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Characterization of the canine desmin (*DES*) gene and evaluation as a candidate gene for dilated cardiomyopathy in the Dobermann

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Abstract

Canine-dilated cardiomyopathy (DCM) in dogs is a disease of the myocardium associated with dilatation and impaired contraction of the ventricles and is suspected to have a genetic cause. A missense mutation in the desmin gene (*DES*) causes DCM in a human family. Human DCM closely resembles the canine disease. In the present study, we evaluated whether *DES* gene mutations are responsible for DCM in Dobermann dogs. We have isolated bacterial artificial chromosome clones (BACs) containing the canine *DES* gene and determined the chromosomal location by fluorescence in situ hybrizidation (FISH). Using data deposited in the NCBI trace archive and GenBank, the canine *DES* gene DNA sequence was assembled and seven single nucleotide polymorphisms (SNPs) were identified. From the canine *DES* gene BAC clones, a polymorphic microsatellite marker was isolated. The microsatellite marker and four informative desmin SNPs were typed in a Dobermann family with frequent DCM occurrence, but the disease phenotype did not associate with a desmin haplotype.

We concluded that mutations in the *DES* gene do not play a role in Dobermann DCM. Availability of the microsatellite marker, SNPs and DNA sequence reported in this study enable fast evaluation of the *DES* gene as a DCM candidate gene in other dog breeds with DCM occurrence.

Keywords: BAC clone; Microsatellite marker; FISH; Sequence; SNP; Heart disease

1. Introduction

Dilated cardiomyopathy (DCM) is a myocardial disease representing an important cause of congestive heart failure (CHF) and sudden death in dogs. It primarily affects large and giant breed dogs with Dobermanns being one of the most frequently affected (Domanjko-Petrič et al., 2002; Sisson and Thomas, 1995). Data regarding the mode of inheritance in the Dobermanns is conflicting. Both autosomal dominant and autosomal recessive modes of transmission have been suggested (Hammer et al., 1996; Meurs, 2002). The high prevalence of DCM in specific breeds suggests a genetic background, but causal muta-

Abbreviations: DCM, dilated cardiomyopathy; BAC, bacterial artificial chromosome; CHF, congestive heart failure; bp, base pairs; kb, kilo base pairs; CFA, *Canis familiaris chromosome*; FISH, fluorescence in situ hybridization; *DES*, desmin gene; DBGSS, Database of the Genome Survey Sequence; DBWGS, Database of the Whole Genome Shotgun Sequence; HSA, *Homo sapiens* chromosome; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; dNTP, deoxyribonucleoside triphosphate.

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tions have not yet been identified. Within each breed, DCM has unique characteristics and, between breeds, it is probably a genetically heterogeneous disease. DCM in the Dobermann is characterised by severe left ventricular dilatation, systolic dysfunction and the so-called occult (preclinical) phase during which ventricular and atrial premature contractions occur. Dobermanns either die suddenly or develop congestive heart failure (Calvert et al., 1997).

Human DCM, which closely resembles the canine form of the disease, is a genetically heterogeneous disease with 13 disease genes identified (Knoll et al., 2002; Schmitt et al., 2003; for review, see Fatkin and Graham, 2002). Most of these genes code for proteins of the cell cytoskeleton and the mechanism by which mutations cause DCM is thought to be impairment of heart force production or transmission (Towbin and Bowles, 2000). One of the identified disease genes is the gene encoding the desmin (*DES*). A missense mutation (Ile451Met) was reported in a family with autosomal dominant DCM without conduction-system disease or skeletal myopathy (Li et al., 1999a). Moreover, *DES* knockout mice exhibit disruption of myofibril organization with ventricular dilatation and impaired systolic contraction (Milner et al., 1999). These findings suggest that a reduction or absence of the *DES* gene function can result in DCM.

The human *DES* gene has been assigned to human chromosome 2q35 and has nine exons that encode a protein of 469 amino acids (Vicart et al., 1996).

In this study, we report on the isolation and characterisation of canine bacterial artificial chromosome (BAC) clones containing the *DES*, chromosomal location of these clones and on the identification of a microsatellite marker residing in the vicinity of the *DES* gene. By searching *Canis familiaris* sequences deposited in the NCBI GenBank trace archive and the 1.5× dog sequence (Kirkness et al., 2003), we determined a partial DNA sequence of the canine *DES* gene and identified seven *DES* single nucleotide polymorphisms (SNPs). To evaluate whether the *DES* gene is involved in the Dobermann DCM, we analyzed the association of the *DES* haplotypes with the DCM phenotype in a family of Dobermanns with frequent DCM occurrence.

2. Materials and methods

2.1. Dogs included in the study and DNA isolation

Blood samples were collected from 18 Dobermanns diagnosed with DCM and 10 DCM free Dobermanns. Dogs were patients of the Department of Clinical Sciences of Companion Animals (Veterinary Faculty, Utrecht University) and of the Clinic for Small Animals and Surgery (Veterinary Faculty, Ljubljana University). The pedigrees of 10 affected and the 10 unaffected Dobermanns included in this study were collected and the familial relationships between these dogs were established. Some relationships were traced with the help of a Dobermann pedigree web page at http://koti.mbnet.fi/dob/. The health status of relatives of investigated Dobermanns was obtained from the owners and breeders. Blood samples were collected and genomic DNA was isolated using a salt extraction method (Miller et al., 1988). DCM diagnosis was based on clinical and/or radiographic symptoms of CHF and echocardiographic evidence of shortening fraction (FS) <25% in the absence of other CHF-related lesions on two dimensional echocardiography.

Echocardiography was performed in conscious dogs, with the dog in right lateral recumbency, using a high definition ultrasound system (HDI 3000, Advanced Technology Laboratories, Woerden, The Netherlands) equipped with a 5–3 MHz broad and phase array transducer. ECG electrodes were placed on the left and right foreleg and the left hind leg for simultaneous ECG recording. All measurements were performed using a trackball driven cursor and ultrasound software. From the right parasternal approach 2D guided M-mode tracings were made for measurements of the interventricular septum, the left ventricular dimension and the left ventricular free wall, in diastole and systole, and of the aortic root and left atrial appendage diameter. These measurements were made from the leading edge of the first endocardial surface to the leading edge of the second endocardial surface. Diastolic measurements were made at the onset of the QRS complex of the ECG and systolic measurements were made at the maximum systolic excursion of the interventricular septum. The diameter of the aortic root was measured at the onset of the QRS complex of the ECG and the dimension of the left atrial appendage was measured at its maximal upward excursion near the end of systole.

From the left ventricular dimension in diastole (LVDd) and systole (LVDs), the fractional shortening (FS) was calculated (FS (%)=[(LVDd−LVDs)/LVDd]×100) and from the diameter of the left atrial appendage and the aortic root the left atrium to aorta ratio (LA/Ao) was calculated. At the Clinic for Small Animals and Surgery (Veterinary Faculty, Ljubljana University), echocardiography was performed as described elsewhere (Domanjko-Petrič et al., 2002). Dobermanns were confirmed as unaffected when they were older than nine years and had no signs or symptoms indicative of DCM.

2.2. Isolation of the canine DES BAC clones

In order to screen the canine BAC library (Li et al., 1999b), two overlapping primers were designed in exon 1 of the human *DES* gene (GenBank accession no. U59167) using Overgo maker program (http://genome.wustl.edu/): Des_Ova: 5′ -ATGAGCCAGGCCTACT**CGTCCAGC**-3′ , Des_Ovb: 5′ -AGGAGGACACGCGCTG**GCTGGACG**-3′ . Bold characters indicate the overlap. The 40-bp probe was synthesized and labelled with α^{32}P-dATP and α^{32}P-

dCTP at 37 °C for 1 h using the overgo technique (Han et al., 2000). The hybridization of the canine BAC library and BAC DNA isolation were performed as described on the BacPac website (http://bacpac.chori.org/).

To confirm the identity of the BAC clones, BAC DNA and canine genomic DNA were digested with *Eco*RI restriction enzyme, separated on a 0.7 % agarose gel and Southern blotted onto Hybond N^+ filters (Amersham). The blots were hybridised overnight at 65 °C with an α^{32}P-dATP-labelled insert of the human 2960632 IMAGE DES cDNA clone (Lennon et al., 1996). The clone was obtained from the Resource Centre of the German Human Genome project (Berlin, Germany) and verified through DNA sequence analysis. The IMAGE clone insert was excised and purified on agarose gel (Quiaquick spin columns, Quiagen). The blots were washed with 2× SSC, 0.1% SDS at 65 °C for 10 min. Genomic DNA blots were exposed to film at −70 °C with intensifying screen overnight and BAC DNA for 5 min.

2.3. Chromosomal localization of the canine DES gene by fluorescence in situ hybrizidation (FISH)

2.3.1. Chromosome preparation

Metaphase chromosomes were prepared from concanavalin A-stimulated peripheral blood lymphocytes from karyotypically normal dogs.

2.3.2. Probes preparation

Total DNA of a canine BAC clone 25J10, which contains the *DES* gene, was isolated and labeled with biotin-16-dUTP or digoxigenin-11-dUTP by nick-translation. For the identification of small dog chromosomes, biotinylated chromosome-specific paints Z, cc and dd, developed by the Animal Health Trust and the Sanger Centre, were used (Breen et al., 1999a).

2.3.3. Fluorescence in situ hybridization

Labeled BAC DNA was hybridized onto un-banded or GTG-banded canine metaphase chromosomes as previously described (Zijlstra et al., 1997). In these experiments, a final probe concentration of 5 ng/μl and a 50- or 100-fold excess of fragmentated total dog DNA were used. Post-hybridization stringency washes were performed in 50% formamide/2× SSC at 42 °C. Biotinylated probe hybridization was detected using avidin-FITC, and two additional layers of biotinylated goat anti-avidin and avidin-FITC. Chromosomes were counterstained with propidium iodide. Hybridization of digoxigenin labeled probe was detected with mouse anti-DIG (1:100, Sigma), followed by a layer of rabbit anti-mouse-TRITC (1:100, Sigma) and a layer of goat anti-rabbit-TRITC (1:100, Sigma). In these experiments, chromosomes were counterstained with DAPI.

Dual-color FISH experiments were performed with the digoxigenin-labeled *DES* probe and the biotinylated chromosome-specific paints Z, cc and dd. In these experiments, for half a slide, 75 ng digoxigenated *DES* probe was precipitated and dissolved in 15 μl hybridization buffer containing biotinylated chromosome-specific paint and competitor DNA (Langford et al., 1995). This probe/paint mixture was denatured for 7 min at 100 °C, pre-annealed for 1.5 h at 37 °C, and applied to denatured slides. Hybridization was carried out overnight at 37 °C and posthybridization washes were performed as described above. Specific sites of hybridization were simultaneously detected by using successive layers of avidin-FITC/mouse anti-DIG, biotinylated goat anti-avidin/rabbit anti-mouse-TRITC and avidin-FITC/goat anti-rabbit-TRITC. Chromosomes were counterstained with DAPI.

Metaphases showing sites of specific hybridization were captured using a Leica DMRA microscope equipped with the GENUS Image Analysis software of Applied Imaging.

2.4. Microsatellite marker isolation in DES BAC clones

DNA from the BAC clones 42C1 and 216E1 was pooled and digested with *Sau*3AI. Adaptors were ligated to the ends of the restriction fragments and the restriction fragments were amplified using adaptor specific primers (Groot and van Oost, 1998). The amplified product was enriched for CA-repeats using 3′ biotinylated $[CA]_{22}$ oligonucleotide. The enriched fragments were amplified and subcloned into the *Bam*HI site of pZErO-1 vector (Invitrogen) after which TOP10F′ competent cells were transformed according to the manufacturer's protocol (Invitrogen). Positive clones were identified by an α-^{32}P-dATP-labelled $[CA]_{22}$ probe. The sequence of the clones was determined using BigDye Terminator cycle sequencing (Applied Biosystems) with M13-forward (5′-GTTTTCCCAGTCACGAC-3′) and M13-reverse (5′-CAGGAAACAGCTATGAC-3′) primers. When the microsatellite marker UU42C1 was observed, flanking primers were designed using Primer 3 software (Rozen and Skaletsky, 2000). Primer sequences were: UU42C1-f: 5′-GAAGAAGCAAGCTGGTGGAC-3′ and UU42C1-r: 5′-GGCTTTCCATAGCAACTCCA-3′. For each polymerase chain reaction (PCR), 25 ng of canine genomic DNA was amplified with 0.33 μM of each primer, 0.6 U of Platinum *Taq* (Invitrogen), 200 μM deoxyribonucleoside triphosphates (dNTPs), 1× Gibco buffer and 3.5 mM $MgCl_2$ in a final volume of 15 μl. The PCR program consisted of a denaturation step of 10 min at 94 °C, followed by 35 cycles of 30 s 94 °C, 30 s 62 °C, 30 s 72 °C and a final extension at 72 °C for 10 min.

2.4.1. Canine DES microsatellite marker typing in the Dobermanns

The forward primer of the UU42C1 marker was labelled with HEX fluorescent dye (Eurogentec). PCR products were generated as described in Section 2.4 and run with GS500 size standards on an automated ABI3100 DNA Analyzer (Applied Biosystems, Foster City, CA). Genescan 3.1 software was used for genotype assessment. UU42C1

microsatellite marker was typed in 10 DCM free Dobermanns and 18 Dobermanns diagnosed with DCM.

2.5. Canine DES DNA and amino acid sequence

The canine *DES* gene sequence was derived from sequences deposited in *C. familiaris* trace archive, Database of the Genome Survey Sequence (DBGSS) and Database of the Whole Genome Shotgun Sequence (DBWGS) of the NCBI GenBank by BLAST search (http://ncbi.nlm.nih.gov/BLAST), using the human DES cDNA sequence (GenBank accession no. NM_001927) as a probe. Canine *DES* sequences were imported as trace (*.scf*) or Editseq (*.seq*) files into Seqman (DNA Star alignment software) and aligned with one another and all nine human *DES* gene exons. Poor quality sequences were eliminated. Canine *DES* DNA sequence, intron–exon borders and the coding region were determined. The 1407-bp canine *DES* coding sequence was compared to the human (GenBank accession no. NM_001927) and the mouse (GenBank accession no. NM_010043) coding region. From the coding region, canine DES amino acid sequence was deduced and compared to its human (GenBank accession no. NP_001918) and mouse (GenBank accession no. NP_034173) counterparts using Blast 2 sequences program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

In order to detect SNPs in the assembled canine *DES* sequence, *C. familiaris* trace archive (NCBI GenBank) sequences were aligned using Seqman (DNA Star alignment software). Six SNPs were located (Table 1). The SNP database of the 1.5× sequence: http://www.ncbi.nlm.nih.gov/SNP/ (Kirkness et al., 2003) was downloaded and searched for SNPs in the canine *DES* (GenBank accession no. BK005142) by local BLAST search.

2.6. DES SNP typing in the Dobermanns

To assess the presence of the *DES* SNPs in 18 DCM and 10 DCM free Dobermanns, primers for SNP testing were designed using Primer 3 software (Rozen and Skaletsky, 2000). Primer sequences and PCR product lengths are given in Table 1. All primers were M13 tailed. For each PCR reaction, 25 ng genomic DNA was used as a template in a 15-μl reaction mixture consisting of 1× Gibco-BRL buffer (Life Technologies), 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.6 U Platinum *Taq* polymerase (Invitrogen) and 0.33 μM of each primer. The PCR program consisted of denaturation for 4 min at 94 °C, followed by 35 cycles of 30 s 94 °C, 30 s 57 °C, 30 s 72 °C and a final extension for 10 min at 72 °C. The reaction was diluted 15× and 1 μl was used in a 10-μl tercycle reaction using 1-μl Big Dye Terminator Ready Reaction Kit (Perkin Elmer ABI), 0.32 μM of the HPLC purified M13 forward primer (5′ -GTTTTCCCAGTCACGAC-3′) in 1× sequence buffer (80 mM Tris, 2 mM $MgCl_2$, pH 9.0). The tercycle consisted of 25 cycles of 30 s at 96 °C, 15 s at 55 °C and 2 min at 60 °C. Tercycle products were purified using multiscreen 96-well filtration plates (Millipore) with Sephadex G-50 (Amersham). Obtained sequences were aligned using Seqman (DNA Star Software) and SNPs were typed by visual examination.

2.7. Definition of haplotypes and association analysis

Haplotypes were constructed for the canine UU42C1 microsatellite marker and four *DES* SNP genotypes that were informative in the Dobermanns. Firstly, all dogs that were homozygous for all five markers were selected which defined two haplotypes. Two additional probable haplotype combinations were derived from heterozygous dogs. Deduced haplotypes were analyzed for the association with the DCM phenotype in the Dobermann family tree.

3. Results

3.1. Selection and confirmation of canine DES BAC clones

Screening of the canine genomic BAC library RP81 with the *DES* exon 1 probe yielded six positive BAC clones: 18L16, 25J10, 42C1, 216E1, 233D5 and 233F5. The *Eco*RI restriction fragment pattern of the BAC clones 25J10, 42C1 and 216E1 are similar and these are grouped. The BAC clones 18L16, 233D5 and 233F5 form a distinct group (Fig. 1).

Southern blots with *Eco*RI restriction enzyme digests of *DES* BAC clones DNA and total dog genomic DNA were

Table 1
Canine *DES* gene SNPs

SNP name	Position in BK005142	Type of change	Forward_primer_5′ –3′	Reverse_primer_5′ –3′	Product length
SNP_Ex3[a]	1808	T/C	TTGCTTGACCACTACCAGGA	AGATGTTCTTAGCCGCGATG	402 bp
SNP_In3[a]	1851	G/C	TTGCTTGACCACTACCAGGA	AGATGTTCTTAGCCGCGATG	402 bp
SNP_In4[a]	2203	A/G	CCAGCTTCAGGAACAACAGG	TGATGTGATGAGAGCCAAGG	497 bp
SNP_In4-1[a]	2249	T/C	CCAGCTTCAGGAACAACAGG	TGATGTGATGAGAGCCAAGG	497 bp
SNP_In6[a]	3249	A/G	CCTGCTCAATGTCAAGATGG	CCTTGGGTACGAGTCTCTGC	498 bp
SNP_In6-1[b]	3423	N	CCTGCTCAATGTCAAGATGG	CCTTGGGTACGAGTCTCTGC	498 bp
SNP_In7[a]	4613	A/C	AACTTCCGAGGTGAGTGCAT	GAGGGTGCCTTGTAGCTCAG	376 bp

[a] SNPs identified in the boxer sequences deposited in the NCBI *Canis familiaris* trace archive (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi).
[b] SNP from the 1.5× standard poodle sequence SNP database: http://www.ncbi.nlm.nih.gov/SNP/ (Kirkness et al., 2003).

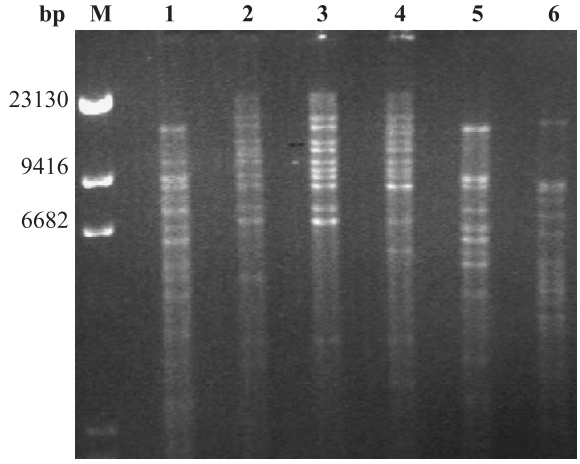

Fig. 1. *Eco*RI restriction fragment patters of BAC clones isolated with a desmin overgo probe. M: Marker lane—500 ng of λ DNA digested with *Hin*dIII; lanes 1–6: 1 μg of BAC DNA digested with *Eco*RI. Lane 1—BAC 18L16, lane 2—BAC 25J10, lane 3—BAC 42C1, lane 4—BAC 216E1, lane 5—BAC 233D5, lane 6—BAC 233F5. Samples were run on 0.7% agarose gel at 20 V for 15 h.

hybridised with a complete human desmin cDNA probe. The probe detected a DNA fragment of the same length in the genomic DNA and in BAC clones 25J10, 42C1 and 216E1 (Fig. 2). These three BAC clones tested all positive for the *DES* sequences amplified with SNP_Ex3 (amplify part of exon 3 and intron 3) and SNP_In7 (amplify part of intron 7) PCR primers. The similar BAC clones 18L16, 233D5 and 233F5 did not hybridise to the DES cDNA probe and were not studied further. We concluded that BAC clones 25J10, 42C1 and 216E1 contain the canine *DES* gene.

3.2. Chromosomal localization of the canine DES gene by FISH

BAC clone 25J10 was used as a probe for FISH analysis. After hybridization of the digoxigenin or biotin labeled *DES* probe to unbanded or GTG-banded chromosomes, clear fluorescent signals were found on the telomeric end of a small chromosome pair (Fig. 3a). Following the chromosome numbering suggested by Reimann et al. (1996), this pair could be pair 31, 33 or 37 on the basis of its G-banding pattern (insert Fig. 3a). Since the small chromosome pairs in the dog are very difficult to reliably identify by G-banding alone, we performed dual-color FISH with the *DES* probe and painting probes specific for CFA31+34 (paint Z), CFA33+36 (paint cc) or CFA37 (paint dd). The results of these experiments clearly show that the hybridization site of the desmin probe does not coincide with the hybridization signals of paint Z (Fig. 3b) or cc (Fig. 3c), but does with paint dd (Fig. 3d). Therefore, the *DES* can be assigned to CFA37qter.

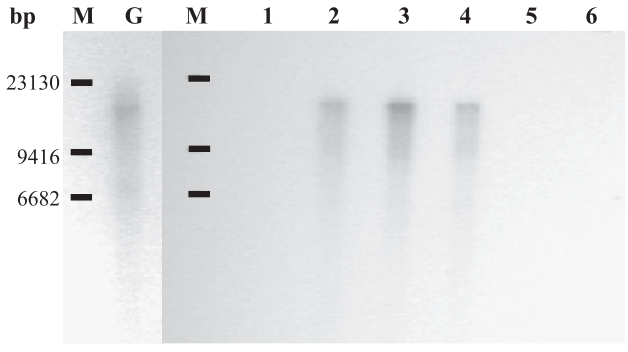

Fig. 2. Auto-radiograms showing Southern blot analysis of dog genomic DNA (lane G) and *DES* BAC clone DNA *Eco*RI digests hybridised with a *DES* I.M.A.G.E. cDNA clone 2960632 (lane 1—BAC 18L16, lane 2—BAC 25J10, lane 3—BAC 42C1, lane 4—BAC 216E1, lane 5—BAC 233D5, lane 6—BAC 233F5). The genomic *DNA* and BAC DNA digests were run on the same gel.

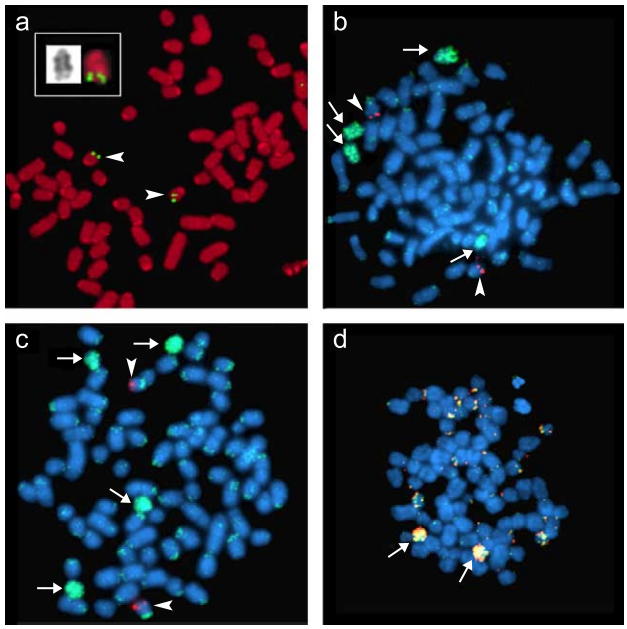

Fig. 3. Chromosomal localization of the canine *DES* gene by FISH. (a) Partial metaphase spread showing hybridization signals (arrowheads) obtained after hybridization with the biotinylated *DES* probe on unbanded chromosomes, insert: G-banded chromosome before (left) and after (right) FISH, b, c and d. Metaphase spreads after dual-hybridization with digoxigenated *DES* probe and biotinylated paint Z (b), paint cc (c) or paint dd (d). Arrowheads indicate hybridization signals obtained with the *DES* probe and arrows indicate hybridization signals obtained with the paints.

3.3. Canine DES polymorphic marker isolation and analysis

A $(CA)_{16}$ microsatellite marker (UU42C1) was isolated from the pooled DNA of the canine *DES* BAC clones 42C1 and 216E1. A PCR specific for UU42C1 with individual BAC DNA as template showed that the microsatellite is located on BAC clones 25J10, 42C1 and 216E1 (Data not shown).

A 5153-bp contig (UU42C1 contig) surrounding the UU42C1 marker region was constructed using canine DNA

Table 2
Canine *DES* SNPs and UU42C1 marker genotypes with derived haplotypes in the Dobermanns

Haplotype	SNP_In3	SNP_In4-1	SNP_In6	SNP_In7	UU42C1[a]
	1851[b]	2249[b]	3249[b]	4613[b]	
1	G	T	G	A	216
2	G	T	G	A	214
3	C	C	A	C	213
4	C	C	A	C	208

[a] The UU42C1 marker position was estimated to be 9.5 kb downstream the *DES* gene.
[b] Position of the SNP in the *BK005142* sequence.

sequences deposited in NCBI trace archives and GenBank. The following sequences from NCBI Genbank were used to make the UU42C1 contig: TI330170432 (trace archive), AACN010025312 and AACN010495151. BLAST analysis of the UU42C1 contig sequence identified several regions of high similarity to the human genomic contig NT_005403 on HSA2, about 9.5-kb downstream from the human *DES* gene. The human region that is similar to the dog DNA sequence around the UU42C1 marker does not contain any known coding or regulatory elements. No significant similarities to the sequences of other species were identified.

The microsatellite marker UU42C1 was typed in 18 DCM and 10 DCM free Dobermanns. The marker exhibited four allele sizes: 208, 213, 214 and 216 bp and was thus polymorphic (Table 2).

3.4. C. familiaris DES sequence

We used data from dog genome projects to reconstruct the nine exons and eight introns of the *DES* gene. The 5′ and 3′ untranslated regions were not determined (Fig. 4). The DNA Sequence was deposited in the NCBI GenBank under accession no. BK005142. The structure of the canine *DES* gene is very similar to the human *DES*, with introns 6 (1293 bp in the dog) and 7 (1683 bp in the dog) being the largest in both species (Fig. 4). The canine *DES* coding sequence is 1407 bp long and shows 92% and 90% similarity with its human and mouse counterparts, respectively. The deduced canine DES amino acid sequence (469 aa) shows a 96% similarity to the human and mouse DES amino acid sequence.

3.4.1. Canine DES SNPs analysis

Seven SNPs were identified in the canine *DES* gene sequence: two in intron 4 and intron 6 and one each in exon 3, intron 3 and intron 7 (Table 1). The codon in exon 3 with the T/C SNP codes for valine in both SNP variants.

The 6 canine *DES* gene SNPs were assessed in 18 DCM and 10 DCM free Dobermanns. SNPs in intron 3 (position 1851), intron 4 (position 2249), intron 6 (position 3249) and intron 7 (position 4613) were polymorphic in this selection of Dobermanns (Table 2).

3.5. DES gene haplotypes and their association with the DCM in the Dobermann pedigree

First, haplotypes were deduced from Dobermanns that were homozygous for all five markers. Two different haplotypes were found: haplotypes 1 and 3 (Table 2). Haplotypes 2 and 4 were identified in dogs homozygous for the four *DES* SNPs and heterozygous for the UU42C1 marker. Haplotype 2 differs from haplotype 1 in the microsatellite and haplotype 4 differs from haplotype 3 also in the microsatellite only. The haplotypes in homozygous dogs exist definitely and haplotypes derived from heterozygous dogs exist most likely, since all the Dobermanns are closely related (Fig. 5). Eight DCM Dobermanns that are not presented in Fig. 5 had the following haplotypes: [1,3] three dogs, [1,4] two, [3,3] two dogs and [2,3] one dog. The actual mode of inheritance could not be determined due to a lack of information regarding the relatives of the analysed dogs (Fig. 5). None of the four identified haplotypes showed association with the DCM phenotype under autosomal dominant or recessive mode of inheritance.

4. Discussion

4.1. Chromosomal localization of the canine DES gene

In the present study, we localized the canine *DES* gene to CFA37qter by FISH analysis. In the human genome, *DES* is located on HSA2q35. Independent comparative painting studies between the human and dog genome indicate that HSA2q35 shares homology with either

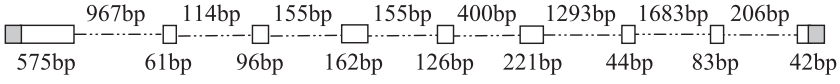

Fig. 4. Canine *DES* gene genomic organization. The blocks represent the nine exons; dashed lines not drawn to scale represent the eight introns of indicated length. The 5′ untranslated region in exon 1 and 3′ untranslated region of exon 9 were not analysed (grey boxes).

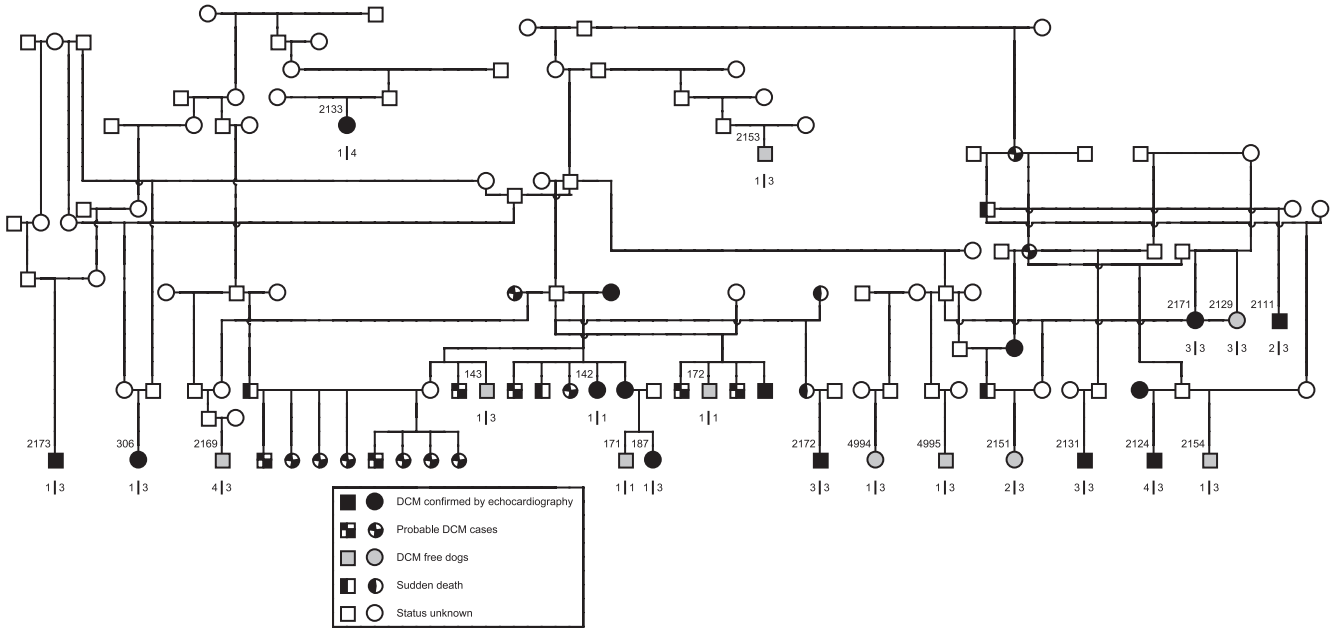

Fig. 5. Pedigree showing the relationship between the analysed Dobermanns; squares—males, circles—females. The haplotypes of the desmin markers are given below the symbols (Table 2).

CFA33 (Yang et al., 1999) or CFA37 (Breen et al 1999b). The discrepancy between these studies is most likely caused by difficulties in the unequivocal identification of the small canine chromosomes. In the present study, this problem was overcome by the use of specific chromosome paints that were assigned to individual dog chromosomes by the Committee for the Standardization of the Dog Karyotype (Breen et al., 1999a).

At present, eight of the genes that have been mapped in the human chromosome region 2q33–2q37 have also been mapped in the dog. These genes are, from proximal to distal *CD28*, *CTLA4*, *FN1*, *SCLC11A1*, *COL4A4*, *COL4A3*, *PDE6D* and *ALPI* (Table 3). The latter four all map to CFA25, whereas the former map to CFA37. In the human genome, *DES* is located between *SLC11A1* and *COL4A4*. The present localization of *DES* on CFA37, indicates that, compared to the human genome, there is a breakpoint between *DES* and *COL4A4* in the canine genome.

4.2. Canine DES gene sequence

Resources recently available from the canine genome project are the 7× redundant dog sequence deposited in the NCBI GenBank trace archive and recently published 1.5× redundant dog genome sequence (Kirkness et al., 2003). These resources enable rapid reconstruction and evaluation of the genes of interest. They have also radically improved the ability to perform comparative mapping and consequent identification of novel genes and regulatory elements. Combination of the NCBI GenBank *C. familiaris* trace archive sequences and the published 1.5× dog sequence (Kirkness et al., 2003) provided the canine *DES* gene

Table 3
Position of the *DES* and its neighbouring genes on the human (HSA) and dog (CFA) chromosomes

Gene name	Gene symbol	HSA[a]	Position human Mb[a]	CFA[b]	Position dog TSP units[b]
CD28 antigen	CD28	2q33	204.773.741	37	1071
Cytotoxic T-lymphocyte-associated protein 4	*CTLA4*	2q33	204.935.054	37	1071
Fibronectin	*FN1*	2q34	216.428.128	37	2736
Solute carrier family 11	*SLC11A1*	2q35	219.450.219	37	2232
Desmin	*DES*	2q35	220.485.644	37[c]	not known
Collagen, type IV, alpha 4	*COL4A4*	2q35–q37	228.072.463	25	not relevant
Collagen, type IV, alpha 3	*COL4A3*	2q36–q37	228.231.820	25	not relevant
Phosphodiesterase 6D, c-GMP specific, rod, delta	*PDE6D*	2q35–q36	232.799.686	25	not relevant
Alkaline phosphate, intestinal	*ALPI*	2q37.1	233.523.404	25	not relevant

[a] Location obtained from LocusLink (http://www.ncbi.nlm.nih.gov).
[b] Location obtained from Guyon et al. (2003).
[c] present study.

sequence. The DES amino acid sequence is strongly conserved between different species; therefore, the observed high degree of similarity between dog and human or mouse was expected (Loh et al., 2000).

Other useful information derived from the dog genome project concern the SNPs. For almost complete *DES* DNA sequence, there were at least two overlapping trace files available. Discrepancies between the DNA sequence files indicated possible SNPs. We could distinguish differences due to poor quality data from true SNPs because the electropherograms are deposited with the trace files. Interestingly, we found 6 SNPs in the 6.4-kb long *DES* sequence from a single boxer, while this breed was selected to be sequenced because it has the lowest level of variation in its genome (http://www.genome.gov/11007358). The use of this data mining is illustrated by the fact that we found 4 out of the 6 SNPs to be informative in a pedigree of Dobermanns. One SNP was found in the poodle SNP database (Kirkness et al., 2003). This could be due to lower coverage (1.5×) of the standard poodle sequence in comparison to the 7× redundant boxer sequence.

4.3. Isolation and location of the UU42C1 microsatellite marker

We isolated a polymorphic microsatellite marker—UU42C1 from the canine *DES* gene BAC clones. The sequence of this marker could not be found in the contig of the canine *DES* gene DNA sequence. It was an interesting finding that parts of a 5153-bp contig sequence surrounding the UU42C1 marker had high homology with segments of human DNA sequence at a 9.5-kb distance from the human *DES* gene because we did not find any similarities of these segments in the mouse genome. This is not surprising, because the similarity between coding and intergenic regions of dog and human genomes is greater than the similarity between the mouse and human or dog. The intergenic conserved sequence blocks we found have not yet been associated with any gene and may contain regulatory elements important for transcription. Between the dog, mouse and human, 45% of the conserved DNA sequence blocks are not associated with any genes and comparative sequence analysis has proven to be a very efficient way for identifying the functional elements encoded within the DNA (Chen et al., 2001; O'Brien and Murphy, 2003).

4.4. Dobermann family and linkage analysis of the DES markers

Several canine genetic diseases have been mapped with the use of microsatellite markers (Acland et al., 1999; Jonasdottir et al., 2000). The newly identified UU42C1 marker and SNPs enabled us a fast evaluation of the *DES* gene as a candidate gene for DCM in the Dobermanns. In the Dobermann family, the *DES* region was polymorphic with four haplotypes, but none of the haplotypes was associated with the DCM phenotype. For example, DCM dogs nos. 2133 and 142 do not have haplotype 3 that is shared by other DCM dogs. Furthermore, two closely related DCM dogs, nos. 142 (haplotypes 1,1) and 2172 (haplotypes 3,3), do not share a single haplotype. Moreover, in the eight singleton Dobermann dogs with DCM, six dogs were heterozygous and none of the haplotypes was present in all affected singleton dogs. It should be stressed that haplotypes 1 and 3 differ in the four SNPs of the haplotype and represent alleles that must have diverged long ago in the ancestral lineage. Therefore, we have excluded the *DES* as a gene responsible for DCM in the Dobermanns under the autosomal dominant and recessive models with a high level of confidence. However, the *DES* gene sequence, SNPs and the microsatellite marker reported in this study are tools that will enable fast evaluation of the desmin gene in breeds other than the Dobermann.

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References

Acland, G.M., Ray, K., Mellersh, C.S., Langston, A.A., Rine, J., Ostrander, E.A., Aguirre, G.D., 1999. A novel retinal degeneration locus identified by linkage and comparative mapping of canine early retinal degeneration. Genomics 59, 134–142.

Breen, M., Bullerdiek, J., Langford, C.F., 1999a. The DAPI banded karyotype of the domestic dog (*Canis familiaris*) generated using chromosome-specific paint probes. Chromosom. Res. 7, 401–406.

Breen, M., Thomas, R., Binns, M.M., Carter, N.P., Langford, C.F., 1999b. Reciprocal chromosome painting reveals detailed regions of conserved synteny between the karyotypes of the domestic dog (*Canis familiaris*) and human. Genomics 61, 145–155.

Calvert, C.A., Hall, G., Jacobs, G., Pickus, C., 1997. Clinical and pathologic findings in Doberman pinschers with occult cardiomyopathy that died suddenly or developed congestive heart failure: 54 cases (1984–1991). J. Am. Vet. Med. Assoc. 210, 505–511.

Chen, R., Bouck, J.B., Weinstock, G.M., Gibbs, R.A., 2001. Comparing vertebrate whole-genome shotgun reads to the human genome. Genome Res. 11, 1807–1816.

Domanjko-Petrič, A., Stabej, P., Žemva, A., 2002. Dilated cardiomyopathy in doberman pinschers, survival, causes of death and pedigree review in a related line. J. Vet. Cardiol. 4, 17–24.

Fatkin, D., Graham, R.M., 2002. Molecular mechanisms of inherited cardiomyopathies. Physiol. Rev. 82, 945–980.

Groot, P.C., van Oost, B.A., 1998. Identification of fragments of human transcripts from a defined chromosomal region: representational

difference analysis of somatic cell hybrids. Nucleic Acids Res. 26, 4476–4481.

Guyon, et al., 2003. A 1-Mb resolution radiation hybrid map of the canine genome. Proc. Natl. Acad. Sci. U. S. A. 100, 5296–5301.

Hammer, T.A., Venta, P.J., Eyster, G.E., 1996. The genetic basis of dilated cardiomyopathy in doberman pinschers. Anim. Genet. 27 (Suppl. 2), 109.

Han, C.S., Sutherland, R.D., Jewett, P.B., Campbell, M.L., Meincke, L.J., Tesmer, J.G., Mundt, M.O., Fawcett, J.J., Kim, U.J., Deaven, L.L., Doggett, N.A., 2000. Construction of a BAC contig map of chromosome 16q by two-dimensional overgo hybridization. Genome Res. 10, 714–721.

Jonasdottir, T.J., Mellersh, C.S., Moe, L., Heggebo, R., Gamlem, H., Ostrander, E.A., Lingaas, F., 2000. Genetic mapping of a naturally occurring hereditary renal cancer syndrome in dogs. Proc. Natl. Acad. Sci. U. S. A. 97, 4132–4137.

Kirkness, E.F., Bafna, V., Halpern, A.L., Levy, S., Remington, K., Rusch, D.B., Delcher, A.L., Pop, M., Wang, W., Fraser, C.M., Venter, J.C., 2003. The dog genome: survey sequencing and comparative analysis. Science 301, 1898–1903.

Knoll, R.. et al., 2002. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. Cell 111, 943–955.

Langford, C.F., Fischer, P.E., Binns, M.M., Holmes, N.G., Carter, N.P., 1995. Chromosome-specific paints from a high-resolution flow karyotype of the dog. Chromosom. Res. 3, 1–10.

Lennon, G.G., Auffray, C., Polymeropoulos, M., Soares, M.B., 1996. The I.M.A.G.E. consortium: an integrated molecular analysis of genomes and their expression. Genomics 33, 151–152.

Li, D., Tapscoft, T., Gonzalez, O., Burch, P.E., Quinones, M.A., Zoghbi, W.A., Hill, R., Bachinski, L.L., Mann, D.L., Robert, S.R., 1999a. Desmin mutation responsible for idiopathic dilated cardiomyopathy. Circulation 100, 461–464.

Li, R., Mignot, E., Faraco, J., Kadotani, H., Cantanese, J., Zhao, B., Lin, X., Hinton, L., Ostrander, E.A., Patterson, D.F., de Jong, P.J., 1999b. Construction and characterization of an eightfold redundant dog genomic bacterial artificial chromosome library. Genomics 58, 9–17.

Loh, S.H., Chan, W.T., Gong, Z., Lim, T.M., Chua, K.L., 2000. Characterization of a zebrafish (*Danio rerio*) desmin cDNA: an early molecular marker of myogenesis. Differentiation 65, 247–254.

Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215.

Milner, D.J., Taffet, G.E., Wang, X., Pham, T., Tamura, T., Hartley, C., Gerdes, A.M., Capetanaki, Y., 1999. The absence of desmin leads to cardiomyocyte hypertrophy and cardiac dilation with compromised systolic function. J. Mol. Cell. Cardiol. 31, 2063–2076.

Meurs, K.M., 2002. Familial Canine Dilated Cardiomyopathy. In *Proceedings*. Advances in Canine and Feline Genomics, St. Louis, Missouri.

O'Brien, S.J., Murphy, W.J., 2003. Genomics. A dog's breakfast? Science 26, 1854–1855.

Reimann, N., Barnitzke, S., Bullerdiek, J., Schmitz, U., Rogalla, P., Niolte, I., Rønne, M., 1996. An extended nomenclature of the canine karyotype. Cytogenet. Cell Genet. 73, 140–144.

Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, pp. 365–386.

Schmitt, J.P., Kamisago, M., Asahi, M., Li, G.H., Ahmad, F., Mende, U., Kranias, E.G., MacLennan, D.H., Seidman, J.G., Seidman, C.E., 2003. Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. Science 28, 1410–1413.

Sisson, D.D., Thomas, W.P., 1995. Myocardial diseases. In: Ettinger, SJ, Feldman, EC (Eds.), Textbook of Veterinary Internal Medicine, fourth ed. WB Sounders Co., Philadelphia, pp. 995–1011.

Towbin, J.A., Bowles, N.E., 2000. Genetic abnormalities responsible for dilated cardiomyopathy. Curr. Cardiol. Rep. 2, 475–480.

Vicart, P., Dupret, J.M., Hazan, J., Li, Z., Gyapay, G., Krishnamoorthy, R., Weissenbach, J., Fardeau, M., Paulin, D., 1996. Human desmin gene: cDNA sequence, regional localization and exclusion of the locus in a familial desmin-related myopathy. Hum. Genet. 98, 422–429.

Yang, F., O'Brien, P.C., Milne, B.S., Graphodatsky, A.S., Solanky, N., Trifonov, V., Rens, W., Sargan, D., Ferguson-Smith, M.A., 1999. A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps. Genomics 62, 189–202.

Zijlstra, C., Mellink, C.H.M., de Haan, N.A., Bosma, A.A., 1997. Localization of the 18S, 5.8S and 28S rRNA genes and the 5S rRNA genes in the babirusa and the white-lipped peccary. Cytogenet. Cell Genet. 77, 273–277.