HOMOZYGOSITY MAPPING IN SHEEP AFFECTED BY CERVICOTHORACIC VERTEBRAL SUBLUXATION: CANDIDATE GENE IDENTIFICATION AND NOVEL MUTATION INVESTIGATION

RACHEL L. TULLOCH[1], BRENDON A. O'ROURKE[2], SHERNAE A. WOOLLEY[1], MEHAR S. KHATKAR[1] and IMKE TAMMEN[1]

Summary

Cervicothoracic vertebral subluxation (CVS) is an inherited disorder affecting Merino sheep. Affected animals exhibit sudden onset of ataxia in sheep from 9 months to 3 years of age, the disorder is of an economic and welfare concern to the industry. The prevalence of this disorder both nationally and internationally is unknown due to its ambiguous phenotype. This is the first study to map investigate genetic variations as a likely cause of CVS in Merino sheep. Twelve clinically affected animals from two separate Australian properties (Riverina, NSW, and Central West, NSW) were sampled and genotyped using the Illumina® OvineSNP50 BeadChip. The *PDS5* homologue B gene was identified as a positional candidate gene using a homozygosity mapping approach. Sanger sequencing of RT-PCR products was utilised to sequence cDNA of the candidate gene in an affected animal. DNA sequencing revealed four variants within the candidate gene when compared to a reference sequence (GenBank® XM_015098134), a 6pb insertion (3619_3620insGTAAGG), two possible missense mutations (3412_C>A; 4342A>T) and an exon deletion (4066del-exon33). The 6bp insertion has been previously catalogued in other reference genomes, the two missense mutations appear to be a novel identification and the exon 33 deletion may represent a splice variant. Further analysis is required to confirm if any variant is pathogenic. A diagnostic test may be developed using information about associated SNP or after confirmation of a disease casing variant in the PDS5B gene.

I. INTRODUCTION

Cervicothoracic vertebral subluxation (CVS) is an inherited disorder that affects the skeletal and neurological systems of the Australian Merino sheep. Pedigree analysis indicates that CVS adheres to autosomal recessive mode of inheritance (personal communication, Aaron McMillian, Animal Genetics and Breeding Unit, Armidale, NSW, Australia), although prior to this study, the genetic mechanism of the disorder has not been explored (Hill et al. 1993). The presence of CVS in commercial flocks results in reduced herd productivity, and presents serious economic and animal welfare concerns. CVS has been identified in Australian Merino sheep on four separate properties, and in one Corriedale flock, a composite breed derived from the Australian Merino (Hartley et al., 1994). The prevalence of the disease in the national flock is unknown due to phenotype ambiguity, careful differential diagnosis is required to identify CVS from other "staggers" like syndromes (Thompson et al. 2008).

The disorder is characterised by the presence of "wedge shaped" hemivertebrae at the last cervical (C7) and/or first thoracic (T1) vertebrae. The base of the vertebra is typically rotated dorsally and the neurological deficits of the hind limbs, or ataxic gait, are attributed resultant compression of the spinal cord (Wong et al., 2005, Hartley et al., 1994). Affected animals exhibit sudden onset tetraplegia in sheep from 9 months to 3 years of age. The skeletal abnormalities and secondary neurological symptoms are often proceeded by recumbency and death. Potential models of CVS have been identified in cattle, dogs, horses, goats and humans (Otero-López et al. 2015, Kramer et al. 2013, Maddox and Cockett 2007).

Autosomal recessive disorders can be caused by homozygous or compound heterozygous mutations (Barbero et al. 2013). Typically, homozygous mutations are detected in animals of consanguineous mating's or in relatively isolated populations (Tryon et al., 2007). Homozygosity mapping in animals suffering from suspected inherited disorders has proven

[1] Faculty of Veterinary Science, School of Life and Environmental Sciences, The University of Sydney, Camden, NSW 2570, Australia
[2] The Elizabeth Macarthur Agricultural Institute (EMAI), NSW Department of Primary Industries, Menangle, NSW 2570, Australia

successful in identifying disease causing genes (Becker et al. 2010, Seelow et al., 2009).

The purpose of this study was to map CVS and identify positional candidate genes. The scope was expanded to incorporate the sequencing of a positional candidate gene. It is anticipated that this study may direct future research towards the development of a commercially available diagnostic test.

II. MATERIALS AND METHODS

a. Sample Acquirement and Preparation

Data and samples were collected from two properties. Tissue and blood samples were collected from 29 affected and 39 suspected carrier animals located on a stud enterprise, in Central West, NSW. In addition, blood and tissue samples were collected from two affected and 13 suspected carrier animals from a second property located in the Riverina, NSW. Pedigree data was provided for 56520 animals on the Central West, NSW stud operation. SNP genotype data were provided for previously genotyped animals, including 101 obligate carriers and 621 wildtype animals, but were not used in this study.

b. Nucleic Acid Extraction

DNA was extracted from whole blood and tissue samples using the DNeasy® Blood & Tissue Kit™ (QIAGEN, CA USA) according to the manufacturer's instructions. DNA was extracted from blood cards using in-house procedures developed by laboratory staff (Molecular Diagnostics Genetics Unit, NSW Department of Primary Industries, Menangle NSW). The quantity and quality of all DNA extracts were determined using the Nanodrop ND-1000™ (Nanodrop Technologies®, DE, USA). RNA was extracted from fibroblast cell culture using the RNeasy Mini Kit™ (QIAGEN, CA USA) according to the manufacturer's instructions.

c. SNP Genotyping and Homozygosity Mapping

DNA extracts from 14 clinically affected Merino sheep and two obligate carriers were outsourced for 50000 (50K) SNP genotyping using the Illumina® OvineSNP50 BeadChip (AGRF, VIC, Australia). The array experiments were performed according to protocols provided by the manufacturer, arrays were scanned and genotypes were called.

The SNP genotypes for 12 affected and two carrier animals were analysed for homozygous regions. Runs of homozygosity (ROH) were computed in PLINK (Purcell et al., 2007). Chromosomal segments were accepted as homozygous if they contained a minimum of 10-200 consecutive homozygous SNPs. A minimum length of 1000kb was also required for a ROH to be identified. The 1% error margin in genotyping calls, due to the use of the Illumina® OvineSNP50 BeadChip, was accounted for by permitting up to one heterozygote on each run. The ROH found on each chromosome were then visualized using the R software program (R Core Team, 2013).

d. Analysis of Homozygous Regions

SNP positions of homozygous regions were transformed from Oar_v1 to Oar_v3.1 based on the UCSC sheep genome browser International Sheep Genome Consortium (ISGC) Oar_v3.1 (Archibald et al., 2010). Boundaries of the shared ROH in affected animals were identified by visual analysis of SNP in EXCEL®. Genes located within the homozygous regions shared by all affected animals were searched for in the Mouse Genome Informatics® database, build MGI 6.05 (Smith et al., 2014). A positional candidate gene was identified based on its position within the detected homozygous region and as a result of documented mutations presenting phenotypically similar in mice compared to CVS in sheep.

e. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and DNA Sequencing

PCR primers were designed using Primer-BLAST® (Ye et al., 2012) and the GenBank® reference sequence for *PDS5B* mRNA (GenBank® XM_015098134). Five overlapping primer pairs (Appendix 1) were designed commencing at the G nucleotide in the ATG start codon and ending 35bp upstream of the TGA stop codon (GenBank® XM_015098134). Primers were designed to cover the coding sequence of *PDS5B* (34 exons), so that the intron-exon junctions as well as the entire exon were sequenced in both directions (Figure A1, Table A1). Cell culture derived RNA from an affected sheep was used for the template RNA in the polymerase chain reaction (PCR). The RT-PCR was performed using the One-Step RT-PCR PCR kit™ (QIAGEN, CA USA) in 50μL volumes containing 15-20ng of total RNA and 3μL of 10μM both forward and reverse primers in accordance with the manufacturer's instructions. A touchdown PCR was performed using the Mastercycler® Pro (Eppendorf, Hamburg, Germany). Reverse transcription was performed at 50°C for 30 min. PCR then commenced with an initial denaturation step at 94°C for 15 min, followed by 11 cycles comprised of denaturation for 1 min at 94°C, annealing for 1 min starting at 65°C and decreasing by 1°C each cycle until 55°C, extension was performed by 72°C for 2 min. An additional 29 cycles were completed with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by a single step at 72°C for 2 min.

PCR products were purified using the QIAquick PCR Purification Kit™ (QIAGEN, CA). To estimate PCR product concentration, 1 μL of each PCR product was loaded onto a 2% (w/v) agarose gel containing 1 x GelRed™ stain (Biotium Inc®, CA) at 85 volts for 45 minutes. The intensity of the PCR product when exposed to UV light was compared to a DNA marker of known concentration. Sanger DNA sequencing was outsourced to Australian Genome Research Facility. DNA sequence data were analysed using Sequencher® 5.4.5, (Gene Codes Corporation, MI USA).

III. RESULTS

a. Homozygosity Mapping and Candidate Gene Identification

Two of the 16 samples sent for 50k SNP genotyping had low call rates at 35% and 43%, these samples were below the Illumina expectations of <0.3 for LogR SD and consequently were excluded from the homozygosity mapping. Both excluded samples had 260/280 ratios of 2.36 (260/280) and low nucleic acid concentration < 45 ng/μL, indicating low quality of the DNA samples (Tryon et al., 2007). The 16 samples were compared in a relationship table (data not shown), to compare the degree of genetic relatedness between the animals genotyped. PLINK analysis identified runs of homozygosity (ROH) and visualization of these identified two regions of homozygosity shared among the affected animals: a region of extended shared homozygosity on chromosome 10 and a second smaller region on chromosome 6 (Appendix B; Figure B1). The region on chromosome 10 was refined after visual assessment of genotyping results in EXCEL to a region of 3,511,206bp (chr10:26,807,907-30,319,112).

The 26 documented genes in this shared region of homozygosity on chromosome 10 were investigated further to determine if mutations in any of these genes were likely to explain the phenotypic skeletal morphology of CVS. Genes which did not document skeletal abnormalities were excluded from further investigation in this study. One gene, *PDS5* cohesin associated factor B (*PDS5B*), was identified as a gene of interest. Mice with the known *PDS5B*^{tm1Jmi} mutation exhibited abnormal vertebral morphology with fusion and transformation of the C7 to T1 vertebrae (Weninger et al., 2014). CVS affected sheep documented in previous studies (Hill et al. 1993, Hartley et al. 1994), presented with a

characteristic phenotype of "wedge shaped" hemivertebrae at the last cervical (C7) and first thoracic (T1) vertebrae.

b. Mutation Identification

Sanger sequencing of 5 overlapping RT-PCR products expected to cover approximately 5150bp of the *PSD5B* gene (~98%) in an affected animal identified four polymorphisms (Table 1, Appendix C). A six base pair insertion was identified 3619bp downstream from the start codon on *PSD5B*, this insertion did not affect the reading frame of the gene and would result in the addition of the amino acids valine and arginine. Two possible missense mutations were identified, however the peak sizes in the chromatogram data for the nucleotides were low. A 111 bp deletion was identified when compared to the reference genome. This deletion corresponds to exon 33 in the human *PDS5B* gene and has been annotated as exon 32 in the ovine *PDS5B* gene. The non-coding first exon of the *PDS5B* gene has not been included in the OARv3.1 assembly.

Table 1: Genomic variations identified during comparative genome analysis

Gene	Type of Variation	Variation Size (bp)	Homozygous Nucleotide Change	Effect	Position - No. bp from Start codon
PDS5B	Missense	-	C>A	Leu>Ile	3412
PDS5B	Insertion	6	+GTAAGG	+Val & Arg	3619
PDS5B	Missense	-	A>T	Arg>Trp	4342
PDS5B	Exon Deletion	111	Exon 33	Unknown	4,066

IV. DISCUSSION

Care must be taken to differentially diagnose CVS from conditions including trauma, thalamic-cerebellar neuropathy, segmental axonopathy, degenerative thoracic myelopathy, Alexander's disease and other 'staggers' syndromes. Due to late onset of clinical signs affected animals might be misdiagnosed as unaffected. The phenotype ambiguity may account for discrepancies between the genotype of suspected carrier or unaffected "normal" animals. Pedigree information and availability of existing SNP genotyping data for relatives was considered when selecting animals for genotyping. Eleven of the 14 samples used in the initial homozygosity mapping were sampled from one property, these animals were between 65% and 91% genetically similar, higher genetic relatedness was associated with an increase length of homozygous regions on chromosome 10 and 6 (Appendix C).

a. Homozygosity Mapping and Candidate Gene Identification

SNP genotyping for homozygosity mapping was used as a preliminary approach to identify a genome region containing the disease causing gene. The two regions of homozygosity were detected in all of the affected samples, this included the outbred population on the second property in Riverina NSW. It is a limitation of the study that only a small sample size was used, however, further Illumina® OvineSNP50 BeadChip data is available for 101 obligate carriers and wildtype animals from the Central West, NSW stud operation. Future research should be directed at including obligate carrier and wildtype animals in a homozygosity or association analysis to confirm the location of the region of homozygosity likely to contain the causative mutation.

The *PDS5* homologue B gene was selected for further candidate gene analysis based on its position within the identified region of interest on chromosome 10 as well as its striking phenotypical similarity between CVS and reported malformations of C7 to T1 vertebrae in

PDS5B^{tm1Jmi} mice (Eppig et al., 2016). *PDS5* is a cohesion protein, required for proper chromosome segregation (Kim et al., 2013), it is highly conserved between humans and sheep (Figure C1). Mutations in cohesion proteins are characterised by multisystem developmental abnormalities due their role in complex gene expression control (Zakari et al., 2015). Skeletal abnormalities have previously been identified in mice who presented with *PDS5B*^{tm1Jmi} mutations, these documented mutations included insertions and intragenic deletions (Eppig et al., 2015). The developmental abnormalities induced by genetic variation in the murine *PDS5B* gene have been directly compared to Cornelia de Lange syndrome (CdLS) in humans (Zhang et al. 2009). Cohesinopathies, or mutations of cohesion proteins, have been associated with other mammalian diseases including Roberts Syndrome (RBS) and CdLS (Zakari et al., 2015, Xu et al., 2014), further analysis is required to determine if CVS in sheep is a potential model for either of these disorders.

The *PDS5B* gene spans across 35 exons and required 5 primer pairs to sequence the 98% of the genes coding sequence (Figure A1). Comparative analysis of the *PDS5B* cDNA between a CVS affected sheep and a wildtype reference sheep genome (International Sheep Genetics Consortium Oar_v3.1/oviAri3) revealed four variants of interest, a six base pair insertion, two missense mutations and an 111bp deletion. The six base pair insertion, identified 3,619bp downstream of the start codon on the affected genome (3619_3620insGTAAGG), does not affect the reading frame of the gene (Table 1, Figure C1). This in-frame insertion codes for the addition of two amino acids, valine and arginine. Further investigation revealed that the 6pb insertion was also present in four of the five available ovine reference sequences (GenBank® XM_015098132, XM_015098133, XM_015098135, XM_015098137), as well as being present in the human reference sequence. Analysis of the two missense mutations revealed low chromatogram call peaks, consequently these variants need to be further investigated.

A 111 bp deletion that corresponds to the deletion of the entire exon 33 was identified when comparing the affected genome to the reference wildtype (International Sheep Genetics Consortium Oar_v3.1/oviAri3 - GenBank® XM_015098134). This variation could represent a splice variant. A BLAST search could identify only one known transcripts in mouflon (GenBank® XM_012148311.2) that also included an exon 33 deletion. Whole exon deletions have been associated with a number of genetic disorders in humans including Alzheimer's disease, early epilepsy, intellectual disability and optic atrophy (Ben-Salem et al., 2015, Hardy et al., 1999). As only one sample from a CVS affected sheep was used for DNA sequencing, the complementary DNA (cDNA) deletion of exon 33 in the *PDS5B* gene on chromosome 10 has not yet been confirmed. The two missense mutations and the possible deletion of exon 33 need to be investigated in genomic DNA of additional affected animal.

Further investigation is required to investigate the two missense mutations and to categorise the exon 33 deletion found between the cDNA genotyping data and the reference genome. If none of these variants are disease causing further investigation of the two identified regions of homozygosity is required. Whole genome sequencing is considered to cover both regions including the non-coding sequences in the *PDS5B* gene and is a direction for future investigation. Non coding regions are known to possess a regulatory role in gene expression, they have the capacity to affect the alternate splicing of mRNA (Jin et al., 2009). It is anticipated that the findings of this study will be used to direct future research in identifying and confirming, a causative variant for CVS, and furthermore, the development of a diagnostic test. Continued sampling of affected and suspected carrier animals will be required to ensure a true disease causing variant is correctly identified.

ACKNOWLEDGMENTS

This work was supported financially by the Australian Wool Education Trust, the University of Sydney and the Elizabeth Macarthur Agricultural Institute (EMAI). Prof Julius van der Werf, Sheep CRC, is acknowledged for making SNP genotyping data for future analysis available.

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Appendix A

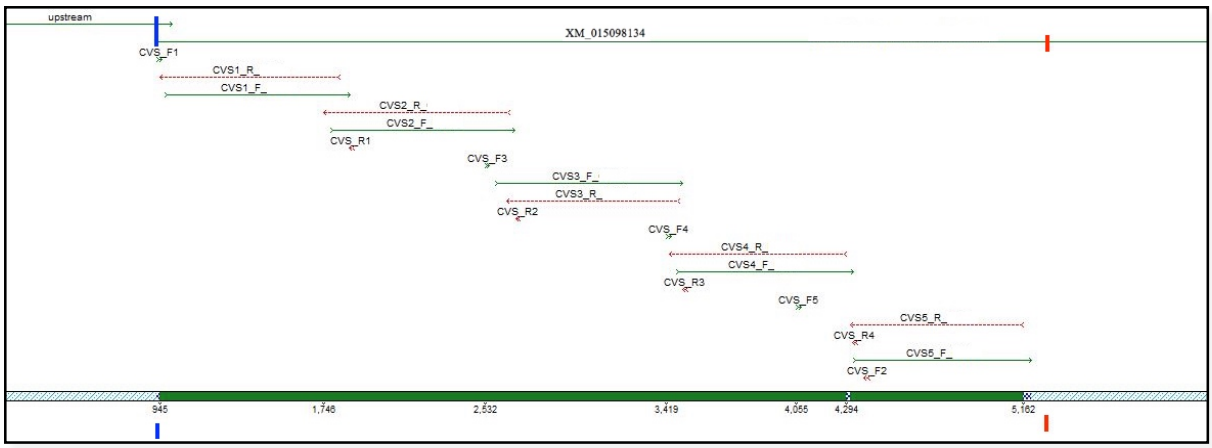

Figure A1: *PDS5B* regions targeted to identify polymorphisms in 1 affected merino sheep. The cDNA region of *PDS5B*, spanning the genomic region chromosome 10: 26,807,907-30,319,112 is represented (Green unbroken line labelled GenBank® XM_015098134). The start codon (Blue), the stop codon (red), and approximately 800bp upstream is shown. The location of each primer (Table A1) used for PCR/sequencing is provided: sense primers are denoted by green arrowheads and antisense primers are denoted by red arrowheads. cDNA sequencing data is represented by solid green lines (sense strands; CVSX_F_) and broken red lines (antisense strands; CVSX_R_).

Table A1: Primer details

Primer ID	Sequence (5'→3')	Strand	Gene	Region	Genomic Location[A]
CVS1_F	GGCTCATTCAAAGACAAGGACC	sense	PDS5B	Start Codon	28687544-28687565
CVS2_R	TGCCCAAGTAACACTGCCAA	antisense	PDS5B	Exon 9	28668483-28668502
CVS2_F	TTGCTGCTCTCAGTTTTACCC	sense	PDS5B	Exon 8	28670723-28670743
CVS2_R	TGTTTGGGGTTGCCCAACTT	antisense	PDS5B	Exon 17	28645095-28645114
CVS3_F	TTGCCAGATCCTGGTAAGGC	sense	PDS5B	Exon 16	28646676-28646695
CVS3_R	GTCAAGTCTCCATCACTGTGC	antisense	PDS5B	Exon 23	28607486-28607506
CVS4_F	GATGGTTCGATGGCTACTTGGA	sense	PDS5B	Exon 23	28607566-28607587
CVS4_R	TGCTGATGAAAGCGGCTTATT	antisense	PDS5B	Exon 30	28586647-28586667
CVS5_F	CCTGGAAAACCCAAAACAACCA	sense	PDS5B	Exon 29	28586635-28586659
CVS5_R	CAAAGCTCTCACACAGAAAGTT	antisense	PDS5B	3' UTR	28572984-28573005

[A] Genomic location refers to position on the *Ovis aries* genome assembly build Oar_v3.1/oviAri3 (released October 2012). Region is based on the assumption that exon 1 is missing from the ovine annotation.

Appendix B

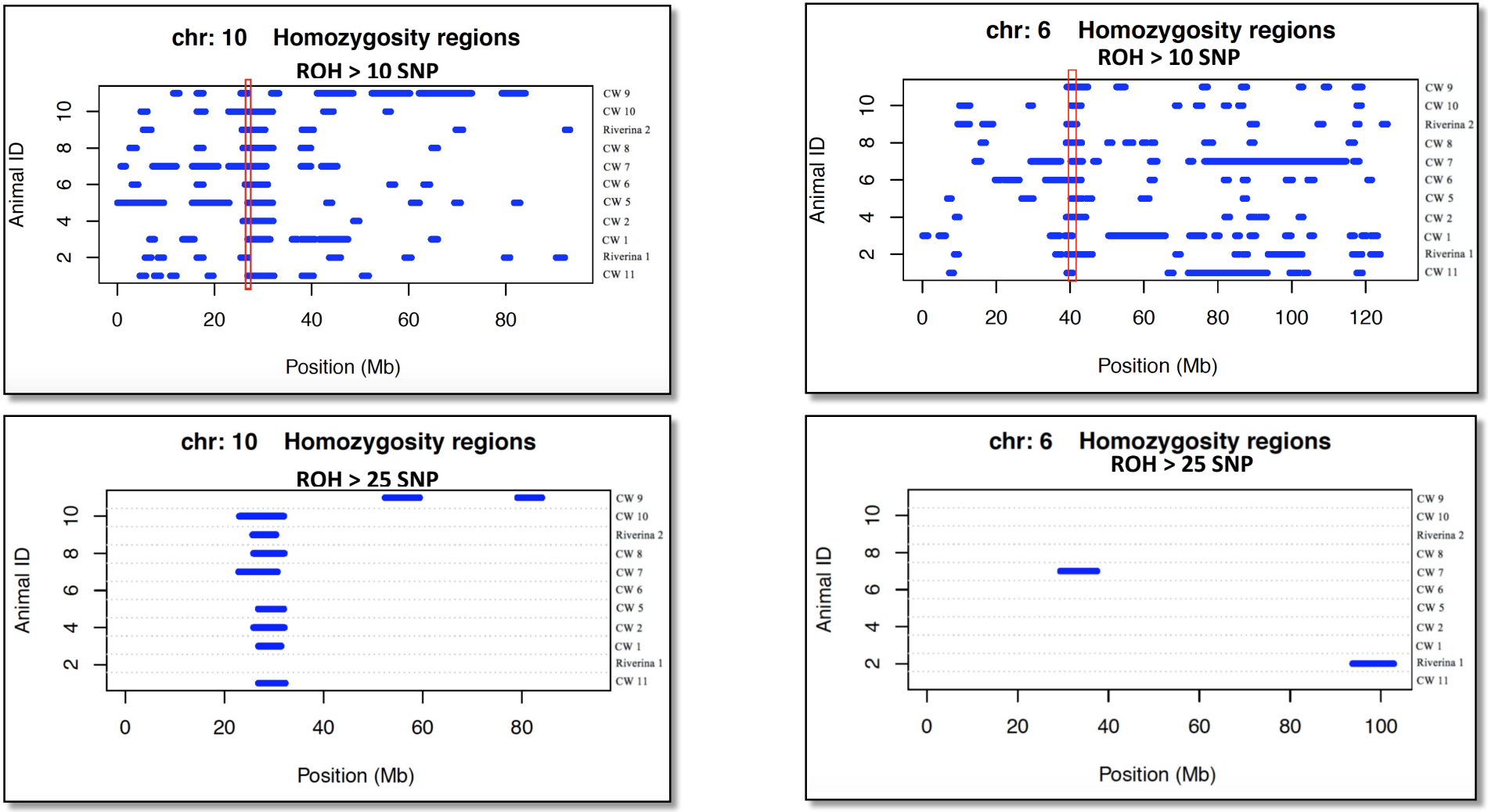

Figure B1: Runs of homozygosity (ROH) on chromosome 10 and 6 visualised using R software program (R Core Team, 2013), different length of ROH (>10 SNP and > 25 SNP) are shown. The region of homozygosity shared among all affected animals is highlighted by a red box.

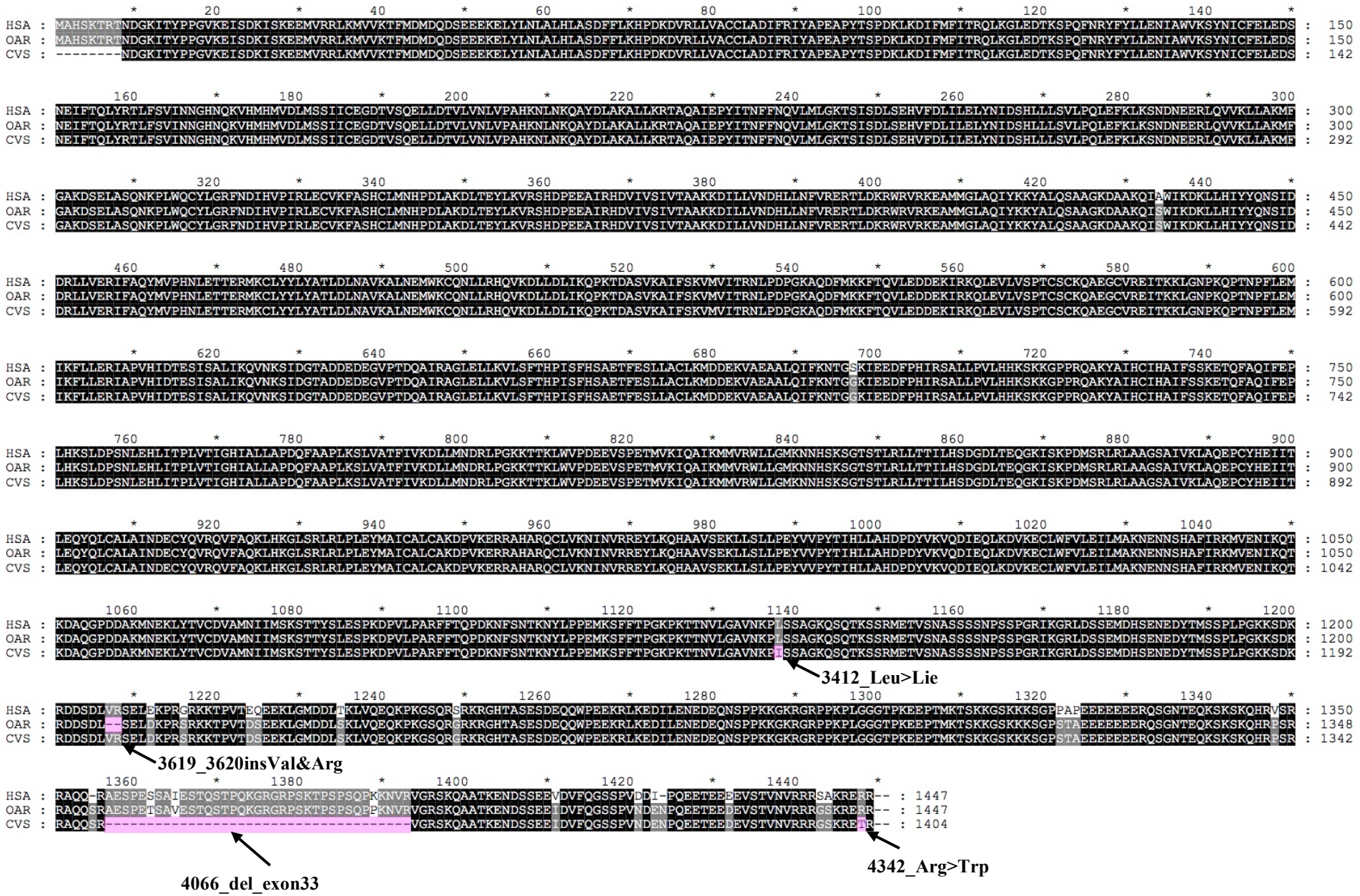

Figure C1: An alignment of human (HSA; Q9NTI5) and sheep (OAR; XP_014953620.1) PDS5B protein sequence and the predicted protein sequence derived from cDNA of a CVS affected sheep (CVS). Variant regions are highlighted in pink; solid arrows denote the position of the variants listed in Table 1. This Image was created using GenDoc® (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html).