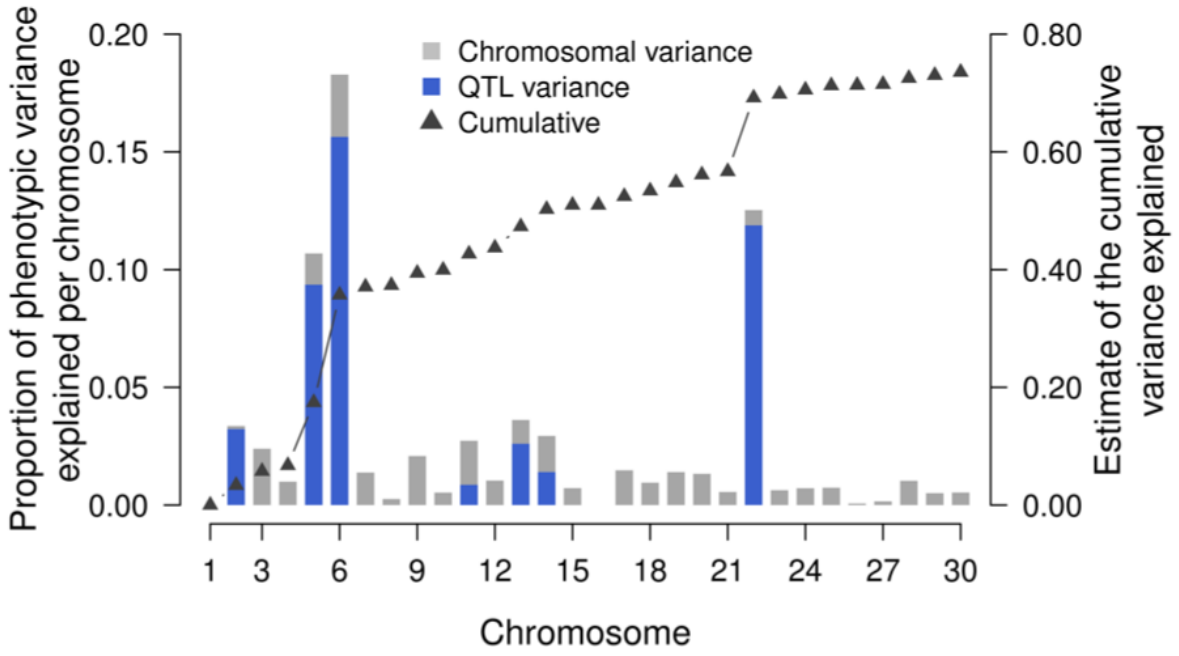

Figure 4. Chromosomal partitioning of the phenotypic variance. The grey and blue bars indicate the fraction of phenotypic variance attributed to a particular chromosome and QTL region, respectively. The triangles represent the cumulative proportion of phenotypic variance attributable to the 30 chromosomes.

Twelve identified QTL regions totally account for 44.96% of the phenotypic variance (*i.e.* 56.91% of the heritability). A major fraction of the phenotypic variance is attributable to the QTL regions encompassing *KITLG* (7.87%), *KIT* (14.56%) and *MITF* (11.33%) on chromosomes 5, 6 and 22, respectively (**Figure 4, Table 3**). While the identified QTL on BTA2 and BTA22 almost account for the entire chromosome variance, the QTL on BTA11 and BTA14 explain only a minor part of the particular chromosome variance. A QTL on BTA22 explains only a marginal fraction of the phenotypic variation.

Table 3: Proportion of phenotypic variance attributable to the twelve identified QTL regions

Chromosome	Candidate gene	QTL-region [Basepairs]	Number of SNPs within the QTL-region	Proportion of phenotypic variance explained [%]
2	*MCM6*	59,128,137 – 64,128,137	1541	1.092
	PAX3	108,706,088 – 113,706,088	1356	2.129
5	*KITLG*	15,706,817 – 20,706,817	1334	7.869
	ERBB3	55,054,914 – 60,054,914	820	1.493
6	*DKK2, LEF1*	16,475,451 – 21,475,451	1501	1.077
	KIT	69,882,208 – 74,882,208	1593	14.560
11	*CRIM1*	16,844,832 – 21,844,832	1514	0.857
13	*ATRN*	49,394,994 – 54,394,994	910	2.598
14	*GSDMC*	9,575,830 – 14,575,830	1384	1.398
22	-	25,431,961 – 30,431,961	1334	0.002
	MITF	29,745,023 – 34,745,023	1356	11.332
	NBEAL2	50,516,253 – 55,516,253	1507	0.548

A 5 Mb interval centred on the most significantly associated SNP was considered as QTL-region. The genomic relationship matrix for each QTL was built based upon SNPs within the 5 Mb interval. The proportion of phenotypic variance explained was then estimated with the effects off all chromosomes and QTL fitted simultaneously.

Discussion

Coat colour phenotypes are routinely recorded for a large number of females, while genotyping is routinely performed in males in the FV breed. To benefit from the large number of cows with phenotypes and the substantial number of bulls with genotypes, the proportion of daughters with ACOP was assessed for a total of 320,186 cows sired by 3579 genotyped artificial insemination bulls. The resulting phenotypes can be considered as breeding values for the bulls. Using pedigree information, the heritability (*i.e.* reliability of the breeding value) for the proportion of daughters with ACOP was estimated as 0.79, which is considerably higher than previous estimates of the heritability for eye-area pigmentation in cattle [44] [17] [45]. A highly heritable phenotype facilitates QTL mapping considerably, especially if the number of genotyped individuals is limited

[46]. Nine of the twelve identified QTL for ACOP account for at least 1% of the trait variance. Although the limited number of genotyped animals restricted the power to detect QTL with small effects, the present study identified three QTL that account for less than 1% of the trait variance.

The coat pigmentation phenotype assessed in this study is a quantitative trait with numerous loci with small effects and few loci with large effects. Twelve identified QTL regions can be considered as the major determinants for ACOP in cattle as they explain 44.96% of the phenotypic variation. The seven chromosomes with identified QTL account for 54.13% of the phenotypic variation. A substantial fraction of the phenotypic variation is attributable to chromosomes without identified QTL. Increasing the number of genotyped animals might enable the detection of additional QTL with minor effect sizes [47], however the number of detectable QTL is limited even in studies with very large sample sizes [48]. The present study nevertheless demonstrates both the leverage potential of progeny phenotyping and the utility of a dense marker map for unravelling the genetic architecture of complex traits in livestock animals. Totally, the 658,385 and 43,029 SNPs accounted for ~93% and ~89% of the heritability. These fractions are distinctly higher than those reported for traits with similar heritability in human genetics [49] [1]. Presumably due to the considerably lower number of independent chromosome segments in cattle, resulting from a small effective population size and concomitant substantial long-range linkage disequilibrium [46]. Our results display that applying dense SNP panels allows to capture most of the genetic variation of complex traits in highly structured livestock populations and thus reduces the *'missing heritability'* [50].

High density genotyping of a minor fraction of the animals of our study population enabled the accurate imputation of ~96% of the genotypes for the remaining animals genotyped at a lower density (Figure S2). This agrees with findings in the American Holstein population [28]. The actual imputation accuracy for the complete study population is supposed to be even higher, since only

half of the animals with high-density genotypes were applied as reference population for the evaluation of imputation accuracy whereas genotypes for the remaining animals were set to missing. Increasing the number of reference individuals with high-density genotypes enables a better resolution of the haplotype structure of the population implicating higher imputation accuracy [51] [52]. Our results demonstrate that the availability of a dense SNP panel and concomitant genotype imputation enables the mapping of QTL for complex traits in livestock populations at a better resolution.

Eight of the twelve identified QTL point to genes, *i.e. MCM6, PAX3, KITLG*, *ERBB3, KIT*, *ATRN*, *MITF* and *NBEAL2,* affecting various coat colour phenotypes in cattle and other species *via* pigment cell genesis and/or pigment formation (*e.g.* [36], [34], and [32] for a review). Interestingly, two of the identified QTL for ACOP in the FV breed are in close vicinity to genes affecting eye morphogenesis during embryonal development, *i.e. DKK2* [37] and *CRIM1* [39]. The QTL on BTA14 points to *GSDMC* (alias *MLZE*) as candidate gene for ACOP in cattle. There are no clues for a direct contribution of *MLZE* to mammalian pigmentation traits or embryonal eye development. However, expression of *MLZE* in growing metastatic melanomas implies a contribution of *MLZE* to melanoma progression [41] [53] and thus possibly to normal melanocyte development.

The identified candidate genes for ACOP interact in a complex fashion, *e.g.* during melanocyte development and melanocyte migration [54],[55]. Two candidate genes for ACOP (*LEF1*, *PAX3*) encode transcription factors regulating the promoter for the BTA22 candidate gene *MITF* [55]. The BTA6 candidate gene *KIT* encodes a transmembrane receptor for the mast cell growth factor encoded by the BTA5 candidate gene *KITLG* [56]. Previous studies evidenced that *KIT* alleles acting in a dominant fashion completely inhibit pigmentation in pigs [57]. A similar mechanism is plausible for eye-area pigmentation in cattle. However, the present study accounts for additive

effects only as the applied phenotype is recorded in the progeny of genotyped artificial insemination bulls. Direct phenotypes for ACOP were not available for the genotyped animals. The proportion of daughters with ACOP is a progeny-derived phenotype for the bulls and acts therefore purely additively. Thus, we cannot dissect non-additive effects, although they are likely to explain a substantial fraction of the genetic variation for complex traits, such as coat colour [58][59]. Assessing phenotypes for ACOP in the genotyped male animals might enable to quantify the extent of non-additive effects. However, the identification of non-additive effects on a genome-wide scale requires large sample sizes and is computationally demanding ([60],[61]). Investigating causal variants directly for non-additive effects overcomes the substantial burden of multiple testing and concomitantly restricts computational costs [62]. However, the present study illustrates the complexity in revealing causal variants in livestock populations. Significantly associated QTL regions might expand over several million base pairs due to extensive linkage disequilibrium (*e.g.* Figure S8), rendering the identification of underlying variants/mechanisms a difficult task. Access to large independent validation populations [63] and comprehensive functional investigations [64], respectively, is indispensable for the fine-mapping of QTL regions in livestock populations.

Pigmentation around the eyes is highly correlated with eye-lid and corneoscleral pigmentation [22]. Corneoscleral pigmentation considerably reduces the susceptibility to bovine infectious keratoconjunctivitis (BIK) [23] and eye cancer (BOSCC) [65]. In the present study, the number of progeny with ACOP increased to > 50% with an increasing number of favourable QTL alleles of the sire. The selection of bulls based on these QTL alleles might rapidly increase the number of progeny with ACOP and thus contribute to reducing the incidence of BIK and BOSCC in areas of increased solar radiation [21]. However, since the twelve identified QTL regions account for 56.9%

of the heritability only, genome-wide evaluation of sires using the entire set of high-density SNPs should allow to most efficiently increase the proportion of progeny with ACOP and thus should reduce the incidence of serious health problems in cattle.

Acknowledgements

The authors acknowledge the Arbeitsgemeinschaft Süddeutscher Rinderzüchter und Besamungsorganisationen e.V. (ASR) and the Förderverein Biologietechnologieforschung e.V. (FBF) for sharing genotype data.

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Supporting information for the

4th Chapter

Supporting Table 1: Number of SNPs not passing the quality control parameters for the medium-density (54K*v1*, 54K*v1*) and the high-density (777K) datasets

Quality parameter	54K*v1*	54K*v2*	777K
SNPs	54,001	54,609	777,962
Individuals	2,545	842	810
SNPs with unknown, Y-chromosomal or Mt-chromosomal position	549	1,075	3,302
SNPs with genotyping rate < 90%	761	256	7,854
SNPs with minor allele frequency < 0.5%	7,579	8,119	112,375
SNPs showing deviation from the Hardy-Weinberg Equilibrium (P<0.001)	704	401	4,578
Individuals with genotyping rate < 90%	6	11	1
Individuals with discrepancies between pedigree and genomic relationship	7	1	7
Duplicate SNPs (identical physical position, distinct SNP-id)	29	30	54
Remaining animals	2,532	830	802
Remaining SNPs	44,759	44,953	653,294

The number of SNPs and animals not passing the applied quality parameters as well as the final number of SNPs and and animals is given for the two medium-density (54K*v1*, 54K*v2*) and for the high-density (777K) dataset, respectively (some SNPs failed for more than one quality control parameter).

Supporting Table 2: Number of SNPs for each of the three datasets after quality control

Dataset	54K*v1*	54K*v2*	777K
54K*v1*	44,759	43,029	40,062
54K*v2*		44,953	40,350
777K			653,294

Numbers along the diagonal represent the final number of SNPs for the two medium-density (54K*v1*, 54K*v2*) and for the high-density (777K) dataset, respectively. Off-diagonal numbers indicate the intersection.

Supporting Table 3: Number of animals for each of the three datasets after quality control

Dataset	54K*v1*	54K*v2*	777K
54K*v1*	2,532	-	521
54K*v2*		830	-
777K			802

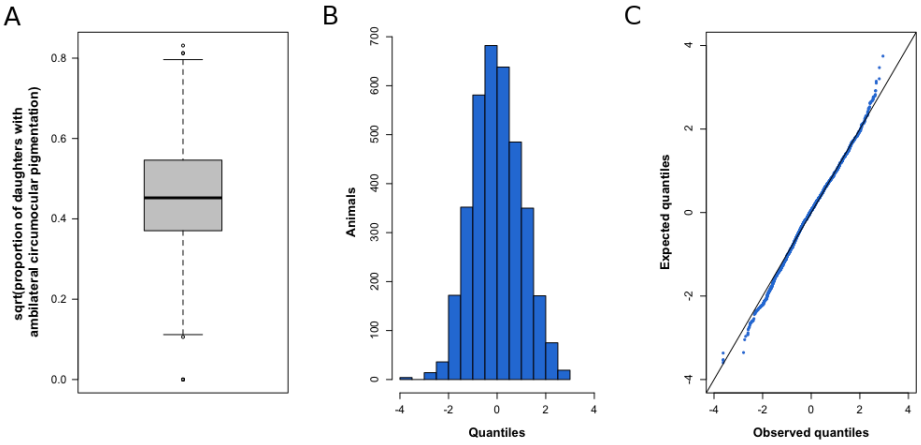
Numbers along the diagonal represent the final number of animals for the two medium-density (54K*v1*, 54K*v2*) and for the high-density (777K) dataset, respectively. Off-diagonal numbers indicate the intersection.

Supporting Table 4: Number of SNPs used for the evaluation of the imputation accuracy

Chromo-some	Chromosome length [Mb]	Number of high-density SNPs	Average distance between two high-density SNPs [bp]	Number of medium-density SNPs	Average distance between two medium-density SNPs [bp]
1	158.34	40,100	3948	2607	60,665
2	137.06	33,962	4024	2070	65,977
3	121.43	30,848	3935	1907	63,520
4	120.83	30,482	3957	1936	62,235
5	121.19	29,777	4069	1648	73,515
6	119.46	30,781	3880	1969	60,474
7	112.64	28,290	3981	1751	64,219
8	113.38	24,935	4546	1812	62,346
9	105.71	26,663	3964	1578	66,877
10	104.3	27,127	3844	1652	62,440
11	107.31	28,544	3758	1691	63,419
12	91.16	22,267	4091	1280	71,106
13	84.24	18,409	4569	1372	61,149
14	84.65	19,369	4339	1418	58,682
15	85.3	21,860	3899	1267	66,809
16	81.72	21,055	3878	1249	65,104
17	75.15	19,808	3793	1223	61,280
18	66	17,556	3751	1008	64,947
19	64.06	17,289	3699	1058	60,115
20	72.04	19,426	3703	1193	60,022
21	71.6	18,336	3903	1056	67,461
22	61.44	16,605	3691	995	61,529

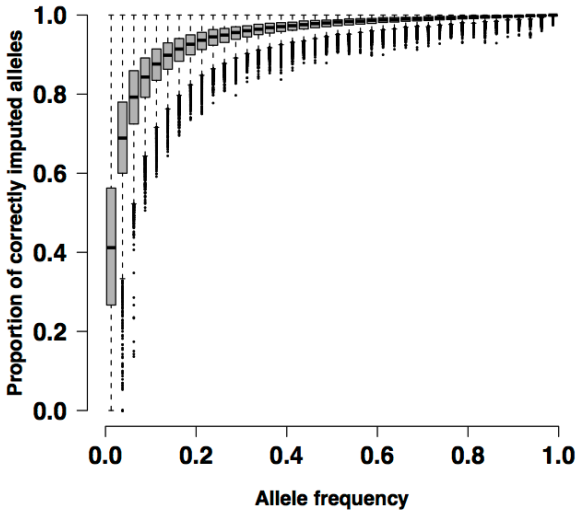
23	52.53	13,751	3815	811	64,475
24	62.71	15,987	3918	973	63,891
25	42.9	11,947	3581	743	57,610
26	51.68	13,932	3707	834	61,168
27	45.41	11,948	3800	735	61,761
28	46.31	11,832	3909	736	62,794
29	51.51	13,302	3864	800	63,958
30	148.82	17,106	8699	690	215,681

Supporting Figure 1: Distribution of the applied phenotype.



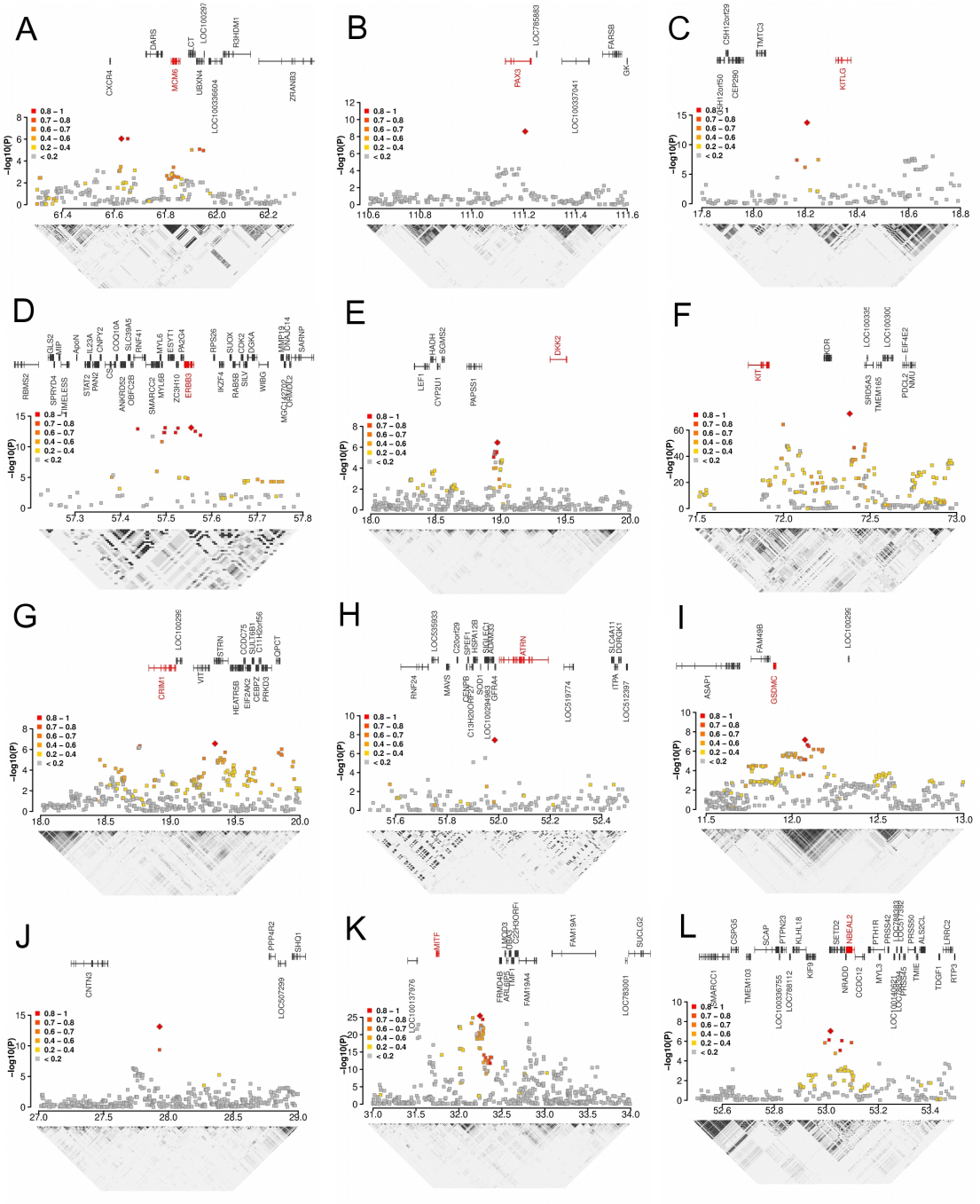
The boxplot (A) and histogram (B) display the distribution of the square root transformed proportion of daughters with ambilateral circumocular pigmentation. The deviation from the expected Gaussian normal distribution is only marginal (C). 66.67%, 95.87% and 99.89% of the values are within one two and three standard deviations, respectively.

Supporting Figure 2: Accuracy of the imputed genotypes.



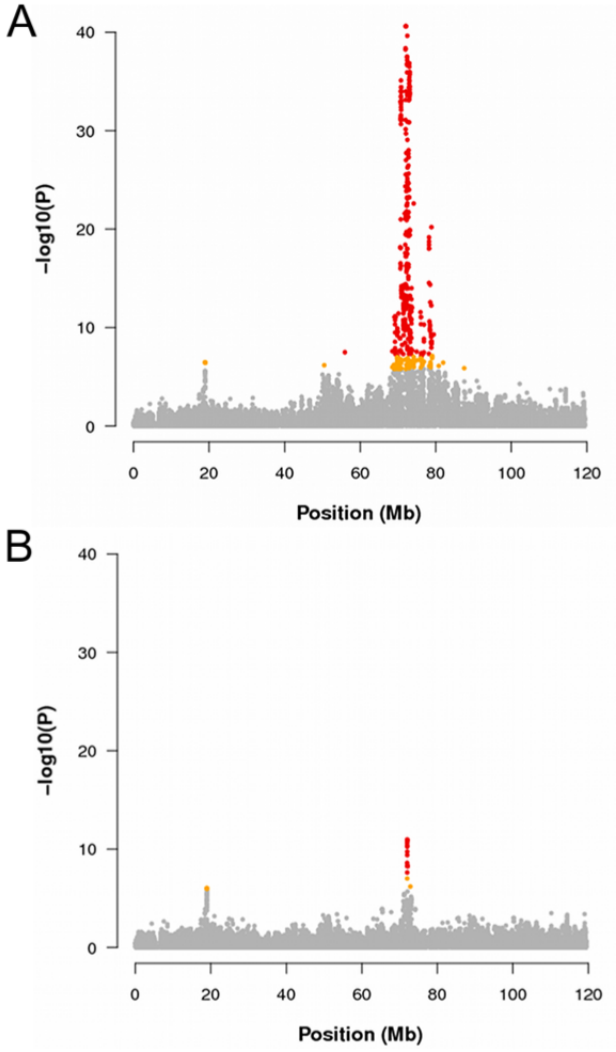
Imputation accuracy was assessed based on genotypes of 613,232 chromosome-wide distributed SNPs of 402 animals. The proportion of correctly imputed alleles is displayed as a function of the allele frequency. The boxplots show the results for allele frequency bins of 2.5%. The concordance between imputed and true allele was poor (41.06%) for rare alleles (*i.e.* alleles with a frequency <2.5%), while imputation of frequent alleles (*i.e.* alleles with a frequency >65%) resulted in an allelic concordance > 99%.

Supporting Figure 3: Detailed overview of the identified QTL



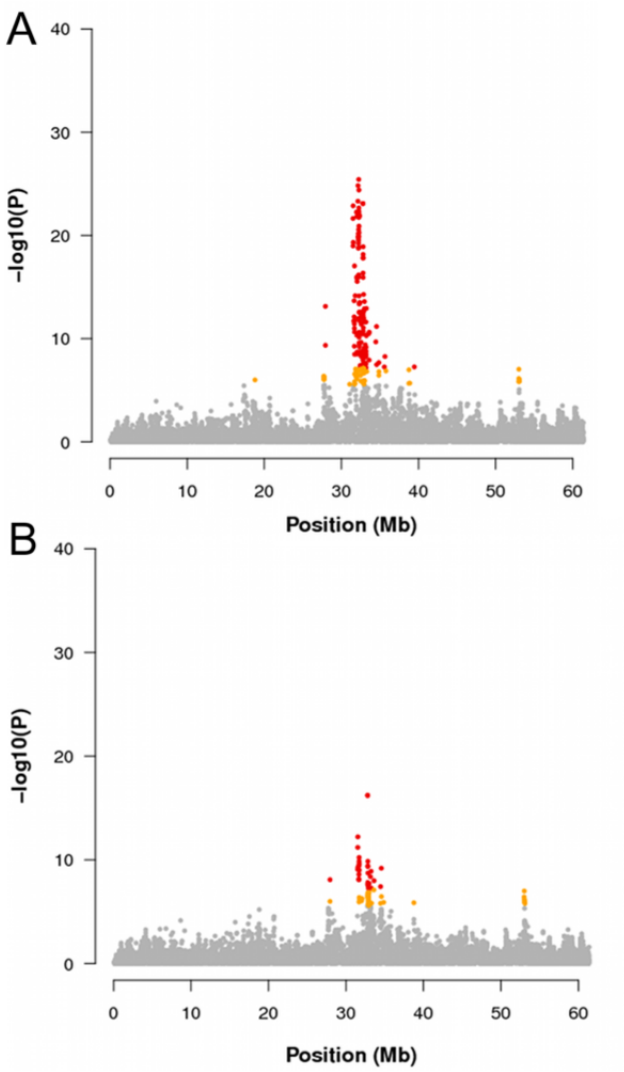
The gene content of the QTL regions on BTA2 (**A,B**), BTA5 (**C, D**), BTA6 (**E, F**), BTA11 (**G**), BTA13 (**H**), BTA14 (**I**) and BTA22 (**J, K, L**) was assessed based on the University of Maryland (UMD3.1) assembly of the bovine genome. Red colour indicates the putative functional candidate gene. The diamond represents the most significantly associated SNP while different colours represent the linkage disequilibrium (r^2) between the most significantly associated SNP and all other SNPs within the displayed region. The heatmap displays the pairwise linkage disequilibrium.

Supporting Figure 4: Association of 30,985 SNPs on chromosome 6 with ambilateral circumocular pigmentation in 3579 animals of the Fleckvieh population.



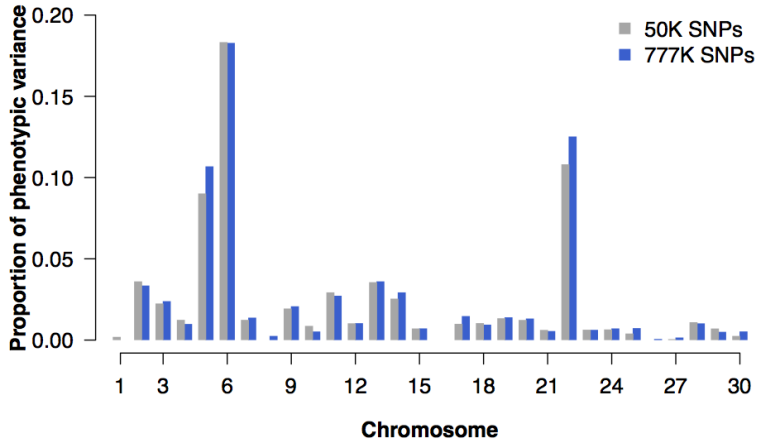
Results for the initial analysis (A) and for the analysis conditional on the *BTB-00263209* SNP (B). Orange dots represent significantly associated SNPs on a chromosome-wide level, red dots represent significantly associated SNPs on a genome-wide level.

Supporting Figure 5: Association of 16,722 SNPs on chromosome 22 with ambilateral circumocular pigmentation in 3579 animals of the Fleckvieh population.



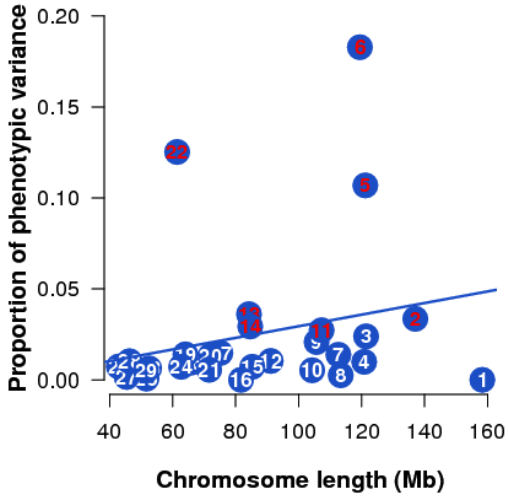
Results for the initial analysis (A) and for the analysis conditional on the *BovineHD2200009208* SNP (B). Orange dots represent significantly associated SNPs on a chromosome-wide scale, red dots represent significantly associated SNPs on a genome-wide scale.

Supporting Figure 6: Effect of different marker densities on the chromosomal partitioning of the genetic variance.



The grey and blue bars indicate the fraction of phenotypic variance attributed to a particular chromosome using the genomic relationship matrices built based on medium-density and high-density SNP information, respectively.

Supporting Figure 7: Correlation between chromosome length and the fraction of phenotypic variance explained.



The estimate of the proportion of phenotypic variance explained by a particular chromosome is displayed as a function of the physical chromosome length (in Mb units). Red numbers indicate chromosomes with identified QTL. The blue line is a linear regression line with slope 3.2 x 10^{-4} (r^2 = 0.06).

5th Chapter

General Discussion

General approach

Genome-wide association studies (GWAS) were performed in the German Fleckvieh (FV) population in an attempt to identify underlying genomic regions for complex traits by using high-density SNP panels. Massive structuring of the study population and the resulting inflation of false positive association signals was accounted for by using principal components analysis and mixed model based approaches, respectively. The utilisation of highly heritable progeny-derived phenotypes facilitated the mapping of 18 quantitative trait loci (QTL) for paternal calving ease, the presence of supernumerary teats and ambilateral circumocular pigmentation in the FV population.

Using progeny-derived phenotypes for genome-wide association analyses

Phenotypes for most economically important traits are recorded in female animals in cattle breeding programs. The genetic value of male animals can be obtained for these traits *via* breeding value estimation [1]. The resulting breeding values are highly heritable as they are assessed based on a large number of progeny records. Georges et al. [2] demonstrated the leverage potential of applying progeny-derived phenotypes for QTL mapping in granddaughter designs. Using highly heritable phenotypes also considerably increases the power to identify QTL in GWAS [3],[4]. In Chapter 2 of the present thesis, the mapping of two QTL affecting calving ease, a trait with low heritability ($h^2 <$ 10% [5],[6]) was facilitated by using estimated breeding values (EBVs) for a comparatively small sample of 1829 artificial insemination bulls of the German FV population. However, using EBVs as phenotypes for GWAS is supposed to cause a substantial inflation of false positive associations as EBVs accumulate family information [7]. A widely-used approach to account for the contribution of relatives is de-regressing the EBVs and weighting the information source appropriately [8]. Nevertheless, daughter yield deviations (DYDs) were claimed to be the phenotypes of choice for QTL studies in pedigreed populations [9],[10]. DYDs reflect the progeny average and are thus not

biased by information of relatives [11]. In order to assess the effect of using different progeny-derived phenotypes, the GWAS for udder clearness (Chapter 3) was repeated with EBVs as phenotypes. However, there was no significant difference in applying either DYDs or EBVs as phenotypes (Figure 1), most likely because of the high reliability of the EBVs for udder clearness ($r^2 = 0.84$). EBVs with high accuracy are appropriate phenotypes for GWAS as the major part of the EBV information results from progeny information and pedigree information contributes less [7]. The average reliability of the EBVs for paternal calving ease (Chapter 2) was even higher ($r^2 = 0.92$). Considering the high reliabilities of the applied EBVs, it seems unlikely that the association signals in the present thesis are inflated due to an accumulation of pedigree information.

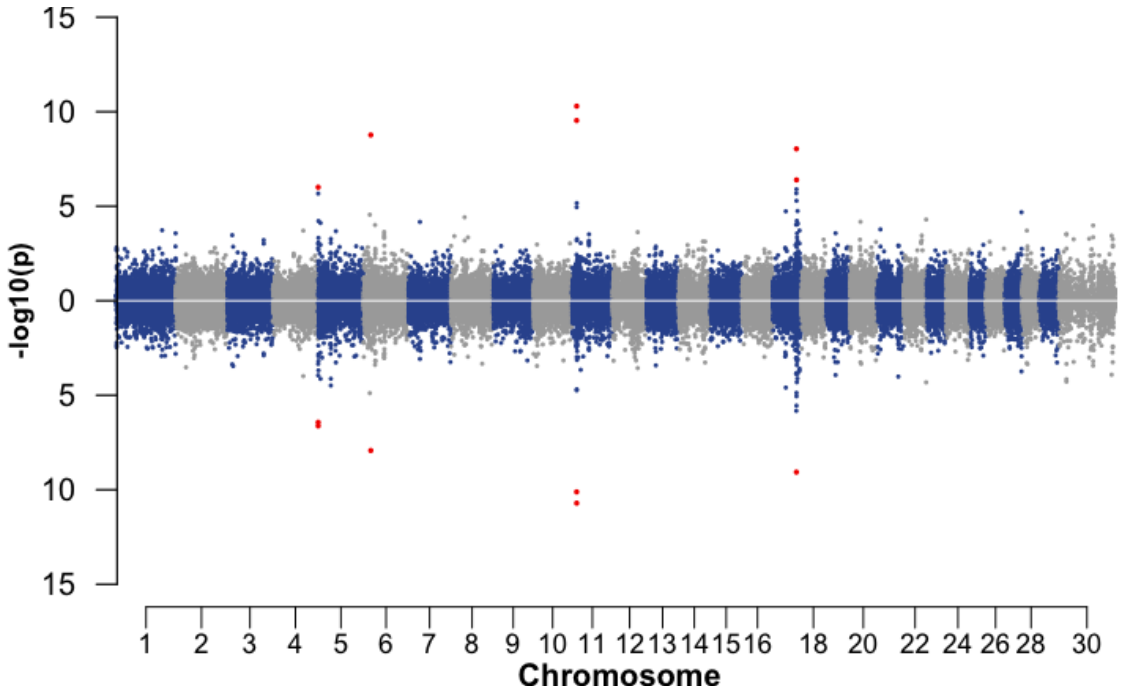


Figure 1: Association of 43,746 SNPs with different progeny-derived phenotypes for udder clearness in 2467 bulls of the German Fleckvieh population. The GWAS was performed using a mixed model based approach to account for population stratification. The results for the scenarios with EBVs and DYDs are shown above and below the horizontal line, respectively. Red symbols represent significantly associated SNPs ($P < 1.14 \times 10^{-6}$).

This thesis provides evidence for the leverage potential of applying progeny-derived phenotypes for the identification of QTL for complex traits in livestock populations. Using EBVs is especially beneficial for the mapping of QTL for functional traits with low heritability such as calving ease (Chapter 2). However, progeny-derived phenotypes act purely additively as they are not directly assessed in the genotyped animals. Therefore, they do not allow to unravel non-additive effects. However, non-additive effects such as epistasis and dominance might substantially contribute to the genetic variation of complex traits [12],[13]. The GWAS in Chapter 4 identified candidate genes for eye-area pigmentation that are well known to interact in a complex fashion, however the data did not allow to quantify the extent of non-additive effects. Access to direct phenotypes and genotyping of female animals, respectively, is indispensable to quantify the extent of non-additive effects for the analysed traits.

Genetic architecture of the analysed traits

The results of the GWAS confirm the infinitesimal model with numerous loci with small effects and only few loci with large effects for three analysed traits [14]. Two, four and twelve major loci were identified for paternal calving ease, the presence of supernumerary teats and the proportion of daughters with ambilateral circumocular pigmentation, respectively. The significantly associated regions explain from 10% to 45% of the respective trait variation and can be considered as the major genetic determinants for the traits in the FV population. Increasing the sample size and applying denser SNP panels might allow for the identification of additional QTL with smaller effects [3], however the number of detectable QTL is even limited in large scale GWAS [15] and depends on the genetic architecture of the trait [16],[17]. In the present thesis, QTL could be identified on chromosomes explaining a large fraction of the trait variation (Figure 2). However, a substantial fraction of the trait variation results from chromosomal regions without identified QTL.

A significant correlation between chromosome length and its explained trait variation was apparent for udder clearness only, while the model fit was poor for paternal calving ease. The linear relationship between chromosome length and its contribution to the genetic variation seems to be more prominent in production traits [18],[19]. However, the approach of partitioning the genetic variation by fitting the effects of all chromosomes separately [20] used in the present thesis has not been applied hitherto in cattle populations, precluding a meaningful comparison with other traits.

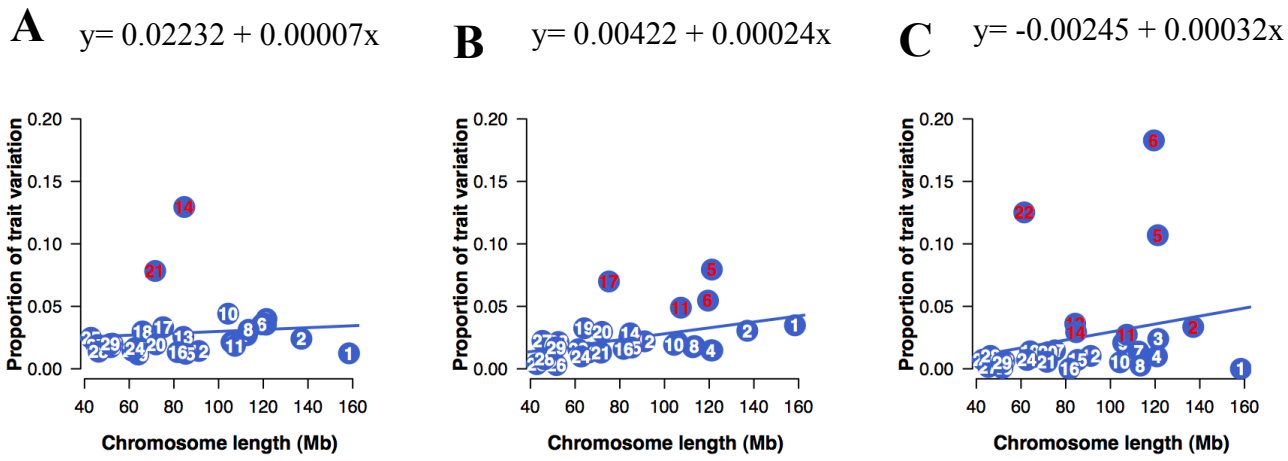


Figure 2: Correlation between chromosome length and the fraction of trait variation explained by a particular chromosome. The proportion of the trait variation explained by a particular chromosome is displayed as a function of its length (in Mb units) for paternal calving ease (A), udder clearness (B) and ambilateral circumocular pigmentation (C). Chromosome-specific relationship matrices were built using totally 658,385 SNPs following VanRaden's approach [48]. The effects of all chromosomes were fitted simultaneously [107]. Red numbers indicate chromosomes with identified QTL. The blue line is a linear regression line. The linear relationship between chromosome length and trait variation explained is only moderate for calving ease (r^2=0.009, P=0.611), udder clearness (r^2=0.165, P=0.029) and ambilateral circumocular pigmentation (r^2=0.055, P=0.219).

The situation is different in humans where the correlation between chromosome length and its explained trait variation is striking, especially for human height, most likely due to a large number of loci with small effects [20]. The results of the present thesis demonstrate that a few loci with large effects (and thus detectable by GWAS) exist for each of the analysed traits in the FV population, which agrees with previous findings (*e.g.* [17]), and which is different from humans, where individual loci often explain a small fraction of the trait variation only (*e.g.* [21],[22]). Several studies attempted to quantify the number of genes underlying complex traits in livestock populations, however the estimates vary greatly and strongly depend on the effective population size [16],[15],[23]. According to Reed et al. [24], it seems very likely that paternal calving ease in particular (which is mainly affected by the size and shape of the calf), is controlled by hundreds of genes with small effects even in heavily selected livestock populations. The distribution of loci with large effects is a major issue for the the appropriate model selection for genomic prediction in cattle populations. Nonlinear methods (*e.g.* BayesB [25]) were shown to clearly outperform 'classical' genomic prediction using GBLUP for traits which are controlled by large-effect genes [23],[26], [27]. Genome-wide association studies offer a powerful tool to gain insights into the genetic architecture of complex traits and concomitantly contribute to explain the suitability of different models for genomic prediction for specific traits.

The benefit of an increasing marker density

Four QTL predisposing to supernumerary teats were identified in a sample of 2467 animals genotyped at 43,698 SNPs (Chapter 3). Increasing the sample size to 3476 animals and applying a high-density panel of 658,385 SNPs did not allow to identify additional QTL (Figure 3), although this design would facilitate to identify QTL accounting for 1% of the trait variation only. The results obtained using the high-density SNP panel are similar to the findings obtained with the medium-

density dataset. This implies that increasing the sample size is more important for QTL detection than increasing the number of SNPs. A number of ~40,000 SNPs seems sufficient for genome-wide analyses in cattle populations [28]. Similar conclusions are drawn in studies considering genomic selection where improving the accuracy of genomic predictions is the most important issue. While enlarging the reference population substantially increases the accuracy of genomic breeding values, applying denser SNP panels results in moderate gain only [29],[30].

The utility of high-density SNP panels for genome-wide analysis of complex traits in cattle has so far been evaluated for the Holstein-Friesian (HF) populations only. However, the characteristics of distinct cattle populations differ considerably [31]. While the effective population size (N_e) for HF is < 100 [32],[33],[34], a N_e of ~140 was estimated for the FV population (see Appendix). N_e is the major determinant for the number of existing independent chromosome segments [23], implying that denser SNP panels are necessary to capture the genetic variation for populations with large N_e. Although increasing the SNP density did not allow for the identification of additional QTL for supernumerary teats, the results presented in Chapter 4 provide evidence for the advantage of applying high-density SNP panels for genome-wide analysis of complex traits in the FV population. The medium-density and the high-density SNP map account for 88.56% and 93.22% of the heritability, respectively. Furthermore, the P-values obtained using the high-density SNP panel are clearly smaller than those obtained using the medium-density SNP panel (Figure 3). These findings provide evidence that the increased density of the 777K-panel allows to pinpoint QTL in cattle populations much more precisely than the 54K-panel. The very dense SNP map enables to capture genetic effects at a better resolution and might result in substantially higher accuracies of genomic breeding values at least in the FV population.

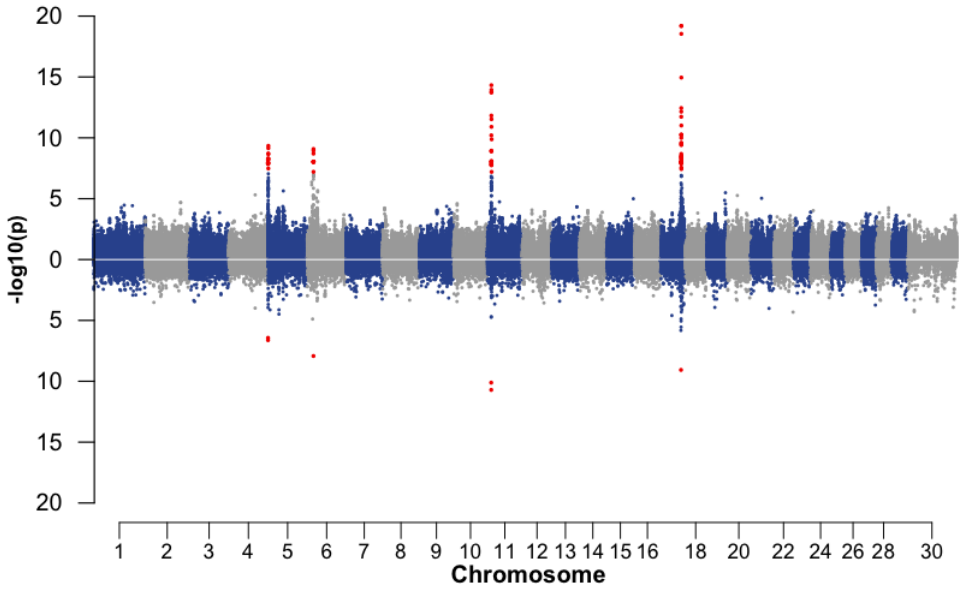


Figure 3: Genome-wide association study for the presence of supernumerary teats in the German Fleckvieh population. The GWAS was performed using a mixed model based approach to account for population stratification and with daughter yield deviations for udder clearness as phenotypes. The results for the high-density dataset comprising 3476 animals and 658,385 SNPs and for the medium density dataset comprising 2467 animals and 43,698 SNPs are shown above and below the horizontal line, respectively. Red symbols represent significantly associated SNPs ($P < 7.59 \times 10^{-8}$ and $P < 1.14 \times 10^{-6}$ respectively).

The phenomenon of the '*missing heritability*'

The phenomenon of the '*missing heritability*' [35] was primarily observed in human genetics where researchers were stunned that high-density SNP panels capture only a fraction of the genetic variation of complex traits. Prototypical for such traits is human height. The heritability of human height is high (h^2~0.8 [36]) and hundreds of variants have been identified so far to contribute to its genetic variation [22],[37],[21]. However, the identified polymorphisms explain only a marginal fraction of the total genetic variation [22],[37],[21]. Furthermore, high-density SNP maps account for ~50% of the heritability of human height only [38]. Similar results were observed for other

complex [20] and mendelian traits [39]. The authors claim that both imperfect linkage disequilibrium (LD) between causal variants and array-based SNPs and rare variants, that are not captured by common genotyping arrays, are the reason for the missing heritability [38]. However, recent investigations also indicate, that genetic interactions might cause '*phantom heritability*', implying an inflation of pedigree-based heritability estimates [40]. The situation in cattle populations seems to be different. Individual loci explain a large fraction of the genetic variation of complex traits (Chapter 4, [17],[41]) and the fraction of genetic variation captured by SNPs is considerably higher in cattle populations than in humans [42],[43],[44]. It is supposed that the number of rare variants contributing to the genetic variation of complex traits is much lower in livestock populations than in humans [43] due to strong artificial selection and a distinctly smaller number of independent chromosomal segments [23]. Goddard and Hayes [45] showed that the number of detectable QTL (QTL with moderate to large effects) remains unchanged while the number of QTL with small effect size decreases considerably under artificial selection. The findings of this thesis support this hypothesis, as a small number of QTL with medium to large effects was identified for each of the analysed traits. The missing heritability for ambilateral circumocular pigmentation was estimated to be only 7% in the FV population when using high-density SNP panels (Chapter 4). The results of the present thesis imply that applying denser SNP panels is beneficial in the FV population (Table 1) which agrees with previous simulation experiments in cattle [46]. However, investigations in humans and model organisms indicate, that increasing the number of SNPs above a certain density does not increase the estimates of the genomic heritability [38],[47], which, again, can be explained by a large N_e and thus a larger number of rare variants contributing to the genetic variation of complex traits in these species.

Table 1: Heritability estimates for three complex traits in the FV population

	NRM	GRM (45K)	GRM (658K)
Ambilateral circumocular pigmentation	0.796	0.699	0.736
Udder clearness (EBV)	0.976	0.694	0.725
Udder clearness (DYD)	0.598	0.560	0.585
Paternal calving ease	0.985	0.814	0.844

The heritabilities were estimated by fitting either the pedigree-based relationship matrix (NRM) or the genomic relationship matrix (GRM) in a random effect model. The relationship matrices were built for 3645 artificial insemination bulls of the FV population. The pedigree relationship was built tracing back pedigree information to 1920. The genomic relationship matrices were built following VanRaden's approach [48] and were based upon medium-density (~45K) and high-density (~658K) SNP information.

Using high-density SNP panels for the identification of mendelian traits in cattle

Besides the genome-wide analysis of complex traits and concomitant genomic evaluation of populations, high-density SNP panels offer an invaluable tool for the identification of mendelian disorders in livestock species [49],[50]. As individual artificial insemination bulls generate tens of thousands of progeny, deleterious alleles may accumulate within few generations and sustainably endanger existing breeding schemes [51],[52]. The availability of high-density genotype information facilitates the systematic identification of segments of extended homozygosity (*i.e.* autozygosity) in affected animals (see Appendix). However, very dense SNP panels are required to detect autozygous segments when a large number of generations separates the affected animals from the founder of the mutation [53],[54]. Thus, the power to pinpoint chromosomal segments underlying emerging congenital defects is considerably increased by using the recently introduced high-density SNP panels comprising more than 650,000 SNPs.

Biological relevance of the identified QTL

The present study identified QTL for three breeding objectives of the FV breeding program. Calving ease is a major breeding objective in cattle populations as calving difficulties (dystocia) are often accompanied with calf losses and thus substantially compromise animal and economical welfare. Furthermore, dystocia seriously compromises milk production and reproductive performance of pertinently affected cows [55],[56],[57],[58]. The present study provides evidence that two major QTL on BTA14 and BTA21 explain a large fraction of the genetic variation of calving difficulties most likely due to enhancing fetal growth. Selection of animals carrying favourable QTL alleles might rapidly reduce the incidence of calving difficulties. However, as the two QTL also explain a substantial fraction of the genetic variation of daily gain and body size, such selection is likely to counteract postnatal growth parameters. Nevertheless, reducing calving difficulties and concomitantly enhancing postnatal growth parameters is possible in cattle populations [59]. A polymorphism ablating a polyadenylation signal of the gene encoding the ribosomal protein S20 (*RPS20*) was supposed to be the underlying QTN for the BTA14 QTL. In the meantime Karim et al. [60] and Littlejohn et al. [61] provided evidence that variants modulating the expression of *PLAG1*, an adjacent gene of *RPS20*, are likely to be the underlying polymorphisms for the growth QTL on BTA14. However, the final proof of causality remains left.

The QTL affecting the development of supernumerary teats (SNTs) pinpoint genes of the highly conserved Wnt signalling pathway. The present study confirms the proposed oligogenic inheritance pattern of SNTs in cattle [62], although the presence of supernumerary nipples/teats has been attributed to different modes of monogenic inheritance in other mammals [63],[64],[65]. The incidence of supernumerary nipples in humans was hitherto exclusively attributed to variations within *TBX3* [66],[67],[68], a gene that was also identified to be associated with SNTs in the present study. Despite the high frequency of supernumerary nipples in humans [69], the identification of

underlying variants was performed in small-sized family designs only. GWAS have not been performed yet. As supernumerary features of the mammary line appear from rudimentary buds to fully developed lactating glands (polymastia) [70], it seems plausible that variation within one gene (*e.g. TBX3*) is required for the development of SNTs while polymorphisms within distinct genes (*e.g. EXOC1*, *LEF1*) determine the shape of SNTs [71]. Accessory mammary tissue accompanies with an increased incidence of urogenital malformations and urogenital cancer in humans [72],[73]. Although the number of investigations is limited, there is evidence for a correlation between the presence of SNTs and fertility traits in cattle [74]. Reducing the incidence of SNTs might thus increase the reproductive performance in cattle, however additional investigations are necessary to confirm this hypothesis. While SNTs in cattle negatively affect milking ability and act as bacteria reservoir [75],[76], teat number and teat morphology are important reproduction traits in swine breeding programs [77]. Several QTL have been identified for teat number and teat malformations in swine [78],[79]. It seems likely that the QTL identified in the present study determine both the teat number and the teat morphology even in other species than cattle.

Chapter 4 reports the first GWAS in cattle based on a high-density map of >650,000 SNPs. The mapping of twelve QTL regions affecting a special aspect of skin pigmentation was facilitated by introducing progeny-derived phenotypes for the bulls. The results confirm that ambilateral circumocular pigmentation (ACOP) is highly heritable in cattle [80]. Applying rather progeny-derived than direct phenotypes for the genotyped bulls even increased the heritability to 79%. ACOP considerably reduces the incidence of serious eye diseases in areas where animals are exposed to increased solar UV radiation [80],[81],[82]. Although the twelve identified QTL regions already account for 56.91% of the heritability, applying the entire high-density SNP panel allows to most efficiently select for animals with ACOP. Currently, EBVs for ACOP are not available for the German FV population. However, especially South African cattle breeding organisations demand

animals with eye-area pigmentation (http://www.wsff.info). Breeding values for ACOP are likely to increase the attractiveness of the FV breed and might contribute to enlarge the FV population beyond Europe. The present study certainly demonstrates the leverage potential of applying progeny-derived phenotypes for efficient selection of animals with eye-area pigmentation.

Quality of the current bovine genome build

The chromosomal position of the SNPs as well as the gene content of associated genomic regions was determined based on the UMD3.1 assembly of the bovine genome [83]. However, as the number of re-sequenced animals is still limited, the current assembly relies on sequence information of a single animal and still contains a significant number of gaps [31]. Thus, the current assembly must still be considered as a draft version. For instance, the lack of detailed knowledge about the genomic organization of the proximal region of BTA21 precluded to further dissect one of the QTLs for paternal calving ease in the present study (Chapter 2). Significant improvements of the bovine assembly are expected in the near future due to numerous finished and ongoing bovine sequencing projects [84],[85],[86],[87]. Furthermore, the applied SNP panels contain both a substantial number of SNPs with undetermined position and a significant number of misplaced SNPs [88],[89] which are uninformative for GWAS. For the high-density dataset, 5039 (0.65%) misplaced SNPs were identified (see Appendix). Although incorrectly placed SNPs are particularly obstructive for haplotype-based analysis such as homozygosity mapping and the identification of selective sweeps [90], the position of significantly associated SNPs should also be validated in GWAS to avoid misinterpretation of significant associations [91].

Reproducibility of the associations

The replication of significantly associated genomic regions in independent populations is crucial to validate identified QTL [92]. However, some phenotype patterns are present in few cattle breeds only (*e.g.* eye-area pigmentation), rendering the access to large validation sets a difficult task. Dividing the mapping population into reference and validation population overcomes this issue [93], albeit at the cost of loosing power to detect association. The present study used progeny-derived phenotypes (EBVs, DYDs), as the recording of direct phenotypes for a large number of animals was not feasible. The replication of association signals resulting from GWAS using EBVs requires similar definitions of EBVs in the discovery and the validation population. However, underlying models for breeding value estimation differ between cattle breeds. Associations in validation populations might be biased when the used phenotypes differ only slightly [94]. This issue is especially apparent considering breeding values for calving traits, where evaluation models differ substantially among cattle breeds [5]. Two QTL explaining a large fraction of the genetic variation of paternal calving ease and growth related traits were identified on BTA14 and BTA21 in the FV population (Chapter 2). However, neither the QTL on BTA14 nor the QTL on BTA21 was identified in a replication study with 2339 HF animals genotyped at 44,245 SNPs (Figure 4). The GWAS in HF revealed strong association at the distal region of BTA18 (position of the most significantly associated SNP (UMD3.1-assembly): 56,561,695 bp) which agrees with the findings of Cole et al. [95]. Neither the GWAS performed in this thesis nor GWAS in the American HF population [95],[96] provide evidence for the presence of a QTL on BTA14 in the HF population. However, in the meantime the QTL for birth weight and growth related traits on BTA14 was validated in numerous independent studies [60],[61],[97],[98]. Several explanations are plausible for the lack of association in the HF population. On the one hand there is the possibility that the QTL is not segregating in the HF population. However, Karim et al. [60] showed that the QTL on

BTA14 indeed segregates in the HF population at high frequency (0.78). On the other hand it seems likely that the QTL accounts for a small fraction of the genetic variation only, requiring larger sample sizes for its detection. However, there is evidence that the QTL accounts for a comparatively large fraction of the genetic variation of growth-related traits in HF [60] and thus should be detectable in the sample consisting of 2339 HF animals. Using high-heritability phenotypes (such as EBVs) should allow to detect QTL accounting for at least 3% of the trait variation in a sample of 2339 genotyped animals (see Chapter 2). Most likely, the QTL remained undetected in the HF sample because of the rather indirect phenotype 'paternal calving ease'. As EBVs for paternal calving traits mainly reflect fetal growth, birth weight would be a more precise phenotype to identify QTL for fetal growth. The heritability for birth weight is considerably higher than for calving traits [99]. However, data collecting for birth weight is expensive and time consuming and thus not standardised in cattle breeding programs. Additionally, Seidenspinner et al. [5] pointed out that parity-specific phenotypes are preferable for the mapping of calving traits in cattle, whereas EBVs for paternal calving traits are currently estimated across parities in the German HF population (http://www.vit.de).

In summary, these findings show that precisely defined phenotypes are indispensable for the replication of association signals in validation populations which is also emphasized by Barendse [94]. Furthermore, this is evidence that QTL explaining a large fraction of the trait variation might remain undetected in validation populations without well-defined phenotypes. Although the replication of associations in validation populations is currently considered as the gold standard to define true variants [92], an unsuccessful replication study should not be considered as *the* criterion for excluding potentially functional variants. Nevertheless, fine mapping of QTL regions and subsequent identification of underlying genetic variants requires independent validation populations to distinguish between true and linked polymorphisms [100].

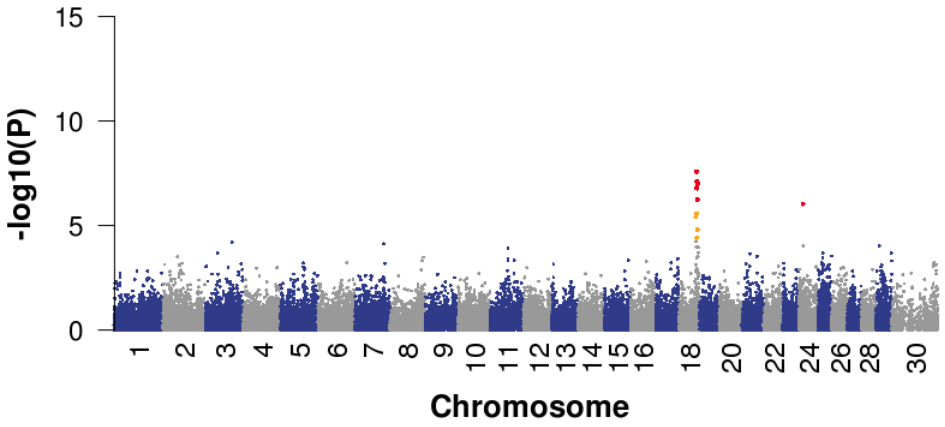


Figure 4: Genome-wide association study for paternal calving ease in the German Holstein-Friesian population. After stringent quality control, 2339 animals and 44,245 SNPs were used for the association analysis. The GWAS was performed using a mixed model based approach to account for population stratification and with estimated breeding values for paternal calving ease as phenotypes. Red symbols represent genome-wide significantly associated SNPs ($P < 1.13 \times 10^{-6}$).

Impacts for practical animal breeding

The integration of underlying genetic variants in breeding programs (*i.e.* marker assisted selection, MAS) enhances genetic gain, especially for low-heritability and heavily accessible traits [101], [102]. Thus, MAS based on the identified QTL for calving ease might add a valuable tool to reduce the incidence of dystocia in cattle. However, the present study provides markers in LD with the QTN only. Addressing the QTN directly would be more efficient and persistent [103],[102]. The success of MAS mainly depends on the fraction of genetic variation explained by the markers. According to Meuwissen and van Arendonk [104], MAS based on two markers explaining approximately 10% of the trait variation should increase genetic gain by 4% in conventional progeny-testing breeding schemes. However, the concept of MAS appears obsolete in the era of genomic selection where genotype information of tens of thousands of SNPs is considered simultaneously to assess the genetic value of individuals [25]. The results in Chapter 4 demonstrate that twelve major QTL indeed account for 56.91% of the heritability, but a substantial fraction of

the heritability is attributable to anonymous SNPs. Genome-wide evaluation of populations utilizes both QTL variation as well as polygenic variation and thus results in higher genetic gain than MAS. Nevertheless, as the reliabilities of genomic breeding values depend on the heritability of the trait [105], the integration of QTN-information in genomic breeding schemes might add a valuable tool to increase the reliabilities for functional traits. Furthermore, using highly informative markers for MAS might be especially useful for improving small livestock populations where a limited number of animals precludes efficient genomic selection [27],[106].

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6th Chapter

Outlook

Outlook

This thesis shows the leverage potential of applying high-density SNP panels for the identification of quantitative trait loci (QTL) in cattle. However, significantly associated genomic regions often extend to several million base pairs due to the small effective population size and concomitant high linkage disequilibrium (LD) in livestock populations. The identified regions often contain numerous functional candidate genes, rendering the identification of the underlying variant(s) a difficult task. Furthermore, causal variants affecting regulatory elements may be located very distantly from the regulated gene, complicating theirs identification considerably [1]. Future genome-wide association studies (GWAS) will exploit whole-genome sequence information and subsequent genotype imputation for the identification of quantitative trait nucleotides (QTN) for complex traits. Re-sequencing a small number of key animals enables to assess a large fraction of the global genomic variation of livestock populations [2],[3]. The subsequent population-wide imputation of whole-genome sequence information of a small number of highly informative individuals is feasible and provides highly reliable genotypes [2],[4] (see Appendix). Using whole-genome sequence information considerably increases the power of GWAS as “true” QTN can be tested directly for association rather than anonymous SNPs in LD with QTN only (Figure 1, Appendix). However, adjacent potential functional variants are often in complete LD, precluding the final proof of causality even after applying a large number of functional investigations [5]. Furthermore, several polymorphic sites might accumulate in form of a composite QTL, rendering the dissection of the underlying genetic mechanisms still a difficult task [6],[7]. Access to large validation populations will be an indispensable prerequisite to differentiate between 'true' and linked effects. The direct investigation and validation of functional candidate QTNs in independent cattle populations will become feasible in the near future by exploiting the large amount of sequence data generated in ongoing collaborations (such as the 1000 bull genomes project).

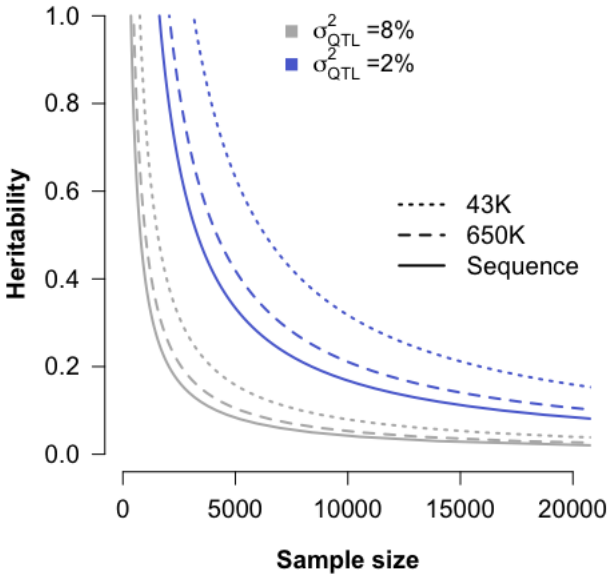


Figure 1: Sample size required to identify a QTL for traits with different heritability. The sample size was calculated based on the formula presented by Goddard and Hayes [11]. The linkage disequilibrium (r^2) between marker and QTN was assumed to be 0.35, 0.65 and 1 for the 43K, the 650K and the sequence data set.

Whole-genome re-sequencing of 43 key animals of the Fleckvieh population [3] and subsequent population-scale imputation using a combination of *Beagle* [8] and *Minimac* [9] enabled to extrapolate the entire sequence information for 3579 animals *via* medium and high-density genotypes. Pre-phasing-based sequence imputation provides highly reliable genotypes even for a small number of animals with high-density genotypes [4]. Thus imputed >12 Mio SNPs facilitated the fine-mapping of a QTL for ambilateral circumocular pigmentation on BTA6 (Figure 2) and furthermore enabled to identify an additional QTL for udder clearness which remained undetected using array-based SNPs only (Figure 3). However the implications for genome-wide analyses of cattle populations by exploiting population-wide sequence information cannot be assessed at present. The realisation of the full potential and the management of this huge amount of data will become a major task for the upcoming years.

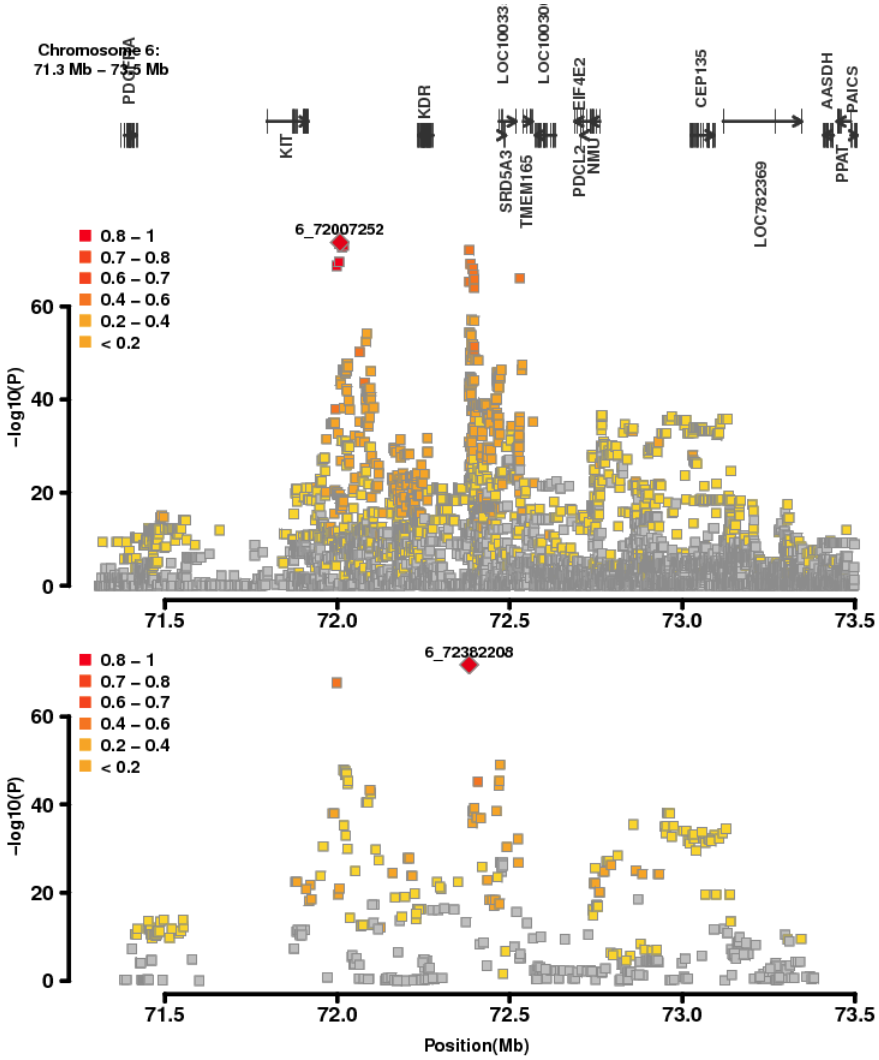
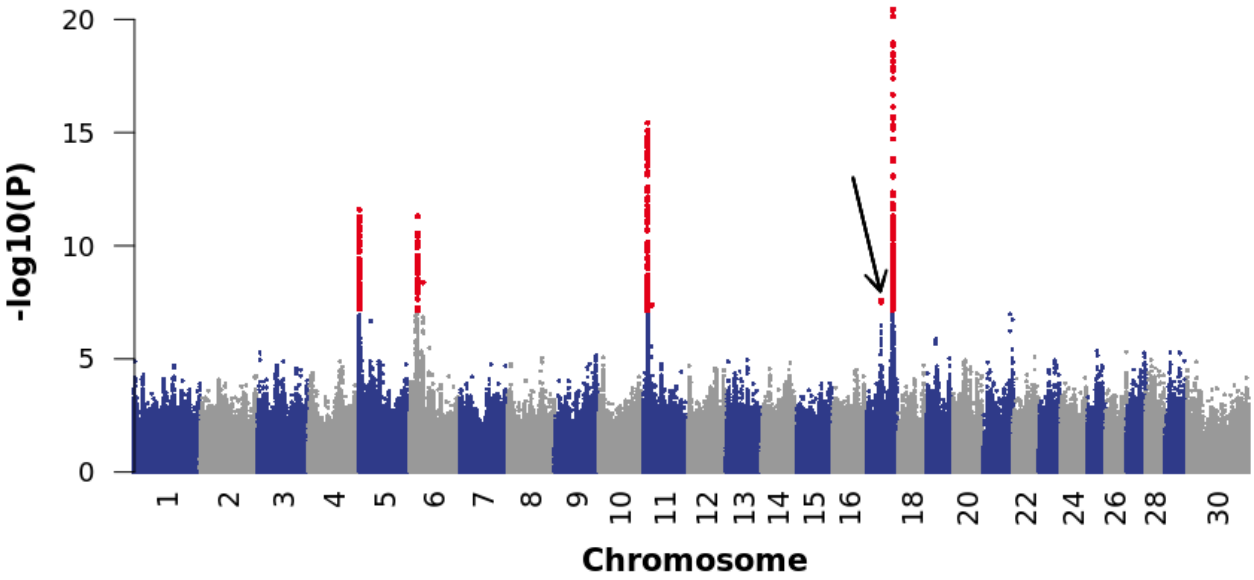


Figure 2: Detailed view of the QTL for ambilateral circumocular pigmentation on chromosome 6 identified by the genome-wide association study in Chapter 4. Whole-genome re-sequencing of 43 key animals resulted in the detection of 8492 SNPs for the BTA6 QTL region (71.4 Mb – 73.5 Mb). These SNPs were imputed for 3579 Fleckvieh bulls applying a combination of *Beagle* [8] and *Minimac [9].* The bottom plot presents the results of the association study using 777K-genotypes only, the top plot presents the results for the association study exploiting whole-genome sequence information. The GWAS indicates the presence of two approximately 0.5 Mb distant albeit linked regions (see also Chapter 4). Remarkably, both association signals result from intergenic regions and indicate regulatory sites as underlying variants. Different colour indicates the linkage disequilibrium (r^2) with the most significantly associated SNP. SNPs and genes were annotated based on the UMD3.1 assembly of the bovine genome [10].



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Summary

The development of high-density single nucleotide polymorphisms (SNP) panels enables to interrogate genotypes for a large number of polymorphic sites simultaneously. Large-scale genotyping of a substantial number of individuals provides comprehensive insights into the genetic variability between as well as within populations. Genome-wide association studies based on genome-wide SNP panels offer a new powerful approach to identify genomic regions underlying phenotypic variation (QTL) of complex traits. Complex traits are determined by a limited number of loci explaining a large fraction of the genetic variation and a large number of of loci with infinitesimal effects. The present study identified two, four and twelve QTL regions for paternal calving ease, the development of supernumerary teats and for eye-are pigmentation in the highly structured German Fleckvieh population.

In chapter 2 the mapping of two QTL for calving traits on Bos taurus autosome (BTA) 14 and 21 ($P = 5.72 \times 10^{-15}$ and $P = 2.27 \times 10^{-8}$, respectively) is reported after careful correction for population stratification. Calving traits are complex as they are influenced by sire-effects through the size of the calf as well as by dam-effects consisting mainly of the pelvic dimensions. The mapping was facilitated by applying breeding values for paternal calving ease to compensate the low heritability of calving traits. A genome-wide association study was performed using a principal components based approach to account for population stratification. Genotypes of 43,863 SNPs were available for 1800 bulls of the German Fleckvieh population. The two identified QTL affect both fetal and postnatal growth parameters. The two loci explain at least 10% of the genetic variation of calving difficulties in the Fleckvieh population.

In chapter 3, the mapping of QTL predisposing to supernumerary teats based on progeny-derived phenotypes for udder clearness is reported. A principal components based and a mixed model based

approach to account for population stratification were compared using real-world data. The study population consisted of 2467 bulls genotyped for 43,698 SNPs. Four QTL on BTA5, BTA6, BTA11 and BTA17 were identified. The four QTL explain a substantial fraction of the genetic variation of supernumerary teats. However, a large fraction of the genetic variation results from chromosomes without identified QTL. The findings confirm the proposed polygenic inheritance pattern of supernumerary teats in cattle. The four QTL pinpoint genes of the highly conserved Wnt-signalling pathway as the major genetic determinants for teat malformations in cattle.

Chapter 4 reports a genome-wide association study for a special aspect of skin pigmentation in cattle. The study population consisted of 3579 bulls of the FV breed with a total of 320,186 progeny with phenotypes for ACOP. The proportion of daughters with ambilateral circumocular pigmentation was applied as phenotype with high heritability ($h^2 = 0.79$). Genotypes were available for 658,385 SNPs. Twelve genomic regions were highly significantly associated. The identified QTL point to *MCM6*, *PAX3*, *ERBB3*, *KITLG*, *LEF1*, *DKK2*, *KIT*, *CRIM1*, *ATRN*, *GSDMC*, *MITF* and *NBEAL2* as underlying genes for eye area pigmentation in cattle. The twelve QTL regions explain 56.91% of the heritability, while the entire SNP map accounts for 93.22% of the heritability. The results support a polygenic inheritance pattern of eye-area pigmentation in cattle and provide the basis for efficient genomic selection of animals that are less susceptible to serious eye diseases.

Zusammenfassung

Die Entwicklung hoch-dichter SNP Arrays ermöglicht die Abfrage einer großen Anzahl von Genotypen für polymorphe Positionen im Hochdurchsatz-Verfahren. Die Genotypisierung einer umfangreichen Anzahl an Individuen liefert umfassende Erkenntnisse über die genetische Diversität innerhalb und zwischen Populationen. Die Verwendung von hoch-dichter SNP Information für genomweite Assoziationsstudien ist ein schlagkräftiger Ansatz zur Identifizierung genomischer Regionen welche die phänotypische Ausprägung komplexer Merkmale maßgeblich beeinflussen (Quantitative trait loci, QTL). Die genetische Architektur komplexer Merkmale wird durch eine kleine Anzahl an QTL mit großen Effekten und eine große Anzahl an QTL mit infinitesimal kleinen Effekten bestimmt. Die vorliegende Arbeit beschreibt die Identifikation von insgesamt 18 QTL für den paternalen Geburtsverlauf, die Euterreinheit und die beidseitige Augenpigmentierung beim Fleckvieh.

In Kapitel 2 wird über die Kartierung von zwei QTL für den paternalen Kalbeverlauf beim Fleckvieh auf Chromosom 14 und Chromosom 21 ($P = 5.72 \times 10^{-15}$ and $P = 2.27 \times 10^{-8}$) berichtet. Die Merkmale des Kalbeverlaufs werden sowohl durch paternale Effekte (die Größe des Kalbes) als auch durch maternale Effekte (Größe und Form des Geburtskanals) beeinflusst. Erst die Verwendung von sicher geschätzten Zuchtwerten für den paternalen Kalbeverlauf kompensierte die niedrigen Heritabilitäten für Kalbemerkmale und ermöglichte die Kartierung der beiden QTL. Der Datensatz bestand aus 1800 Bullen mit Genotypen für 43,698 SNPs. Die Populationsstruktur wurde über einen Haupkomponenten-basierten Ansatz berücksichtigt. Die beiden QTL beeinflussen sowohl prä- als auch postnatale Wachstumsparameter beim Rind und erklären mindestens 10% der genetischen Variation für Kalbeschwierigkeiten in der Fleckvieh Population.

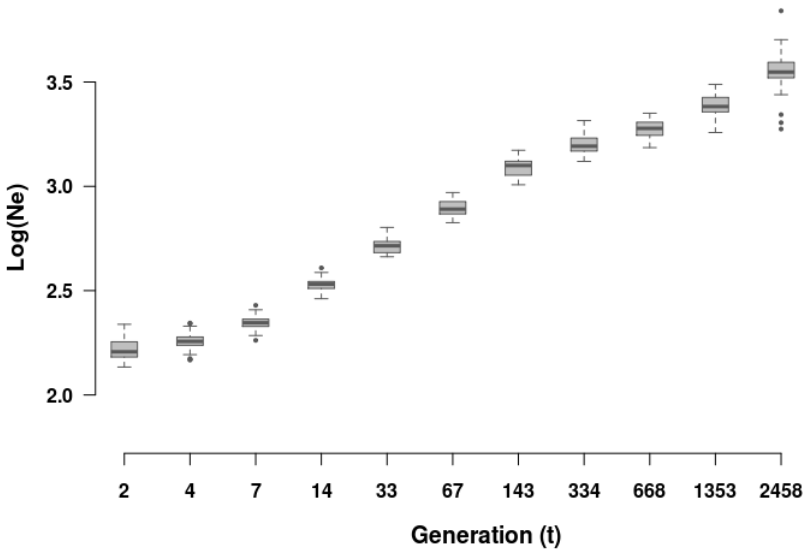
Kapitel 3 beschreibt die Kartierung von QTL für die Entwicklung überzähliger Zitzen unter Verwendung von nachkommen-basierten Phänotypen. In einem realen Datensatz wurde ein Hauptkomponenten-basierter mit einem auf einem gemischten Modell beruhenden Ansatz zur Berücksichtigung von Populationsstruktur verglichen. Der Datensatz umfasste 2467 Bullen die an 43,698 SNPs genotypisiert wurden. Vier QTL auf BTA5, BTA6, BTA11 und BTA17 konnten identifiziert werden. Die vier QTL erklären einen beträchtlichen Anteil der genetischen Variation für die Ausprägung überzähliger Zitzen beim Fleckvieh. Jedoch resultiert ein Großteil der genetischen Variation von Chromosomen, für die keine QTL identifiziert werden konnten. Die Ergebnisse bestätigen die Vermutung dass es sich bei der Euterreinheit beim Rind um ein polygenes Merkmal handelt. Gene des hoch konservierten Wnt-Signalwegs konnten als Haupt-Einflussfaktoren für Zitzenmissbildungen beim Rind ausgemacht werden.

In Kapitel 4 wurde eine genomweite Assoziationsstudie für eine besondere Erscheinungsform der Fellpigmentierung durchgeführt. Die analysierte Population bestand aus 3579 Bullen von denen insgesamt 320,186 Nachkommen hinsichtlich der beidseitigen Augenpigmentierung phänotypisiert waren. Der Anteil an Töchtern mit beidseitiger Augenpigmentierung wurde als Phänotyp mit hoher Heritabilität ($h^2 = 0.79$) für die Bullen verwendet. Hoch-dichte Genotypen für 658,385 SNPs waren verfügbar. Insgesamt konnten zwölf signifikant assoziierte QTL identifiziert werden. In unmittelbarer Nähe zu den QTL liegen *MCM6*, *PAX3*, *ERBB3*, *KITLG*, *LEF1*, *DKK2*, *KIT*, *CRIM1*, *ATRN*, *GSDMC*, *MITF* und *NBEAL2*. Die zwölf QTL erklären 56.91% der Heritabilität, jedoch erklären die gesamten SNPs insgesamt 93.22% der Erblichkeit. Die Ergebnisse lassen vermuten, dass es sich bei der beidseitigen Augenpigmentierung um eine polygenes Merkmal handelt und dienen als Basis für die effiziente genomische Selektion von weniger anfälligen Tieren für schwerwiegende Augenerkrankungen.

Appendices

1. Estimation of the effective population size

These results are part of the following publication:
Pausch H, Aigner B, Emmerling R, Edel C, Götz K-U, Fries R (2012) Imputation of high-density genotypes in the Fleckvieh population. submitted to Genetics Selection Evolution



A maximum effective population size (Ne) of 3529 was estimated at ~2500 generations ago. However, the Ne decreased considerably within the last generations. While the past Ne was estimated to ~340, ~222 and ~181 for 14, 7 and 4 generations ago, respectively, the past Ne ~2 generations ago was estimated to 161 (±22.8) only. Individual estimates for the 29 autosomes range from 136 (BTA14) to 218 (BTA29) for ~2 generations ago.

The past effective population size (N_e) was estimated based on a comprehensive dataset comprising 2842 FV bulls born between 1998 and 2005 genotyped with the Illumina BovineSNP 50K Bead chip. After stringent quality control genotypes for 42,237 autosomal SNPs were considered. The marker-based N_e was estimated using $r^2 = \frac{1}{4 N_e c + 1} + e$ ((Sved 1971),(Tenesa et al. 2007)), where r^2 is the pairwise linkage disequilibrium and c is the genetic distance. Physical distances were converted to genetic distances (Arias et al. 2009) under the assumption of constant recombination rates. N_e at generation t was estimated for all autosomes separately within different marker distance bins (<0.025, 0.025-0.050, 0.050-0.1, 0.1-0.2, 0.2-0.5, 0.5-1, 1-2, 2-5, 5-10, 10-15, 15-30 cM) using non-linear least squares as implemented in the R function *nls*(). The generation t was calculated as t = 1/2c, where c was averaged across all corresponding pairwise marker distances.

Arias, J.A. et al., 2009. A high density linkage map of the bovine genome. *BMC Genetics*, 10, p.18.

Sved, J.A., 1971. Linkage disequilibrium and homozygosity of chromosome segments in finite populations. *Theoretical Population Biology*, 2(2), pp.125-141.

Tenesa, A. et al., 2007. Recent human effective population size estimated from linkage disequilibrium. *Genome Research*, 17(4), pp.520-526.

2. Fine-mapping of the bovine Arachnomelia-Syndrome
(Poster presented at the EAAP Annual Meeting 2011, Stavanger)

Using Microsatellites for Fine-Mapping of Recessive Traits: an Obsolete Principle?

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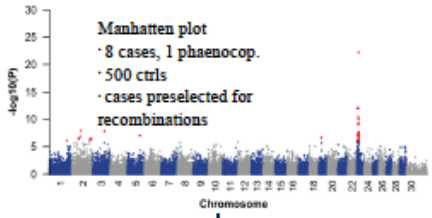
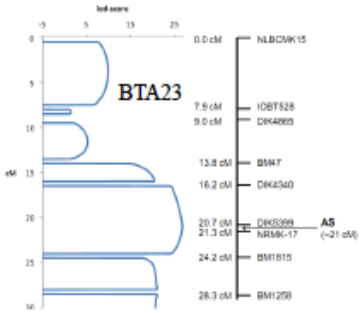
Arachnomelia (AS) in Cattle

- Recessive inherited
- 88 cases from 4 half-sib families
- Arachnomelia mapped using Microsatellites *versus* 50k chip

WGS using 50k chip

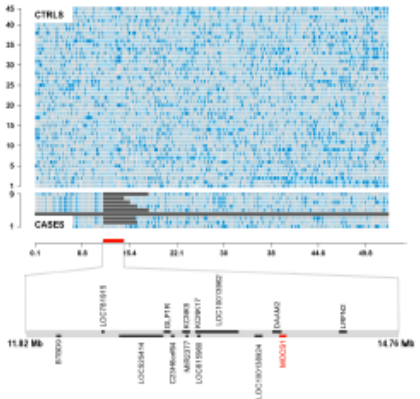


Whole Genome Scan using microsatellites

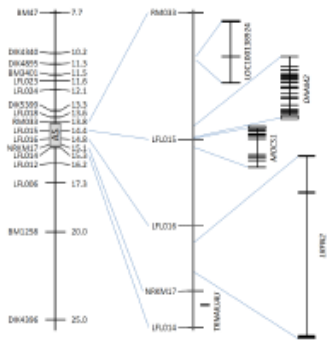


Linkage analysis using 88 cases
Selection of recombined haplotypes

Refinement of AS Region using Homocygosity Analysis



Refinement of AS Region using regional microsatellites



Conclusions

Both methods reliably identified the AS candidate region
Microsatellite mapping results in smaller AS candidate region:
AS region DNA Chip – 2.9 Mb
Mircosatellites – 1.8 Mb (due to mutated MS allele)

3. Imputation of high-density genotypes

These results are part of the following publication:
Pausch H, Aigner B, Emmerling R, Edel C, Götz K-U, Fries R (2013) Imputation of high-density genotypes in the Fleckvieh cattle population. GSE 45: 3.

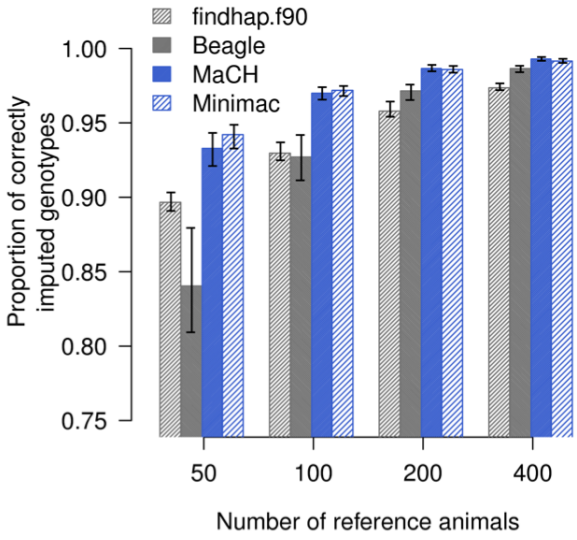


Figure 1: Proportion of correctly imputed genotypes.The barplots display the proportion of correctly imputed genotypes averaged over six chromosomes for four different scenarios with increasing sizes of the reference population. The black lines represent the minimum and maximum imputation accuracy for six chromosomes.

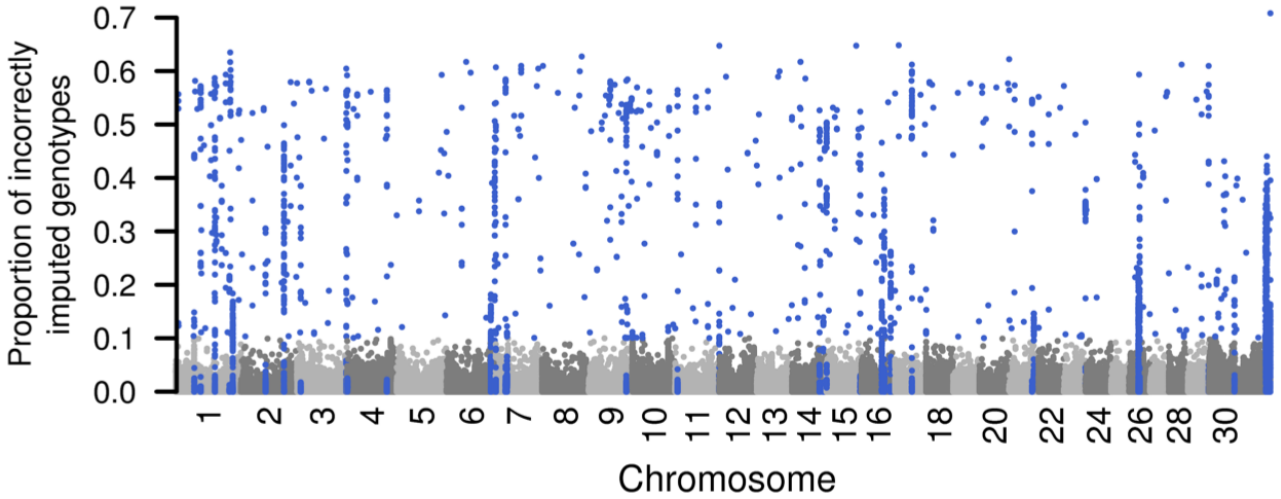


Figure 2: Genome-wide distribution of the proportion of correctly imputed genotypes. Genotypes of 599,535 SNPs were imputed for 397 animals based on haplotype information of 400 reference animals using Minimac. Blue dots represent 5039 SNPs within regions of poor imputation quality probably representing misplaced SNPs.

4. Imputation of whole-genome sequence information

(Poster presented at the ISAG conference 2012, Cairns)

Chair of Animal Breeding

Synbreed

Imputation of whole-genome sequence information for QTL fine-mapping in the Fleckvieh population

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Background

Genome-wide association studies (GWAS) have been successfully applied to identify quantitative trait loci (QTL) for important traits in livestock species. However, the identification of underlying variants remains a difficult task. Access to **whole-genome re-sequencing data** of key animals and subsequent **population-wide imputation** improves QTL mapping considerably as causal variants can be directly tested for association.

Population-wide imputation of sequence information

Medium- and high-density genotypes were available for 3668 bulls of the German Fleckvieh (FV) population. **Whole genome re-sequencing of 43 key animals** and subsequent multi-sample variant calling yielded **genotypes at 17.3 million sites**. We used a combination of *Beagle* [1] and *Minimac* [2] for the stepwise imputation of sequence information *via* medium- and high-density genotypes (**Figure 1**). This enabled to highly accurately extrapolate **12 million SNPs** and **1.5 million InDels** for **3668 animals**.

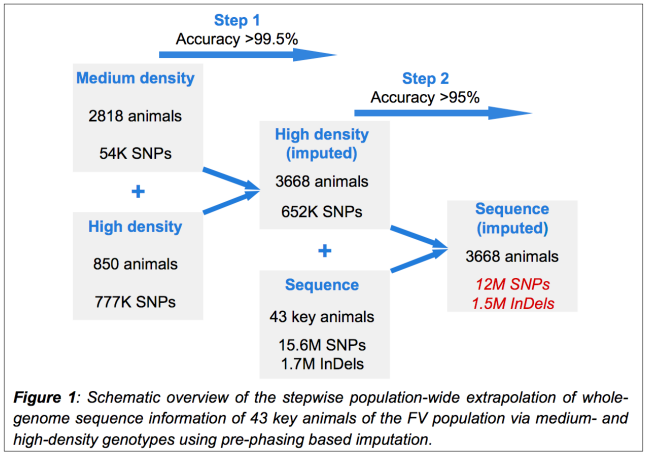

***Figure 1**: Schematic overview of the stepwise population-wide extrapolation of whole-genome sequence information of 43 key animals of the FV population via medium- and high-density genotypes using pre-phasing based imputation.*

Genome-wide association study

The **13.5 million imputed genotypes** were tested for association with progeny-derived phenotypes for **milk-fat content** (FC) in early and late lactation (lactation days 8-12 and 303-308, respectively) using a mixed-model based GWAS [3]. We identified **nine QTL** (**Figure 2A & 2B**), among them a QTL on **BTA27** close to *GPAT4* which is associated with **early lactational FC** only. The association resulting from imputed whole-genome sequence information is much stronger than that obtained from using array-based SNPs only (**$P=2.45 \times 10^{-18}$** *vs.* $P=3.63 \times 10^{-11}$)(**Figure 2C**). The most significantly associated SNP is located in the 3'-UTR of *GPAT4* in a putative microRNA binding site. The SNP reached high significance ($P=4.01 \times 10^{-17}$) in an **independent validation study** with 2327 animals of the German Holstein-Friesian population.

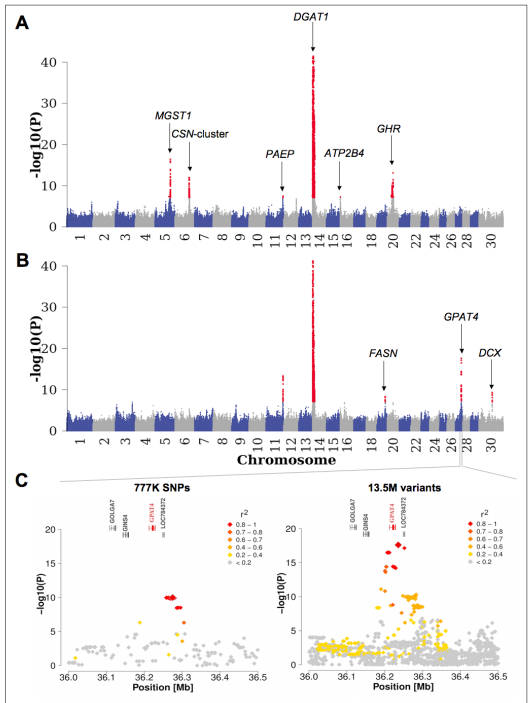

***Figure 2**: Manhattan plots for two sequence-based GWAS for late lactational (A) and early lactational (B) milk-fat content. Red dots represent significant SNPs. The association of the sequenced-based GWAS is much stronger than that obtained using array-based SNPs only (C). Different color indicates the extent of LD with the top SNP.*

Chromosome partitioning of the genetic variation

Chromosome-specific relationship matrices were fitted to estimate the proportion of variation explained per chromosome [4]. While **BTA27** accounts for a substantial fraction of the genetic variation for **early lactational FC**, its contribution to late lactational FC is negligible (**Figure 3**). Using relationship matrices built based on imputed sequence information **enables to capture genetic effects at a better resolution** in the FV population.

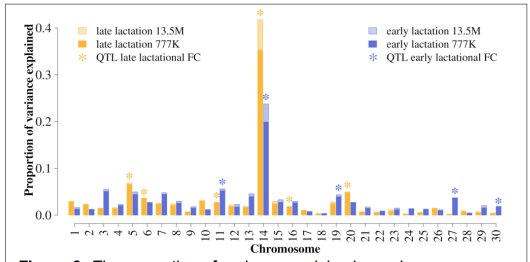

***Figure 3**: The proportion of variance explained per chromosome was estimated based on 777K and 13.5M genotypes, respectively [4]. The contribution of chromosomes varies considerably for early and late lactational FC. Asterisks represent chromosomes with identified QTL.*

Conclusion

The highly accurate **population-wide extrapolation of sequence information** resulting from re-sequencing a small number of key animals allows for the fine-mapping of QTL regions at maximum resolution. This enabled to pinpoint a polymorphism within a **regulatory site** as potentially underlying variant for a QTL for **early lactational milk-fat content on BTA27** which segregates in the Fleckvieh and Holstein-Friesian populations.

Federal Ministry of Education and Research
This study is funded by the Germany Federal Ministry of Education and Research (BMBF) within the AgroClustEr "Synbreed-Synergistic plant and animal breeding"

[1] Browning & Browning, 2009, AJHG 84(2), pp 210-223
[2] Fuchsberger et al., 2012, *submitted*
[3] Kang et al., 2010, Nat Genet 42(4), pp 348-354
[4] Yang et al., 2011, AJHG 88(1), pp 76-82

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Acknowledgements

First of all I owe my deepest gratitude to my supervisor Prof. Dr. Ruedi Fries for giving me the opportunity to work in his group, for supporting my work in any respects and for providing me with all facilities needed for the completion of this thesis.

Secondly, I'd like to thank the Bundesministerium für Bildung und Forschung (BMBF) and the Förderverein für Biotechnologieforschung (FBF) for financial support within the projects GenoTrack and Synbreed.

I want to thank Prof. Dr. Aurélien Tellier for chairing the examination board and Prof. Dr. Chris-Carolin Schön, Assoc. Prof. Dr. Ben Hayes and Prof. Dr. Thomas Meitinger for acting as co-referees of this thesis.

I also want to express my gratitude to Dr. Kay-Uwe Götz, Dr. Reiner Emmerling, Dr. Christian Edel and Dr. Dieter Krogmeier from the Bavarian State Research Center, Institute of Animal Breeding (LfL-ITZ) for providing any kind of data and for numerous fruitful discussions.

Science is team work – so I'd like to extend my thanks to all my colleagues from the Chair of Animal breeding, not mentioned by name, who supported me and my work in any respects. I especially want to thank you, Sandra, Simone and Tini for your friendship, for numerous discussions and for encouraging and supporting me during the last three years. I also want to mention Birgit Hoffmann for her help in administrative matters.

Last but not least, special thanks to all my friends and especially to my family. You always supported me without reserve and you remind me that life consists of much more than work alone.