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Genomic Characterisation, Polymorphism Analysis and Association Studies of Candidate Genes for BSE Susceptibility

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Abbreviations

Aa	amino acid
Acc	Accessionnumber
ADAM-TS	A disintegrin and metalloproteinase with thrombospondin motifs
ADP	adenosin diphosphate
ATP	adenosine triphosphate
ARIH1	Ariadne-1 protein homolog (Drosophila) gene
BAC	bacterial artificial chromosome
Baylor HGSC	Baylor College of Medicine-Human Genome Sequencing Center
BLAST	basic local alignment search tool
bp	base pair
BRUNOL6	Bruno-like 6, RNA binding protein (Drosophila) gene
BSE	Bovine Spongiform Encephalopathy
BTA10	Bovine chromosome 10
Bta	*Bos Taurus taurus*
Bti	*Bos Taurus indicus*
cDNA	coding DNA
CELF	CUG-Binding Protein and Embryonic Lethal Abnormal vision-type RNA-binding protein –like factor
CJD	Creutzfeld-Jakob-Disease
cM	centi Morgan
cR_{5000}	centi Ray (number refers to radiation dose: 5000 rads)
DDBJ	DNA Database of Japan
DMSO	dimethyl sulfoxid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
ddNTPs	dideoxynucleotides
EBI	European Bioinformatics Institute
EDTA	ethylendiamintetraacetat
ELISA	Enzyme-linked Immunosorbent assay
EM	estimation maximisation
EMBL	European Molecular Biology Laboratory
EST	expressed sequence tag
GB	German Brown
GC	Genomic Control
GF	German Fleckvieh
GH	German Holstein
GSDB	Genome Sequence Data Base
HSA15	Human chomosome 15
hexA	Hexosaminidase A (enzyme)
HEXA	alpha subunit of HexA gene
HEXB	beta subunit of HexA gene
hME	homogenous MassExtend™
HSPA8	Heat-shock protein 70
HWE	Hardy-Weinberg-Equilibrium
indel	Insertion Deletion polymorphism
IREB2	Iron responsive binding protein 2
kb	kilo base pairs
LAMR1	Laminin receptor 1 gene
LD	Linkage Disequilibrium
MAF	Minor Allele Frequency

MALDI-TOF MS	Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry
Mb	Mega bases
MIH	Maternal Inherited Haplotype
mRNA	messenger ribonucleic acid
N	A, C, G, T, U
NCBI	National Center of Biotechnology Information
OR	Odds Ratio
ORF	Open Reading Frame
PAR	Population Attributable Risk
PARP6	Poly-(ADP-ribose) Polymerase 6 gene
PCR	polymerase chain reaction
PLG	Plasminogen gene
PRNP	prion protein gene
PrP^c	cellular prion protein
PrP^{sc}	scrapie prion protein
QTL	quantitative trait loci
RACE	rapid amplification of copyDNA ends
RFLP	restriction fragment length polymorphism
RH	radiation hybrid
Rpm	rounds per minute
SAP	Shrimp Alkaline Phosphatase
SBE	single base extension
SDS	sodium dodecylsulfat
SNP	Single nucleotide polymorphism
SP1	Stimulating Protein 1 (transcription factor)
SQL	Structured Query Language
TBE	Tris Borate EDTA buffer
TDT	Transmission Disequilibrium Test
TE	Tris EDTA buffer
TGF	Transforming Growth Factor
THSD4	thrombospondin, type 1, domain containg 4 gene
TNE	Tris Natr EDTA buffer
Tris	Tris (hydroxymethyl) aminomethane
TSE	Transmissible spongiforme encephalopathy
UK	United Kingdom
UTR	Untranslated Region
vCJD	variant CJD

1 Introduction and Goals

Bovine Spongiform Encephalopathy (BSE) and its human form, variant Creutzfeld-Jakob disease (vCJD) belong to a group of fatal neurodegenerative prion diseases that are known as Transmissible Spongiform Encephalopathies (TSE). Most likely, BSE originates from Scrapie, the TSE of sheep, which was transmitted from sheep to cattle via the ingestion of infected meat and bone meal, a substantial protein source for cattle in the UK. Disease specific isoforms of the prion protein (PrP^{sc}) cause BSE by interaction with normal host prions (PrP), resulting in its conversion to PrP^{sc}. The misfolded PrP^{sc} later accumulates in the brain leading to neurodegeneration with formation of spongiform vacuoles (Prusiner, 1998a). In the disease process, the host's own PrP plays a crucial role: knockout mice experiments showed that mice lacking the prion protein never develop TSE. A notable feature of TSEs is a varying susceptibility of individuals to succumb to the diseases that is influenced by the genetic make-up and in particular, their prion protein gene (*PRNP)* genotype. Only human beings homozygous for a *PRNP* variant are currently known to have developed the variant Creutzfeldt-Jakob disease. In sheep, three codons in *PRNP* are strongly associated with the resistance or susceptibility to Scrapie. However to date, the genetic background of BSE in cattle is still unclear. Efforts to identify variants in the bovine *PRNP* that influence the susceptibility of cattle to BSE did not give a clear answer. So far, no convincing association between polymorphisms in the coding sequence of *PRNP* gene with the BSE incidence could be demonstrated. Nevertheless, a recent study investigating a small number of diseased animals from different breeds showed significant association of polymorphisms in the *PRNP promoter* and BSE (Sander et al., 2004). It is quite possible that these variants are influencing the expression levels of *PRNP* and are so intervening in PrP^{c} procession. The role of *PRNP* as a functional candidate gene for BSE susceptibility is remarkable and encourages to investigate the bovine *PRNP* in more detail by studying the promoter polymorphisms in a large sample of BSE diseased animals and controls.

In order to identify positional candidates, whole genome marker scans were performed in a set of BSE diseased and control animals revealing several marker loci that were significantly associated with BSE (Hernandez-Sanchez et al., 2002) (Zhang et al., 2004). Assuming a continuous trait, which can be measured (e.g., the individual incubation time), the regions encompassing the associated marker loci can be considered as so called Quantitative Trait Loci (QTL). One of these QTL regions on cattle chromosome 10 contains the gene encoding the alpha subunit of the lysosomal enzyme beta-hexosaminidase A (hexA), *HEXA*. Recently, a QTL study in mice linked the prion incubation time to the homologous region containing

H*exa* (Stephenson et al., 2000). The enzyme HexA plays a central role in the degradation of glyco-proteins of nerve cell membrane in the lysosome. In humans, polymorphisms in *HEXA* cause the neurodegenerative Tay-Sachs disease with accumulation of glyco-proteins in the lysosome. Further, *HEXA* was functionally connected to TSEs by showing that gene expression is elevated in Scrapie infected brains of mice (Kopacek et al., 2000). Thus, the chromosomal region of *HEXA* was postulated as a potential candidate region for BSE susceptibility.

In this work, genetic factors which influence the susceptibility to BSE in cattle were identified and assessed by candidate gene analyses and association studies of the coding and promoter regions of *PRNP*, *HEXA* and the adjacent region of *HEXA*.

The specific goals of this thesis were

1. **Characterisation of candidate genes for BSE susceptibility on bovine chromosome 10**
   - Sequence and structure analysis of the bovine *HEXA* gene and its promoter
   - Elucidation of 300 kb region centred on the *HEXA* gene
2. **Screening for polymorphisms in *HEXA* and neighbouring genes**
3. **Investigation of the potential role of candidate genes for BSE susceptibility by association studies in different breeds**
   - Genotyping of *null-loci* as Genomic Control to assess putative population stratification.
   - Genotyping of polymorphisms in the extended *HEXA* region in BSE diseased and control animals.
   - Genotyping of polymorphisms in the promoter region of *PRNP* in BSE diseased and control animals.

2 Literature review

2.1 Bovine Spongiform Encephalopathy (BSE) and Prion Diseases

2.1.1 Incidence of BSE and surveillance strategy in the United Kingdom (UK)

After the first case of BSE was established in the UK in 1987, the number of affected animals increased and peaked in 1992 with 36 682 cases per year (Figure 2.1). Due to introduction of a feeding ban of ruminant protein to ruminants (feed ban) in July 1988, the number of BSE cases per year declined from 1993. Since August 1996, the feeding of meat and bone meal from all mammals to all farmed livestock was prohibited to prevent any possibility of contaminating feed for ruminants (real feed ban). After implementation of the real feed ban, only 120 BSE cases have been confirmed in UK. Experts predicted a small number of cases born after the 1st of August 1996 because of exposure to BSE infected feed originating from before the real feed ban. Some animals might also have been exposed to BSE through maternal transmission, but were not taken under the offspring cull because the dam was slaughtered before it developed clinical signs of disease and therefore was not diagnosed as a BSE case. Furthermore, there might be additional, not yet identified infection routes (e.g., through suckler milk, fertilizer or cross-contaminated feed). However, affected animals born after the real feed ban might be genetically more susceptible than those born before due to the smaller dose of infectious material in circulation.

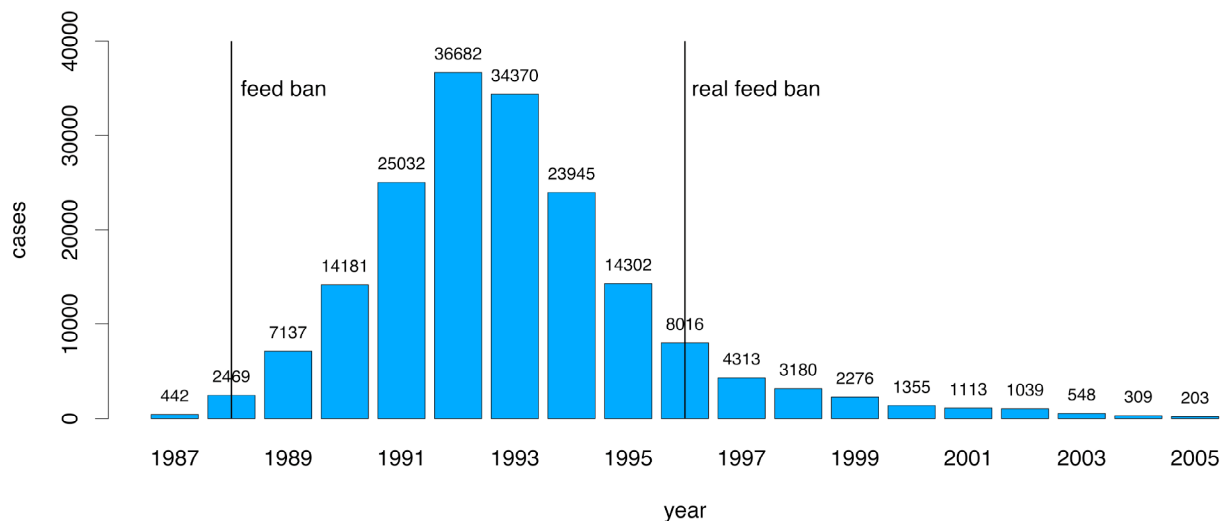


Figure 2.1 - Development of the number of BSE-diseased animals in the UK (state 12/05).

The average annual incidence rate of BSE in the UK cattle population is 0.2% (http://www.oie.int/eng/info/en_esbincidence.htm). The strategy of monitoring BSE cases in the UK is divided in passive and active surveillance. Passive surveillance comprises the sim-

ple recording of sick animals. This method identified 179 130 BSE positive animals to the end of 2005. Active surveillance identified 1 678 BSE positive animals by testing brain samples using EU approved rapid testing procedures based on the presence of proteinase K resistant prion protein fragments. The following categories of cattle are tested for BSE:

- All cattle destined for human consumption aged over 30 months,
- All fallen stock aged over 24 months,
- All emergency slaughter animals,
- Animals found sick at ante mortem inspection aged over 24 months,
- All cattle born between August 1, 1995 and August 1, 1996 entering the Older Cattle Disposal System.

The programme results are published regularly on the web page http://www.defra.gov.uk/animalh/bse/statistics/incidence.html.

2.1.2 Incidence of BSE and surveillance strategy in Germany

In Germany, the number of BSE incidences is orders of magnitudes smaller than in the UK cattle population. The first confirmed BSE case occurred November 26th 2000. Figure 2.2 shows the annual distribution of 403 confirmed cases until the end of November 2006. The BSE status of German animals was diagnosed in clinically suspect animals and by testing all slaughtered animals older the 24 months in certified local laboratories based on the presence of proteinase K resistant prion protein fragments. The initial diagnosis had to be confirmed by the German National BSE Reference Laboratory at the Friedrich-Löffler Institute (Riems Island). If suitable tissue was available, the diagnosis was histopathologically confirmed. The annual incidence rate is 0.0012% among cattle of all breeds (http://www.oie.int/eng/info/en_esbincidence.htm). In contrast to the UK, the cattle feed in Germany did not contain meat and bone meal systematically, but traces might be intermingled in the concentrate. This was due to the fact that factories producing concentrated feed, did not insist on a strict division between ruminant and pig feed, which contained animal protein. Also another source of animal protein for ruminants was the suckler milk that contained animal fat. This is reflected in the contribution of the BSE cases showing higher incidence rates in those regions, where farmers are not able to produce their own concentrate, prevailing in the grasslands. The southwest region of Bavaria is mostly grassland, a preferential dairy cow keeping area, where the main concentrates have to be bought. In this area a cluster falls with an above-average number of cases (LMU-Project-Network-BSE-Risk, 2004).

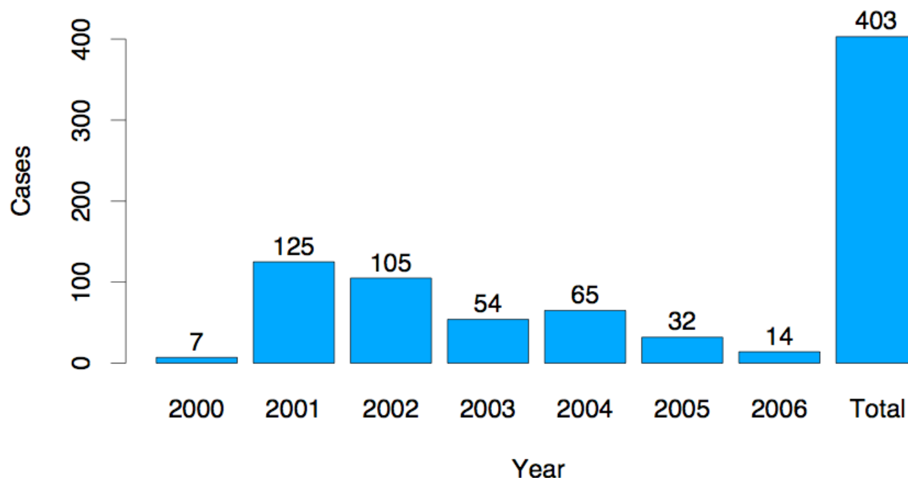


Figure 2.2 - Development of the number of BSE-diseased animals in Germany (state 11/06)

2.1.3 Basics and genetics of prion diseases

Transmissible Spongiform Encephalopathies (TSEs) are fatal neurodegenerative disorders in humans and other mammals that can be experimentally transmitted to individuals of the same and other species. The morbid agent is the so called Scrapie prion protein (PrP^{sc}); the name origins from the archetype TSE of sheep (Scrapie) and is used for all TSE pathogens. In contrast to other classical pathogens, PrP^{sc} has no own genome and consists of an abnormal conformation of the cellular and hosts own prion protein (PrP^{c}) (Prusiner, 1982).

PrP^{c} is a glycoprotein attached by a C-terminally linked glycosyl-phosphat-idylinositol (GPI) anchor on the plasma membrane of neuronal cells, predominantly on presynaptic membranes (Herms et al., 1999). Due to copper-binding ability assigned by an octapeptide repeat, possible copper-specific neuroprotective mechanisms have been proposed (Roucou et al., 2004; Vassallo and Herms, 2003).

PrP^{sc} converts PrP^{c} into PrP^{sc} by forcing a conformational change as shown in Figure 2.3, which in turn acts as template for others, thus creating a protein-folding chain reaction. This conformational change might involve other proteins, which yet have to be identified. The ability of self-replication of conformational information enables prions to transmit disease, encode heritable phenotypic traits or encrypt molecular memories (Shorter and Lindquist, 2005). The exponential multiplication of the pathogen leads to deletion of cells in the brain and in progressed stage to the spongiform neurodegeneration ending in the death of the affected individual (Prusiner, 1991; Prusiner, 1998b). PrP^{sc} is resistant against proteinase K digestion whereas PrP^{c} can be completely digested. Therefore, the pathogen can be detected by tests, based on proteinase K digestion and ELISA-technique.

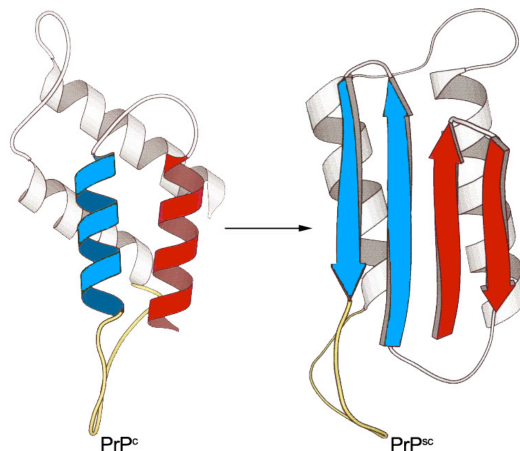


Figure 2.3 - Hypothetical protein structure models for PrP^c (left) and PrP^{sc} (right).
Conversion of α-helices into β-sheet structure by formation of the pathogenous form.

Scrapie, the archetype prion disease, is a natural disease of sheep and goats and its characteristics include a long incubation or preclinical period, progressive ataxia, astrocytosis, tremor and death. Scrapie has been recognized in Europe for over 200 years ago and is present in many countries worldwide. The typical histopathological changes are prior in the brain that include vacuolation from cell death, neuronal loss and, in some cases amyloid plaque formations. In sheep, variations at positions 136, 154 and 171 of the prion protein sequence play an important role for the susceptibility to Scrapie. In dependence of the arrangement of the genotypes at the three loci, the sheep is more or less liable to come down with Scrapie (Hunter et al., 1997). Testing the individual Scrapie resistance by genotyping has become an important issue in the sheep production. In the European Union a breeding programme for TSE resistance in purebred sheep exists forcing the member states and their sheep flocks to follow the selection for resistance to TSE (Commission-of-the-European-Union, 2003).

In human beings, several distinctable prion diseases are known (Prusiner, 1991; Prusiner, 1998b) (Collinge, 2001). The sporadic Creutzfeld-Jakob-Disease (CJD), the most frequent form of human prion diseases affects about one individual per million. In the United Kingdom, there are fifty to sixty deaths per year due to sporadic CJD. In the affected individuals, the PrP^c undergoes a spontaneous conformation change to the abnormal form and leads to the disease. Kuru, the prion disease among the Fore folk in Papua New Guinea, first recognized in the 1950s, was transmitted from human to human during cannibalistic feasts (Gajdusek et al., 1966). Initially misfolded prion proteins were migrating from the dead to the tribesman alive, consuming the brains of the affected people. A novel form of human TSE, variant CJD (vCJD), was first reported in the United Kingdom in 1996 (Will et al., 1996) (Collinge and Rossor, 1996). VCJD is said to be the human form of BSE, transmitted by passing the infec-

tious prions from cattle to human in the form of brain tissue contaminated beef.

Mutations in the prion protein predispose for the outbreak of human prion diseases. A mutation at codon 129 (M129V) causing a methionine (M) to valin (V) amino acid change is strongly associated with vCJD, sporadic CJD and Kuru. Today, all but one cases of the vCJD in the UK were homozygous for methionine (MM) as shown in Figure 2.4. Individuals with genotypes containing at least one V allele might have longer incubation times or are much more resistant to the pathogen. If the incubation time were pre-longed for a V allele, cases of vCJD patients would appear in the next future, owing the genotypes MV or VV. One patient, heterozygous at the codon 129, died from a different disease, five years after he received a blood transfusion from a donor who developed vCJD. The heterozygous patient was in pre-clinical stadium of vCJD; protease resistant prion protein was detected in the spleen, but not in the brain (Peden et al., 2004). In contrast to the common nationwide genotype frequency of the M129V polymorphism in the UK, the affected persons of sporadic CJD have remarkably shifted genotype ratios. Palmer et al. (1991) reported that homozygous individuals for both directions (MM or VV) are overrepresented in the sporadic CJD patient group (Figure 2.4). Also for Kuru, an influence of the M129V polymorphism was observed. Heterozygosis at the mutation site confers a relative resistance to the prion disease (Mead et al., 2003). Elderly survivors of the epidemic who had multiple exposures at cannibalistic feasts are in contrast to younger unexposed Fore, predominantly M129V heterozygotes. The authors assumed, that strong balancing selection with an advantage to survive for the heterozygote individuals occurred.

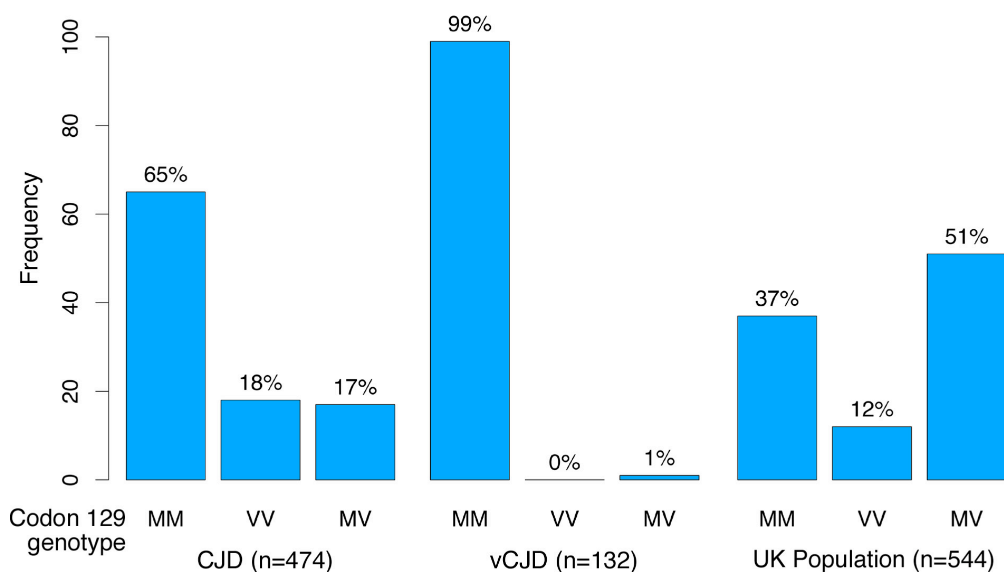


Figure 2.4 - Genotype Frequencies at human Prion protein polymorphism (M129V).
Distribution of the genotypes at M129V in the human prion protein gene in patients suffering from sporadic CJD, vCJD and among the UK population (National-CJD-Surveillance-Unit, 2004)

2.1.4 Genetic Background of BSE in cattle

In 1990, Wijeratne and Curnow showed by segregation analysis in families with BSE-members that 73% of the affected animals had first or second degree relatives also affected. The Mendelian model for an autosomal recessive inheritance could not be excluded. But the piled occurrence of bulls as ancestors of BSE animals could also arise from the international breeding strategy. Studies of the progenies of BSE affected cows indicated, that there may be a genetic influence on the susceptibility in cattle, because of the apparent higher risk of developing BSE in off-spring of BSE-affected cows compared with uninfected controls (Donnelly et al., 1997; Ferguson et al., 1997; Wilesmith et al., 1997). The gene encoding the prion protein, *PRNP*, has been suggested as a candidate locus for BSE susceptibility. However, no clear relationships between bovine *PRNP* polymorphisms and susceptibility to BSE have been revealed so far. The study of Neibergs et al. (1994) showed that an octapeptide repeat in the amino acid sequence is associated with BSE, but this was not confirmed in a similar study of Hunter et al. (1994). Moreover, two insertion / deletion (indel) polymorphisms in the promoter region of *PRNP* were subject of an association study of Sander et al. (2004), revealing that both the 23-bp indel (Sander et al., 2004) in the promoter region and the 12-bp indel (Hills et al., 2001) in intron 1 are tentatively associated with the BSE status. In vitro analysis showed that both indel sides affect binding sites for transcription factors (RP58 and SP1, respectively), and in vivo and in vitro investigations suggest that the two polymorphisms affect PRNP expression, albeit with the direction on the effects remaining to be clarified (Sander et al., 2005).

In order to identify loci responsible for susceptibility to BSE, Hernandez-Sanchez et al. (2002) performed a genome-wide search for markers associated with BSE, by using a Transmission Disequilibrium Test (TDT). In this study, 358 BSE affected and 172 healthy half-sibs of four fathers were genotyped at 146 informative microsatellite markers loci. Significant segregation distortion was found on cattle chromosomes 5, 10 and 20. Putative candidates located in these regions are e.g. both subunits of the enzyme hexosaminidase A (hexA), *HEXA* and *HEXB* on cattle chromosomes (BTA) 10 and 20, respectively. To map quantitative trait loci (QTL) for BSE susceptibility, a whole-genome scan was conducted by Zhang et al. (2004), including 173 microsatellite markers and four half-sib families with affected and unaffected members. Two genome-wide significant QTL (BTA17 and X/Y_{ps}) and four genome-wide suggestive QTL (BTA 1, 6, 13 and 19) were revealed. A potential candidate for BSE susceptibility, which falls within the 95% C.I. of the QTL on BTA 19 is the gene encoding neurofibromin 1 *NF1*. A microsatellite flanking this gene was genotyped in a panel of BSE-affected and control animals by Geldermann et al. (2006), revealing significant association in one of four investigated cattle breeds.

2.2 Single Nucleotide Polymorphisms

A Single Nucleotide Polymorphism (SNP) refers to a position in the DNA sequence at which two alternative bases occur at appreciable frequency (>1%). SNPs are the most common type of variations found in mammalian genome. In human beings, SNPs occur with a mean frequency of one SNP per kb (Wang et al., 1998). For cattle, Werner et al. (2004) identified on average one SNP every 180 bp by randomly screening of 91 kb bovine genomic DNA. In this study, SNP screening was performed in a panel of animals belonging to the taurine breeds German Holstein, German Brown, German Fleckvieh, Kerry and Angus and to the indicine breeds Sahiwal and Hariana. Bovine DNA from seven US breeds was used to screen 5.2 kb of nine loci of the chemokine gene family by Heaton et al. (2001). The authors detected on average one variable site every 143 bp. First, SNPs were used as genetic markers in analysing Restriction Fragment Length Polymorphisms (RFLPs) (Botstein et al., 1980). Often without knowing the sequence data, this was an indirect method of genotyping SNPs, because the RFLPs were caused by point mutations, which determine the cutting site of an enzyme. However, this method is still in use and appropriate to genotype few SNPs with known sequence information. Due to the abundance and distribution of SNPs, the interest of using SNPs as genetic markers increased in the last decade. As a by-product of the whole genome sequencing projects, thousands of SNPs were identified in the different species. Nowadays, SNPs are being genotyped in disease association studies and pharmacogenetics, etc. (Gray et al., 2000) and the application of SNPs is ranging from forensic fingerprinting where individuals can be identified by determining the genotypes at a panel of 34 SNPs (Li et al., 2006) to whole genome association studies where pooled DNA can be genotyped parallel at 500 000 SNPs (Papassotiropoulos et al., 2006). Other biallelic variation occuring with considerable frequency are insertion-deletion (indel) polymorphisms. At an indel polymorphism the DNA sequence of one to thousand or more bases are either existent or not. Indels can be genotyped with the methods of SNP typing if the first base of insertion and first base after insertion are different nucleotides.

Various methods of genotyping SNPs are established. The appropriate genotyping approach depends on the number of samples and SNPs. Common SNP typing chemistries currently available are based on differential hybridisation, allele specific amplification, primer extension with allele specific nucleotide incorporation and allele specific DNA-cleavage. Each chemistry has its appropriate detection system. Some of the current technologies of high throughput genotyping SNPs are fluorescent microarray-based systems (e.g., Gene Chips from Affymetrix, Santa Clara, CA, USA), fluorescent bead-based technologies (e.g., BeadArray, Illumina, San Diego, CA, USA), automated enzyme-linked immunosorbent (ELISA) assays (SNP-IT, Orchid, Princeton, NJ, USA) and the technique used in context of this thesis,

mass spectroscopy detection (e.g., iPlex, Sequenom, San Diego, CA, USA). This technique is described in detail in Chapter 3.14 - Genotyping SNPs with MALDI-TOF MS.

2.3 Challenge of Association studies

Case-Control-Studies based on the comparison of affected (= cases) and unaffected (= controls) individuals from a population is called a population-based association study. The allele A at a gene locus is said to be associated with a phenotype, if it occurs with a significantly higher or lower frequency in the affected group compared to the unaffected (Lander and Schork, 1994). Mainly, biallelic DNA polymorphisms were used to perform association studies including SNPs and insertion-deletion polymorphisms with minor allele frequencies of above 5%. After genotyping of a panel of cases and controls, the statistical analysis include the Test for Hardy-Weinberg-Equilibrium (as shown in Chapter 3.15.2 – Test for Hardy-Weinberg-Equilibrium), allele and genotype frequency based calculation of the test statistic (as shown in Chapter 3.15.3 – The Armitage Trend test) and logistic regression analysis for risk calculations (as shown in Chapter 3.15.4 – Logistic regression analysis). Afterwards, Linkage Disequilibrium (LD) and haplotype analysis were performed to elucidate putative combined effects on the trait (as shown in Chapters 3.15.5 and 3.15.6 – Measuring LD and tagging SNPs and Inferring haplotypes, respectively). Recent advances of the availability of many genetic markers, in particular of SNPs and the increasing speed and capacity of genotyping machines, faciliate the performance of association studies.

Association of a marker allele with a phenotype might have different origins. First, the SNP is causal and the individual genotype influences the phenotype (e.g., predisposition to a disease). In this case, the same direction and effect size of the association would be expected to occur in different populations. Hence, the association can be replicated in other studies. The involvement of the polymorphism in the phenotype can be confirmed by other approaches like expression analysis or verification of the function or malfunction in transgenic animal models. Second, the allele does not cause or change the trait, but is in LD with the causative polymorphism. I.e., the associated allele tends to occur on those chromosomes, which carry the causative/trait changing mutation, due to the haplotype configuration of the ancestor, where the mutation debuted. Depending on the genetic distance and the age of the mutation, recombination events reduce the LD between marker and causative mutation. A genetic distance of 1cM corresponds to a probability of recombination in meiosis of 1%. Here, the results for different isolated populations (e.g., cattle breeds) can be oppositional. In one population the first allele can be associated with the trait and in the other population the second allele, due to specific recombination events in the ancestry of the populations. Consequentially, no allelic association can be found if these populations were pooled for the study.

The third cause for appearance of association is population stratification as shown in Figure 2.5. This scenario occurs if cases and controls consist of subpopulations and if the proportion of cases and controls sampled from each subpopulation vary. Subsequently, if the subpopulations have variable frequencies at a SNP locus, the allele or genotype frequencies of cases and controls differ, too. Hence, the frequencies between cases and controls do not differ because the locus is involved in the trait characteristics but rather due to unbalanced selection and the association is an artefact of population admixture. A concrete example for population stratification was published by Lander and Schork (1994): In San Francisco, the ability to eat with chopsticks should be genetically investigated, by performing an association study with the HLA complex. The population of San Francisco consists of two subpopulations: the Caucasian and the Asian. The tendency to select chopstick-eaters from the Asian subpopulation suggests itself. One allele would turn out to be associated with the ability to eat with chopsticks, not because of a functional influence of HLA on the dexterity, but simply because of the higher frequency of that apparently associated allele in the Asian population.

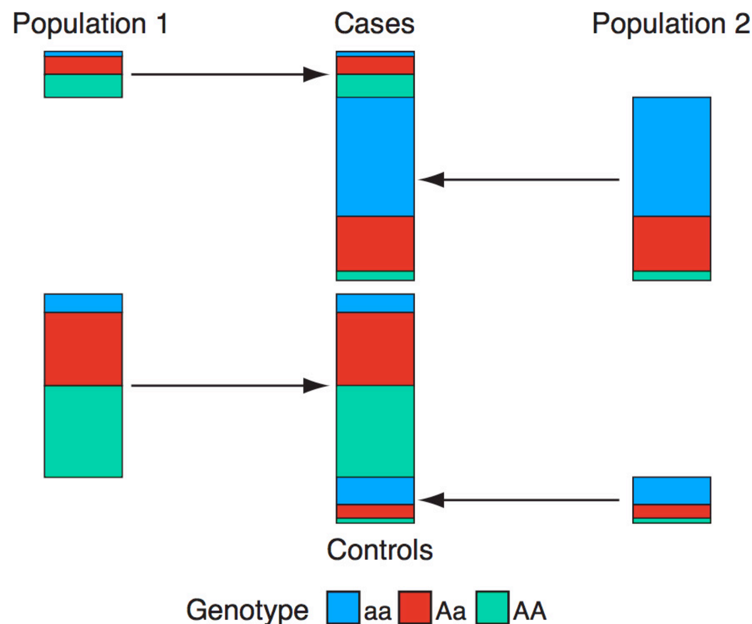


Figure 2.5 - Effect of Population Stratification at a SNP locus.
Exemplary for population stratification with two populations: The cases have an excess of individuals of population 2, whereas the controls are abundantly recruited from population 1. The frequency of allele A is substantially lower in population 2 than in population 1. This leads to spurious association, the structure mimics the signal of association with significant difference in allele and genotype frequencies between cases and controls (modified from Marchini et al. (2004)).

This example rationalizes that an adequate choice of a control group is pivotal for a successful association study. Therefore, a study should only be performed within a homogenous population. Furthermore, for a valid comparison between cases and controls, individuals of both

groups should differ preferable only in the investigated attribute. Thus, a comparison of individuals from different origins (e.g., cattle breeds) in the case and control group is not useful and must be avoided. If origin, parentage and relatedness of the samples are unknown or population substructure is expected (e.g., in inbred populations), the population stratification should be detected and corrected. An appropriate approach is the Genomic Control (GC) (Devlin and Roeder, 1999). For GC, additional loci to the candidates' -so called *null-loci*- have to be genotyped. The *null-loci* are unlinked polymorphisms, considered having no effect on the susceptibility or the value of the trait. By calculating the Armitage Trend test statistic for the *null-loci*, the inflation factor λ is inferred as the empirical median divided by its expectation under the χ^2-distribution. If $\lambda > 1$, the test statistics of the investigated candidate loci are divided by λ, in order to correct for stratification.

The analysis of multiple polymorphisms in a candidate region inflates the global type 1 error rate. Adjustment for multiple testing is a basic problem of association studies performed with many markers. The test wise type 1 error rate (alpha-level) has to be corrected downwards to consider false positives, if more than one test in a particular study was made. Usually, the alpha-level is set to 0.05, which denotes that one of twenty tests shows a false positive association. The probability of finding at least one association rises in line with the number of performed tests. Therefore, a correction for multiple testing is required. Customarily, this can be performed by order of Bonferroni-Holm correction, False Discovery Rate approach or permutation procedure.

3 Animals, Methods and Material

3.1 Animals

3.1.1 Animals used for comparing sequencing and SNP detection

For direct sequencing and SNP search, a random sample representing taurine and indicine cattle breeds was chosen as shown in Table 3.1. The DNA of 31 bulls of the three German main breeds German Holstein, German Fleckvieh and German Brown, unrelated in the last three generations, were selected. In order to detect all variations in the sequence, additionally ten Sahival, eight Tharparkar and seven Hariana, representing *Bos taurus indicus* were used. To facilitate searching and evaluation of SNPs, DNA pools were prepared for each taurine breed and for all indicine breeds by adding equal amounts of DNA of the individual samples and sequenced. This also allowed a direct preliminary estimation of the SNPs' allele frequencies in the different populations by visual assessment of the sequencing traces (as shown in Chapter 3.12 – Estimation of allele frequencies in the populations). SNP search was performed in a panel of 16 samples containing four pools (Holstein Friesian, German Fleckvieh, German Brown and Bos indicus) and three individual animals of each breed and Bos indicus cattle, respectively.

Table 3.1 - Animals used for sequencing and SNP search

Species	Breed	Number of animals
Bos taurus taurus (cattle)	German Fleckvieh	31
	German Brown	31
	German Holstein	31
Bos taurus indicus (zebu)	Sahival	10
	Tharparkar	8
	Hariana	7

3.1.2 Animals used for genotyping

3.1.2.1 German Fleckvieh, German Brown and German Holstein

To estimate the allele frequencies of the genotyped SNPs in the three German main breeds, the sequencing panel animals consisting each of 31 animals, were genotyped (see Chapter 3.1.1 – Animals for comparing sequencing and SNP detection).

3.1.2.2 German BSE-Animals

BSE status of German animals was diagnosed in clinically-suspect animals and by testing all slaughtered animals 24 months of age or older in certified local laboratories based on the presence of proteinase K resistant prion protein fragments. The initial diagnosis had to be

confirmed by the German National BSE Reference Laboratory at the Friedrich-Löffler-Institut of Novel and Emerging Infectious Diseases (FLI-INNT) (Riems Island) formerly known as *Bundesforschungsanstalt für Viruserkrankungen*. DNA was extracted using the QiAamp DNA Mini Kit (Qiagen Valencia, CA) after tissue decontamination with 13.5 M guanidine chloride and kindly provided by the FLI-INNT. A total number of 276 cases, collected from November 2000 until the end of 2005, was available. Based on the farmer's declaration, 127 of these belong to German Holstein, 106 to German Fleckvieh and 43 to German Brown breed. This separation into different populations is required, because of the genetical difference due to distinct selection strategies and breeding goals depending on the utilisation. To date there are 403 BSE cases recorded in Germany, including at least ten different breeds. The breed information was provided by the Friedrich-Löffler-Institut of Epidemiology in Wusterhausen. German Holstein black pied -BSE cases (n=73) with diagnosis in the years 2001 and 2002 were used for further investigations at the *HEXA* locus. For the association study at the prion protein polymorphisms, all available BSE cases until the end of 2005 of the main breeds German Holstein, German Brown and German Fleckvieh were investigated.

3.1.2.3 German Control Animals

Control animals for the German Holstein breed consisted of 627 paternal half-sibs, whose DNA have been isolated in the framework of a QTL-mapping project by the Institute of Animal Breeding at the Christian-Albrechts-University of Kiel, by the Department of Animal Breeding and Genetics at the Justus-Liebig-University of Gießen and by the Research Unit Molecular Biology at the Research Institute for the Biology of Farm Animals. The half-sib animals were approximately contemporary with the BSE animals and geographically similarly distributed. Only the maternally inherited alleles were considered (see Chapter 3.15.10 - Inferring allele- and genotype frequencies from half-sibs). For the association study at the *PRNP* promoter polymorphisms control animals were selected as follows: German Brown bulls (n =90) used for artificial insemination were selected so that their pedigree was representative for the German Brown population. The German Fleckvieh control (n = 137) consisted of bulls kept at the experimental Station Hirschau of the Technical University of Munich. They were purchased on markets throughout Bavaria and can be considered to be representative for the German Fleckvieh population. DNA was isolated by proteinase K and chloroform-phenol extraction of blood (German Fleckvieh controls) or bull sperm (German Brown control), provided by several Bavarian insemination stations.

3.1.2.4 UK Animals

Samples of BSE-affected UK animals were collected between 1990 and 1993. Animals were identified as BSE suspects by veterinary diagnosis of live animals and the initial diagnosis confirmed by histopathological examination. Blood samples were obtained from a total of 365

BSE affected and 276 BSE unaffected age-matched paternal half-sib offspring, born on the same farms as the BSE affected animals. The proportion of affected to unaffected animals was similar in each of the 37 half-sib groups. None of the controls was recorded in the BSE case database of the UK-Department for Environment, Food and Rural Affairs (Defra) at a later date. DNA was obtained from blood via proteinase K digestion and phenol-extraction at the Veterinary Laboratories Agency in Weybridge, UK.

3.2 DNA Handling

Genomic DNA was extracted from frozen bull semen for the individuals of the sequencing panel and the German Brown control animals in our laboratory. Further, DNA of the German Fleckvieh control animals was prepared from blood samples by proteinase K digestion and chloroform-phenol extraction. DNA samples of German BSE diseased animals, German Holstein control animals and UK animals were obtained from different other laboratories (see Chapter 3.1.2 – Animals used for genotyping). Depending on further use, different DNA concentrations were required. DNA for sequencing was calibrated to a working solution of 25ng/μl, while DNA for genotyping by MALDI-TOF MS was adjusted to 1ng/μl.

To control the quantity and quality of the DNA, 1-2 μl of the solution were tested by gel electrophoresis on a 0.8% agarose gel in 1/2 TBE buffer together with lambda DNA standard (SD0011; MBI Fermentas, St. Leon-Rot, Germany) of known concentration. The quantity was further assessed using fluorometric methods by mixing 2μl of each DNA with 1x TNE buffer containing fluorescent Hoechst Dye (H33258, Fluka) incorporating into the DNA in a special cuvette. The solution was measured by a fluorometer (DyNA-quant 200, Hoefer Scientific, San Francisco, CA, USA). The DNA solutions of the samples for genotyping were measured by PicoGreen dsDNA Quantification Kit (Molecular Probes, Leiden, Netherlands) with a flourometry plate reader (GeniOS Fluorescence Plate Reader, TECAN, Crailsheim, Germany). The advantage of this system is the high-throughput analysing of the plate reader, the favoured binding of the Pico Green to double stranded (ds) DNA and the sensitive detection of quantities as little as 25pg/ml of ds DNA. After quantification of the DNA solutions, 200 ng of DNA was diluted with TE to 200 μl to aim for a final concentration of 1ng/μl. 5μl of each DNA dilution (1ng/μl) was dispensed by pipetting station (TECAN, Crailsheim, Germany) over a 384-sample plate and dried at room temperature.

3.3 The Basic Local Alignment Search Tool (BLAST)

The Basic Local Alignment Search tool (BLAST) (Altschul et al., 1990) is a powerful method to identify similar and/or homologues sequences. BLAST finds regions of local similarity

between sequences by comparing nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. The tool is available on the web-pages of the National Center of Biotechnology Information in Rockville Pike, USA (NCBI) http://www.ncbi.nlm.nih.gov/ or the web-page of the European cooperation of Welcome Trust Sanger Institute and the European Bioinformatics Institute (EBI) http://www.ensembl.org/. The programme and sequence databases can be downloaded and installed locally on a desktop computer to reduce processing times because of independence from the official server.
The tool was used for different purposes:

- Analysis of exon-intron structures by *BLAST search* of mRNA sequences against genomic sequences,
- Identifying bovine sequences (genomic or ESTs) by using homologous human sequences as query input,
- Newly sequenced bovine sequences were approved and assigned
- Alignment of two sequences with *bl2seq* (NCBI BLAST).

Sources for human sequences are, e.g. *nr* (non redundant)-, *RefSeq* and *EST*-Division of the Nucleotide database of NCBI and the cow or human division of Ensembl web-page which contains sequence data from *GenBank* (NCBI), *EMBL* (European Molecular Biological Laboratory in Hinxton Hall, UK), *GSDB* (Genome Sequence Data Base in Santa Fe, USA) and *DDBJ* (DNA Database of Japan in Mishima, Japan).

The gene structure and the neighbouring genes were detected with the tool *Ensembl Mart View*, where gene information can be exported (http://www.ensembl.org/Multi/martview). The region of interest can be chosen, different filters can be applied and various output information can be requested (e.g. RefSeq ID, strand, start and end position, external gene ID, etc.).

3.4 Excursus: Changes in bovine genomic sequence availability

The sources of bovine sequence data have enlarged many-fold over the last four years. The following excursus should show how the increased sequence yield has changed the working strategies for analysing candidate genes.

3.4.1 Sequence detection by BAC–DNA (Primer walking)

In case where no bovine sequence is available for the gene of interest, an initial bovine PCR system and its primers need to be derived from human or mouse sequences. It is especially advantageous to use coding sequences of the gene for this purpose (e.g. human mRNA) because exons are more highly conserved between species than are introns. Using the initial

bovine PCR system, as verified by sequencing and BLAST comparison, a bovine Bacterial Artificial Chromosome (BAC) library is screened for clones containing gene-inserts. After BAC-DNA isolation, the insert is used as a template for Primer-Walking, where the BAC-DNA is sequenced directly without a previous PCR-amplification by e.g., one up-primer or from a primer of the BAC ends known vector sequence (BAC end sequencing). Initial primers for BAC end sequencing were derived from T7 and SP6 promoter sites, which were located on the pBACe3.6 vector flanking the insert. After determination of the sequence, the next up-primer can be designed from it. This process is repeated iteratively: a direct sequencing step is applied, the new sequence is determined and this sequence is used for primer design. To identify SNPs in the newly determined gene sequence, region-specific PCR primers have to be designed and applied to the DNA of both pooled and individual animals. In summary, the steps that need to be performed for each part of the gene are, in order, primer walk sequencing, applying a PCR-system and finally sequencing of individuals to find sequence variations.

3.4.2 Bovine Expressed Sequence Tags (ESTs)

The first abundant bovine sequences that could be identified by BLAST search were expressed sequence tags (ESTs). Inserts of cDNA clones were partially sequenced from one or both ends performing single-pass automated sequencing read. ESTs are characterised by being short (400 – 600 bases) and relatively inaccurate (around 2% error) (Adams et al., 1991). In the current context, a human reference sequence (mRNA) was used to blast search the *EST-other* division (dbEST) of GenBank to find bovine sequences for the candidate genes of interest. In Figure 3.1, the EST sequencing approach is shown. By comparison of the human exon structure and the EST sequences, the bovine structure could be inferred and used for primer design spanning the introns as well. For the obtained PCR fragment, the size could be estimated by agarose gel electrophoresis. Sequencing of the PCR fragments resulted in one up and one downstream read of around 450 to 500 bases, which were assembled if the sequences overlapped in the intron. This was the case when the fragment was smaller than 900 bp, for larger fragments the missing region was elucidated by primer walk on the PCR fragment by primer design at the end of the resolved region. In summary, a conserved gene structure between human and cattle was postulated and the human exon pattern was applied on the EST sequence to identify exon-intron-boundaries. Than PCR systems were designed to amplify the intron sequence, which were subsequently sequenced.

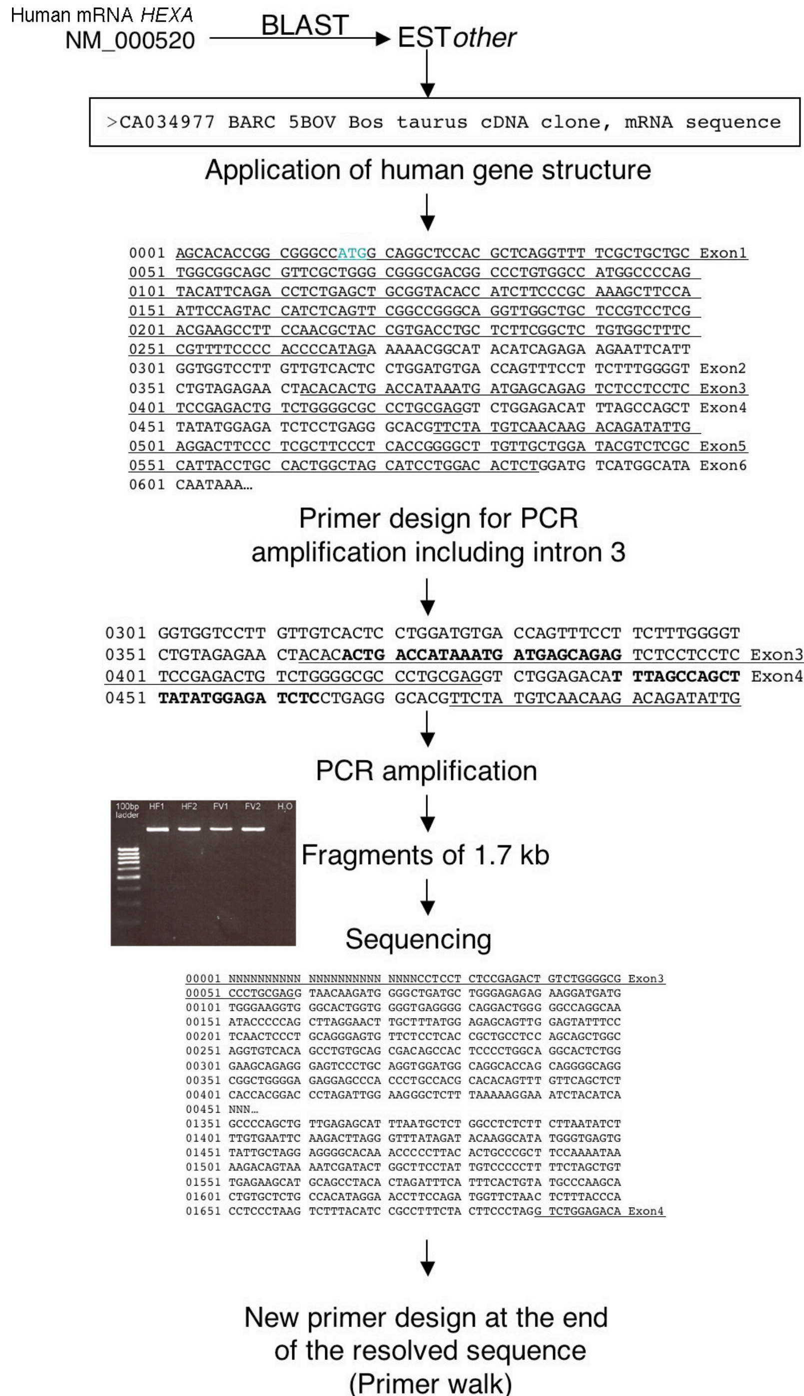


Figure 3.1 - Exemplified EST sequencing approach.
As first step, human mRNA of *HEXA* was used to BLAST the EST other division of NCBI and a homologous bovine EST sequence was obtained. In the second step, the exon pattern from human known gene structure was applied on the EST sequence (exons alternating underlined / not underlined). In the third step, a PCR system was designed to amplify intron 3 with up and downstream primers (highlighted by bold letters) in exon 3 and 4, respectively. The size of the fragment obtained by PCR was visualised by agarose gel electrophoresis (1.7 kb). Subsequently, the PCR fragment was sequenced with the same primers, used for amplification. The reads from both primers did not overlap; hence the intron was partially sequenced, only. To elucidate the missing region, primer walk on the PCR fragment was performed. Therefore, new primers at the end of the resolved sequence were designed and sequenced.

3.4.3 *Btau 1.0*: First genomic cattle sequence

In October 2004, the first preliminary genomic sequence for cattle was published and available at the Ensembl Web-service (www.ensembl.org; Pre!Cow v.25, *Btau 1.0*). A whole genome shotgun approach was employed and clones containing small DNA inserts of one Hereford bull were sequenced. About 15 million reads were assembled by the Atlas genome assembly system (Havlak et al., 2004) at the Baylor College of Medicine Human Genome Sequencing Center (Baylor HGSC) (http://www.hgsc.bcm.tmc.edu/projects/bovine/). This sequence represent about 9 Gb and a 3x coverage of the clonable bovine genome. If both end sequences from one clone did not overlap, a contig was build by filling the missing base pairs with an appropriate number of Ns as estimated by the insert size. Two contigs were assembled if they contained partly the same sequence. The total length of the scaffolds was 2.26 Gb and they ranged in size from 1 kb to 30 kb with an N50 size of 13.5 kb (i.e. 50% of all assembled scaffolds are greater than or equal to this length). This first assembly of the bovine draft sequence was of fragmentary nature with relatively short scaffolds in respect to the estimated mean gene size of 27 kb (Wong et al., 2001), leading to single genes being distributed across two or more scaffolds. By BLAST search, the scaffolds containing the candidate gene could be identified. The availability of these genomic sequences allowed optimisation of primer design and PCR for sequencing. Moreover, the scaffold sequences simplified primer design within the genes as well as for the 5'- and 3'- ends and the segments between the genes, e.g. for selective PCR amplification and sequencing of exons. To obtain the sequence of exons, primers were placed in intronic sequences, spanning the PCR system over the exon. However due to the incomplete nature of the scaffolds, gaps still existed not only between genes, but also within genes.

3.4.4 *Btau 2.0*: Second assembly of the cow genome

In June 2005, the Baylor HGSC published the second version of the bovine draft sequence (*Btau 2.0*). The new chromosomal linear scaffolds were produced by the Atlas genome assembly system (Havlak et al., 2004) with contigs of whole shotgun sequences and BAC end sequences of one Hereford bull. About 23 million reads were assembled, representing about 17.7 Gb of sequence and about 6.2x coverage of the bovine genome. The total length of all scaffolds was 2.62 Gb, with the N50 size of the scaffolds being 434.7 kb (i.e. 50% of all assembled scaffolds are greater than or equal to this length). Including the gaps in the scaffolds, the total length was inflated to 3.1 Gb. Therefore, 0.48 Gb or 15 % of the sequence were gaps filled with Ns. The scaffolds were composed of contigs that could be ordered and oriented with respect to one other by sequence accordance and were annotated in a 5'-3' direction from the centromere to the telomere of the chromosomes. In the cow genome released at the Ensembl web service (www.ensembl.org; *Btau 2.0*; v.39, June 2006) the contigs were assembled chromosome-wise and moreover, genes were annotated based on evidence derived from

known proteins of cDNA and EST sequences by the Ensembl Automatic Gene Annotation System (Curwen et al., 2004). Genes, which are not yet annotated in cattle but, e.g. in human or other species, were identified by orthologous prediction searching for similarity between the species.

3.4.5 *Btau 3.1*: Chromosomal assembly of the cow genome

In August 2006, the third draft assembly of the bovine genome (*Btau 3.1*) was released by the Baylor HGSC. Produced in combination of the Whole Genome Shotgun approach and the hierarchical approach using BAC sequences, the release was assembled from BAC shotgun reads and whole genome shotgun reads from small insert libraries as well as BAC end sequences. About 26 million reads were fitted which yields 7.0x sequence coverage. The total length of the scaffolds was 2.73 Gb with the N50 size being 997.5 kb. Additionally, contigs not overlapping and therefore not annotated on chromosomes exist with a N50 size of 48.7 kb. However, most of the sequence was annotated chromosome-wise and the number of Ns has been reduced in respect to the previous version. The total length of the sequence counted up to 2.73 Gb and including gaps to 2.87 Gb. Hence, 0.14 Gb or 5% of the sequence is filled with Ns (*Btau 2.0* contained 15 % unknown sequence).

3.5 Handling repetitive sequences

Stretches of highly repetitive DNA sequences are uniformly distributed over the bovine genome. These repetitive sequences might be similary represented in cattle and human, and are estimated to represent up to 50% of the whole genome. Different repetitive sequences are known: short simple repeats (e.g., AAAA), tandem repeats (e.g., $(CA)_N$ -usable as genetic markers-), segmental duplications of 10 - 300 bp blocks, processed pseudo-genes (non-functional re-integrated RNA copies), SINES (Short Interspersed Elements), DNA Transposons, Retroviral Retrotransposons or LINES (Long Interspersed Elements). Repetitive sequences cause technical problems during PCR due to unspecific primer binding (it binds maybe at more than one locus). Therefore, non-specific amplification could occur, which is often visible in double bands when the probe runs an agarose gel, followed by ambiguous sequencing results with highly polymorphic sequences. Variations found in repetitive sequences are inappropriate for genotyping because the origin of the different alleles is not clear. Genotyping in repetitive regions could entail an excess of heterozygous individuals because of disguising unspecific PCR fragments. Thus, it is important for sequencing and essential for genotyping procedures to check for repetitive sequences before designing primer assays. A tool to find repetitive sequences is *RepeatMasker* (Smit et al., 1996-2004). The programme identifies repetitive sequences and substitutes the corresponding sequences with Ns.

By using the N-masked output file to design primers, the risk of unspecific amplification is reduced.

3.6 Primer design

Primers were designed using the Primer3 software available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Rozen and Skaletsky, 2000). Default parameters for PCR primers were melting temperature of 60°C ± 3°C and optimal primer size of 20 bp with a range from 18 bp to 27 bp. Primers were purchased from MWG (Ebersberg, Germany). The SpectroDESIGNER™ software (SEQUENOM, Hamburg, Germany) was used to design the primer assays for genotyping with MALDI-TOF MS. All primers used for PCR, sequencing and genotyping are listed in Appendix 11.2 (Primers).

3.7 Screening of BAC Libraries

In order to obtain Bacterial Artificial Chromosomes (BACs), the male bovine BAC Library RPCI-42 (Warren et al., 2000) supplied by the Children's Hospital Oakland Research Institute (BACPAC Resources, Oakland, CA, USA) was screened with gene specific PCR products. The library was constructed from partially *Eco*RI digested and size selected DNA of Holstein Bull white blood cells. The DNA was cloned between the *Eco*RI sites of the pBACe3.6 vector and the ligation products were transformed into DH10B electro component cells. The BAC library was spotted onto 22x22 cm filters for hybridization screening with radio labelled DNA probes. The characterisation of the BAC library used is shown in the Table 3.2.

Table 3.2 - Characterisation of bovine BAC library

Segment	Cloning Vector	DNA	Total Plates	Total Clones	Average Insert size	Genomic Coverage
1	pBACe3.6	Holstein Bull White Blood Cell	288	108 776	165 kb	6.0 x
2	pBACe3.6	Holstein Bull White Blood Cell	288	107 663	163 kb	5.9 x
Total Library			576	216 439	164 kb	11.9 x

Generation of radio labelled probe

Cleaned PCR fragments amplified by gene specific primers with a concentration of 20ng/μl were used as input for generation of radio labelled probe. The PCR fragments were radio labelled using Megaprime DNA labelling system (RPN1604 Amersham Biosciences, Freiburg, Germany) and desoxy adenosine 5'-(α-^{32}P) triphosphate (AA0004-250 Amersham Biosciences, Freiburg, Germany). 40 ng of PCR products were adjusted to a volume of 21μl and together with 5μl random nanomer primer mix (Amersham Biosciences, Freiburg, Germany)

denatured at 95°C for five minutes. The reaction was chilled and the following reagents were added: 5 μl reaction buffer (10 x), 4μl of dGTP, dCTP and dTTP, 2μl Klenow Enzym and 5μl $dATP^{32}$ (1.85 kBq). After incubation for 15 minutes at 37°C, the reaction was stopped by adding 10μl of 0.2 M EDTA (pH 8.0). The radio labelled probes were denaturised at 95°C for seven minutes.

Dot-Blot as positive control

Two μl of each, PCR product and its ten- and hundred-fold dilution were blotted onto nylon membranes (Hybond-N+, PRN303B; Amersham Bioscience Freiburg, Germany), which were saturated with 0.4N NaOH. After five minutes, the dot-blot was shaken for one minute in 5x SSC buffer.

Hybridization

Two filters of the BAC library were applied to a roller bottle and pre-hybridised at 67 °C for 30 minutes together with a dot-blot control and 15-20 ml of Church buffer (Church and Gilbert, 1984). Subsequently, 17 μl of radio labelled probe and 10ml of Church buffer was added in each bottle and incubated for hybridisation at 67°C overnight (14-16 hours). Filters were washed for 20 minutes at 63°C twice in 2x SSC and a third time in 0.5x SSC + 0.1% SDS. Finally, each filter was rinsed in 2x SSC, wrapped in plastic film and placed together with medical X-ray film NewRX (03E220; FUJIFILM Medical Systems, Stamford, CT, USA) in cassettes. X-ray films were exposed for eight hours at –80°C. After the development of the X-ray films, the positive clones could be identified. From the position on the filter the positive clones can be named by filter number, plate number and position on the plates and ordered. A colony PCR with the primers used for the screening attested the containing of the right insert in the clones.

3.8 Preparation of BAC DNA

The starter culture was made from a single colony picked after laying out the clones on a selective lurid broth (LB) agar plate (containing 12.5μg/mL chloramphenicol (CHL) 0634433; Roche Diagnostics, Mannheim, Germany). The colony was applied in 3ml LB medium (12.5μg/mL CHL) and strongly shaken with 300rpm for 8 hours at 37°C. For the culture 100ml LB medium (12.5μg/mL CHL) was inoculated with 500μl of the starter culture and incubated overnight at 37°C with shaking at 300rpm. For BAC DNA preparation, the Qiagen Plasmid Midi Kit (12462; Qiagen, Hilden, Germany) was used according to manufactures manual. The DNA pellet was re-dissolved in 500μl 10mM Tris-Cl (pH 8.0) and desalted using Microcon YM-100 filters (42413, Millipore, Eschborn, Germany). Samples were centrifuged at 7000rpm for 15 minutes and another 10 minutes after adding 250μl 10 mM Tris-Cl (pH

8.0). After adding 200μl Tris-Cl (pH 8.0) an upside down centrifugation of the filters for 1 minute at 7000rpm recovered the cleaned BAC DNA. The concentration of the DNA was measured with a fluorometer (DyNA Quant200; Hoefer Pharmacia BiotechInc, San Francisco, CA, USA).

3.9 Polymerase Chain Reaction (PCR)

3.9.1 Standard PCR

Standard PCR reaction was made in a volume of 20 μl per sample. Master Mix was prepared containing 0.5 Units of Qiagen Taq Polymerase (1005476, Qiagen Hilden Germany), 1x Qiagen PCR buffer (containing 1,5 mM MgCl), 200 μM of each nucleotide and 0.5μM of forward and reverse primer (MWG) and 50 ng of DNA was amplified in a T-Gradient Thermocycler (Biometra, Göttingen, Germany) under the following conditions: initial denaturation at 94 °C for three minutes, followed by 30 cycles consisting of 30 seconds at 94°C for denaturation, 60 seconds at 60 °C for primer annealing and 60 seconds at 72 °C for extension. This was followed by a final extension step for three minutes.

3.9.2 Primer optimisation

Each primer system was tested with bovine DNA with an annealing temperature of 60 °C in three different reactions: standard PCR reaction without any additives (plain), the standard PCR reaction with 5% DMSO (dimethyl sulfoxid) and the standard PCR reaction with 1x Q-solution (Qiagen). The conditions supplying the best amplification results were applied for the sequencing panel. In case of unspecified amplification a gradient PCR with varying annealing temperature was performed. PCR products together with a DNA marker (Genruler 100 bp ladder: SM024; MBI Fermentas, St. Leon-Rot, Germany) were tested by 2 %-agarose gel electrophoresis.

3.9.3 Long range PCR

In order to amplify fragments larger than 2 kb, a long range PCR protocol was used. In addition to AmpliTaq Polymerase (201207; Qiagen, Hilden, Germany), ProofStartTaq (202203; Qiagen, Hilden, Germany) as proofreading polymerase was used for the amplification. Proofreading is achieved by a 3' to 5' exonuclease activity where the polymerase recognizes and removes incorrectly incorporated desoxynucleotides. The exonuclease activity can lead to primer degradation during PCR setup, which result in unspecific amplification products. Qiagen ProofStartTaq polymerase has been chemically modified for initial temporally inactivation. The enzyme is activated by the initial denaturing step at 95°C. The reaction was performed in a volume of 25 μl with an input of 50ng DNA, 1x PCR buffer (including $MgSO_4$),

1μM of each primer, 300μM of each nucleotide, 1 Unit AmpliTaq and 0.1 Unit ProofStartTaq Polymerase in a T-Gradient Thermocycler (Biometra, Göttingen, Germany) under the following conditions: initial denaturation at 94 °C for three minutes, followed by 35 cycles consisting of 10 seconds at 94°C for denaturation, 60 seconds at 60 °C for primer annealing and depending on the expected fragment-length 60 seconds per kb at 68 °C for extension. The last extension step at 68°C for 180 seconds was completing the reaction before cooling.

3.10 DNA Sequencing

The sequencing was performed according to Sanger et al. (1977) (chain-termination-method) with fluorescent didesoxy-nucleotides. The generated PCR-products were sequenced by BigDye® Terminator Cycling Kit v1.1 (BigDye) (Applied Biosystems, Foster City, CA, USA). The products were separated by polyacrylamid gel electrophoresis with the help of an automated sequencer ABI 377 (Applied Biosystems, Foster City, CA, USA).

Sequencing reaction for BAC DNA

Primerwalking on BAC DNA was performed in total 10 μl volume, by adding 150 ng of BAC-DNA, 4μl BigDye and 2.5 pM of either up or down primer. For BAC-end sequencing, the initial primers were derived from the T7 and SP6 promoter sites, which were located on the pBACe3.6 vector flanking the insert. For BAC sequencing the conditions for temperature cycling was initial denaturation for five minutes at 96°C, followed by 100 cycles being 20 seconds at 96°C, 20 seconds at 58°C and four minutes at 60°C.

Sequencing reaction for PCR products

In order to sequence PCR-fragments, they were purified with Multi-Screen-Filtration-Plates (MANU03010; Millipore, Eschborn, Germany) under vacuum (MAVM0960R; Millipore, Eschborn, Germany). Products were eluated with 40μl Tris-HCl (10mM) and quantity was tested together with a DNA marker (Genruler 100 bp ladder: SM0241; MBI Fermentas, St. Leon-Rot, Germany) by 2 %-agarose gel electrophoresis. Subsequently, the sequencing reaction was performed in a 10μl volume. 10-20 ng of purified PCR fragment, 2 - 4μl BigDye and 2.5 pM of either up or down primer was added. Initial denaturation for 15 seconds at 96°C, followed by 35 cycles of 10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C was applied to the reactions.

Cleaning of sequencing reactions

Sequencing reactions were cleaned from unincorporated dye terminators by gel filtration. The purifying step was done with MultiScreen filtration plates (MAHVN4510; Millipore, Eschborn, Germany), which were loaded with 45 μL of Sephadex G-50 Fine (G-50-50; Sigma-

Aldrich Chemie GmbH, Deisenhofen, Germany) and with 300 μL of double distilled water. After three hours incubation at room temperature, the columns were swelled and excessive water was removed by centrifugation at 900 g for five minutes. The sequencing reactions were applied on the top of the columns and by centrifugation at 900 g for five minutes it was filtered through and collected in a reaction plate. The samples were dried in a vacuum centrifuge (Speed Vac Plus, SC110A; Thermo Savant, Holbrook, NY, USA) and resolved in formamide (47670, Fluka, Buchs, Switzerland) coloured by dextran blue dye (31393, Fluka, Buchs, Switzerland). Finally, denaturation for two minutes at 96°C was performed.

Seperation of sequencing reactions

For separating the sequencing products, a 36 cm WTR (well to read) polyacrylamid gel with following composition was used: 21g of urea, 20 ml of water (HPLC grade), 8.4 ml of 30% acrylamide/bisacrylamide (29:1), 6.0 ml of 10x TBE buffer, 20µl of TEMED and 300 µl of 10%-ammonium persulfate solution. Samples were run on the automated sequencer ABI 377 (Applied Biosystems, Foster City, CA, USA) in 1x TBE using the module *SeqRun 36E-1200* with a collection time of 8 hours.

3.11 Analysis of the sequences and SNP detection

The traces of the separated sequencing products were processed by the ABI Sequencing Analysis Software (v. 3.1; Applied Biosystems, Foster City, CA, USA). The chromatogram output files were reanalysed for base calling, sequence assembly and polymorphism detection by Phred/Phrap/Polyphred software suite (Ewing and Green, 1998; Ewing et al., 1998; Nickerson et al., 1997). Consed (Gordon et al., 1998) was used for viewing and editing the sequence assemblies. In Figure 3.3 the Consed view of 15 aligned sequences with a polymorphic site at position 221 is shown.

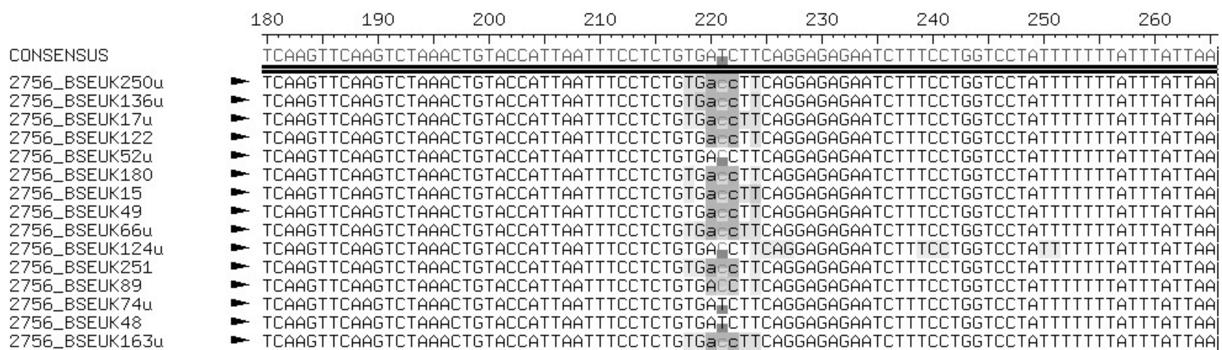


Figure 3.3 - Consed view of aligned sequence traces

Sequence alignment of 15 samples indicating a SNP polymorphism (C/T) at position 221.

3.12 Estimation of allele frequencies in the populations

Allele frequencies were preliminary estimated by comparing sequencing traces of pooled DNA samples with sequencing traces of individual animals (Kwok et al. 1994). The peak heights at the SNP position of the sequencing traces from a heterozygote and these of the DNA pools were compared (see Figure 3.4) and the allele frequency of the pools were approximately estimated. Later genotyping of all individuals of each pool revealed the exact allele frequencies, showing that these estimations are appropriate to determine the approximative allele frequencies.

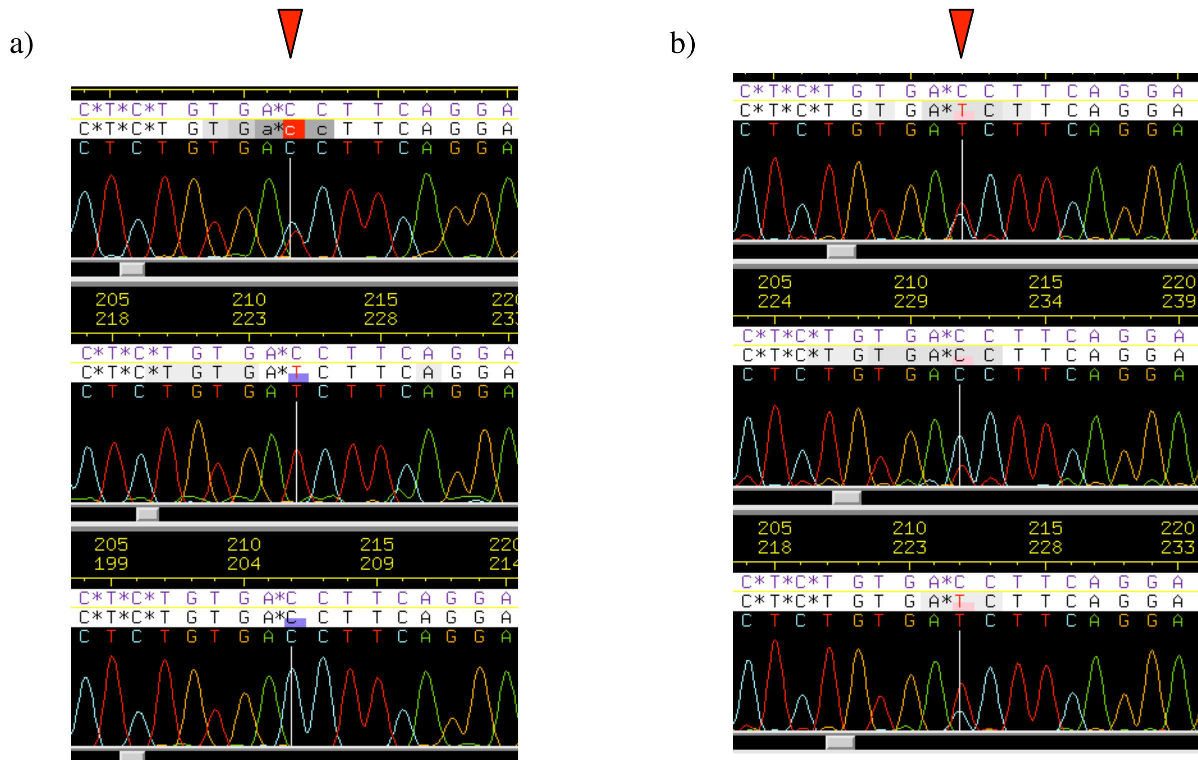


Figure 3.4 - Comparison of sequencing traces of a) individual animals and b) pooled DNA
From top to bottom are shown in a) a heterozygote CT, a homozygote T and a homozygote C in b) pool traces from German Holstein, German Fleckvieh and German Brown. From the peak heights of the pool traces, preliminary allele frequencies for the T allele were approximately estimated: here frequencies were suggested being around 0.65, 0.30 and 0.80 for HF, GF and GB, respectively. (Genotyping of the pool animals revealed the exact allele frequencies for each pool, 0.73, 0.35 and 0.68 for HF, GF and GB, respectively. The approximative estimations differ between 0.05 and 0.12 from the real frequencies.)

3.13 Selection of SNPs / polymorphisms for genotyping

3.13.1 SNPs for the Genomic Control (GC)

For the GC approach a SNP panel initially developed for paternal testing and identity control (Werner et al., 2004) was used. The panel consists of 37 SNP markers, which were allocated over the whole genome and are considered as being polymorphic in the European cattle breeds Holstein Friesian, German Fleckvieh and German Brown.

3.13.2 SNPs for the Association Study

SNPs in the candidate genes *ARIH1, HEXA*, *BRUNOL6* and *PARP6* were selected for genotyping in BSE diseased and control animals. The SNPs were derived by comparing sequencing of DNA pools and individual animals of different breeds.

SNPs were selected for genotyping considering the following criteria:

- SNP is not located in repetitive sequence.
- Exonic SNPs and SNPs in regulatory regions (Splice sites, 5'-UTRs, promoter regions) were preferentially chosen, subsequently intronic SNPs.
- Pre-estimated allele frequency of the rare allele is >10% in German Fleckvieh, German Holstein and UK Holstein animals.
- SNPs are evenly distributed over the gene.
- Extension primer region (up to 50 bases around the SNP) is free of variation.

Additionally, two insertion/deletion polymorphisms (indel) in the regulatory region of the prion protein gene (*PRNP*) were selected because it has been shown in a small case-control study (Sander et al., 2004) that these variants were tentatively associated with BSE. The indel polymorphisms were genotyped with the first base of the insertion and the first base after the insertion representing the insertion and deletion alleles, respectively.

Futhermore, eight SNPs in four additional candidate genes have been selected because the genes encoding Plasminogen (*PLG*), Heat-shock protein 70 (*HSPA8*), Iron-responsive binding element (*IREB2*) and the Laminin Receptor (*LAMR1*) have been postulated as candidate genes for BSE susceptibility in Juling (2002).

3.14 Genotyping SNPs with MALDI-TOF MS

Matrix Assisted Laser Desorption/Ionisation (MALDI) is a pulsing technique, which can analyse molecule mass with the help of a Time of Flight (TOF) analysator. The probe is co-crystallised with an excess of suitable matrix, most frequently an organic acid (e.g. 3-hydroxypicolinic acid) and is hit by a very short laser pulse in the mass spectrometer under high vacuum. The laser ionizes the analyt, consisting of DNA molecules and matrix, and introduces it into the flight tube. The role of the matrix is to absorb the laser energy during subsequent collisions with the DNA molecules resulting in the formation of DNA ions. Furthermore it secures the probe from photolytic damages and from interaction of the analytes among each other. The generated ions are all accelerated to the same potential in an electric field causing them to fly through a field - free drift region, also known as flight tube, to the detector, where they are separated according to their mass-over-charge ratio. Ions with a higher mass-over-charge ratio pass slower through the flight tube and achieve the detector later than ions with a small mass-over-charge ratio. Lighter ions (smaller DNA fragments) travel faster, than heavier (larger DNA fragments), leading to a separation of the fragments based on their mass difference. The electric measurement of the time of flight from the start of the ions to the arrival at the detector is a Time Of Flight Mass Spectrometer (TOF MS).

3.14.1 The hME (homogeneous MassExtend™) method

The principle of the hME method (homogenous MassExtend™, Sequenom, Hamburg, Germany) is shown in Figure 3.5. In order to genotype SNPs with this method, first, the genomic sequence in a scope of 200 to 500 bp around the SNP locus has to be amplified. The PCR assays can be multiplexed up to ten PCR primer systems. The PCR primers are modified at the 5'- end by adding a ten base pair tag in order that they are not disturbing the measurement of the MALDI-TOF analysis by being out of the range of the extension primers. Excessive and unbound nucleotides were degraded by incubation with SAP (Shrimp Alkaline Phosphatase) since they could be built in the extension products and falsify the results. During the hME reaction, the extension primers anneal adjacent to the SNP and the addition of a DNA polymerase along with a mixture of nucleotides and a terminator nucleotide allows extension through the polymorphic site and generates allele specific extension-products, each having a unique molecular mass. As shown in Figure 3.5, hME uses 1 base in conjunction with 2-3 bases extension products for genotyping in order to create large mass differences between the allelic specific products. Before the extension products can be spotted on a microchip (SpectroChip™, Sequenom, Hamburg, Germany) by a nano-dispenser (SpectroPoint™ Nanoliter Pipetting System, Sequenom, Hamburg, Germany), the conditioning of the products by treating with a special ion exchanger resin (SpectroClean™, Sequenom, Hamburg, Germany) is necessary.

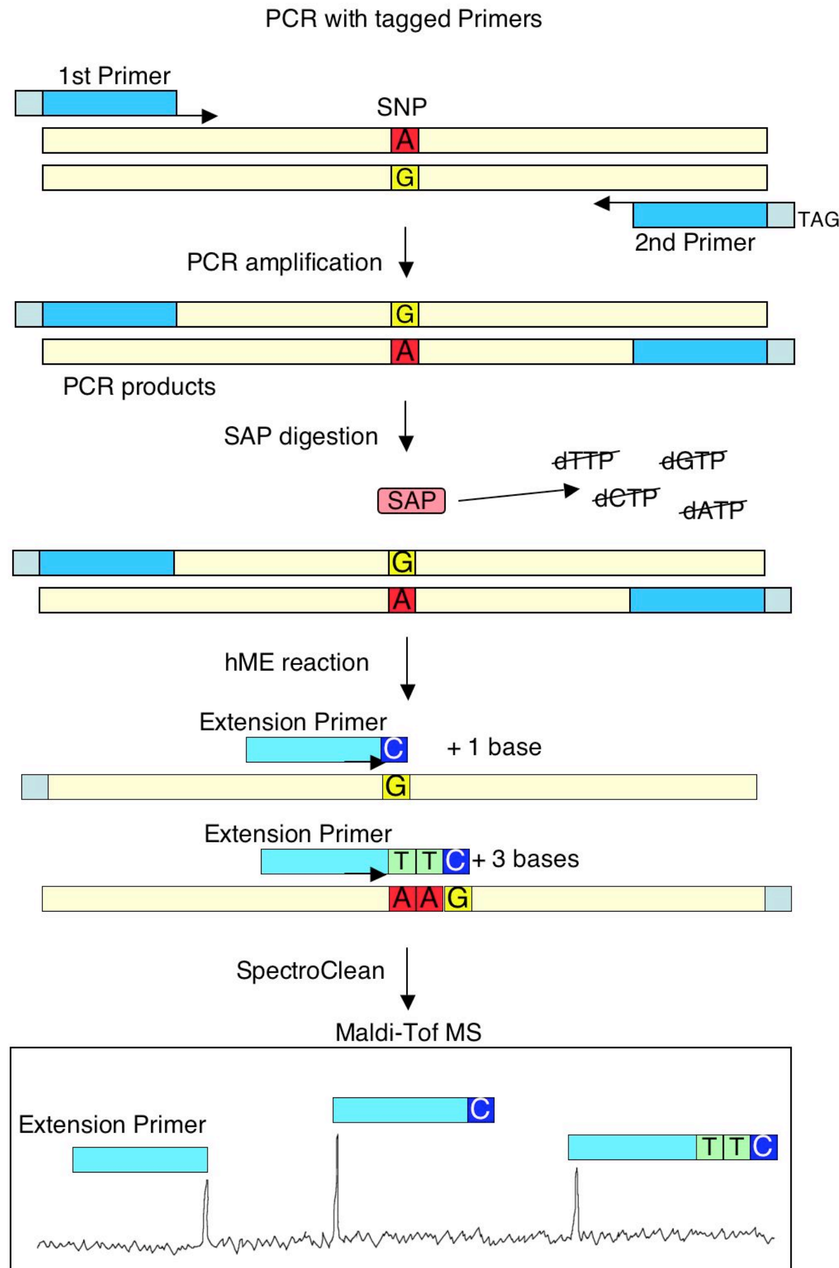


Figure 3.5 - Principle of the hME method.
After PCR amplification with tagged primers, the unbound nucleotides were dephosphorylated by SAP digestion. The hME reaction contains a so-called stop-mix (e.g. dTTPs and ddCTPs that terminates the elongation). Extension primers were elongated depending on the SNP allele. The two extension products can be discriminated by their mass with MALDI TOF MS.

3.14.1.1 PCR reaction for the hME method

The total volume used in the PCR reaction was 6μl in a 384-microtiter plate, each well containing 5ng over-night-dried DNA. For dispensing of the master mix, the pipetting station Genesis RSP 150 Workstation (Tecan, Crailsheim, Germany) was utilized. A single reaction

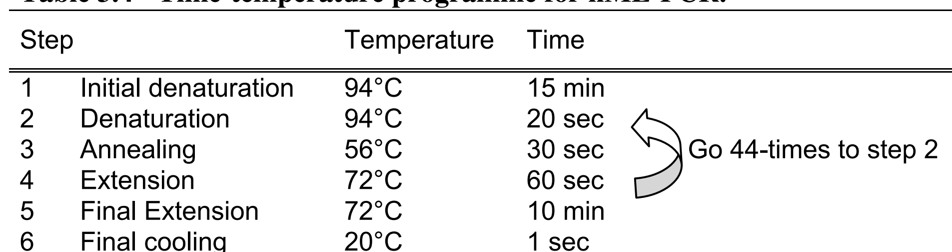
included the PCR reagents (HotStar Taq™, buffer and $MgCL_2$ from Abgene, Hamburg, Germany) as shown in Table 3.3.

Table 3.3 - Master Mix for hME-PCR (single reaction).

Volume (µl)	Reagent	Concentration
0.60	PCR-Buffer	10 x
0.60	dNTPs	2mM
0.60	MgCl2	25mM
0.01	Primer up	100pmol/µl
0.01	Primer dn	100pmol/µl
0.02	HotStar Taq™-Polymerase	5U/µl
4.16	H_2O	
6.00		

For thermo cycling, a PCR PTC 225 Tetrad, Peltier Thermal Cycler of MJ Research (Boston, USA) was used with the time-temperature-programme as shown in Table 3.4 with 45 cycles.

Table 3.4 - Time-temperature programme for hME-PCR.



Step		Temperature	Time	
1	Initial denaturation	94°C	15 min	
2	Denaturation	94°C	20 sec	
3	Annealing	56°C	30 sec	Go 44-times to step 2
4	Extension	72°C	60 sec	
5	Final Extension	72°C	10 min	
6	Final cooling	20°C	1 sec	

3.14.1.2 SAP-digestion

For the Shrimp Alkaline Phosphatase (SAP, Amersham Biosciences, Freiburg, Germany) treatment to dephosphorylate unincorporated dNTPs, one reaction with a total volume of $2\mu l$ contained reagents as shown in Table 3.5.

Table 3.5 - Mix for SAP-Treatment (single reaction).

Volume (µl)	Reagent
0.17	hME buffer
0.30	SAP
1.53	H_2O
2.00	

$2\mu l$ of the solution was dispersed in the wells over the plates with a Multimek96 Automated 96-Channelpipettor (Beckmann Coulter, Fullerton, USA) and the incubation and SAP-deactivation was done in a PCR PTC 225 Tetrad Peltier Thermal Cycler of MJ Research (Boston, USA) as shown in Table 3.6.

Table 3.6 - Incubation time-temperature programme for SAP treatment

Step		Temperature	Time
1	Incubation	37°C	20 min
2	Deactivation of SAP	85°C	5 min
3	Final Cooling	20°C	1 sec

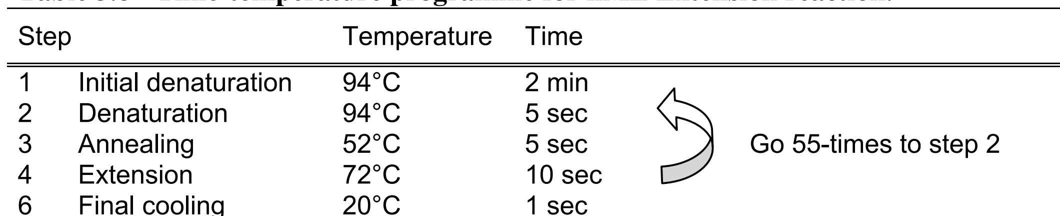
3.14.1.3 Primer extension reaction for the hME method

Primer extension was performed with the MassExtend enzyme (Sequenom, Hamburg, Germany) which is a Thermosequenase and the hME Extend Mix, which contains Buffer and dideoxy-nucleotides and deoxynucleotides, respectively) 2μl of the hME-Mix Cocktail (see Table 3.7) was dispersed with a Multimek96 Automated 96-Channelpipettor (Beckmann Coulter, Fullerton, USA) into the wells of the 384 well plate. The time-temperature programme as used for the Primer-extension reaction in a PCR PTC 225 Tetrad Peltier Thermal Cycler of MJ Research (Boston, USA) is shown in Table 3.8.

Table 3.7 - Master Mix for hME Extension reaction (single reaction).

Volume (2µl)	Reagent	Concentration
0.200	hME Extend Mix	10 x Buffer + 2.25 mM of each d/ddNTP
0.054	hME Primers	100 pmol/µl
0.018	MassExtend enzyme	32U/µl
1.728	H_2O	
2.000		

Table 3.8 - Time-temperature programme for hME Extension reaction.



Step		Temperature	Time	
1	Initial denaturation	94°C	2 min	
2	Denaturation	94°C	5 sec	
3	Annealing	52°C	5 sec	Go 55-times to step 2
4	Extension	72°C	10 sec	
6	Final cooling	20°C	1 sec	

3.14.2 The iPLEX method

The iPLEX method (Sequenom, Hamburg, Germany) is shown in Figure 3.6. Similar to the hME procedure (see Chapter 3.14.1 – The hME method), the first step is the PCR amplification of the region around the SNP with tagged primers. IPLEX allows multiplexing the PCR reaction up to 29 different SNP loci. Than, unbound nucleotides were dephosphorylated by incubation with SAP since they could be built in the extension products and falsify the results. The most significant difference relative to the hME method is that all reactions for the iPLEX assay are terminated by a single base extension (SBE), meaning that the differences between the two allelic specific products arise only from the different mass of the modified nucleotides. Table 3.9 shows the mass differences between the SBE products of the iPLEX assay.

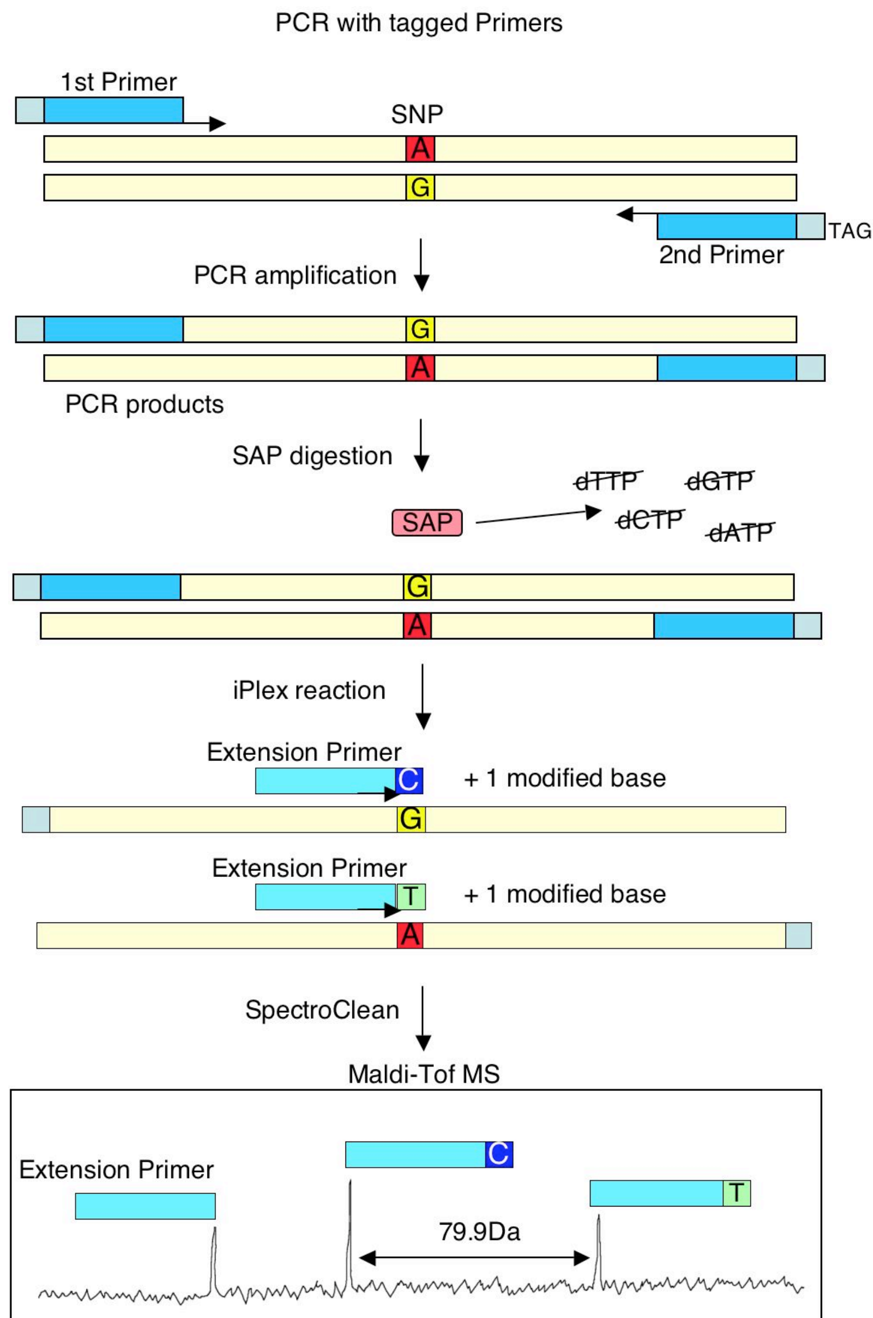


Figure 3.6 - Principle of the iPLEX method.
After PCR amplification with tagged primers, the unbound nucleotides were dephosphorylated by SAP digestion. The iPLEX reaction contains chain terminating nucleotides. Extension primers were elongated by one base depending on the SNP allele. The two extension products can be discriminated by the mass differences of the incorporated bases with MALDI TOF MS.

Characteristic for the MassARRAY® MALDI-TOF MS is the inverse relationship between product signal-to noise ratio and increased size of product mass. Therefore, the larger the extension product, the smaller the signal-to-noise ratio visualised as peaks. To compensate for

this effect in the high multiplexed reaction, it is suggested to load 2x the concentration of the oligomers that occupy the large mass portion. The loading concentration was stepwise adjusted according to the mass, i.e. for a light extension product, the half amount of primer was used compared to a heavy product.

Table 3.9 - Mass differences between iPLEX extension products
Mass differences (dalton) between the extension products depend on the incorporated base.

Terminator	A	C	G	T
A	0	-24	16	55.9
C	24	0	40	79.9
G	-16	-40	0	39.9
T	-55.9	-79.9	-39.9	0

3.14.2.1 PCR reaction for the iPLEX method

The total volume for an iPLEX PCR-reactions was 5μl in a 384-microtiter plate, each well containing 5ng over-night-dried DNA. The pipetting station Genesis RSP 150 Workstation (Tecan, Crailsheim, Germany) was used to dispend the master mix. Due to high multiplexing level (up to 29) primers were pooled to the required concentration of 0.5μM for the forward and reverse primer, respectively. HotStar Taq-Polymerase, PCR-Buffer and $MgCl_2$ from Qiagen (Hilden, Germany) was used and a single reaction contained as shown in Table 3.10.

Table 3.10 - Master Mix for iPLEX-PCR (single reaction).

Volume (µl)	Reagent	Concentration
0.625	PCR-Buffer	10 x
0.100	dNTPs	25 mM
0.325	$MgCl_2$	0.5 µM
1.000	Primer Mix pooled	100 pmol/µl
0.100	HotStar Taq™-Polymerase	5 U/µl
2.650	H_2O	
5.000		

The same time-temperature programme as for the hME-PCR (see Chapter 3.14.1.1 - PCR reaction for the hME method; Table 3.8) as well as the same thermal cycler was employed. SAP treatment to dephosphorylate unincorporated dNTPs was performed as shown in Chapter 3.14.1.2 – SAP digestion.

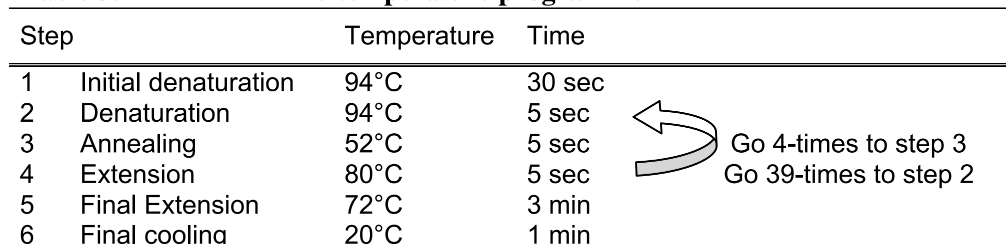
3.14.2.2 Primer extension for the iPLEX method

The iPLEX reagent kit (including Shrimp Alkaline Phosphatase and buffer; iPLEX-Enzyme mix and buffer, iPLEX Termination mix and a calibrant for the MALDI-TOF measurement) was used. For each multiplexed assay, the extension primers have to be pooled in a primer mix. A single iPLEX primer extension reaction contained reagents as shown in Table 3.11.

Table 3.11 - Master Mix for iPLEX-Extension reaction (single reaction).

Volume (µl)	Reagent
0.200	iPLEX Buffer (10x)
0.200	iPLEX Termination Mix
0.054	Primer mix
0.041	iPLEX Enzyme
0.755	H_2O
2.000	

Table 3.12 - iPLEX Time-temperature programme



Step		Temperature	Time	
1	Initial denaturation	94°C	30 sec	
2	Denaturation	94°C	5 sec	
3	Annealing	52°C	5 sec	Go 4-times to step 3
4	Extension	80°C	5 sec	Go 39-times to step 2
5	Final Extension	72°C	3 min	
6	Final cooling	20°C	1 min	

3.14.3 Processing of the extension products for MALDI-TOF MS

Special ion exchanger resin (SpectroClean™, Sequenom, Hamburg, Germany) was used to clean up the extension products. Therefore, 3mg of resin and 16 μl water were added directly to the extension products and the plates were incubated for 10 min on a rotator to mix and then centrifuged for 5 min at 1600rpm.

3.14.4 MALDI-TOF MS Analysis

To analyse the extension products by MALDI –TOF MS, 2 nl of the reaction was spotted onto a silicium chip (SpektroCHIP™, Sequenom, Hamburg, Germany) using a nanodispenser (SpectroPoint Nanoliter Pipetting System™, Sequenom, Hamburg, Germany). On these chips, 384 spots with a crystal matrix (3-hydroxypicolinic acid) and 10 more matrix spots for the calibrant (Sequenom, Hamburg, Germany) were arranged. The calibrant is consisting of three different oligos with a known mass to level the analysis system. After the spotting, the chip was infiltrated into the vacuum lock of the MassARRAY™ mass spectrometer (Sequenom, Hamburg, Germany). Calibration of the system, measuring of the extension products and the analysis was done automatically under standard settings.

3.15 Processing the genotype data and statistical analysis

3.15.1 Data handling

The dynamic object-oriented programming language *Python* (http://www.python.org/) and a MySQL database (http://www.mysql.com) was used to handle the large amount of data. Database loadings and database queries for statistical analysis was achieved by standardised pro-

cedures with the help of *Python* scripts and *SQL* (structured query language) queries. The Open-Source-Software R (http://www.r-project.org/) was mainly used for statistical analysis and creating figures. R is a language and environment for statistical computing and graphics by providing a wide variety of statistical features and graphical techniques. So-called packages -ready-made tools for different applications- are accessible to download on the web page: e.g., for genetic implementations the package *genetics (v.1.2.0)* was used.

3.15.2 Test for Hardy-Weinberg-Equilibrium

Deviations from Hardy-Weinberg-Equilibrium (HWE) can indicate problems in the genotyping procedure. HWE describes the condition that in absence of mutation, migration, selection and non-random mating the genotype frequencies at any locus are a simple function of the allele frequencies. For a biallelic SNP marker with the alleles A and B the basic formula for the HWE is $n = np^2 + 2npq + nq^2$, where n is the number of animals, p is the frequency for allele A and q is the frequency for allele B. np^2 is the number of homozygous genotypes AA, $2npq$ the number of heterozygous genotypes AB and nq^2 the number of homozygous genotypes BB. By knowing the genotype frequency the corresponding allele frequency can be estimated and vice versa. If the observed genotypes do not accord with the estimated, the results deviate from HWE. In this study, deviations from HWE were tested by the Exact test statistic (Wigginton et al., 2005) that is implemented in the Software *Haploview (v.3.3)* (Barrett et al., 2005). By default, a deviation from HWE was assumed, if the P-value was below 0.001.

3.15.3 The Armitage Trend test

Sasieni (1997) suggest that the allele frequency based calculation of the test statistic (χ^2) is less robust against deviations from HWE than a trend test statistic such as the Armitage Trend test (Armitage, 1955; Sasieni, 1997) using the genotype information in a 2x3 contingency table. In this thesis, genotyping results were discarded only with strong deviation from HWE (< P-value of 0.001). Therefore, the Armitage Trend test was performed in order to test, if the allele A of a SNP occurs with a significantly higher or lower frequency in the affected group compared to the unaffected. The data for each biallelic marker are given in a 2x3 contingency table of genotypes by cases and controls as shown in Table 3.13.

Table 3.13 - 2x3 Contingency table for the Armitage Trend test.

	AA	AB	BB	Total
Cases	r_{AA}	r_{AB}	r_{BB}	R
Controls	s_{AA}	s_{AB}	s_{BB}	S
Total	n_{AA}	n_{AB}	n_{BB}	N

Thus, the chi-square test for no association between disease and genotype, calculated under an additive genetic model, is

$$Y^2_{trend} = \frac{N\{N(r_{AB} + 2r_{BB}) - R(n_{AB} + 2n_{BB})\}^2}{R(N-R)\{N(n_{AB} + 4n_{BB}) - (n_{AB} + 2n_{BB})^2\}}.$$

The trend values (Y^2) follow the chi-square distribution with one degree of freedom. The significance thresholds for 5% and 1% type 1 error rate are 3.84 and 6.62, respectively. The Armitage Trend test statistic was implemented in the *R* package association created by R.Fries.

3.15.4 Logistic regression analysis

Logistic regression is part of a category of statistical models called generalized linear models and is used to model dichotomous (0 or 1) outcomes, such as the case-control status. This technique models the log odds of an outcome defined by the values of covariates in the model. For logistic regression, the *R* package 'logistf', version 1.05 (Heinze and Ploner, 2004) was applied, which fits parameters based on penalized maximum likelihood estimation. Likelihood ratio test were applied as proposed by North et al. (2005) to investigate both the mode of allelic action (additive vs. dominance effects) and for possible epistatic effects (additive by additive, additive by dominance, dominance by dominance interactions) for the *PRNP* polymorphism data.

3.15.5 Measuring Linkage Disequilibrium and *tagging* SNPs

Pairwise Linkage Disequilibrium (LD) is a non-random association between two loci. LD represents a condition in which some combinations of marker alleles occur more or less frequently in a population than would be expected from a random arrangement of haplotypes from the allele frequencies. The two most common measures of LD are the Lewontin's standardized disequilibrium coefficient D' (Lewontin, 1964) and the square of correlation coefficient r^2 (Hill and Robertson, 1968). Both methods are implemented in *Haploview (v3.3)* (Barrett et al., 2005).

Measuring the LD between SNPs is of particular importance in preliminary studies which aim at selecting a marker set that represents all existent haplotypes. SNPs which are in strong LD are redundant, i.e. genotyping of one of these SNPs is enough to determine the genotypes of the other SNPs. From the redundant SNPs, those are chosen for further genotyping which cover all known variation. This selection is called *tagging* SNPs and the SNPs are called *tag* SNPs. By genotyping these *tag* SNPs the haplotype can be uniquely determined. In this study, SNPs were *tagged* by using the software *Tagger* (de Bakker et al., 2005) that combines a pairwise r^2 method with a multi-marker haplotype approach and is implemented in *Haploview*

(v3.3) (Barrett et al., 2005). The *tagging* can be influenced by changing the thresholds for r^2 and LOD for the multi-marker tests and by forcly in- and excluding of specific SNPs.

3.15.6 Inferring haplotypes

The analysis of multiple SNPs was carried out with the software *Haploview (v3.3)* (Barrett et al., 2005), which infers haplotypes based on the expectation maximization method. *Haploview* infers the haplotype frequencies within cases and controls and performes χ^2-tests to identify frequency differences. *Phase (v2.1.1)* (Stephens and Donnelly, 2003; Stephens et al., 2001), which applies a Bayesian statistical framework, was used to derive individual haplotypes and diplotypes. Diplotypes with posterior probabilities lower than 0.9 were excluded from further analysis.

3.15.7 Correction for Multiple Testing

The correction for multiple testing was performed by the permutation procedure, which is implemented in *Haploview (v.3.3)* (Barrett et al., 2005). This procedure was applied to the genotyping results of 38 SNPs in the extended *HEXA* region in the UK Holsteins, first involving only the single marker associations. The haplotype association analysis required a second permutation procedure. Each permutation shuffles an association test of the null-hypothesis that there are no differences between two marker alleles. The higher the number of permutations the more compact the distribution curve and therefore a better fitting for inferring the empirical (permuted) P-values. Depending on the number of permutations performed and the number of empirical P-values exceeding the highest observed (non-permuted) P-value, the empirical P-value is calculated by the integral area below the distribution curve exceeding the observed P-value for each SNP. SNPs having an empirical P-value < 0.05 are significantly associated with the trait. In Figure 3.7, the distribution curve after 10^5 permutations is displayed.

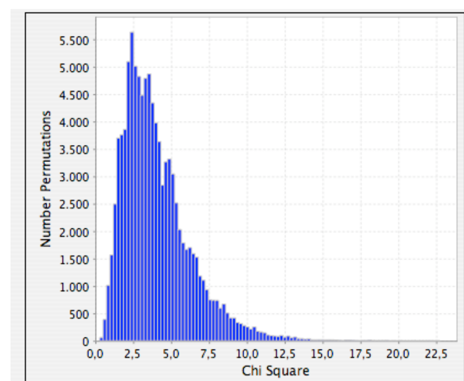


Figure 3.7 - Distribution curve of 10^5 permutation tests
Permutations shuffled an association test that there are no differences between two marker alleles (view from *Haploview (v3.3)* (Barrett et al., 2005)).

For the German Holstein association study, the permutation procedure of *Haploview* could not be used. The German control animals consists of paternal half-sibs and the maternal inherited alleles were inferred and genotype data for the maternal population estimated. Hence, the single genotype data, all used for the controls were not assigned to individuals, but used as maternal population genotype frequency (see Chapter 3.15.10 – Inferring allele and genotype frequencies from half-sibs). Therefore, Bonferroni- Holm correction for multiple comparisons was applied to the German Holstein data or else when required: For this approach, the global alpha-value ($alpha_g$) is normally set to 0.05 and the p-values of n comparison were sorted from the lowest to the highest. The alpha value for the lowest p-value is set equal to $alpha_g/n$. For the 2nd lowest p-value, the alpha value is set equal to $alpha_g/(n-1)$. For the next p-value, alpha value is set equal to $alpha_g/(n-2)$, etc.

3.15.8 Inferring the Inflation factor as Genomic Control (GC)

GC (Devlin and Roeder, 1999) was performed to identify population stratification. A GC approach attempts to find an inflation factor λ that, approximately,

$$\frac{Y_{test}^2}{\lambda} \sim \chi_1^2(0)$$

where $\chi_1^2(0)$ is the central chi-square distribution with 1 degree of freedom. λ considers the excess of variance in the chi-square distributed test statistic. To infer λ, GC uses a panel of unlinked *null-loci*, which were regarded as having no effect. By inferring the median of the *null-loci* trend statistic between the cases and controls, λ of the population is calculated expressing the value for population stratification. For λ Devlin and Roeder (1999) propose the robust estimator

$$\hat{\lambda} = \frac{median(Y_1^2, Y_2^2, ..., Y_L^2)}{0.456},$$

where $Y_1^2, Y_2^2, ..., Y_L^2$ denote the calculated values of the Armitage Trend statistic at the *null-loci* and 0.456 is the median of the $\chi^2(0)$- distribution. For correction of the test-statistic at the candidate loci, the trend test statistic has to be divided by λ.

3.15.9 Inferring Population Attributable Risk (PAR)

PAR is calculated after Greenland (1998) and Greenland and Rothman (1998) by substracting the incidence rate among non-carriers of the risk allele (I_u) from the total incidence rate of the population (I_p) divided by the total incidence rate (I_p):

$$PAR = \frac{I_p - I_u}{I_p},$$

where

$$I_u = \frac{p(AA_{cases}) \cdot I_p}{p(AA_{cases}) \cdot I_p + p(AA_{controls}) \cdot (1 - I_p)}$$

and

$$p(AA_{cases}) = genotype\ frequency\ no\ risk\ allele-carriers\ among\ cases$$

$$p(AA_{controls}) = genotype\ frequency\ no\ risk\ allele-carriers\ among\ controls$$

$$I_p(UK) = 0.002$$

$$I_p(Germany) = 0.000012$$

(Incidence rates after World-Organisation-of-Animal-Health (2006))

3.15.10 Inferring allele and genotype frequencies from half-sibs

The German Holstein control animals were paternal half-sibs. Thus, the actual control frequencies were those of the maternally inherited alleles. Depending on the sire genotype at the loci, different calculation strategies were used: If the sire was heterozygous, only progenies with homozygous genotypes could be considered because only these genotypes allowed unequivocally determining the origin of each allele (Table 3.14). If the sire was heterozygous, all progenies could be considered. Maternal genotype frequencies were estimated based on the inferred maternal allele frequencies, assuming HWE as shown in Table 3.15.

Table 3.14 - Calculation of allele frequencies of dams of half-sibs.
Allele frequencies are differently calculated depending on the sire's genotype.

Genotype Sire	Possible Genotypes Daughters[a]			Allele Frequencies in Dams[b]	
	n_1	n_2	n_3		
AA	AA	AB		$p(A)=\frac{n_1}{n_1+n_2}$	$p(B)=\frac{n_2}{n_1+n_2}$
AB	AA	AB	BB	$p(A)=\frac{n_1}{n_1+n_3}$	$p(B)=\frac{n_3}{n_1+n_3}$

[a] n_1, n_2, n_3 indicate the number of half-sibs of genotype AA, AB and BB, respectively.
[b] p(A), p(B) indicate frequency of Allele A and B, respectively.

Table 3.15 - Calculation of the genotype frequencies of the dams of German Holstein half-sibs.

Genotypes	Genotype Frequencies in Maternal Population[a,b]		
	AA	AB	BB
Formula	$n \cdot p(A)^2$	$n \cdot 2p(A) \cdot p(B)$	$n \cdot p(B)^2$

[a] n indicate the number of all half-sibs with known genotype
[b] p(A), p(B) indicate frequency of Allele A and B, respectively.

4 Results

4.1 Characterisation of the bovine *HEXA* gene

The enzyme hexosaminidase A (HexA) is involved in the lysosomal degradation of intercellular glycolipids, the GM2 gangliosides which are glyco-sphingolipids predominantly occurring in the tissues of the central nervous system. HexA is composed of two subunits alpha and beta, which were encoded by the genes *HEXA* and *HEXB*, respectively (Proia, 1988; Proia and Soravia, 1987). Together with a ganglioside binding co-factor, the GM2-activator protein, HexA catalyses the degradation of GM2 gangliosides (Chavany and Jendoubi, 1998) as shown in Figure 4.1.

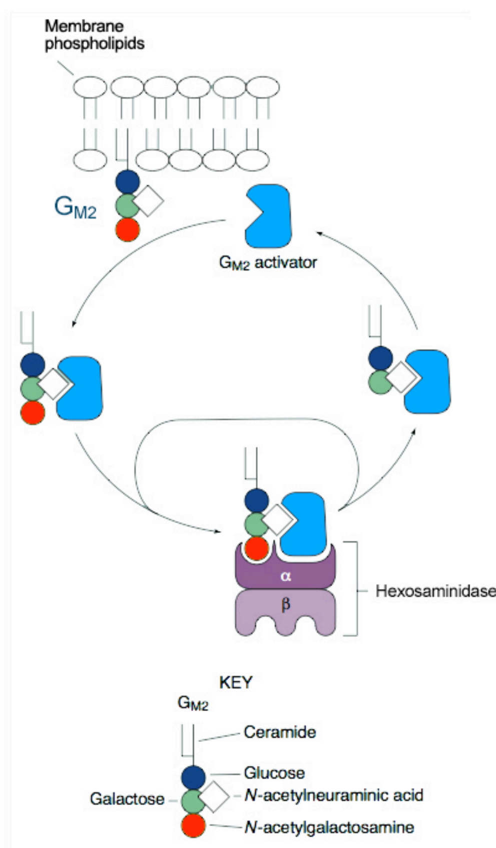


Figure 4.1 - Model for the lysosomal function of HexA
Hexosaminidase (HexA) is a heterodimer composed of one alpha and one beta chain. The GM2 activator protein extraxcts the glyco-lipids (GM2), and the resulting activator-lipid complex is the substrate for the enzymatic reaction catalysed by HexA, where the terminal N-acetylgalactosamine from GM2 is removed (modified after Chavany and Jendoubi (1998)).

In human, reduced activity or lack of HexA due to polymorphisms in the *HEXA* or *HEXB* loci are leading to neurodegenerative disorders like Tay-Sachs or Sandhoffs disease, respectively. In these fatal autosomal recessive disorders, GM2 gangliosides accumulate and so mainly result in neuronal distortion and severe neurological degeneration (Chavany and Jendoubi,

1998). Tay-Sachs and Sandhoffs disease have almost the same clinical symptoms, which include onset in infancy, rapid progression, and death usually before age of 4 years. Over 50 different types of Tay-Sachs disease are described, all caused by different sequence variants in *HEXA* resulting in defected or inefficient enzyme. In animals, the disorders are called Gangliosidoses, occurring naturally in cats (Cork et al., 1978) and dogs (Singer and Cork, 1989). The human and animal diseases are also called Lysosomal Storage Diseases, because of the accumulation of intercellular components in the lysosome. The storage of ‚waste' in the lysosome of nerve cells can be observed in prion diseases, too. The turnover of PrP^{c} to PrP^{Sc} generates protease-resistant components, which are indigestible for the lysosomal enzymes. Therefore, lysosomal enzyme efficiency could influence BSE development.

Furthermore, Kopacek et al. (2000) showed an increased expression of *HEXA* - mRNA in PrP^{Sc} infected brains of mice as well as an increasing expression of *HEXB* - mRNA during the infection. The up-regulation of both, *HEXA* and *HEXB* correlated well with the spongiform degeneration in the brain after infection. Similar results were obtained in a study using gene expression array technology. Xiang et al. (2004) identified an emerged expression of *HEXB* in Scrapie- infected brains of mice compared to uninfected. This leads to the assumption that prion diseases either directly or indirectly influence HexA.

In cattle, the *HEXA* gene is located close to a marker on BTA 10 that showed significant segregation distortion in BSE affected animals analysed by Transmission-Disequilibrium Tests (TDTs) (Hernandez-Sanchez et al., 2002). Moreover, the homologous region in mice located on chromosome 9 seems to affect the incubation time after experimental challenge with Scrapie (Stephenson et al., 2000). Thus, both functional and positional data made *HEXA* a promising candidate gene for BSE susceptibility in cattle.

For investigation of potential linkage of the *HEXA* gene and BSE, first the homologues bovine region was identified and the structure compared with the human and mouse *HEXA* locus. In order to obtain sequences of bovine *HEXA*, the human reference mRNA of *HEXA* (Acc NM_000520, NCBI) was subject to a BLAST search of the *ESTothe*r database division of NCBI. Three bovine Expressed Sequence Tags (ESTs) could be identified and are listed in Table 4.1. The corresponding sequences are covering the translated region of the *HEXA* gene, except for a gap of 35 basepairs in exon 6. The EST sequencing approach is shown in Figure 3.1 of Animal, Material and Methods section. The re-sequenced DNA and the derived exonic sequence are almost identical to the EST sequences and all discrepancies are located at the ends of the sequences and were presumably caused by poor sequence quality of the EST sequences.

Table 4.1 - Bovine ESTs for the *HEXA* gene
GeneBank Accession numbers, clone library, sequence size, homologous position, sequence identity to human mRNA, covered exons and sequence identity with the bovine re-sequenced DNA for each EST are listed.

GenBank Accession	Clone library	Size (bp)	Homologue human	Identity with human mRNA (bp)	Covered exons	Identity with bovine mRNA (bp)
CA034977	BARC 5 BOV	607	20-617	82.5% (494/599)	Ex1 – Ex6	99.67% (605/607)
AW653034	MARC 1 BOV	550	652-1192	90.4% (490/542)	Ex6 – Ex10	99.81% (549/550)
BE484497	BARC 5 BOV	504	1194-1618	86.8% (370/426)	Ex10 – Ex14	100% (504/504)

For SNP screening (see Chapter 4.3 - Polymorphism analysis in the HEXA region), the bovine *HEXA* gene was completely sequenced with the exception of intron 1, which has an approximate size of 20.5 kb. Further, 5.3 kb of the 5'-end and 2.5 kb of the 3'-end sequence was analysed. The entire *HEXA* spans 32.3 kb in cattle and 32.6 kb in human. In both species the Open Reading Frame (ORF) is organised in 14 exons (see Table 4.2) and the coding sequences contain 1587 bases, corresponding to 529 amino acids in the protein sequence. A graphical overview of the bovine *HEXA* gene is shown in Figure 4.2.

Table 4.2 - Exon and intron organisation of bovine *HEXA* gene.

Exon				Intron		
No	Size (bp)	Position in cDNA	5'splice donor … 3'splice acceptor [a]	No	Size (bp)	Human (bp)
			…gggccATGGC…			
1	225	1-225	CATAG**gt**gag … ttgc**ag**AAAAA	1	20 511[b]	19102
2	93	226-319	GAACT**gt**aag … tttc**ag**ACACA	2	1013	900
3	66	348-414	GCGAG**gt**aac … ccct**ag**GTCTG	3	1630	1821
4	47	415-462	GCACG**gt**gag … acac**ag**TTCTA	4	507	512
5	111	463-574	CTCTG**gt**aca … ctat**ag**GATGT	5	866	1833
6	102	575-677	AAAAG**gt**atg … tttc**ag**GGGTC	6	822	482
7	133	678-811	GCCAG**gt**aag … cctt**ag**GTGTC	7	570	1258
8	181	812-993	TGCTG**gt**atg … attc**ag**GAAGT	8	1111	944
9	87	994-1081	CAGAC**gt**gag … ctct**ag**GCTAC	9	148	289
10	73	1082-1155	TAAAG**gt**gag … tgac**ag**GTTCG	10	650	975
11	184	1156-1340	TGAAG**gt**aaa … cctc**ag**GTAGC	11	143	201
12	91	1341-1432	CTCTG**gt**aag … ccgc**ag**GCCTA	12	918	684
13	105	1433-1538	CTGAG**gt**gag … ttgc**ag**GCGTG	13	1017	1305
14	64	1539-1603	…CCTGAgcaag…			

[a] Intron sequences are indicated by lowercase letters, exon sequences by capital letters, the splice site sequences are bolded and start and stop codon are marked as red triplets.
[b] Intron 1 in cattle could not be resolved by sequencing. The size was estimated from bovine genomic draft contig Accesion number GenBank NW_928085

Figure 4.2 - Graphical overview bovine *HEXA*.
The blue and white colour shows the translated and the un-translated regions of the exons, which are represented by the bars. The black thin lines between the exons are representing the introns with a length approximately corresponding to the intron length.

Conservation of the catalytic hydrolase domain of HexA across species was analysed by sequence alignment of human, murine and bovine HexA protein sequences. The alignment was carried out with the software *ClustalX* (v. 1.83) (Thompson et al., 1997) and shaded by *BoxShade* 3.21, (http://www.ch.embnet.org/software/BOX_form.html) (Figure 4.3). From known human HexA (Acc. NP_000511, NCBI) domain structure consist of a N-terminal signal sequence (amino acids 1 - 22), followed by the glycosyl hydrolase family 20, domain 2 (amino acids 35-165; pfam02838) and a C-terminal glycosyl hydrolase family 20, catalytic domain (amino acids 167-488; pfam00728). The HexA protein is conserved across species with an overall amino acid identity of 84.5% between man and cattle. This emphasizes the important role, which HexA and the gangliosides degradation pathway in mammals play.

```
Human     1 MTSSRLWFSLLLAAAFAGRATALWPWPQNFQTSDQRYVLYPNNFQFQYDVSSAAQPGCSVLDEAFQRYRDLLFGSGSWPRPYLTGKRHTLEKNVLVVSVV
Cattle    1 MAGSTLRFSLLLAAAFAGRATALWPWPQYIQTSELRYTIFPQSFQFQYHLSSAAQVGCSVLDEAFQRYRDLLFGSVAFRFPHPIEKRHTSEKNSLVVLVV
Mouse     1 MAGCRLWVSLLLAAALACLATALWPWPQYIQTYHRRYTLYPNNFQFRYHVSSAAQAGCVVLDEAFRRYRNLLFGSGSWPRPSFSNKQQTLGKNILVVSVV

Human   101 TPGCNQLPTLESVENYTLTINDDQCLLLSETVWGALRGLETFSQLVWKSAEGTFFINKTEIEDFPRFPHRGLLLDTSRHYLPLSSILDTLDVMAYNKLNV
Cattle  101 TPGCDQFPSLGSVENYTLTINDEQSLLLSETVWGALRGLETFSQLIWRSPEGTFYVNKTDIEDFPRFPHRGLLLDTSRHYLPLASILDTLDVMAYNKFNV
Mouse   101 TAECNEFPNLESVENYTLTINDDQCLLASETVWGALRGLETFSQLVWKSAEGTFFINKTKIKDFPRFPHRGVLLDTSRHYLPLSSILDTLDVMAYNKFNV

Human   201 FHWHLVDDPSFPYESFTFPELMRKGSYNPVTHIYTAQDVKEVIEYARLRGIRVLAEFDTPGHTLSWGPGIPGLLTPCYSGSEPSGTFGPVNPSLNNTYEF
Cattle  201 FHWHLVDDSSFPYESFTFPELTKKGSYNPATHIYTAQDVKEVIEYARLRGIRVLAEFDTPGHTLSWGPGVPGLLTPCYSGSHPSGTFGPVNPALNNTYEF
Mouse   201 FHWHLVDDSSFPYESFTFPELTRKGSFNPVTHIYTAQDVKEVIEYARLRGIRVLAEFDTPGHTLSWGPGAPGLLTPCYSGSHLSGTFGPVNPSLNSTYDF

Human   301 MSTFFLEVSSVFPDFYLHLGGDEVDFTCWKSNPEIQDFMRKKGFGEDFKQLESFYIQTLLDIVSSYGKGYVVWQEVFDNKVKIQPDTIIQVWREDIPVNY
Cattle  301 MSTFFLEISTVFPDFYLHLGGDEVDFTCWKSNPDIQAFMKKKGFGDDFKKLESFYIQTLLDIVSAYGKGYVVWQEVFDNKVKVRPDTIIQVWREEIPVKY
Mouse   301 MSTLFLEISSVFPDFYLHLGGDEVDFTCWKSNPNIQAFMKKKGF-TDFKQLESFYIQTLLDIVSDYDKGYVVWQEVFDNKVKVRPDTIIQVWREEMPVEY

Human   401 MKELELVTKAGFRALLSAPWYLNRISYGPDWKDFYVVEPLAFEGTPEQKALVIGGEACMWGEYVDNTNLVPRLWPRAGAVAERLWSNKLTSDLTFAYERL
Cattle  401 VKELALVTRAGFRALLSAPWYLNHITYGPDWKEIYLVEPLAFEGSPEQKALVIGGEACMWGEYVDSTNLVPRLWPRAGAVAERLWSNKMVSNLDFAFKRL
Mouse   400 MLEMQDITRAGFRALLSAPWYLNRVKYGPDWKDMYKVEPLAFHGTPEQKALVIGGEACMWGEYVDSTNLVPRLWPRAGAVAERLWSSNLTTNIDFAFKRL

Human   501 SHFRCELLRRGVQAQPLNVGFCEQEFEQT
Cattle  501 AHFRCELLRRGVQAQPLSVGYCDMEFEQT
Mouse   500 SHFRCELVRRGIQAQPISVGYCEQEFEQT
```

Figure 4.3 - Alignment of HexA protein sequences from different species.
Sequence alignment of HexA protein from Homo sapiens (human) (Acc. NP_000511, NCBI), Bos taurus (cattle) (translated from predicted mRNA sequence) and Mus musculus (mouse) (Acc. NP_034551, NCBI). Numbers on the left site indicate the amino acid position. Black and grey backgrounds of letters indicate identical and conserved amino acids, respectively. The lines below the alignment highlight the domain structure of the enzyme: signal sequence (dark-blue), glycosyl hydrolase domain 2 (pfam02838) (blue) and the glycosyl hydrolase catalytic domain (light-blue). The alignment was annotated using BoxShade 3.21, (http://www.ch.embnet.org/software/BOX_form.html).

The 5'-ends contain genetic regulatory elements, which potentially can be identified by sequence conservation in this region. Therefore, human and bovine sequences were compared with regard to find conserved regions and putative transcription factor binding sites. 1 kb sequence upstream the translation start of both species were aligned and analysed with the tool *Consite* (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/). As shown in Figure 4.4, the conservation cut-off was adjusted to sequence identity of 80 %. Within the conserved regions (>80 % identity), transcription factor binding sites were detected: three AP2alpha binding sites, which are known to have enhancing function, six GATA-1 binding sites, one cEBP and one HNF-3beta TF. Variations within the transcription factor binding sites have not been identified. However, an effect of transcription factors on the expression level has to be analysed in functional promoter analyses. No conserved promoter sequences (e.g., TATA or CAAT box) were found by the *Consite* tool.

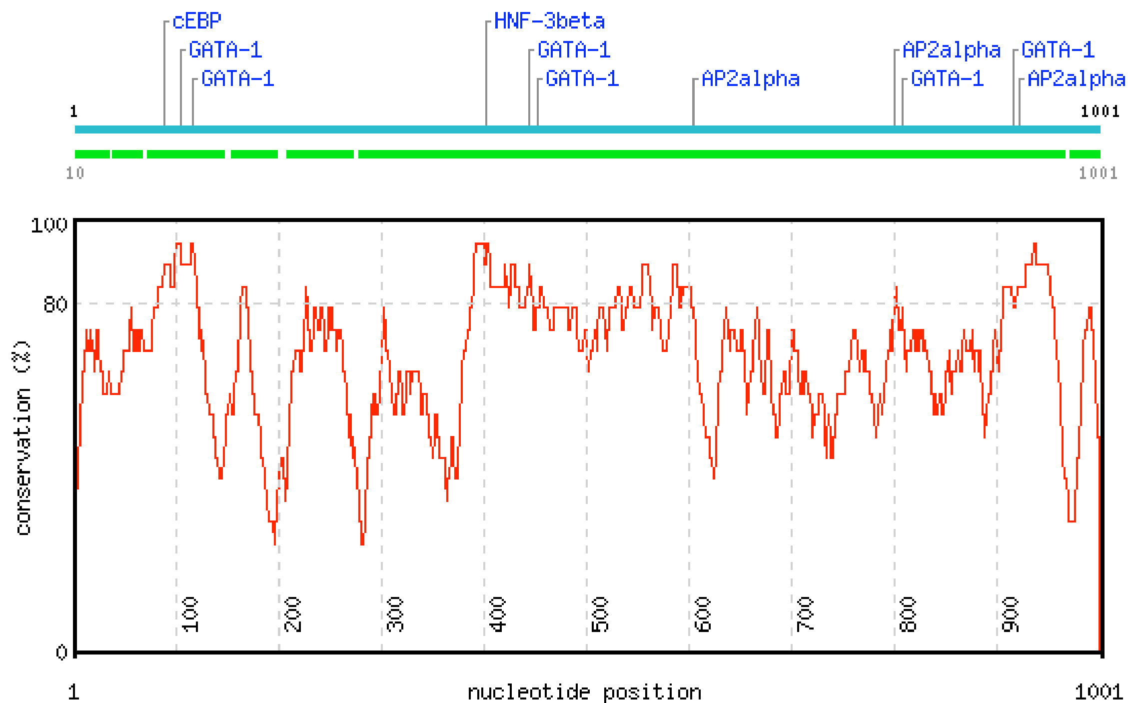


Figure 4.4 - Conservation profile of the aligned bovine and human 5'-region of *HEXA*.
The red line shows the identity of both sequences. All transcription factor binding sites (blue) situated in conserved regions (≥ 80% identity) are displayed.

Subsequently, putative transcription start sites were identified by the software *Neural Network Promoter Prediction* (v.2.2) provided by *Berkeley Drosophila Genome Project* (http://www.fruitfly.org/seq_tools/promoter.html) (Reese and Eeckman, 1995; Reese et al., 1996). The programme uses the data of 429 eukaryotic RNA Polymerase II promoters and was used on the 5'-region of cattle, human and mouse. Figure 4.5 shows the putative bovine promoter sequence with the predicted transcription start site located at bp position -780 (relative to the A of the translation start codon (ATG)) with a score value of 0.97. Promoter spe-

cific sites, such as the TATA-Box and CAAT-Box were identified at 20 bp and 90 bp upstream the transcriptions start, respectively. In contrast, in the human sequence, no promoter specific sites could be found. Here, the prediction tool assigned with a score value of 0.87 the transcription start at –31 bp, which rather corresponds to the cluster of transcription start sites between positions –26 and –10 identified by Norflus et al. (1996) by rapid amplification of cDNA ends (RACE) analysis. In the murine sequence, no transcription start with a score value of > 0.50 was found. But Wakamatsu et al. (1994) assigned a transcription start cluster to positions –42 to –21 bp from the first in-frame ATG codon of the murine *hexa* gene. In this study no TATA or CAAT box in the murine 5'-region was observed. Remarkably, the human and murine transcription start sites correspond to similar sites and the gene seems to be regulated by similar elements, whereas the promoter sequence of bovine *HEXA* may indicate a different regulation.

Start	End	Score	Promoter Sequence
-877	-774	0.97	TTCAATTTCCTACTGTACTAAGTCCTGATTCTCGGACTTTCTATGTAAACCC CTGCAGAATTTCTCTCTGGGTATAAGACCTGTCATCTCTGGGTGTTCTCCAG

Figure 4.5 - Predicted bovine *HEXA* promoter sequence
The putative transcription start of *HEXA* is marked blue and the putative promoter specific elements are framed. The start and end positions are relative to the adenine of the translation start-triplet (ATG). The Score value indicates the accuracy of the prediction (with 0 being the minimum and 1 the maximum accuracy) (Reese, 2001).

In general, mammalian promoters can be separated in two classes, the TATA box promoters and the CpG rich promoters (Carninci et al., 2006). CpG rich promoters are marked by unmethylated DNA regions with a high frequency of CpG di-nucleotides relative to the whole genome, the so-called CpG islands. CpG islands are defined as regions with a G+C content of more than 50% and a value of at least 0.6 for the ratio of observed versus expected CpG content (Gardiner-Garden and Frommer, 1987). To evaluate putative CpG islands, the bovine, murine and human *HEXA* 5'- end sequences were analysed with the programme *cpgplot* from the Emboss web-service http://www.ebi.ac.uk/emboss/cpgplot/ (Larsen et al., 1992). Prediction of CpG islands in the three sequences revealed putative CpG islands in the 5'-region of *HEXA* for all species as shown in Figure 4.6. The largest CpG rich region was observed in the bovine sequence with a length of 372 bp. In human and mouse, sequences of 336 bp and 133 bp corresponds to CpG islands, respectively. Relative to the translation start, the CpG islands ends at –55 bp ± 1 bp in all sequences. These findings lead to the suggestion that *HEXA* is regulated by a CpG rich promoter in all three species, albeit a TATA and CAAT box have been identified in the bovine sequence. Carninci et al. (2006) showed that the classical TATA

box promoter architecture with single dominant transcription start represents a minority of the promoters found in mammalian genomes. Additionally, CpG rich promoters represent the majority of mammalian promoters, which are associated with broad transcription start site regions. The experimentally identified broad clusters of transcription start sites in human (Norflus et al., 1996) and mouse (Wakamatsu et al., 1994) suggest CpG rich promoter for *HEXA* in these species. Moreover, the study of Carninci et al. (2006) highlighted that the sequences of TATA box containing promoters are much more conserved between species compared to CpG rich promoters, which seem to be evolve rapidly in mammals. No TATA box conservation and the finding of CpG islands in all three species argue against the hypothesis that *HEXA* is differently regulated in cattle.

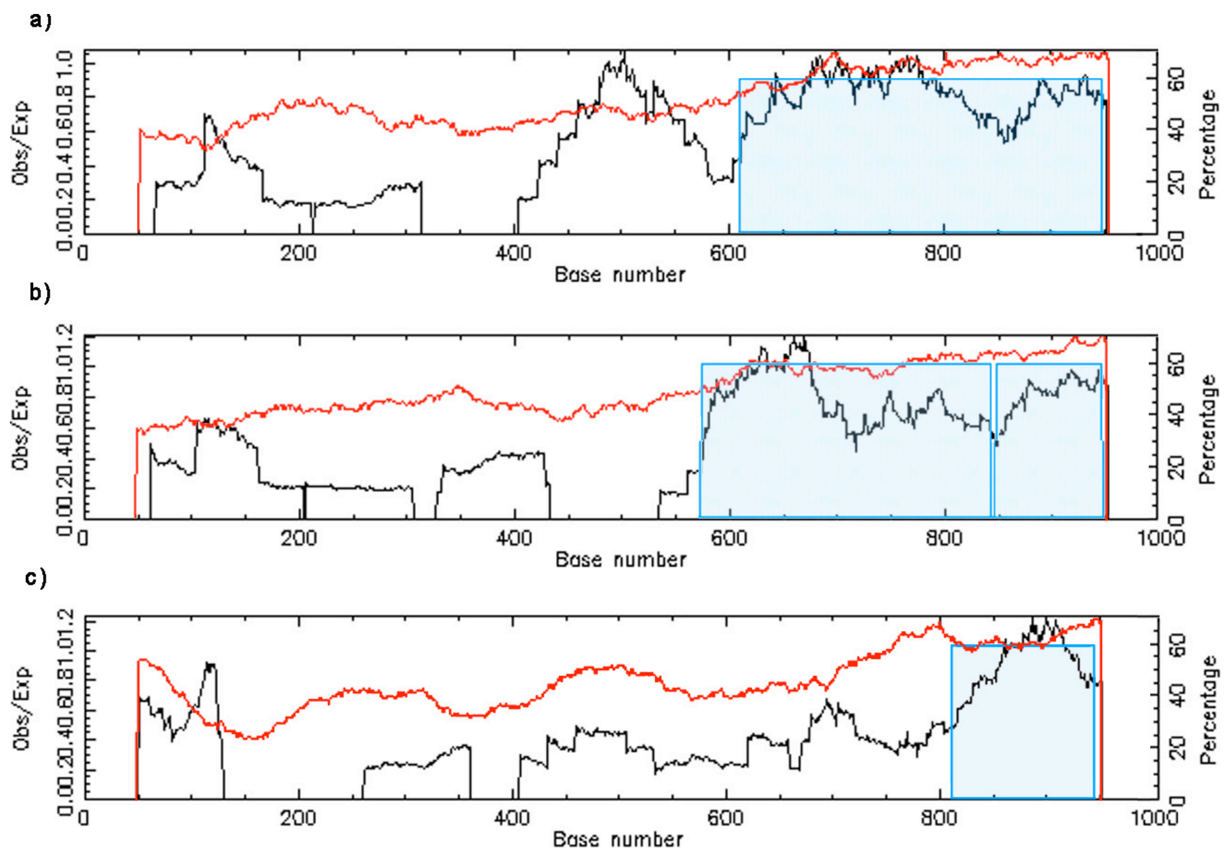


Figure 4.6 - Predicted CpG islands in the 5'-end of *HEXA* from a) man b) cattle and c) mouse. The upstream sequence (1 kb) of each species was analysed by cpgplot http://www.ebi.ac.uk/emboss /cpgplot/ (Larsen et al., 1992). The ratios of observed versus expected CpG content are plotted in black on the primary axis on the left. In red are the relative C+G contents displayed on the secondary axis on the right of each plot. The horizontal axis shows the bases relative to the translation start (=1000). The CpG island is highlighted by the blue box.

4.2 Fine-mapping of the region surrounding *HEXA*

4.2.1 Elucidation of the structure of the region surrounding *HEXA*.

4.2.1.1 Isolation of BAC clones for the bovine HEXA region

Screening the bovine RPCI-42 BAC library with a *HEXA*-specific PCR-product revealed six BAC-clones (Table 4.3). Colony-PCR with several primer systems covering the entire gene showed that each clone contained *HEXA* from exon 1 to exon 14. Using the corresponding human genomic draft sequence as shown in Contig View in Ensembl (v39) (http://www.ensembl.org/ Homo_sapiens/), the genes were identified within a 300-kb region centred on *HEXA*. BAC-end sequencing and testing the BACs with specific primers from the genes that were listed in the human region allowed building a BAC-contig. For this, the BAC end sequences were subjects of BLAST searches against the human and bovine draft sequences, which allowed assigning their corresponding position on HSA 15 and BTA 10 as shown in Figure 4.7. The bovine genomic region and the corresponding region in human beings is oriented from centromere to telomere. Therefore, *PARP6*, *BRUNOL6* and *HEXA* are here assigned on the negative strand (reverse-complement).

Analysis of the BLAST results revealed that the T7 end sequences of TUM910 andTUM924 are identical and the T7 end of TUM916 is located 200 bp upstream. All three BAC end sequences start in *BRUNOL6*, intron 2. Both BAC ends of TUM909 (SP6) and TUM 920 (T7) are situated in *PARP6*, intron 15 and intron 8, respectively. Except for the SP6 end of TUM910, which end in *ARIH1* intron 2, all annotated BAC ends are located between *HEXA* and *ARIH1*. TUM907 BAC ends showed no similarities to sequence entries for the extended *HEXA* region, neither for the human nor the bovine draft sequence (*Btau 2.0*). A possible explanation for this could be that the end sequences of TUM907 do not match with the existing sequences because the corresponding part has not been sequenced yet and the sequence contains Ns at that position. The newest assembly of the cow genome (*Btau 3.0*) was finally used to annotate the TUM907 BAC ends: BLAST hits were found for SP6 on an unnotated scaffold and for T7 on BTA26. This suggests that TUM907 is a chimerical clone containing an insert composed of two or three different sequence fragments.

Table 4.3 - Identified BAC clones containing a *HEXA* specific insert
For each BAC are shown: the BAC-Id, assigned in the internal database with the corresponding plate address in the bovine RPCI-42 library, insert length obtained from blasting BAC end sequences against bovine genomic draft sequences (*Btau 2.0*) and the corresponding sequence position on BTA 10.

BAC-Id	Plate address RPCI-42	Insert length BAC end	BLAST hits on BTA 10 (*Btau 2.0*) SP6 end	T7 end
TUM907	23O4		-	-
TUM909	66C17	195.7	10 630 790	10 826 459
TUM910	144L318	198.6	10 894 371	10 695 729
TUM916	152A6	122.4	10 817 784	10 695 528
TUM920	244L16	172.6	10 811 781	10 638 759
TUM924	285B13	132.2	10 827 491	10 695 729

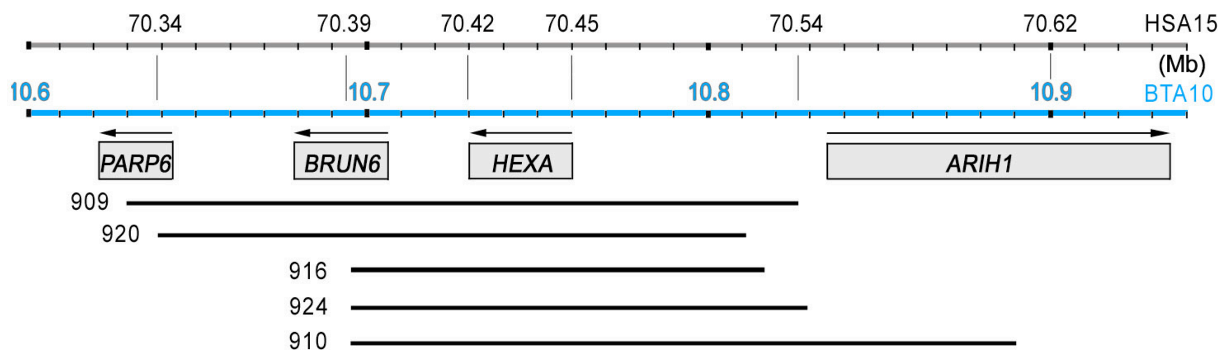


Figure 4.7 - BAC-Contig encompassing the bovine *HEXA* region.
BAC contig from the region of bovine chromosome 10 (BTA 10, *Btau 2.0*) containing a 300-kb region centred on *HEXA* with the corresponding part of human draft sequence (HSA 15q23). The contig is oriented from telomere to centromer (from the left to the right). BAC inserts are shown as black bold horizontal lines, with the length of lines reflecting their size. The name of each clone corresponds to the number assigned in the internal database. Names of genes are written in the grey rectangles indicating the genes size. The arrows above the rectangles show the orientations of the genes, which correspond to the human Contig View in Ensembl v.39 June 2006 (www.ensembl.org). The blue horizontal scale indicates the bovine draft sequence in Megabases (Mb) whereas the dark grey scale stands for the homologous human draft sequence (Mb).

4.2.1.2 Using Btau 1.0

In October 2004, the first bovine genomic sequence was available at the Ensembl web-service (www.ensembl.org; Pre! Cow v.25, *Btau 1.0*). By the date of completion of this work, the preliminary version of the bovine draft sequence was not accessible anymore at the Ensemble web service. However, the first release had an extreme impact on resolving the region because primer design was facilitated and therefore sequencing could be optimised and accelerated. Until then, for instance for the 5'-end, re-sequencing was only possible with preceding primer walk on BAC DNA. This approach could be skipped with the release of the scaffolds. Further, the PCR systems and their fragment length could be improved easily by an adjustment of the fragment length to the ideal size for sequencing of 1000 bp.

The bovine draft sequences for the four genes of the extended *HEXA* region was obtained by blasting the corresponding human reference mRNAs. Subsequently, twelve scaffolds ranging in size from 0.95 kb to 28.7 kb could be identified (Table 4.4). For *HEXA,* two scaffolds were found, one spanning from the 5'-end to intron 1 with a size of 15.3 kb (SCAFFOLD95258) and the second spanning from intron 1 to the 3'-end with a size of 28.7 kb (SCAFFOLD5566). The sequences of both did not overlap and the gap in intron 1 could not be closed by PCR systems with primers designed at the putative transition of the scaffolds, respectively. A possible reason for the failure of the primer system is the unreliability and imprecision of the sequences at the beginning and end of the scaffolds. For *ARIH1*, located upstream of *HEXA*, one scaffold (SCAFFOLD295439) was found containing the 5'-end and exon 1. For *BRUNOL6* and *PARP6*, both located downstream of *HEXA,* two and six scaffolds were identified, respectively (Table 4.4).

In order to reveal the structure of the bovine genes and to identify the coding regions, the scaffolds were compared with the homologous human sequence and corresponding gene structure was applied. The scaffolds were mainly used to re-sequence the adjacent region of *HEXA* and the neighbouring genes.

Table 4.4 - Bovine scaffolds for *ARIH1*, *HEXA*, *BRUNOL6* and *PARP6* (*Btau 1.0*)
For each scaffold the human BLAST template, the Ensembl scaffold number from *Btau 1.0* (Pre!Cow v.25), the sequence size, the section of the gene contained by the corresponding scaffold, numbers of gaps and numbers of Ns (not resolved regions) are displayed.

Gene	BLAST template [a]	Ensembl Scaffold Number	Size (bp)	Segment of Gene [b]	Number of gaps	Number of Ns (bp)
ARIH1	NM_005744	SCAFFOLD295439	21062	5'-end to I1	3	400
HEXA	NM_000520	SCAFFOLD95258	15052	5'-end to I1	4	1500
		SCAFFOLD5566	28610	I1 to 3'-end	3	500
BRUNOL6	NM_052840	SCAFFOLD305639	16952	5'-end to I2	3	1540
		SCAFFOLD125719	14289	I3 to 3'-end	3	240
PARP6	NM_020214	SCAFFOLD310354	7279	5'-end to I4	2	190
		SCAFFOLD3547	3547	I5 to I8	-	
		SCAFFOLD196836	4678	I10 to I15	1	50
		SCAFFOLD184260	1240	I15 to E17	-	
		SCAFFOLD338507	947	I17 to I18	-	
		SCAFFOLD135442	9410	I18 to 3'-end	3	1550

[a] Human mRNA reference sequences for each gene (Acc from NCBI)
[b] I and E stand for intron and exon, respectively

4.2.1.3 Using Btau 2.0

From the new release of the cow genome (*Btau 2.0*), one linear scaffold could be identified, including all four genes for the extended *HEXA* region. Due to its chromosomal position, the contig got the initial name ‘BTA Chr10.23’ with the accession number NW_928085 in dbNucleotide of NCBI (Version 30.09.2005). The contig size is about 707.1 kb and contains 75.3 kb unresolved sequence, which is indicated by Ns. By the release date of *Btau 2.0,* the SNP analysis in the extended *HEXA* region was already finished. Therefore, the contig was not used to design primers for re-sequencing. However, the contig allowed to confirm the 5'-3' reading direction of the genes and the position of the genes to each other (Table 4.5).

Table 4.5 - *PARP6*, *BRUNOL6*, *HEXA*, *ARIH1* mapped on bovine contig BTA 10.23 (*Btau 2.0*)
The position of the gene on contig BTA 10.23 (NW_928085) and corresponding location on human chromosome 15.

Gene	Contig location (bp)	Orientation[a]	Gene size (kb)	Location on HSA 15 (bp)	Orientation[b]
PARP6	69 663 – 89 968	-	22.4	70 320 594 – 70 350 446	-
BRUNOL6	125 488 – 152 757	-	29.4	70 400 583 – 70 364 122	-
HEXA	176 636 – 208 196	-	31.4	70 455 393 – 70 422 833	-
ARIH1	277 936 – 381 489	+	103.6	70 553 721 – 70 662 877	+

[a] Orientation of genes within the contig: + sense strand; - antisense strand.
[b] Orientation of genes on chromosome 15: + sense strand; -antisense strand.

4.2.1.4 Using Btau 3.1

In August 2006, the third version of the cow genome (*Btau 3.1*) was published (see Chapter 3.4.5 – *Btau 3.1*: Chromosomal assembly of the cow genome). The sequence was used to confirm the annotation results for the genes in the *HEXA* region by an automated annotation approach. Therefore, a 400-kb segment of BTA 10 (from bp 16 400 001 to 16 800 000) that putatively contained *PARP6, BRUNOL6, HEXA* and *ARIH1* was used to run *GenomeThreader* (Gremme et al., 2005), a tool for gene structure prediction. The tool compares known ESTs from different species with the input sequence and produces gene annotation models. The output of *GenomeThreader* is an XML-file that can be viewed by *Apollo* (Lewis et al., 2002), a sequence annotation editor (see Figure 4.8). The genes *ARIH1*, *HEXA* and *BRUNOL6* could be annotated by these tools without manual correction. *PARP6* had to be manually annotated because the *Btau 3.1* assembly contained a wrong arrangement in this gene region. Using *PARP6* exon 1 for a BLAST search against *Btau* 3.1 revealed a hit with 100 % identity on BTA 10 at position 18 969 195 which is >2.5 Mb apart from the inferred location at 16 473 774. To handle this assembly problem, a 19.5-kb segment of the linear scaffold of *Btau 2.0* (NW_928085) (from bp 65 001 to 84 500) containing the missing part of

PARP6 was merged with a 350-kb segment of BTA 10 (*Btau 3.1* from bp 16 450 001 to 16 800 000) and used as the input sequence for *GenomeThreader*. Subsequently, bovine *PARP6* could be annotated with an approximately gap of 25 bp in exon 9 that is due to missing sequence in NW_928085. The annotations of the genes are shown in Table 4.6.

Table 4.6 - *PARP6*, *BRUNOL6*, *HEXA*, *ARIH1* mapped on BTA10 (*Btau 3.1*).

Gene	Contig location [a](bp)	Gene size (kb)	Location on BTA 10 (bp) (*Btau 3.1*)
PARP6	3 216 – 30 760	27.5	n.d. - 16 501 318
BRUNOL6	46 541 – 75 081	28.5	16 545 639 - 16 517 099
HEXA	98 855 – 130 939	32.1	16 569 413 - 16 601 397
ARIH1	201 866 – 303 856	102.0	16 672 324 - 16 774 314

[a] Contig contains 19.5 kb of NW_928085 (from bp 65 001 to 84 500) and 350 kb segment of *Btau 3.1*, BTA10 from bp 16 450 001 to 16 800 000, n.d = not determined

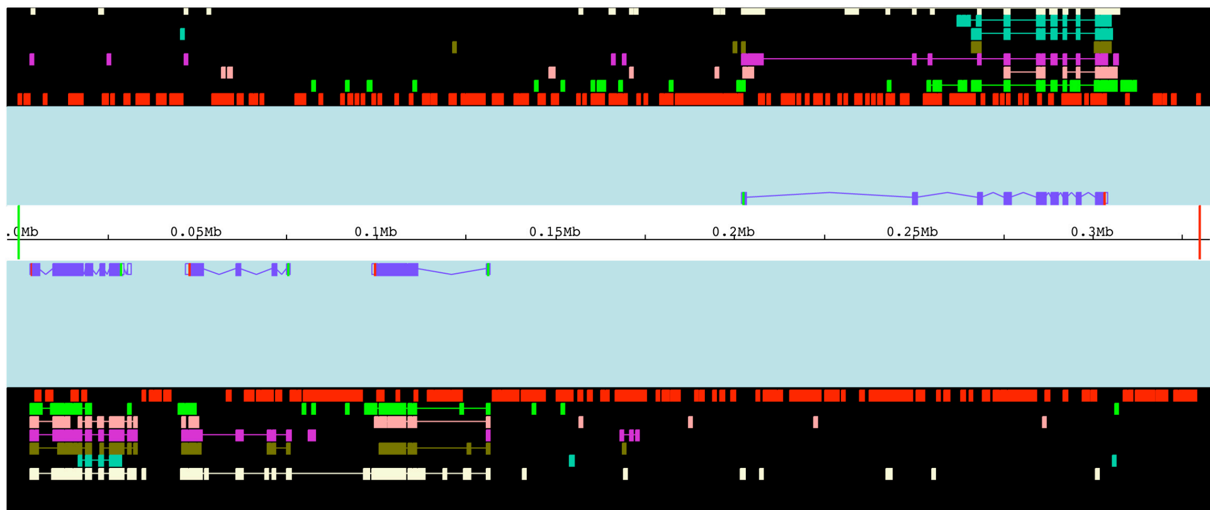


Figure 4.8 - Encompassing region of *HEXA* in Apollo view (Lewis et al., 2002).
The horizontal axis (sequence panel) in the middle shows a 370-kb segment of BTA 10. The annotations (blue) on the forward strand are shown above the white sequence panel (*ARIH1*) and those on the reverse strand are shown below it (containing from left to right *PARP6, BRUNOL6* and *HEXA*). Within the annotations of the genes, green and red vertical dashes represent the translation start and stop, respectively. The results of the GenomeThreader (Gremme et al., 2005) are shown on the black backgrounds on the top and bottom for the forward and reverse strand, respectively: EST homologies of different species are bright green (*Bos taurus*), rose (*Sus scrofa*), pink (*Mus musculus*), brown (*Rattus norvegicus*), dark green (*Canis familiaris*) and white (*Homo sapiens*). Red rectangles mark repetitive sequences as predicted by RepeatMasker implemented in the GenomeThreader tool.

4.2.2 Characterisation of *HEXA*-neighbouring genes

4.2.2.1 Ariadne-1 protein homolog (ARIH1)

The gene upstream of *HEXA* encodes the homologue of drosophila ariadne-1 gene (*ARIH1)*. *ARIH1* is a member of the ubiquitin pathway, which plays a fundamental role in maintaining many basic cellular processes (Hershko and Ciechanover, 1986). Ubiquitinylation (ubiqitin-labbeling of proteins) marks the start for degradation of normal and abnormal intracellular proteins, in particular of transcriptions factors (Ciechanover et al., 1999) and growth modulators (Tan et al., 2000). At least three essential components are required for ubiquitinylation of a protein: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin-protein ligase (E3) (Hershko and Ciechanover, 1998). *ARIH1* represents a component of the E3 complex. Members of the ubiquitin pathway are involved in several neurodegenerative disorders like Parkinson's or Alzheimer's disease (Mori et al., 1987). The functional domain of *ARIH1* contains a RING motif, which stands for Really Interesting New Gene. RING is little described as a cystein-rich motif with zinc-binding capacity (Freemont, 1993). The biological importance of the drosophila ariadne-1 gene is implied by the observation that mutations in this gene result in severe neuronal defects (Aguilera et al., 2000). These data are linking *ARIH1* to neurodegenerative TSEs and therefore are making the homologues bovine gene to a functional candidate for BSE susceptibility.

The complete structure of bovine *ARIH1* was assigned by using GenomeThreader (Gremme et al., 2005) and the Apollo Genome Annotation and Curation Tool, v 1.6.4 (Lewis et al., 2002). The entire gene spans 102 kb in cattle and 109 kb in human. The coding sequence of *ARIH1* in cattle and man is organised in 14 exons (see Table 4.7 and Figure 4.9) and the translated sequence from start to stop codon contains 1668 bp (cattle) and 1671 bp (man), corresponding to a protein sequence of 556 and 557 residues, respectively.

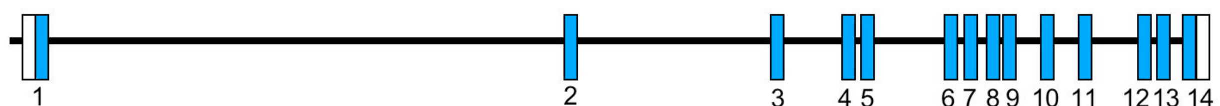


Figure 4.9 - Graphical overview of bovine *ARIH1*
The blue and white colour shows the translated and the untranslated regions of the exons, which are represented by the bars. The black thin lines between the exons represent the introns, with a length approximately corresponding to the intron length.

Table 4.7 - Exon and intron organisation of bovine *ARIH1* gene.

Exon			5'splice donor … 3'splice acceptor[a]	Intron		
No	Size (bp)	Position in cDNA		No	Size (bp)	Human (bp)
			…gcgccATGGA…			
1	369	1-369	TCCAGgtgag … tttagAATCC	1	47034	43053
2	68	370-437	GAAAGgtaag … tgcagGTACT	2	18198	26686
3	145	438-582	ACTCGgtgag … tgcagTATTT	3	7248	10307
4	93	583-675	GACAGgtaaa … tccagACTAT	4	506	489
5	56	676-731	GTTATgtaag … ttcagGCGCC	5	8309	5576
6	67	732-798	TAGAGgtaag … ttcagTGCAA	6	1292	1845
7	107	799-905	TTTTGgtaag … aacagCTTTA	7	2718	3063
8	43	906-948	GTAAGgtgag … aacagTGGTT	8	527	501
9	72	949-1020	CAAAGgttgg … catagGAATG	9	2730	3000
10	131	1021-1151	GCCTGgtagg … ttcagGTACA	10	3419	1801
11	58	1152-1209	AGGAGgtaag … gacagAGATC	11	5348	8566
12	261	1210-1470	TTGAGgtagg … tgcagAATAA	12	1053	1084
13	113	1471-1583	TACAGgtaag … tacagATACT	13	983	1021
14	84	1584-1669	…ACTGAgaatg…			

[a] Intron sequences are indicated by lowercase letters, exon sequences by capital letters, the splice site sequences are bolded, red triplets stand for start and stop codon.

Tan et al. (2000) reported that the human *ARIH1* protein sequence is 98% identical with the murine sequence. The conservation of *ARIH1* in man, mouse and cattle was analysed by sequence alignments of their amino acid sequences using the software *ClustalX (v. 1.83)* (Thompson et al., 1997). Figure 4.10 displays the complete alignment annotated by *BoxShade 3.21*, (http://www.ch.embnet.org/software/BOX_form.html).

Figure 4.10 - Alignment of protein sequences of *ARIH1* of different species.
Human (NP_005735, NCBI), murine (NP_064311, NCBI) and bovine sequences (Apollo-output) are highly conserved. The region containing the RING-motif is marked by the blue line below the alignment and is 100 % identical between the three species.

The 5'-end of man and cattle were compared to find conserved promoter sites. Therefore, 1 kb sequence upstream of the translation start of both species were aligned and analysed with the tool *Consite* (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/). As shown in Figure 4.11, the conservation cut-off was adjusted to sequence identity of 98 %. Within the highly conserved region (> 98 % identity) which is located in 350-bp region upstream the translation start, 21 transcription factor binding sites were detected including ten conserved AP2alpha binding sites, which are known to have enhancing function and four Stimulating Protein 1 (SP1) bindings sites. No conserved promoter sequences (e.g., TATA or CAAT box) were found by the *Consite* tool.

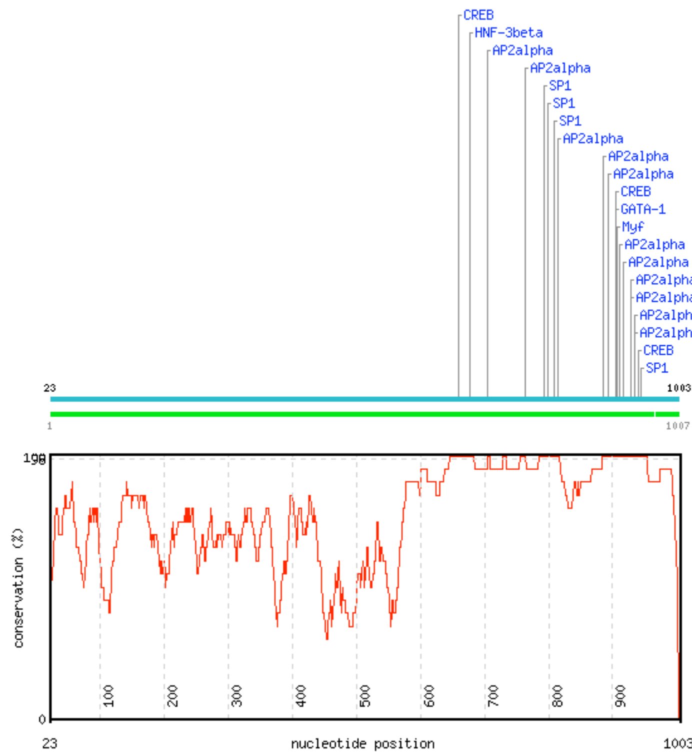


Figure 4.11 - Conservation profile of the aligned bovine and human 5'-region of *ARIH1*
The red line shows the identity of both sequences. All transcription factor binding sites (blue) situated in conserved regions (≥ 98% identity) are displayed.

To evaluate putative CpG islands, the *ARIH1* 5'-end sequences of cattle and man were analysed with the programme *cpgplot* from the Emboss web-service http://www.ebi.ac.uk/emboss/cpgplot/ (Larsen et al., 1992). Prediction of CpG islands in both sequences revealed putative CpG islands in the 5'-end of *ARIH1* (see Figure 4.12) with sizes of 551 and 525 bp in cattle and man, respectively. The CpG islands correspond to the highly conserved region from –180 to –400 bp upstream the translation start as revealed by the Consite tool. Relative to the translation start, the CpG islands ends at –52 bp and –58 bp in cattle and man, respectively. These findings lead to the suggestion that *ARIH1* is regulated by a CpG rich promoter in both species.

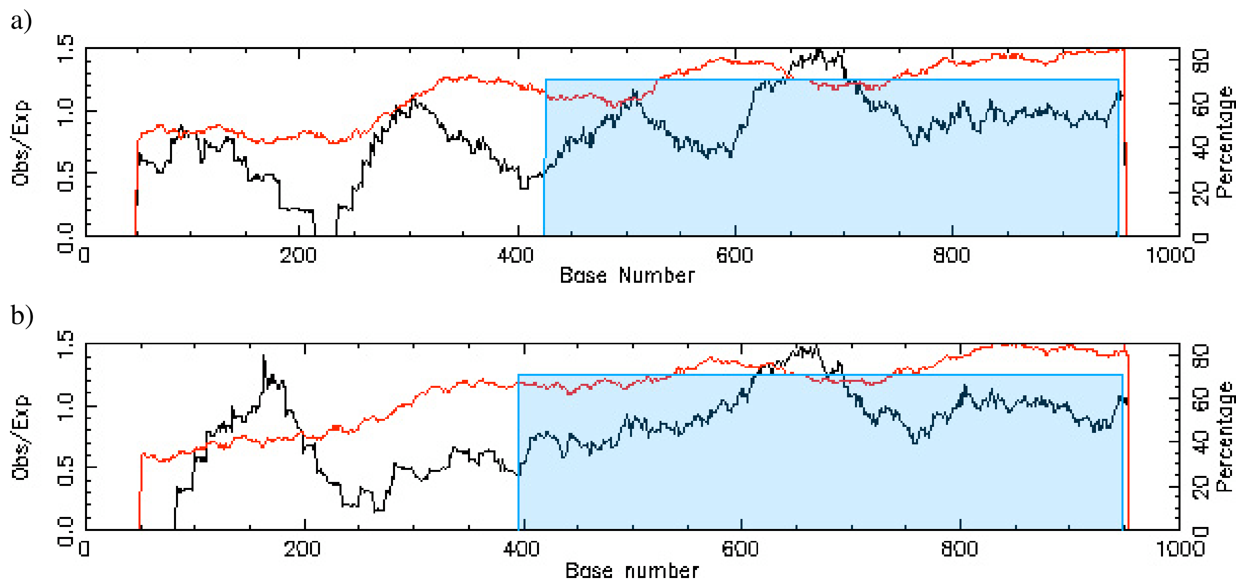


Figure 4.12 - Predicted CpG islands in the 5'-end of *ARIH1* in a) man and b) cattle
The upstream sequence (1 kb) of each species was analysed by *cpgplot* http://www.ebi.ac.uk/emboss/cpgplot/ (Larsen et al., 1992). In black and on the left primary axis are plotted the ratios of observed versus expected CpG content. In red and on the right primary axis the relative C+G contents in percent are plotted. The horizontal axis shows the bases relative to the translation start (=1000). The CpG island is highlighted by the blue box.

4.2.2.2 Bruno-like 6, RNA binding protein (Drosophila) (BRUNOL6)

Downstream of *HEXA*, the gene encoding Bruno-like 6, RNA binding protein (Drosophila) (*BRUNOL6*) is located. *BRUNOL6* belongs to a gene family of RNA binding proteins influencing muscle specific splicing and enhancer specific alternative splicing of different genes (Ladd et al., 2004). Several alternative splicing events are regulated either positively or negatively by BRUNOL family members, depending on the pre-mRNA targets (Han and Cooper, 2005). Members of this protein family may also be involved in mRNA editing and translation. These genes are also called CUG-Binding Protein (CUG-BP) and Embryonic Lethal Abnormal Vision-Type RNA-Binding Protein 3 (ETR3) -like factors (CELFs) (Ladd et al., 2004). Disruption of the *CELF6/BRUNOL6* function may play a role in myotonic muscle dystrophy (Timchenko et al., 1996) and contributes to insulin resistance a clinical manifestation of this disease (Savkur et al., 2001). *BRUNOL6* contains three RNA binding domains and a divergent domain with unknown function. It is strongly expressed in the kidney, brain and testis and expressed at very low levels in most other tissues.

The structure of bovine *BRUNOL6* was assigned by using GenomeThreader (Gremme et al., 2005) and the Apollo Genome Annotation and Curation Tool, v 1.6.4 (Lewis et al., 2002). The gene from translation start to poly-A signal spans 28.5 kb in cattle and 35.5 kb in humans. The cDNA sequence of *BRUNOL6* in cattle and man is organised in 13 exons (see Ta-

ble 4.8 and Figure 4.13), the translated sequences from start to stop codon contains 1446 bp (cattle) and 1440 bp (man) corresponding to exon 1 to 12 and 480 and 482 amino acid residues in the protein sequences.

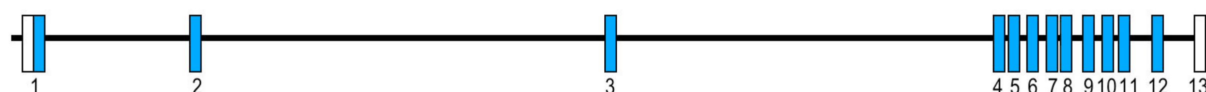


Figure 4.13 - Graphical overview of bovine *BRUNOL6*
The blue and white colour shows the translated and the untranslated regions of the exons, which are represented by the bars. The black thin lines between the exons represent the introns and their lengths correspond approximately to the intron length.

Table 4.8 - Exon and intron organisation of bovine *BRUNOL6* gene.

Exon			5'splice donor … 3'splice acceptor [a]	Intron		
No	Size (bp)	Position in cDNA		No	Size (bp)	Human (bp)
			…gggatATGGC…			
1	262	1-262	CAAAG**gt**gcg … ccc**ag**GCTGT	1	3918	3685
2	83	263-345	CAGGG**gt**aag … ttc**ag**ATGAA	2	10263	11050
3	49	346-394	AGGAG**gt**aac … ccc**ag**AGGAC	3	10310	14490
4	129	395-523	TAAAG**gt**gac … ccc**ag**GCTGT	4	103	108
5	80	524-603	TGGCG**gt**gag … ccc**ag**GGCGC	5	161	168
6	144	604-747	CCGCG**gt**agg … caa**ag**ATCCT	6	117	119
7	133	748-880	GGCAG**gt**act … tgc**ag**CCAAC	7	98	87
8	147	881-1027	CCCAG**gt**ggg … ccc**ag**CCCAG	8	208	207
9	63	1028-1090	CGCAG**gt**ttc … tcc**ag**CAGCC	9	253	249
10	81	1091-1171	AGAAG**gt**gag … tcc**ag**GCCCC	10	87	86
11	144	1172-1315	TTTTG**gt**gag … tcc**ag**GGTTT	11	562	915
12	155	1316-1440	…TTACTGA**tctgt**…	12	819	893
13	261	-	3'-UTR			

[a] Intron sequences are indicated by lowercase letters, exon sequences by capital letters, the splice site sequences are bolded, red triplets stand for start and stop codon.

In order to analyse the extent of conservation of the gene product and its RNA binding motifs, the protein sequences of man, cattle and mouse were aligned using *ClustalX* (see Figure 4.14). The RNA recognition motifs (Acc. NP_443072, NCBI) are highly conserved between the species. The sequences of man, mouse and cattle differ in one amino acid whereas the sequences of human and cattle differ in two amino acids. Hence, the identity of the sequences is > 99% between the three species.

Figure 4.14 - Alignment of *BRUNOL6* protein sequences of different species.
Sequence alignment of BRUNOL6 protein from Homo sapiens (Acc NP_780444, NCBI), Bos taurus (Apollo-output) and Mus musculus (Acc NP_443072, NCBI). Numbers on the left site indicate the amino acid position. Black and grey backgrounds of letters indicate identical and conserved amino acids, respectively. Blue lines below the alignment mark the in the three species highly conserved RNA binding motifs.

In order to find conserved promoter sites for *BRUNOL6,* the 5'-end sequences of man and cattle were compared. Therefore, 1 kb sequence upstream of the translation start of both species were aligned and analysed with the tool *Consite* (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/). As shown in Figure 4.15, the conservation cut-off was adjusted to sequence identity of 94 %. Within the highly conserved region (> 94 % identity), which starts 700 bp upstream of the translation start, 16 transcription factor binding sites were detected including six conserved AP2alpha binding sites and four SP1 bindings sites. No conserved promoter sequences (e.g., TATA or CAAT box) were found by the *Consite* tool.

The human transcription start is located at position –310 relative to the adenine of the ATG start triplet (Acc. NM_052840, NCBI). For the bovine gene the putative transcription start was predicted by the software *Neural Network Promoter Prediction* (v.2.2) provided by *Berkeley Drosophila Genome Project* (http://www.fruitfly.org/seq_tools/promoter.html) (Reese and Eeckman, 1995; Reese et al., 1996). The predicted transcription start site is located at position - 209 bp (relative to the adenine of the translation start triplet (ATG)) with a score value of 0.77. Again, promoter specific sites, such as the TATA-Box and CAAT-Box could not be identified. Hence, the gene might be regulated by CpG rich promoter region.

The human and bovine 5'-end sequences of *BRUNOL6* were analysed for CpG islands with the programme *cpgplot* from the Emboss web-service http://www.ebi.ac.uk/emboss/cpgplot/ (Larsen et al., 1992). Prediction of CpG islands in both sequences revealed putative CpG islands in the 5'-end of *BRUNOL6* (see Figure 4.16) with sizes of 743 bp and 483 bp in man and cattle, respectively. Relative to the translation start, the CpG islands ends at –52 bp and –58

bp in man and cattle, respectively. These findings lead to the suggestion that *BRUNOL6* is regulated by a CpG rich promoter in both species.

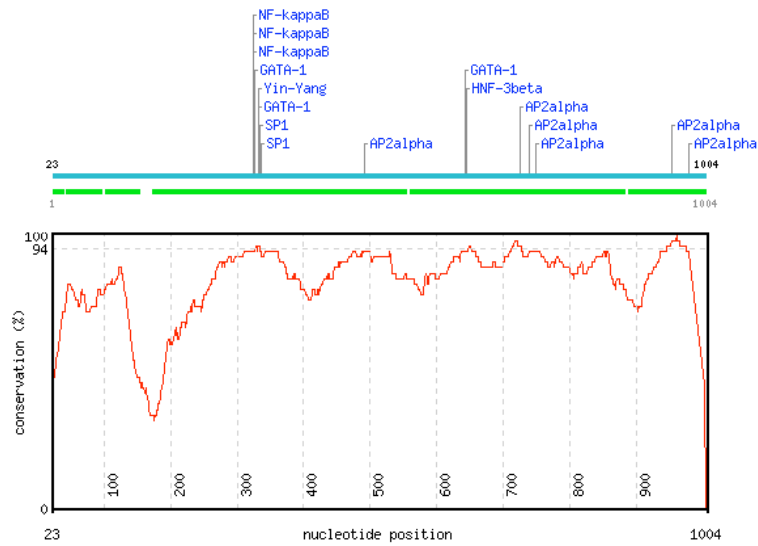


Figure 4.15 - Conservation profile of the aligned bovine and human 5'-region of *BRUNOL6*.
The red line shows the identity of both sequences. All transcription factor binding sites (blue) situated in conserved regions (≥ 94% identity) are displayed.

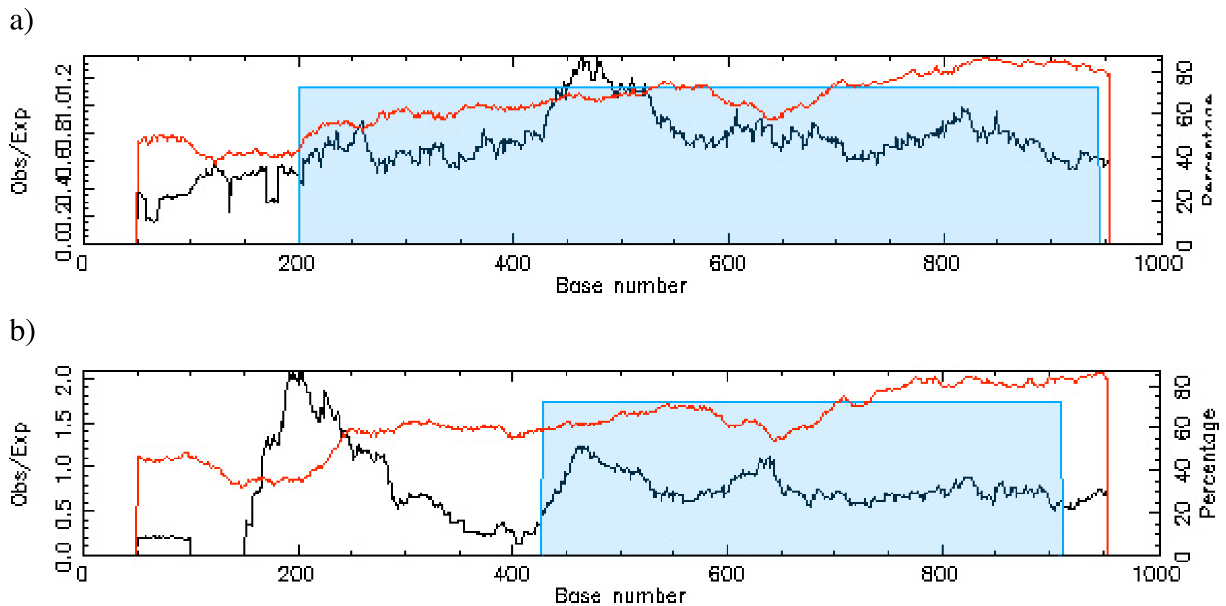


Figure 4.16 - Predicted CpG islands in the 5'-end of *BRUNOL6* in a) man and b) cattle.
The upstream sequence (1 kb) of each species was analysed by *cpgplot* http://www.ebi.ac.uk/emboss/cpgplot/ (Larsen et al., 1992). In black and on the left primary axis are plotted the ratios of observed versus expected CpG content, In red and on the right primary axis is plotted the relative C+G contents in percent The horizontal axis shows the bases relative to the translation start (=1000). The CpG island is highlighted by the blue box.

4.2.2.3 Poly- (ADP-ribose) Polymerase 6 (PARP6)

Downstream of *BRUNOL6,* the gene encoding the Poly-(ADP-ribose)-Polymerase 6 (*PARP6*) is located. This belongs to a gene family composed of at least 18 members encoding proteins that share homology with the catalytic domain of the founder gene *PARP1*. The *PARP* family gene products are said to have a central importance in a wide variety of biological processes, e.g. DNA repair, transcriptional regulation, centromere function and apoptosis (Diefenbach and Burkle, 2005). Poly- (ADP)-ribosylation represents an immediate enzymatically activated response to DNA damage. Especially for *PARP6,* no detailed function is known yet. The protein contains three RNA-binding motifs and Ame et al. (2004) proposed that *PARP6* is a member of the subgroup of *PARP1*, which is the most extensively studied gene of the family. In three independent *PARP1*-deficient mouse models, the animals showed hypersensitivity to DNA damaging radiation and agents (Shall and de Murcia, 2000). Although, *PARP6* has no obvious connection to BSE, it was analysed to cover a larger region.

The structure of bovine *PARP6* was assigned by using GenomeThreader (Gremme et al., 2005) and the Apollo Genome Annotation and Curation Tool, v 1.6.4 (Lewis et al., 2002). The gene from translation start to poly-A signal spans 28.5 kb in cattle and 35.5 kb in man. The cDNA sequence of *PARP6* in cattle is organised in 23 exons (see Table 4.9 and Figure 4.17) and all exons except the 9th, could be annotated on the bovine sequence. Without the missing basepairs, the bovine translated sequence from start to stop codon contains 1815 bp corresponding to exon 2 to 23 that translate into 605 amino acids residues in the protein sequence. The human sequence consists of 1890 bp, which correspond to 630 amino acids in the protein sequence. The difference of 25 amino acids between man and cattle originate from the missing basepairs of bovine exon 9.

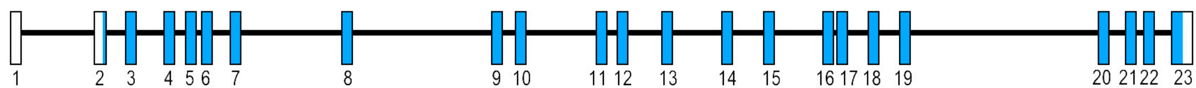


Figure 4.17 - Graphical overview bovine *PARP6.*
The translated and untranslated regions of the exons are depicted as bars in blue and white, respectively. The introns are shown as black thin line with a length approximately corresponding to the intron length.

Table 4.9 - Exon and intron organisation of bovine *PARP6* gene.

Exon			5'splice donor … 3'splice acceptor [a]	Intron	
No	Size (bp)	Position in cDNA		No	Size (bp)
			…cttGTGAA…		
1	190	-	TGAAG**gt**gaa … tgc**ag**GACAG	1	1877
2	207	1-3	CAATG**gt**gat … atc**ag**GACAT	2	632
3	78	4-81	TTCAG**gt**gag … ctc**ag**GGAAG	3	729
4	95	82-176	ATCAG**gt**ggg … tat**ag**AGAAT	4	429
5	61	177-237	TGGAT**gt**aag … tgt**ag**GAGGA	5	229
6	91	238-328	ACCGG**gt**aaa … tgc**ag**AACCA	6	736
7	67	329-395	AAAAA**gt**aag … ttt**ag**AATCC	7	2446
8	150	396-545	CCCAA**gt**aag … **unknown**	8	3357
9	136	546-681 [b]	CTTTG**gt**aca … atc**ag**GTCAG	9	539
10	54	682-735 [b]	TCCAG**gt**aat … ccc**ag**ATCAT	10	1796
11	96	736-831 [b]	TCAAG**gt**ata … ttc**ag**CCAGC	11	303
12	93	832-924 [b]	CAGAG**gt**atg … cta**ag**GTGGT	12	1039
13	126	925-1050 [b]	CTAAG**gt**ata … cac**ag**AAGAA	13	1278
14	66	1051-1116 [b]	CCCAG**gt**att … tac**ag**GGCTC	14	952
15	68	1117-1184 [b]	CAGTG**gt**atg … cct**ag**GATCA	15	1286
16	49	1185-1233 [b]	GCAGG**gt**aag … agc**ag**CTGAA	16	248
17	113	1234-1343 [b]	TTCCA**gt**gag … tac**ag**TGGGT	17	710
18	73	1344-1416 [b]	TGCAG**gt**gag … gca**ag**CTGCA	18	700
19	70	1417-1486 [b]	CTCAG**gt**aag … gac**ag**GAATG	19	4882
20	80	1487-1566 [b]	CCCAG**gt**att … tct**ag**ACCCG	20	395
21	64	1567-1630 [b]	TGAAG**gt**agg … ccc**ag**TGATT	21	172
22	85	1631-1715 [b]	TTTGT**gt**aag … ggc**ag**ATATG	22	336
23	384	1716-1815 [b]	…ACAAACTGAGGGGG…		

[a] Intron sequences are indicated by lowercase letters, exon sequences by capital letters, the splice site sequences are bolded, red triplets stand for start and stop codon.
[b]Exclusively missing bp in exon 9

The homology of *PARP6* and in particular its RNA binding motifs, between man, mouse and cattle was analysed by sequence alignment using ClustalX (v1.83) (Thompson et al., 1997). This shows a sequence identity of both ADP-ribosyl domains between the human (Acc. NP_064599, NCBI) and bovine sequence of 99% (Figure 4.18).

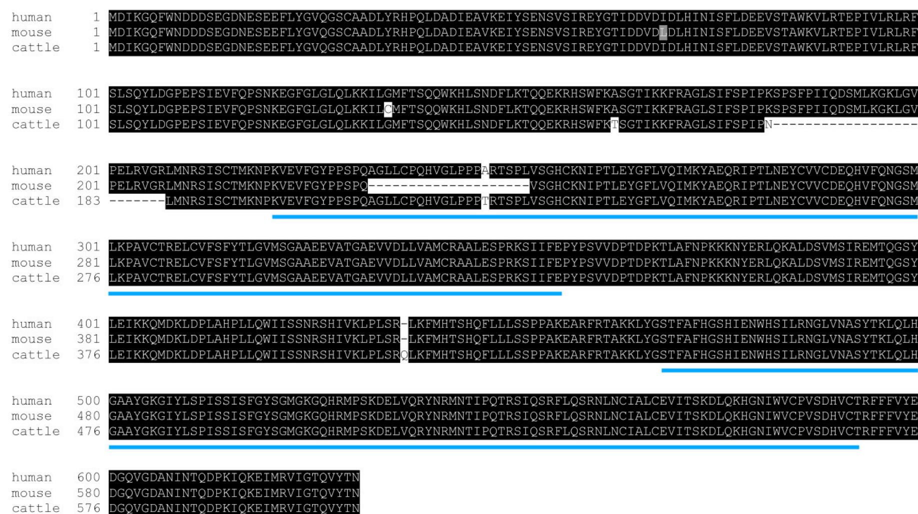


Figure 4.18 - Alignment of *PARP6* protein sequences of different species.
The sequence alignment shows extensive conservation of *PARP6* between man (Acc. NP_064599, NCBI), mouse (Acc. NP_084198, NCBI) and cattle sequence (Apollo-output). The blue lines below the alignment mark the regions containing the ADP-ribosyl-motifs.

The translation start of *PARP6* is located in the second exon. Potential gene regulatory elements were identified by analysing the putative promoter region of *PARP6*, the region 6 kb upstream the translation start (including 3 kb upstream exon 1, exon 1 and intron 1) of the human and bovine sequence with the programme *cpgplot* from the Emboss web-service http://www.ebi.ac.uk/emboss/cpgplot/ (Larsen et al., 1992). Putative CpG islands were identified in the 5'-end sequence upstream of exon 1 with sizes of 731 and 489 bp in cattle and human, respectively. Therefore, the analysis was repeated using only 3kb of the sequence upstream of exon 1 and the result is shown in Figure 4.19. The CpG islands are located approximately 1.4 to 1.9 kb and 1.2 to 1.9 kb upstream of exon 1 in man and cattle, respectively.

The human and bovine sequence from 3kb upstream of exon 1 was further analysed for conserved promoter sites using Consite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/). As shown in Figure 4.20, a conservation cut-off was adjusted to sequence identity of 84 %. A highly conserved region (> 84 % identity) could be identified located about 1.2 to 1.5 kb upstream of exon 1 containing twenty-two conserved AP2alpha binding sites, which are known to have enhancing function and four SP1 bindings sites. The conserved region corresponds to the predicted CpG island. No conserved promoter sequences (e.g., TATA or CAAT box) were found by the Consite tool.

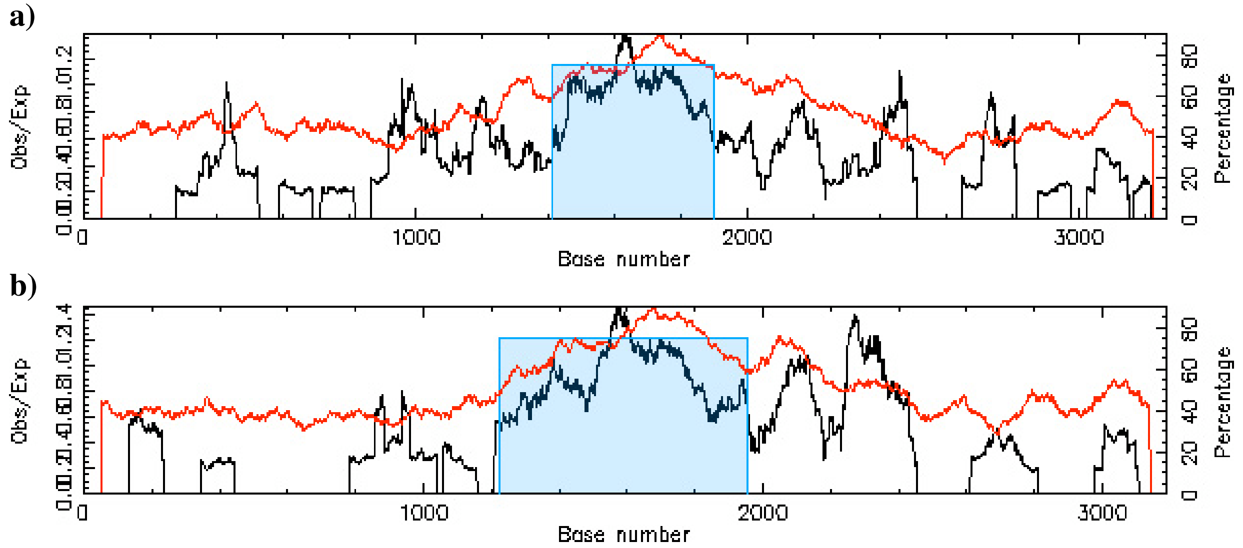


Figure 4.19 - Prediction of CpG islands in the 5'-end of *PARP6* in a) man and b) cattle.
The corresponding sequence of man and cattle (from 3 kb upstream of exon 1 to end of exon 1) was analysed by *cpgplot* http://www.ebi.ac.uk/emboss/cpgplot/ (Larsen et al., 1992). In black and on the primary axis on the left, the ratios of observed versus expected CpG content are plotted. In red and on the right secondary axis, the relative C+G contents are shown. The horizontal axis shows the bases relative to the end of exon 1 (3001 = approx. transcription start). The CpG island is illustrated by the blue box, and encompasses in man and cattle 0.5 and 0.7 kb, respectively.

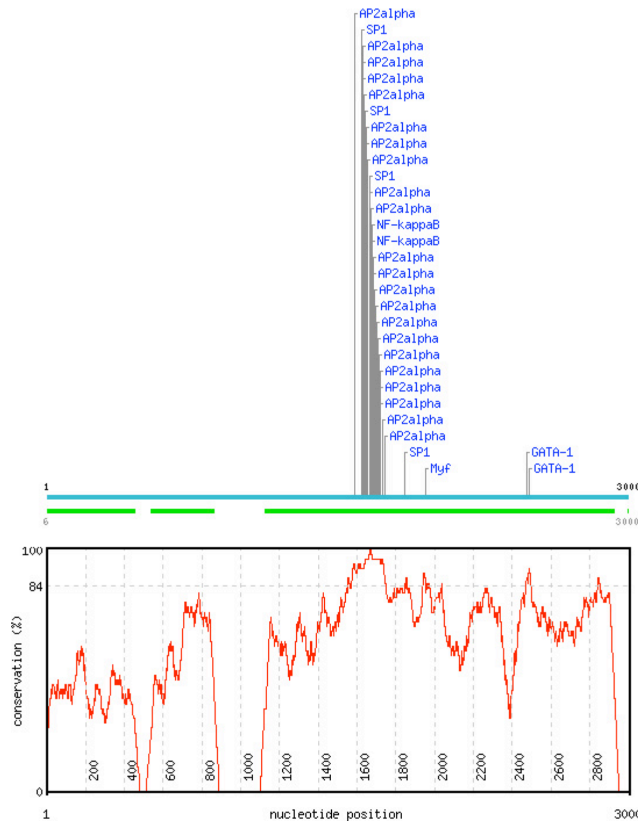


Figure 4.20 - Conservation profile of the aligned bovine and human 5'-region of *PARP6*
The red line shows the identity of both sequences. All transcription factor binding sites (blue) situated in conserved regions (≥ 84% identity) are displayed.

4.3 Polymorphism Analysis in the *HEXA* region

Polymorphism analysis was performed by sequencing a panel of pooled and individual DNA samples from animals of different breeds of taurine (Bta) and indicine cattle (Bti). In total, 57.2 kb DNA sequence was screened and 201 SNPs and nine Indel polymorphisms were found (see Table 4.10). For both the taurine and indicine breeds all SNPs in coding region are synonymous. All variations found in the region centered on the *HEXA* gene along with the location and the corresponding polymorphic breeds are shown in Appendix 11.3 (Polymorphisms identified in the extended HEXA region by re-sequencing).

The entire *HEXA* gene except the large intron 1 but comprising regions up- and downstream was re-sequenced. In total, 23.4 kb sequence was generated and 167 polymorphisms were found. From these variations, seven are insertion/deletion polymorphisms and 160 are SNPs. Fifty-one variations are polymorphic in indicine cattle only, or between both subspecies. In the exonic sequence (1.6 kb), four synonomous taurine SNPs have been found. In the putative regulatory region (1000 bp upstream the translation start) four taurine SNPs have been identified, which are not affecting putative transcription factor binding sites. This indicates a SNP, on average, approximately every 200 bp within intronic and flanking and every 400 bp within exonic sequence.

Partially re-sequencing (11.4 kb) of *ARIH1* revealed eight SNPs (three within Bti) in non-coding intronic and flanking regions and one SNP in the putative regulatory region with no effect on transcription factor binding sites. For the taurine breeds, on average, this indicates a SNP every 1.9 kb. For bovine *BRUNOL6*, DNA fragments with a total size of 13.8 kb were re-sequenced for SNP search. Within the coding sequence (1.24 kb), four indicine SNPs (synonymous) and within intronic sequences (6.9 kb) eight taurine and 12 indicine SNPs have been identified. In the putative regulatory region no variation was found in neither species. For the taurine breeds, this indicates one SNP aproximately every 1.7 kb. In total, 8.6 kb of bovine *PARP6* was re-sequenced. For the taurine breeds six and for the indicine breeds two SNPs have been identified in intronic regions. This indicates a SNP approximately every 1.1 kb in the taurine breeds (only intronic regions).

Table 4.10 - SNPs and insertion/deletion (indel) polymorphism found in *HEXA* region.

	All		**Upstream**		**cDNA**		**Intronic**		**Downstream**	
	SNPs	Indels	SNPs	Indels	SNPs	Indels	SNPs	Indels	SNPs	Indels
ARIH1	11.4 kb		5.7 kb		0.5 kb		5.2			
Btau	6		4				2			
Bti	3		2				1			
All	9		6				3			
HEXA	23.4 kb		5.3 kb		1.6 kb		14 kb		2.5kb	
Btau	111	5	33	2	4		65	2	9	1
Bti	49	2	15	1	1		30	1	3	
All	160	7	48	3	5		95	3	12	1
BRUNOL6	13.8 kb		2.4 kb		1.2 kb		7.6 kb		2.6 kb	
Btau	8						8			
Bti	16	2			3		12	2	1	
All	24	2			3		20	2	1	
PARP6	8.6 kb				1.0 kb		6.9 kb		0.7 kb	
Btau	6						6			
Bti	2						2			
All	8						8			
Region	57.2 kb		13.4 kb		4.3 kb		33.7 kb		5.8 kb	
Btau	131	5	37	2	4		81	2	9	1
Bti	70	4	17	1	4		45	3	4	
All	201	9	54	3	8		126	5	13	1

4.4 Genomic control (GC)

In order to detect possible population stratification among cases and controls of UK and German Holsteins, GC after Devlin and Roeder (1999) was performed. This methodology allows correcting the significance threshold according to the extent of population stratification detected by genotyping a panel of neutral SNPs. This avoids excess in the rate of false positives results in the presence of population stratification. For this purpose both cases and controls were genotyped by the hME method and MALDI-TOF for a panel of 37 SNPs that was initially developed for paternal testing and identity control (Werner et al., 2004). These SNPs are considered as so-called *null-loci* having no effect on the phenotype. After further examination of the genotypes, eight SNPs were excluded from the calculations due to monomorphism (two SNPs), strong deviance from HWE caused by an excess of heterozygous genotypes (three SNPs) and unspecific calling in the genotyping procedure (three SNPs). In Figure 4.21, the minor allele frequencies for the remaining 29 SNPs are presented for the cases and controls of both, the UK and the German Holstein animals. The corresponding trend values are obtained by Armitage Trend test, which tests for differences in the genotype frequencies between cases and controls. The median of the trend values across all 29 SNPs is similar for both populations: 0.48 and 0.47 for the UK and the German Holsteins, respectively. In Appendices 11.5, 11.6 and 11.7, the genotyping results for the null-loci for the UK and German Holsteins and for the sequencing panel animals consisting of German Fleckvieh, German Brown and German Holstein are presented, respectively.

The inflation factor λ is used to correct the significance threshold for the detected extend of population stratification. λ is calculated by dividing the median value of the Armitage Trend statistic across all *null-loci* by 0.456 (see Chapter 3.15.8 – Inferring the inflation factor as GC). Virtually no stratification could be detected for both populations. As shown in the Table 4.11, the inflation factors constitute 1.05 and 1.03 for the UK and German Holstein animals, respectively. Therefore, the significance threshold of the corresponding type 1 error rate of 5 % increases only slightly from 3.84 to 4.03 and from 3.84 to 3.96 for UK and German Holsteins, respectively. The detected effect of inflation is marginal in both populations, indicating that the groups are not stratified. Therefore, the changes in the P-values of the association test due to correction of the χ^2-test statistics by the inflation factors 1.05 (UK Holstein) and 1.03 (German Holsteins) are only marginally.

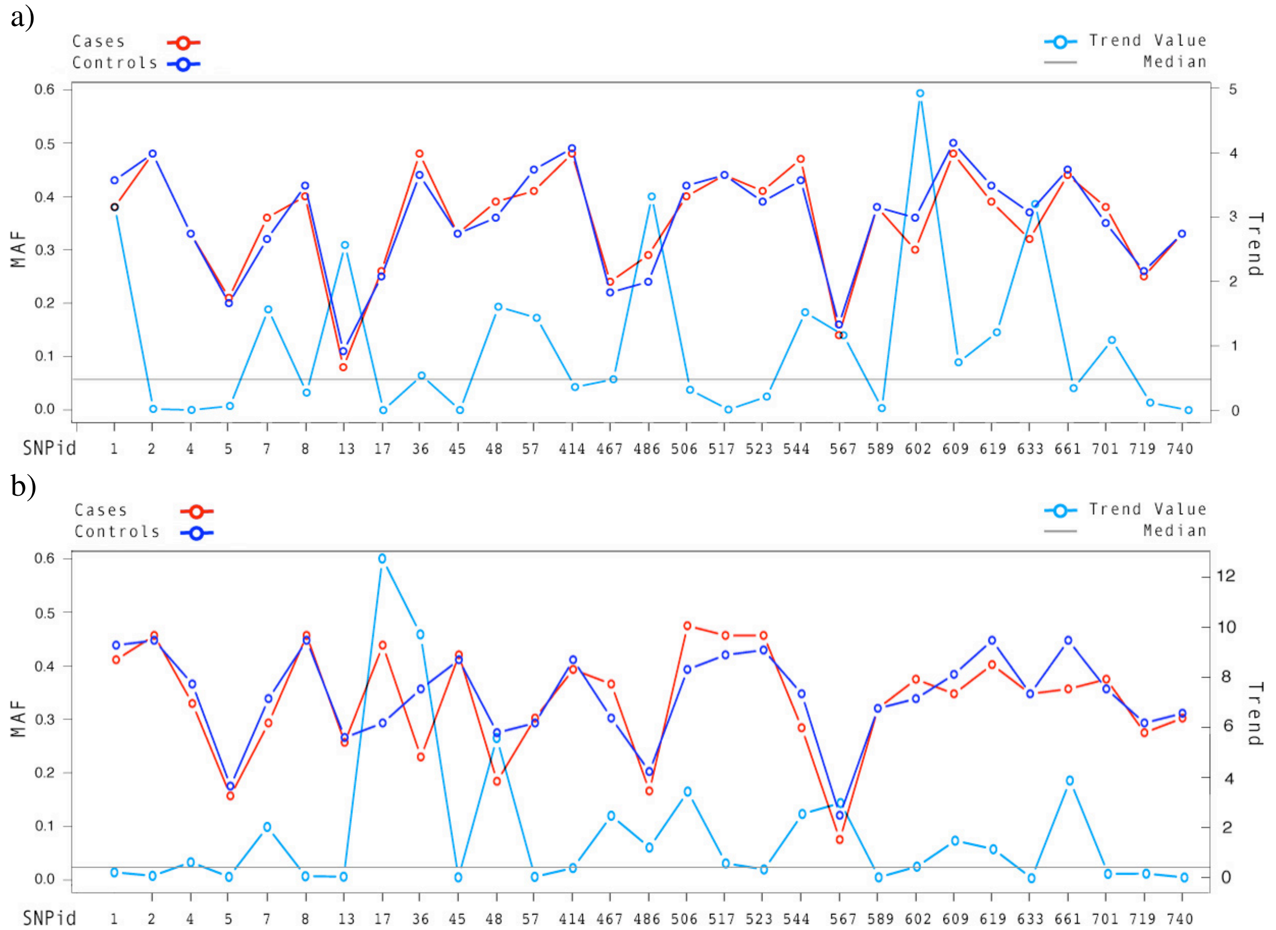


Figure 4.21 - Minor allele frequencies (MAF) and Trend Values of the *null-loci* used for calculation the inflation factors for a) UK Holsteins b) for the German Holsteins

The primary axis on the left displays the MAF of the *null-loci* of cases and controls. The secondary axis on the right shows the differences in genotype frequencies between cases and controls as trend values, obtained from Armitage Trend test. The SNPids on the horizontal axis indicate the SNP identification numbers assigned in the internal database. The median of the trend values across all 29 SNPs is displayed as grey horizontal line.

Table 4.11 - Results of GC approach in the UK and German Holstein animals.

Population	Inflation factor λ	λ * 3,84 Significance threshold	Corrected test statistic
UK Holstein	1.05	4.03	$\frac{\chi^2_{test}}{1.05} \sim \chi^2(0)$
German Holstein	1.03	3.96	$\frac{\chi^2_{test}}{1.03} \sim \chi^2(0)$

In order to evaluate the GC method and the estimated population stratification between cases and controls within both populations, the same method was used to calculate stratification

between UK and German Holsteins. For this purpose, cases and control animals were jointly compared between the populations. In Figure 4.22, the combined minor allele frequencies of each population are presented for all 29 SNPs. Allele frequency differences between the UK and German Holsteins, results in high Trend values. The median of the Trend values across all 29 SNPs is 8.91 and the corresponding inflation factor is 19.54, which indicates extensive stratification between populations, although both populations are from the same breed.

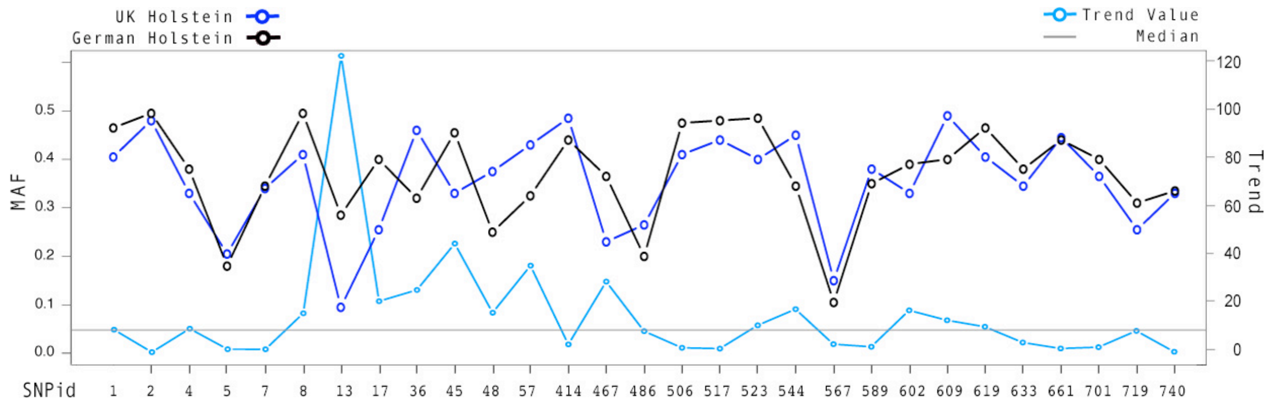


Figure 4.22 - MAF of *null-loci* of UK and German Holsteins and corresponding Trend values. The primary axis on the left displays the MAF of the populations. The secondary axis on the right shows the Trend values, obtained from Armitage Trend test, testing differences in the genotype frequencies between UK and German Holsteins. The SNPids on the horizontal axis indicate the SNP numbers assigned in the internal database. The grey horizontal line stands for the median trend values across all 29 SNPs.

4.5 Genotyping of SNP with MALDI-TOF MS

Fifty-six polymorphisms in the genes *ARIH1*, *HEXA*, *BRUNOL6*, *PARP6*, *PRNP* and in additional candidates were selected for genotyping in a panel of BSE-cases and controls of UK and German Holsteins. Criteria for the selection are presented in Chapter 3.13 – Selection of SNPs/Polymorphisms for Genotyping.

In the extended *HEXA* region (including the neighbouring genes), 41 SNPs were selected and analysed in different genotyping assays. At first, four SNPs in the *HEXA* gene (418, 400, 276 and 417) were genotyped by the hME-method and MALDI-TOF MS (as shown in Chapter 3.14.1 – The hME method). The calling rate, which is defined as the fraction of obtained genotypes relative to possible genotypes (376 / plate) ranged from 96-100% and could therefore be considered as on a very high level. Control experiments comparing randomly selected genotypes obtained by hME method and MALDI-TOF MS with genotypes from the same individuals obtained from sequencing showed perfect agreement. In addition, concordant results were obtained for duplicated samples on the DNA plate.

Using the iPLEX method (see Chapter 3.14.2 – The iPLEX method), 37 SNPs of the extended *HEXA* region were genotyped in three assays (one 27-plex and two 16-plex assays). Additionally, SNPs from other candidate genes, including *LAMR1*, *PLG*, *HSPA8* and *IREB2* and two insertion/deletion polymorphisms at the *PRNP* locus were genotyped with this method. Primer design procedure for the iPLEX method allowed combining three SNPs located in *ARIH1*, 26 SNPs selected in *HEXA*, four SNPs in *BRUNOL6* and *PARP6*, respectively and two SNPs detected in *LAMR1, IREB2* and *PLG,* respectively in a 27-plex and a 16-plex assay. In the 27-plex assay, genotyping of five SNPs failed because of bad spectrum quality possibly caused by insufficient PCR amplification. Three SNPs had acceptable (>90%) and 19 SNPs had high calling rates (>95%). In the 16-plex assay, 14 SNPs revealed high calling rates (>95%). For two SNPs the results could not be analysed due to poor spectrum quality.

An additional iPLEX (16-plex) assay was performed, including six SNPs, which showed insufficient quality in one of the earlier assays. Again for one of these SNPs, no reliable genotypes could be obtained. Comparison of the results of the hME with the iPLEX method was carried out by including one SNP showing good results by hME. The iPLEX genotypes for each individual were identical with the genotypes obtained from hME method and the calling rate in both experiments was >95 %. This comparison indicates that both systems worked appropriately. Additionally, nine SNPs that had not been genotyped before were included in this assay: one SNP from *HEXA*, *BRUNOL6* and *PARP6*, respectively, two insertion-deletion polymorphism in the *PRNP* promoter region and four SNPs from the *HSPA8* gene. One of

these SNPs was not polymorphic in the Holstein animals. Therefore, these genotypes were not included in further analyses. For the remaining SNPs, the genotyping results were of sufficient quality to be used in association studies. Table 4.12 summarises the SNP genotyping with MALDI-TOF MS.

Table 4.12 - Summary of SNP genotyping with MALDI-TOF MS.
Number of successfully genotyped SNPs with different methods (hME and I-plex) and detection of mass differences by MALDI-TOF MS.

	hME	I-plex				Successfully genotyped with		
	1-4 plex	27-plex	16-plex	16-plex				
Gene				Rerun	Add	hME	I-plex	∑
ARIH1		2	1	1			2	2
HEXA	4	16	10	3	2	4	24	28
BRUNOL6		2	2	1	1		4	4
PARP6		4		1	1		4	4
PRNP					2		2	2
HSPA8					4		4	4
PLG			2				2	2
LAMR1		1	1				1	1
IREB2		2					2	2
GC SNPs	37					29		29

A panel of 41 SNPs in the encompassing region of *HEXA* was genotyped revealing that 38 SNPs could be further analysed. The arrangement of the SNPs over the region is shown in Figure 4.23. The genotypes were tested for HWE (as shown in Chapter 3.15.2 - Test for Hardy-Weinberg-Equilibrium): Departures from the assumption of HWE were not observed for the UK animals. The test for HWE for the German Holstein controls (all half-sibs of one sire) was obsolete, since their genotypes were deduced assuming HWE (see Chapter 3.15.10 – Inferring allele and genotype frequencies from half-sibs). However, test for HWE discovers genotyping errors if the genotyping assay worked insufficiently.

Figure 4.23 - SNPs successfully genotyped in the extended *HEXA* region.
The blue line indicates the chromosomal region analysed, with genes and partially exons (E) displayed on top. Arrows indicate the 5'-3' reading direction. Thin lines mark SNPs with the corresponding SNP ids as assigned in the internal database.

4.6 Association of single markers with BSE

4.6.1 SNPs in the extended *HEXA* region

UK and German Holstein

The minor allele frequencies for both, the UK and the German BSE cases and controls for the 38 SNPs in the *HEXA* region and the corresponding Trend-values obtained from Armitage Trend test are demonstrated in Figure 4.24. The allele frequencies of the German Holsteins are similar to the frequencies of the UK Holstein, whereas significant differences between cases and controls within each population were only found in the UK animals. The single marker analysis by Armitage trend test revealed that the genotypes of six intronic SNPs (276, 796, 956, 988, 989 and 995) were significantly different between cases and control animals of UK Holstein at the SNP-wise 5% type I error rate with correction for population stratification. The genotyping and association results for the candidate SNPs are presented in Appendices 11.8 and 11.9, respectively.

Description Figure 4.24 (page 72):

Figure 4.24 - MAF and Trend values of the SNPs genotyped in (a) the UK and (b) German Holsteins BSE-cases and controls.
The primary axis on the left displays the MAF of cases and controls. The secondary axis on the right shows the Trend values, obtained from Armitage Trend test, testing differences in the genotype frequencies between cases and controls. The SNP ids on the horizontal axis indicate the SNP numbers as assigned in the internal database and thin lines mark the SNP position on the chromosomal region. Arrows indicate the 5'-3' reading direction of the genes. The Figure is not to scale according to the physical map locations; the SNPs are equidistant to allow easy visualisation of the association test results. The significance threshold (p = 0.05) corrected for population stratification is displayed as grey horizontal line.

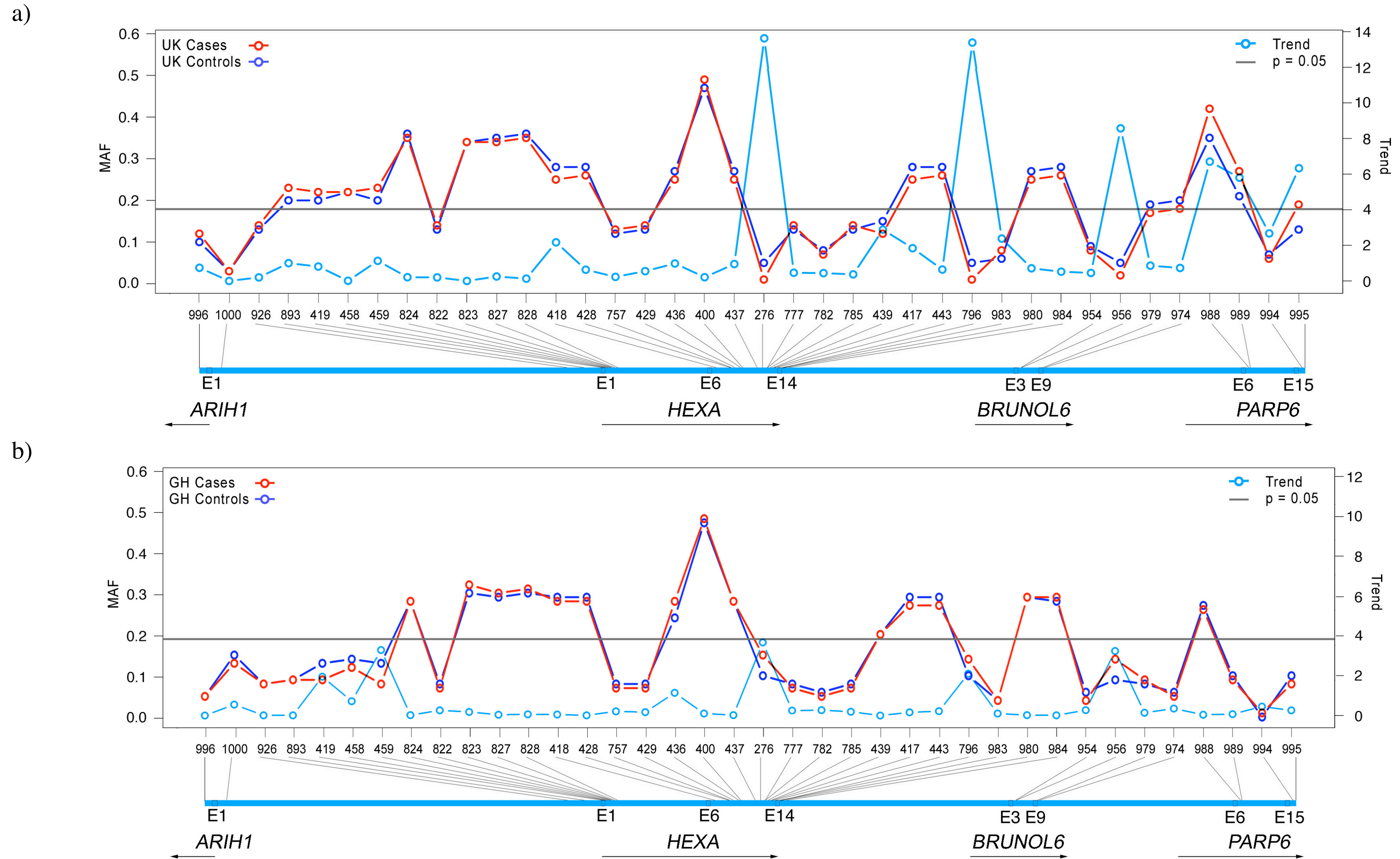


Figure 4.24 - MAF and Trend values of (a) UK and (b) German Holsteins BSE-cases and controls. (For description see page 71).

Multiple testing was corrected for the UK animals by the permutation procedure as implemented in the software *Haploview (v3.3)* (Barrett et al., 2005) (as shown in Chapter 3.15.7 – Correcting for Multiple Testing). 10^5 permutations including all 38 SNPs were performed revealing three SNPs with ids 276, 796 and 956 significantly associated with the BSE status with permuted P-values of 0.002, 0.005 and 0.05, respectively. The associations result from an overrepresentation of the rare alleles in the control animals, e.g. the frequency of the T alleles for both, the SNP 276 (G/T) and the SNP 796 (C/T) constitute 1% in cases and 5% in control animals. This indicates that the rare alleles at the three SNPs are protective and most of the animals carry the susceptibility alleles. The marginal effect of population stratification was not accounted for, when multiple testing was applied.

Logistic regression analysis was performed by modelling a regression of the number of susceptibility alleles on the BSE risk. Results of the analyses are presented in Odds Ratios (OR) for individuals with different genotypes. OR are defined as the ratio of the probability to develop BSE for individuals with one genotype versus the probability to succumb to BSE for animals carrying another genotype. The inferred OR increases proportional with the number of susceptibility alleles. In contrast to heterozygous carriers, possess homozygous carriers of the susceptibility alleles relatively higher risks of having BSE with OR ranging from 3.62 to 2.64 for the significant SNPs (Table 4.13). No UK animals with homozygous genotype for the protecting alleles were identified in this study. Assuming HWE and a mean allele frequency of 3 %, only 0.09 % of the individuals of the UK population are expected to be homozygous for the protecting allele at SNP 276. One could speculate that the homozygous genotype at SNP 276 is highly protective.

Table 4.13 - Results of single marker analyses in the UK Holstein animals.
Rare alleles are represented as 'A's and the frequent alleles as 'B's.

	Alleles		Genotypes Cases			Genotypes Controls			Permuted	Logistic Regression Analysis		
SNPid	A	B	AA	AB	BB	AA	AB	BB	P-value[a]	P-value[b]	OR AB [c]	OR BB
276	T	G	-	10	340	-	27	246	2×10^{-3}	6.5×10^{-4}	1	3.62
796	T	C	-	10	347	-	26	243	5×10^{-3}	7.2×10^{-4}	1	3.60
956	G	T	-	13	330	-	26	245	5×10^{-2}	5.4×10^{-3}	1	2.64

[a] P-value obtained from χ^2-testing and corrected for multiple testing by 10^5 permutations.
[b] P-value from logistic regression, modelling the number of B alleles on BSE status.
[c] reference genotype

For the German Holstein animals, no association of a SNP in the extended *HEXA* region with the BSE status could be identified. The direction of the allele frequencies for the SNPs sig-

nificantly associated with BSE in the UK animals differs in the German animals (see Table 4.14). The rare '*UK-protecting*' alleles at the SNPs 276, 796 and 956 tend to be more frequent in the German cases. However, these observations were not significant with P-values obtained from Armitage Trend test being 0.053, 0.15 and 0.072 for SNPs 276, 796 and 956, respectively (Figure 4.24) without correction for multiple testing. However, logistic regression analysis showed that the risk for BSE decreases proportional to the number of '*UK-susceptible*' alleles (see Table 4.14). Thus, the effect of the SNP alleles seems to be different between UK and German Holstein animals. Nevertheless, the results for Holstein Friesian are not significant, possible due to the small number of BSE affected animals in this group.

Table 4.14 - Results of single marker analyses in the German Holstein animals.
Rare alleles are represented as 'A's and the frequent alleles as 'B's

	Alleles		Genotypes Cases			Genotypes Controls[d]				Logistic Regression Analysis			
SNPid	A	B	AA	AB	BB	AA	AB	BB	P-value[a]	P-value[b]	OR AA [c]	OR AB	OR BB
276	T	G	4	12	51	6	108	507	5.3×10^{-2}	4.7×10^{-2}	1	0.60	0.37
796	T	C	3	10	50	6	112	512	1.5×10^{-1}	1.4×10^{-1}	1	0.67	0.45
956	G	T	3	13	49	5	98	493	7.2×10^{-2}	6.3×10^{-2}	1	0.61	0.37

[a] P-value obtained from χ^2-testing, not corrected for multiple testing
[b] P-value from logistic regression, modelling the number of B alleles on the BSE status.
[c] reference genotype
[d] Control consists of half-sibs (see Chapter 3.15.10 - Inferring the allele and genotype frequencies from halfsibs)

Allele and genotype frequencies for all SNPs that have been genotyped in BSE cases and controls of UK and German Holstein animals in the *HEXA* region are summarised in Appendices 11.8 and 11.9, respectively.

German Fleckvieh, German Brown and German Holstein

Allele frequencies of the genotyped SNPs in the German main breeds German Holstein, German Fleckvieh and German Brown were estimated by genotyping 31 bulls of the sequencing panels, which were unrelated in the previous three generations for each breed. Figure 4.25 shows clearly the differences in the MAFs for the three populations. German Fleckviehs MAFs average 13 % higher compared to the MAFs of German Holstein for all SNPs in the *HEXA* gene (from id 926 to id 984). Unlike German Holstein and German Fleckvieh, the German Brown animals are mainly not polymorphic at the extended *HEXA* region, except for two SNPs (ids 400 and 988), which showed MAFs above 5%. Therefore, the SNPs chosen in the extended *HEXA* region appeared to be inappropriate for an association study in German Brown. The genotyping results are presented in the Appendix 11.10.

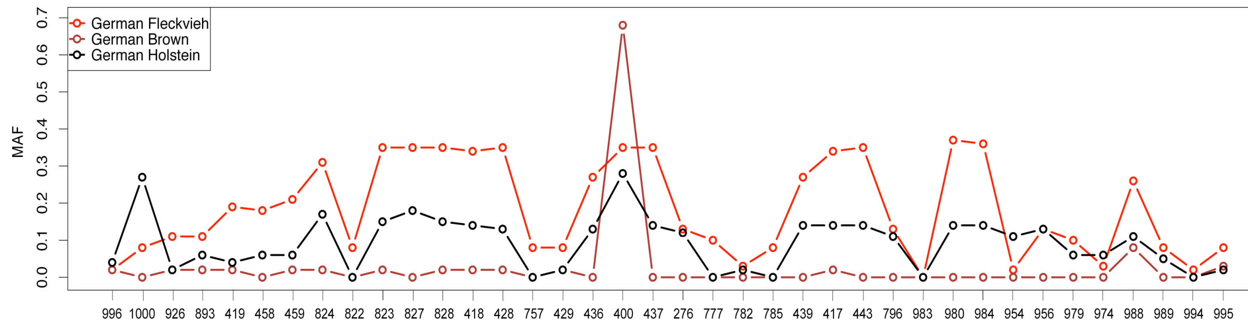


Figure 4.25 - MAF in German Fleckvieh, German Brown and German Holsteins bulls.
The MAF of the SNPs genotyped in the extended *HEXA* region.The vertical axis displays the MAF. The SNP ids on the horizontal axis indicate the SNP numbers assigned in the internal database.

The SNPs with ids 276, 796 and 956 that were associated with the BSE status in the UK animals show similar allele frequencies ranging from 10 % to 13 % in both, the German Fleckvieh and the German Holstein bulls. In German Fleckvieh, homozygous individuals carrying both alleles were found at these SNPs, whereas in German Holstein, only animals that are homozygous for the more frequent allele were identified. In German Brown, no '*UK-protecting'* alleles were found at the SNPs.

Appendix Table 11.10 summarises the results for German Fleckvieh, German Holstein and German Brown, including allele and genotype frequencies for all genotyped SNPs of the *HEXA* region.

4.6.2 SNPs in additional candidate genes

SNPs in four additional candidate genes have been genotyped in UK and German Holstein BSE diseased animals and controls: the genes encoding Plasminogen (*PLG*), Heat-shock protein 70 (*HSPA8*), Iron-responsive binding element (*IREB2*) and the Laminin Receptor (*LAMR1*) have been postulated as candidate genes in Juling (2002). Association was tested by Armitage Trend tests. As shown in Figure 4.26, no significant association has been detected for one of the SNPs in the UK animals and therefore, no further analysis such as correction for multiple testing or population stratification was applied. In German Holstein animals, the intronic SNP with id 392 in *HSPA8* shows significant association, after correction for population stratification with a P-Value of 0.029. But Bonferroni correction for multiple testing suggests, that this finding is due to chance with corrected P-value of 0.21. Thus, genotyping of SNPs in additional candidates in BSE diseased and control animals revealed no significant differences in the allele frequencies and no association. However, the weak coverage of SNPs in the additional investigated genes does not allow dismissing these genes from the list of potential candidate genes for BSE susceptibility.

Genotyping and association results for the additional SNPs are summarised in Appendices 11.8 and 11.9 for UK and German Holstein, respectively.

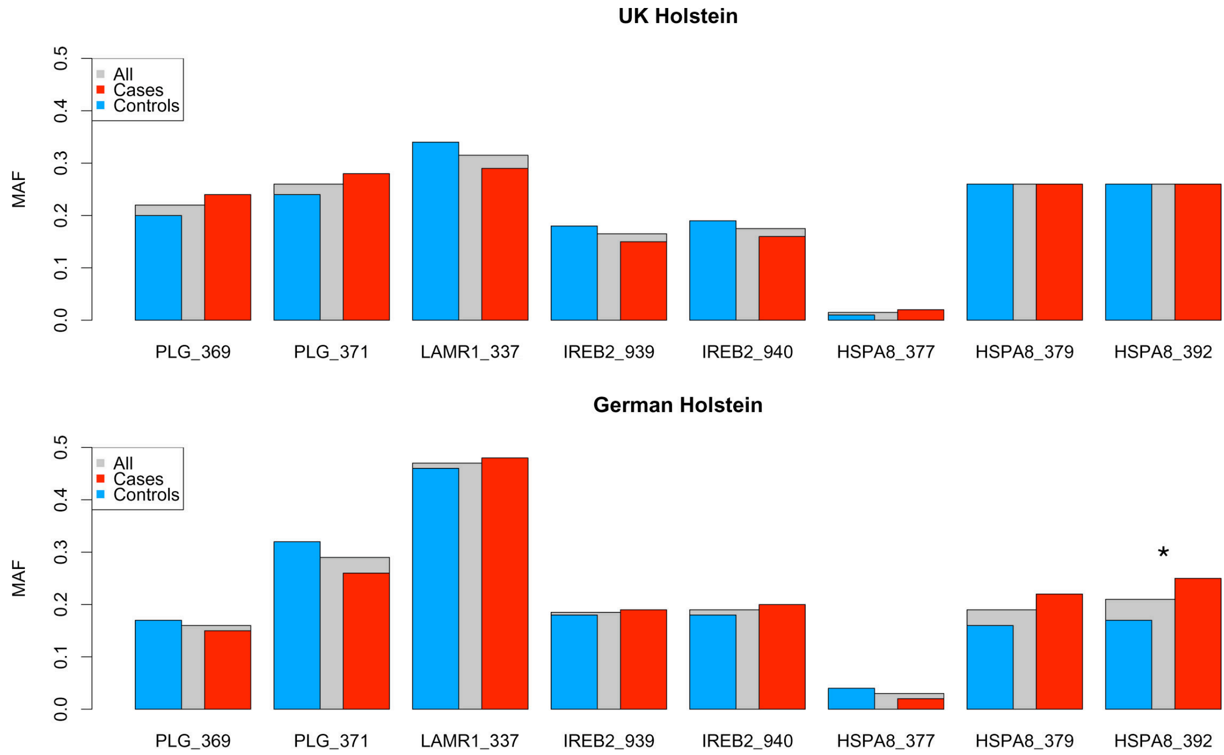


Figure 4.26 - Minor Allele Frequencies (MAF) of eight SNPs in additional candidate genes.
MAF from UK and German Holstein Friesian BSE affected (red) and control (blue) animals. The asterisk indicates significance with a nominal P-value of 0.029. Bonferroni correction for multiple testing revealed an corrected P-value of 0.21.

4.7 Analysis of the *HEXA* region

4.7.1 LD in *HEXA* region among UK animals

Genotypes of 38 SNP markers obtained in the previously carried out single marker analysis allowed an intensive analysis to identify LD between markers and haplotype pattern in the extended *HEXA* region. As presented in Chapter 4.6.1 – SNPs in the extended HEXA region, the frequencies at six SNPs of the extended *HEXA* region differ significantly between BSE cases and controls in the UK Holstein animals and three SNPs were associated after correction for multiple testing. In order to see whether the association of these SNPs is resulting from one or several SNPs, the pairwise LD was calculated between SNPs. Pairwise LD is defined as non-random association between two loci. Analysis of the LD was done with *Haploview (v3.3)* (Barrett et al., 2005). In Figure 4.27, the *HEXA* region and pairwise LD between the genotyped SNPs in UK animals are presented, measured by D' and r^2 (see Chapter 3.15.5 – Measuring of Linkage Disequilibrium and tagging SNPs). Strong LD exists across the whole region and therefore some of the makers are redundant. Particularly between the three SNPs (276, 796 and 956) showing significant association in the previous single marker analysis, strong LD is measured with r^2 between 0.97 and 1. One could argue that one of these SNPs is causal for the association and the single marker association of the other two is due to LD with the causal mutation. Alternatively, all three SNPs may be in LD with the putative causal polymorphism more up- or downstream of this region. However, since genes were not completely sequenced and screened for polymorphisms, additional polymorphisms in coding and non-coding, but regulative regions may exist.

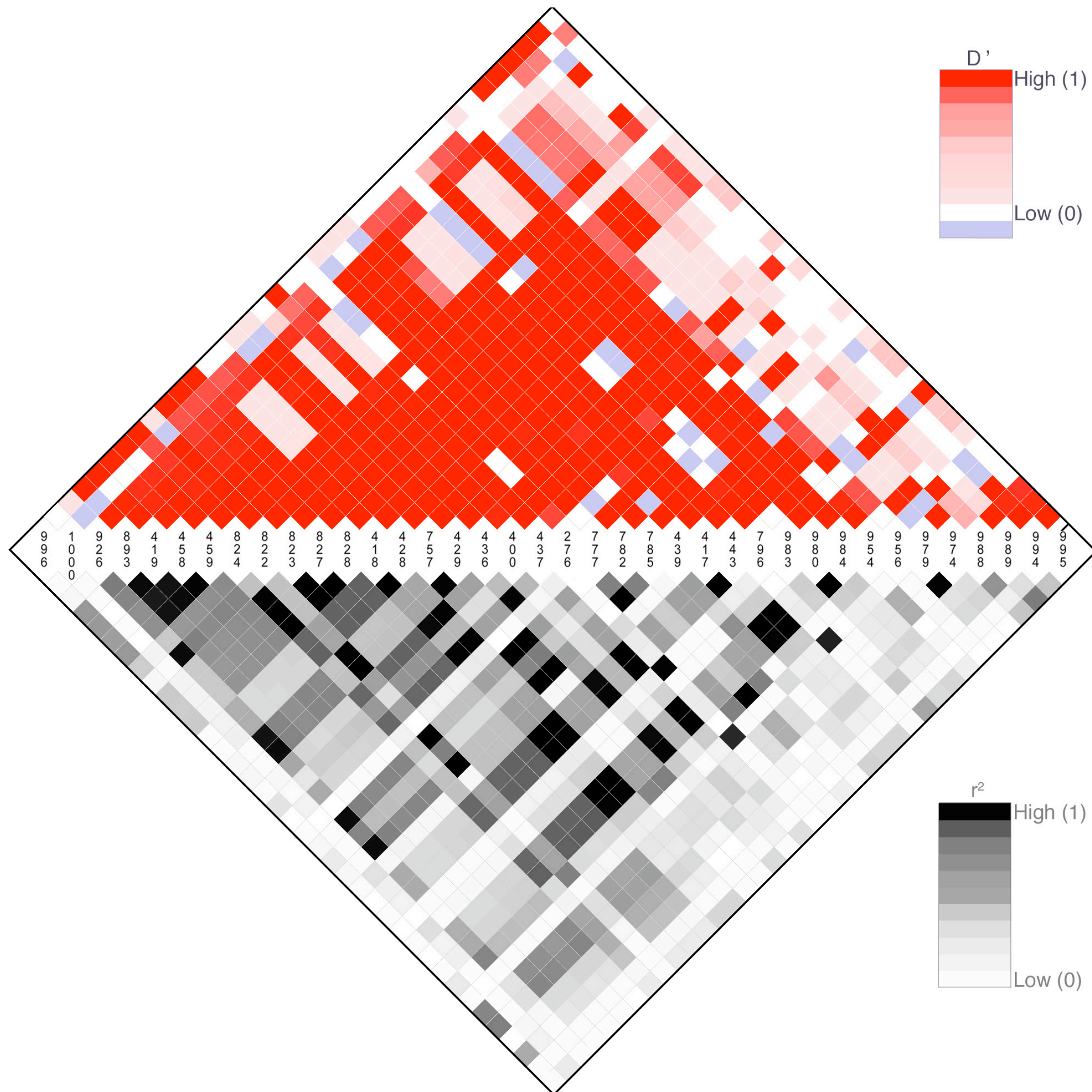


Figure 4.27 - LD plot of the extended *HEXA* region (208 kb) among UK Holsteins.
The triangle on the top shows the LD measured by D' while the lower triangle visualises LD measured by r^2. Regions of high and low LD are presented by red and white shading for D' and black and white shading for r^2, respectively. The darker the colour of the squares, the higher are the values for D' and r^2 and therefore the stronger the LD between both corresponding markers. The SNP ids on the horizontal axis indicate the SNP numbers assigned in the internal database.

4.7.2 Haplotype analysis and SNP *tagging* for UK animals

In order to elucidate the potential effect of combinations of SNPs on the BSE risk, a haplotype analysis was performed. Haplotypes were estimated using *Haploview (v3.3)* (Barrett et al., 2005) that uses an accelerated EM algorithm similar to the partition/ligation method described in Qin et al. (2002). Including all genotyped SNPs and assuming no haplotype block structure, twelve haplotypes with a frequency of > 1 % were identified in total (see Figure 4.28). This analysis revealed that all '*UK-protecting*' alleles of SNPs 276, 796 and 956, repre-

senting the rare alleles T-T-G, respectively, occur on the same haplotype (red bold letter haplotype 11 in Figure 4.28).

Strong LD was observed between SNPs over the extended HEXA region. Therefore, SNPs were *tagged* by the software *Tagger*, implemented in *Haploview (v3.3)* (Barrett et al., 2005), which eliminates redundant SNPs being in strong LD to each other. The software selects randomly from equivalent SNPs within a haplotype, hence the selection could differ for each *tagging* operation. The pairwise *tagging* including 38 SNPs with a r^2 -threshold of 0.8 picked 18 SNPs with the three associated SNPs (276, 796, 956) included manually. Applying the procedure without manually including, 16 SNPs were *tagged* inclusively one out of the SNPs 276, 796 and 956. These SNPs are in strong LD and therefore one would be enough to define the others. However, the SNPs were not eliminated from further analysis.

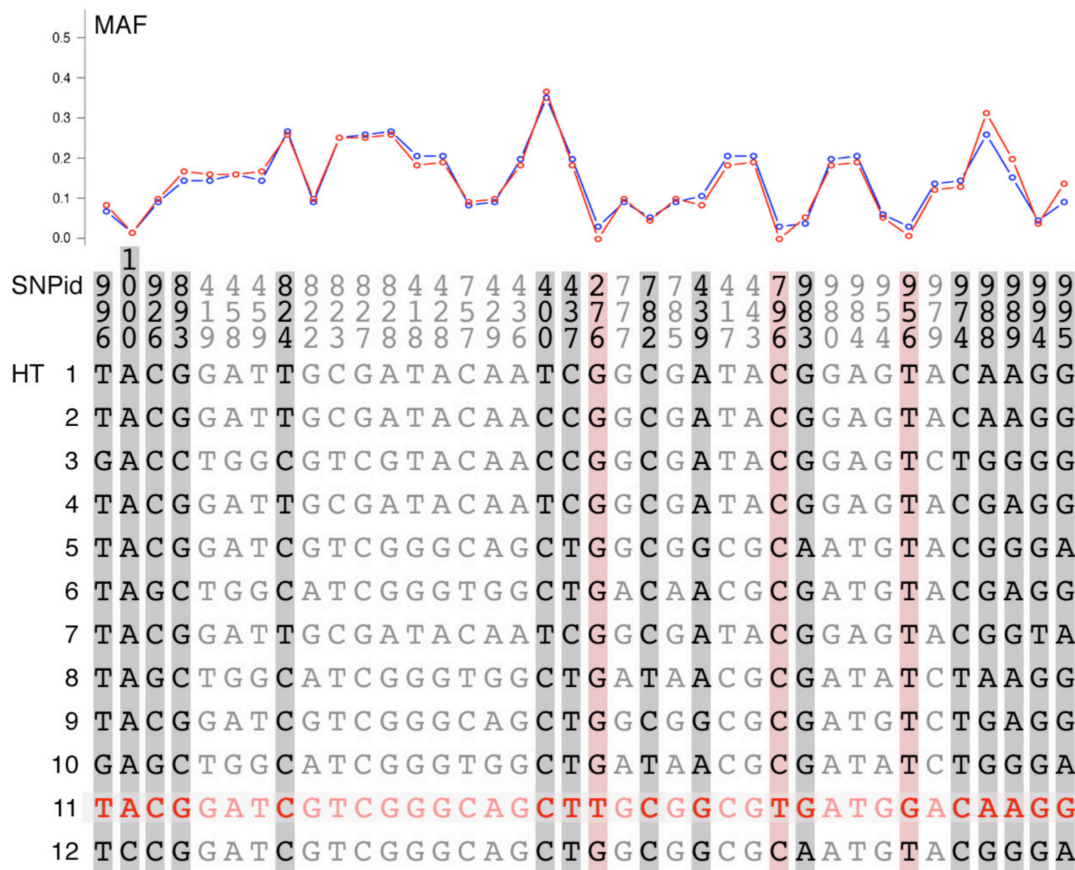


Figure 4.28 - Haplotypes in the extended *HEXA* region including 38 SNPs.
The line graph shows the minor allele frequencies (MAF) for each SNP, with the SNPids displayed on the horizontal axis. For each SNP the alleles are displayed in rows below of the corresponding SNPid. Hence, twelve haplotypes (HT) were generated from 38 SNPs genotyped. SNPs that were selected by *tagging* are shaded grey. Red shading marks SNPs that were associated with BSE status and have been manually included in the *tagging* procedure. The red bold haplotype contains the three *'UK-protecting'* alleles.

After the *tagging* procedure, 18 SNPs remained to derive haplotypes. Again, twelve haplotypes with minor frequencies > 1% were predicted. Subsequently, χ^2-tests were carried out, testing the number of observations of one haplotype against the pooled observations of all remaining haplotypes. These tests revealed that two haplotypes were significantly associated with BSE (Figure 4.29). Haplotype number 11 (red shaded in Figure 4.29) containing all three '*UK-protecting*' alleles T-T-G was significantly overrepresented in the controls compared to the cases (4.7% versus 1.3%) with a P-value of 0.002 after correction for multiple comparisons by 10^5 permutations. This suggests that the haplotype association with BSE is caused by the rare alleles at the SNPs 276, 796 and 956, which were considered to be the protecting alleles. However, the second significantly associated haplotype number 9 (pink shaded in Figure 4.29) was also less frequent in BSE cases compared to the controls (4.1% versus 1.6%, permuted P-value 0.043). Surveying the loci that were associated with the BSE status revealed that no '*UK-protecting*' allele appeared within this haplotype. This result indicates additional loci being involved in the BSE development which are in LD with the investigated SNPs.

SNPid	996	1000	926	893	824	400	437	276	782	439	796	983	956	974	988	989	994	995					
Haplotype																			Cases	Controls	χ^2	P-Value	Per-P
1	T	A	C	G	T	T	C	G	C	A	C	G	T	C	A	A	G	G	0.355	0.369	0.26		
2	T	A	C	G	T	C	C	G	C	A	C	G	T	C	A	A	G	G	0.171	0.167	0.03		
3	G	A	C	C	C	C	C	G	C	A	C	G	T	T	G	G	G	G	0.082	0.075	0.21		
4	T	A	C	G	T	T	C	G	C	A	C	G	T	C	G	A	G	G	0.063	0.054	0.41		
5	T	A	C	G	C	C	T	G	C	G	C	A	T	C	G	G	G	A	0.061	0.045	1.63		
6	T	A	G	C	C	C	T	G	C	A	C	G	T	C	G	A	G	G	0.061	0.041	2.48		
7	T	A	C	G	T	T	C	G	C	A	C	G	T	C	G	G	T	A	0.057	0.034	3.68		
8	T	A	G	C	C	C	T	G	T	A	C	G	T	T	A	A	G	G	0.037	0.050	1.44		
9	T	A	C	G	C	C	T	G	C	G	C	G	T	T	G	A	G	G	0.016	0.041	7.15	0.0075	0.043
10	G	A	G	C	C	C	T	G	T	A	C	G	T	T	G	G	G	A	0.033	0.025	0.64		
11	T	A	C	G	C	C	T	T	C	G	T	G	G	C	A	A	G	G	0.013	0.047	12.5	0.0004	0.002
12	T	C	C	G	C	C	T	G	C	G	C	A	T	C	G	G	G	A	0.020	0.017	0.20		

Figure 4.29 - Haplotype analysis in the extended *HEXA* region including 18 *tag*SNPs.
Numbers on top of the table indicate the ids of the *tagged* SNPs. Grey shading highlights SNPs that were significantly associated in the single marker analysis. Further shown are haplotype frequencies for cases and controls, the χ^2-test statistic, testing the corresponding haplotype against all others, the corresponding P-value for those haplotypes which yielded P < 0.05 and the P-value after 10^5 permutation (Per-P). Haplotype 11 (containing the rare ‚*UK-protecting*' alleles) and haplotype 9 are significantly overrepresented in the control animals (marked by red and pink shading).

Haplotype blocks refer to sites of closely located SNPs, which are inherited in blocks. The block structure was assessed manually according to the genetic distances between loci. All 18 SNP markers were assigned to a total of four haplotype blocks: Three blocks were assigned to

the SNPs in *ARIH1*, to the markers in the upper part of *HEXA* and to the SNPs in *PARP6* as shown in Figure 4.30. The SNPs 276, 796 and 956 were combined in one haplotype block (Block 3) because of the high pairwise LD between these SNPs.

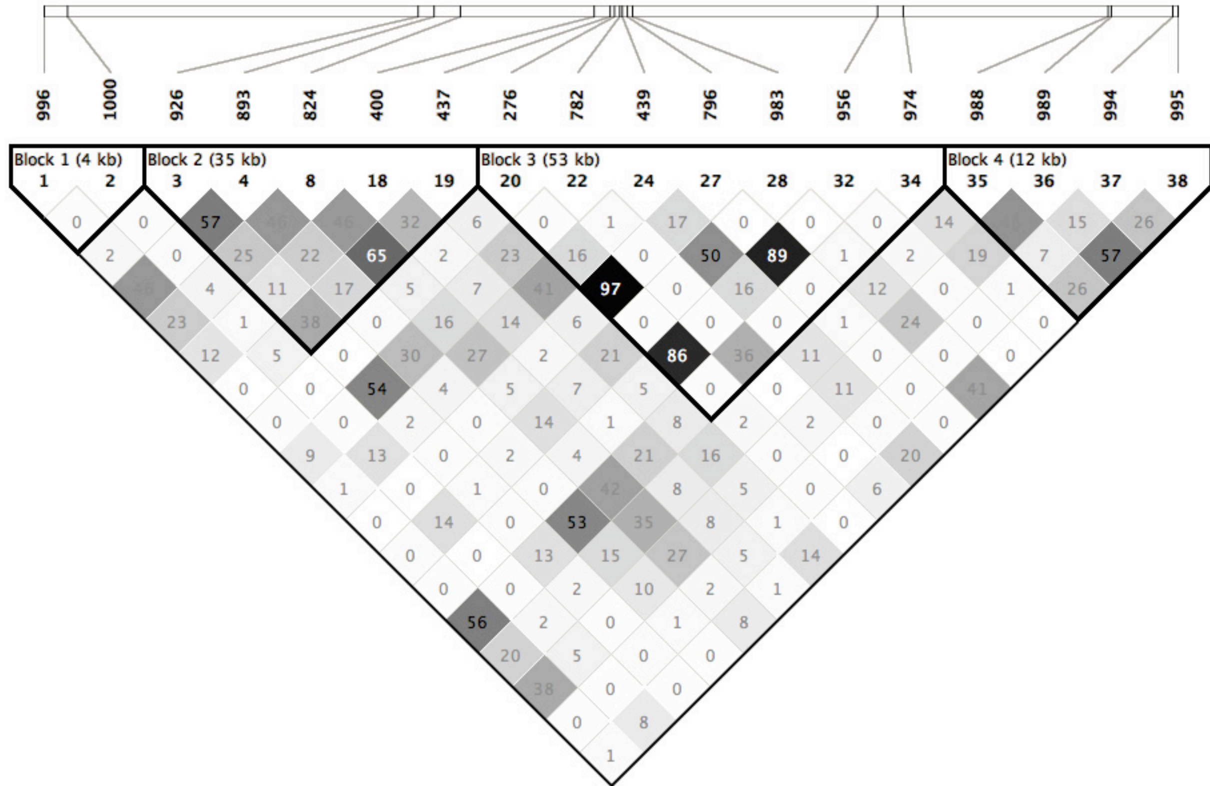


Figure 4.30 - LD plot and haplotype block structure for *tagged* SNPs.
SNPids on the horizontal axis on the top of the Figure indicate the SNP ids as assigned in the internal database. Lines above indicate the location of the SNP in the extended *HEXA* region. The plot shows the LD measured by r^2 for *tagged* SNPs, with the individual r^2 shown by numbers in the squares. The darker the shading of squares, the higher is the pairwise LD between the two corresponding SNPs. The block structure is marked by bold triangles.

The block structure and recombination rate between neighbouring haplotype blocks are shown in Figure 4.31. The haplotypes are compartmentalised in smaller fragments and the block structure of the associated haplotypes was identified. For the first two blocks, both haplotypes that are significantly associated with BSE share the same alleles, whereas in block 3 and 4 the haplotypes are different (see red and pink shaded haplotypes in Figure 4.31). To identify the haplotype blocks that are responsible for the association, χ^2-tests were carried out (Table 4.15). Block 1, consisting of two SNPs in *ARIH1,* shows no significant association. The haplotype of block 2, which is represented in both BSE associated haplotypes, is over-represented in the controls. However, after correction for multiple testing, no significant differences could be found. Notably, in block 3, the haplotype containing the three *'UK-protecting'* alleles of the BSE associated SNPs is significantly associated with BSE with a

permuted P-value of 0.008. However, the block 3 haplotype that is assigned to the second BSE-associated haplotype is not significantly associated as well as all haplotypes from block 4. The block analysis confirmed the hypothesis that the main effect on BSE development in the extended *HEXA* region is assigned to the three *'UK–protecting'* alleles, which are located in haplotype block 3.

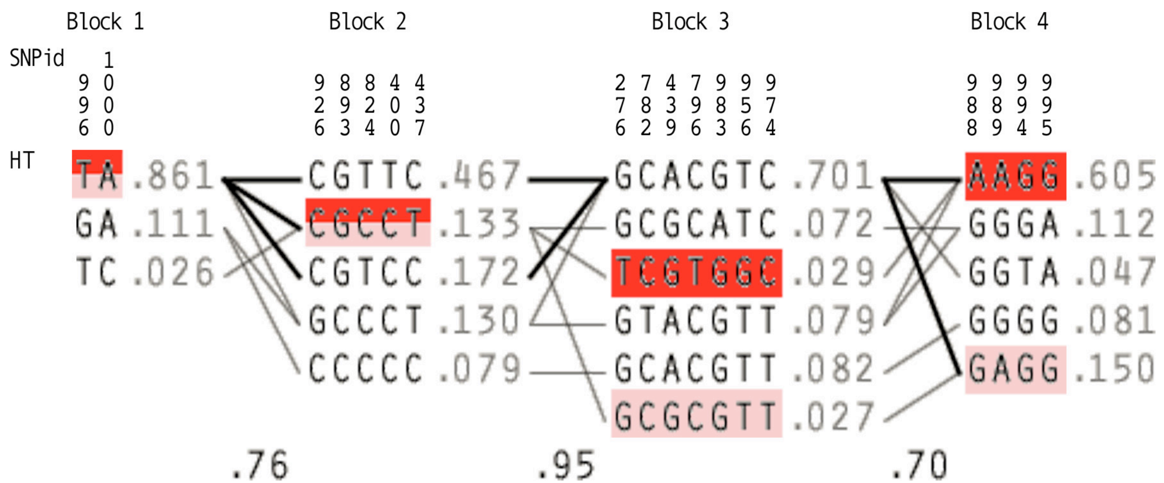


Figure 4.31 - Haplotype blocks for the *tagged* SNPs of the extended *HEXA* region
This Figure shows the block haplotypes with the corresponding population frequencies and all observed combinations between the block-haplotypes. Bold lines between two haplotypes are highlighting the combination frequencies >10 % while thin lines are indicating frequencies from 1 % to 10 %. Between the blocks, D' is displayed, representing the extent of LD between two neighbouring blocks. The BSE associated haplotypes are highlighted by red and pink shadows as shown in Figure 4.29.

Table 4.15 - Analysis of association within haplotype blocks in the UK Holstein animals. Frequencies of haplotypes for all animals and in the case and control group are displayed. Furthermore, the results for the χ^2-test, testing the corresponding haplotype against the pooled observations of all remaining haplotypes in one block and the corresponding P-values before and after 10^5 permutations (Per-P) are presented. The block haplotypes that are presented in the BSE associated haplotypes 11 and 9 are referenced by the corresponding numbers.

SNPid	Represented in Haplotype		Haplotype Frequencies All	Cases	Controls	χ^2	P-value	Per-P
Block 1								
996 1000								
T A	11	9	0.861	0.856	0.868	0.387	0.5341	
G A			0.111	0.116	0.104	0.473	0.4915	
T C			0.026	0.025	0.027	0.056	0.8125	
Block 2								
926 893 824 400 437								
C G T T C			0.467	0.475	0.457	0.413	0.5205	
C G T C C			0.172	0.172	0.173	0.002	0.9615	
C G C C T	11	9	0.133	0.115	0.157	4.778	0.0288	0.3483
G C C C T			0.130	0.136	0.122	0.514	0.4734	
C C C C C			0.079	0.085	0.073	0.597	0.4397	
G C T T C			0.006	0.004	0.008	0.968	0.3251	
Block 3								
276 782 439 796 983 956 974								
G C A C G T C			0.701	0.714	0.683	1.436	0.2308	
G C A C G T T			0.082	0.087	0.076	0.502	0.4784	
G T A C G T T			0.079	0.075	0.084	0.343	0.5580	
G C G C A T C			0.072	0.081	0.060	2.209	0.1372	
T C G T G G C	11		0.029	0.013	0.049	15.04	0.0001	0.0008
G C G C G T T		9	0.027	0.017	0.040	6.539	0.0106	0.1072
Block 4								
988 989 994 995								
A A G G	11		0.605	0.578	0.641	5.149	0.0233	0.2829
G A G G		9	0.150	0.154	0.144	0.271	0.6028	
G G G A			0.112	0.124	0.097	2.24	0.1345	
G G G G			0.081	0.084	0.078	0.186	0.6665	
G G T A			0.047	0.056	0.035	3.167	0.0751	

4.7.3 Haplotype analysis for the German Holstein animals

Haplotype frequencies for the German Holstein animals were derived with the software *Phase (v2.1.1)* (Stephens et al., 2001) (Stephens and Donnelly, 2003). The advantage of this programme over *Haploview (v3.3)* is that *Phase* deduces diplotypes (haplo-genotypes) for each individual by application of a Bayesian statistical framework. The German Holstein controls were paternal half-sibs, thus, the actual haplotype frequencies were those of the maternally inherited haplotypes. Therefore, the diplotypes have to be corrected for the parental haplotype to reveal the maternally inherited haplotypes. The analysis involving all 38 SNPs revealed

that the sire was homozygous for one haplotype. Hence, the maternal inherited haplotypes could be derived directly by subtracting the paternal haplotype. Subsequently, maternal haplotypes were used to compare haplotype frequencies with the haplotypes obtained for the German Holstein BSE diseased animals (Table 4.16). Six haplotypes were found with frequencies > 3% in cases and controls, respectively. The rare haplotypes with frequencies < 3 % in both groups were excluded from further analyses, because of insufficient numbers of haplotypes to conduct statistical analysis. Only four of the six haplotypes were also identified and analysed in the UK Holsteins. Haplotypes 1 and 2 are the most frequent haplotypes in both populations, whereas the other haplotype frequencies differ between both populations.

χ^2-testing revealed that haplotypes 11 and 13 are significantly associated with the BSE status at a haplotype-wise 5% type I error rate with P-values being 0.034 and 0.019, respectively. However, after Bonferroni-Holm adjustment (to account for multiple comparisons) these findings are rather suggestive. It has been shown in the previous analysis that haplotype 11 is highly associated in the UK animals resulting from an overrepresentation of this haplotype in the control animals. For the German animals, this haplotype is remarkably overrepresented in the BSE diseased animals. Haplotype 13 was not found in the UK animals. Nevertheless, associations of haplotypes in both population, albeit in different directions, imply a putative mutation that is in LD to the investigated polymorphisms.

Table 4.16 - Haplotype analysis in the German Holstein animals.
Haplotypes 1,2,10 and 11 are named by numbers assigned from the haplotype analysis in the UK animals. Haplotype 13 and 16 are shown below the table. All haplotypes with minor frequencies < 3% are combined in Residues. For the controls, the number (n) of maternal inherited haplotypes (MIH) among control animals were derived and compared with the number (n) of haplotypes in the BSE diseased animals (cases) and tested for association with BSE by χ^2-test statistic, testing the corresponding haplotype against the pooled observations of all remaining haplotypes. Bonferroni-Holm correction was applied to account for multiple testing.

Haplotype	Frequency of Haplotypes				χ^2	P-value	Bonferroni-Holm corrected P-value
	Controls (MIH)	(n)	Cases	(n)			
1	42.9	261	34.9	44	2.755		
2	13.5	82	11.9	15	0.228		
11	8.9	54	15.1	19	4.476	0.034	0.16
16[a]	7.9	48	11.1	14	1.396		
13[b]	4.9	30	10.3	13	5.484	0.019	0.11
10	4.1	25	4.8	6	0.048		
Residues	-	108	-	15			

[a]Ht 16: TCCGGATTGCGATACAATCGGCGATACGGAGTACAAGG
[b]Ht 13: TACGGATTGCGATACAACCGGCGATACGGAGTACGAGG

4.8 Association of polymorphisms in *PRNP* promoter with BSE

4.8.1 Characterisation of the bovine *PRNP* gene

Endogenous prion protein (PrP^c) is known to play a central role in the pathogenesis of transmitted prion diseases (Bueler et al., 1993; Hunter, 1999). The gene encoding PrP^c, *PRNP*, has therefore been suggested as a candidate locus for disease susceptibility. Variations of *PRNP* in humans, mice and sheep, have been shown to have major effects on the susceptibility to and incubation time of such diseases (Carlson et al., 1988; Collinge et al., 1996; Hunter et al., 1997; Mead et al., 2003; Palmer et al., 1991; Windl et al., 1996). However, no clear relationships between bovine *PRNP* polymorphisms and susceptibility to bovine spongiform encephalopathy (BSE) have been revealed so far (Hunter et al., 1994; Nakamitsu et al., 2006; Neibergs et al., 1994; Sander et al., 2004). See also Chapters 2.1.3 - Basics and genetics of prion diseases and 2.1.4 - Genetic Background for BSE in cattle, respectively.

The human and bovine *PRNP* genes have been characterised previously (Puckett et al., 1991; Choi et al., 2006; Hills et al., 2001; Horiuchi et al., 1998; Inoue et al., 1997; Lee et al., 1998; Mahal et al., 2001). The bovine *PRNP* gene is organised in three exons, separated by two introns (see Table 4.17 and Figure 4.32) as compared with two exons and one intron in humans. The ORF length of the bovine gene is 795 bp in contrast to 762 bp in humans corresponding to 264 and 253 amino acids in the protein sequence, respectively. The entire genes span 20.2 kb (cattle) and 15.2 kb (man), respectively. Choi et al. (2006) showed that the sequence identity is 83% at the nucleotide level and 86% at the amino acid level.

Table 4.17 - Exon and intron organisation of bovine *PRNP* gene.

Exon				5'splice donor … 3'splice acceptor [a]	Intron	
No	Size (bp)	Position in mRNA	cDNA		No	Size (bp)
				…cctagttGCCAGTCG…		
1	53	1-52		AGCAGgtaaa…ttaagACTTC	1	2436
2	98	53-151		CAGATgtagg…tgcagATAAG	2	13551
3	4091	152-4244	162-956	ATCATGGTG…GGATAGGGG		
				…AAAAGGAGtatctt…		

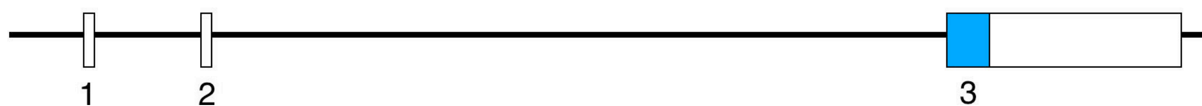


Figure 4.32 - Graphical overview bovine *PRNP* gene.
The blue and white colour shows the translated and un-translated region. Exons are represented by bars. The black thin lines between the exons represent the introns, whereas its length corresponds approximately to the intron length.

Previously, a promoter analysis for the bovine *PRNP* gene was carried out revealing the transcription start by a modified method of RACE (Inoue et al., 1997). The 5'- end contained no potential CAAT or TATA sequences. Four potential binding sites for SP1 have been identified in the 5'-end and in intron 1. Functional promoter analysis revealed two putative promoter regions of the bovine *PRNP*, one in the 5'-end and one in intron 1. The region –88 to –30 bp and 123 to 831 bp relative to the transcription start, were identified as regions that contribute promoter activity. In order to confirm the putative promoter regions of *PRNP*, the region 5.5 kb upstream of exon 2 (containing 3 kb of the 5'-end, the complete exon 1 and the complete intron 1) were analysed with the programme *cpgplot* from the Emboss web-service (http://www.ebi.ac.uk/emboss/cpgplot/) (Larsen et al., 1992). This analysis identified putative CpG islands of *PRNP* (see Figure 4.33) in the region corresponding to the promoter sites of (Inoue et al., 1997).

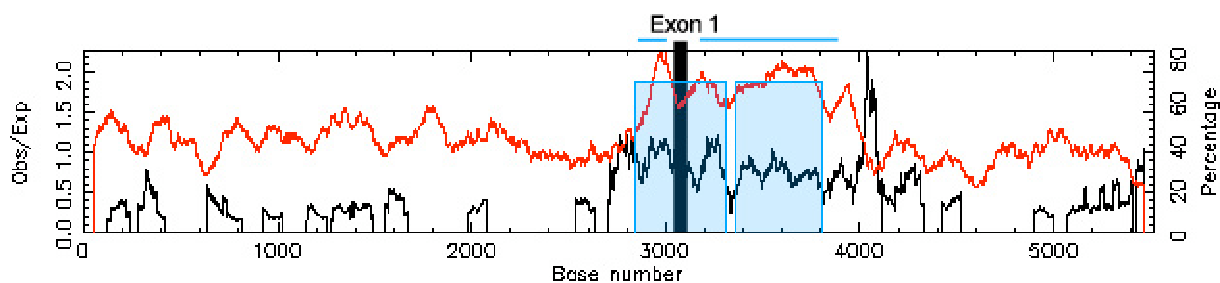


Figure 4.33 - Predicted CpG islands in bovine *PRNP*.
The sequence 5.5 kb upstream relative to exon 2 was analysed by *cpgplot* http://www.ebi.ac.uk/ emboss/cpgplot/ (Larsen et al., 1992). The black graphs plot the ratios of observed versus expected CpG content, which is displayed on the primary axis on the left. The red graphs plot the relative C+G contents, which are shown on the secondary axis on the right of the plot. The CpG island is illustrated by the blue box. The promoter regions as identified by Inoue et al. (1997) are represented by the blue bars above the plot. The black vertical bar marks exon 1.

The promoter region of *PRNP* was partially resequenced to confirm two previously described insertion/deletion polymorphisms and see if the variants are polymorphic in the UK and German cattle populations. Both, the 23-bp indel (Sander et al., 2004) in the promoter region and the 12-bp indel (Hills et al., 2001) in intron 1 affect binding sites for transcription factors

(RP58 and SP1, respectively), and thus might affect the expression of *PRNP* (Sander et al., 2005). Both polymorphisms showed tentative association with BSE in a small association study (Sander et al., 2004) and were therefore selected for association analysis in the BSE cases and control animals of four different populations: Holstein-Friesian cattle from the United Kingdom (UK Holstein) and Germany (German Holstein), German Brown and German Fleckvieh.

4.8.2 Single Marker Analyses

Genotyping of both indel polymorphism and χ^2 testing revealed significant associations of each polymorphism with the BSE status in all populations except German Fleckvieh. Genotypes with deletion alleles and deletion alleles of both loci are generally overrepresented in the affected animals of all breeds (see Table 4.18, 4.19 and Figure 4.34). Using Fisher's combined probability test, highly significant associations between the deletion allele and the BSE cases were found for both the 23-bp ($P = 2.01 \times 10^{-3}$) and the 12-bp indel ($P = 8.66 \times 10^{-5}$) over all populations. A combined logistic regression analysis involving all breeds also revealed highly significant associations between both indels and BSE ($P = 5.7 \times 10^{-5}$ and $P = 1.2 \times 10^{-7}$, respectively). Modelling the genotype data shows that the BSE risk tends to increase in line with the number of deletion alleles at both loci (Table 4.19). Heterozygous and homozygous carriers of the 12-bp deletion allele, when compared with non-carriers, have relatively higher risks of BSE, ranging from 1.32 to 4.01 and 1.74 to 3.65 in the different breeds. The corresponding population attributable risks (Greenland, 1998; Greenland and Rothman, 1998), *i.e.* the portion of the BSE incidence in the population that is due to the risk allele (12-bp deletion allele), range from to 0.35 to 0.53 (Table 4.19).

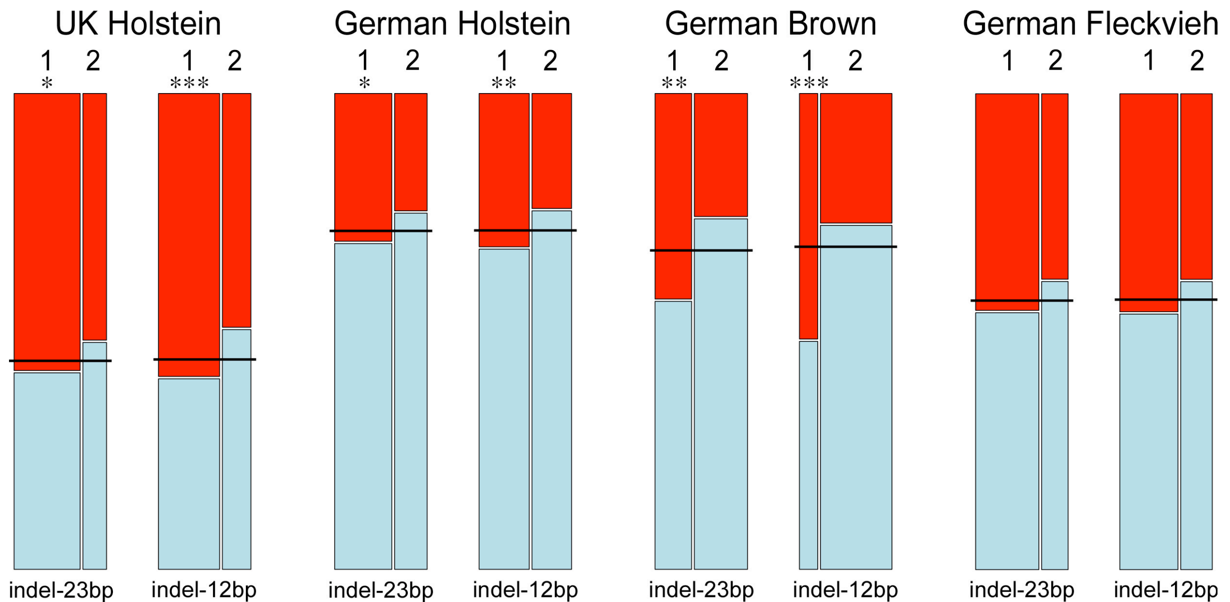


Figure 4.34 - Allele frequencies of the *PRNP* polymorphisms
Allele frequencies of the 23- and 12-bp insertion / deletion polymorphisms in UK Holstein, German Holstein, German Brown and German Fleckvieh BSE affected (red) and control (light-blue) animals. "1" and "2" above the columns represent the deletion and insertion alleles, respectively. The horizontal black lines indicate the expected proportions of cases and controls in each class. The thickness of the columns is proportional to the allele frequency. Asterisks indicate the level of significance: * < 0.05, ** < 0.01, *** < 0.001.

Table 4.18 - Genotype frequencies and association analyses at *PRNP* promoter polymorphisms

UK Holstein	23-bp indel Frequency of Genotypes					12-bp indel Frequency of Genotypes				
	Cases[a]	(n)	Controls[a]	(n)	P-Value[c]	Cases[a]	(n)	Controls[a]	(n)	P-Value[c]
del/del	55.4	(201)	46.4	(128)	2.4×10^{-2}	49.4	(173)	37.0	(100)	2.1×10^{-3}
del/ins	41.0	(149)	48.9	(135)	4.7×10^{-2}	45.4	(159)	51.9	(140)	1.1×10^{-1}
ins/ins	3.6	(13)	4.7	(13)	4.7×10^{-1}	5.1	(18)	11.1	(30)	5.8×10^{-3}
German Holstein[b]										
del/del	45.7	(58)	38.0[b]	(119)[b]	1.4×10^{-1}	40.0	(50)	28.2[b]	(87)[b]	1.6×10^{-2}
del/ins	46.5	(59)	47.3[b]	(148)[b]	8.8×10^{-1}	45.6	(57)	49.8[b]	(154)[b]	4.2×10^{-1}
ins/ins	7.9	(10)	14.7[b]	(46)[b]	5.2×10^{-2}	14.4	(18)	22.0[b]	(68)[b]	7.2×10^{-2}
German Brown										
del/del	20.9	(9)	13.8	(12)	2.9×10^{-1}	7.0	(3)	3.3	(3)	3.4×10^{-1}
del/ins	65.1	(28)	41.4	(36)	1.1×10^{-2}	51.2	(22)	22.2	(20)	7.8×10^{-4}
ins/ins	14.0	(6)	44.8	(39)	5.0×10^{-4}	41.9	(18)	74.4	(67)	2.5×10^{-4}
German Fleckvieh										
del/del	53.8	(57)	46.3	(63)	2.5×10^{-1}	45.3	(48)	39.4	(54)	3.6×10^{-1}
del/ins	39.6	(42)	43.4	(59)	5.6×10^{-1}	46.2	(49)	45.3	(62)	8.8×10^{-1}
ins/ins	6.6	(7)	10.3	(14)	3.1×10^{-1}	8.5	(9)	15.3	(21)	1.1×10^{-1}

[a] Represents frequency (%) of genotypes; (n) indicates the number of genotypes.
[b] Control consists of half-sibs (see Chapter 3.15.10 - Inferring allele and genotype frequencies from half-sibs).
[c] P-values of χ^2-test against the both remaining genotypes

Table 4.19 - Allele frequencies and association analyses at *PRNP* promoter polymorphisms

	Frequency of Deletion					Logistic Regression Analysis			
23-bp indel	Cases[a]	(n)	Controls[a]	(n)	P-Value[c]	P-Value[d]	OR ins/del[e]	OR del/del[e]	PAR[f]
UK Holstein	75.9	(363)	70.8	(276)	4.2×10^{-2}	2.9×10^{-2}	1.36	1.84	0.24
German Holstein	68.9	(127)	61.8[b]	(335)	4.3×10^{-2}	4.4×10^{-2}	1.38	1.91	0.46
German Brown	53.5	(43)	34.5	(87)	3.3×10^{-3}	5.4×10^{-3}	4.74	4.62	0.69
German Fleckvieh	73.6	(106)	68.0	(136)	1.8×10^{-1}	1.9×10^{-1}	1.31	1.71	0.36
Combined	(639)		(834)		2.0×10^{-3} [g]	5.7×10^{-5}	1.43	2.04	
12-bp indel									
UK Holstein	72.1	(350)	63.0	(270)	5.8×10^{-4}	3.2×10^{-4}	1.61	2.60	0.53
German Holstein	62.8	(125)	53.2[b]	(357)	9.1×10^{-3}	1.1×10^{-2}	1.48	2.20	0.35
German Brown	32.6	(43)	14.4	(90)	5.9×10^{-4}	1.1×10^{-2}	4.01	3.65	0.44
German Fleckvieh	68.4	(106)	62.0	(137)	1.5×10^{-1}	1.5×10^{-1}	1.32	1.74	0.44
Combined	(624)		(854)		8.7×10^{-5} [g]	1.2×10^{-7}	1.58	2.50	

[a] Represents frequency (%) of deletion allele; (n) indicates the number of individuals.
[b] Control consists of half-sibs (see Chapter 3.15.10 - Inferring allele and genotype frequencies from half-sibs).
[c] P-values of χ^2-test.
[d] P-values from logistic regression, modelling the number of deletion alleles on BSE status.
[e] Odds Ratios with reference genotype: ins/ins.
[f] Population Attributable Risk
[g] P-Values of Fisher's Combined Probability test.

4.8.3 Inferring haplotype and diplotype frequencies from half-sibs at the *PRNP* polymorphisms

Diplotypes of the half-sib animals were obtained using *Phase (v2.1.1)* (Stephens and Donnelly, 2003; Stephens et al., 2001). The sire transmitted either haplotype 23del-12del or 23ins-12ins to progeny (see Table 4.20). Thus, except for daughters with the diplotype 23del-12del/23ins-12ins, the maternal haplotype could be directly determined by subtraction of the paternal haplotype. Because of the large number of animals with diplotype 23del-12del/23ins-12ins the frequency of maternally contributed haplotypes were indirectly calculated from these diplotypes as shown in Table 4.21. Diplotype frequencies in the dam population were estimated based on the maternal haplotype frequencies, assuming HWE (see Table 4.22). Haplotype 4 (23ins-12del) occurred with a frequency <1% and was therefore excluded from the estimation of diplotype frequencies in the dams. Calculation of the approximative numbers of diplotypes were based on half the number of half-sibs because each animal contributed only one haplotype observation.

Table 4.20 - Calculation of haplotype frequencies in dams of half-sibs.
The haplotype frequencies are inferred on observed diplotypes in German Holstein half-sibs.

Diplotype	Number of Diplotype Observations		Maternal Haplotype 1 23del-12ins	2 23del-12del	3 23ins-12ins	4 23ins-12del
23del-12ins[a]/ 23ins-12ins [b]	$n(1/3)$	40	40			
23del-12ins[a]/ 23del-12del [b]	$n(1/2)$	16	16			
23del-12del[a]/ 23del-12del [b]	$n(2/2)$	181		181		
23del-12del/ 23ins-12ins[c]	$n(2/3)$	253		$253 \cdot p(2)_e$ [d] 145.98	$253 \cdot p(3)_e$ [d] 107.02	
23ins-12ins[a]/ 23ins-12ins [b]	$n(3/3)$	125			125	
23del-12del [b]/ 23ins-12del[a]	$n(2/4)$	3				3
∑	n	618	56	327	232	3
P_D (x)[e]			**0.0906**	**0.5291**	**0.3754**	**0.0049**

[a] maternal haplotype.
[b] paternal haplotype.
[c] origin of haplotypes cannot be directly determined.
[d] $p(2)_e$ and $p(3)_e$ indicate the frequency of maternally inherited haplotype 23del-12del and 23ins-12ins among half-sibs with diplotype 23del-12del / 23ins-12ins, respectively (see Table 4.21).
[e] $p_D(x)$ indicate frequencies of haplotype x in dams.

Table 4.21 - Calculation of the frequency of maternally inherited haplotypes.
Calculation of the frequency of maternally inherited haplotypes (MIH) in the German Holstein half-sibs with 23del-12del / 23ins-12ins (2/3) diplotype.

Number of Paternally plus Maternally Inherited Haplotypes 23del-12del (2) and 23ins-12ins (3)[a]	Total Frequency of Haplotypes[b]	Approximative Frequency of Maternally Inherited Haplotypes[c]
$n(2) = n(1/2) + 2 \cdot n(2/2) + n(2/3) + n(2/4)$	$p(2) = 0.539$	$p(2)_e = (p(2) - 0.5) \cdot 2 + 0.5$
$n(2) = 634$		$p(2)_e = 0.577$
$n(3) = n(1/3) + n(2/3) + 2 \cdot n(3/3)$	$p(3) = 0.461$	$p(3)_e = (p(3) - 0.5) \cdot 2 + 0.5$
$n(3) = 543$		$p(3)_e = 0.423$

[a] n(x) indicate number of observations of haplotype x.
[b] p(x) indicate frequency of haplotype x.
[c] $p(x)_e$ indicate approximative frequency of maternally inherited haplotype x.

Table 4.22 - Calculation of approximative numbers of diplotypes of German Holstein
Numbers of diplotypes calculated assuming HWE.

	Diplotypes					
	1/1	1/2	1/3	2/2	2/3	3/3
	23del-12ins 23del-12ins	23del-12ins 23del-12del	23del-12ins 23ins-12ins	23del-12del 23del-12del	23del-12del 23ins-12ins	23ins-12ins 23ins-12ins
Formula[a,b]	$\frac{n}{2} \cdot (p_D(1))^2$	$\frac{n}{2} \cdot 2 p_D(1) p_D(2)$	$\frac{n}{2} \cdot 2 p_D(1) p_D(3)$	$\frac{n}{2} \cdot (p_D(2))^2$	$\frac{n}{2} \cdot 2 p_D(2) p_D(3)$	$\frac{n}{2} \cdot (p_D(3))^2$
Inferred observations	3	30	21	87	123	44

[a] n indicate the total number of diplotype observations (Table 3).
[b] $p_D(x)$ indicate haplotype frequencies as calculated in Table 4.20.

4.8.4 Haplotype analyses

To estimate the combined effect of both polymorphisms, haplotype analysis was performed. Strong LD was present in all populations with the most frequent haplotypes being 23del-12del, 23ins-12ins and 23del-12ins. Haplotype 23ins-12del occurs in < 1% in all populations and was therefore excluded from further analyses. The χ^2-test indicated significant deviations from the expected haplotype frequencies in cases and controls of all populations except German Fleckvieh (Figure 4.35 and Table 4.23) and logistic regression modelling revealed significant contrasts in the ORs. The 23del-12del haplotype is associated with an increased risk of BSE incidence, whereas the 23ins-12ins haplotype seems to be protective. The effect of the 23del-12ins haplotype depends on the population, but it does not differ significantly from that of the 23ins-12ins reference haplotype in any population. Additionally, the effect of the 23del-12del haplotype in the combined analysis is highly significantly different from that of the remaining major haplotypes ($P = 3.7 \times 10^{-6}$ and $P = 9.6 \times 10^{-4}$ for the 23ins-12ins and 23del-12ins haplotypes, respectively).

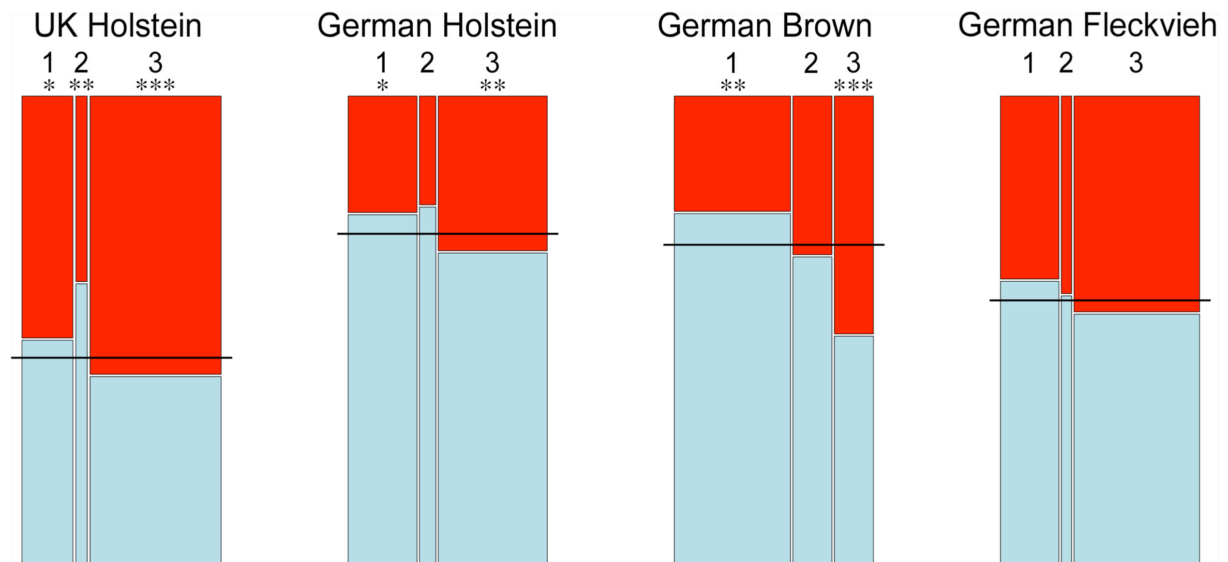


Figure 4.35 - Haplotype Frequencies of *PRNP* polymorphisms
Haplotype frequencies in UK Holstein, German Holstein, German Brown and German Fleckvieh BSE affected (red) and control (light blue) animals. Numbers above the columns represent the haplotypes: (1) 23ins-12ins, (2) 23del-12ins and (3) 23del-12del. The horizontal black lines indicate the expected proportions of cases and controls in each class. The thickness of the columns is proportional to the haplotype frequency. Stars indicate the level of significance: * < 0.05, ** < 0.01, *** < 0.001.

Table 4.23 - Haplotype frequencies and association analyses at *PRNP* promoter polymorphisms

	Frequency of Haplotypes					Logistic Regression Analysis		
UK Holstein	Cases[a]	(n)	Controls[a]	(n)	P-Value[c]	OR[d]	P-Value 23ins-12ins[e]	P-Value 23del-12ins[f]
23ins-12ins	24.2	(171)	29.1	(159)	5.1×10^{-2}	1		6.6×10^{-2}
23del-12ins	4.1	(29)	8.1	(44)	3.1×10^{-3}	0.62	6.6×10^{-2}	
23del-12del	71.7	(506)	62.8	(343)	8.9×10^{-4}	1.37	1.6×10^{-2}	1.3×10^{-3}
German Holstein								
23ins-12ins	30.2	(77)	39.1[b]	(232)	1.4×10^{-2}	1		8.1×10^{-1}
23del-12ins	7.2	(17)	9.1[b]	(56)	3.9×10^{-1}	0.93	8.1×10^{-1}	
23del-12del	62.2	(162)	51.3[b]	(327)	7.5×10^{-3}	1.49	1.4×10^{-2}	1.1×10^{-1}
German Brown								
23ins-12ins	45.0	(39)	66.1	(119)	1.3×10^{-3}	1		1.8×10^{-1}
23del-12ins	22.5	(18)	19.4	(35)	5.7×10^{-1}	1.58	1.8×10^{-1}	
23del-12del	32.5	(27)	14.4	(26)	7.8×10^{-4}	3.14	5.4×10^{-4}	8.4×10^{-2}
German Fleckvieh								
23ins-12ins	26.8	(58)	32.6	(90)	1.7×10^{-1}	1		7.5×10^{-1}
23del-12ins	5.1	(11)	5.4	(15)	8.7×10^{-1}	1.15	7.5×10^{-1}	
23del-12del	68.1	(147)	62.0	(171)	1.7×10^{-1}	1.33	1.5×10^{-1}	7.2×10^{-1}
Combined								
23ins-12ins						1		5.0×10^{-1}
23del-12ins						0.90	5.0×10^{-1}	
23del-12del						1.50	3.7×10^{-6}	9.6×10^{-4}

[a] Represent frequency (%) of haplotypes; (n) indicates the number of haplotypes
[b] Control consists of half-sibs (see Chapter 4.8.3 - Inferring haplotype and diplotype frequencies from half-sibs at the *PRNP*-polymorphisms).
[c] P-values from χ^2-test against pooled observations of all remaining haplotypes.
[d] Odds Ratios with reference haplotype 23ins-12ins.
[e] P-values from logistic regression, testing the risk effect compared to reference haplotype 23ins-12ins.
[f] P-values from logistic regression, testing the risk effect compared to reference haplotype 23del-12ins.

4.8.5 Diplotype analyses

We attempted to assess the risk of BSE infection associated with each diplotype. Five common diplotypes were found (Figure 4.36 and Table 4.24), with the sixth, 23ins-12del / 23ins-12del, being relatively rare (< 3 %); as such, it was excluded from further analyses. For German Holstein control animals, the diplotypes were inferred from estimated haplotype frequencies (as shown in Chapter 4.8.3 - Inferring haplotype and diplotype frequencies from half-sibs at the *PRNP*-polymorphisms). In both Holstein populations, the 23del-12del / 23del-12del diplotype is significantly overrepresented in affected animals as revealed by χ^2-testing (P = 2.5×10^{-3} and P = 9.2×10^{-3} for UK and German Holstein, respectively). Significant association was also observed in the German Brown breed, where the 23ins-12ins / 23ins-12ins diplotype is highly overrepresented in the controls (P = 4.7×10^{-4}), and the 23del-12del / 23ins-

12ins diplotype occurs significantly more often in the cases (P = 6.0 x 10^{-4}). Analysis over all populations indicates that the highest risk is conferred by the 23del-12del / 23del-12del diplotype (Table 4.24). Figure 4.37 shows the diplotype conferred ORs obtained from logistic regression analysis of the combined data, accounting for population effects, and the data of each individual population. The ORs were calculated using the absolute risks averaged across all five diplotypes as reference in order to allow for a meaningful comparison of populations. The analyses of the individual populations and the combined analysis suggest that risk of BSE in a population tends to increase in line with the number of deletion alleles at the 12-bp indel.

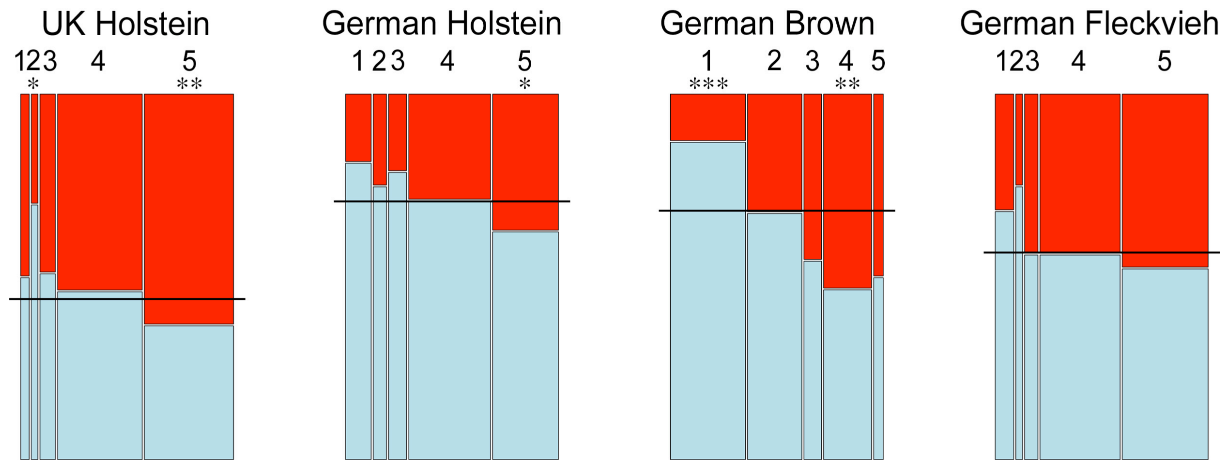


Figure 4.36 - Diplotype Frequencies of the *PRNP* polymorphisms
Diplotype frequencies in UK Holstein, German Holstein, German Brown and German Fleckvieh BSE affected (red) and control (light blue) animals. Numbers above the columns represent the haplotypes: (1) 23ins-12ins / 23ins-12ins, (2) 23ins-12ins /23del-12ins, (3) 23del-12ins / 23del-12del, (4) 23ins-12ins / 23del-12del and (5) 23del-12del / 23del-12del. The horizontal black lines indicate the expected proportions of cases and controls in each class. The thickness of the columns is proportional to the diplotype frequency. Asterisks indicate the level of significance: * < 0.05, ** < 0.01, *** < 0.001.

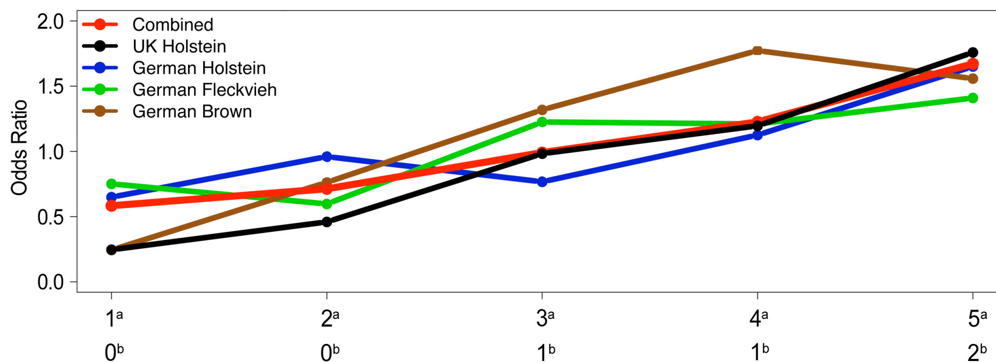


Figure 4.37 - Odds Ratios of Diplotypes
Odds ratios (ORs) conferred by the diplotypes in each population and over all populations. The ORs were calculated using the absolute risks averaged across all five diplotypes as reference. [a] Numbers refer to diplotypes: (1) 23ins-12ins / 23ins-12ins, (2) 23ins-12ins /23del-12ins, (3) 23del-12ins / 23del-12del, (4) 23ins-12ins / 23del-12del and (5) 23del-12del / 23del-12del. [b] Numbers indicate number of deletion alleles at the 12-bp indel locus.

Table 4.24 - Diplotype frequencies and association analyses at *PRNP* polymorphisms.

	No. of 12-bp Deletion	Frequency of Diplotypes					
UK Holstein		Cases[a]	(n)	Controls[a]	(n)	P-Value[d]	OR[e]
23del-12ins / 23del-12ins	0	0	(0)	<1.0	(3)	-	
23ins-12ins / 23ins-12ins[c]	0	3.7	(13)	4.8	(13)	4.8×10^{-1}	1[c]
23del-12ins / 23ins-12ins	0	1.7	(6)	5.1	(14)	1.4×10^{-2}	0.45
23del-12ins / 23del-12del	1	6.5	(23)	8.8	(24)	2.7×10^{-1}	0.96
23ins-12ins / 23del-12del	1	39.0	(139)	43.6	(119)	2.4×10^{-1}	1.17
23del-12del / 23del-12del	2	48.7	(172)	36.6	(100)	3.6×10^{-3}	1.72
German Holstein							
23del-12ins / 23del-12ins	0	<1.0	(1)	<1.0	(3)[b]	-	
23ins-12ins / 23ins-12ins[c]	0	7.8	(10)	15.3	(47)[b]	6.1×10^{-2}	1[c]
23del-12ins / 23ins-12ins	0	5.5	(7)	7.1	(22)[b]	6.0×10^{-1}	1.48
23del-12ins / 23del-12del	1	6.3	(8)	9.4	(29)[b]	2.4×10^{-1}	1.18
23ins-12ins / 23del-12del	1	39.1	(50)	40.6	(125)[b]	3.4×10^{-2}	1.73
23del-12del / 23del-12del	2	40.6	(52)	26.6	(82)[b]	1.2×10^{-2}	2.54
German Brown							
23del-12ins / 23del-12ins	0	2.4	(1)	3.3	(3)	-	
23ins-12ins / 23ins-12ins[c]	0	14.3	(6)	45.6	(41)	3.7×10^{-4}	1[c]
23del-12ins / 23ins-12ins	0	26.2	(11)	25.6	(23)	9.6×10^{-1}	3.12
23del-12ins / 23del-12del	1	11.9	(5)	6.7	(6)	3.1×10^{-1}	5.40
23ins-12ins / 23del-12del	1	38.1	(16)	15.6	(14)	4.3×10^{-3}	7.27
23del-12del / 23del-12del	2	7.1	(3)	3.3	(3)	-	6.38
German Fleckvieh							
23del-12ins / 23del-12ins	0	<1.0	(1)	0	(0)	-	
23ins-12ins / 23ins-12ins[c]	0	6.5	(7)	10.9	(15)	2.4×10^{-1}	1[c]
23del-12ins / 23ins-12ins	0	1.9	(2)	4.3	(6)	-	0.79
23del-12ins / 23del-12del	1	6.5	(7)	6.5	(9)	1	1.63
23ins-12ins / 23del-12del	1	38.9	(42)	39.1	(54)	1	1.61
23del-12del / 23del-12del	2	45.4	(49)	39.1	(54)	2.9×10^{-1}	1.88
Combined							
23del-12ins / 23del-12ins	0						
23ins-12ins / 23ins-12ins[c]	0						1[c]
23del-12ins / 23ins-12ins	0						1.22
23del-12ins / 23del-12del	1						1.70
23ins-12ins / 23del-12del	1						2.10
23del-12del / 23del-12del	2						2.86

[a] Represents the frequency (%) of diplotypes; (n) indicates the number of individuals

[b] Calculated assuming HWE from haplotype frequency estimates (as shown in Chapter 4.8.3 - Inferring haplotype and diplotype frequencies from half-sibs at the *PRNP*-polymorphisms).

[c] Diplotype taken as reference.

[d] P-values from χ^2-test against pooled observations of all remaining diplotypes.

[e] Odds Ratios from logistic regression.

4.8.6 Modelling the modes of allelic action and relative contribution of both polymorphisms

Likelihood ratio tests were applied as proposed by North et al. (2005) to investigate both the modes of allelic action for both loci (additive versus dominance effects) and for possible epistatic effects (additive by additive, additive by dominance and dominance by dominance interactions). The results indicate that for the individual populations as well as for the combined analysis neither the inclusion of dominance effects nor inclusion of epistatic effects leads to a significantly better fit compared to a model that accounts for additive effects only. Hence, likelihood ratio tests were performed to verify whether fitting an additive effect for either indel locus reduces the likelihood significantly compared to a full model that includes additive effects of both polymorphisms. This analysis revealed that exclusion of the 23-bp indel does not reduce the likelihood significantly in any of the individual populations or in the combined data. However, exclusion of the 12-bp indel polymorphism reduces the likelihood significantly in UK Holstein ($P = 1.04 \times 10^{-3}$) and in the combined analysis ($P = 6.89 \times 10^{-4}$). Thus, the main effect on BSE susceptibility seems to result from the 12-bp indel. This is supported by the haplotype analysis, which showed that the effect of the 23del-12ins haplotype does not differ significantly from that of 23ins-12ins, whereas the 23del-12del haplotype is significantly associated with higher BSE risk in the Holstein populations and in German Brown (Table 4.23).

5 Discussion

5.1 Characterisation of the extended *HEXA* region

In this study, fine mapping of a 300-kb genomic region encompassing four genes was conducted in cattle. The region has been initially proposed by Hernandez-Sanchez et al. (2002) to be a promising candidate region of BSE susceptibility because of putative linkage to a marker on BTA 10, which showed significant segregation distortion in BSE affected animals. Moreover, the homologous region in mice has been identified to show suggestive evidence of QTL affecting prion incubation time after experimental challenge (Stephenson et al., 2000). The central candidate gene in the 300-kb region was *HEXA*, the gene encoding the alpha subunit of the lysosomal enzyme hexosaminidase A (HexA). Various polymorphisms in *HEXA* cause Tay-Sachs disease, a autosomal recessive, progressive neurodegenerative disorder in human belonging to the Lysosomal Storage Diseases caused by accumulation of intercellular components in the lysosome (Chavany and Jendoubi, 1998). This storage of ‚waste' in the lysosome of nerve cells can also be observed in prion diseases. The turnover of PrP^{c} to PrP^{Sc} generates protease-resistant components (Prusiner, 1998a), which are indigestible for the lysosomal enzymes. Therefore, lysosomal enzyme efficiency could influence BSE development.

Starting with sequencing and characterisation of the *HEXA* gene, SNPs were identified and as a preliminary study, four intronic SNPs were genotyped in a panel of BSE affected and control animals from UK and Germany. One of these SNPs showed highly significant association with BSE in the UK animals. Subsequently, a 300-kb region centred on *HEXA* with the genes *PARP6, BRUNOL6* and *ARIH1* was characterised, partially re-sequenced and SNPs identified within the genes. Fine mapping of this region in cattle showed that the gene order on BTA10 coincidences with the homologous region in man on HSA 15. By BAC sequencing it has been established that the locus order is *PARP6* (-) *BRUNOL6* (-), *HEXA* (-) and *ARIH1* (+). This gene order is consistent with this of the chromosomal annotation in *Btau 2.0* (June 2006) gene assembly. The newest version of bovine gene assembly *Btau 3.1* (August 2006) contains misassembled sequences at the *PARP6* locus. The sequence publisher at the Baylor College of Medicine warn of typical errors in the draft genome sequence including misassembles, but the number of errors in the bovine sequence assembly is difficult to predict.

Comparison of the protein sequences encoded by the genes within the contig across species showed high conservation with a sequence identity of > 84.5 %. Thus, it can be assumed that the function for *HEXA, PARP6, BRUNOL6* and *ARIH1* is also conserved in human, mouse and cattle. Homologues of *ARIH1* display the highest sequence identity of > 99% between cattle and man and as proposed by Tan et al. (2000) of > 98% between mouse and man, re-

spectively. Partially re-sequencing of the *ARIH1* gene in cattle identified approximately one SNP every 1900 bp in the non-coding sequence which differs from a previous bovine study, in which one SNP every 180 bp was detected by sequencing a similar panel of animals (Werner et al., 2004). The low level of polymorphism within cattle and the highest conservation between species underlines the potentially important function of *ARIH1*. As previously shown, *ARIH1* function can be linked to neurodegenerative diseases and thus, may be involved in TSE development. Mutations in the functional homologue (*ariadne* gene) from Drosophila cause severe neuronal defects highlighting its crucial biological function (Aguilera et al., 2000). The gene product plays a putative role in ubiquitylation (Tan et al., 2000), which is the first step of intracellular protein degradation. Strong sequence conservation of *BRUNOL6* and *PARP6* notably in the functional domains have also been observed. *BRUNOL6* belongs to a gene family of RNA binding proteins influencing muscle specific splicing and enhancer specific alternative splicing of different genes (Ladd et al., 2004). However, *BRUNOL6* also expressed in the brain could indirectly influence TSE development by alternative splicing of yet unidentified genes, which are involved in prion diseases. *PARP6s* specific function is unknown, but as a member of the PARP gene family which is involved in many biological processes (e.g. DNA repair, apoptosis or transcriptional regulation) (Diefenbach and Burkle, 2005) it could be involved in TSE development. If *PARP6* plays a role in neurodegenerative diseases it might be a indirect, e.g. as an interactor with other genes or gene products. Although connections of both genes to TSE diseases are rather speculative, their gene structure and regulation were analysed to cover a larger genomic region.

The promoter analyses for the investigated candidate genes revealed CpG (Cytosin-phosphatidyl-Guanosin) rich regulatory elements in all 5'-ends. CpG islands are mostly unmethylated DNA regions and are thus accessible for transcription factors. CpG rich promoters represent the majority of mammalian promoters, which are associated with broad transcription start site regions (Carninci et al., 2006).

5.2 SNP analysis

5.2.1 SNP identification and selection

One goal of this dissertation was to identify polymorphisms in candidate genes, which are presumably influencing BSE susceptibility. Therefore, sequence information for designing PCR systems and re-sequencing for SNP identification was necessary. The increasing number of accessible bovine sequence data in recent years facilitates primer design for re-sequencing tremendously. But still the limiting element in this study was the cost and time consuming re-

sequencing and SNP search compared to the later carried out genotyping. Usage of an automated sequencer by poly-acrylamid gel electrophoresis with parallel detection of 96 samples is comparable with the daily throughput of a 4-capillary automated sequencer, but requires a trained handling with the harmful acrylamid and routine in pouring the gels. The costs per sequencing reaction is mainly depending on the input of sequencing dye, containing the flourescence labelled di-desoxy nucleotides. During this study, the input of sequencing dye could be halved (from 4 to 2 μl/reaction) and the costs including PCR, PCR cleanup and sequencing reaction cleanup ranged from 4.90 to 2.90 Euro. These expenses could be further reduced considerably to 1 - 1.50 Euro per reaction by using a capillary sequencer which only requires 0.25 - 0.5 μl sequencing dye per reaction.

SNP search was performed in a panel of pooled and individual DNA from animals of the taurine breeds German Holstein, German Fleckvieh and German Brown and from indicine breeds. A total of 136 taurine DNA variations (131 SNPs and 5 Indels) were identified in the extended *HEXA* region, 83 of them in intronic regions, 39 in the 5'-end with five of them in putative regulatory regions, eight in the 3' end and four synonymous SNPs in coding regions. From these SNPs, an informative panel had to be selected in order to genotype BSE affected and control animals to identify association of SNP alleles and haplotypes with BSE. Initially, the number of SNPs had to be minimized while simultaneously maintaining the information provided by all other SNPs (= SNP *tagging*). Therefore, preliminary determination of pairwise LD and haplotypes is necessary (Byng et al., 2003). However, an automated approach to derive provisional LD and haplotypes over the whole region including all SNPs could not be conducted, because the genotype data of 136 SNPs from nine individuals (three of each breed German Holstein, German Fleckvieh and German Brown) were not sufficient. Due to lack of DNA for a few individuals, those had to be replaced while screening the region. Moreover, the German Brown animals were mostly not polymorphic at the identified SNPs in the extended *HEXA* region and therefore actually less individuals remained for deriving LD. Thus, 41 SNPs were manually selected by the following scheme. First, all exonic SNPs, although synonymous, and all SNPs in regulatory regions were chosen. Finally intronic SNPs were selected such that they represent evenly distributed markers over the whole region. If pairwise LD and haplotype phases were clearly recognised for neighbouring SNPs by comparison of the genotypes of the animals, these SNPs would be treated as redundant. Nevertheless, *tagging* after successful genotyping of 38 from 42 SNPs in a larger sample revealed that 16 *tag*SNPs were representing all information. This shows that the preliminary SNP selection was rather inefficient, but required for maintaining the overall information. Despite rapid advances in cattle genetics, in comparison, substantial progress has been made in human genetics by the HapMap project, supplying a catalogue of already *tagged* common human genetic variants, from which their organisation on the chromosome is known (HapMap-Consortium,

2003; HapMap-Consortium, 2005). This project is conducted by the HapMap consortium consisting of at least 25 institutes and 14 genotyping centres all contributing to the work. In a first step, 269 individuals from four populations were genotyped at more than 1 million SNPs which was aimed to be at least one common SNP every 5 kb. From these data, the *tag*SNP and common haplotypes for the human genome were derived which can be used now for high efficient SNP selection for association studies and comparative haplotype analysis.

5.2.2 SNP genotyping with MALDI-TOF MS

The genotyping of the SNPs was performed with a high-throughput genotyping platform, using the mass differences of allele specific extension products (hME and iPLEX, Sequenom, Hamburg, Germany) detected by Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectroscopy (MALDI-TOF MS). Compared to other genotyping methods, e.g. restriction enzyme digestion of Restriction Fragment Length Polymorphism (RFLP) or sequencing, the costs are at least one order of magnitude lower with MALDI-TOF MS because of the possibility to multiplex up to 27 fold and parallel analysis of 376 samples with this method. The expenses for the analysis of one genotype with iPLEX and MALDI-TOF MS range from 0.05 and 0.10 Euro, whereas genotyping by RFLP assays costs on average 0.50 Euro and the sequencing of one sample costs at least 1.50 Euro. In addition to the cost benefit and the advantage of the high-throughput system of MALDI-TOF MS, the amount of sample DNA per animal needed, regardless of the multiplexing grade is 10 fold less than common genotyping assays using PCR (5ng versus 50ng). DNA input per genotype is even lower when genotyping with the MALDI-TOF MS and iPLEX method with a multiplexing grade from 16 to 27, which requires only 0.18 - 0.31 ng DNA per animal and genotype. Further, all steps can be performed with automated pipetting stations.

5.3 Genomic control (GC)

Correction for population stratification by GC (Devlin and Roeder, 1999) was performed to prevent spurious association and false positives due to cryptic substructures. The impact of population stratification on case-control association studies is discussed in numerous publications by different authors (Marchini et al., 2004; Thomas and Witte, 2002; Wacholder et al., 2002). Population stratification affects association studies when cases and controls disproportional represent genetic subpopulations, so that cases and controls have differences in disease prevalence and variations in allele frequency. This can then bias the statistical evidence towards an association (spurious association) between allele and phenotype. Population stratification is probably the most cited reason for non-repeatability of association results.

The GC suggests that in presence of population stratification the standard χ^2-statistic used in case-control studies is inflated by a multiplicative factor, which is proportional to the degree of stratification. This factor is called inflation factor λ and is estimated from a set of unlinked genetic markers (so-called *null-loci*) scattered around the genome. λ is used to correct the test statistic of the candidate loci if $\lambda > 1$.

In this thesis, the GC approach was conducted in cattle for the first time. As *null-loci*, a set of 37 SNP markers was chosen and genotyped in two cattle populations. Due to the exclusion of eight SNPs with unreliable results, 29 SNPs were used for the estimation of λ. It was inferred for the German and the UK Holstein Friesians revealing values of 1.03 and 1.05, respectively. Thus, the effect of inflation is only marginal in both populations suggesting no stratification within both groups.

As Freedman et al. (2004) compared the genotypes of 35 to 40 non-coding SNPs of European, African and Asian Americans to assess stratification levels and to evaluate the SNPs used, GC was performed between the UK and the German Holstein population. Unexpectedly, strong population stratification was observed. The inflation factor between the cases and controls of both groups constituted at a level of no stratification, whereas the comparison of the pooled populations consisting each of both, cases and controls revealed that the populations are genetically different, resulting in a high inflation factor ($\lambda = 19.54$). Despite a intense international exchange of breeding stock in Holstein Friesian, the revealed genetic distinctions between UK and German Holstein animals highlight that both groups should not a priori be merged for an association study. Moreover, the term ‘different populations’ for the UK and German Holstein animals is apparently correct in the context of genetic association studies. However, the minor inflation factors found between cases and controls within each population shows that controls in both populations are well suited as reference for an association study.

5.4 Association of SNPs in the extended *HEXA* region with BSE

In this study, for the first time, remarkably association of SNPs and haplotypes in a candidate region not belonging to the prion gene complex with BSE was shown. Successfully genotyping of 38 SNPs of the extended *HEXA* region (including the genes *ARIH1, HEXA, BRUNOL6* and *PARP6*) in BSE diseased and control animals from UK Holstein cattle population revealed three SNPs associated with the BSE status after correction for multiple testing. The rare alleles of the three SNPs are all presented on one haplotype, which is significantly overrepresented in the controls (5% versus 1% with an permuted p-value of 0.002) and may constitute a factor that protects against BSE infection or are associated with such a factor. The alleles and the haplotype were postulated as the *UK-protecting* alleles or haplotype, respec-

tively. A second haplotype, significantly overrepresented in the control animals, was identified, which does not contain any *UK-protecting* allele, but confers protecting effects. However, these findings could not be confirmed in the German Holstein animals. Here, albeit not significant, the *UK-protecting* alleles are represented more often in the BSE cases, suggesting the SNPs are not causal but in LD with the putative causal mutation. It is likely that in the German animals the putative risk allele was transferred to one or more haplotypes by crossing over events and the effect of the risk allele are not measurable in this population. Also the second BSE-associated haplotype in the UK animals, which does not contain any protecting allele of the single markers, could have emerged from a crossing-over event, where the putative causal variant was transferred to the haplotype.

A promising potential functional candidate gene can be found in the upstream region, the gene encoding for the metalloproteinase thrombospondin, type I, domain containing 4 (THSD4), which contains the spacer and TSP1 repeat domains of the ADAM-TS (a disintegrin and metalloproteinase domain, with thrombospondin type I motifs) protein family. These domains are required to bind and cleave substrates, (e.g. the Transforming Growth Factor (TGF)-beta) and may regulate the function of ADAM-TS enzymes (Hirohata et al., 2002). These enzymes are catalysing the hydrolysis of polypeptides in the extra-cellular matrix. In a transgenic Alzheimer disease mouse model, it has been shown that one member of the metalloproteinase family (ADAM10) reduced the amyloid plaque formation and hippocampal defects when it was moderately over-expressed in mice (Postina et al., 2004). Another metalloproteinase can breakdown the aggregated amyloid fibrils, which are preliminarily thought to be resistant to degradation in Alzheimer disease. The plaque formation in Alzheimer disease is similar to the prion plaque formation in TSEs (Meyer-Luehmann et al., 2006). Earlier studies indicate that over-expression of TGF-beta may initiate or promote amyloidogenesis risk factor for developing Alzheimer's disease (Wyss-Coray et al., 1997). The TGF-beta binding and activating capacity of thrombospondin links the gene product to the neurodegenerative disorders. Therefore, metalloproteinases are promising candidates for BSE susceptibility.

5.5 Association of SNPs in the promoter region of *PRNP*

Analysis of the *PRNP* promoter showed significant association between *PRNP* promoter indel polymorphisms (23-bp and 12-bp) and the BSE status in three cattle populations, the UK and German Holsteins and German Brown, respectively but no significant association in the German Fleckvieh population. However, like in the other populations, the deletion alleles are overrepresented in BSE-affected animals of German Fleckvieh. Furthermore, including the German Fleckvieh population into the combined analyses leads to more significant overall

association. Thus, it can be assumed that the effect of the indel polymorphisms on BSE susceptibility in German Fleckvieh is equivalent to the other three populations. Haplotype analysis and likelihood ratio tests identified that the main effect on BSE susceptibility is due to the 12-bp indel. Although suggestive, the findings cannot directly verify a causal role for either or both indel polymorphisms in the aetiology of BSE. The observed associations could result from another, genetic variant in LD with the indel polymorphisms, although a functional role for these two polymorphisms is supported by their location in regulatory regions of *PRNP*. In vitro analyses have shown that the 12-bp deletion disrupts binding of the SP1 transcription factor and in vivo and in vitro investigations suggest that the two polymorphisms affect *PRNP* expression, albeit with the direction of the effects remaining to be clarified (Sander et al., 2005). One could speculate that, all other factors being equal, susceptibility to BSE might be reduced with a lowered expression of endogenous prion protein. Elevated expression of the prion protein, in contrast, would result in more substrate for conversion into the pathogenic form and possibly higher BSE susceptibility.

The calculated population attributable risks allow assessing the effect of the indel polymorphisms on the BSE epidemic. For example, 53% and 35% of the Holstein BSE cases in UK and Germany, respectively, can be explained by the 12-bp deletion allele. Thus, if the UK Holstein population had been fixed for the alternative 12-bp insertion allele, roughly 90,000 BSE cases would have occurred instead of the 184,000 cases that were recorded. Similarly, the number of cases in German Holstein would have been reduced from 138 to about 90. Although the two populations have similar frequencies of the risk increasing deletion alleles, the BSE-incidence is orders of magnitude smaller in the German than in the UK population, which is most likely explained by differences in environmental exposure like the common usage of animal protein in the feed in UK. Thus, although the indel polymorphisms, and particularly the 12-bp indel, are associated with the BSE risk, they are not the main risk factors. Instead, the decisive factor is environmental: whether or not an animal is sufficiently exposed to the infectious agent. Eliminating or minimizing exposure to the infectious agent should therefore be considered to be the primary measure to reduce BSE incidence, even in the light of the strong genetic component of BSE susceptibility as shown in this thesis. However, selective breeding to reduce the frequency of the susceptibility alleles would still bring an additional protective component that should not be discounted.

6 Conclusions and Outlook

The present study suggests that variation on BTA 10 confers protective effects for BSE susceptibility. The examination of a 300-kb region centred on the *HEXA* gene revealed three intronic SNPs with the rare alleles significantly overrepresented in the control animals compared with BSE affected animals from the UK. These rare alleles are all combined on one haplotype. However, the association could not be confirmed in the panel of German Holstein BSE affected and control animals. Thus, it can be concluded that the association seems to result from a putative mutation which is in LD with the associated SNPs. Additional SNP analyses and association studies in the direction of the centromer might help to clarify the origin of the protective effects in the UK animals.

Futhermore, it has been shown that two indel polymorphism are strongly associated with the BSE status in three of four investigated cattle populations. It can be concluded, that the main effect on BSE susceptibility result from the 12-bp indel. The causality of either or both polymorphism cannot be verified in this thesis, however the data highlight that the 12-bp deletion allele is associated with increased risk for an animal to succumb to feed-borne BSE. Thus, the findings show a substantial genetic component for the susceptibility of cattle to BSE and, conferred by variants in the regulatory region of *PRNP*, a gene that has been shown to be involved in prion disease susceptibility/resistance in other species. However, the different dimensions of the BSE epidemic in UK and German cattle, on the one hand and the similar frequencies of the *PRNP*-associated susceptibility alleles in UK and German cattle on the other, indicate, that the main BSE risk factor for a cattle is environmental, i.e. exposure to contaminated feed, and not genetic. Additional association studies with polymorphisms in the promoter and in the coding region of *PRNP* might be appropriate to go further in the elucidation of the association's origin. The causality of the polymorphisms might be confirmed by expression studies testing differences in *PRNP*-transcript levels depending on genotype or diplotype of individuals.

There might be additional loci affecting BSE susceptibility or prion incubation time. In order to identify these regions and promising candidate genes, whole genome association studies will be performed at the institute in the next future. Therefore, a dense set of 10 000 SNP markers spread over the whole genome will be genotyped in a panel of 100 BSE affected and 100 control animals and analysed for association. At date, for cattle, two SNP sets containing 10 000 and 25 000 SNP markers, respectively are available from Affymetrix (Santa Clara,

CA, USA) and next year Illumina (San Diego, CA, USA) will provide bovine SNP sets comprising 40 000 to 75 000 markers.

7 Summary

Bovine Spongiform Encephalopathy (BSE) belongs to a group of neurodegenerative diseases, which are known as transmissible prion diseases. BSE is most likely caused by the ingestion of feedstuffs contaminated with infectious prions leading to a progressive degeneration of the brain of the affected animals. In humans, mice and sheep, coding variants of *PRNP*, the gene encoding the endogenous prion protein, have been shown to have major effects on the susceptibility to and incubation time of such diseases. However, up to date, the effects of polymorphisms in the coding and regulatory regions of bovine *PRNP* on BSE susceptibility have been considered marginal or non-existent. Hence, it has been suggested that additional regions and therein located genes with their gene products might be involved in the disease development. Recently, whole genome marker scans have been conducted and additional putative candidate regions have been identified. In this thesis, such a candidate region on BTA 10 which is located close to a marker on BTA 10 with significant segregation distortion in BSE affected animals (Hernandez-Sanchez et al., 2002) and the *PRNP*-Promoter region have been investigated for association with BSE susceptibility in cattle.

From the candidate region on BTA 10, a 300-kb region encompassing four genes (*ARIH1*, *HEXA, BRUNOL6* and *PARP6*) has been characterised and screened for polymorphisms, which were subsequently used for an association study with BSE. Therefore, BSE affected and control animals of Holstein Friesian from UK and Germany have been analysed. Successful genotyping of 38 SNPs in the UK Holsteins (350 diseased and 270 control animals), revealed three intronic SNPs as associated with the BSE status with permuted p-values ranging from 5×10^{-2} to 2×10^{-3} conferring potential BSE protecting effects. The three SNPs are in strong LD with the rare alleles presented on the so-called ‚UK-protective' haplotype, which is significantly overrepresented in the controls with a permuted p-value of 2×10^{-3}. However, these findings could not be confirmed in the panel of German Holstein animals (73 diseased and 627 controls). Albeit not significant, the protecting haplotype and alleles are slightly overexpressed in the German Holstein BSE affected animals. Thus, it is rather inprobable that one of the three SNPs is causative. It is more likely that a putative causal mutation is in LD to the associated SNPs and is probably located downstream of *PARP6* in the direction of the centromer. However, SNP analyses in this region and additional association studies might help to clarify the origin of the protective effects assigned to the associated haplotype in the UK animals.

Furthermore in this thesis, polymorphisms in the bovine *PRNP* promoter region have been investigated for association with BSE. It has been shown that two previously reported promoter insertion/deletion polymorphism (23-bp and 12-bp, respectively) are significantly associated with the BSE status in three of four cattle populations. Genotyping of BSE-diseased and control animals of UK Holstein (350 cases vs. 270 controls), German Holstein (127 cases vs. 627 controls), German Brown (43 cases vs. 90 controls) and German Fleckvieh (106 cases vs. 137 controls) revealed a significant overrepresentation of the deletion alleles at both polymorphic sites in diseased animals. The main effect on susceptibility is associated with the 12-bp indel polymorphism. Compared with non carriers, heterozygous and homozygous carriers of the 12-bp deletion allele posess relatively higher risks of having BSE, ranging from 1.32 to 4.01 and 1.74 to 3.65 in the different breeds. These values correspond to population attributable risks ranging from 35% to 53%. The result demonstrate a substantial genetic *PRNP* associated component for BSE susceptibility in cattle, which have not been shown before in this form and impact and over different population. However, the findings cannot verify a causal role for either or both indel polymorphisms in the aetiology of BSE. On the one hand, a functional role is supported by their location in the regulatory CpG rich region. Moreover, the indel-polymorphisms affect putative transcription factor binding sites, suggesting that they influence directly *PRNP* expression. On the other hand, the observed association could result from a putative causal variant in LD with the indel polymorphism. This variant might be located in the regulatory or in the coding sequence of *PRNP*. More association studies with additional polymorphisms and expression studies in vitro and in vivo could help to elucidate the origin of the association. However, although the BSE risk conferred by the deletion allele is considerable, the main risk factor for BSE in cattle is environmental, i.e. exposure to feedstuffs contaminated with the infectious agent.

8 Zusammenfassung

Bovine Spongiforme Enzephalopathie (BSE) gehört zu einer Gruppe neurodegenerativer Erkrankungen, die als transmissible Prionenkrankheiten bezeichnet werden. BSE wird sehr wahrscheinlich durch eine über das Futter aufgenommene krankmachende Form des Prionproteins verursacht und führt zu einer fortschreitenden Degeneration des Gehirns erkrankter Rinder. Bei anderen Spezies, wie bspw. beim Mensch, bei der Maus und beim Schaf konnte gezeigt werden, dass Polymorphismen im Prionprotein-Gen (*PRNP*) die Anfälligkeit gegenüber oder die Inkubationszeit der Prionenkrankheiten beeinflussen. Bis heute konnten jedoch beim Rind keine oder nur marginale Effekte nachgewiesen werden, die auf Polymorphismen in der kodierenden bzw. regulatorischen Region des *PRNP* zurückzuführen wären. Daher wird angenommen, dass zusätzliche Genomregionen und die darin befindlichen Gene und deren Genprodukte in das Krankheitsgeschehen verwickelt sind. Mittels Mikrosatellitenmarkern wurden kürzlich weitere Regionen beim Rind identifiziert, die einen Einfluss auf die BSE-Entwicklung haben könnten. In der vorliegenden Arbeit wurde solch eine Kandidatengenregion auf BTA 10 nahe eines BSE assoziierten Markers (Hernandez-Sanchez et al., 2002) und der *PRNP*-Promoterbereich nach einer Assoziation mit BSE hin untersucht.

Von der Kandidatengenregion auf BTA 10 wurden ca. 300 Kilobasen analysiert, die darin enthaltenen vier Gene *ARIH1*, *HEXA*, *BRUNOL6* und *PARP6* charakterisiert, DNA Variationen gesucht und diese auf Assoziation mit BSE hin untersucht. Die hier gezeigte Assoziationsstudie wurde in BSE betroffenen und Kontroll-Tieren der Rasse Holstein Friesian aus Großbritannien (UK) und Deutschland durchgeführt. Nach dem erfolgreichen Genotypisieren von 38 SNPs in den UK Holsteins (350 BSE-betroffene und 270 Kontrolltiere) wurden drei intronische SNPs identifiziert, die signifikant mit dem BSE Status assoziiert sind mit permutierten P-Werten von 5×10^{-2} bis 2×10^{-3} und deren seltenen Allele einen potenziell schützenden Effekt übertragen. Die drei SNPs liegen im starken Kopplungsungleichgewicht, wobei sich die jeweils selteneren Varianten alle auf einem, dem sogenannten „UK-schützenden“ Haplotypen befinden, der signifikant häufiger bei den Kontrolltieren auftritt mit einem permutierten P-Wert von 2×10^{-3}. Diese Assoziation konnte allerdings in einer Auswahl von deutschen Holstein Tieren (73 BSE-betroffene und 627 Kontrollen) nicht reproduziert werden. Das Ergebnis ist zwar nicht signifikant, aber bei dieser Tiergruppe ist der „UK-schützende“ Haplotyp in den BSE Tieren leicht übervertreten. Daher erscheint es eher unwahrscheinlich dass einer der drei SNPs ursächlich für die Assoziation ist. Es ist eher vorstellbar, dass sich die potentielle kausale Mutation im Kopplungsungleichgewicht zu den assoziierten SNPs und stromabwärts von *PARP6* in Richtung des Zentromers befindet. Weitere

SNP-Analysen in dieser Region und zusätzliche Assoziationsstudien könnten den Ursprungs des schützenden Effekts, der dem assoziierten Haplotypen zugewiesen wurde, aufklären.

Des weiteren wurden in dieser Arbeit Polymorphismen in der *PRNP*-Promoter Region auf eine Assoziation mit BSE hin untersucht. Dabei konnte gezeigt werden, dass zwei bekannte Insertions-Deletions-Polymorphismen (23-bp und 12-bp Indel) signifikant mit dem BSE Status in drei von vier untersuchten Rinderrassen assoziiert sind. Die Genotypisierung von BSE-betroffenen und Kontrolltieren der Rassen UK Holstein Friesian (350 BSE-Fälle und 270 Kontrollen), Deutsche Holstein (127 BSE-Fälle und 627 Kontrollen), Deutsches Braunvieh (43 BSE-Fälle und 90 Kontrollen) und Deutsches Fleckvieh (106 BSE-Fälle und 137 Kontrollen) ergab, dass die Deletionsallele an beiden Polymorphismen über alle BSE-Tiere hinweg signifikant häufiger vorkommen als in den gesunden Kontrollen. Der stärkste Einfluss auf die BSE-Anfälligkeit scheint jedoch vom 12-bp Indel Polymorphismus auszugehen. Im Vergleich zu Individuen die kein 12-bp Deletionsallel besitzen, haben heterozygote und homozygote Träger ein relativ höheres Risiko an BSE zu erkranken (von 1.32 bis 4.01 bzw. von 1.74 bis 3.65 in den verschiedenen Rassen). Das dem 12-bp Deletionsallel zuzuschreibende Risiko für die Population (Population Attributable Risk) reicht von 35 % bis 53 % in den verschiedenen Rassen. Die Ergebnisse veranschaulichen eine substantielle genetische Komponenete für die BSE Anfälligkeit, die in dieser Form und Stärke und über mehrere Populationen hinweg vorher noch nicht gezeigt werden konnte. Über die Frage, ob einer oder beide Polymorphismen eine funktionale Rolle in der Ätiologie von BSE spielen, konnte in dieser Arbeit allerdings nur spekuliert werden. Da die Indel-Polymorphismen Transkriptionsfaktorbindungsstellen verändern und sie sich in CpG-reichen also putativen regulatorischen Regionen befinden, könnten Sie einen direkten, also kausalen Einfluss auf die Genexpression haben. Falls keiner der Indel-Polymorphismen einen direkten Effekt übertragen sollte, dann lägen beide im Kopplungsungleichgewicht zu der ursächlichen Mutation, die möglicherweise noch im Promoterbereich oder stromabwärts in der kodierenden Sequenz zu vermuten wäre. Weitere Assoziationsstudien an zusätzlichen Polymorphismen und Expressionsstudien in vitro und in vivo könnten zur Aufklärung dieser Frage betragen. Es bleibt anzumerken, dass obwohl das von den Deletionsallelen übertragene genetische Risko beträchtlich ist, der Hauptrisikofaktor für eine BSE-Erkrankung die Umwelt darstellt, d.h. vor allem die Erregerexposition durch verseuchtes Futter.

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11 Appendices

11.1 Buffers

Church		(5% SDS, 1 mM EDTA, 0.341 M Na_2HPO_4 and 0.159 M NaH_2PO_4)
TE		(10mM Tris-Cl, 1mM EDTA, pH 8)
TBE	10x	(900 mM Tris-Cl, 900 mM Borate, 20 mM EDTA, pH 8.3)
TNE	10x	(100 mM Tris-Cl 10mM EDTA NaCl 2M; pH 7.4)
SSC	20x	(3M NaCl, 0.3 M Na-Citrate)

11.2 Primers

PCR-Primers for bovine *ARIH1*

Gene	Primer	Pair	Lab-name	Sequence	Region
ARIH1	3157	3158	ARI_3157up	CGTTCCCGACCTCTACCC	Ex1
ARIH1	3158	3157	ARI_3158dn	TCCACCATGTGTTGCAGAAT	Ex1
ARIH1	3159	3160	ARI_3159up	CGGACGAGGGCTACAACTAC	Ex1
ARIH1	3160	3159	ARI_3160dn	CTGGATGACCTCGTTGACCT	Ex1
ARIH1	3162	3163	ARI_3162up	TTTCACTGGCCTTGAATGTG	Ex4
ARIH1	3163	3162	ARI_3163dn	ACCATGAGCAGGACACGAA	Ex5
ARIH1	3164	BAC	ARI_3164up	TCTGCAACACATGGTGGAAT	Ex1
ARIH1	3165	BAC	ARI_3165up	CGGACGATGATAACCTGGAT	Ex1
ARIH1	3166	BAC	ARI_3166dn	GTTATCATCGTCCGGCTCAT	Ex1
ARIH1	3167	BAC	ARI_3167dn	CTCTTCGTCCTCGTCGAACT	Ex1
ARIH1	3229	BAC	ARI_3229dn	GCCCAGATCCAGGTTATCAT	Ex1
ARIH1	3230	BAC	ARI_3230dn	GTCCTCCTCGCTGCACTCT	Ex1
ARIH1	3231	BAC	ARI_3231dn	GTCCTCGTCCTCCTCCTCCT	Ex1
ARIH1	3232	BAC	ARI_3232up	ATTACCGCTACGAGGTGCTC	Ex1
ARIH1	3233	BAC	ARI_3233up	TGAGCAGGAGGAGGATTACC	Ex1
ARIH1	3234	3235	ARI_3234up	CGCCTGATCACAGATTCAAA	Ex6
ARIH1	3235	3234	ARI_3235dn	GCAGCGAACAGGTTTAGCAT	Ex7
ARIH1	3299	seq	ARI_3299up	ACTGCTTTTGTGCTGCAGTG	I6
ARIH1	3300	seq	ARI_3300dn	TCAGGGAAGGAGTTCATATGCT	I6
ARIH1	3558	3559	ARI_3558up	GTACTCAATTAAGACAAGCCATGA	SCA295439(rc)
ARIH1	3559	3558	ARI_3559dn	CTCTTCGTCCTCGTCGAACT	SCA295439(rc)
ARIH1	3560	3561	ARI_3560up	AGTGGTCAGCTGCTCCGTAT	SCA295439(rc)
ARIH1	3561	3560	ARI_3561dn	TACTCTCCGGACATGCTGTG	SCA295439(rc)
ARIH1	3562	3563	ARI_3562up	TGGGTCGGGAATAAATGAAA	SCA295439(rc)
ARIH1	3563	3562	ARI_3563dn	AGGTAGTTGCCACACCTGGA	SCA295439(rc)
ARIH1	3601	3602	ARI_3601up	GCATGCCAATTTTCTTCCTT	SCA295439
ARIH1	3602	3601	ARI_3602dn	AGTGGAGCCAACAGATTTGC	SCA295439
ARIH1	3603	3604	ARI_3603up	ATGCTTTCAGACCCATCACC	SCA295439
ARIH1	3604	3603	ARI_3604dn	AAGCAAAGGGCAATTTACGA	SCA295439

ARIH1	3605	3606	ARI_3605up	GGTGTACAGTTGCCCCAAGT	SCA295439
ARIH1	3606	3605	ARI_3606dn	TAGGGTCTGGCCTCAGTGAT	SCA295439
ARIH1	3607	3608	ARI_3607up	GTTGGCTCAGTAGCGGTAGC	SCA295439
ARIH1	3608	3607	ARI_3608dn	ACTTGGGGCAACTGTACACC	SCA295439
ARIH1	3609	3610	ARI_3609up	AGGACAAATGTTCCGTGGTC	SCA295439
ARIH1	3610	3609	ARI_3610dn	GGCCTCCGAGAGAAAACAGT	SCA295439
ARIH1	3611	3612	ARI_3611up	CCTCCTGCCTGGTAAAACAC	SCA295439
ARIH1	3612	3611	ARI_3612dn	AAGTCACCTTGCATCTTTGG	SCA295439
ARIH1	3613	3614	ARI_3613up	GTGAAAGCGCTGGTGAAACT	SCA295439
ARIH1	3614	3613	ARI_3614dn	CTGTGTTTTACCAGGCAGGAG	SCA295439
ARIH1	3637	3166	ARI_3637up	CGGAAAAAATCCACCACCTG	SCA295439
ARIH1	3166	3637	ARI_3166dn	GTTATCATCGTCCGGCTCAT	SCA295439
ARIH1	3638	3639	ARI_3638up	TACTCTGTTCGCTGCTGAGG	SCA295439
ARIH1	3639	3638	ARI_3639dn	GTCCTCCTCGCTGCACTCT	SCA295439
ARIH1	3640	3641	ARI_3640up	CTGGGGACTGTTTTCTCTCG	SCA295439
ARIH1	3641	3640	ARI_3641dn	GTCTGCTCTATCCCCAGCAG	SCA295439
ARIH1	3642	3643	ARI_3642up	GTCAACGAGGTCATCCAGGT	SCA295439
ARIH1	3643	3642	ARI_3643dn	AACCCAAACGAACAGCAAAC	SCA295439
ARIH1	3644	3645	ARI_3644up	GGGCAGTGGTATTTCTTTCG	SCA295439
ARIH1	3645	3644	ARI_3645dn	AAGGCACAATCAACATTTGCTA	SCA295439
ARIH1	3646	3647	ARI_3646up	CCCAGCTATATTTAACTCACATTG	SCA295439
ARIH1	3647	3646	ARI_3647dn	CGCTTCAGTCGTGTCTGACT	SCA295439
ARIH1	3648	3649	ARI_3648up	GCAACCCACTCCAGTGTTCT	SCA295439
ARIH1	3649	3648	ARI_3649dn	AGGGGTGAAGAAAACCCAAC	SCA295439
ARIH1	3650	3651	ARI_3650up	CCAACATGATCAAAAGCTTGG	SCA295439
ARIH1	3651	3650	ARI_3651dn	AGTTTCACCAGCGCTTTCAC	SCA295439
ARIH1	3698	3699	ARIH_3698up	TGAGGCATACAACCTCAATTTC	SCA295439

PCR-Primers for bovine *BRUNOL6*

Gene	Primer	Pair	Lab-name	Sequence	Region
BRUNOL6	3139	3140	BRUN6_3139up	GTGGCACTAGTGGAGGAGG	
BRUNOL6	3140	3139	BRUN6_3140dn	CAACAGGAAAGGCAGAGGAG	
BRUNOL6	3141	3141	BRUN6_3141up	CAACAGGAAAGGCAGAGGAG	
BRUNOL6	3142	3142	BRUN6_3142dn	TTTCAGATTGGCATGAAACG	
BRUNOL6	3152	3153	BRUN6_3152up	AGAAGCACCTCGGATAAGCA	E15
BRUNOL6	3153	3152	BRUN6_3153dn	GGCTGTGACAACAGACTTGG	E15
BRUNOL6	3154	3155	BRUN6_3154up	GCGAATCTTCCAAGTCTGTTG	E15
BRUNOL6	3155	3154	BRUN6_3155dn	GCTGAGCTGAGACACCTCCT	E15
BRUNOL6	3156		BRUN6_3156up	CCCCTTCTCTCAATGGCAGT	E15
BRUNOL6	3186	3187	BRUN6_3186up	GCCATGAATGGCTTTCAGAT	E12
BRUNOL6	3187	3186	BRUN6_3187dn	TGCTCAAGCATTTCTGTGCT	E13
BRUNOL6	3188	3189	BRUN6_3188up	AGGCCGCTTTAAGGATTGTT	
BRUNOL6	3189	3188	BRUN6_3189dn	CAGAAGGTGGTCTGGAGCAA	
BRUNOL6	3596	3597	BRUN_3596up	ACTCCAGCCATCTTCCCTCT	SCA125719
BRUNOL6	3597	3596	BRUN_3597dn	CGCTTCCTATGGGAGTGGTA	SCA125719
BRUNOL6	3599	3600	BRUN_3599up	CACAGCATTGACAGGCAGAT	SCA125719
BRUNOL6	3600	3599	BRUN_3600dn	GGAGCTTGGGTTCCTAATCC	SCA125719
BRUNOL6	3787	3788	BRUN_3787up	CCATCTGCAGTGATTTTGGA	5’-end
BRUNOL6	3788	3787	BRUN_3788dn	GACTGTGGCTCAGACCATGA	5’-end

BRUNOL6	3789	3790	BRUN_3789up	CACTGCCTTCTGTGCAATGT	5'-end
BRUNOL6	3790	3789	BRUN_3790dn	TGTTCAAGAGCAGCAGTAGCA	5'-end
BRUNOL6	3791	3792	BRUN_3791up	GCTGCTCTTGAACAGTCATCA	5'-end
BRUNOL6	3792	3791	BRUN_3792dn	GCACACACCTCCAATCACTG	E1
BRUNOL6	3793	3794	BRUN_3793up	GAGGACTGGCTTCGGTAGTG	E1
BRUNOL6	3794	3793	BRUN_3794dn	AGCACCGTCAGCTCGTAGAT	E1
BRUNOL6	3795	3796	BRUN_3795up	ACGCCATCAAGCTTTTCGT	E1
BRUNOL6	3796	3795	BRUN_3796dn	GACCATGGTGCCTGTCTCTT	I1
BRUNOL6	3797	3798	BRUN_3797up	TAGTCCTGGGAGGGTTGATG	I1
BRUNOL6	3798	3797	BRUN_3798dn	AAGGGTACAGGTAAGTCCTCTGC	I2
BRUNOL6	3799	3800	BRUN_3799up	TGGCCTTGAGTTTGTCAGTG	I1
BRUNOL6	3800	3799	BRUN_3800dn	CTGAGGCCTGGAATTGGATA	I2
BRUNOL6	3834	3833	HEXA_3834dn	CCTTTTCATCCCCTTCCTCT	5'-end
BRUNOL6	3836	3835	HEXA_3836dn	CCTCTTGGAAAACCCAGAGAA	5'-end
BRUNOL6	3839	3838	HEXA_3839dn	TTAATCCCATAGGTCCCTGCT	5'-end
BRUNOL6	3185	3184	BRUN6_3185dn	ATAGCTGTCTGGGCACTGGT	E12
BRUNOL6	3184	3185	BRUN6_3184up	TCAGGAGTTTGGTGATGCAG	E11

PCR-Primers for bovine *HEXA*

Gene	Primer	Pair	Lab-name	Sequence	Region
HEXA	2584	2585	HEXA_2584up	ACTCTGGGTCTCACCCCTCT	E8
HEXA	2585	2584	HEXA_2585dn	CACCAAAGCCTTTCTTCTTCA	E9
HEXA	2712	2713	HEXA_2712up	ACCCAGATATCCAGGCCTTT	E9
HEXA	2713	2712	HEXA_2713dn	CAAACACCTCCTGCCACAC	E10
HEXA	2714	2715	HEXA_2714up	ACTCCTGGCCACACTCTGTC	E7
HEXA	2715	2714	HEXA_2715dn	CAACCTCATCTCCTCCAAGG	E8
HEXA	2716	seq	HEXA_2716up	ACGGAAACCAGAATCCAGTG	I8
HEXA	2717	seq	HEXA_2717up	TGGATCTGGGGAAAGAGTTG	I8
HEXA	2754	2716	HEXA_2754dn	CACTCCCGCTATGTCCTCAC	I9
HEXA	2755	2716	HEXA_2755dn	AGGACTCTGAAGGACGGTGA	I9
HEXA	2756	2757	HEXA_2756	CGAGAGCTTCACTTTTCCAGA	E6
HEXA	2757	2756	HEXA_2757	AAGCCGTGCATATTCAATCA	E7
HEXA	2796	BAC	HEXA_2796	CTTTGCTAGCAGGGCCATAG	I6
HEXA	2952	2953	HEXA_2952	TGTGTTTTGTGCCTTTCAGG	E7
HEXA	2953	2952	HEXA_2953	GGGCATGCTACATCCAAGAG	E7
HEXA	2954	2955	HEXA_2954up	ACCTCTGAGCTGCGGTACAC	E1
HEXA	2955	2954	HEXA_2955dn	ACCCCAAAGAAGAAACTGG	E2
HEXA	2956	2957	HEXA_2956up	CTCTGTGGCTTTCCGTTTTC	E2
HEXA	2957	2956	HEXA_2957dn	CAGTCTCGGAGAGGAGGAGA	E3
HEXA	2958	2959	HEXA_2958up	ACTGACCATAAATGATGAGCAGAG	E3
HEXA	2959	2958	HEXA_2959dn	GAGATCTCCATATAAGCTGGCTAAA	E4
HEXA	2960	2961	HEXA_2960up	CTGGAGACATTTAGCCAGCTT	E4
HEXA	2961	2960	HEXA_2961dn	TGTCCAGGATGCTAGCCAGT	E5
HEXA	2962	2963	HEXA_2962up	GGGGCTTGTTGCTGGATAC	E5
HEXA	2963	2962	HEXA_2963dn	TCTGGAAAAGTGAAGCTCTCG	E6
HEXA	2964	2965	HEXA_2964up	GGCATCTGGTCGATGACTCT	E6
HEXA	2965	2964	HEXA_2965dn	GACAGAGTGTGGCCAGGAGT	E7
HEXA	2966	2967	HEXA_2966up	GCTCCACGCTCAGGTTTTC	E1
HEXA	2967	2966	HEXA_2967dn	GAAGAGCAGGTCACGGTAGC	E1

HEXA	2990	seq	HEXA_2990up	CTGCAGGGAGTGTTCTCCTC	I3
HEXA	2991	seq	HEXA_2991dn	TCCTATGTGGCAGAGCACAG	I3
HEXA	2992	seq	HEXA_2992up	TTAGGATTCTGGGGTGATGG	I5
HEXA	2993	seq	HEXA_2993dn	TCAGAGCACACACTGTGAACC	I5
HEXA	2994	2995	HEXA_2994up	CTGTGCTCTGCCACATAGGA	I3
HEXA	2995	2994	HEXA_2995dn	GGGACCATCTCAAGCCACTA	I4
HEXA	2997	2998	HEXA_2997up	CCTCTCGGAACTGAGCAAAC	E10
HEXA	2998	2997	HEXA_2998dn	ACCAGTGCCAACTCCTTCAC	E11
HEXA	2999	3000	HEXA_2999up	ATCATCCAGGTATGGCGAGA	E11
HEXA	3000	2999	HEXA_3000dn	CAGGTTTGTGCTGTCCACAT	E12
HEXA	3001	3002	HEXA_3001up	GGCCTGTATGTGGGGAGAGT	E12
HEXA	3002	3001	HEXA_3002dn	CAGCTCACAGCGGAAGTGT	E13
HEXA	3003	3004	HEXA_3003up	AGGCTGTGGAGCAACAAGAT	E13
HEXA	3004	3003	HEXA_3004dn	CTCTCCTAGGGCCAGTACCC	E14
HEXA	3005	3006	HEXA_3005up	ACGCACACAGTTTGTTCAGC	I3
HEXA	3006	3005	HEXA_3006dn	TGCCCCTCCTAGCAATACAC	I3
HEXA	3007	3008	HEXA_3007up	TTCTCTGAACAAGGGCAGCTA	I5
HEXA	3008	3007	HEXA_3008dn	CCCCAGCTTCTTGCTAAGTG	I5
HEXA	3009	3010	HEXA_3009up	CTGGGTTCACAGTGTGTGCT	I5
HEXA	3010	3009	HEXA_3010dn	CTTTGCTAGCAGGGCCATAG	I6
HEXA	3011	3012	HEXA_3011up	GTTGAGGCCTGTCTTTGCTT	I11
HEXA	3012	3011	HEXA_3012dn	GAGAGCCCATGCCACAAG	I12
HEXA	3013	seq	HEXA_3013up	AGAGGGTCAGTTCCCTGGTT	I13
HEXA	3014	3015	HEXA_3014up	CCCCATAGAAAAACGGCATA	E2
HEXA	3015	3014	HEXA_3015dn	GACAGTCTCGGAGAGGAGGA	E3
HEXA	3044	3045	HEXA_3044up	CACTGGACCACCAGGAAAGT	I2
HEXA	3045	3044	HEXA_3045dn	CTCCCTCTGCTTCCCAGAGT	I3
HEXA	3046		HEXA_3046up	GCGTTTTGGAGAAACAGCAT	I3
HEXA	3059	3060	HEXA_3059up	ACGTGGGGCTTCTATCCTCT	5’-UTR
HEXA	3060	3059	HEXA_3060dn	ACTGGAATTGGAAGCTTTGC	E1
HEXA	3061	3062	HEXA_3061up	ACAGAAGCAGGGAGGCATT	E14
HEXA	3062	3061	HEXA_3062dn	CTCCAGGGGCAGATACTGAG	3’-UTR
HEXA	3063	2955	HEXA_3063up	ATGGCCCCAGTACATTCAGA	E1
HEXA	3064	3065	HEXA_3064up	AGCTGCGGTACACCATCTTC	E1
HEXA	3065	3064	HEXA_3065dn	CCAGACAAGTGCTGGCTTCT	E2
HEXA	3078	3079	HEXA_3078up	AACCGCCTGAACTTGAACC	5´-end
HEXA	3079	3078	HEXA_3079dn	CAGAGGAAGGGCGTTTCAG	I1
HEXA	3080	BAC	HEXA_3080up	TCTGTAATCCTGCCCTCCTG	I1
HEXA	3125	SBE	HEXA_3111dn	TGCTCAGAGATGGTAAGGAACTC	I13
HEXA	3112	SBE	HEXA_3112up	AACTGTACCATTAATTTCCTCTGTGA	I6
HEXA	3113	SBE	HEXA_3113dn	AACTCTCCTCAGGCCATTTCAT	I11
HEXA	3114	SBE	HEXA_3114dn	GGGCTCCAGGCCTAGCAT	I2
HEXA	3115	3116	HEXA_3115up	AAGCCAGCACTTGTCTGGAG	I2
HEXA	3116	3115	HEXA_3116dn	TCAGACGCTGGTTAGAGTGC	I2
HEXA	3117	3118	HEXA_3117up	ACCAGCCAGTTTAAGGGACA	I6
HEXA	3118	3117	HEXA_3118dn	CCATGATGCCAGTGAGACAA	I6
HEXA	3119	3120	HEXA_3119up	ACTCTGGGGAAACAGCTTCA	I13
HEXA	3120	3119	HEXA_3120dn	ATCTCAGCCTCACCAGTTCC	I13
HEXA	3126	3127	HEXA_3126up	AGGAGCTGTAGCGGGTCAC	E1
HEXA	3127	3126	HEXA_3127dn	GAGCCCGGGAACGAGACT	E1

HEXA	3128	BAC	HEXA_3128dn	GACCGAAAGCAGTCCAGAAG	5´-end
HEXA	3130	3129	HEXA_3130up	GCGTAGGCTACTGTGACATGG	3´-end
HEXA	3129	3130	HEXA_3129dn	GGGGTGTGTGTCTGGACTCT	3´-end
HEXA	3132	3131	HEXA_3132up	CTCAGTATCTGCCCCTGGAG	3´-end
HEXA	3131	3132	HEXA_3131dn	TTTGTGGAAGAGGGGTTCAC	3´-end
HEXA	3133	BAC	HEXA_3133up	TGAACGCGGAATAAAAGGTC	3´-end
HEXA	3134	3135	HEXA_3134up	TCTCTAGCTGAGCCCATTCTG	5´-end
HEXA	3135	3134	HEXA_3135dn	AGAGAGAACCCGAAGCTCCT	5´-end
HEXA	3136	BAC	HEXA_3136dn	CCAGGGAACTTCAGAAGCAA	5´-end
HEXA	3137	BAC	HEXA_3137up	GGGTCTTTGGGGACTCTCTC	3´-end
HEXA	3138	BAC	HEXA_3138up	GTGAACCCCTCTTCCACAAA	3´-end
HEXA	3147	3148	HEXA_3147up	CCTAGCCTGTTTGTGGCATT	5´-end
HEXA	3148	3147	HEXA_3148dn	GTGACCCGGGAGTACTGACT	5´-end
HEXA	3149	BAC	HEXA_3149dn	TGCAAGCGATAAGACATTGC	5´-end
HEXA	3150	BAC	HEXA_3150up	ATGCCTCTCCTAGCTCACCA	3´-end
HEXA	3151	BAC	HEXA_3151dn	GGCTGGGAACGAAACTTGTA	3´-end
HEXA	3161	3064	HEXA_3161dn	TCGGTGATTTTCCTCTCACA	I1
HEXA	3190	3191	HEXA_3190up	GGTTGCAAAGAGCTGCTG	I1
HEXA	3191	3190	HEXA_3191dn	GACGGTTCTGGGCACTTG	I2
HEXA	3192	BAC	HEXA_3192dn	GGTGCCACCTAGTCACCTGT	I1
HEXA	3474	3475	HEXA_3474up	CCAGAGATTGACAGGGTGGT	5'-end (SCA95258)
HEXA	3475	3474	HEXA_3475dn	CCTGAGGAAGTGGTTTCCAA	5'-end (SCA95258)
HEXA	3476	3477	HEXA_3476up	TGATTCCACTTTATGAGCCAAA	5'-end
HEXA	3477	3476	HEXA_3477dn	TGCCATAACTCCCCTTTCTG	5'-end
HEXA	3478	3479	HEXA_3478up	CTGGGAAAACTGGACAGCTC	SCA95258
HEXA	3479	3478	HEXA_3479dn	CCACGCACCAAAACTACTGA	SCA5566
HEXA	3480	3481	HEXA_3480up	CACAGCACGTGAGAATGGTC	5’-end (SCA95258)
HEXA	3481	3480	HEXA_3481dn	TGCCTGTCCATTTGTCTGAG	5’-end (SCA95258)
HEXA	3482	3483	HEXA_3482up	ATTCAGCTCGCCCTCTCTC	3’-end (SCA5566)
HEXA	3483	3482	HEXA_3483dn	GCTAAGTATCAGATTTCCAGAGGA	3’-end (SCA5566)
HEXA	3484	3485	HEXA_3484up	TGGAACCCACTCAGAAAAGG	3’-end (SCA5566)
HEXA	3485	3484	HEXA_3485dn	CCTCAGTGAGAGCAGCCTTC	3’-end (SCA5566)
HEXA	3486	3487	HEXA_3486up	GCATTGGCCCAAAGACTACT	SCA184260
HEXA	3487	3486	HEXA_3487dn	CCCTTTCTAGCCTGTTTTGC	SCA184260
HEXA	3488	3489	HEXA_3488up	CTCATCCAGCGCTCTTTAGC	SCA15309
HEXA	3489	3488	HEXA_3489dn	GAATCCCATCGTCTGACTGC	SCA15309
HEXA	3496	3497	HEXA_3496up	CCGAAGACCTTGGGAAGAAT	SCA125719
HEXA	3497	3496	HEXA_3497dn	GGATGCAATCTGGGTGTCTT	SCA125719
HEXA	3498	3499	HEXA_3498up	GCATTGTGCTGGTGTACAGA	SCA125719
HEXA	3499	3498	HEXA_3499dn	TGACAGCCTTTCCCGTAGAA	SCA125719
HEXA	3500	3501	HEXA_3500up	AGTTTTGCTGCCCAACTCAT	SCA95258
HEXA	3501	3500	HEXA_3501dn	AAGTTGCCTTGTGGGAAAAC	SCA95258
HEXA	3502	3503	HEXA_3502up	ACCAGAAGGCGACAGAAAGA	SCA5566
HEXA	3503	3502	HEXA_3503dn	GGGGAACCTGAGACCATTTT	SCA5566
HEXA	3526	3527	HEXA_3526up	ACAGCCCCAAACTGAGGATT	SCA125719
HEXA	3527	3526	HEXA_3527dn	CAGGAGTTTGGTGATGCAGA	SCA125719
HEXA	3528	3529	HEXA_3528up	GGCAGGAATGTCTGGATGAG	SCA125719
HEXA	3529	3528	HEXA_3529dn	CCAGGCTCGGACAGTCTCTA	SCA125719
HEXA	3530	3531	HEXA_3530up	AGACTGTCCGAGCCTGGTT	SCA125719
HEXA	3531	3530	HEXA_3531dn	GAGGAGTGCACCGTCTTACG	SCA125719

HEXA	3532	3533	HEXA_3532up	CAGGGGTCAGCTCAAGTCAC	SCA125719
HEXA	3533	3532	HEXA_3533dn	TAGGTGGCTACTGGGTCTGC	SCA125719
HEXA	3538	3539	HEXA_3538up	CAGTTCCACATGGTGTATGAAGA	SCA125719
HEXA	3539	3538	HEXA_3539dn	CCAAACTGCCATAGTTCAACC	SCA125719
HEXA	3652	3653	HEXA_3652up	GGAGAAGGGAACAGCTACCC	5'-end
HEXA	3653	3652	HEXA_3653dn	ATGAAGAAGCTGGACCCAAA	5'-end
HEXA	3654	3655	HEXA_3654up	GATCCGCTGGTGAAGGAATA	5'-end
HEXA	3655	3654	HEXA_3655dn	TAATTCCCTGGTGAGGGATG	5'-end
HEXA	3656	3657	HEXA_3656up	AAGCAACCTAGGTGCATCGT	5'-end
HEXA	3657	3656	HEXA_3657dn	GTGTGTTGCCATTTCCTCCT	5'-end
HEXA	3658	3659	HEXA_3658up	AGGAGGAAATGGCAACACAC	5'-end
HEXA	3659	3658	HEXA_3659dn	AGGAAACGGGCTCTGAGAAT	5'-end
HEXA	3660	3661	HEXA_3660up	GGCATAGAAGACTTGGGTTCA	5'-end
HEXA	3661	3660	HEXA_3661dn	CTGGGCATATCTCCTTGGAA	5'-end
HEXA	3662	3663	HEXA_3662up	CCAGGGCTAGGAATACAGCA	5'-end
HEXA	3663	3662	HEXA_3663dn	TCCACCCCATGGACTATACA	5'-end
HEXA	3801	3802	HEXA_3801up	ATGCCTCTCCTAGCTCACCA	3'-end
HEXA	3802	3801	HEXA_3802dn	CGAGCTCAGCTTTCCCTATG	3'-end
HEXA	3833	3834	HEXA_3833up	TGAACTGAACAGCACCATGA	3'-end
HEXA	3835	3836	HEXA_3835up	TGCTCCATCATCAAGTCCTG	3'-end
HEXA	3837	3836	HEXA_3837up	CCCCCATTAAAGGACAAAGA	5'-end
HEXA	3838	3839	HEXA_3838up	AGCTCTAGCAAGATGCATGACT	5'-end

PCR Primers for bovine *PARP6*

Gene	Primer	Pair	Lab-name	Sequence	Region
PARP6	3490	3491	HEXA_3490up	ATGCGCTTCCTGCCATAAG	SCA184260
PARP6	3491	3490	HEXA_3491dn	GTCCCCAACCGTGGTTTC	SCA184260
PARP6	3492	3493	HEXA_3492up	TTCTTCCCCTTCTCCTCAGC	SCA3547
PARP6	3493	3492	HEXA_3493dn	GCCACCAGACTGACTTACACC	SCA3547
PARP6	3494	3495	HEXA_3494up	GCCTTTGAAGACTCATGTTTGA	SCA3547
PARP6	3495	3494	HEXA_3495dn	ACCCCAAAGAGCCTAATGTC	SCA3547
PARP6	3534	3535	HEXA_3534up	TTTCTGACATGTGCCCAAGA	PARP6
PARP6	3535	3534	HEXA_3535dn	TTCATCCCACAAACTTGTTCC	PARP6
PARP6	3536	3537	HEXA_3536up	GTGAGTGATTGGCCACAAAG	PARP6
PARP6	3537	3536	HEXA_3537dn	AATTCAGGCTTGGAGACTGC	PARP6
PARP6	3615	3616	PARP_3615up	TCCCTGATCTCCCATTTCTG	SCA184260
PARP6	3616	3615	PARP_3616dn	GCACGTTCATCGCTAGCTT	SCA338507
PARP6	3618	3615	PARP_3617dn	GTGTGAGAATGGGTGGGAAT	SCA338507
PARP6	3746	3747	PARP_3746up	TCCCTGATCTCCCATTTCTG	E17
PARP6	3747	3746	PARP_3747dn	CAGACTTCCCCGACATGAGT	E18
PARP6	3748	3749	PARP_3748up	GGAGTCTTGTTCCCAATGCT	E6
PARP6	3749	3748	PARP_3749dn	TGATGGTTCTGCAAAGAGAAGA	E7
PARP6	3750	3751	PARP_3750up	CCCTTATTTCTTGTTTTTCATCA	E3
PARP6	3751	3750	PARP_3751dn	CCCCTACCATCCCTGAAGTA	E4
PARP6	3752	3753	PARP_3752up	GGAGAAATAACTGGGGATAAAGA	E2
PARP6	3753	3752	PARP_3753dn	TACTGTTTCCATGCCCCATC	E2
PARP6	3754	3755	PARP_3754up	CAGAAAGCAAGCAGCAACAA	E1
PARP6	3755	3754	PARP_3755dn	TCAAGCACAAAAGGCAGAAG	E1
PARP6	3756	3757	PARP_3756up	TGAAGAAACCAAGGCTCAAGT	E11

PARP6	3757	3756	PARP_3757dn	TTAGGGCATGAGTTTGATCC	E12
PARP6	3758	3759	PARP_3758up	AATGTGTTCATTTCTGCTACACAG	E13
PARP6	3759	3758	PARP_3759dn	GCCTGGTACTTCTCAGCCTTC	E14
PARP6	3760	3761	PARP_3760up	TGGGTGAATGAACTGGTTTG	E19
PARP6	3761	3760	PARP_3761dn	TACCCATCCTCATCCTCCAA	E20
PARP6	3762	3763	PARP_3762up	TTGGAGGATGAGGATGGGTA	E22
PARP6	3763	3762	PARP_3763dn	AATAGCTGGGAGGAGGAGGA	E22
PARP6	3764	3765	PARP_3764up	AGAGATAATGCCCCCTTGCT	E16
PARP6	3765	3764	PARP_3765dn	GGAGGAACTGGTGTGAGGTG	E17

Primers for direct sequencing of BAC DNA

BAC	Primer No	Lab-name	Sequence	Region
All	1658	SP6	TTTGCGATCTGCCGTTTC	vector pBACe3.6
All	1659	T7	CCGCTAATACGACTCACTATAGGG	vector pBACe3.6
909	3429	909_3429up	TGCTGCTGCTGCTAAGTCAC	909_T7
920	3430	920_3430up	TGGTTCCTCTGCCTTTCCTA	920_SP6
907	3431	907_3431up	CATTCTTGGCAACATGGCTA	907_SP6
909	3434	909_3434up	GGAAGGGCTTGAGACAGAGA	909_SP6
910	3435	910_3435up	AGGGAATAAGGCGATAGGG	910_SP6
907	3438	907_3438up	GCAGTCAGACGATGGGATTC	907_T7
910	3439	910_3439up	CCACTAGGGGGCTAGGGTAG	910_T7
910	3440	910_3440up	TGGATTTAGATCCTGGCTTCA	910_T7
920	3441	920_3441up	TACTTGGGGATGGGTGAGAA	920_T7
920	3442	920_3442up	TGATGGTACCGCTTGTCTTG	920_T7

Primers used for genotyping (hME), SNPid as assigned in the internal database.

SNPid	Lab_id	Forward PCR Primer	Reverse PCR Primer	Extension Primer
8	FS_8	ACGTTGGATGCATGGATGAATCTTGAGGAC	ACGTTGGATGTTGACTGTTCTGGATACCTC	GAGGACATTATATTAAGTGAA
246	FS_246	ACGTTGGATGTTCTCGCCAGTATGGATTCG	ACGTTGGATGCATATATGCGTGGAGTGTGG	GCTTTTTGAGCTCTGATGG
4	FS_4	ACGTTGGATGATTTCCAGGTCTCAGTCTGG	ACGTTGGATGCAACCTCCAAAACCCTCAAG	GTGCCCCTGTTTCTGACT
2	FS_2	ACGTTGGATGTTGACATATGTGGAGCCCAG	ACGTTGGATGGTTCCTCCAGACTTGAAGAG	GCCTCATGTGCCATGCTAGGC
7	FS_7	ACGTTGGATGAGAAGTCAACCTGGGAACAG	ACGTTGGATGATACTCACAGGGACAGTACC	TGCGGCTCAGGTAGTGA
414	FS_414	ACGTTGGATGCCAAACCTCTTCTCAGAATC	ACGTTGGATGGGCCTTGTAATCTTATATTGC	CACTTCAAAGGAGCCTTC
5	FS_5	ACGTTGGATGTTCCCCTCCTCCTACAGACTC	ACGTTGGATGAGGGCTGCACAGTACTGTGG	CTCCTACAGACTCACTGAAC
16	FS_16	ACGTTGGATGCTGTGTGGTTTGTTTACCAG	ACGTTGGATGCCTCATTCTCCTACTTACTG	TAGTTCAATGAAGACTCTGA
57	FS_57	ACGTTGGATGTGAGTAGGGAATGTGACCAG	ACGTTGGATGTGTGTGCACCTGTGTGATTG	ACTTCAGAGTTTTGCCTGAGCTT
48	FS_48	ACGTTGGATGCACTAAGTGGAATGAGCCTG	ACGTTGGATGGCTCATAGGTTGTAGTCAAG	TGGAATGAGCCTGAATTGTATATC
523	FS_523	ACGTTGGATGAAGCCCCTCCAGCATAAAAG	ACGTTGGATGCTTTCCGTTCTGCCAATGTC	GTAGTCTTCTGTCATATGCAAA
1	FS_1	ACGTTGGATGATCTGCCAAGTGGTGACATC	ACGTTGGATGAAGGAAGGAGGTGATGTAGG	AGTGGTGACATCAACTGTGA
211	FS_211	ACGTTGGATGACCTCATGGACTCTAGCATG	ACGTTGGATGAGTGTCACTGACTCAAATGG	TGACCTTCACCACCTCC
45	FS_45	ACGTTGGATGGCTCTCAACTGCTTCCAAAG	ACGTTGGATGTTTGACCTTTCTCCCTTCCG	CTGCTTCCAAAGGGCTGT
467	FS_467	ACGTTGGATGACCCACTCTACTCTCACTTC	ACGTTGGATGGAAGACCTGGAGGGATTTTG	TTCAACAGATGCTCAACTCT
602	FS_602	ACGTTGGATGGGTTTTGTGGTTAAGAAACC	ACGTTGGATGATATAGGAGGCCCCTATAAG	TTAAGAAACCTGCTCTCTTG
486	FS_486	ACGTTGGATGATCATGCTTTCCCTGAAGGG	ACGTTGGATGCAGCAATTGTCAGCAACACC	TTCCCTGAAGGGTCCAGGA
506	FS_506	ACGTTGGATGGGCTCTTGATGGATTTTCAG	ACGTTGGATGGTGACCTTGATTCTTCTGGG	GATTTTCAGCAACCCTTGGGTAGA
517	FS_517	ACGTTGGATGGGAAGCTTCTGGAAGTTCTG	ACGTTGGATGATGGAGGGACCAGAACTCTG	TGGCCTGGGAGACTTTG
544	FS_544	ACGTTGGATGCTGCAAGAGAGAAGTGTGTC	ACGTTGGATGATCATGCAGTGAGATGACCC	CAAGAGAGAAGTGTGTCTGTGCC
685	FS_685	ACGTTGGATGTAAGTCGCTTCAGTCGTGTC	ACGTTGGATGTCTTCCCTAGAGAATCCCAG	GCGACCCCATAGACGGCAG
17	FS_17	ACGTTGGATGTGGGCCAAAGCAGAAATAAC	ACGTTGGATGGTAGAGGTAGTTCTCAGTTC	ATAACATTCAGTTGTGGATGT
13	FS_13	ACGTTGGATGCTCTAGGTTTAGCAAGTTTGC	ACGTTGGATGTCCCTCCTAGGTTCTTAAAG	TTGCAAAAACATAATAAAAATTAA
619	FS_619	ACGTTGGATGTAGTTTTCTTTGGCGTTCCC	ACGTTGGATGAATAGATTCCAAGAAGGCAG	TTCCCACTTCTCCCTTAAT
661	FS_661	ACGTTGGATGAACAAAAGGCCTCCACATCC	ACGTTGGATGTTTGGTGCCAGTTGAAAGCC	CTCCCTGTAAGGTCTTTTAGAT
9	FS_9	ACGTTGGATGCAATGTAATCCAGACAACTG	ACGTTGGATGCTTTGTGAGTTTTATGATCAAC	GGTATGTATATATATTATACATA
609	FS_609	ACGTTGGATGTTTGATACCGGGAGAAGCAG	ACGTTGGATGAGGAAAGGCCTTTCTAGGGC	TGGAGGGCTGCCAGTGGCCC
633	FS_633	ACGTTGGATGAGTAGCTGAACTCATGGTGC	ACGTTGGATGCTAAACCGTACAATACTGGC	AGGTTAAATGCTTTAGTGAGGACA
701	FS_701	ACGTTGGATGAGGCAGAATTGCCATAGTGG	ACGTTGGATGATCACAGCTATTGAGCCCTC	CCATAGTGGGCATCAAGA
706	FS_706	ACGTTGGATGATGGATAGTGTGCCCATTCC	ACGTTGGATGTGAGAGAGTGCTGATCAGTC	TGCCCATTCCTTTCATTTC
719	FS_719	ACGTTGGATGTTAACACACCATTTAGTCAC	ACGTTGGATGTGTTCGTGTATATACACATG	ACCATTTAGTCACATATACAC
740	FS_740	ACGTTGGATGATGCACACACACGTCTTCTG	ACGTTGGATGTAGAATCCAAGGTCTGGATG	CTTCTGGCTCTGTCTGCT
14	FS_14	ACGTTGGATGGCTAATTTAAGAGTATAATGCC	ACGTTGGATGTAATTAGCACAGTTTGCAAG	AAGAGTATAATGCCATCCAA
589	FS_589	ACGTTGGATGTTCACTTGGGCCCTGTTCCG	ACGTTGGATGGTTGCTCACATTACTGTGGG	CTGCGCTGCCTCCCGCG

696	FS_696	ACGTTGGATGAGCATCCCGACCTGTTCATG	ACGTTGGATGAAGCAATGCTGGAGAGGTGG	TCCCTGTTCTGTCCTGCCC
567	FS_567	ACGTTGGATGTGAATCTGGGCGATATGGAG	ACGTTGGATGGTTCTGGATGTTGAAGCCTG	ATGGAGCTGGGAGAGAG
36	FS_36	ACGTTGGATGAACTAGAATCAGAGAAGTCC	ACGTTGGATGCTGTGTGAGCCTCGATCCTG	GACTCCATAGGAACTGTAG
418	HEXA_418	ACGTTGGATGTCTAACTCCAGATGGCCTGC	ACGTTGGATGTTGACGGTTCTGGGCACTTG	GCCTGCCCTCAGTTGCA
400	HEXA_400	ACGTTGGATGGGACCAGGAAAGATTCTCTC	ACGTTGGATGATATAGGAGAAGGGCACTGG	GAAAGATTCTCTCCTGAAG
417	HEXA_417	ACGTTGGATGAGTTATCTGCTCAGAGATGG	ACGTTGGATGCTGTCTAATTTACTGGGTGAC	GCTCAGAGATGGTAAGGAACTC
276	HEXA_276	ACGTTGGATGAAGCAAAGACAGGCCTCAAC	ACGTTGGATGAGGAGAGTTGGACATTTCAG	TCTCCTCAGGCCATTTCAT

Primers used for genotyping (Iplex assays)

	SNPid	Forward PCR Primer	Reverse PCR Primer	AMP_LEN	Extension Primer
Well1	ARIH_1000	ACGTTGGATGATTTAAGGTCACCCAGGTGC	ACGTTGGATGCCTGAGACTGAAAGGGAAAG	100	GGGGCACCCAGGTGCTAAAACAT
	ARIH_998	ACGTTGGATGTTCTTGGGCTCAATTCCCAG	ACGTTGGATGAGAACTATCACTGGACTGGC	115	GGGACTCAATTCCCAGAGATTGTTAT
	BRUN_956	ACGTTGGATGACAGTGCACGTTAGCATCAG	ACGTTGGATGAGAGGGCCAGCCTGTAATTC	108	CGATGATCCAGAAATGGTGGGATA
	BRUN_979	ACGTTGGATGTTGACCAATTCAGCCAGTCC	ACGTTGGATGAGGTTAGGCTCTGTTCTGAG	113	TGCAGCCAGTCCACCCCTATAAAGGTC
	HEXA_436	ACGTTGGATGGGAAAGAAGAGTCATCGACC	ACGTTGGATGGTGCTCTGACCTATAGGATG	100	GGTGGAAAACGTTGAATTTATTGTA
	HEXA_437	ACGTTGGATGGCTTCTGCATGGATACAGAG	ACGTTGGATGTCGGTTCATAGTGAGGGAAG	99	CTCTGAGCCCTGAGTG
	HEXA_439	ACGTTGGATGTGAGGCACAGAGAAGTTCAG	ACGTTGGATGAAGAGCACAGACAGTGAAGG	99	CCCCCCCAAAGTTGCTCACAACC
	HEXA_443	ACGTTGGATGGTGGAGAAGATGTTGCTGG	ACGTTGGATGGCCTGCAAGAGGATGTTAAG	101	GTCCTAAAACTAACTGCCTTC
	HEXA_458	ACGTTGGATGAGCAGCAGCGAAAACCTGAG	ACGTTGGATGTCACCTGACCAGGCTGTCAC	117	GATTGCCCGCCGGTCTGCTTCC
	HEXA_757	ACGTTGGATGCTAACTCTTTACCCACCTCC	ACGTTGGATGTGGCTAAATGTCTCCAGACC	93	CCTCCCTAAGTCTTTACATC
	HEXA_777	ACGTTGGATGGGCAGCTGTTCTCAAAATG	ACGTTGGATGTAAGACTCCTCTCTGCTGTC	110	ATCAAAATGTAAACTCTACACAGC
	HEXA_785	ACGTTGGATGTCTTCCTGTCAGGAAGGGAT	ACGTTGGATGTAAACGTCTGGCACACTTCC	109	ATCTGCCCATGGCCTGCT
	HEXA_822	ACGTTGGATGCCCAATGGATAAAACCCCAC	ACGTTGGATGGTGCTTTGGATATGGGCTTC	118	GGAGAAAAATTGCCAGCC
	HEXA_827	ACGTTGGATGCTAGGGCCTAAAATAATCCTG	ACGTTGGATGTGACAGGTGTTTCTGCCAAC	98	GAAGTTTAACAACATGTAACACATA
	HEXA_893	ACGTTGGATGCTTGAAAGCTAAGCTAAAG	ACGTTGGATGCCCATTACTCTTCAGCTAAG	107	AATTGTATTGAGATTTTATTCTTAAGT
	HEXA_899	ACGTTGGATGCCAAACTCCCTAACTATCCC	ACGTTGGATGAGAGAAAACAATCATATATA	100	TGACAAAAACAATGACACAGA
	HEXA_926	ACGTTGGATGCTTCATTACTGTCTTCCAAC	ACGTTGGATGAGTCATGCATCTTGCTAGAG	113	GTATTTACCTTTTCATTGCTACT
	HEXA_980	ACGTTGGATGGAGCAAGGAACTGAAGGATG	ACGTTGGATGTTCCAATCTCGTGGATACAG	117	TTTTCAGGCCTGCCTTCC
	HEXA_983	ACGTTGGATGAGTTCTTTTACCTGGTCCTG	ACGTTGGATGCCACGAGATTGGAAACTTTC	99	AAGGACAATGGAGAGGTACA
	HEXA_984	ACGTTGGATGAGACAAAGACAATTCCTAC	ACGTTGGATGCACTTATCAATCAGTACAGGC	110	GTTAGGAAAAAAAGTACATGAAATAC
	IREB_939	ACGTTGGATGATCCGTGAGCCAGCTTATAG	ACGTTGGATGGGCTTCCAAATTGCAGCTGA	102	TCCTTCATAATGAATAGAGACG
	IREB_940	ACGTTGGATGGCTGAATACTTTTCCAGTGC	ACGTTGGATGCCACTAAGTAACATTACAAG	103	AAAAACATCTCACATGAGTG
	LAMR_216	ACGTTGGATGCGGTGAACTCTCAAGTGTAG	ACGTTGGATGAGAGACCCTTGTGGCGTTAG	90	GTAGTGTGCTACACGAG
	PARP_995	ACGTTGGATGAGGCATGCAAGATGTGTGTG	ACGTTGGATGTGGGATTGTTTGAAGCGTGG	106	CGTGTGTGTATGGCTAACC
	PARP6_989	ACGTTGGATGATCCTCCTGGGAGTCCAAAG	ACGTTGGATGCAGAGGTTTTCGTTTTCCAC	115	CAAAGCCCCAGTTCAG
	PARP6_991	ACGTTGGATGGGGTACTGATACTGTTAGGC	ACGTTGGATGTGATCAAGGACAGGTGAAAG	117	TATTGTTTCCAACTTGACTAGTCTT
	PARP6_994	ACGTTGGATGGCAAGGCTGCTGCTTTGTTT	ACGTTGGATGCCTGTTGCTAGAGATGATCC	119	TTGTTGCTGCTTTGTTTAGGATG
Well2	ARIH_996	ACGTTGGATGCTAGGTTAATAAAACAACGG	ACGTTGGATGTGAAACATCAGTCGTACAGG	118	AAACAACGGTTTTTAATTTACTT
	BRUN_954	ACGTTGGATGGAATTACAGGCTGGCCCTCT	ACGTTGGATGATGCCTCAGCCCTCAAAATC	90	CCTGGCCCTCTGAGAGTAGC
	BRUN_975	ACGTTGGATGATTGCAGTTGCAGTTGGAGG	ACGTTGGATGGGTTAGGCTTCAGAATCTGG	99	TGGAGGGAATCTCTGA
	HEXA_419	ACGTTGGATGCTGGTGCGCATATAGTTCTG	ACGTTGGATGTTCAAGTTCAGGCGGTTCCC	114	TGTGGAGGTGCATTAGC
	HEXA_420	ACGTTGGATGAAAGCCACAGAGCCGAAGAG	ACGTTGGATGTCCAGTACCATCTCAGTTCG	117	AGGTCACGGTAGCGTTGGAAGGC
	HEXA_428	ACGTTGGATGTGCCCCTCCTAGCAATACAC	ACGTTGGATGTAATGCTCTGGCCTCTCTTC	116	GCAATACACTCACCCA
	HEXA_429	ACGTTGGATGAGACGTATCCAGCAACAAGC	ACGTTGGATGGGTCCACACAGTTCTATGTC	103	AGGGAAGTCCTCAATATC
	HEXA_782	ACGTTGGATGTTTACTGAAGATGAAGCGGG	ACGTTGGATGGGGATGTGACCGACACTTTA	105	CGCCCTCCAGTGAGCTG

	HEXA_796	ACGTTGGATGCCCTTGTCTCTAGGCACAG	ACGTTGGATGGGCCAGGCTTCCACTGCAC	94	CTAGGCACAGGGGCACGTGGGCAA
	HEXA_823	ACGTTGGATGTTGGATATGGGCTTCATCTG	ACGTTGGATGATCTCCCAATGGATAAAACC	117	AGGTGGTTACTGAAAATTGTG
	HEXA_824	ACGTTGGATGCTGTGAGAGGAAAATCACCG	ACGTTGGATGGGCCAATTATATCCACCACC	105	CCGATCACTTCCATAAGGAAA
	HEXA_828	ACGTTGGATGCTACGGCTTCAATCAACTAG	ACGTTGGATGGGAGTTAATAAAAGTCCAGG	118	CTCAAGAAAAGCAAAGAAGAA
	HEXA_900	ACGTTGGATGCTGCTAGGCTCTGAATATTT	ACGTTGGATGCTCTGACAAGTATATCAGAC	117	TTAATTGGACTCTGATGATTTTT
	LAMR_255	ACGTTGGATGCACCTCTCCTTAGAATCATAC	ACGTTGGATGTTTTCGCTAACGCCACAAGG	108	GACTATCCAACTCCACTCA
	PLG_369	ACGTTGGATGAGAGGGTAGGACGAATTGAG	ACGTTGGATGGCATCTTCCCACTAGTTATC	100	TAGCATTGACATATATACACTATTA
	PLG_371	ACGTTGGATGCCGGCGACAAGGATCAAATG	ACGTTGGATGTGTGTTCCAAAGGACGTGAC	94	CAAGGATCAAATGTGGCAG
Well3	ARIH_998	ACGTTGGATGTTCTTGGGCTCAATTCCCAG	ACGTTGGATGAGAACTATCACTGGACTGGC	115	GCTCAATTCCCAGAGATTGTTAT
	BRUN_974	ACGTTGGATGGACCCTGCTGAGTGCTATAC	ACGTTGGATGTTGTGAGGCTCCCTACAAAC	105	ATTGACTTCCGGAGGC
	BRUN_979	ACGTTGGATGTTGACCAATTCAGCCAGTCC	ACGTTGGATGAGGTTAGGCTCTGTTCTGAG	113	CTCCACCCCTATAAAGGTC
	HEXA_276	ACGTTGGATGAAGCAAAGACAGGCCTCAAC	ACGTTGGATGAGGAGAGTTGGACATTTCAG	106	CATCTCCTCAGGCCATTTCAT
	HEXA_459	ACGTTGGATGAAAGCCACAGAGCCGAAGAG	ACGTTGGATGTCCAGTACCATCTCAGTTCG	117	GGGTCGAGGACGGAGCAGCC
	HEXA_893	ACGTTGGATGCTTGAAAGCTAAGCTAAAG	ACGTTGGATGCCCATTACTCTTCAGCTAAG	107	TTGTATTGAGATTTTATTCTTAAGT
	HEXA_899	ACGTTGGATGCCAAACTCCCTAACTATCCC	ACGTTGGATGAGAGAAAACAATCATATATA	100	ACAAAAACAATGACACAGA
	HEXA_926	ACGTTGGATGCTTCATTACTGTCTTCCAAC	ACGTTGGATGAGTCATGCATCTTGCTAGAG	113	TATTTACCTTTTCATTGCTACT
	HSC70_377	ACGTTGGATGTGCTAAAGCCCTTAACACAC	ACGTTGGATGCAACTGGCTGGATAAGAACC	102	CACACAAAACCCCATAC
	HSC70_379	ACGTTGGATGTCCTTCCTTGGATGTCTGAG	ACGTTGGATGTGATGCAATCAACCTGACAC	99	TCTGAGTGACCCAAGA
	HSC70_387	ACGTTGGATGGCCAAACGACTTATTGGACG	ACGTTGGATGTCATTCACCACCATGAAGGG	103	CCTGATGATGCTGTTGTCCA
	HSC70_392	ACGTTGGATGACCCAAGTGAGTATCTCCAG	ACGTTGGATGGGGTGGCACTTTTGATGTGT	105	CTGTAGATTTGACCTCAAAGAT
	PARP6_988	ACGTTGGATGTTAGGTGATAGGGAATGAAG	ACGTTGGATGAGGAACAAAGCCTTTTGGTG	109	GAGAAGACTGAAAATCTCCTA
	PARP6_991	ACGTTGGATGTGATCAAGGACAGGTGAAAG	ACGTTGGATGGGGTACTGATACTGTTAGGC	117	ACAGGTGAAAGAACTGA
	PRP_1031	ACGTTGGATGAGGAGAGCTCCATTTACTCG	ACGTTGGATGGCTAGATTCCTACACACCAC	98	TGTACTCGGAATGTGGGC
	PRP_819	ACGTTGGATGAATTCCAACTCCTAGCTATC	ACGTTGGATGTAGGCCAAAGAGTTGGACAG	107	GCCTAGCTATCACGTCAA

11.3 Polymorphisms identified in the extended HEXA region by re-sequencing

All SNPs with flanking sequence are stored in the internal database. Description for the next tables: SNPid and Primers (see Appendix 11.2) as assigned in the internal database, location (I and E stands for intron and exon, respectively), breeds mean SNP in German Holstein (GH), German Fleckvieh (GF), German Brown (GB), UK Holstein (UK) and within the Bos Indicus breeds (BI). *SNP synon* stands for synonymous SNP.

Polymorphisms identified in the bovine HEXA gene (including 5'- and 3'-end)

		Primer		Bases			
SNPid	Location	Left	Right	A	B	Breeds	Polymorphism
276	I11	2999	3000	G	T	GH,GF	SNP
400	I6	2756	2757	T	C	GH,GF,BI,GB	SNP
401	I9	2712	2713	G	A	GB	SNP
402	I9	2712	2713	A	G	GH,GF,BI,GB	SNP
403	I6	2756	2757	A	G	BI	SNP
404	I6	2756	2757	T	C	BI	SNP
405	I9	2712	2713	A	G	GF,BI	SNP
406	I9	2712	2713	G	C	GF,BI	SNP
407	I9	2712	2713	C	T	BI	SNP
408	I9	2712	2713	C	T	GH,GF	SNP
413	I9	2712	2713	C	T	GF	SNP
415	I9	2712	2713	A	C	GF,BI	SNP
417	I13	3003	3004	C	T	GH,GF,BI,GB	SNP
418	I2	3014	3015	T	G	GH,GF,BI	SNP
419	5'-end	3059	3060	G	T	GF	SNP
420	E1	3078	3079	A	G	GF,BI,UK	SNP synon
421	I2	3014	3015	C	T	GH,GF	SNP
422	I2	3014	3015	G	A	GH,GF,BI	SNP
424	I3	3044	3045	C	T	GH,GF	SNP
428	I3	2990	2991	A	G	GH,GF,GB,BI	SNP
429	E5	2960	2961	A	G	GH,GF,GB,BI	SNP synon
430	I5	3007	3008	C	T	GH,GF,BI	SNP
436	E6	3009	3010	A	G	GH,GF,BI	SNP synon
437	I10	2997	2998	T	C	GH,GF,BI	SNP
438	I10	2997	2998	C	T	GH,GF	SNP
439	I13	3003	3004	A	G	GH,GF,BI	SNP
440	I13	3003	3004	T	C	GH,GF,BI	SNP
441	I13	3003	3004	A	G	GH,GF,BI	SNP
442	I13	3003	3004	T	C	GH GF,BI	SNP
443	I13	3003	3004	A	G	GH GF,BI	SNP
458	5'-end	3078	3079	A	G	GF,BI	SNP
459	E1	3126	3127	T	G	GF,BI,UK	SNP synon
460	I2	3114	3115	G	A	GF	SNP
470	I2	3114	3115	G	A	BI	SNP
471	I3	3044	3045	G	A	GF	SNP
555	I3	3044	3045	C	T	GH	SNP
751	I3	3005	3006	G	C	BI	SNP
752	I3	3046		C	G	BI	SNP
753	I3	3005	3006	T	C	BI	SNP

754	I3	3005	3006	G	A	GF,BI	SNP
755	I3	3005	3006	G	T	BI	SNP
756	I3	2990	2991	A	G	BI	SNP
757	I3	2994	2995	C	T	GF,BI,UK	SNP
758	I5	2962	2963	A	G	GF,BI	SNP
759	I5	2962	2963	G	A	GF,BI	SNP
760	I5	3007	3008	G	C	GF,BI	SNP
761	I5	3007	3008	A	G	GF	SNP
762	I5	3007	3008	G	A	GF,BI	SNP
763	I5	3007	3008	C	T	BI	SNP
764	I5	3007	3008	T	C	BI	SNP
765	I5	3007	3008	C	T	GF	SNP
766	I5	2962	2963	G	C	GH,GF	SNP
767	I5	2962	2963	A	C	BI	SNP
768	I10	2997	2998	G	T	BI	SNP
769	I10	2997	2998	T	C	GH,GF,BI	SNP
770	I10	2997	2998	T	C	GF,BI	SNP
771	I10	2997	2998	A	C	GF,BI	SNP
772	I10	2997	2998	G	A	BI	SNP
773	I12	3001	3002	G		BI	INDEL
774	I12	3001	3002	C	A	GF,BI	SNP
775	I12	3001	3002	A	G	GF,BI	SNP
776	I12	3001	3002	G	A	BI	SNP
777	I12	3001	3002	G	A	GF	SNP
778	I12	3001	3002	A	G	BI	SNP
779	I12	3001	3002	C	T	BI	SNP
780	I12	3001	3002	T	G	GH	SNP
781	I12	3001	3002	T	A	GH	SNP
782	I12	3001	3002	T	C	GH,GF	SNP
783	I12	3001	3002	C	T	GF	SNP
784	E13	3001	3002	C	T	BI	SNP synon
785	I13	3003	3004	G	A	BI	SNP
786	I13	3003	3004	C	T	BI	SNP
787	I13	3003	3004	T	G	BI	SNP
788	I13	3003	3004	G	A	BI	SNP
789	I13	3003	3004	T	G	BI	SNP
790	I13	3003	3004	A	G	BI	SNP
791	I13	3003	3004	A	G	GH,GF,BI	SNP
792	3’-end	3061	3062	C	T	GF,BI	SNP
793	3’-end	3061	3062	T	C	GF,BI	SNP
794	3’-end	3130	3129	T	G	GF,BI	SNP
795	3’-end	3130	3129	G	C	BI	SNP
796	3’-end	3130	3129	C	T	GF,GH	SNP
797	3’-end	3130	3129	A	C	BI	SNP
798	3’-end	3130	3129	C	T	BI	SNP
799	I13	3003	3004	A	G	BI	SNP
800	I13	3003	3004	A	G	BI	SNP
801	I13	3003	3004	G	T	BI	SNP
821	5’-end	3059	3060	C	T	BAC	SNP
822	I1	3261	3262	G	A	GF,UK,BI	SNP
823	I1	3261	3262	C	T	GF,UK,GH,BI	SNP
824	I1	3242	3243	T	C	GF,GH,BI	SNP
825	I1	3180	3181	C	T	BI	SNP
826	I1	3180	3181	G	A	BI	SNP

827	I1	3180	3181	G	A	GF,GH,BI	SNP
828	I1	3180	3181	A	G	GF,GH,BI	SNP
829	I1	3180	3181	C	T	BI	SNP
884	5’-end	3658	3659	A	G	BI	SNP
885	5’-end	3658	3659	T	G	GF,UK,BI	SNP
886	5’-end	3658	3659	C	T	GH,GF,UK,BI	SNP
887	5’-end	3658	3659	A	G	GH,GF,UK,BI	SNP
888	5’-end	3658	3659	G	A	UK,GF,BI	SNP
889	5’-end	3658	3659	A	G	BI	SNP
890	5’-end	3658	3659	C	T	GF,BI	SNP
891	5’-end	3658	3659	G	A	GH,GF,UK,BI	SNP
892	5’-end	3658	3659	C	G	BI	SNP
893	5’-end	3658	3659	G	C	GH,GF,UK,BI	SNP
894	5’-end	3660	3661	C	A	BI	SNP
895	5’-end	3660	3661	T	C	GH,UK	SNP
896	5’-end	3652	3653	G	A	BI	SNP
897	5’-end	3652	3653	C	T	BI	SNP
898	5’-end	3652	3653	A	C	BI	SNP
899	5’-end	3652	3653	A	G	UK,GH	SNP
900	5’-end	3652	3653	T	A	UK,BI	SNP
901	5’-end	3652	3653	T	C	BI	SNP
902	5’-end	3652	3653	G	A	UK,BI	SNP
903	5’-end	3284	3285	C	T	BI	SNP
904	5’-end	3284	3285	G	A	BI	SNP
905	5’-end	3255	3256	A	G	GF,BI	SNP
906	5’-end	3255	3256	A	T	GF,BI	SNP
907	5’-end	3255	3256	T	C	GF,BI	SNP
908	5’-end	3257	3258	A	G	GF,BI	SNP
909	5’-end	3257	3258	C		BI	INDEL
910	5’-end	3257	3258	G	A	GF,BI	SNP
911	5’-end	3257	3258	A	G	BI	SNP
912	5’-end	3257	3258	C	T	GF,BI	SNP
913	5’-end	3257	3258	A	C	BI	SNP
914	5’-end	3236	3237	C	T	GF,BI	SNP
915	5’-end	3236	3237	T	C	GF,BI	SNP
916	5’-end	3238	3239	G	A	BI	SNP
917	5’-end	3238	3239	T	C	GF,BI	SNP
918	5’-end	3238	3239	T	C	BI	SNP
922	5’-end	3654	3655	C	T	UK,BI	SNP
923	5’-end	3654	3655	T	C	UK	SNP
924	5’-end	3654	3655	C	T	UK	SNP
925	5’-end	3654	3655	A	G	BI	SNP
926	5’-end	3654	3655	C	G	UK	SNP
927	5’-end	3654	3655	T	A	UK	SNP
928	5’-end	3654	3655	G	T	UK	SNP
929	5’-end	3654	3655	G	C	UK	SNP
930	5’-end	3654	3655	C	T	UK	SNP
931	5’-end	3654	3655	C		UK	INDEL
932	5’-end	3654	3655	A	T	UK	SNP
933	5’-end	3654	3655	T		UK BI	INDEL
934	5’-end	3654	3655	C	A	UK	SNP
950	I1	3261	3262	G	T	GF,BI	SNP
951	I1	3278	3279	T	C	GF,GH,BI	SNP
952	I1	3278	3279	A	G	GF,GH,BI	SNP

953	I1	3278	3279	C	T	GF,GH,BI	SNP
980	3’-end	3801	3802	G	A	UK	SNP
981	I13	3003	3004	T	C	BI	SNP
982	I13	3013	3004	G	A	GF	SNP
983	3’-end	3801	3802	G	A	UK	SNP
984	3’-end	3482	3483	A	T	GH,GF,UK	SNP
985	3’-end	3482	3483	T	C	UK	SNP
986	3’-end	3482	3483	A	G	GF,UK	SNP
987	3’-end	3482	3483	C	TACT	GF,UK	INDEL
1005	I1	3500	3501	C	G	GH,GF	SNP
1006	I1	3500	3501	T	G	GH,GF	SNP
1007	I1	3500	3501	A	C	GH,GF	SNP
1008	I1	3500	3501	A	G	GH,GF	SNP
1009	I1	3500	3501	G		GH,GF	INDEL
1010	I1	3500	3501	T	C	GH,GF	SNP
1011	I1	3500	3501	A	G	GH,GF	SNP
1012	I1	3502	3503	T	C	UK,GF	SNP
1013	I1	3502	3503	C		UK	INDEL
1014	I1	3502	3503	A	G	GH,GF,UK	SNP
1015	I1	3502	3503	C	T	GH,GF,UK	SNP

Polymorphisms identified in the bovine ARIH1 gene (including 5’-end)

		Primer		Bases			
SNPid	Location	Left	Right	A	B	Breeds	Polymorphism
882	5'-end	3601	3602	A	C	GF	SNP
883	5'-end	3601	3602	G	A	UK	SNP
996	I1	3644	3645	T	G	UK	SNP
997	I1	3644	3645	A	G	BI	SNP
998	I1	3646	3647	C	A	GH,GF,UK	SNP
999	5'-end	3609	3610	G	A	UK,FV	SNP
1000	5'-end	3560	3561	A	C	GH,UK	SNP
1001	5'-end	3611	3612	A	T	BI	SNP
1002	5'-end	3611	3612	G	T	BI	SNP

Polymorphisms identified in the bovine PARP6 gene

		Primer		Bases			
SNPid	Location	Left	Right	A	B	Breeds	Polymorphism
988	I6	3748	3749	G	A	GH,GF,UK	SNP
989	I6	3748	3749	A	G	GH,GF,UK	SNP
990	I6	3534	3535	A	G	BI	SNP
991	I7	3536	3537	T	C	GH,GF,UK	SNP
992	I15	3487	3486	G	A	FV, BI	SNP
993	I15	3764	3765	T	A	BI	SNP
994	I15	3764	3765	G	T	GH,GF,UK	SNP
995	I17	3746	3747	G	A	GH,GF,UK	SNP

Polymorphisms identified in the bovine BRUNOL6 gene

SNPid	Location	Primer Left	Primer Right	Bases A	Bases B	Breeds	Polymorphism
954	I3	3539	3538	G	A	GH,GF,UK	SNP
955	I3	3539	3538	C	T	BI	SNP
956	I3	3539	3538	T	G	GH,GF,UK	SNP
957	I3	3596	3597	T	A	BI	SNP
958	I3	3596	3597	G	A	BI	SNP
959	I3	3596	3597	G	C	BI	SNP
960	I3	3596	3597	A		BI	INDEL
961	I3	3596	3597	C	T	BI	SNP
962	I3	3596	3597	T	C	BI	SNP
963	I3	3599	3600	T	C	BI	SNP
964	I3	3533	3532	G	T	FV,BI	SNP
965	I3	3533	3532	A	T	BI	SNP
966	I3	3531	3530	T	C	BI	SNP
967	I5	3531	3530	C	T	BI	SNP
968	E6	3531	3530	T	C	BI	SNP
969	I7	3531	3530	G	C	BI	SNP
970	E8	3531	3530	G	A	BI	SNPsynon
971	E8	3531	3530	C	T	BI	SNPsynon
972	I11	3184	3185	G	A	BI	SNP
973	I11	3184	3185	C	A	BI	SNP
974	I11	3184	3185	C	T	UK	SNP
975	I11	3184	3185	G	C	GH,GF,UK	SNP
976	I11	3184	3185	A		BI	INDEL
977	I11	3184	3185	A	G	FV,BI	SNP
978	I9	3529	3528	C	T	FV	SNP
979	I9	3529	3528	A	C	GH,GF,UK	SNP

11.4 Polymorphisms in additional candidate genes selected for genotyping

SNPid	Gene	Location	Primer Left	Primer Right	Bases A	Bases B	Breeds	Polymorphism
312	LAMR1	I4	2545	2546	T	C	GH,GF,GB,UK	SNP
337	LAMR1	I4	2545	2546	A	G	GH,GF,GB,UK	SNP
369	PLG	I11	2720	2721	T	C	GH,GF,GB,UK	SNP
371	PLG	I11	2720	2721	G	A	GH,GF,GB,UK	SNP
377	HSPA8	I8	2730	2731	G	A	GH	SNP
379	HSPA8	I8	2730	2731	C	T	GH,GB,UK	SNP
387	HSPA8	E3	2617	2618	G	A	GH,GB,UK	SNP synon
392	HSPA8	E5	2829	2830	A	G	GH,GB,UK	SNP synon
939	IREB2	E12	3223	3222	T	C	GH,GF,UK	SNP non synon*
940	IREB2	I21	3389	3390	C	G	GH,GF,UK	SNP

*Isoleucin / Threonin amino acid exchange

11.5 Genotyping results for the null-loci (UK Holstein animals)

Listed are the internal SNPid, the chromosomal location, the bases of the SNP, the genotypes for cases and controls, the allele frequencies for cases and controls, the trend value (Y2) obtained from Amitage Trend test and result of exact test for deviation from Hardy-Weinberg equilibrium: ok = p > 0.01

		Bases		Genotypes						Allele			
				Cases (+ve)			Controls (-ve)			frequency			
SNPid	Location	A	B	AA	AB	BB	AA	AB	BB	+ve	-ve	Y^2	HWE
1	4 q22-24	G	A	59	153	143	44	150	82	0.38	0.43	3.151	ok
2	10 q14-15	G	A	73	184	87	52	158	61	0.48	0.48	0.019	ok
4	1 q43-44	C	G	36	149	153	34	110	127	0.33	0.33	0.003	ok
5	7 q15-21	A	G	14	118	217	11	89	173	0.21	0.20	0.065	ok
7	9 q17-19	A	G	46	154	144	26	124	122	0.36	0.32	1.565	ok
8	5 q23-25	A	G	47	163	108	44	129	86	0.40	0.42	0.272	ok
9	4 q13-14			361			274			not polymorphic			
13	2 q33-36	C	T	4	50	294	5	49	211	0.08	0.11	2.565	ok
14	17 q24-26			26	318		22	241					9×10^{-123}
16	3 q31-32			7	351		3	272					5×10^{-171}
17	14 q23-24	A	G	24	130	195	23	92	156	0.26	0.25	0.000	ok
36	24 q21-22	T	G	80	179	96	63	125	87	0.48	0.44	0.538	ok
45	9 q11-12	G	A	32	168	155	22	136	118	0.33	0.33	0.001	ok
48	16 q12-13	T	C	54	166	130	43	108	122	0.39	0.36	1.606	ok
57	23 q12-13	G	C	56	178	117	58	128	87	0.41	0.45	1.434	ok
211	20 q11			unspecific calling									
414	X q26-31	G	A	60	215	76	60	151	64	0.48	0.49	0.359	ok
467	20 q21-23	A	G	19	132	203	13	97	165	0.24	0.22	0.479	ok
486	6 q11	C	T	23	157	172	20	92	160	0.29	0.24	3.318	ok
506	3 q11	C	T	53	173	121	54	112	97	0.40	0.42	0.316	ok
517	16 q13-14	G	A	66	176	109	56	122	87	0.44	0.44	0.009	ok
523	1 q11	C	T	55	179	121	48	120	106	0.41	0.39	0.211	ok
544	19 q12	C	T	78	173	102	44	145	81	0.47	0.43	1.520	ok
557	21 q12-14			unspecific calling									
567	11 q11-12	T	G	6	87	261	5	79	192	0.14	0.16	1.163	ok
589	12 q21	G	A	45	177	130	49	111	112	0.38	0.38	0.031	ok
602	18 q21-24	C	T	32	148	173	32	130	180	0.30	0.36	4.922	ok
609	29 q11-13	C	T	75	190	92	66	142	66	0.48	0.50	0.743	ok
619	25 q21-23	A	C	51	171	125	49	122	88	0.39	0.42	1.209	ok
633	1 q33-34	G	A	31	166	160	38	125	111	0.32	0.37	3.201	ok
661	28 q12-13	G	A	70	168	115	49	150	75	0.44	0.45	0.341	ok
685	21 q24			358			275			not polymorphic			
696	26 q13-14	C	T	1	352			272					9×10^{-185}
701	12 q12-13	G	A	44	177	131	34	120	116	0.38	0.35	1.089	ok
706	8 q23-24			unspecific calling									
719	2 q44-45	T	C	19	137	194	14	113	146	0.25	0.26	0.117	ok
740	18 q11	T	C	34	166	154	31	119	124	0.33	0.33	0.000	ok

11.6 Genotyping results for the null-loci (German Holstein animals)

Listed are the internal SNPid, the chromosomal location, bases of the SNP, the genotypes from halfsibs, sire genotype and inferred genotypes from maternal inherited alleles (MIA), allele frequencies, the trend value (Y2) obtained from Amitage Trend test and result of exact test for deviation from Hardy-Weinberg equilibrium: ok = p > 0.01

				Genotypes													
				Controls (-ve)							Cases (+ve)			Allele-Frequency			
		Bases		Halfsibs				MIA									
SNP	Location	A	B	AA	AB	BB	*Sire*	AA	AB	BB	AA	AB	BB	-ve	+ve	Y^2	HWE
1	4 q22-24	G	A	147	319	162	*AG*	70	154	85	15	29	21	0.48	0.45	0.203	ok
2	10 q14-15	G	A	153	304	161	*AG*	75	157	83	15	29	15	0.49	0.50	0.064	ok
4	1 q43-44	C	G	124	308	184	*CG*	50	148	110	7	29	23	0.40	0.36	0.609	ok
5	7 q15-21	A	G	57	318	244	*AG*	11	92	198	1	20	44	0.19	0.17	0.029	ok
7	9 q17-19	A	G		227	391	*GG*	83	287	247	2	28	20	0.37	0.32	2.026	ok
8	5 q23-25	G	A		288	302	*AA*	141	295	155	15	26	15	0.49	0.50	0.057	ok
9	4 q13-14			not polymorphic													
13	2 q33-36	C	T	93	313	224	*CT*	27	131	158	7	23	35	0.29	0.28	0.04	ok
14	17 q24-26	G	C	55	445	82					4	48	5				2×10^{-45}
16	3 q31-32	C	T		624	13						60	6				3×10^{-179}
17	14 q23-24	A	G	202	426		*AA*	65	274	289	16	31	19	0.32	0.48	12.76	ok
36	24 q21-22	T	G		243	388	*GG*	94	299	239	3	26	36	0.39	0.25	9.73	ok
45	9 q11-12	G	A	279	343		*GG*	125	308	189	16	29	22	0.45	0.46	0.02	ok
48	16 q12-13	T	C	92	323	210	*CT*	28	128	146	4	18	43	0.30	0.20	5.59	ok
57	23 q12-13	C	G		199	429	*GG*	63	272	293	6	31	29	0.32	0.33	0.044	ok
211	20 q11			unspecific calling													
414	X q26-31	G	A	285	343		*GG*	129	311	187	11	35	21	0.45	0.43	0.398	ok
467	20 q21-23	A	G	106	301	217	*AG*	35	142	146	10	32	23	0.33	0.40	2.49	ok
486	6 q11	C	T		141	489	*TT*	32	219	380	3	18	45	0.22	0.18	1.22	ok
506	3 q11	C	T	131	300	177	*CT*	56	151	102	14	34	12	0.43	0.52	3.46	ok
517	16 q13-14	G	A	157	334	135	*AG*	62	145	84	17	30	17	0.46	0.50	0.59	ok
523	1 q11	T	C	161	270	181	*CT*	76	170	96	21	21	21	0.47	0.50	0.35	ok
544	19 q12	C	T		239	391	*TT*	90	296	242	7	26	32	0.38	0.31	2.57	ok
557	21 q12-14			unspecific calling													
567	11 q11-12	T	G		82	550	*GG*	11	143	479		10	55	0.13	0.08	3.01	ok
589	12 q21	G	A		225	409	*AA*	79	290	264	11	23	31	0.35	0.35	0.04	ok
602	18 q21-24	T	C	121	305	203	*CT*	45	152	127	14	24	26	0.37	0.41	0.47	ok
609	29 q11-13	C	T	269	368		*CC*	114	311	213	11	24	26	0.42	0.38	1.51	ok
619	25 q21-23	A	C	129	204	132	*AC*	64	130	67	12	25	19	0.49	0.44	1.17	ok
633	1 q33-34	A	G	119	324	191	*AG*	46	147	118	11	28	27	0.38	0.38	0.012	ok
661	28 q12-13	G	A	156	311	164	*AG*	76	160	84	8	36	22	0.49	0.39	3.92	ok
685	21 q24			not polymorphic													
696	26 q13-14			unspecific calling													
701	12 q12-13	G	A		248	389	*AA*	97	303	238	16	22	28	0.39	0.41	0.19	ok
706	8 q23-24			unspecific calling													
719	2 q44-45	T	C	100	318	217	*CT*	32	137	149	7	25	34	0.32	0.30	0.20	ok
740	18 q11	T	C	104	331	199	*CT*	36	137	131	5	34	27	0.34	0.33	0.049	ok

11.7 Genotyping results for the null-loci (Fleckvieh, Brown and Holstein)

Listed are the internal SNPid, the chromosomal location, both alleles of the SNP (A and B), genotypes, allele frequencies and result of exact test for deviation from Hardy-Weinberg equilibrium: ok = $p < 0.01$, dev = $p > 0.01$ for German Fleckvieh (GF), German Brown (GB) and German Holsteins (GH).

SNP	Location	Alleles		Genotypes									Allele Frequencies			HWE
				GF			GB			GH						
		A	B	AA	AB	BB	AA	AB	BB	AA	AB	BB	GF	GB	GH	
1	4 q22-24	G	A	4	11	16	9	8	14	4	16	9	0.31	0.42	0.41	ok
2	10 q14-15	G	A	10	14	6		2	28	4	11	10	0.57	0.03	0.38	ok
4	1 q43-44	C	G	2	10	19		1	26	8	10	6	0.23	0.02	0.54	ok
5	7 q15-21	A	G		14	17		5	24	3	6	19	0.23	0.09	0.21	ok
7	9 q17-19	A	G		7	23	10	13	8	5	10	11	0.12	0.53	0.38	ok
8	5 q23-25	A	G	7	8	11		11	13	8	8	5	0.42	0.23	0.57	ok
9	4 q13-14					31			31			29		not polymorphic		
13	2 q33-36	C	T	1	11	16		2	29	6	9	14	0.23	0.03	0.36	ok
14	17 q24-26	C	G	3	27		8	23		3	25					dev
16	3 q31-32	T	C	1	27			31		7	22					dev
17	14 q23-24	A	G		8	22		10	21	12	10	7	0.13	0.16	0.59	ok
36	24 q21-22	T	G	5	12	14	2	15	13	2	11	16	0.35	0.32	0.26	ok
45	9 q11-12	G	A	5	15	10	3	12	16	5	16	8	0.42	0.29	0.45	ok
48	16 q12-13	T	C	1	2	28	3	21	5	4	14	10	0.06	0.47	0.39	ok
57	23 q12-13	G	C	1	11	19	1	7	21	22	4	2	0.21	0.16	0.86	ok
211	20 q11							unspecific calling								
414	X q26-31	G	A	6	17	8	6	19	4	4	10	14	0.47	0.53	0.32	ok
467	20 q21-23	A	G	4	9	17	2	5	24	2	12	15	0.28	0.15	0.28	ok
486	6 q11	C	T	8	11	12	10	15	6	3	10	15	0.44	0.56	0.29	ok
506	3 q11	C	T	18	8	2	7	17	7	7	11	7	0.79	0.50	0.50	ok
517	16 q13-14	G	A	5	15	11	18	11	2	2	16	9	0.40	0.76	0.37	ok
523	1 q11	C	T	8	12	11	21	8	2	5	13	11	0.45	0.81	0.40	ok
544	19 q12	C	T	5	21	5		15	16	2	15	11	0.50	0.24	0.34	ok
557	21 q12-14							unspecific calling								
567	11 q11-12	T	G		4	27	2	10	19		5	24	0.06	0.23	0.09	ok
589	12 q21	G	A	8	14	9		12	19	1	14	13	0.48	0.19	0.29	ok
602	18 q21-24	C	T	3	15	13	3	10	18	7	19	2	0.34	0.26	0.59	ok
609	29 q11-13	C	T	19	11	1	17	13	1	4	14	11	0.79	0.76	0.38	ok
619	25 q21-23	A	C	7	13	7	19	11	1	7	12	9	0.50	0.79	0.46	ok
633	1 q33-34	G	A	3	11	17	17	12	2	10	13	6	0.27	0.74	0.57	ok
661	28 q12-13	G	A	3	12	16	7	11	13	3	16	10	0.29	0.40	0.38	ok
685	21 q24													not polymorphic		
696	26 q13-14				31			31			28					dev
701	12 q12-13	G	A	2	10	19	5	15	11	6	18	5	0.23	0.40	0.52	ok
706	8 q23-24							unspecific calling								
719	2 q44-45	T	C	1	8	19	6	11	14	1	14	14	0.18	0.37	0.28	ok
740	18 q11	T	C	5	13	12	3	21	7	1	12	15	0.38	0.44	0.25	ok

11.8 Genotyping results for the candidate SNPs (UK Holstein animals)

Listed are the locus, the internal SNPid, the intra-gene position (Pos), the rare and frequent allele (A and B, respectively), the number of genotypes in BSE cases and controls, the allele frequencies, the trend value (Y^2), obtained from Armitage Trend test, the corresponding p-Value, the permutated p-Value and the p-value for the HWE test (Exact test).

SNP		Al-leles		Genotypes Cases (+ve)			Genotypes Controls (-ve)			Frequency p(A)					
	Pos	A	B	AA	AB	BB	AA	AB	BB	+ve	-ve	Y^2	p-value	Permp-value	HWE p-value
ARIH1															
996	I1	G	T		83	265	1	54	213	0.12	0.1	0.74			$3x10^{-3}$
1000	Up	C	A		19	324		15	256	0.03	0.03	0.00			$<1x10^{-1}$
HEXA															
926	Up	G	C	1	97	253	8	56	208	0.14	0.13	0.20			$<1x10^{-1}$
893	Up	C	G	9	140	200	9	93	170	0.23	0.2	1.00			$<1x10^{-1}$
419	Up	T	G	7	141	201	9	87	164	0.22	0.2	0.81			$5x10^{-3}$
458	Up	G	A	6	129	187	10	92	157	0.22	0.22	0.01			$5x10^{-3}$
459	E1	G	T	8	145	200	9	92	168	0.23	0.2	1.13			$3x10^{-3}$
824	I1	C	T	37	170	145	37	117	112	0.35	0.36	0.21			$<1x10^{-1}$
822	I1	A	G	1	91	249	7	55	208	0.14	0.13	0.20			$<1x10^{-1}$
823	I1	T	C	35	173	145	30	119	112	0.34	0.34	0.00			$<1x10^{-1}$
827	I1	C	G	31	170	139	36	116	113	0.34	0.35	0.25			$<1x10^{-1}$
828	I1	G	A	35	172	143	36	117	113	0.35	0.36	0.13			$<1x10^{-1}$
418	I2	G	T	15	146	188	26	102	146	0.25	0.28	2.17			$<1x10^{-1}$
428	I3	G	A	17	145	183	24	99	140	0.26	0.28	0.63			$<1x10^{-1}$
757	I4	T	C	1	92	259	7	54	212	0.13	0.12	0.23			$<1x10^{-1}$
429	E5	G	A	1	97	257	6	55	206	0.14	0.13	0.55			$<1x10^{-1}$
436	E6	G	A	15	136	182	26	92	144	0.25	0.27	0.98			$<1x10^{-1}$
400	I6	T	C	84	174	94	60	132	76	0.49	0.47	0.21			$<1x10^{-1}$
437	I10	T	C	12	147	184	25	95	145	0.25	0.27	0.95			$<1x10^{-1}$
276	I11	T	G		10	340		27	246	0.01	0.05	13.6	$2x10^{-4}$	$2x10^{-3}$	$<1x10^{-1}$
777	I12	A	G	1	91	240	7	53	204	0.14	0.13	0.46			$<1x10^{-1}$
782	I12	T	C		53	305	3	39	224	0.07	0.08	0.44			$<1x10^{-1}$
785	I13	A	G	1	93	250	7	54	208	0.14	0.13	0.37			$<1x10^{-1}$
439	I13	G	A	5	72	269	8	65	193	0.12	0.15	2.88			$<1x10^{-1}$
417	I13	C	T	15	140	184	25	100	145	0.25	0.28	1.84			$<1x10^{-1}$
443	I13	G	A	15	148	182	26	96	144	0.26	0.28	0.64			$<1x10^{-1}$
796	3'end	T	C		10	347		26	243	0.01	0.05	13.3	$3x10^{-4}$	$5x10^{-3}$	$<1x10^{-1}$
983	3'end	A	G	2	53	292	1	30	239	0.08	0.06	2.38			$<1x10^{-1}$
980	3'end	A	G	16	139	184	24	97	144	0.25	0.27	0.71			$<1x10^{-1}$
984	3'end	T	A	17	141	178	27	95	145	0.26	0.28	0.52			$<1x10^{-1}$
BRUNOL6															
954	I3	A	G		54	303	2	42	224	0.08	0.09	0.45			$<1x10^{-1}$
956	I3	G	T		13	330		26	245	0.02	0.05	8.57	$3x10^{-3}$	$5x10^{-2}$	$<1x10^{-1}$
979	I9	C	A	8	100	237	8	85	173	0.17	0.19	0.86			$<1x10^{-1}$
974	I11	T	C	8	111	237	8	91	173	0.18	0.2	0.73			$<1x10^{-1}$
PARP6															
988	I6	G	A	53	191	109	34	122	115	0.42	0.35	6.70	$1x10^{-2}$	$3x10^{-1}$	$<1x10^{-1}$
989	I6	G	A	18	153	183	10	94	167	0.27	0.21	5.80	$2x10^{-2}$	$4x10^{-1}$	$<1x10^{-1}$

994	I15	T	G	1	37	300		20	247	0.06	0.07	2.67			$<1x10^{-1}$
995	I17	A	G	12	107	228	7	58	203	0.19	0.13	6.33	$1x10^{-2}$	$2x10^{-1}$	$<1x10^{-1}$
PLG															
369	I11	C	T	18	135	201	8	93	166	0.24	0.2	2.57			$<1x10^{-1}$
371	I11	A	G	27	143	186	12	102	152	0.28	0.24	2.58			$<1x10^{-1}$
LAMR1															
255	I4	A	G	31	146	178	25	132	111	0.29	0.34	3.2			$<1x10^{-1}$
IREB2															
939	E12	C	T	13	101	230	3	74	189	0.15	0.18	2.54			$<1x10^{-1}$
940	I21	G	C	13	104	229	3	78	189	0.16	0.19	2.27			$<1x10^{-1}$
HSPA8															
377	I8	A	G		8	349		10	263	0.02	0.01	1.13			$<1x10^{-1}$
379	I8	T	C	18	148	188	23	97	151	0.26	0.26	0.03			$<1x10^{-1}$
392	E5	G	A	18	145	191	23	95	153	0.26	0.26	0.03			$<1x10^{-1}$

11.9 Genotyping results for the candidate SNPs (German Holsteins animals)

Listed are the locus, the internal SNP Id, the intra-gene position, the rare and frequent allele (A and B, respectively), the number of genotypes in the cases and in the control halfsibs, genotypes inferred from the maternal inherited alleles, the allele frequencies, the trend value (Y^2), obtained from Armitage Trend test

SNP	Pos	Alleles		Cases (+ve)			Controls Half-Sibs			Controls Inferred (-ve)			Frequency p(A)		Y^2
		A	B	AA	AB	BB	AA	AB	BB	AA	AB	BB	-ve	+ve	
ARIH1															
996	I1	G	T	1	5	60		34	599	2	63	565	0.05	0.05	0.00
1000	5'end	C	A	2	13	51		93	510	14	156	430	0.15	0.13	0.55
HEXA															
926	5'end	G	C		11	55		51	576	4	94	529	0.08	0.08	0.01
893	5'end	C	G	1	10	55		55	570	5	100	520	0.09	0.09	0.01
419	5'end	T	G		12	55		85	556	11	146	480	0.13	0.09	1.95
458	5'end	G	A	3	9	53		84	500	12	142	426	0.14	0.12	0.72
459	E1	G	T	1	8	56		83	541	11	144	469	0.13	0.08	3.29
824	I1	C	T	6	25	34		177	459	49	254	329	0.28	0.28	0.02
822	I1	A	G		9	56		50	552	4	90	504	0.08	0.07	0.26
823	I1	T	C	8	26	32		188	439	56	262	305	0.30	0.32	0.18
827	I1	C	G	6	27	32		174	423	50	245	297	0.29	0.30	0.04
828	I1	G	A	7	26	32		188	445	56	263	311	0.30	0.31	0.06
418	I2	G	T	5	27	34		183	452	53	262	321	0.29	0.28	0.05
428	I3	G	A	5	27	33		183	450	53	259	318	0.29	0.28	0.01
757	I4	T	C		9	59		50	589	4	92	540	0.08	0.07	0.21
429	E5	G	A		9	56		51	583	4	92	534	0.08	0.07	0.17
436	E6	G	A	5	26	33		143	459	34	218	347	0.24	0.28	1.14
400	I6	T	C	15	32	17		296	334	139	314	177	0.47	0.48	0.10
437	I10	T	C	5	28	34		176	462	49	254	332	0.28	0.28	0.02
276	I11	T	G	4	12	50		62	575	6	108	507	0.10	0.15	3.76
777	I12	A	G		9	57		48	551	4	88	504	0.08	0.07	0.25
782	I12	T	C		6	59		37	598	2	68	561	0.06	0.05	0.27

785	I13	A	G		9	55		50	556	4	90	508	0.08	0.07	0.19
439	I13	G	A	3	20	41		124	495	25	198	392	0.20	0.20	0.00
417	I13	C	T	4	28	34		183	451	53	261	320	0.29	0.27	0.16
443	I13	G	A	4	28	35		173	431	50	246	305	0.29	0.27	0.22
796	3’end	T	C	3	12	50		62	572	6	112	512	0.10	0.14	2.10
983	3’end	A	G		5	62		26	580	1	50	552	0.04	0.04	0.10
980	3’end	A	G	5	28	32		173	432	49	246	306	0.29	0.29	0.02
984	3’end	T	A	5	27	32		170	429	48	242	305	0.28	0.29	0.01
BRUNOL6															
954	I3	A	G		6	61		36	600	2	66	564	0.06	0.04	0.27
956	I3	G	T	3	12	49		54	546	5	98	493	0.09	0.14	3.24
979	I9	C	A	3	5	56		47	573	4	87	530	0.08	0.09	0.14
974	I11	T	C	1	4	59		38	590	2	71	554	0.06	0.05	0.35
PARP6															
988	I6	G	A	5	24	37		166	459	44	244	337	0.27	0.26	0.04
989	I6	G	A	1	10	55		63	574	6	112	515	0.10	0.09	0.07
994	I15	T	G			66		2	600		4	588	0.00	0.01	0.45
995	I17	A	G		11	55		59	547	6	105	492	0.10	0.08	0.26
PLG															
369	I11	C	T	4	11	47		111	522	19	183	430	0.17	0.15	0.36
371	I11	A	G	7	20	38		201	430	64	274	293	0.32	0.26	1.75
LAMR															
255	I4	A	G	16	31	19	288	340	0	132	312	184	0.46	0.48	0.17
IREB2															
939	E12	C	T	4	18	45		107	493	19	176	405	0.18	0.19	0.20
940	I21	G	C	4	19	43		106	496	19	175	409	0.18	0.20	0.62
HSPA8															
377	I8	A	G		3	64		24	606	1	46	583	0.04	0.02	0.85
379	I8	T	C	3	23	39		103	523	17	172	437	0.16	0.22	2.86
392	E5	G	A	4	24	37		105	522	18	175	435	0.17	0.25	4.94

11.10 Genotyping results for the candidate SNPs (Fleckvieh,Brown and Holstein)

Listed are the gene, the internal SNP Id, the intra-gene position, the rare and the frequent allele (A and B, respectively), the genotypes and allele frequencies for German Fleckvieh (GF), German Brown (GB) and German Holstein (GH) sequencing panel animals.

SNP	Loc	Alleles		GF			GB			GH			Frequency p(A)		
		A	B	AA	AB	BB	AA	AB	BB	AA	AB	BB	GF	GB	GH
ARIH1															
996	I1	G	T		1	30		1	22		2	21	0.02	0.02	0.04
1000	5'end	C	A	1	3	27			29	2	11	15	0.08	0.00	0.27
HEXA															
926	5'end	G	C		7	24		1	30		1	26	0.11	0.02	0.02
893	5'end	C	G		7	24		1	30		3	24	0.11	0.02	0.06
419	5'end	T	G	2	8	21		1	22		2	21	0.19	0.02	0.04
458	5'end	G	A	1	9	21			28		3	24	0.18	0.00	0.06
459	E1	G	T	2	8	19		1	30		3	24	0.21	0.02	0.06
824	I1	C	T	5	8	16		1	22		7	14	0.31	0.02	0.17
822	I1	A	G		5	26			29			28	0.08	0.00	0.00
823	I1	T	C	5	12	14		1	22		7	16	0.35	0.02	0.15
827	I1	C	G	5	12	14			28		10	18	0.35	0.00	0.18
828	I1	G	A	5	12	14		1	22		7	16	0.35	0.02	0.15
418	I2	G	T	5	10	14		1	30		8	20	0.34	0.02	0.14
428	I3	G	A	4	13	13		1	21		6	17	0.35	0.02	0.13
757	I4	T	C		5	26			31			28	0.08	0.00	0.00
429	E5	G	A		5	26		1	21		1	22	0.08	0.02	0.02
436	E6	G	A	3	11	17			29		7	21	0.27	0.00	0.13
400	I6	T	C	4	11	12	10	14	1	1	14	14	0.35	0.68	0.28
437	I10	T	C	5	11	14			31		8	20	0.35	0.00	0.14
276	I11	T	G	2	4	25			31		7	22	0.13	0.00	0.12
777	I12	A	G		6	25			29			28	0.10	0.00	0.00
782	I12	T	C		2	29			23		1	22	0.03	0.00	0.02
785	I13	A	G		5	26			29			28	0.08	0.00	0.00
439	I13	G	A	4	9	18			31		8	20	0.27	0.00	0.14
417	I13	C	T	5	10	14		1	29		8	20	0.34	0.02	0.14
443	I13	G	A	5	12	14			29		8	20	0.35	0.00	0.14
796	3'end	T	C	2	4	25			23		5	18	0.13	0.00	0.11
983	3'end	A	G			31			29			28	0.00	0.00	0.00
980	3'end	A	G	5	12	13			29		8	20	0.37	0.00	0.14
984	3'end	T	A	5	11	13			29		8	20	0.36	0.00	0.14
BRUNOL6															
954	I3	A	G		1	30			23		6	21	0.02	0.00	0.11
956	I3	G	T	2	4	24			28		7	21	0.13	0.00	0.13
979	I9	C	A	1	4	25			31		3	24	0.10	0.00	0.06
974	I11	T	C		2	29			31		3	24	0.03	0.00	0.06

PARP6															
988	I6	G	A	4	8	19	1	3	27		6	21	0.26	0.08	0.11
989	I6	G	A	1	3	27			31		3	25	0.08	0.00	0.05
994	I15	T	G		1	30			29			28	0.02	0.00	0.00
995	I17	A	G	1	3	27	1		29		1	27	0.08	0.03	0.02
PLG															
369	I11	C	T	1	12	18		6	17	2	5	16	0.23	0.13	0.20
371	I11	G	A	5	15	11	2	13	7	4	9	10	0.40	0.39	0.37
LAMR															
337	I4	G	A	5	17	9	13	9	1	8	12	3	0.44	0.76	0.61
IREB2															
939	E12	C	T		3	28		1	28	2	11	15	0.05	0.02	0.27
940	I21	G	C		3	28		1	28	2	11	15	0.05	0.02	0.27
HSPA8															
377	I8	A	G			31			31		5	22	0.00	0.00	0.09
379	I8	T	C		1	29	2	13	16		6	21	0.02	0.27	0.11
392	E5	G	A		2	29	2	13	16		6	21	0.03	0.27	0.11

Curriculum vitae

KATRIN JULING

Birthday	July 31, 1976
Birthplace	Offenbach / Main, Germany

EDUCATION AND STUDY

1982-1986	Primary school - Basic education in Eching, Munich, Germany
1986-1996	Secondary school - Dom-Gymnasium, Freising, Germany
1996	Abitur (German university-entrance diploma)
1996	three month stay in Berkeley, CA, USA
1997-2002	Study of Agricultural Science with focus on Animal Science at the Life Science Centre of the Technical University of Munich
2001	Instructor qualification
2002	Diploma thesis: *SNP-Analyse in Kandidatengenen für BSE-Prädisposition* (Chair of Animal Breeding, TUM)
2002	Diploma degree (Dipl. Ing. agr. Univ.)

POST GRADUATE EDUCATION

2002-2007	PhD position at the Chair of Animal Breeding of TUM Topic: BSE-Genetics
2005	Project leader qualification and delegate for bio safety (§§ 15 and 17 of *Gentechniksicherheitsverordnung*)
2007	Promotion (Dr.rer.nat.) Dissertation thesis: Genomic Characterisation, Polymorphism Analysis and Association studies of candidate genes for BSE susceptibility