

mucus cell metaplasia (MCM). Furthermore, apoptosis of goblet cells caused by the delay of BCL2 upregulation may lead to MCM persistence.^{3,10} Because of the potential role of BCL2 in equine RAO and lymphoid tissue neoplasia, we have identified a fragment of equine BCL2 cDNA and designed primers that will allow us to study gene expression and investigate the role of this gene in equine chronic respiratory and neoplastic diseases.

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SNP detection and association analyses of candidate genes for rupture of the cranial cruciate ligament in the dog

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Source/description: Rupture of the cranial cruciate ligament (RCCL) is the most common cause of lameness in the dog and leads to arthritis formation. Previous studies have identified RCCL as a genetic disease with a possible partially penetrant, recessive mode of inheritance.¹ Using a biological candidate gene approach, polymorphisms in genes that were associated with joint hypermobility in cattle and primary arthritis formation in humans were tested for associ-

ation with the RCCL phenotype.^{2,3} The genes chosen were *fibrillin 1* (FBN1), *collagen type 9 alpha 1*, (COL9A1), *collagen type 9 alpha 2* (COL9A2) and *cartilage oligomeric matrix protein* (COMP).

Primer sequences: Human FBN1 sequence (GenBank accession no. NM_000138) was used to design primers (Table 1), with the forward primer designed to force incorporation of the recognition sequence for the restriction enzyme *HincII* (New England Biolabs, Beverly, MA, USA). To determine the canine sequence for COL9A1, COL9A2 and COMP, the respective human mRNA sequences NM_001851, NM_001852 and NM_000095 were compared using the BLAST algorithm against the dog whole-genome shotgun sequence (National Center for Biotechnology Information, Bethesda, MD, USA). The sequence was then aligned using human exonic/intronic boundaries as determined by Ensembl (European Bioinformatics Institute and Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK). Primers were designed using Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA) or Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). All information is included in Table 1.

PCR conditions: For determination of sequence polymorphisms, a 10- μ l PCR volume contained 12.5 ng canine genomic DNA, 2.5 pmol of each primer, 1.25 mM dNTP, 1 μ l 10X PCR buffer (Promega, Madison, WI, USA) for FBN1 or 2 μ l GoTaq buffer (Promega) for COL9A1, COL9A2 and COMP, the respective amount (see Table 1) of MgCl₂ (Promega), either 0.35 U Taq polymerase for FBN1 or 0.25 U GoTaq polymerase and distilled water to equal 10 μ l. The thermal cycling protocol was 3 min at 94 °C, 35 cycles of 94 °C for 30 s, respective primer annealing temperature (see Table 1) for 45 s and 72 °C for 45 s, with a final extension of 72 °C for 10 min.

SNP detection and polymorphisms: Single nucleotide polymorphisms (SNP) were detected using pooled DNA samples from normal ($n = 3$) and RCCL-affected Newfoundlands ($n = 3$). The sequence data were analysed using Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test for each SNP was designed for a 10- μ l reaction by incubating 3 μ l of PCR product with the respective restriction enzyme and secondary substrates (see Table 2) according to the manufacturer's directions for at least 5 h. The digested PCR products were separated on agarose gels (see Table 2) stained with ethidium bromide.

Association analyses: Pedigrees, determination of cranial cruciate ligament (CCL) status (normal or affected with rupture of the CCL), and DNA were collected from Newfoundland dogs in a study performed at Iowa State University. Newfoundland dogs were classified as RCCL affected based on signs of pain on hyperextension of the knee, knee

Table 1 Primers, conditions for polymerase chain reaction amplification and SNP location of canine genes tested for involvement in rupture of the cranial cruciate ligament.

Gene	Primer sequence, 5' → 3' (forward/reverse)	Annealing temperature (°C)	MgCl ₂ (mM)	Fragment size (bp)	Location of primers	Location of SNP, type	dbSNP local identifier (NCBI_ss#)
<i>FBN1</i>	TATATTTATAATGCTTAAGAGATATGGTCAA CAGATGGTTTTTGTGGAT	55.4	15	303	Intron 19 to exon 20	Intron 19, C ↔ T	<i>FBN1</i> (dbSNP: ss38343205)
<i>COL9A1</i>	CTGGAGTCAGGGGAGGTGTA TGGGATTTTCTGGAGAGCAG	60	0 ¹	466	Intron 2	A ↔ T	<i>COL9A1</i> (dbSNP: ss38343206)
<i>COL9A2</i>	TAGTGGTGCCCCGTTAAACAT CCCCCGTTTACAGATGAGAA	57	0 ¹	176	Intron 30	bp 121, 122 T ↔ G, C ↔ G	<i>COL9A2-1</i> (dbSNP: ss38343207) and <i>COL9A2-2</i> (dbSNP: ss38343208)
<i>COMP</i>	GCCCTAGGGTACCCAACCTCA GTTGAGCACCACCCAGTTG	62	10 ¹	1131	Exons 12–15	bp 631 T ↔ G	<i>COMP</i> (dbSNP: ss38343209)

SNP, single nucleotide polymorphism; dbSNP, database of single nucleotide polymorphisms.

¹15 mM MgCl₂ included in GoTaq buffer.

Table 2 Polymerase chain reaction-restriction fragment length polymorphism conditions and single nucleotide polymorphism (SNP) patterns.

Gene	Enzyme, amount (U/reaction)	Substrates ¹	Digestion temperature (°C)	Fragment sizes (bp) based on SNP	Gel (%) ²
<i>FBN1</i>	<i>HincII</i> (5)	BSA, NEB3	37	C: 274, 29 T: 303	4
<i>COL9A1</i>	<i>FokI</i> (3)	NEB4	37	T: 283, 129, 54 A: 283, 183	4
<i>COL9A2</i>	<i>BstNI</i> (3)	BSA, NEB2	60	T,C: 144, 33 G,G: 119, 33, 25	4
<i>COMP</i>	<i>BstEII</i> (3)	BSA, NEB3	60	G: 1130, 90 T: 631, 500, 90	2

¹BSA, bovine serum albumin. Buffers NEB2, NEB3 and NEB4 were purchased from New England BioLabs (Ipswich, MA, USA).

²The gel used is agarose.

effusion, decreased range of motion, positive cranial drawer sign or cranial tibial thrust, radiographic evidence of stifle effusion and osteoarthritis, and/or surgical confirmation of a ruptured cranial cruciate.⁵ From this pedigree, affected and normal dogs were chosen for association analyses based on their likelihood of being genetically either homozygous normal (all unaffected, $n = 45$) or homozygous recessive (all affected, $n = 45$).⁴ The PCR-RFLP test was performed for one SNP in each gene for all 90 dogs, and chi-squared analyses were performed to test for association of SNP genotype and allele frequency with RCCL status using JMP 5.1 (SAS Institute Inc., Cary, NC, USA). No significant

association was found between any SNP and RCCL status of the dogs (see Table 3), although for *COL9A1* the association with allele frequency and RCCL status was close to significance (power >0.7).

Comments: Although there were no statistical associations between the identified SNPs and RCCL status, the PCR-RFLP tests described here may be useful for association studies involving dogs that are affected with other musculoskeletal diseases, such as hip or elbow dysplasia. Mapping was not conducted because the chromosomal location of the genes was determined through the use of the previously men-

Table 3 Pearson chi-squared probability results.

Gene	Status	1,1 genotype (number)	1,2 genotype (number)	2,2 genotype (number)	Chi-squared value, P -value	1 Allele (number)	2 Alleles (number)	Chi-squared value, P -value
<i>FBN1</i>	Normal	5	15	22	1.14, 0.56	25	59	1.30, 0.25
	Affected	3	13	27		19	67	
<i>COL9A1</i>	Normal	6	19	18	2.97, 0.23	31	55	2.76, 0.10
	Affected	2	17	24		21	65	
<i>COL9A2</i>	Normal	10	31	4	1.44, 0.49	51	39	0.02, 0.89
	Affected	12	26	7		50	40	
<i>COMP</i>	Normal	10	27	7	0.55, 0.76	47	41	0.36, 0.55
	Affected	7	27	8		41	43	

tioned whole genome shotgun sequence. *FBN1*, *COL9A1*, *COL9A2* and *COMP* are expected to map on dog chromosomes 30, 12, 15 and 20, respectively, based upon the human-dog comparative map (<http://idefix.univ-rennes1.fr:8080/Dogs/RH3270-page.html>). Further sequencing of these genes is needed for identification of additional SNPs that can then be tested for association with RCCL.

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A PCR-RFLP method for genotyping the *myostatin* locus in Piedmontese cattle

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Description: In Piedmontese cattle the double-muscling phenotype has been increasingly favoured by artificial selection since the beginning of the 20th century, so that at present this phenotype is widely spread throughout the Piedmontese breed in Italy. However, animals with the normal phenotype still exist and the frequency of the *mh* allele in the breed is uncertain. Therefore, identification of the *mh* genotype is of practical importance for selection. The mutation in the *myostatin* gene responsible for double muscling in Piedmontese has been identified; it is a guanine-to-adenine transition at position 938 in exon 3, resulting in a cysteine-to-tyrosine substitution that negatively affects the functional properties of the myostatin protein.^{1,2} Polymerase chain reaction (PCR) methods for genotyping the *myostatin* locus in Piedmontese cattle are available and based on the use of

fluorescent allele-specific primers and subsequent automated sequencing of the amplified products.^{3–5} Within this study we describe an alternative PCR restriction fragment length polymorphism (RFLP) method. As the G938A mutation does not alter any restriction enzyme recognition site, we designed the forward primer based on the published bovine *myostatin* cDNA sequence (GenBank accession no. AF019761) with a purposeful mismatch (G for A at position 19) that, in conjunction with G938, introduces a recognition site for the endonuclease *Bst*F5I in the wild-type allele.

Primer sequences: Forward: 5'-CCA ATT ACT GCT CTG GAG GAT-3'

Reverse: 5'-GGA GAC ATC TTT GTA GGA GTA CAG C-3'

PCR conditions: The PCR reaction contained 50–100 ng genomic DNA, 1X PCR buffer II, 2.5 mM MgCl₂, 200 µM each dNTP, 0.2 µM each primer, and 1.25 U Red *Taq* DNA polymerase (Sigma, St Louis, MO, USA) in a total volume of 25 µl. The amplification was performed under the following conditions: 5 min at 97 °C, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were digested with *Bst*F5I (New England BioLabs, Beverly, MA, USA) according to the manufacturer's instructions. The digested fragments were electrophoresed on a 3.5% agarose gel, stained with ethidium bromide and visualised under UV light.

Comments: The ability of this method to distinguish wild and mutant *myostatin* alleles was tested using nine animals of known genotypes: three double-muscling Piedmontese bulls (homozygous for the mutant allele), three crossbred Piedmontese × Friesian (heterozygous) and three purebred Friesian cows (homozygous for the wild-type allele). The PCR produced amplicons of the expected size (124 bp, extending from nt 917 to nt 1040). The wild-type allele, which contains the restriction enzyme *Bst*F5I recognition sequence, yielded two fragments of 100 and 24 bp, while the mutant allele gave a single fragment of 124 bp. The analysis of an additional 30 animals confirmed that the test is effective in distinguishing three genotypes. Compared with the procedures previously proposed, this alternative method has the advantage of requiring only basic equipment and reagents, making it possible for almost any laboratory to routinely genotype the *myostatin* locus in Piedmontese cattle.

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