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Thesis abstract

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Abstract (the text has to fit into the space below)

Cardiomyopathies are diseases of the myocardium that lead to cardiac dysfunction, heart failure, arrhythmia and sudden death. In human medicine, cardiomyopathies frequently warrant heart transplantation in patients at diverse age.

Bovine dilated cardiomyopathy (BDCMP) is a progressive degenerative heart muscle disorder that has been observed in the past thirty years in cattle of Canadian Holstein origin. BDCMP is a hereditary disease genetically related to the Holstein-Friesian sire "ABC Reflection Sovereign". In Switzerland the disease affects Swiss Fleckvieh and Red Holstein breeds. The age at onset of BDCMP vary from 2 to 4 years however, the disease has also been diagnosed in a vastly younger or older individuals. BDCMP is characterized by a global heart enlargement, ventricular remodelling leading to a chamber dilatation with reduced wall thickness and a decrease of systolic function. Ascites and pleural effusion together with chronic congestion in the liver and kidneys accompany the disease. Typical histological signs are myocardial degeneration, swollen mitochondria, initial cardiomyocyte necrosis and myocardial fibrosis.

A strong evidence for an autosomal recessive mode of inheritance for BDCMP has been proposed and thereafter confirmed by segregation analysis based on an experimental BDCMP pedigree. Nevertheless, other environmental factors such as gestation, parturition, lactation or obesity may play a role in the age at onset of the disease. Presently, BDCMP is sporadically diagnosed in the slaughterhouses in Switzerland. The frequency of the mutant allele causing the disease remains to be established.

A whole-genome scan using 199 microsatellite markers and one SNP revealed the location of the BDCMP-locus on bovine chromosome 18 (BTA18). The initial interval of interest on BTA18 was flanked by microsatellite markers *MSBDCMP06* and *BMS2785* and corresponded to a physical distance of 6.7 Mb. Based on the available sequence information from humans and cattle it has been shown that the homologous region of the initial interval matches to the human chromosome 19 (HSA19). Since the cardiac troponin I gene (*TNNI3*) was located in very close vicinity to the 6.7-Mb interval and its biological role is essential for the myocardial contractility, it was worth to investigating this functional candidate gene for BDCMP. The sequencing of *TNNI3* gene revealed a SNP in intron 6 (c.378+315G>A). A significant LOD score value of 3.37 was calculated for this transition. However, sequencing results of an extended pool of BDCMP-affected and BDCMP-unaffected animals revealed three possible genotypes. Thereby the c.378+315G>A variation was not in the perfect linkage disequilibrium with the disease status. Moreover, an expression analysis of the *TNNI3* gene by real-time PCR and Northern blotting experiments did not show significant differences in the *TNNI3* gene expression levels between BDCMP-cases and controls. In consequence, this gene was excluded as a candidate gene for BDCMP. A positional cloning procedure was further used to narrow down the region of interest. A combined approach of homozygosity mapping and association study was performed and thereby allowed us to limit the interval with the disease-locus to 1.0 Mb. This interval spanned between microsatellite markers *DIK3006* and *MSBDCMP51* is significantly associated with the disease. To identify potential recombinant events and reduce further the interval of question additional BDCMP-affected animals were genotyped with other SNP markers. Preliminary results allowed us to narrow down the critical interval to a physical distance of 240 kb. This region is encompassed by the vasodilator-stimulated phosphoprotein gene (*VASP*) and the radial spokehead-like 1 gene (*RSHL1*). The *VASP* gene was further investigated because of its role in heart function and development. Two SNPs in exon 7 (c.760C>A) and in intron 7 (c.830+310A>G) of this gene were identified but they were not in the perfect disequilibrium with BDCMP status. Therefore these variations could be excluded as being causative for BDCMP. In view of the fact that within the interval of interest no known heart specific candidate gene is located we expect to identify a novel function of one or several genes in the heart.

Positional cloning of the causative mutation for bovine dilated cardiomyopathy

Graduate School for Cellular and Biomedical Sciences

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PhD Thesis

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SUMMARY

Cardiomypathies are diseases of the myocardium associated with cardiac dysfunction. They lead to heart failure, arrhythmia and sudden death. Moreover, they are a main reason for heart transplantations in children. Four categories of cardiomyopathies, hypertrophic (HCM), dilated (DCM) and restrictive (RCM) cardiomyopathies and right ventricular dysplasia (ARVD) were evaluated according to morphological and functional criteria. The report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force defines DCM as a dilatation and impaired contraction of the left ventricle or both ventricles.

Bovine dilated cardiomyopathy (BDCMP) is a progressive degenerative disorder of the myocardium. BDCMP has been observed worldwide in cattle of Canadian Holstein origin over the last 30 years. It is a hereditary disease traced back genetically to the red factor carrier Holstein-Friesian bull “ABC Reflection Sovereign”. BDCMP affected animals are mostly 2 to 4 year old animals, however calves and old cows have also been diagnosed as BDCMP affected. Typical symptoms of the disease are a global heart enlargement, tachycardia, ventricular remodelling and as a consequence chamber dilatation with decreased wall thickness. Ascites and pleural effusion together with chronic congestion in the liver and kidneys accompany the disease. Histological hallmarks such as myocardial degeneration, swollen mitochondria, increased number of Z-bands, cardiomyocyte necrosis and further myocardial fibrosis are frequently found.

Segregation analysis has revealed strong evidence for an autosomal recessive mode of inheritance of the disease. Initially, a whole-genome scan using 199 microsatellite markers and one SNP revealed the position of the disease-causing locus on bovine chromosome 18 (BTA18). The previously fine-mapped interval on BTA18 corresponded to a physical distance of 6.7 Mb (Btau 3.1) and was flanked by

microsatellite markers *MSBDCMP06* and *BMS2785*. Based on available sequence information on humans and cattle it was shown that the homologous region of the interval of interest corresponds to the human chromosome 19 (HSA19). This is a gene-rich chromosomal fragment; however, none of these genes was considered to be an obvious candidate gene for BDCMP. Since the cardiac troponin I gene (*TNNI3*) was located in the close vicinity of the 6.7-Mb interval and its biological role is important in the contractile apparatus of the myocardium, it was worth to investigating it as a functional candidate for the disease. Moreover, a significant LOD score value of 3.37 was calculated for the SNP in intron 6 of the *TNNI3* gene justifying to scrutiny of this gene. The sequencing of this gene in a larger number of BDCMP-affected animals and control animals confirmed the presence of a single SNP in intron 6 (c378+315G>A). This transition was not associated with the BDCMP. Moreover, a real-time PCR *TNNI3* analysis together with a Northern blotting experiment did not reveal a significant difference in expression levels of *TNNI3* gene between examined groups of BDCMP-affected and BDCMP-unaffected individuals. Consequently the *TNNI3* gene was excluded as a candidate gene for BDCMP. No other obvious functional candidate genes led us to further narrow down the region of interest. A combined strategy of homozygosity mapping and association study was used and thereby allowed us to limit the interval to 1.0 Mb (Btau 4.0). It was demonstrated that the chromosomal region flanked by microsatellite markers *DIK3006* and *MSBDCMP51* is significantly associated with BDCMP. To explore potential recombinant individuals we genotyped additional SNP markers. By this means the crucial interval could be reduced to a physical distance of 240 kb (Btau 4.0). We expected to identify a novel function of a gene in the heart because no known heart specific candidate gene is located in the region of interest.

In conclusion we successfully reduced the critical interval to a size that enables the re-sequencing of the entire region.

INTRODUCTION

1. Cardiomyopathies

1.1 Historical background and classification of cardiomyopathies.

The conception of myocardial diseases has an important and evolving history. In the 1850s chronic myocarditis was the only described cause of heart muscle diseases. Initially, the term “primary myocardial disease” was introduced at the beginning of 1900. The term “cardiomyopathy” was introduced in 1957 (Abelmann, 1984). With the passage of time, an increased awareness and understanding of these diseases developed a number of definitions of cardiomyopathies. In 1968, the World Health Organisation (WHO) characterised these disorders as “diseases of different and often unknown aetiology in which the dominant feature is cardiomegaly and heart failure” (Abelmann, 1984). Later in 1980 the WHO report classified cardiomyopathies only as “heart muscle diseases of unknown cause”. Cardiomyopathies are a severe and heterogeneous group of diseases and according to the recent 1995 WHO report are defined as “diseases of the myocardium associated with cardiac dysfunction”. They are classified according to morphological and functional criteria into four categories: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic right ventricular dysplasia (ARVD) and restrictive cardiomyopathy (RCM) (Richardson *et al*. 1996). Many conditions characteristic for one form of a cardiomyopathy may progress into another condition. Cardiomyopathies were also divided into two groups, primary and secondary myocardial disorders, respectively. Nonetheless, as the aetiology of previously idiopathic diseases has been determined this difference has become tenuous (Elliott *et al*. 2008). Recently, an update of the

1995 WHO report has been recommended by the European Society of Cardiology Working Group on Myocardial and Pericardial diseases (Elliott *et al*. 2008) as well as by the American Heart Association (Maron *et al*. 2006). According to the European classification cardiomyopathy should be defined as “a myocardial disorder in which heart muscle is structurally and functionally abnormal in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease”. Moreover, a statement of the American Heart Association canters on the expanded classification of cardiomyopathies into a familial/genetic and non-familial/non-genetic type (Thiene *et al*. 2008).

1.2 Characteristics and genetic background of dilated cardiomyopathy in humans

Dilated cardiomypathy is characterized by dilatation of the left ventricle or both ventricles as well as by left ventricular systolic dysfunction (Richardson *et al*. 1996). Right ventricle dilatation and dysfunction can be present, however they are not essential for the diagnosis (Elliott *et al*. 2008). The disease has various causes like idiopathic, familial/genetic, viral and/or immune, alcoholic/toxic or associated with a cardiovascular disorder in which the degree of myocardial dysfunction is not explained by abnormal loading conditions or the extent of ischemic damage.

Idiopathic dilated cardiomyopathy (DCM) is the third most frequent cause of heart failure and shows left ventricular dilatation, impaired systolic function, increased myocardial mass and a reduction in ventricular wall thickness. Patients affected by DCM suffer from heart failure, arrhythmia and are at risk of premature death. Idiopathic DCM has in the United States a prevalence of one case per 2500 individuals with an incidence of 7/100000 per year. However, these features may be underestimated due to fact that many subjects remain asymptomatic until marked

ventricular dysfunction has occurred (Taylor *et al.* 2006). Idiopathic DCM is the most common form of cardiomyopathy and an indication for cardiac transplantation in children (Towbin *et al.* 2006). It is also the primary reason for heart transplantation in patients below the age of 40 years.

DCM presents different modes of inheritance. About one quarter to one third of DCM cases are inherited as autosomal dominant, autosomal recessive, X-linked and maternal patterns (Felker et al. 2000; Schönberger and Seidman 2001; Fatkin and Graham 2002). No less that 25% of DCM patients in Western populations present evidence for a familial type of the disease with an autosomal dominant mode of inheritance (Mestroni *et al*. 1999; Burkett *et al*. 2005). Familial dilated cardiomyopathy (FDC) is defined as dilated cardiomyopathy of unknown cause occurring in two or more closely related family members (Kushner *et al.* 2006). It is mainly caused by mutations in genes coding for cytoskeletal and sarcomeric proteins (Burkett and Hershberger 2005; Hughes and McKenna 2005; Taylor *et al.* 2006; Morimoto 2008). Murphy *et al*. (2004) reported a recessive mutation in the cardiac troponin I gene (*TNNI3*) leading to the DCM in human. X-linked cardiomyopathy is a rapidly progressive primary myocardial disease resulting in death or transplantation within 1-2 years. An early age at onset and congestive heart failure are characteristic for males while manifesting female carriers present with late onset and slow progression of heart failure (Berko and Swift 1987; Towbin *et al*. 1993). To date only mutations in the dystrophin gene (*DMD*) have been identified as causing this type of cardiomyopathy (Towbin and Bowles 2000). Rare forms of maternal inherited DCM are caused by mutations in the mitochondrial DNA (Suomalainen *et al*. 1992; Brackett *et al*. 1995).

1.3 Dilated cardiomyopathy in different species

Dilated cardiomyopathy is a frequent disease of the heart muscle and it also occurs in many species of animals. Hereditary forms of DCM were reported in hamster (Sakamoto *et al*. 1997; Escobales and Crespo 2008), turkey (Genao *et al*. 1996; Reed *et al*. 2007), pig (Hendrick *et al*. 1990), cat (Kittleson *et al*. 1999) sheep (Moainie *et al*. 2002), goat and cattle (Tontis *et al*. 1990; Tontis *et al*. 1992; Eschenhagen *et al*. 1995). A novel locus for juvenile DCM in Portuguese water dogs was mapped to a region of 3.9 Mb on canine chromosome 8 (Werner *et al*. 2008). The canine 3.9-Mb interval is homologous to human chromosome 14. However, the human counterpart lacks of known genes that are involved in any cardiomyopathies. Fifteen candidate genes for DCM in Newfoundland dogs were investigated by Wiersma *et al*. (2008). Examination of all coding sequences of these genes revealed no indication that they are involved in DCM.

Several studies have reported DCM causing human gene mutations in transgenic mouse models. Du *et al*. (2007) created a knock-in mouse model for cardiac troponin T (*TNNT2*) and found that Ca^{2+} desensitization of cardiac filament causes DCM. The imobendan, a positive inotropic agent, prevented cardiac enlargement, heart failure and sudden death by directly increasing the Ca^{+2} sensitivity of this myofilament. Lombardi *et al*. (2008) generated transgenic mice expressing either cTnT-Q92 or cTnT-W141 in the heart. They found by co-immunoprecipitation and Ca^{2+} sensitivity assays that differential interaction among sarcomeric proteins containing cTnT-Q92 or cTnT-W141 are responsible for the respective HCM and DCM phenotypes. Recently, mutations in the *SCN5A* gene that encodes for the major cardiac sodium channel Nav1.5 were found to be associated with DCM. To repress *Scn5a* gene expression in mouse Hesse *et al*. (2007) produced transgenic mice that ectopically express the transcriptional repressor Snail in their heart. These transgenic animals

show severe DCM indicating that decreased *Scn5a* expression and thus a significant reduction in sodium current can result in DCM. Their findings support the hypothesis that mutations in the human *SCN5A* gene can cause DCM.

1.4 Bovine dilated cardiomyopathy

Bovine dilated cardiomyopathy (BDCMP) is a heart muscle disease observed over the last three decades in cattle of Holstein-Friesian origin. The first cases of BDCMP were reported in the late 1970s in Switzerland (Martig *et al*. 1982), Japan (Sonda *et al*. 1982) and Canada (Baird *et al*. 1986). In Switzerland, the pure Simmental population was enhanced by introduction of Red Holstein genes since 1968. These crosses created a separate cattle breed referred to as Swiss Fleckvieh. BDCMP affects animals belonging to the Swiss Fleckvieh and Red Holstein breed (Martig *et al*. 1982; Martig 1992; Graber and Martig 1993). This disease is characterized by a global cardiac enlargement, chamber dilatation with reduced wall thickness and systolic dysfunction. BDCMP-affected individuals all demonstrate signs of congestive heart failure, particularly subacute edema within the brisket and to a lesser extent in the jaw and ventral abdominal region. An increased pulse rate, up to 140 beats/min, tachycardia with galloping rhythm of the heart and congestion of the jugular veins are commonly observed in BDCMP-affected animals. It is a slowly progressive terminal disease however; developing within a few days or weeks clinical symptoms lead to sudden death of affected animals or they have to be culled shortly after disease onset (Martig 1982; Sonda *et al*. 1982; Martig and Tschudi 1985; McLennan and Kelly 1990; Tontis *et al*. 1990; Bradley *et al*. 1991; Kümper and Bahnemann 1992; Danzl 1995). The age at onset of BDCMP is between 2 and 4 years, nonetheless BDCMP-affected calves as well as old BDCMP-affected cows have also been

diagnosed (Martig and Reusser 1988; Martig 1992; Graber and Martig 1993). Regularly, observed characteristics at necropsy are cardiomegaly, hypertrophy of all parts of the myocardium and myocardiofibrosis together with myofibrillar degeneration. Typical post-mortem signs are presented by hydrothorax, interstitial edema in the lungs, ascites and congestion in the liver and kidneys (Losiger *et al*. 1985; Tontis *et al*. 1990; Bradley *et al*. 1991; Martig 1992). Transmural fibrosis precedes extensive myocardial degeneration and initial cardiomyocyte necrosis in all sections of the myocardium (König *et al*. 1990). It is known that at first the left ventricle fails to compensate and this subsequently leads to pulmonary hypertension followed by hypertrophy of the right ventricle. In time, a progressive remodelling of the heart causes the right heart failure (Lobsiger *et al*. 1985; Matig and Tschudi 1985). To date, there is no medical treatment for this disease. Moreover, BDCMP can be misdiagnosed as traumatic pericarditis or valvular endocarditis (Martig *et al*. 1982).

2. Genes involved in dilated cardiomyopathies

2.1 Mutations in different genes cause dilated cardiomyopathy in humans

Olson *et al*. (1998) identified the first gene, cardiac actin (*ACTC*), for the pure form of DCM. This gene is located on human autosome 15 (HSA15q14). Identified missense mutations in the *ACTC* gene affect its conserved amino acids in domains that attach to Z bands and intercalated discs. It is likely that the altered function of actin rather than loss of its function causes the idiopathic dilated cardiomyopathy (DCM). The identification of the missense mutation Ile451Met in the desmin gene (*DES*) that causes the idiopathic DCM (Li *et al*. 1999) together with the mutations in *ACTC* strongly support the hypothesis that genes encoding for proteins which transmit force

might be responsible for the familial form of DCM (Li *et al*. 2001). In the past ten years a number of mutations in various genes which are responsible for the DCM phenotype increased prominently (Table 1). Haghighi *et al*. (2006) detected the Arg14Del mutation in the human phospholamban gene (*PLN*) in the large family associated with DCM. They used a mouse model to recap the disease phenotype. An interruption in a constitution of the *PLN* gene possibly resulted in a super-inhibited activity of SERCA2a. Since this mutation was identified in patient of mild and late onset forms, the association with a variable phenotype was implied (DeWitt *et al*. 2006). Subsequently, Haghighi *et al*. (2008) found that a mutation at a position -36 bp relative to the *PNL* transcriptional start site thereby increases its transcription and may function as a genetic modifier in DCM. *In vitro* experiments revealed that the genetic variant increases *PLN* expression levels by changing the glucocorticoid nuclear receptor (GR)-mediated regulation of transcription. Recently, mutational hotspots in the lamin A/C gene (*LMNA*) were identified (Perrot *et al*.2008). The p.R190W *LMNA* mutation hot spot is located in codon 190 and it was initially detected in a small Italian family with a severe form of DCM (Arbustini *et al*. 2002). Perrot *et al*. (2008) detected four mutations in *LMNA* hot spots (p.R190W, p.E161K, p.E203G, p.R644C) and identified one mutation (p.K219T) located in a novel codon. Moreover, they confirmed that all patients with mutations in the *LMNA* gene present a homogenous severe DCM phenotype.

It is also speculated that many more mutations in the coding as well as in the non-coding regions of different genes might be involved in DCM (Haghighi *et al*. 2008).

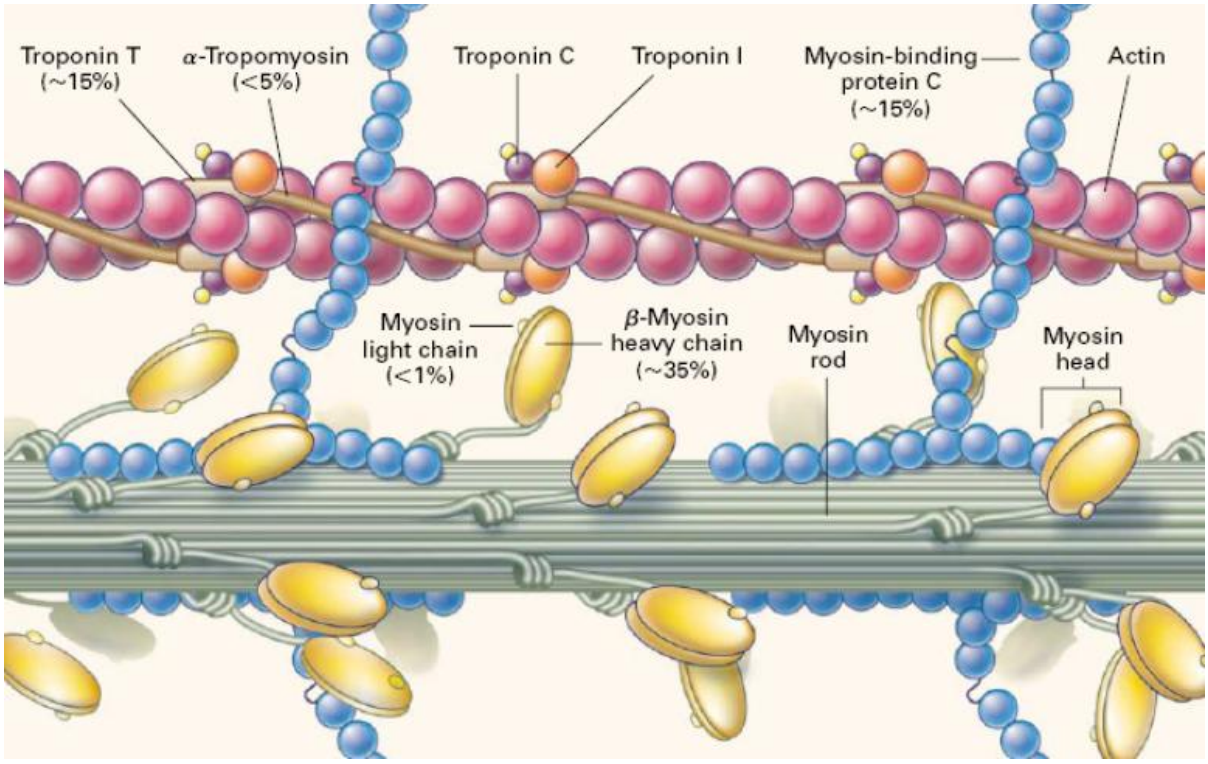
Table 1. Gene mutations identified for dilated cardiomypathy in humans

Human chromosome HSA	location	Mode of inheritance	Gene designation	Gene abbreviation	References
HSA1	1q21.2	AD	Lamin A/C	*LMNA*	Perrot *et al*. 2008
HSA1	1q32	AD	Cardiac troponin T2	*TNNT2*	Durand *et al*. 1995 Li *et al*. 2001
HSA1	1q42.1	AD	Presenilin 2	*PSEN2*	Li *et al*. 2006
HSA2	2q24.3	AD	Titin	*TTN*	Siu *et al*. 1999 Gerull *et al*. 2002
HSA2	2q35	AD	Desmin	*DES*	Li *et al*. 1999
HSA5	5q33-q34	AD	Sarcoglycan, delta	*SGCD*	Tsubata *et al*. 2000
HSA6	6q22.1	AD	Phospholamban	*PLN*	Haghighi *et al*. 2006 Haghighi *et al*. 2008
HSA11	11p11.2	AD	Cardiac myosin binding protein C	*MYBPC3*	Daehmlow *et al*. 2002
HSA11	11p15.1	AD	Cysteine and glycine-rich protein 3	*CRP3*	Knöll *et al*. 2002
HSA12	12p12.1	AD	ATP-binding cassette, sub-family C	*ABCC9*	Bienengraeber *et al*. 2004
HSA14	14q12	AD	Cardiac muscle myosin, heavy polypeptide 7	*MYH7*	Kamisago *et al*. 2000 Käkkäinen *et al*. 2004
HSA14	14q24.3	AD	Presenilin 1	*PSEN1*	Li *et al*. 2006
HSA15	15q14	AD	Cardiac muscle actin alpha	*ACTC*	Olson *et al*. 1998
HSA17	17q12	AD	Titin-cap (telethonin)	*TCAP*	Knoll *et al*. 2002
HSA19	19q13.4	AR	Cardiac troponin I	*TNNI3*	Murphy *et al*. 2004
Chr X	Xp21.2	XR	Dystrophin	*DMD*	Towbin *et al*. 1993
Chr X	Xq28	XR	Tafazzin	*TAZ*	D'Adamo *et al*. 1997

2.2 Cardiac troponin I gene (*TNNI3*) as a candidate gene for bovine dilated cardiomyopathy

A regulatory thin-filament protein, troponin I (TnI) is a member of the troponin complex. In conjunction with other troponin subunits and tropomyosin it contributes to calcium dependent muscle activation (Zot and Potter 1987; Lehrer, 1994; Spirito *et al*. 1997) (Figure 1). Three different TnI genes that encode specific muscle twitch type isoforms are known (Koppe *et al*. 1989; Murphy *et al*. 1991; Wilkinson *et al*. 1991). The fast skeletal, slow skeletal and cardiac isoforms are encoded by genes *TNNI1*, *TNNI2* and *TNNI3*, respectively (Cummins and Perry 1978; Tiso *et al*. 1997). Several mutations in the *TNNI3* gene responsible for various types of cardiomyopathies have been reported. Kimura *et al*. (1997) identified in the human *TNNI3* gene 14 polymorphisms and six disease-associated mutations in patients with HCM. Furthermore, six novel mutations in conserved and functional regions of *TNNI3* gene were detected in RCM cases (Mogensen *et al*. 2003). Recently, the first recessive mutation in *TNNI3* gene causing idiopathic DCM was found (Murphy *et al*. 2004). A homozygous C-to-T substitution in exon 1 of the gene leads to the A2V amino acid exchange. The authors strongly suggest that the A2V amino acid substitution may cause alterations in the protein organization and thereby obstruct interaction with cardiac troponin T (cTnT). This impaired protein-protein interaction leads to reduced contractility of the heart and in this manner to the disease (Murphy *et al*. 2004). The reported human causative recessive mutation as well as the identification of *TNNI3* gene in close vicinity to the interval of interest on BTA18 and a significant LOD score value of the *TNNI3* SNP in intron 6 inclined us towards further investigation of this putative gene for BDCMP. The human *TNNI3* gene mapped to HSA 19q13.3-q13.3 (Bermingham *et al*. 1995). This location is in agreement with well determined synteny between HSA19 and BTA18 (Goldammer *et al*. 2002, Mömke *et*

al. 2005). The bovine *TNNI3* gene as well as its human counterpart contain eight exons. The comparison of coding sequences of these human and cattle genes revealed an identity of 88.5%. Preliminary sequencing results of the gene revealed a SNP in intron 6 (c.378+315G>A) (Guziewicz, 2004; Guziewicz *et al*. 2007). To validate the association between c378+315G>A substitution and the disease an extended number of BDCMP-affected and control animals were genotyped. All possible genotypes within the group of BDCMP-affected individuals were found. In accordance with the hypothesized recessive mode of inheritance for the disease these results exclude the G-to-A transition in intron 6 of *TNNI3* gene as a causative mutation for BDCMP. Additionally, expression analysis and Northern blot analysis of the *TNNI3* gene strongly supports the aforementioned exclusion (Owczarek-Lipska *et al*. 2009). Moreover, the current interval of 240 kb definitively eliminates the bovine *TNNI3* gene as a candidate gene responsible for BDCMP.



(Spirito *et al.* 1997)

Figure 1

Components of the contractile apparatus of the myocardium. Binding of calcium to the subunits of troponin complex and α-tropomyosin enables myosin-actin interaction and thereby cardiac muscle contraction. Actin stimulates ATPase activity in the myosin head. This results in the accumulation of force along actin filaments. Cardiac myosin-binding protein C binds myosin and modulates contraction. Mutations in the genes encoding the members of this complex may impair protein-protein interactions and result in the insufficient muscle contraction. In consequence, these changes may lead to different types of cardiomyopathies (Spirito *et al*. 1997).

3. Genetics of bovine dilated cardiomyopathy

3.1 Hereditary nature and a mode of inheritance for bovine dilated cardiomyopathy

In the first report about BDCMP it was speculated that this disease was inherited. Afterwards, pedigree analyses of BDCMP-affected reported animals in Canada (Baird *et al*. 1986), Japan (Satoh *et al*. 1988) and Switzerland (Martig *et al*. 1982; Martig and Reusser 1988) revealed a red factor carrier Holstein-Friesian sire “ABC Reflection Sovereign”. Since the 1950s this bull and its offspring were used extensively worldwide in breeding. The first hypothesis about an autosomal recessive mode of inheritance for BDCMP was proposed by Satoh (1988). Furthermore, Dolf *et al*. (1998), based on results from segregation analysis performed on an experimental pedigree, confirmed the genetic nature of this disease. The comparison of three genetic models, a major gene model, a mixed inheritance model and an environmental model, indicated the presence of a single biallelic major gene responsible for BDCMP (Dolf *et al*. 1998). Additionally, environmental factors like gestation, parturition, lactation and obesity, which overstress the myocardium, may play an important role in the onset of this disorder (Van Vleet and Ferrans 1986; Martig and Reusser 1988; Tontis *et al*. 1990; Dolf *et al*. 1998).

3.2 Mapping of the BDCMP locus on bovine chromosome 18

Dilated cardiomyopathy is a disease known in various species and many causative mutations in a large number of genes have been identified (Table 1). The majority of these genes encode for cytoskeletal and sarcomeric proteins in the cardiac myocytes. Because all of these genes should be considered for BDCMP, the whole genome scan was considered for use in narrowing down the critical interval. The idea of a whole genome scan is aimed at finding the chromosomal region that is linked to

the disease. This is performed by covering the genome with evenly spread polymorphic markers to find those which are linked to the phenotype. Guziewicz (2004), using on the experimental BDCMP pedigree information, performed a two-point linkage analysis using a set of 199 microsatellite markers. Genetic maps were constructed for all autosomal chromosomes. Using the same genetic linkage map the multipoint linkage analysis was performed. Both results from the two-point linkage analysis and from the multipoint linkage analysis were in agreement and strongly supported the location of the BDCMP locus on bovine chromosome 18 (BTA18) between microsatellite markers *MSBDCMP06* and *BMS2785* (Guziewicz, 2004; Guziewicz *et al*. 2007). This interval corresponds to a physical distance of 6.7 Mb (Btau 3.1). Furthermore, it has been shown that the interval on BTA18 (BTA18q24-q26) is syntenic to the human chromosome 19 (HSA19q13.4). This is a gene-rich region and contains more than 200 annotated genes. Nevertheless, none of these genes was previously described as involved in the heart function (Guziewicz *et al*. 2007). Interestingly, the cardiac troponin I gene (*TNNI3*) was identified in a telomeric vicinity of the *MSBDCMP06-BMS2785* interval. Moreover, a point mutation (c.378+315AG) found in intron 6 of this gene revealed a significant two-point LOD score value of 3.37 (Guziewicz *et al*. 2007). In consequence, it was worth considering this gene as a functional candidate gene for BDCMP due to its important role in the contractile apparatus of the myocardium (Perry, 1999; Murphy *et al*. 2004).

3.3 Fine mapping of the disease-causing locus

The identified 6.7-Mb interval of interest on BTA18 is absent in obvious functional candidate genes for BDCMP (Guziewicz *et al*. 2007). A fine mapping approach was

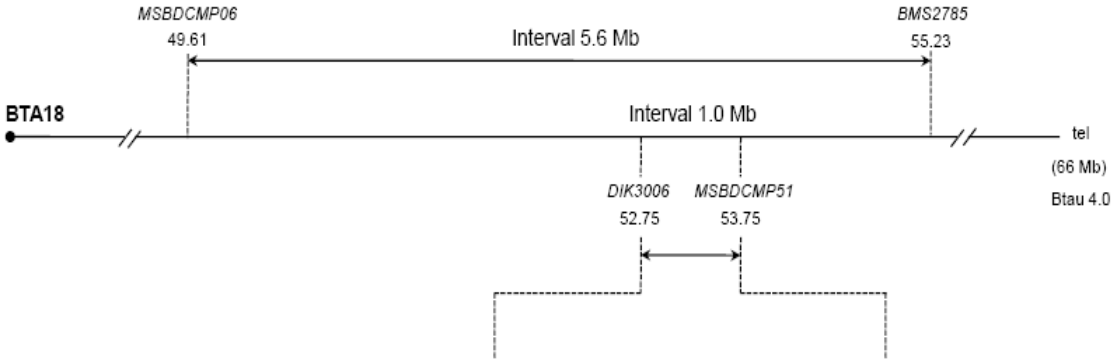
chosen to narrow down the interval of interest. For this purpose a combined strategy of homozygosity mapping and association-based analysis was used.

Homozygosity mapping was first proposed as a way to map human recessive traits with the DNA of affected inbred children (Lander and Botstein, 1987). This method aims to identify the autosomal recessive disease-causing locus by virtue of the fact that the adjacent DNA sequence is identical by descent (IBD).

Since all BDCMP cases traced back to a common ancestor, “ABC Reflection Sovereign”, we reasoned that they share a common IBD haplotype. For this purpose additional BDCMP-affected and control animals were genotyped with a panel of nine randomly selected microsatellite markers within the interval (Guziewicz *et al*. 2007). All BDCMP-affected individuals were homozygous at the microsatellite markers *DIK3006*, *MSBDCMP54*, *MSBDCMP53*, *MSBDCMP42*, *MSBDCMP25* and *RME01*. These results emphasized the IBD concept. Moreover, in regard to this theory recombinant chromosomes were considered for an individual that is heterozygous for markers located distal of the interval *MSBDCMP06-BMS2785* and homozygous for contiguous markers located within this interval. Eleven presumably recombinant chromosomes on the centromeric and nine on the telomeric site of the interval *MSBDCMP06-BMS2785* were found. The homozygosity mapping allowed us to narrow down the interval of interest to 4.1 Mb between microsatellite markers *MSBDCMP06* and *MSBDCMP51*. Thereafter, fifteen newly developed microsatellite markers were included in the analysis. Markers evenly distributed within the interval were genotyped with an enlarged set of BDCMP-affected individuals. The identification of an additional three and 18 recombinant chromosomes from the centromeric and telomeric side, respectively, reduced the interval containing the disease-causing locus to 1.0 Mb. The 1.0-Mb region is flanked by microsatellite markers *DIK3006* and *MSBDCMP51* (Figure 2). Subsequently, to support the

position and further fine map the disease-causing locus the association-based strategy using SNP markers was performed. SNP markers from the publicly available database (http:// http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp) as well as newly developed ones were used. Additionally, on each distal side of the interval SNP markers located in genes apolipoprotein E (*APOE*) and neuronal PAS domain protein 1 (*NPAS1*) were included to test the region outside of the *DIK3006* and *MSBDCMP51* interval for association with the disease. Two groups of BDCMP-affected and BDCMP-unaffected animals were genotyped. The results were analyzed using two independent softwares, SAS Genetics and Haploview 4.0 (Barrett *et al*. 2005). A minimal minor allele frequency (MAF) of 20% was used in this study. The association analysis revealed significant association between markers and BDCMP over the whole interval *DIK3006-MSBDCMP51*. Furthermore, three highly associated regions within the interval were identified (Owczarek-Lipska *et al*. 2009). The human homologous region on HSA19 of this 1.0-Mb interval contains 47 genes and 18 annotated loci. In the mouse this region was found on chromosome 7 (MMU7) but the gene order is inverted in comparison with that in human and cattle. For two of these genes, the fukutin-related protein gene (*FKRP*) and the vasodilator-stimulated phosphoprotein gene (*VASP*), a role in heart function and development was reported by Müller *et al*. (2005) and Eigenthaler *et al*. (2003), respectively. However, to further narrow down the 1.0-Mb interval a pool of additional BDCMP-affected animals was screened with SNP markers. Identification of a further two recombinant chromosomes, one on centromeric and one on telomeric side, of the *DIK3006-MSBDCMP51* interval reduced the crucial chromosomal region to 240 kb. Simultaneously, the fine mapping of the interval excluded the aforementioned *FKRP* gene (Figure 2).

The current 240-kb interval corresponds to the human chromosome 19 (HSA19) and the mouse chromosome 7 (MMU7) between 50.70 and 50.99 Mb (build 36.3) and 19.64 and 19.85 Mb (build 37.1), respectively. It is encompassed from the centromeric side by the vasodilator-stimulated phosphoprotein gene (*VASP*) and from the telomeric side by the radial spokehead-like 1 gene (*RSHL1*). This region contains nine other genes (*OPA3*, *GPR4*, *EML2*, *GIPR*, *SNRPD2*, *QPCTL*, *SIX5*, *DMPK* and *DMWD*). Moreover, one annotated locus (*LOC388553*) on the human counterpart and one predicted gene (*EG243866*) on the mouse counterpart correspond to the annotated hypothetical locus (*LOC100140560*) on the bovine genome assembly (Btau 4.0). Furthermore, there is an annotated microRNA (*MIRN330*) on human and mouse counterpart and additional microRNA (*MIRN642*) on human genome assembly (build 36.3). The bovine *VASP* gene as a plausible candidate gene for BDCMP was deeper investigated. Sequencing of the gene in 33 BDCMP-affected and 154 BDCMP-unaffected individuals revealed a SNP in exon 7 (c.760C>A) and a SNP in intron 7 (c.830+310A>G). Both of the identified variations were not in perfect disequilibrium with BDCMP mutation and can be excluded as being causative. None of the remaining genes within the 240-kb interval has been previously described as an obvious gene causing dilated cardiomyopathy (Figure 3).



ID	Status	MS_06		MS_31		MS_22		DIK3006		MS_54		MS_53		MS_42		MS_25		RME01		MS_51		MS_48		IDVGA55	
61	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
P233	BDCMP	225	232	181	185	135	143	100	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
223	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
370	BDCMP	225	232	181	208	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
434	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
495	BDCMP	223	232	181	187	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	232	215	217	195	197
497	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
1013	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
38	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
506	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	197
527	BDCMP	223	232	181	187	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	232	215	217	195	197
145	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
P251	BDCMP	225	232	181	185	135	143	100	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
147	BDCMP	232	232	181	181	143	143	104	104	139	139	128	128	116	116	142	142	161	161	226	226	217	217	195	195
676	Control	223	232	181	185	136	143	100	100	139	139	128	134	116	120	140	142	159	161	216	226	215	217	195	197
3339	Control	223	232	181	185	136	143	100	102	139	139	128	134	116	120	140	142	159	161	216	226	215	217	195	197
6418	Control	232	232	181	181	143	145	100	102	139	153	128	134	116	116	142	142	159	161	226	226	215	217	195	197
806	Control	225	229	181	181	132	145	100	100	153	167	136	136	112	116	140	154	159	159	220	222	211	215	197	205
809	Control	225	225	181	185	145	145	100	100	153	153	134	138	116	128	142	154	159	159	220	220	213	215	195	205
811	Control	225	225	175	185	132	145	100	100	153	155	124	136	112	126	140	140	159	159	218	222	215	221	197	197
814	Control	225	225	185	185	145	145	100	100	155	155	124	124	126	126	140	140	159	159	218	218	221	221	197	197
826	Control	223	225	187	187	136	145	100	100	139	167	128	134	114	118	140	158	159	159	224	226	213	235	197	197
829	Control	225	229	185	193	145	145	100	100	139	149	134	140	122	122	142	154	159	159	216	230	209	209	197	197
837	Control	225	225	175	175	132	145	100	100	153	155	124	136	112	126	140	140	159	159	218	222	215	221	197	197
842	Control	225	229	187	187	136	145	100	100	139	153	128	136	114	114	142	158	159	159	222	226	213	215	197	205
920	Control	223	229	181	181	138	145	100	100	139	149	134	140	122	126	140	142	159	159	222	234	215	217	197	197
970	Control	223	223	183	187	136	143	100	100	139	149	142	142	124	126	140	142	159	159	226	228	215	229	197	197
972	Control	225	232	185	185	136	136	100	100	139	149	134	140	124	126	142	142	159	159	216	222	215	217	197	197

Figure 2

Homozygosity mapping of the BDCMP locus on BTA18. The initial interval of 6.7 Mb between microsatellite markers *MSBDCMP06* and *BMS2785* based on Btau 3.1 corresponds to a 5.6 Mb on Btau 4.0. The narrowed down 1.0-Mb interval of interest is flanked by microsatellite markers *DIK3006* and *MSBDCMP51*. The table below presents a random selection of genotyping results from 14 BDCMP-affected and 14 control animals. Abbreviations MS and P refer to microsatellite markers MSBDCMP and to paraffin- embedded heart samples, respectively.

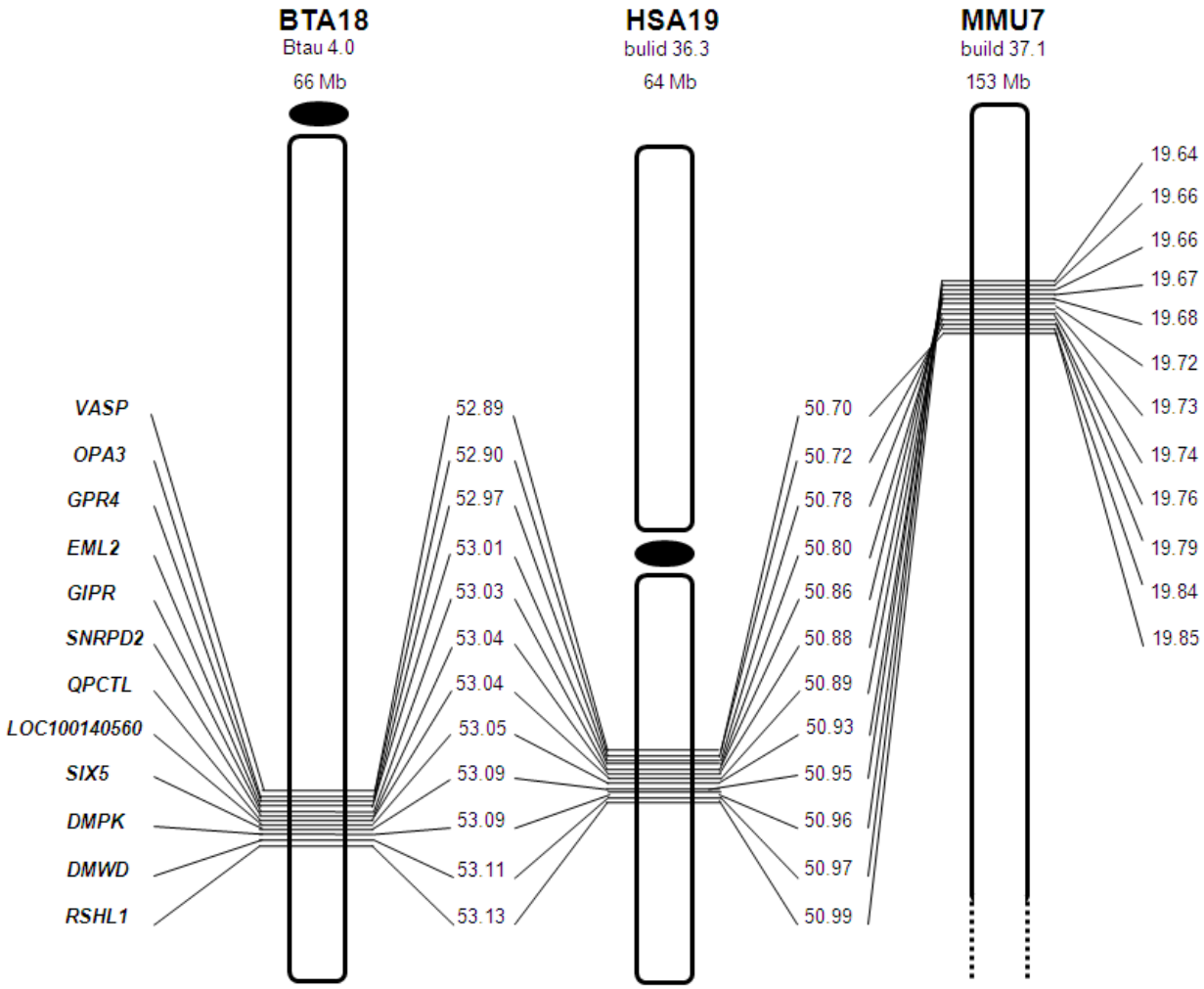


Figure 3

Comparative map of BTA18, HSA19 and MMU7. The chromosomal positions are indicated for the genes and given in Mb distances based on the bovine (Btau 4.0), human (build 36.3) and mouse (build 37.1) genome assemblies. The 240-kb interval on BTA18 containing the disease-causing locus is encompassed by the *VASP* and *RSHL1* genes, respectively. The hypothetical locus (*LOC100140560*) on Btau 4.0 corresponds to the annotated locus (*LOC388553*) and predicted gene (*EG243866*) on human and mouse counterparts, respectively.

3.4 Bovine genome assemblies and a bacterial artificial chromosome contig on bovine chromosome 18

An assessed by Guziewicz *et al*. (2007) interval of 6.7 Mb based on the previous bovine genome assembly Btau 3.1. The draft sequence Btau 3.1 was an improved assembly of Btau 2.0. but was found to be still erroneous and incomplete. Recently, the Human Genome Sequence Centre at the Baylor College of Medicine released an updated and improved bovine genome assembly Btau 4.0.

The Btau 4.0 assembly was constructed based on the 7.15X mixed assembly of whole genome shotgun (WSG) libraries and bacterial artificial chromosome (BAC) sequences. A female of the Hereford breed was used as a DNA source. In August 2008 the map viewer of the *Bos taurus* build Btau 4.0 was released in the NCBI (http://www.ncbi.nlm.nih.gov/Genomes/). It contains the 7.15X WGS Btau 4.0 assembly together with a complete mitochondrial genome obtained from a Korean native cow (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9913).

Furthermore, the existing *Bos taurus* assembly version 1.5 created by a group of researchers from the Centre for Bioinformatics and Computational Biology at the University of Maryland was improved and the 2.0 version was released. The 2.0 bovine genome assembly includes about 54 Mb of additional sequence as compared to version 1.5 (ftp://ftp.cbcb.umd.edu/pub/data/assembly/Bos_taurus/Bos_taurus_UMD_2.0). In order to find the best marker order we decided to construct an experimentally derived BAC contig. Furthermore, the BAC contig would allow us to re-sequence every region of interest including gaps existing in the publicly available sequence. Initially, a publicly available BAC contig listing CHORI BAC clones from the Children's Hospital Oakland Research Institute, USA was used to construct the BAC contig surrounding the BDCMP locus. Nevertheless, when the overlapping genomic sequences were

determined in the aforementioned list, it was unfeasible to PCR amplify part of these sequences and thereby close the BAC contig. Strikingly, a bioinformatics analysis based on Btau 3.1 revealed that the chromosomal region containing the BDCMP locus and an additional region were inverted as compared to the homologous chromosomal regions on HSA19. Moreover, the BAC contig contained gaps around breakpoints of these inversions. An introduction of supplementary BAC clones form the Laboratoire de Génétique Biochimique et de Cytogénétique, France (Eggen *et al*. 2001) and the rearrangement of a part of the CHORI BAC clones enabled us to construct a comprehensive BAC contig (Figure 4). A comparative bioinformatics analysis using the bovine genome assembly Btau 4.0 confirmed that the order of genetic makers located in our BAC contig fully corresponds to that on HAS19. The BAC contig is considered to be an excellent tool in locating the DNA markers and fine mapping the interval of interest.

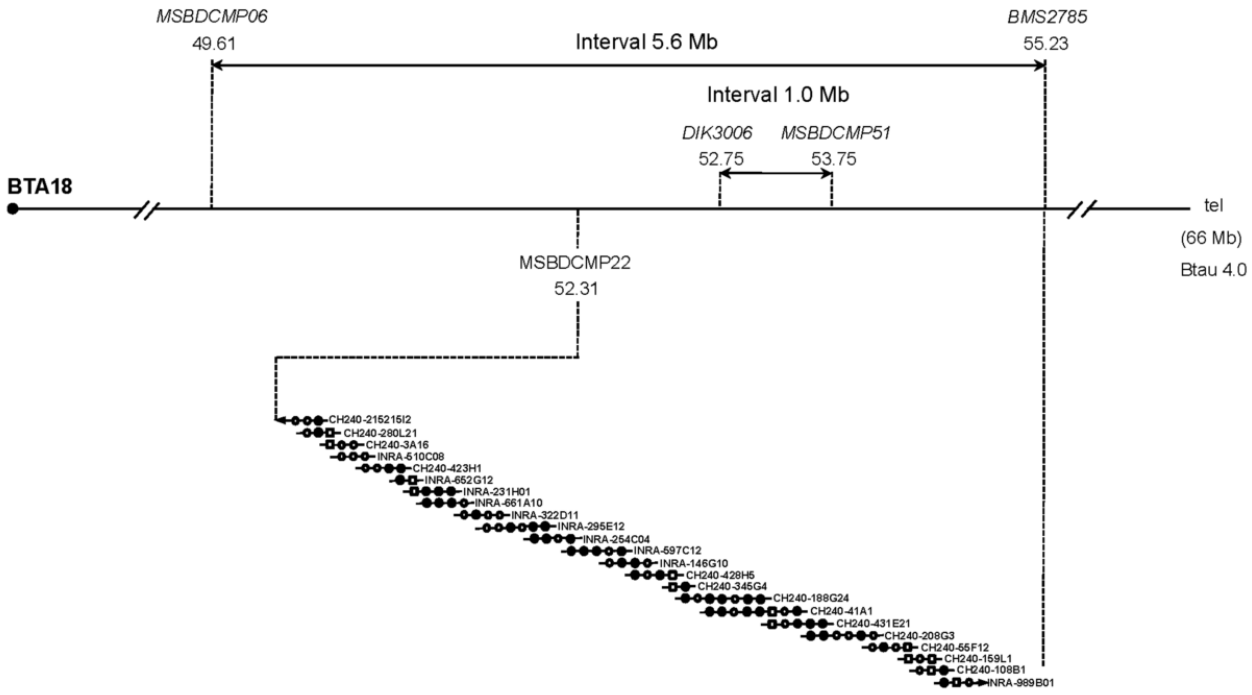


Figure 4

BAC contig on BTA18 encompassed by microsatellite makers *MSBDCMP22* and *BMS2785*. This contig composed of 23 tiled BAC clones covers the 1.0-Mb interval of interest. Microsatellite markers, genes and PCR products with known positions on BTA18 are represented by open circles, filled circles and open squares, respectively.

4. Conclusions and perspectives

Bovine dilated cardiomyopathy (BDCMP) is a progressive degenerative disease of the heart muscle leading to congestive heart failure and in consequence to death.

Dilated cardiomyopathy in cattle of Canadian Holstein origin (Martig et al. 1982; Tontis et al. 1990), cardiomyopathy in Japanese Black cattle (Watanabe et al. 1979) and cardiomyopathy in the Hereford breed (Morrow and McOrist 1985; Storie et al. 1991) have been reported. In Switzerland BDCMP-cases are periodically diagnosed in Swiss Fleckvieh cattle but the frequency of the mutant allele responsible for the disease remains unknown (Owczarek-Lipska *et al*. 2009). The elimination of sire descendants from the "ABC Reflection Sovereign" sire population dramatically decreased the occurrence of this disorder and practically eliminated it in the Fleckvieh population. However, in the mid-1990s in Denmark three incidents of bovine cardiomyopathy were identified. All of them presented with similar symptoms to the BDCMP-cases reported in Switzerland (Leifsson and Agerholm, 2004).

Idiopathic DCM in human is the third most common cause of heart failure in humans. The aetiology of the disease remains unknown, although genetic factors seem to be a predominant reason for the disease (Michels *et al*. 1992). Therefore, an improved knowledge of the genetic background and the mechanism that leads to DCM is highly relevant and may lead to an early treatment of patients as well as an enhanced therapy. Although there are several animal models reported (Gwathmey and Davidoff, 1993; Reed *et al*. 2007) a further animal model that could contribute to a better understanding of human DCM would be very valuable. A proper animal model for human DCM should closely imitate the human disease, be easy to repeat, be appropriate for measurements of cardiac traits and be economically beneficial to permit therapeutic tracing (Guziewicz, 2004). BDCMP shows almost all of the typical characteristics that have been described in human DCM. If the predisposition follows

an autosomal recessive mode of inheritance this model will allow the study of animals that are clinically unaffected but carry the genetic defect (Eschenhagen *et al*. 1995). Moreover, it is worth recalling that cattle families are large, containing several generations at the same time, which is why the disease spread more rapidly (Furuoka *et al*. 2001; Nart *et al*. 2004). BDCMP has thus been proposed as a key biochemical model in cardiovascular research (Weekes *et al*. 1999).

The exclusion of a functional candidate gene *TNNI3* and the lack of any obvious functional candidates within the interval of interest on BTA18 indicates that most likely a novel gene, which was not previously reported to be involved in cardiomyopathy can be identified. Presently we are sequencing the genes within the 240-kb interval, in which the potential mutation must to be in perfect disequilibrium with BDCMP. The complex phenotype and the wide range of age at onset of the disease suggest that BDCMP may be caused by a regulatory mutation. To demonstrate the causality of this type of mutation electrophoretic mobility shift assays (EMSA) and transient transfection assays using a dual-luciferase reporter assay might be suitable in *in vitro* experiments. Studies investigating gene expression and protein function will potentially provide additional evidence for the causality of the identified mutation. Additional experiments to prove the causality of the mutation depend on its characteristics. It is suggested that cardiomyocyte primary cell cultures be used to study relevant gene(s) involved in BDCMP. We plan to challenge them by RNA interference using small interfering RNAs but also by using pharmaceutical chemicals. It might also be of interest to study the function of BDCMP causing gene(s) during development, since factors responsible for the onset of the disease remain unknown. For this approach the collection of heart tissue samples from BDCMP-affected animals belonging to different age classes would be necessary.

The identification of the causative mutation for BDCMP is prerequisite to providing insight into the understanding of the mechanism of onset and development of the disease. It is of obvious interest to verify whether the mutation causing BDCMP also occurs in DCM human patients.

Moreover, the identification of the BDCMP causing mutation would provide a possibility to detect carrier animals in the Swiss Fleckvieh population. In so doing, it would help to establish a marker assisted selection (MAS) program against this disease and thus to improve the health status of this cattle breed.

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CHAPTER ONE

Part I

Bovine cardiac troponin I gene (*TNNI3*) as a candidate gene for bovine dilated cardiomyopathy

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Bovine cardiac troponin I gene (*TNNI3*) as a candidate gene for bovine dilated cardiomyopathy

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Abstract

The cardiac troponin complex, which is an important component of the contractile apparatus, is composed of the three subunits troponin I (TnI), troponin C (TnC) and troponin T (TnT). Troponin I is the inhibitory subunit and consists of three isoforms encoded by *TNNI1*, *TNNI2* and *TNNI3* genes, respectively. Due to the different types of cardiomyopathies caused by mutations in the *TNNI3* gene and its fluorescence *in situ* hybridization (FISH) mapping on bovine chromosome 18q26, which was shown to be linked to the recessively inherited bovine dilated cardiomyopathy (BDCMP), bovine *TNNI3* was considered as candidate gene for BDCMP.

Real-time polymerase chain reaction (PCR) *TNNI3* expression analysis resulted in a significant difference between BDCMP affected and unaffected animals when normalized to *ACTB* gene expression, but there was no significant difference in expression when normalized to *GAPDH*. Northen blotting experiment was in agreement with the expression analysis and did not reveal a significant difference between the group of BDCMP affected and unaffected animals. Sequencing of the bovine *TNNI3* gene revealed a single nucleotide polymorphism in intron 6 (c.378+315G>A), but this single nucleotide polymorphism (SNP)was present regardless of the BDCMP status. In summary our data provide evidence to exclude the bovine *TNNI3* gene as a candidate for BDCMP.

Keywords: troponin complex, cardiac troponin I gene (*TNNI3*), bovine dilated cardiomyopathy (BDCMP), Swiss Fleckvieh, cattle

Zusammenfassung

Bovines Cardiac Troponin I Gen (*TNNI3*) als Kandidatengen für die bovine dilatative Kardiomyopathie

Der Cardiac Troponin Komplex, welcher eine wichtige Komponente des kontraktilen Apparates ist, setzt sich aus den drei Untereinheiten Troponin I (TnI), Toponin C (TnC) und Troponin T (TnT) zusammen. Troponin I ist die inhibitierende Untereinheit und besteht aus drei Isoformen, die durch die Gene *TNNI1*, *TNNI2* und *TNNI3* kodiert sind. Verschiedene Formen der Kardiomyopathie beim Menschen werden durch Mutationen

im *TNNI3* Gen verursacht. Das bovine *TNNI3* Gen wurde auf dem Chromosom 18q26, welches mit der rezessiv vererbten bovinen dilatativen Kardiomyopathie (BDCMP) gekoppelt ist, durch Fluoreszenz-*in-situ*-Hybridisierung (FISH) physikalisch kartiert und als Kandidatengen für BDCMP untersucht.

Die Echtzeit-Polymerase-Kettenreaktion (Real-Time-PCR) *TNNI3* Expressionsanalyse ergab einen signifikanten Unterschied zwischen BDCMP kranken und gesunden Tieren bei einer Normalisierung mit der *ACTB* Genexpression, aber es zeigte sich kein signifikanter Unterschied bei einer Normalisierung mit der *GAPDH* Genexpression. Northern Blotting Experimente bestätigten die Resultate der Expressionsanalyse und zeigten ebenfalls keine signifikanten Unterschiede in der *TNNI3* Genexpression zwischen BDCMP kranken und gesunden Tieren. Die Sequenzierung des bovinen *TNNI3* Gens zeigte eine Punktmutation im Intron 6 (c.378+315G>A), diese kam jedoch unabhängig vom BDCMP Status bei den untersuchten Tieren vor. Zusammenfassend erlauben unsere Untersuchungen, das das bovine *TNNI3* Gen als Kandidat für die BDCMP ausgeschlossen werden kann.

Schlüsselwörter: Troponinkomplex, Cardiac Troponin I Gen (*TNNI3*), bovine dilatative Kardiomyopathie (BDCMP), Schweizerisches Fleckvieh, Rind

Introduction

The troponin complex is composed of the three subunits: troponin I (TnI), troponin C (TnC) and troponin T (TnT), which interact during muscle contraction and relaxation with actin via tropomyosin (GOMES *et al.* 2002). TnI consists of three isoforms. Two of them, coded by the genes *TNNI1* and *TNNI2*, are present in slow-twitch and in fast-twitch skeletal muscles, and the third form coded by the *TNNI3* gene is present in heart muscle (CUMMINS and PERRY 1978, TISO *et al.* 1997). Human cardiac TnI protein has an animo-terminal extension with the RRRSS sequence. This sequence is also present in rat and cattle counterparts (VALLINS *et al.* 1990, MITTMANN *et al.* 1992). Phosphorylation on both serines (S) results in a reduction of the interaction between troponin I and troponin C and this leads to an increase in heart contractility (LIAO *et al.* 1992). It has been shown that the majority of mutations found in the *TNNI3* gene are responsible for hypertrophic cardiomyopathy (KIMURA *et al.* 1997, MOGENSEN *et al.* 2004). However, MURPHY *et al.* (2004) reported a rare *TNNI3* mutation causing idiopathic dilated cardiomyopathy in human.

Bovine dilated cardiomyopathy (BDCMP) is a severe and terminal heart disease. BDCMP affects animals belonging to the Red Holstein breed and to Red Holstein × Simmental crosses (Swiss Fleckvieh) (GRABER and MARTIG 1993). DOLF *et al.* (1998) confirmed an autosomal recessive inheritance of a major gene for BDCMP by segregation analysis using an experimental pedigree. In a recent study we showed by linkage analysis that the BDCMP locus maps to bovine chromosome 18 (BTA18) (GUZIEWICZ 2007).

Here we present conclusive evidence that the bovine *TNNI3* gene can be excluded as a candidate gene for BDCMP.

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Material and methods

Cloning and physical mapping of the bovine TNNI3 gene

The following primers to amplify exon 7 of *TNNI3* were used to screen a bovine genomic DNA BAC library comprising 105984 clones (EGGEN *et al.* 2001): forward primer TNNI3ex7f 5′-TGA CCT TCG AGG CAA GTT TA-3′ and reverse primer TNNI3ex7r 5′-TCC TCC TTC TTC ACC TGC TT-3′. The primers were deduced from the orthologues DNA sequence of human *TNNI3* with the acc. no. X90780.

A single colony from BAC clone 388A05, containing the *TNNI3* gene, was inoculated into the 5 ml LB medium with 12.5 µl/ml chloramphenicol and incubated at 37 °C with shaking (300 rpm) during 6-8 h. Afterwards, the culture was diluted (1/1000) into selective LB medium and incubated at 37 °C with shaking over 12-16 h. The BAC DNA (7-15 µg) was extracted according to the manufacture's recommendations using a NucleoBond PC-Kit (Macherey-Nagel AG, Oensingen, Switzerland).

The DNA from the BAC clone 388A05 was digested with Sau3AI restriction enzyme. The obtained fragments, were labeled with biotin-16-dUTP (Roche Diagnostics, Rotkreuz, Switzerland) by the random priming DNA labeling method with Prime-It Fluor Fluorescence Labeling Kit according to the manufacturer's protocol (Stratagene, Amsterdam, The Netherlands). Bovine mitotic metaphase chromosomes from fibroblast culture were QFQ stained and hybridized with the labeled BAC probe (SOLINAS-TOLDO *et al.* 1995). FISH results were analyzed with a fluorescence light microscope under the 63x oil immersion objective and photographed using Quantix Camera (Photometrics, Tucson, USA).

Sequencing of the bovine TNNI3 gene

The DNA from three BAC clones (388A05, 477A02 and 428H02) containing at least part of the *TNNI3* gene were pooled, partially digested with Sau3AI and cloned into pUC19 vector (Roche Diagnostics, Rotkreuz, Switzerland). White positive colonies were picked and transferred in 96-well deep well plates (Milian Instruments SA, Geneva, Switzerland) with each well containing 1 ml LB with 10% glycerol and ampiciline (100 µg/ml). After incubating for 20 h at 37 °C with vigorous shaking (300 rpm) about 200 µl suspension of each clone were transferred into 96-well microtiter plate and stored at −80 °C for further analysis. This bovine plasmid sub-library from the three BACs was first screened by using human *TNNI3* gene exon 7 specific primers, mentioned above, and following DNA sequencing with the corresponding bovine specific primers to identify clones harbouring part of the bovine *TNNI3* gene. Plasmid DNA of positive clones was sequenced using the Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Biosciences, Dübendorf, Switzerland) according to the manufacturer's recommendations. The sequencing reactions were run on 4.3% denaturing polyacrylamide gels on an automated DNA Sequencer LI-COR 4200 (LI-COR, Bad Homburg, Germany) and analysed with a software packages eSeq and AlignIR (LI-COR).

Association analysis of SNP in intron 6 of the bovine TNNI3 gene

DNA from 170 blood samples and paraffin embedded tissues were extracted according to standard protocols. Samples from 136 BDCMP affected animals of Swiss Fleckvieh and 34 unaffected animals consisting of 16 Swiss Fleckvieh, 10 Holstein-Friesian and 8 Simmental were used. These animals were genotyped for the SNP in intron 6 of the bovine *TNNI3* gene (c.378+315G>A).

PCR products surrounding the SNP were obtained with primers TNNI3intron6_f 5'-GGG ATT CTC CAG ACA AGA ACA C-3' and TNNI3intron6_r 5'-CCA CAC TTG AGC TGA CTT ACC A-3' and sequenced form the both sides on an ABI 3730 capillary sequencer (Applied Biosystems, Rotkreuz, Switzerland). Sequencing results were analyzed using the Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, USA).

RNA extraction and first cDNA strand synthesis

Total RNA from heart tissues of 4 BDCMP affected and 9 BDCMP unaffected animals was extracted using TRIZOL Reagent (Invitrogen, Basel, Switzerland) and reverse transcribed using the First-Strand cDNA Synthesis Kit (GE Healthcare, Basel, Switzerland).

5'-RACE experiment

The 5' rapid amplification of cDNA ends of the bovine *TNNI3* gene (5'-RACE) was performed using the FirstChoice RLM-RACE Kit according to the manufacturer's protocol (Ambion, Rotkreuz, Switzerland).

Expression analysis of the bovine TNNI3 gene

PCR products from the coding regions of bovine *GAPDH* (glyceraldehydes-3-phosphate dehydrogenase), *ACTB* (actin beta) and *TNNI3* (cardiac troponin I) genes were cloned and transformed into *E. coli* cells (TOPO TA Cloning Kit, Invitrogen, Basel, Switzerland). The plasmids were used to create RNA probes for the Northern analysis. Primers and TaqMan probes for these genes were designed using Applied Biosystems' primer express software (Applied Biosystems, Rotkreuz, Switzerland) and based on the sequence of the cloned PCR products. Sequences of primers and probes are listed in Table 1.

Table 1
Primers and TaqMan probes used for the real time PCR experiment
Primer und TaqMan Proben, die für das Real-Time-PCR Experiment verwendet wurden

Gen	Oligonucleotide (5' to 3')
ACTB	F: CGG ACA GGA TGC AGA AAG AGA R: GGG CGC GAT GAT CCT GAT Probe: AAA GAG ATC ACT GCC CTG GCA CCC A
GAPDH	F: CCC ACT CCC AAC GTG TCT GT R: CCA CCT TCT TGA TCT CAT CAT ACT TG Probe: CTG ACC TGC CGC CTG GAG AAA CCT
TNNI3	F: CGC ACG CCA CGC AAA AGT R: CTG CAG CAT CAG GGT CTT CA Probe: AAG ATC TCC GCC TCA AGG AAA CTG CAG

The real time PCR experiment was performed according to the guidelines form Applied Biosystems, on a 7300 Real-Time PCR System. The real time PCR data were analyzed by the relative quantification $\Delta\Delta$ C_T method (SCHMITTGEN and LIVAK 2008). The expression level is given as fold difference compared to a calibrator sample.

Northern blot analysis and detection of antisense transcript of the TNNI3 gene

Northern analyses were performed according to standard protocols. RNA probes were synthesized using the MAXIscript Kit (Ambion, Rotkreuz, Switzerland) with a DY-681-aaUTP conjugate (DYOMICS, Jena, Germany). Hybridization was carried out according to the protocol accompanying the ULTRAhyb hybridization buffer (Ambion, Rotkreuz, Switzerland). The Northern blot was analyzed on an Odyssey Infrared Imaging System (LI-COR).

Bioinformatics analyses

The amino-acid sequences from the cardiac troponin I protein (cardiac TnI) encoded by *TNNI3* gene were compared between bovine (UniProt acc. no. P08057), equine, human, mouse, chicken, African clawed frog and zebrafish (GenBank acc. no. NP_001075373, NP_000354, NP_033432, NP_998735, NP_001088122, NP_001008613, respectively) using the ClustalW2 multiple alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html). To estimate the percentage of identity between the amino-acid sequence of the bovine cardiac TnI and amio-acids sequences from the cardiac TnI protein from the above maintained species BLAST analysis (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi) was performed.

Results and discussion

Physical mapping and sequencing of the bovine TNNI3 gene

Three bovine clones (388A05, 477A02 and 428H02) containing the *TNNI3* gene were isolated from the bovine BAC library for mapping and sequencing purposes. The FISH experiment performed with BAC clone 388A05 as a probe on bovine metaphase chromosomes revealed the position of the bovine counterpart gene on BTA 18q26 (Figure 1). BERMINGHAM *et al.* (1995) mapped the human *TNNI3* gene to HSA 19q13.3-q13.4. This chromosomal position is in accordance with the well established synteny between human chromosome 19 and bovine chromosome 18 (GOLDAMMER *et al.* 2004, MÖMKE *et al.* 2005). Both, the human *TNNI3* and the bovine *TNNI3* gene are composed of eight exons (Figure 2). The comparison between the human (GenBank acc. no. NM_000363) and bovine (GenBank acc. no. AJ842179) coding sequences revealed an identity of 88.5%. Similar to the human *TNNI3* gene an ATG start codon and a TGA stop codon were identified in the respective exon 1 and exon 8 of the bovine ortholog (Figure 2). The transcription start site of the bovine *TNNI3* gene was confirmed by a 5'-RACE experiment and is in agreement with the reported sequence of bovine fetal liver mRNA (GenBank acc. no. BC102731). The transcribed *TNNI3* mRNA encodes a 212 amino-acid protein. The bovine cardiac TnI protein sequence shares 92% identity to human, 93% and 91% identity to, equine and mouse, respectively. These three species are almost completely identical in

the conserved domains. Additionally, more divergent species, like the chicken, the African clawed frog and the zebrafish, show a amino-acid identity of 65%, 73% and 63%, respectively (Figure 3).

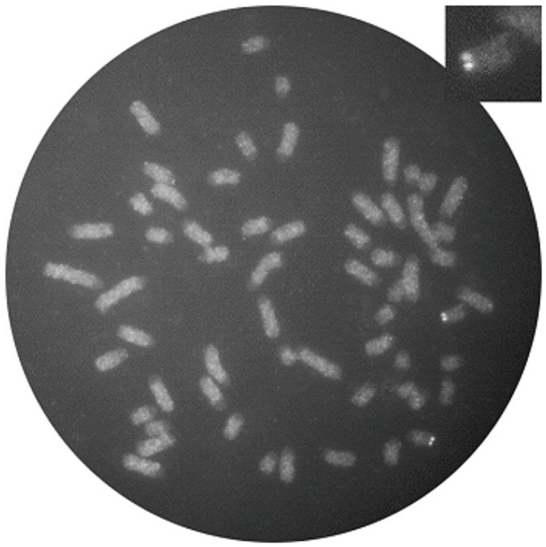

Figure 1
FISH mapping of the bovine *TNNI3* gene. The biotin-16-dUTP labeled BAC clone 388A05 hybridized to QFQ-banded methaphase chromosomes and identified the position of the bovine *TNNI3* gene on BTA18q26. Chromosomes were analysied using the 63x oil immersion objective.

FISH Kartierung des bovinen TNNI3Gens. Der biotin-16-dUTP markierte BAC Klon 388A05 hybridisiert an die QFQ-gebänderten Metaphasenchromosmen und identifiziert die Position des bovinen TNNI3 Gens auf dem BTA18q26. Die Chromosomen wurden mit einem 63x Ölimmersionsobjektiv analysiert.

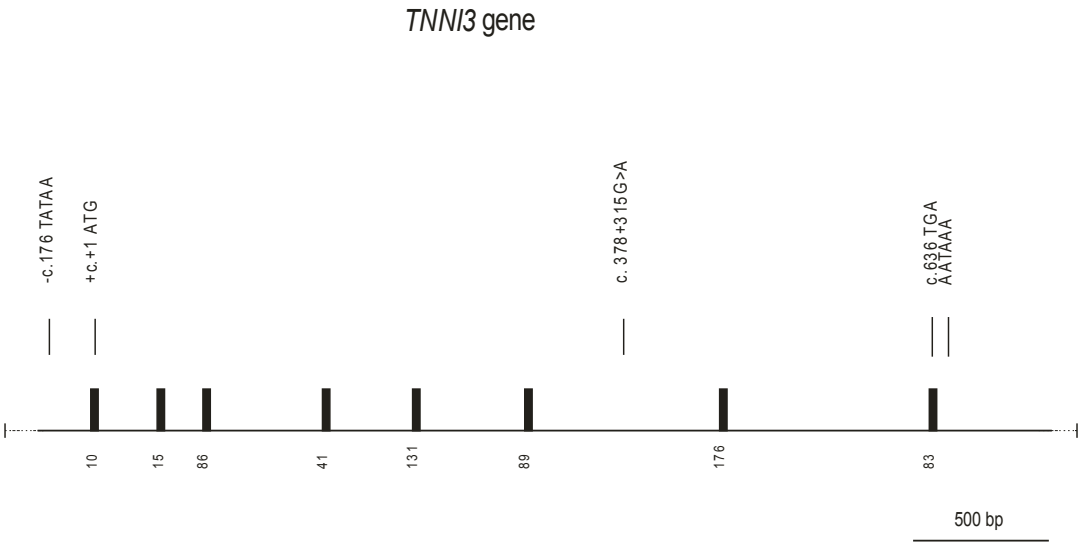

Figure 2
Bovine *TNNI3* gene structure including the position of SNP in intron 6. ATG start codon, TGA stop codon, TATAA sequence and polyadenylation signal are indicated. The exons are shown in black boxes and their sizes are given below.

TNNI3 Gen Struktur mit der Position der Punktmutation im Intron 6. ATG Startkodon, TGA Stopkodon, TATAA Sequenz und Polyadenylationssignal sind angegeben. Die Exons sind in schwarzen Boxen mit darunter stehenden Größenangabe aufgezeigt.

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Bos taurs       MADR-------------------------SGGSTAGDTVPAPPPVRRRSSANYRAYATEP 35
Equus caballus  MADQ-------------------------SGN-------AAPPPVRRRSSANYRAYATEP 28
Mus musculus    MADE-----------------------SSD-AAGEPQPAPAPVRRRSSANYRAYATEP 34
Homo sapiens    MADG-----------------------SSD-AAREPRPAPAPIRRRSS-NYRAYATEP 33
Gallus gallus   MAEE-----------------------EEP--------KPPPLRRKSSANYRGYAVEP 27
Xenopus laevis  MSDEEEVTYEEEEEDYVEEEEEEEEVVAPEPPKPAPPPAAPPPLIRRRSSANYRAYATEP 60
Danio rerio     ------------------------------------------------------MP 2
                                                                       *

Bos taurs       HAKKKSKISASRKLQLKTLMLQIAKQELEREAEERRGEKGRALSTRCQPLELAGLGFAEL 95
Equus caballus  HAKKKSKISASRKLQLKTLMLQIAKQELEREAVERRGEKGRALSTRCQPLELAGLGFEEL 88
Mus musculus    HAKKKSKISASRKLQLKTLMLQIAKQEMEREAEERRGEKGRVLRTRCQPLELDGLGFEEL 94
Homo sapiens    HAKKKSKISASRKLQLKTLLLQIAKQELEREAEERRGEKGRALSTRCQPLELAGLGFAEL 93
Gallus gallus   HAKRQSKISASRKLQLKTLLLQRAKRELERREEQERAGEKQRHLGELCPPPELEGLGVAQL 87
Xenopus laevis  QVKIKPKISASRKLQLKSLMLQIAKAEMEHEEEERALEKERYLAEQCQPLQLSGLSLSEL 120
Danio rerio     EQEKKSKISASRKLMLKSLMVAKAKEELEQELADKEDEKEKYLSEKAPQLQTSGMSFAEL 62
                . : :.******** **:*::  ** *:*:*  ::  ** : *   .   :  *:.. :*

Bos taurs       QDLCRQLHARVDKVDEERYDVEAKVTKNITEIADLNQKIFDLRGKFKRPTLRRVRISADA 155
Equus caballus  QDLCRQLHARVDKVDEERYDVEAKVTKNITEIADLNQKIFDLRGKFKRPTLRRVRISADA 148
Mus musculus    QDLCRQLHARVDKVDEERYDVEAKVTKNITEIADLTQKIYDLRGKFKRPTLRRVRISADA 154
Homo sapiens    QDLCRQLHARVDKVDEERYDIEAKVTKNITEIADLTQKIFDLRGKFKRPTLRRVRISADA 153
Gallus gallus   QELCRELHARIGRVDEERYDMGTRVSKNMAEMEELRRRVAG--GRFVRPALRRVRLSADA 145
Xenopus laevis  QDLCRELHARIDVVDEERYDMEAEVNKNITEIEDLNLKIFDLRGKFKKPNLRRVRLSADA 180
Danio rerio     QELCRELHAKIDVVDEERYDIEAKVLHNTREIKDLNIKVLDLRGKFKRPTLRRVRVSADA 122
                *:***:***::. *******: :.* :*  *: :*  :: .  *:* :* *****:****

Bos taurs       MMQALLGARAKETLDLRAHLKQVK---KEDTEKE-NREVGDWRKNIDALSGMEGRKKKFE 211
Equus caballus  MMQALLGTRAKETLDLRAHLKQVK---KEDTEKE-NREVGDWRKNIDALSGMEGRKKKFE 204
Mus musculus    MMQALLGTRAKESLDLRAHLKQVK---KEDIEKE-NREVGDWRKNIDALSGMEGRKKKFE 210
Homo sapiens    MMQALLGARAKESLDLRAHLKQVK---KEDTEKE-NREVGDWRKNIDALSGMEGRKKKFE 209
Gallus gallus   MMAALLGSKHRVGTDLRAGLRQVR---KDDAEKE-SREVGDWRKNVDALSGMEGRKKKFE 201
Xenopus laevis  MMRALLGTKHKVSMDLRASLKQVKQTKKEDVDKD-IREVGDWRKNVDALSGMEGRKKKFE 239
Danio rerio     ILRSLLGSKHKVSMDLRANLKSVK---KEDTEKEKTVEVSDWRKNVEAMSGMEGRKKMFD 179
                :: :***:: :   **** *:.*:   *:* :*:   **.*****::*:******** *:

Bos taurs       G------ 212
Equus caballus  G------ 205
Mus musculus    G------ 211
Homo sapiens    S------ 210
Gallus gallus   APGGGQG 208
Xenopus laevis  STGAVAV 246
Danio rerio     AAQ---- 182
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Figure 3
Multiple alignment of the bovine cardiac TnI protein (coded by *TNNI3* gene) with the orthologous sequence of horse, mouse, human, chicken, African clawed frog and zebrafish. The amino-acid seqences from six different species were derived from GenBank. Residues, depending on to the similarity between the cardiac TnI protein in diverse species, are presented by asterisks (identical) and colons or dots (very similar or similar). Conserved domains of the cardiac TnI protein are indicated in bold.

Vergleich der bovinen Cardiac TnI Proteinsequenz (kodiert durch das TNNI3 Gen) mit den orthologen Sequenzen des Pferdes, der Maus, des Menschen, des Huhns, des Afrikanischen Krallenfrosches und des Zebrafisches. Die Sequenzen der sechs verschieden Spezies wurden der GenBank entnommen. Die Aminosäuren sind entsprechend der Ähnlichkeit zwischen dem Cardiac TnI Protein der verschiedenen Spezien mit einem Stern (identisch), mit einem Doppelpunkt (sehr ähnlich) oder einem Punkt (ähnlich) markiert. Konservierte Domänen des Cardiac TnI Proteins sind fett gedruckt.

Analysis of the transition G>A in intron 6 of the bovine TNNI3 gene

Sequencing of the bovine *TNNI3* gene in six affected and in six unaffected individuals revealed only a SNP in intron 6 (c.378+315G>A) (GUZIEWICZ 2004). In order to verify the association between the c.378+315G>A transition and BDCMP we genotyped 136 BDCMP affected and 34 unaffected animals of unknown ancestry. Within the group of BDCMP affected animals 70 (51.5%) were homozygous AA, 9 (6.6%) were homozygous GG and 57 (41.9%) were heterozygous AG. The corresponding genotype frequencies for the group of unaffected animals, 4 (11.8%) were homozygous AA, 10 (29.4%) were homozygous GG and 20 (58.8%) were heterozygous AG were observed. The allele frequencies between

the two groups were significantly different (chi-square test, $P>0.001$) indicating that the c.378+315A allele is closely associated with BDCMP. In this context it is worth to mention that the majority of animals (152) from the Swiss Fleckvieh breed were genotyped. Nevertheless, since all three genotypes were present in the group of affected animals the c.378+315G>A transition cannot be the causative mutation for BDCMP.

Expression analysis, the Northern blotting and detection of antisense transcript of the TNNI3 gene

We then further quantified the *TNNI3* gene expression in heart tissues of the right ventricle. In the real time PCR experiment we used *ACTB* and *GAPDH* genes for data normalization. Amplification efficiencies of 99.8% for *ACTB*, 98.4% for *GAPDH*, and 99.9% for *TNNI3* genes were calculated. Expression of the *TNNI3* gene was analyzed in heart tissue from four affected and eight unaffected Swiss Fleckvieh animals and compared to a calibrator sample from a healthy adult individual. No significant difference in the *TNNI3* gene expression was found between affected and unaffected animals when it was normalized to the *GAPDH* gene expression (Wilcoxon two-sample test, two sided, $P=0.50$). In contrast, the expression of the *TNNI3* gene differed in the two groups when it was normalized to the *ACTB* expression (Wilcoxon two-sample test, two sided, $P=0.02$) (Figure 4).

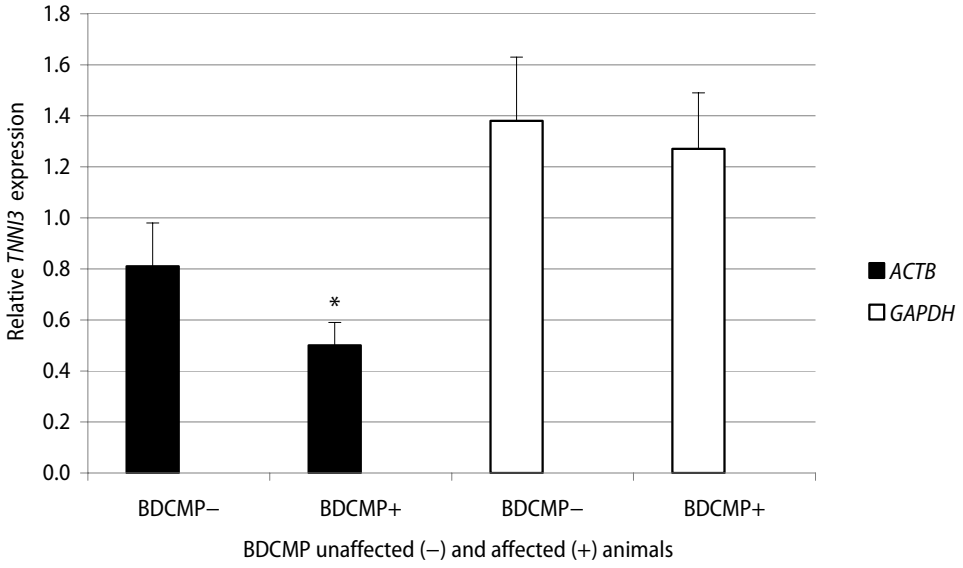

Figure 4
Relative quantification of real-time PCR results of *TNNI3* gene expression for unaffected (–) and BDCMP affected (+) animals. *TNNI3* gene expression normailized to *ACTB* gene expression was significantly different between the two groups ($P=0.02$) whereas no significant difference between the groups was found when normalized to *GAPDH* gene expression ($P=0.50$).

Relative Quantifizierung der Real-Time-PCR Resultate der TNNI3 Genexpression für gesunde (–) und BDCMP erkrankte (+) Tiere. Die TNNI3 Genexpression war zwischen den beiden Gruppen signifikant verschieden, wenn zur ACTB Genexpression normalisiert wurde (P=0.02). Dagegen wurde bei einer Normalisierung mit der GAPDH Genexpression (P=0.50) kein signifikanter Unterschied zwischen den beiden Gruppen gefunden.

According to the achieved results we assume that the discrepancies in *ACTB* and *GAPDH* expression might be caused by changes in the composition of cells in BDCMP affected

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and unaffected heart tissues. During the disease development an increased amount of collagen fibers and a transmural myocardiofibrosis are observed (TONTIS *et al.* 1990). Differences in the abundance of *ACTB* and *GAPDH* in fibroblasts and cardiomyocytes might be a reason of this inconsistency. However, the small sample number should also be considered.

In addition to the real time PCR experiment we performed a Nothern analysis. The mean expression ratio between *TNNI3* and *GAPDH* genes in heart tissues of three affected and three unaffected animals were 1.6 and 2.1, respectively. However, it was found that the difference is not significant (Wilcoxon two-sample test, two sided, P=0.1) which is in agreement with the real time PCR experiment. The *TNNI3* transcripts in affected and unaffected animals did not show any distinctive features as judged by the Northern blot (Figure 5). Furthermore we could not detect any TNNI3 gene antisense transcript as found in human and rat heart (BARTSCH *et al.* 2004). This indicates that there are no TNNI3 antisense transcripts present in bovine heart tissues or it is much less abundant then in human and rat heart.

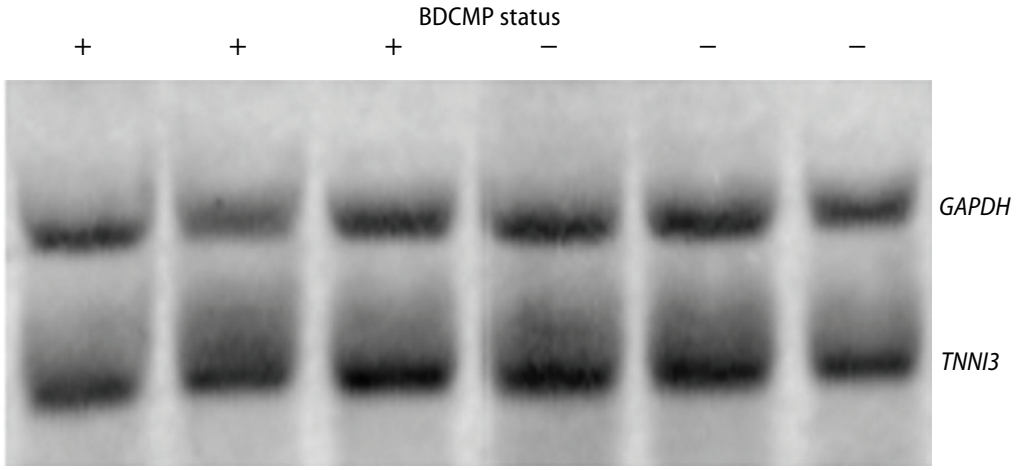

Figure 5
Northern blot analysis of the *TNNI3* gene expression in three BDCMP unaffected (–) and three BDCMP (+) affected animals. *TNNI3* gene expression was normalized to *GAPDH* gene expression.
Northern Blot Analyse der TNNI3 Genexpression in drei gesunden (–) und drei BDCMP erkrankte (+) Tiere. Die TNNI3 Genexpression wurde zur GAPDH Genexpression normalisiert.

In conclusion, the bovine *TNNI3* gene, due to its chromosomal position and an essential function in contractile apparatus, was strongly suggested to be a plausible candidate gene for BDCMP. The recently fine mapping of BDCMP locus within a 6.7 Mb interval, between microsatellite markers MSBDCMP06 and BMS2785 (GUZIEWICZ *et al.* 2007) excludes *TNNI3* gene as positional candidate gene. However, the *TNNI3* gene could not be conclusively excluded as in the study's BDCMP pedigree a high LOD score of 3.37 was obtained at that locus. Therefore in the present study we described and thoroughly investigated the bovine *TNNI3* gene. A single intronic polymorphism in *TNNI3* gene was found, but not in perfect disequilibrium with BDCMP mutation. Thus, we could exclude this mutation as being causative. Furthermore, the position of *TNNI3* on the current

bovine genome assembly (build 4.0) is more distal from the 6.7 Mb interval than it was on the previous version of the bovine genome sequence (Btau 3.1) supporting the exclusion of this candidate gene. Additionally, the examination of the *TNNI3* expression strongly supports that mutations in the bovine *TNNI3* gene do not cause BDCMP.

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CHAPTER TWO

Part I

The locus for bovine dilated cardiomyopathy maps to chromosome 18

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The locus for bovine dilated cardiomyopathy maps to chromosome 18

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Summary

Bovine dilated cardiomyopathy (BDCMP) is a severe and terminal disease of the heart muscle observed in Holstein–Friesian cattle over the last 30 years. There is strong evidence for an autosomal recessive mode of inheritance for BDCMP. The objective of this study was to genetically map BDCMP, with the ultimate goal of identifying the causative mutation. A whole-genome scan using 199 microsatellite markers and one SNP revealed an assignment of BDCMP to BTA18. Fine-mapping on BTA18 refined the candidate region to the *MSBDCMP06–BMS2785* interval. The interval containing the *BDCMP* locus was confirmed by multipoint linkage analysis using the software LOKI. The interval is about 6.7 Mb on the bovine genome sequence (Btau 3.1). The corresponding region of HSA19 is very gene-rich and contains roughly 200 genes. Although telomeric of the marker interval, *TNNI3* is a possible positional and a functional candidate for BDCMP given its involvement in a human form of dilated cardiomyopathy. Sequence analysis of *TNNI3* in cattle revealed no mutation in the coding sequence, but there was a G-to-A transition in intron 6 (AJ842179:c.378+315G>A). The analysis of this SNP using the study's BDCMP pedigree did not conclusively exclude *TNNI3* as a candidate gene for BDCMP. Considering the high density of genes on the homologous region of HSA19, further refinement of the interval on BTA18 containing the *BDCMP* locus is needed.

Keywords bovine dilated cardiomyopathy, BTA18, genome scan, *TNNI3*.

Introduction

Bovine dilated cardiomyopathy (BDCMP) is a heart muscle disease observed worldwide in Holstein–Friesian cattle during the last 30 years. The first cases of BDCMP were reported in the late 1970s in Switzerland (Martig *et al.* 1982), Japan (Sonoda *et al.* 1982) and Canada (Baird *et al.* 1986). In Switzerland, the introduction of Red Holstein genetics into the Simmental population began in 1968. These crosses are referred to as Fleckvieh and form a breed separate from Simmental and Red Holstein. BDCMP affects Fleckvieh and Red Holstein cattle but not the Simmental cattle of the Swiss Fleckvieh breed (Martig *et al.* 1982; Martig 1992; Graber & Martig 1993). The disease is characterized by a global cardiac enlargement and ventricular remodelling leading to a chamber dilatation with decreased wall thickness and a diminution in systolic function. A subacute oedema within the brisket and to a lesser extent in the jaw and the ventral abdominal region is usually observed. Moreover, an increased pulse rate, up to 140 beats/min, tachycardia with gallop rhythm of the heart and distention with congestion of the jugular veins are common in BDCMP-affected animals. The typical age at onset of BDCMP is between 2 and 4 years but BDCMP was also diagnosed in calves younger than 2 months and in cows older than 8 years (Martig & Reusser 1988; Martig 1992; Graber & Martig 1993). Although BDCMP is a slowly progressive terminal disease, distinct clinical symptoms develop within a few days or weeks, and all affected animals die or have to

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be culled shortly after disease onset (Martig *et al.* 1982; Sonoda *et al.* 1982; Martig & Tschudi 1985; McLennan & Kelly 1990; Tontis *et al.* 1990; Bradley *et al.* 1991; Kümper & Bahnemann 1992; Danzl 1995). Initial cardiomyocyte necrosis often occurs in all parts of the heart followed by transmural fibrosis (König *et al.* 1990). First, the left ventricle fails to compensate, leading to pulmonary hypertension, followed by hypertrophy of the right ventricle, which finally causes the clinically manifest right heart failure (Lobsiger *et al.* 1985; Martig & Tschudi 1985). BDCMP can be easily misdiagnosed as traumatic pericarditis or valvular endocarditis (Martig *et al.* 1982). To date, there is no treatment known for BDCMP.

In the first reports, it was speculated that this form of bovine cardiomyopathy was inherited. Later, it was shown that all reported cases in Canada (Baird *et al.* 1986), Japan (Satoh 1988) and Switzerland (Martig *et al.* 1982; Martig & Reusser 1988) traced back to a red factor carrier Holstein–Friesian bull, ABC Reflection Sovereign, strongly suggesting a hereditary nature of the disease. Satoh (1988) proposed an autosomal recessive mode of inheritance. Further segregation analysis based on an experimental BDCMP pedigree confirmed the genetic basis of an autosomal recessive mode of inheritance for the disease (Dolf *et al.* 1998). In addition, environmental factors such as gestation, parturition, lactation or obesity may play a role in the age at onset of BDCMP (Martig & Reusser 1988; Satoh 1988; Tontis *et al.* 1990; Graber & Martig 1993; Danzl 1995; Dolf *et al.* 1998). Recently, BDCMP cases were reported in Denmark (Leifsson & Agerholm 2004). BDCMP is periodically diagnosed in the slaughterhouses in Switzerland, but the frequency of the mutant allele responsible for the disease remains unknown.

The aim of this study was to genetically map the *BDCMP* locus leading to the identification of the causative mutation. Furthermore, we characterized a potential candidate gene, *TNNI3*, which encodes cardiac troponin I.

Materials and methods

Animals

In 1989, we established a herd segregating for BDCMP. Two Fleckvieh AI bulls, which were reported to be affected by BDCMP, were used as founders. Later, another three Fleckvieh AI bulls were added to the pedigree. These three AI bulls were known BDCMP carriers as they each had at least six affected offspring and their mothers were purebred Simmental. In 1994, three Angus AI bulls and two Angus cows were used to introduce polledness into the herd. The final pedigree consisted 114 animals across extended full- and half-sib families produced by embryo transfer (Fig. 1).

Tissue samples

Blood samples for all animals except the AI bulls were collected in 10 ml EDTA vacutainer tubes and stored at −80 °C. Semen samples for the AI bulls were provided by Swissgenetics (formerly Swiss Association for Artificial Insemination), and DNA was isolated using standard protocols. Lymph nodes were collected at slaughter and stored at −80 °C as additional DNA sources. For the pathological diagnoses, heart, kidney, liver and lung samples were collected at slaughter and conserved as paraffin blocks for most of the animals in the pedigree except the AI bulls and cows from private breeders.

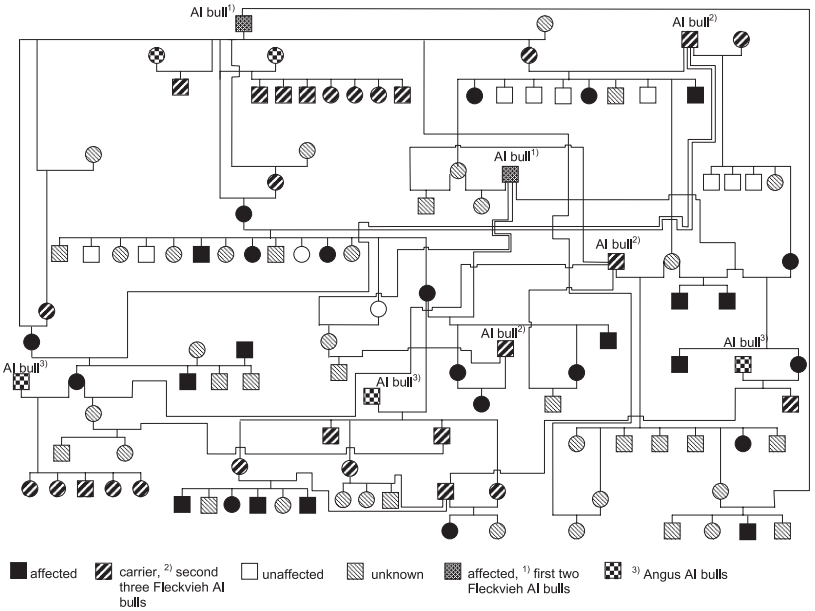

Figure 1 The experimental bovine dilated cardiomyopathy pedigree.

BDCMP diagnosis

Macroscopic and microscopic examination of heart, lung, liver and kidney was performed on all animals except the AI bulls and cows from private breeders. Based on necropsy findings in 400 BDCMP animals (Tontis *et al.* 1990), criteria were established to classify animals in one of four diagnostic groups (Dolf *et al.* 1998). In the first group, animals were slaughtered because of clinically manifested BDCMP that was confirmed at necropsy. The second group showed strong evidence for BDCMP without clinical signs but at necropsy there was strong evidence for the disease. The third group included animals with no clinical signs at necropsy of BDCMP and only a focal scale-like myocardial fibrosis. The fourth group consisted of animals without any pathological findings related to BDCMP. The 114 animals of the BDCMP pedigree were assigned as follows: 30 affected animals (first and second group), 43 unaffected animals (fourth group) and 41 animals of unknown status (third group and unknown cases) (Fig. 1).

DNA extraction and microsatellite genotyping

DNA was extracted from blood using Chelex® 100 (BioRad) or the High Pure PCR Template Preparation Kit (Roche). The latter method was also used for DNA isolation from lymph nodes. DNA extraction from semen was based on the guanidine–HCl extraction method (Bahnak *et al.* 1988). Amplified marker sequences were separated on 8% denaturing polyacrylamide gels (6% SequaGel XR, Milian, complemented with 50% Long Ranger Gel Solution, Bioconcept) using LI-COR DNA sequencer models 4000 and 4200 (LI-COR Biosciences).

Genotyping of microsatellite *MSBDCMP06* and the *TNNI3* SNP

Microsatellite *MSBDCMP06* was developed by amplifying a tandem repeat in the bovine genome sequence (Btau 3.1) from BTA18 using primers that spanned nucleotides at positions 47232048 to 47232073 and 47232241 to 47232269 respectively. Two *TNNI3* alleles were identified by direct sequencing of a PCR product amplified with the TNNI3int6f (5′-AGGAGAGATACGACGTGGAGGCGAAAG-3′) and TNNI3int6r (5′-CGCAGAGTGGGCCGCTTAAACTTG-3′) primers.

Genome scan and linkage analysis

A total of 199 microsatellite markers were genotyped on animals from the BDCMP pedigree. Chromosomal maps were constructed by ordering the markers using CRI-MAP version 2.4 (Green *et al.* 1990), supported by the physical map of the markers deduced from the draft whole-genome bovine sequence (Btau 3.1). Two-point linkage analyses between the disease phenotype and the markers were performed using FASTLINK version 4.0p (Cottingham *et al.* 1993). Parameters characterizing the biallelic locus underlying BDCMP were estimated previously (Dolf *et al.* 1998). The frequency of the mutant allele (a) was estimated as 0.684 in the pedigree. Penetrances for the three genotypes (*AA*, *Aa* and *aa*) were set as 0.000, 0.000 and 1.000 respectively. Multipoint analyses were carried out using LOKI, version 2.4.6 (Heath 1997). The model included one major gene. For each analysis, 110 000 iterations were calculated, of which the first 9999 were discarded. Start values of 7 were chosen for the mean and the residual variance. Tau_beta and tau_mode were set to 2, which made the variance of the normal prior distribution of the trait loci effects tau_beta x the current residual variance at each iteration. The total map length was set to 3500 Kosambi cM. The maximal number of trait loci was set to 10, with a mean of 2 and a start value of 3.

TNNI3 sequence

Three clones (388A05, 477A02 and 428H02) that contained at least part of *TNNI3* were isolated from a bovine BAC library (Eggen *et al.* 2001), pooled, partially digested with *Sau*3AI and cloned into pUC19 (Roche Diagnostics) (Eggen *et al.* 2001). DNA of clones positive for *TNNI3* was sequenced using the Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Biosciences). The sequencing reactions were run on 4.3% denaturing polyacrylamide gels on a LI-COR 4200 (LI-COR) and analysed with eSeq and AlignIR (LI-COR).

Results and discussion

Two-point linkage analysis of 199 microsatellite markers and one SNP with the *BDCMP* locus resulted in an average LOD score of 0.26 ± 0.32. The LOD score threshold of 1.22 was calculated as the average LOD score plus three times the standard deviation. LOD scores above the threshold were found for *BMS4008* (BTA1, LOD = 1.62), *BMS1290* (BTA9, LOD = 1.33), *BMS1758* (BTA11, LOD = 1.69) and *BMS7109* (BTA18, LOD = 2.84). These results suggested that the *BDCMP* locus was located on BTA18. Therefore, animals in the pedigree were genotyped for additional markers on BTA18. A genetic map with a subset of 22 of the 63 typed BTA18 markers and their two-point LOD scores are given in Table 1. High LOD score values were obtained for markers *MSBDCMP06*, *DIK3006*, *DIK3005*, *RME01* and *BMS2785*, as well as for the SNP in *TNNI3* intron 6, which was telomeric to the microsatellite interval.

Genetic maps were constructed for all autosomal chromosomes using CRI-MAP. On each chromosome, 3–13 microsatellite markers were mapped using the BDCMP pedigree information. These maps, together with the BTA18 genetic map presented in Table 1, were used to perform the multipoint linkage analysis with LOKI. The multipoint

Table 1 Recombination fraction, genetic linkage map positions (sex averaged) and two-point LOD scores for 22 markers on BTA18.

Marker	LOD score	Θ^1	Position (cM)[2]
BMS1355	0.01	0.449	0.0
BMS1322	0.00	0.55	10.5
ABS013	0.13	0.398	13.3
INRA121	0.52	0.169	30.0
BM8151	1.26	0.147	39.0
BM7109	2.69	0.079	47.0
BMS2914	1.03	0.126	48.0
BMS2639	1.82	0.096	53.2
DIK4232	1.06	0.001	56.0
MSBDCMP06	5.36	0.001	64.1
BB710	4.00	0.01	69.2
DIK2464	2.75	0.001	71.5
DIK3006	5.48	0.001	72.6
DIK3005	5.62	0.001	75.2
IDVGA55	4.37	0.001	81.4
RME01	5.48	0.001	82.7
DIK4823	3.15	0.001	84.6
RM128	2.91	0.075	87.0
BMS2785	4.58	0.001	88.2
TNNI3 SNP	3.37	0.001	93.5
TGLA227	1.14	0.206	104.5
DIK5235	1.48	0.107	109.4

[1]Recombination rate between BDCMP and marker.
[2]Kosambi cM.

linkage analysis using LOKI produced a Bayes Factor (BF) (posterior/prior ratio) for each chromosome and then the chromosome was split into bins (default 1 cM). The bin with the highest BF was considered to be the location score. In the multipoint linkage analysis, BTA18 was implicated with a BF of 23.76 (next best chromosome BF < 0.25). On BTA18, the bin with the highest BF of 380.79 was located between *DIK3005* and *IDVGA55*.

The results from the multipoint linkage analysis were in agreement with the two-point linkage analysis and provided convincing evidence that the *BDCMP* locus is located on BTA18 in the interval between markers *MSBDCMP06* and *BMS2785*. We further analysed the pedigree divided into 12 nuclear families. These tests showed that two out of the 12 nuclear families had two-point LOD score values discordant with the other ten families. Two-point and multipoint analyses without those two families significantly improved the test statistics. However, the *BDCMP* locus still mapped within the *MSBDCMP06* and *BMS2785* interval.

The interval between *MSBDCMP06* and *BMS2785* corresponds to a physical distance of 6.7 Mb (Btau 3.1). The homologous region on HSA19 is very dense and contains more than 200 annotated genes. No gene is obviously involved in heart function. Interestingly, the *troponin I* gene (*TNNI3*) telomeric to this interval is a functional candidate for BDCMP because of its role in the contractile apparatus of the heart muscle (Perry 1999). A recessive mutation identified in this gene leads to dilatative cardiomyopthie in humans (Murphy *et al.* 2004). Histological findings of heart included myofibrillar loss, hyperchromatic nuclei and myocyte hypertrophy.

The bovine *TNNI3* gene was sequenced in this study (AJ842179). Genomic DNA from three to six affected and unaffected individuals was sequenced for the whole gene, including 497 bp 5′ of the translation initiation site and 84 bp 3′ of the polyA signal. There were no differences between the sequences of the eight exons of the cardiac *TNNI3* gene from affected and non-affected animals. However, a single G-to-A transition was found in *TNNI3* intron 6 (c.378+315G>A). This SNP was genotyped across the BDCMP pedigree. The two-point LOD score was significant at 3.37 (Table 1) and thus significant. Of the 28 BDCMP affected animals in the pedigree, 16 were homozygous c.378+315AA and 12 were heterozygous c.378+315AG. We concluded that the c.378+315G>A is not causative based on the proposed recessive mode of inheritance. However, the complex manifestation of the disease and its variable age of onset complicates accurate phenotyping of the animals. Also, there is the possibility that regulatory non-coding mutations of the *TNNI3* gene were not identified in our sequence and therefore, not tested for association with the trait. In order to pursue additional candidate genes for BDCMP, it will be essential to narrow the BTA18 interval given the high density of genes on HSA19.

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CHAPTER TWO

Part II

The bovine dilated cardiomyopathy locus maps to a 1.0-Mb interval on chromosome 18

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The bovine dilated cardiomyopathy locus maps to a 1.0-Mb interval on chromosome 18

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Abstract Cardiomyopathies are myocardial diseases that lead to cardiac dysfunction, heart failure, arrhythmia, and sudden death. In human medicine, cardiomyopathies frequently warrant heart transplantation in children and adults. Bovine dilated cardiomyopathy (BDCMP) is a heart muscle disorder that has been observed during the last 30 years in cattle of Holstein-Friesian origin. In Switzerland BDCMP affects Swiss Fleckvieh and Red Holstein breeds. BDCMP is characterized by a cardiac enlargement with ventricular remodeling and chamber dilatation. The common symptoms in affected animals are subacute subcutaneous edema, congestion of the jugular veins, and tachycardia with gallop rhythm. A cardiomegaly with dilatation and hypertrophy of all heart chambers, myocardial degeneration, and fibrosis are typical postmortem findings. It was shown that all BDCMP cases reported worldwide traced back to a red factor-carrying Holstein-Friesian bull, ABC Reflection Sovereign. An autosomal recessive mode of inheritance was proposed for BDCMP. Recently, the disease locus was mapped to a 6.7-Mb interval *MSBDCMP06-BMS2785* on bovine Chr 18 (BTA18). In the present study the BDCMP locus was fine mapped by using a combined strategy of homozygosity mapping and association study. A BAC contig of 2.9 Mb encompassing the crucial interval was constructed to establish the correct marker order on BTA18. We show that the disease locus is located in a gene-rich interval of 1.0 Mb and is flanked by the microsatellite markers *DIK3006* and *MSBDCMP51*.

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Introduction

Cardiomyopathies are diseases of the myocardium associated with cardiac dysfunction. In human medicine they are classified as dilated cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy (Richardson et al. 1996). Many conditions characteristic for one form of a cardiomyopathy may progress to another condition. Cardiomyopathy frequently results in heart failure, arrhythmia, and sudden death. The histologic findings in such a dysfunctional myocardium are nonspecific, with hypertrophic myocytes, cellular necrosis, and fibrosis.

Bovine dilated cardiomyopathy (BDCMP) is a primary disease of the heart muscle observed worldwide in Holstein-Friesian cattle during the last 30 years. In Switzerland the introduction of Red Holstein genetics into the Simmental population began in 1968. These crosses are referred to as Fleckvieh and form a section apart from Simmental and Red Holstein. BDCMP affects Fleckvieh and Red Holstein cattle but not the Simmental cattle of the

Swiss Fleckvieh breed (Graber and Martig 1993; Martig 1992; Martig et al. 1982). The disease is characterized by a global cardiac enlargement and ventricular remodeling leading to a chamber dilatation with decreased wall thickness and a diminution in systolic function. A subacute subcutaneous edema within the brisket and to a lesser extent in the mandibular and the ventral abdominal region is usually observed. Moreover, an increased pulse rate, up to 140 beats/min, tachycardia with gallop rhythm of the heart, and congestion of the jugular veins are common in affected animals. The typical age at onset of BDCMP is between 2 and 4 years but BDCMP was also diagnosed in calves younger than 2 months and in cows older than 8 years (Graber and Martig 1993; Martig 1992; Martig and Reusser 1988). Although BDCMP is a slowly progressive terminal disease, distinct clinical symptoms develop within a few days or weeks and all affected animals die or have to be culled shortly after disease onset (Bradley et al. 1991; Danzl 1995; Kümper and Bahnemann 1992; Martig and Tschudi 1985; Martig et al. 1982; McLennan and Kelly 1990; Sonoda et al. 1982; Tontis et al. 1990). Initially, cardiomyocyte necrosis in all parts of the heart followed by transmural fibrosis often occurs (König et al. 1990). First, the left ventricle fails to compensate, leading to pulmonary hypertension, followed by hypertrophy of the right ventricle, which finally causes the clinically manifest right heart failure (Lobsiger et al. 1985; Martig and Tschudi 1985). BDCMP can be easily misdiagnosed as traumatic pericarditis or valvular endocarditis (Martig et al. 1982). The pathologic findings are cardiomegaly with an extensive dilatation and hypertrophy of all sections of the myocardium, as well as myocardial fibrosis, partially accompanied by myofibrillar degeneration. To date, there is no treatment known for BDCMP.

All reported cases in Canada (Baird et al. 1986), Japan (Satoh 1988), and Switzerland (Martig and Reusser 1988; Martig et al. 1982) were traced back to a red factor-carrying Holstein–Friesian bull, ABC Reflection Sovereign, which strongly suggests a hereditary nature of the disease. A segregation analysis based on an experimental BDCMP pedigree confirmed the genetic basis of an autosomal recessive mode of inheritance for the disease (Dolf et al. 1998). Recently, BDCMP cases were reported in Denmark (Leifsson and Agerholm 2004). BDCMP is periodically diagnosed in slaughterhouses in Switzerland, but the frequency of the mutant allele responsible for the disease remains unknown.

The locus for bovine dilated cardiomyopathy was mapped to Chr 18 (BTA18) in the *MSBDCMP06–BMS2785* interval (Guziewicz et al. 2007). In present study we fine-mapped the BDCMP locus to a 1.0-Mb interval by a combined homozygosity mapping and association-based strategy.

Material and methods

BAC libraries

Two bovine BAC libraries were explored to establish a BAC contig around the BDCMP locus on BTA18 (CHORI-240: bovine BAC library, Children's Hospital Oakland Research Institute, USA and the INRA bovine BAC library) Eggen et al. 2001). CHORI BAC clones were selected based on the contig information of BTA18 which was released together with the bovine genome assembly Btau 3.1 (http://www.hgsc.bcm.tmc.edu/projects/bovine/). The INRA BAC library was PCR-screened to identify the corresponding BAC clones. In addition, we used the web-based BAC contig information tool (http://genome.jouy.inra.fr/WebAGCoL/CarteBovinGenet/WebFPC/).

DNA extraction from BAC clones

BAC DNA was isolated according to the rapid alkaline lysis miniprep method developed by the Children's Hospital Oakland Research Institute (http://bacpac.chori.org/bacpacmini.htm).

BAC clones screening

BAC DNA from clones of the CHORI BAC library was tested by PCR. BAC DNA pools from the INRA BAC library were first PCR-screened and subsequently positive clones were examined by PCR-based strategy for the presence of specific markers and genes. The PCR amplification mixture contained 1.5 µl of BAC DNA (25 ng), 0.4 µl of the primer (10 pmol/µl, forward and reverse, respectively), 0.2 µl of dNTPs mix (5 mM), 1 µl of 10 × PCR buffer, and 0.05 µl of AmpliTaqGold polymerase (5 U/µl), and deionized water was added to a final volume of 10 µl (Applied Biosystems, Rotkreuz, Switzerland). The amplification was performed with an initial denaturation step at 95°C for 20 min, followed by 30 cycles with a denaturation step at 94°C for 30 sec, an annealing step at 60°C for 1 min, an extension step at 72°C for 2 min, and a final extension phase at 72°C for 10 min.

The amplicons were separated on 1% agarose gels, stained with ethidium bromide, and visualized under UV light. Primer pairs were designed with the PRIMER3 software and are listed in Supplementary Table 1.

To screen BAC clones, 20 microsatellite markers, 7 PCR products with known location on BTA18, and 27 PCR products of orthologous genes located on human counterpart Chr 19 (HSA19) were used.

DNA extraction

DNA from 49 blood samples was isolated using Chelex® 100 (BioRad, Reinach BL, Switzerland) or a High Pure PCR Template Purification Kit (Roche Diagnostics, Rotkreuz, Switzerland). DNA from 304 formalin-fixed and thereafter paraffin-embedded myocardial samples from BDCMP-affected animals was isolated and purified using a DNeasy Blood & Tissue Kit (Qiagen, Hombrechtikon, Switzerland).

Genotyping with microsatellite markers

In the present study, samples from 353 animals were genotyped for 24 microsatellite markers (MS) in the interval between *MSBDCMP06* and *BMS2785*, whereof 9 were selected from publically available data and 15 represent newly developed markers taken from the bovine genome sequence assembly Btau 4.0 (Supplementary Table 2).

The volume of a multiplex amplification reaction was 10 µl and contained 2 µl of DNA (20 ng), 0.2 µl of each primer (10 pmol/µl, forward and reverse, respectively), 5 µl of Multiplex PCR Master Mix (Qiagen), and 1 µl of 5 × Q-solution. Water was added to reach the final volume. Most of the multiplex reactions contained a combination of five different primer pairs. The PCR was conducted under the following conditions: initial denaturation step at 95°C for 15 min, 30 cycles with a denaturation step at 94°C for 30 sec, an annealing step at 60°C for 1 min, and an extension step at 72°C for 45 sec. The final extension step was carried out for 30 min at 60°C. PCR products were initially examined on 2% agarose gels and, subsequently, 1 µl of each PCR was combined with 10 µl of genotyping mix (980 µl of HiDi formamide and 20 µl of GeneScan™-500 LIZ™ Size Standard, Applied Biosystems). After a 2-min denaturation at 95°C and chilling on ice, samples were genotyped on an ABI 3730 capillary sequencer (Applied Biosystems). The data were processed using the GeneMapper® software ver. 4.0 (Applied Biosystems).

SNP genotyping

Annotated SNPs (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp) were selected according to their location within the interval of interest on BTA18. Additional SNP markers were developed in order to increase the marker density in the critical interval. These SNPs in the 1.0-Mb region between microsatellites markers *DIK3006* and *MSBDCMP51* flanked by the distal genes *APOE* and *NPAS1* were genotyped using a panel of 18 BDCMP-affected animals and 18 controls. In total, 38 PCR products were obtained according to standard protocols (Supplementary Table 3). These 38 PCR products each contained one to eight SNPs resulting in a total of 123 SNPs. Amplicons were purified with rAPid alkaline phosphatase (Roche) and exounuclease I (N.E.B., Bioconcept, Allschwil, Switzerland). Both strands were directly sequenced on an ABI 3730 capillary sequencer (Applied Biosystem) using the BigDye® Terminator Cycling Kit (Applied Biosystems). Sequencing data were analyzed using the Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistical analysis

The statistical analysis including genotypes of 123 SNP markers was initially performed with SAS Genetics 9.1.3 software (SAS Institute Inc., Cary, NC, USA) using the procedure *allele*. The established minor allele frequency (MAF) was 20%. In total, 86 SNP markers were analyzed with the SAS Genetics 9.1.3 software procedure *casecontrol* and with the Haploview 4.0 software (Barrett et al. 2005) (http://www.broad.mit.edu/mpg/haploview/download.php). A permutation test was carried out with 1000 permutations and the single marker procedure was selected.

Results and discussion

BAC contig construction

The comparison between the cattle and the human maps revealed that the critical region on bovine Chr 18 (BTA18q) is homologous to human Chr 19 (HAS19) (Guziewicz 2004). Recently, Guziewicz et al. (2007) demonstrated in an experimental pedigree segregating for BDCMP that the disease locus maps to a 6.7-Mb region encompassed by MS markers *MSBDCMP06* and *BMS2785* on BTA18 (Btau 3.1). This interval corresponds now to 5.6 Mb in the bovine genome sequence assembly Btau 4.0. The publicly available BAC contig listing of bovine CHORI BACs was initially used to build the contig around the BDCMP locus. Although the information of the overlapping genomic sequence was indicated in the BAC contig provided by the bovine genome project, several overlaps could not be verified by PCR-based STS content mapping. Therefore, additional BAC clones were introduced from the INRA bovine BAC library in the attempt to close the remaining gaps. The combination of clones from the two BAC libraries allowed us to construct a 2.9-Mb BAC contig spanning the interval between MS markers *MSBDCMP22* and *BMS2785* (Fig. 1b). The minimal tiling path of this contig consists of 23 tiled BAC clones. We found that the gene order on a chromosomal segment

between positions 51.11 and 52.12 Mb was inverted in Btau 3.1 as compared to our BAC contig and the human counterpart HSA19. This finding was then fully confirmed in the recent cattle genome assembly (Btau 4.0), where the gene order in the critical interval is in agreement with that in our BAC contig and human Chr 19. The BAC contig is an excellent tool toward fine mapping the critical interval, to map the DNA markers, and to explore the still existing and numerous DNA sequence gaps in the present version of the bovine genome.

Homozygosity mapping of the BDCMP locus

This approach aims to identify the autosomal recessive disease-causing locus by virtue of the fact that the adjacent DNA sequence is homozygous by descent. It is evident that all BDCMP-affected animals in the Swiss Fleckvieh population trace back to the Canadian bull ABC Reflection Sovereign (Dolf et al. 1998). Therefore, alleles adjacent to the BDCMP locus are considered identical by descent and share extended haplotypes. In an initial step 25 BDCMP-affected and 24 unaffected Fleckvieh animals were genotyped with nine MS markers randomly distributed within the interval between MS markers *MSBDCMP06* and *BMS2785*, previously defined by Guziewicz et al. (2007). All the affected animals were homozygous at the markers *MSBDCMP22*, *DIK3006*, *DIK3014*, *MSBDCMP25*, and *RME01* supporting the hypothesized identity by descent. Chromosomes were considered recombinant for an individual that is heterozygous for markers located distal of the interval *MSBDCMP06–BMS2785* and homozygous for adjacent markers located within this interval. We found 11 presumably recombinant chromosomes on the centromeric site and 9 on the telomeric site of the interval *MSBDCMP06–BMS2785*. This homozygosity mapping narrowed the critical interval down to about 4.1 Mb between markers *MSBDCMP06* and *MSBDCMP51*. An additional 15 evenly distributed MS markers were selected within the interval *MSBDCMP06–BMS2785* based on the bovine genome sequence assembly Btau 4.0. The markers were chosen so that they were evenly distributed in the interval. DNA from 304 paraffin-embedded heart tissue samples of cattle with a positive BDCMP diagnosis, including some cases with uncertain diagnoses, were available for genotyping. The advantage of these paraffin-embedded samples is that the BDCMP diagnosis can be reevaluated based on histologic criteria by an experienced pathologist. Furthermore, these paraffin-embedded samples were collected during a period of about 20 years at two different locations in Switzerland and the BDCMP animals were originally diagnosed by different pathologists. The reevaluation is important as BDCMP can be easily misdiagnosed. We genotyped all these samples with 24 MS markers in the interval *MSBDCMP06–BMS2785*. The identification of an additional 3 and 18 recombinant chromosomes from the centromeric and telomeric side, respectively, allowed us to reduce the critical interval from 4.1 to 1.0 Mb. The current 1.0-Mb interval is flanked by MS markers *DIK3006* and *MSBDCMP51* (Fig. 1a). It is striking that the BDCMP-affected animals, previously assumed to be unrelated, are all homozygous for the markers that were also homozygous in the BDCMP animals in the experimental pedigree. This finding strongly indicates that indeed all affected animals are descendents from a single founder animal.

Association study

In this study we combined homozygosity mapping with an association-based strategy to further fine map the BDCMP locus in the interval between MS *DIK3006* and *MSBDCMP51* (Fig. 1a). We included on each distal site of this interval additional SNP markers located in *APOE* and *NPAS1* genes, respectively. The association study was performed including these markers and indicates that the region outside of *DIK3006* and *MSBDCMP51* is not associated with BDCMP. In this interval a total of 36 PCR products containing one to eight SNP markers were genotyped by means of DNA sequencing for 18 BDCMP-affected and 18 unaffected animals. In addition, two families comprising eight individuals from the experimental pedigree were included in the genotyping procedure to deduce the disease haplotype. BAC clones were tested by PCR to map the SNP markers to our BAC contig. However, one of these PCR products containing six SNPs (Supplementary Table 3) was thereafter excluded from the association study because we could not amplify it on any of the BAC clones. This emphasizes that the BAC contig can be a useful tool in the fine mapping of disease genes.

The association study was initially performed using SAS Genetics software. A minimal MAF of 20% was applied in the analysis resulting in the exclusion of 31 SNP markers. The SNP in *NPAS1* gene was therefore excluded from the final analysis and is not presented in Fig. 1c. The genotypes of 86 remaining SNP markers were thereafter analyzed using the Haploview 4.0 software. The best observed χ^2 was 60.4 and the best permutation χ^2 (1000 permutations, single marker) was 21.4. The analyses revealed significant association between markers and disease over the whole interval *DIK3006-MSBDCMP51* and showed three highly associated regions (Fig. 1c).

The human HSA19 counterpart of this 1.0-Mb interval corresponds to a region between 50.50 and 52.11 Mb (Build 36.6). This is a gene-rich region and contains 47 genes and 18 annotated hypothetical loci. In the mouse this region was found on Chr 7 (MMU7) between 17.07 and

19.99 Mb (Build 37.1) but the gene order is inverted compared to that in human and cattle.

An *in silico* evaluation showed that among 47 genes on HSA19, *CKM*, *VASP*, *PPP1R13L*, *DMPK*, and *AP2S1* genes are highly expressed in the cardiovascular system; moderately expressed are the *GIPR*, *PNMAL2*, and *GRLF1* genes, and *GPR4*, *IGFL3*, *HIF3A*, *PRKD2*, *STRN4*, and *FKRP* are reported to be expressed in the heart (http://www.dsi.univ-paris5.fr/genatlas/, http://www.ncbi.nlm.nih.gov/). An association between two of these genes, the fukutin-related protein gene (*FKRP*) and the vasodilator-stimulated phosphoprotein gene (*VASP*), and dilated cardiomyopathy was reported. Müller et al. (2005) found evidence that dilated cardiomyopathy in human may be associated with a C826A mutation in the *FKRP* gene. Although mutations in this gene are mostly responsible for limb-girdle muscular dystrophy (LGMD2I), they described three siblings showing the C826A substitution and an unusual phenotype with dilated cardiomyopathy and without changes in skeletal muscles. Our preliminary results indicate that there are no differences in the *FKRP* sequence between BDCMP-affected and unaffected animals. Eigenthaler et al. (2003) described that a disruption of cardiac Ena-VASP protein in mice may play a crucial role in intercalated disk function at the interface between cardiac myocytes and thereby might cause dilated cardiomyopathy. However, to our knowledge no mutation in the *VASP* gene in any species has yet been reported to be associated with cardiomyopathy.

In conclusion, using the combined strategy of homozygosity mapping and association study, we were able to fine map the BDCMP locus to a region of about 1.0 Mb. At

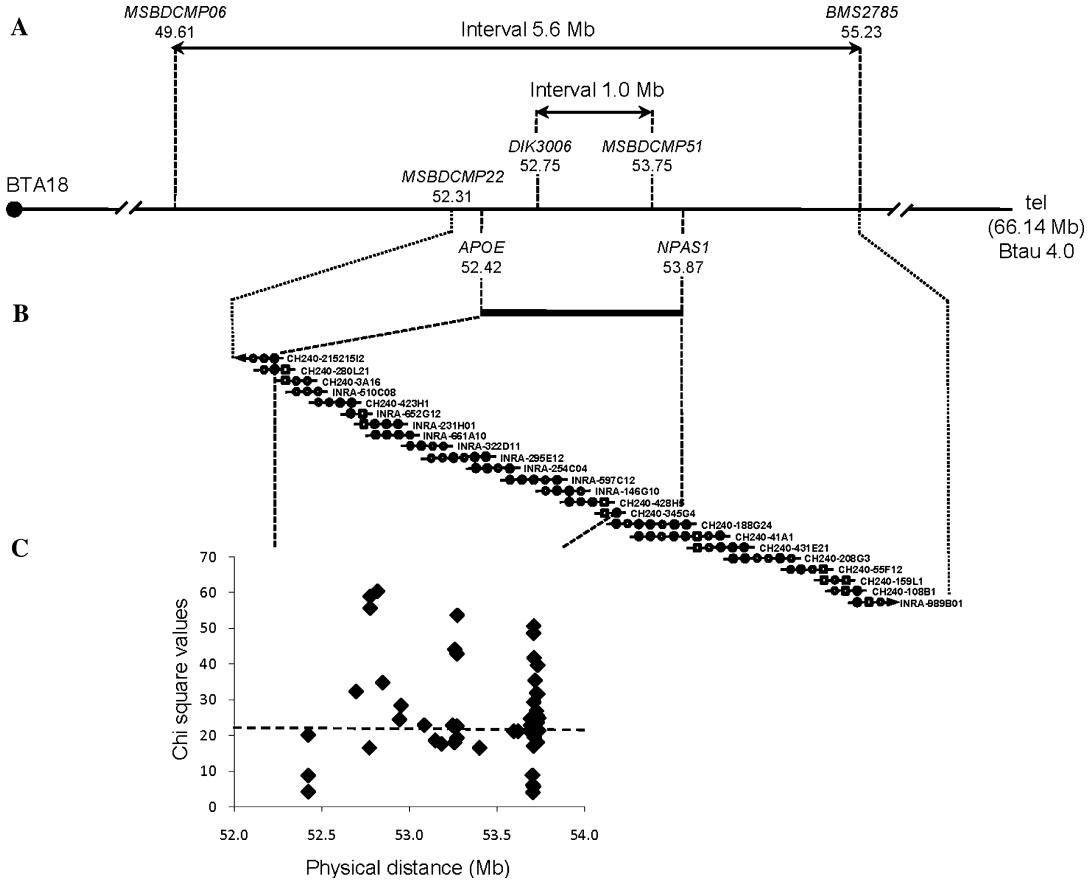

Fig. 1 Genetic mapping of the BDCMP locus. **a** Initial interval defined to 6.7 Mb between MS markers *MSBDCMP06* and *BMS2785* based on Btau 3.1 corresponds to a physical distance of 5.6 Mb on Btau 4.0. Current interval of 1.0 Mb is flanked by MS markers *DIK3006* and *MSBDCMP51*. The association study was performed in the 1.0-Mb interval surrounded by the distal genes *APOE* and *NPAS1*. **b** BAC contig of 2.9 Mb between MS markers *MSBDCMP22* and *BMS2785* composed of 23 BAC clones. Open circles, filled circles, and open squares indicate MS markers, genes, and PCR products with known location on BTA18, respectively. **c** Schematic presentation of the association analysis (Haploview 4.0 software). The best observed χ^2 was 60.44. The dashed line indicates the significance threshold obtained by permutation testing (best permutation χ^2 was 21.45, 1000 permutations). χ^2 values are plotted against the physical distance on BTA18. In total, 86 SNP markers with MAF above 20% were analyzed

present we are screening recombinant animals with additional SNP markers to further narrow down the interval. In addition, we will further investigate those genes that are expressed in the cardiovascular system. It is still possible that genes with not yet known function as well as regulatory mutations or chromosomal rearrangements may play an important role in the development of BDCMP.

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