Chapter 11

Identification of Polymorphism in the Keratin Genes (KAP3.2, KAP6.1, KAP7, KAP8) and Microsatellite BfMS in Merino Sheep Using Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) Analysis

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Additional information is available at the end of the chapter

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1. Introduction

Wool production is a major agricultural industry world-wide, the most important wool-growing countries include Australia, China, New Zealand, South Africa and countries within South America. In Australia for example, the world's largest producer of wool accounting for ~ 30% of the world production, wool industry is among the top industries in export revenue. While Australia has long been associated with the production of high-quality wool, the importance of this industry and the value of wool exports have been steadily declining.

1.1. Challenges facing the wool industry

The wool industry is faced with many challenges that require innovative solutions. The major competitors to the wool industry, cotton and synthetics, have developed new fibres that meet consumer needs such as being lightweight, soft and easy to care. These competitors have also made better productivity gains than wool, which has resulted in lower prices for all textile products. Today, there is much instability in wool prices, with a major problem facing the industry in faulty wool production. It has been observed that considerable variation exists both within and between fleeces across sheep breeds, as well as within inbred lines of sheep. Since the efficiency of wool processing is dependent on the consistency of wool fibre, it is of prime importance to wool producers that this variation is controlled. The wool characteristics that are

of economic importance include fibre diameter (or fineness), grease and clean fleece weight, fleece strength and length, colour, yield, crimp and bulk. For Merino and halfbred wools, fibre diameter is the major factor that contributes to price variation as it significantly influences both fibre processing properties and ultimate product quality. The colour of wool is also important because superior colour (bright and white) can be dyed to the maximum range of shades and consequently is worth more than poorer coloured wool. Furthermore, the quantity of wool is important in overall wool production and in the efficiency of the production system.

1.2. Classical selective breeding – Not a simple solution

For many years, farmers have been using classical selective breeding, where by selection of breeding animals was traditionally based on the phenotype (that is appearance) of the individual animal, a rather slow method of selection. Each animal is assigned a breeding value (BV), which describes the future genetic potential of an animal. The BV is calculated by adjusting phenotype to exclude factors such as birth rank, lambing status and sex in order to give an estimate of the genetic merit. The desired goal of this strategy is the accumulation of "good" forms of genes for that particular trait in the population, over time. This has resulted in many breeds that are commercially important today. The domestic sheep *Ovis aries* today comprises over 500 different domestic breeds. However, wool characteristics, like many production traits (such as milk yield, growth rate, meat tenderness), do not exhibit simple Mendelian inheritance patterns (recessive or dominant). Instead, they are controlled by not only many genes, but also the interaction of these genes, each having small additive effects on the phenotype observed. Environmental and management factors also play a role. Thus, wool traits are quantitative and show continuous variation in phenotype, a fact that makes it difficult to deduce the genotype of an animal from its phenotype, and to relate genetic variation to differences in the phenotype. In other words, genetic improvement breeding programme select for "phenotypic superior" animals, without the knowledge of the actual genes that are being selected – which I will term as "**blind selection**" in this paper. Furthermore, other strategies to control environmental factors such as nutrition, time of shearing or mineral supplementation tend to be costly. In addition, wool production traits tend to only be fully expressed when an animal is mature, at least three years old, and therefore genetic progress using phenotypic selection and pedigree information is relatively slow.

1.3. Identification of gene markers: A possible solution

The answer to sidestepping this "**blind selection**", inaccuracy in describing the genetic potential of an animal and slow progress may lie in identifying specific genetic markers that are associated with wool production traits. Some sheep consistently produce quality or faulty wool, suggesting that genetic factors are an important key in determining wool characteristics. In addition, estimates for the heritability (h^2) of most wool traits are generally high (h^2 = 0.3 - 0.6), indicating that wool traits are under genetic control and that they can be selected for. A gene is a segment of DNA that provides the genetic information necessary to produce a protein. For almost all of the genes, there are two copies (alleles), one

inherited from the mother and the other from the father. In any population of animals, there can be many different alleles. This is termed polymorphism or genetic variation. Polymorphism results from DNA mutation. It is this polymorphism that is taken advantage of, in order to identify genetic markers. A genetic marker for a particular characteristic can be defined as a piece of DNA that directly affects a phenotype and shows polymorphism. It can also be a piece of DNA that is closely linked to another piece of DNA that affects a phenotype. Genetic markers can either be genes or non-functional DNA segments such as microsatellites or minisatellites.

A number of different types of genetic markers are commonly used, including restriction fragment length polymorphisms (RFLPs), microsatellite and minisatellite DNA, and polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) variants. Restriction fragment length polymorphism results from the alteration of the restriction site(s) recognised by a specific restriction endonuclease or by the insertion or deletion of sequence between two restriction sites. The variation in fragment lengths is detected using gel electrophoresis. Although RFLPs were the first genetic markers developed, they are losing popularity as a screening method to identify genetic markers because they have the disadvantages of not identifying all of the polymorphism with a length of DNA, are time-consuming and restriction enzymes and consumables tend to be expensive. Simpler marker systems have subsequently been developed, many of these systems are now based on satellite DNA sequences.

Throughout the genome of higher eukaryotic organisms, there are a variety of different short DNA sequence repeats known as satellite DNA. These sequences do not code for protein and are highly variable from individual to individual in both the number and type of repeats (Groth *et al*., 1987). Microsatellites are composed of DNA repeats in tandem at each locus. The tandem repeats are usually simple, and consist of either a single nucleotide or dinucleotide such as (CA)n, with each dinucleotide repeated about ten times. Minisatellites have longer repeated sequences than microsatellites, such as (ACTG)n. Since microsatellites and minisatellites show a substantial amount of polymorphism, they can serve as useful markers for the identification of genetic variation of value to animal breeding. Although the variation in the number of repeats can sometimes be detected using RFLP, PCR is generally used to amplify the polymorphic region and the amplimer analysed for length variation (a technique referred to amplified fragment length polymorphism – AFLP).

1.4. Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) as a preferred type of genetic marker

PCR is also used in conjunction with SSCP. The PCR-SSCP technique offers a rapid, sensitive and relatively inexpensive way to screen for sequence variation with minimal sequencing. First described by Orita *et al*. (1989), this technique has become one of the preferred methods for screening samples to detect polymorphism because it is both simple and sensitive. In this techniques, regions of the gene of interest are amplified using PCR and the products denatured and then cooled rapidly to promote the formation of secondary structures due to internal base-pairing, which are in turn sequence dependent (Orita *et al*., 1989). The folded

single-stranded DNA molecules are separated by polyacrylamide gel electrophoresis under non-denaturing conditions. The folded secondary structures are affected by physical conditions such as temperature, percentage of polyacrylamide, ionic strength of the electrophoretic buffer, glycerol concentration (Spinardi *et al*., 1991), ratio of acrylamide to bis-acrylamide, run length and run voltage. This can be exploited when optimising an SSCP protocol so that maximum variation can be detected in a given section of DNA. Molecules that differ by even a single nucleotide may form different conformers under a given set of conditions and, upon electrophoresis in a non-denaturing polyacrylamide gel, migrate differently. Many methods for viewing the folded DNA conformers have been described. These include the radioactive labelling of primers followed by autoradiography (Orita *et al*., 1989), silver staining (Sanguinetti *et al*., 1994), ethidium bromide staining (Yap and McGee, 1993) and more recently the use of fluorescently labelled primers and fluorescent dyes.

1.5. Methods used to identify genetic markers

There are several ways to identify genetic markers, but the two approaches most commonly used are the genome scanning or linkage analysis and the candidate gene approach. In the genome scan approach, the whole genome is searched to identify Quantitative Trait Loci (QTL) that affect any given trait. These are not necessarily the genes that are responsible for trait variation, but give an indication of where such genes may lie. Linkage analysis is an involved process. A map of the chromosomes, laying out the location, phase and order of genes and markers, and the distance between them, is required before linkage analysis can be performed. Firstly, a selection of about 200 markers distributed throughout the genome are genotyped, in the sire of the animals. Only the informative markers are genotyped in the progeny and each marker tested for suggestive linkage. Regions showing suggestive linkage are then studied by saturating the region with markers to identify those that are tightly linked. Phenotypic variation is then linked to the segregation of DNA markers within a population. Once the gene locus is identified by the tightly linked markers, the DNA can be sequenced. Linkage analysis can be an expensive and lengthy process requiring access to full chromosome libraries and arrays of markers.

In the candidate gene approach, known genes or gene markers that are thought to be responsible for the phenotypic variance of a trait are targeted for investigation. In this case, knowledge of the understanding of the genes that are likely to affect wool quality. The method requires a good knowledge of the physiological and biochemical processes of the gene product and can be a more direct method than the gene mapping approach, provided the right initial assumptions are made. One of the limitations of this approach is its "hit and miss" nature. A targeted gene may not be polymorphic in a population or genetic variation within the targeted gene may not affect the trait (Goddard, 2002). For the candidate gene approach to be useful, a quick and relatively inexpensive way to screen the target gene for polymorphism is essential.

The wool fibre is a complex structure composed primarily of proteins from the keratin family, which are the keratin intermediate-filament proteins (KRTs) and the keratin intermediate-filament associated proteins (KAPs). The KRTs form the skeletal structure of

the wool fibre (microfibrils) and are embedded in a matrix of KAPs (Powell and Rogers, 1986), the different proteins being connected through disulphide cross-linkages (Powell, 1996). Therefore, genes that code for the KAPs and KRTs proteins are potential candidate genes in the identification of genetic markers associated with wool quality traits.

1.6. Half sib analysis

Half-sib analysis is a tool that allows genetic effects to be ascertained from field trials while controlling for environmental and management effects. Firstly, the gene being targeted must be polymorphic, with at least two alleles. A good sire is selected and mated to many ewes (at least 200 in number), that are selected at random from a range of environments, in order to maximize phenotypic variation in wool traits. The sire must be informative at each locus that is being investigated (i.e., the genotype of the sire must be heterozygous). If not, then the progeny does not get genotyped for those loci that the selected sire is homozygous. For those loci that the sire is heterozygous, the progeny born are genotyped soon after birth, and allowed to grow until their wool measurements can be taken at (12, 24 and 36 months of age). Suppose a sire has the genotype AB at the K33 locus, then all the progeny that have inherited the A allele from the sire are put in one group, and those that have inherited the B allele from the sire are put in another group. The means of the wool measurements from both groups are then compared. If the group of progeny that inherited the B allele from their sire are found to for example have a significantly stronger staple strength than those progeny that inherited the A allele from their sire, then this would give an indication that the K33 B allele might be associated with stronger staple strength.

1.7. Previously published association of genetic markers with wool traits

Numerous studies have described variation within both the KAP and KRT genes, including the work of Rogers *et al*. (1994a); Parsons *et al*. (1994a; 1996); McLaren *et al*. (1997); Beh *et al*. (2001); Itenge-Mweza *et al*. (2007). There are some reports associating variation in the KRT and KAP genes with variation in wool traits. Parsons *et al*. (1994b) and Beh *et al*. (2001) reported associations between variation in KAPs and mean fibre diameter in Merino sheep, while Rogers *et al*. (1994b) reported association between staple strength in Romney sheep and the region spanning the KAP1.1/KAP1.3/K33 loci on ovine chromosome 11. Itenge *et al*. (2009; 2010) reported association between variation in the KAP1.1 gene with variation in yield. In one of the half-sib families studied, variation in the K33 gene was associated with variation in staple strength. Markers, other than the KRT and KAP genes associated with wool traits have also been reported and these, together with reported keratin gene markers are summarised in Table 1.1.

1.8. Gel electrophoresis

Gel electrophoresis is the process in which an electrical current is applied to a gel to separate large molecules such as nucleic acids, from a mixture of similar molecules, based on differences or how they react to the electrical current. The technique relies on the fact that

Trait[1]	Analysis	Breed	Chromosome	Marker(s)	Gene(s)	Reference
Yield	Candidate gene Candidate gene	Merino Merino	11 11	- -	KAP1.1 KAP1.3	Itenge *et al.* (2009; 2010) Itenge *et al.* (2009)
FDSD	Candidate gene	Merino	11	-	K33	Itenge *et al.* (2009)
MFD	Candidate gene Genome Scan Genome Scan	Peppin Merino Merino Not specified	1 1 Not specified	- *Oar*DB6/RM65 Not specified	KAP6 and KAP8 - Not specified	Parsons *et al.* (1994a) Beh *et al.* (2001) Henry *et al.* (1998)
Staple strength	Candidate gene Candidate gene Candidate gene	Merino Romney Merino	11 11 11	- - -	K33 K33, KAP1.1 & KAP1.3	Itenge *et al.* (2009; 2010) Rogers *et al.* (1994a) Itenge *et al.* (2009)
Staple length	Candidate gene Candidate gene Candidate gene Segment mapping	Merino Merino Merino Synthetic breed INRA 401[2]	11 11 11 3, 7 & 25	- - - BMC1009, ILST005 & IDGVA8-IDGVA88	KAP1.1 KAP1.3 K33 - -	Itenge *et al.* (2009; 2010) Itenge *et al.* (2010) Itenge *et al.* (2009) Ponz *et al.* (2001)
CVD	Segment mapping	Synthetic breed INRA 401[2]	4, 7 & 25	McM218, ILST005 & IDGVA8-IDGVA88		Ponz *et al.* (2001)
Yellowness (Y-Z)	Candidate gene Genome Scan	Merino MRM[3] backcross	11 3	- TGLA77	KAP1.3 -	Itenge *et al.* (2009) McKenzie *et al.* (2001)
Brightness (Y)	Genome Scan	MRM[3] backcross	11	*Oar*FCB193	-	McKenzie *et al.* (2001)
Challenge colour	Genome Scan	Corriendale	11	*Oar*FCB193	-	McKenzie *et al.* (2001)
White colour	Candidate gene Candidate gene	Merino Merino	11 Not specified	- Not specified	KAP1.3 Not specified	Itenge *et al.* (2010) Benavides and Maher (2000)
CFW	Selected markers	Merino	Not specified	BMS and DRB1	MHC region	Bot *et al.* (2003)

[1]MFD = mean fibre diameter; CVD = coefficient of variation of fibre diameter.
[2]A composite Romanov (prolific breed) and Berrichon du Cher (meat breed)
[3]MRM = Merino X Romney X Merino backcross

Table 1. Potential genetic markers for wool quality traits reported by various researchers.

nucleic acids are negatively charged because of the phosphate groups on the phosphodiester backbone of the nucleic acid strands (Nicholl, 1994). Nucleic acid molecules will migrate from the negative (black) terminal to the positive (red) terminal if put in solution and an electric field is applied, due to the net negative charge in solution. The gel matrix adds a sieving effect so that particles can be characterized by both charge and size.

Agarose is a macromolecular substance that is derived from seaweed. It can be purified to a whitish granular powder which, when mixed with water and heated, can be left to set like a jelly. This is called a gel and it acts like a sieve for the DNA molecules. To separate DNA molecules that are different lengths, agarose is used to produce a molecular sieve. The speed that the DNA travels through the gel is inversely proportional to the size of the DNA. In other words, small DNA particles migrate faster than large DNA molecules, as they are less physically restrained by the gel matrix. The length of a piece of DNA can be determined by comparing it to a molecular weight ladder. Agarose gel electrophoresis can be affected by:

1. The percentage of agarose, which affects the sieving of the DNA molecules.
2. The voltage applied during the electrophoresis, which cause the DNA molecules to move.

Typically, 1000 – 50,000 bp can be separated by 0.3% agarose, and 300 – 6000 bp can be separated by 1.4% agarose, while base pairs less than 500 are better separated using polyacrylamide gel, with gel percentage between 10-20. The polyacrylamide gel electrophoresis works under non-denaturing conditions.

After the electrophoresis is complete, the molecules in the gel can be stained to make them visible. Ethidium bromide, silver, or coomassie blue dye may be used for this process. Other methods may also be used to visualize the separation of the mixture's components on the gel. If the analyte molecules fluoresce under ultraviolet light, a photograph can be taken of the gel under ultraviolet lighting conditions, often using a Gel Doc. A molecular weight marker (MM) is often included on the gel to give an indication of the fragment size.

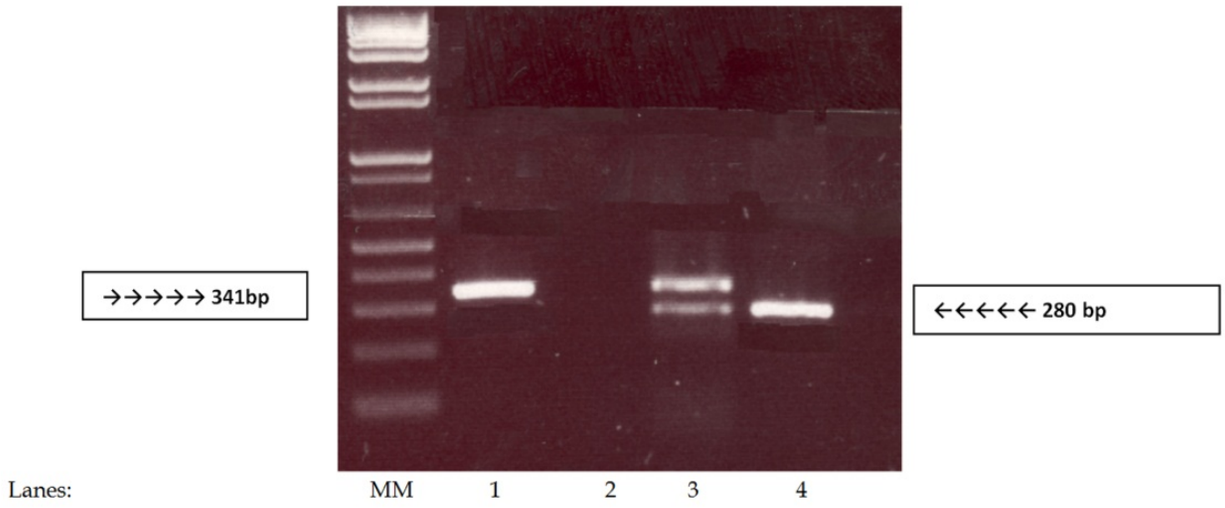


Figure 1. An example of a gel photo. MM is the molecular marker. Lane 1 has 341 bp DNA, while lane 4 has 280bp DNA. Lane 2 is blank.

1.9. Aim and objective of this paper

This paper discusses the identification of genetic variation in the KAP3.2, KAP6.1, KAP7, KAP8, KRT2.10 and BfMS loci in Merino sheep using polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis. Polymorphism within these loci is likely to be in part responsible for the observed variation in wool characteristics and could result in the identification of gene markers to be used in gene marker-assisted selection programmes within the wool industry.

2. Materials and methods

2.1. Sheep used in the study

This study used two half-sib families referred to as Sire Line 1 (SL1) and Sire Line 2 (SL2). The SL1 half-sib was produced by mating a fine wool producer Merino ram to 150 Merino ewes, selected at random from a range of New Zealand environments, in order to maximise phenotypic variation in wool traits. In year one, the SL1 consisted of 131 pure New Zealand Merino lambs, with 128 of these surviving to the second shearing at 24 months. Following the second shearing the wether lambs and some of the ewe lambs were culled and only the remaining ewe lambs (n = 37) were shorn at 36 months of age. The SL2 half –sib consisted of

35 lambs (Merino x Romney ram x Merino ewes). Half-sib groups were kept as single flocks to minimise environmental variation between individual progeny and provide control. All lambs were tagged at birth to their dam and their gender and birth rank were recorded.

2.2. Wool shearing and sampling

Mid-side wool samples were collected at 12, 24 and 36 months of age for SL1 and at 12 months of age for SL2. Except for greasy fleece weight (GFW) which was determined at shearing, wool measurements were performed by the New Zealand Wool Testing Authority Ltd (NZWTA), Napier, New Zealand according to International Wool Textile Organisation (IWTO) standards. Measurements included comfort factor or the percentage of fibres of diameter greater than 30 μm (F<30), mean fibre diameter (MFD, IWTO-12-03), fibre diameter standard deviation (FDSD, IWTO-12-03), coefficient of variation of fibre diameter (CVD, IWTO-12-03) and curvature, were all measured using a Sirolan™ Laserscan Fibre Diameter analyser while the mean staple length (MSL, IWTO-30) and mean staple strength (MSS, IWTO-30) of each sample was determined using Automatic tester for Length and Strength (ATLAS). The colour (MY-Z) and brightness (MB) of the wool was measured using a reflectance spectrophotometer, where the tristimulus values Y-Z indicate the yellowness of the wool and the tristiulus value Y represents the brightness of the wool. The yield of wool, the weight of clean wool after impurities such as vegetable matter have been removed, expressed as a percentage of greasy wool weight was mathematically derived for the wool base (IWTO-19) measurements. Once yield measurements were obtained from the NZWTA, clean fleece weight (CFW) was calculated as the product of GFW and yield.

2.3. Blood sampling on FTA™ cards and DNA isolation

Blood samples (containing DNA) were collected from the progeny and sires onto FTA™ cards (Whatman, Middlesex, UK). These were stored at room temperature (See Figure 2.1). A small punch (1.2 mm in diameter) was taken from the blood on the FTATM cards using a Harris Micro Punch (Whatman International Ltd, UK) and put into a 200 μL tube. The DNA on the punches was isolated following a modified manufacturer's protocol. 200 μL of FTA™ reagent was added to each tube containing a 1.2 mm punch of FTA™ paper, containing the sample DNA. The tubes were incubated at room temperature for 60 minutes. Each tube was vortexed three times for about five seconds at the start of the incubation, half-way through the incubation, and after the incubation period. The FTA™ reagent was aspirated, and the cards were washed with 200 μL of TE buffer (1 M Tris and 0.5 M Na_2EDTA) for two minutes. The TE buffer was aspirated and the tubes were left open, but covered with a tube holder and stored at 4 ^{o}C and used for the subsequent PCR reaction.

2.4. Amplification of the loci using PCR

The PCR conditions for the loci that are described in the literature were initially used. However, re-optimisation was necessary for amplification in an i-Cycler PCR machine (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR protocols were optimised by using a

temperature gradient (to determine annealing temperature) coupled with a magnesium titration.

All the primer sequences used in the study were obtained from the literature (Table 2.1), and were synthesized by Invitrogen New Zealand Limited, Penrose, Auckland, New Zealand. PCR amplifications were performed in a reaction mixture containing ~ 50 ng of genomic DNA on a washed 1.2 mm punch of FTA™ paper, 1× PCR reaction buffer with 1U Taq polymerase (Qiagen, GmBH, Hilden, Germany). Table 2.2 lists the total reaction volume used along with the specific dNTP, primer, magnesium, and Q concentrations for each locus.

Amplification consisted of 1 minute denaturation at 95 °C, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at temperatures specified in Table 2.3 for 1 minute and extension at 72 °C for 1 minute, with a final extension of 72 °C for 7 minute. All the primer sequences used in the study were obtained from the literature, and were synthesised by Invitrogen New Zealand Limited, Penrose, Auckland, New Zealand.

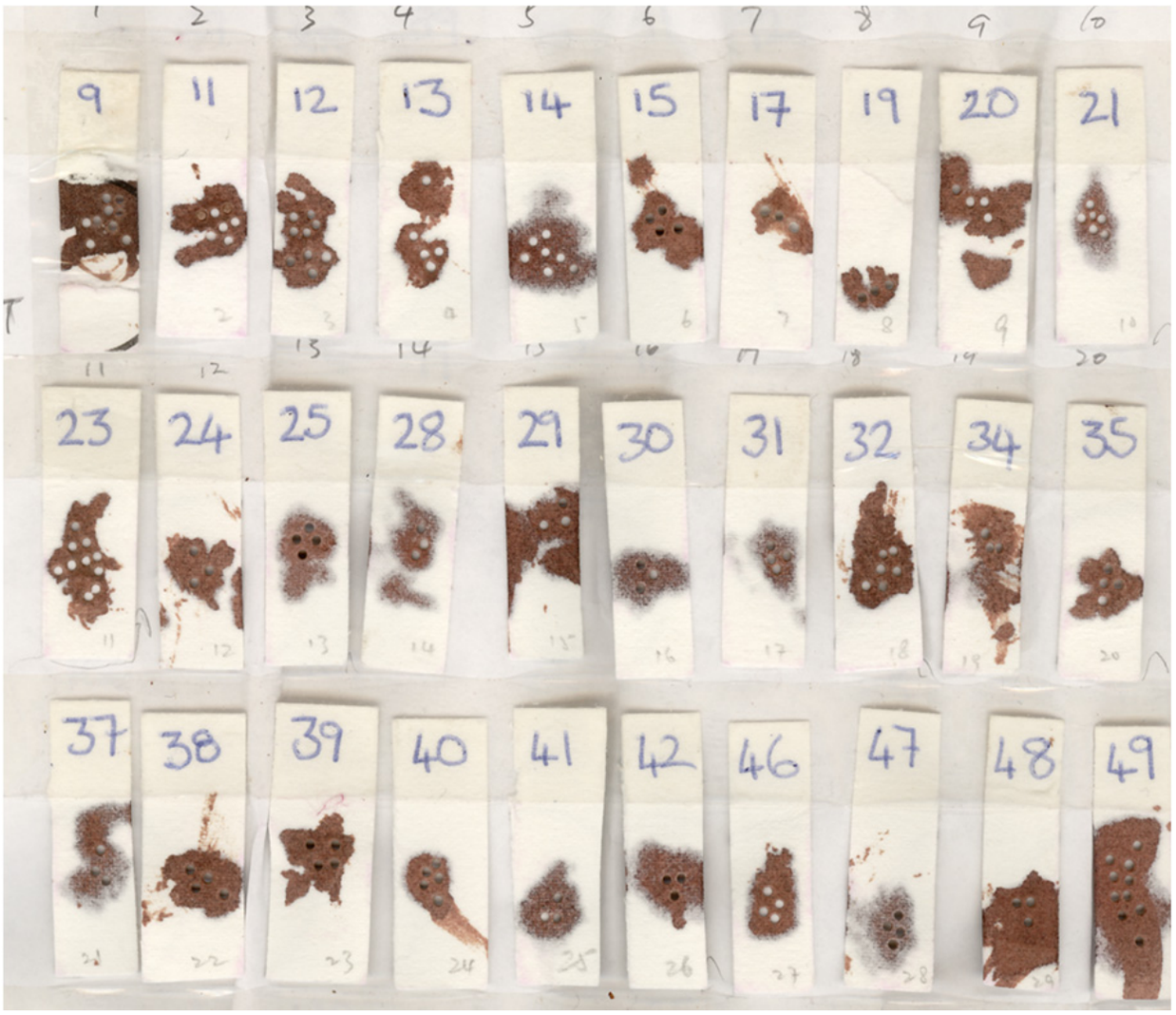


Figure 2. FTA™ cards of blood samples collected from the progeny of sire line 1

Locus	Primer Sequence	Source
Keratin genes		
KAP3.2	5′- CGAGACACCAAGACTTCTCTCATC-3′ 5′- AGTGAGTGTTGAAGGCCAGATCAC-3′	McLaren *et al.* (1997)
KAP6.1	5′- CCAATGGCATGAAGGTGT-3′ 5′- AAAAAGGGAAGGGTTGGTG-3′	McLaren *et al.* (1997)
KAP7	5′- CATCGGACAGCTTGAGGTAT-3′ 5′- ACAGAGAATTGAGGGCGG-3′	McLaren *et al.* (1997)
KAP8	5′- GGACGCAACTGAGGACGCAACTG-3′ 5′- ACACTTGGAATTCAATAAATATGTGTTGG-3′	Wood *et al.* (1992)
KRT2.10	5′- ATGGCCTGCCTGCTCAAGGAGTAC-3′ 5′- CTTAGGACTGAGACTAGGATGAGG-3′	Rogers (1994)
Microsatellites		
BfMS	5′- CAACGGTCTGCAACCGAATTACC-3′ 5′- CAATCCGTGGGTTGGAACACAA-3′	Bot *et al.* (2003)

Table 2. Primer sequences and source references for each locus investigated.

Locus	Total volume (μL)	Primer concentration (nM)	dNTP concentration (μM)	Mg^{2+} concentration (mM)	Q Concentration (×)
KAP3.2	25	350	175	1.0	1×
KAP6.1	25	400	200	1.0	1×
KAP7	25	350	175	1.0	1×
KAP8	25	350	175	1.0	1×
KRT2.10	20	400	200	1.0	-
BfMS	25	350	175	1.0	1×

Table 3. Optimised PCR conditions for each locus investigated.

Locus	Annealing temperature (°C)	Amplimer size (bp)
KAP3.2	58	424
KAP6.1	62	528
KAP7	63	413
KAP8	62	124*
KRT2.10	65	191
BfMS	58	200*

* = Variable length as amplification of a microsatellite region
# = Amplified with primers by Rogers *et al.* (1994b)

Table 4. Optimised annealing temperatures and predicted amplimer sizes for each locus investigated.

2.5. Agarose gel electrophoresis

Amplimers were analysed in 1.0% w/v SeaKem® LE agarose (FMC Bioproducts, Rockland, Maine, USA) gels prepared with 1× TBE buffer (89 mMTris, 89 mM orthoboric acid, 2 mM Na_2EDTA; pH 8) containing 0.1 mg/L ethidium bromide. Five μL of PCR product was added to 2.5 μL of loading dye (0.2% bromophenol blue, 0.2% xylene cyanol, 40% (w/v) sucrose) and the gels were electrophoresed at a constant 10 Vcm^{-1} for 30 minutes. A molecular weight marker (Invitrogen Life Technologies) was included on the gel to give an indication of the fragment size. DNA bands were viewed on a UV transilluminator (254 nm) and a photograph taken for records.

2.6. Optimisation of SSCP gels

PCR-SSCP conditions were available in the literature for KAP3.2 (McLaren *et al*., 1997), however these were deemed to be insufficiently stringent. For this reason, the PCR-SSCP protocols used in this study were established empirically using template DNA from two small half-sib families (to observe inheritance of allele-specific banding pattern) and DNA samples of other unrelated Merino sheep (for increased genotypic variation). Many different gel conditions (gel percentage, voltage, time of running, temperature, addition of glycerol) were assessed to determine the optimum combination of conditions to resolve allele specific banding patterns in a reproducible manner. Amplimers from sires of the SL1 and SL2 and their selected progeny were also included on the optimising gels in order to ascertain allele banding patterns by following inheritance, and to determine whether the sires were heterozygous, and therefore informative, for the locus genotyped. Alleles were named in the order they were identified using letters of the alphabet.

2.7. Detection of sequence variation using PCR-SSCP

Each locus used specific SSCP gel conditions, and these are summarised in Table 2.4. Polyacrylamide (37.5:1 acrylamide / bis-acrylamide, Bio-Rad Laboratories, Hercules, Ca, USA) vertical gels (Protean II 16 x 16 cm, 1.0 mm thick spacers, 28 well comb, Hoefer, Inc., San Francisco, Ca, USA) were prepared containing 0.5× TBE (44.5 mMTris, 44.5 mM orthoboric acid, 1 mM Na_2EDTA [pH 8.0]) and polymerised using 10% ammonium persulphate and TEMED. Gels were pre-electrophoresed at running temperatures and voltage for one hour. Amplimers were mixed with 50 μL loading dye (95% formamide, 10 mM Na_2EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol), denatured by heating at 95 ^oC for five minutes and immediately placed on wet ice before loading 15 μL aliquots. The gels were then electrophoresed at the optimum gel conditions with 0.5× TBE running buffer, followed by silver-staining according to the method of Sanguinetti *et al*. (1994).

2.8. Cloning of allele standards

For KAP3.2, KAP7 and KRT2.10 loci, genomic DNA was obtained from the sire and this DNA was amplified using the PCR conditions described above and the amplimers were

Locus	Gel % (37.5:1)[1]	Run length (hours)	Temperature[2](°C)	Voltage (V)
KAP3.2	8	17	30	250
KAP7	10	4	20	200
KAP8	10	4	20	200
BfMS	12	7	15	300

[1] Acrylamide/Bis-acrylamide ratio
[2] The vertical gel electrophoresis tanks were connected to a circulating water chiller to maintain a constant gel temperature. This is the temperature listed in the above table.

Table 5. Optimised SSCP conditions for the loci investigated.

subsequently cloned using the Promega pGEM® - T Easy Vector System I (Promega Corporation, Madison, WI, USA). Since each plasmid can only accept one molecule of DNA and therefore only one allele. Ligation reactions were performed in a total reaction volume of 10 μL containing three units T4 DNA ligase, 50 ng of plasmid DNA and 1× ligation buffer, and incubated overnight at 4 °C. Constructs were transformed into competent *E. coli* cells (Invitrogen™, One Shot™, INVαF') using the manufacturer's protocol. Sixty μL and 150 μL of the transformation mix were spread on labelled LB (0.5 % casein hydrolysate, 0.25 % yeast extract, 85.6 mM NaCl; pH 7.0) agar plates containing 100 μg/mL ampicillin that had been spread with 40 μL of 40 mg/mL X-Gal (BDH Laboratory Suppliers, Poole, England). The plates were incubated overnight at 37 °C. Six colonies for each representative allele were selected and cultured overnight in terrific broth (Invitrogen Corporation, Paisley, Scotland, UK), supplemented with 50 μg/mL ampicillin, for plasmid isolation. Colonies were screened for the correct alleles using a rapid boiling-PCR method, where by Fifty μL aliquot of an overnight culture (bacterial cells with gene of interest cultured in terrific broth) was centrifuged at 13,000 rpm for 2 minutes, the supernatant was discarded and 30 μL TE (1 M Tris, 0.5 M Na_2EDTA) buffer added, boiled for 10 minutes, centrifuged at 13000 rpm for 2 minutes and then 1μL of the supernatant was used as the template for the appropriate PCR. Amplimers were run on 2% agarose gels next to the original genomic PCR amplimers for comparison. Plasmid DNA was then isolated from clones, which had banding patterns corresponding to the original banding pattern seen from amplimers of genomic DNA, using the FastPlasmid™ Mini Kits (Eppendorf, Hamburg, Germany) following the manufacturer's instructions. These amplified plasmid DNAs were subsequently sequenced and used as standards for scoring unknown genotypes.

2.9. DNA sequencing

Plasmid standards were sequenced in the forward and reverse directions using the M13 forward and reverse primers at the Waikato University DNA Sequencing Facility, University of Waikato, New Zealand or Lincoln University Sequencing Facility, Lincoln, New Zealand. The sequences were compiled using DNAMANTM version 4.0 (Lynnon Biosoft, Quebec, Canada) and the electropherograms. To minimise the likelihood of PCR and sequencing errors, sequence data was derived from four separate colonies, at least two of which were from independent PCR amplifications. When sequencing data was consistent, the sequences were submitted to NCBI GenBank (http://www.ncbi.nlm.hih.gov). These were *Ovis aries* keratin intermediate-

filament Type II (KRT2.10) gene: Accession number AY437406; *Ovis aries* high-sulphur keratin IF-associated protein 3.2 (KAP3.2) gene: Accession number AY483216 and *Ovis aries* high-glycine/tyrosine Type II keratin protein 6.1 (KAP6.1) gene: Accession number AY483217.

2.10. Statistical analyses

In order for any of the loci to be informative, they have to be heterozygous in the chosen sires allowing the segregation of the sire alleles to be followed in the progeny and segregation analyses performed. Segregation of the sire alleles within SL2 was observed and a chi-square goodness of fit test performed to ascertain whether the sire alleles inherited by the progeny occurred in a 1:1 ratio within the population. Any progeny which had the same genotype as both its sire and dam was excluded from the association analysis since it was not possible to determine which of the alleles had been inherited from the sire. The association of alleles of KAP8 with all measured wool traits (MFD, FDSD, CVD, curvature, yield, yellowness, brightness, comfort factor, staple length, staple strength, GFW and CFW) was then analysed for each year of phenotypic data using an analysis of variance (ANOVA) tests using SPSS version 13 (SPSS Science Inc., Chicago, IL, USA). The ANOVA model included sire allele and gender as factors and a full factorial model was used. The analysis used assumed that the ewe's alleles effects were distributed randomly in progeny. The date of birth was not included in the ANOVA because the progeny were half-sibs born in a five weeks period, and it was assumed that variation in birth date was balanced across the half-sib in the segregation analyses, and that none of the genes analysed had a significant effect on gestation length.

3. Results

Six loci (KAP3.2, KAP6.1, KAP7, KAP8, KRT2.10 and BfMS) were included in the study. All of them were amplified successfully using PCR and polymorphism was detected in three loci (KAP3.2, KAP8 and BfMS). Of the loci which were polymorphic, only KAP8 was heterozygous for SL2 (Tables 3.1), and thus potentially informative as a genetic marker. The remaining loci appeared to be homozygous in the sires, and thus uninformative. Table 3.2 shows the genotype of SL2 progeny at KAP8 locus.

Locus	No. of alleles detected	SL1 genotype	SL2 genotype	Informative[1] (Yes / No)
KAP3.2	3	AA	AA	No
KAP6.1	1	AA	AA	No
KAP7	1	AA	AA	No
KAP8	4	AA	AB	Yes[2]
KRT2.10	1	AA	AA	No
BfMS	3	AA	CC	No

[1]Heterozygous = informative; homozygous = uninformative
[2]Informative for SL2 only.

Table 6. Genotype results for the loci investigated in the study, indicating whether the sire genotype was informative (heterozygous) or non-informative (homozygous).

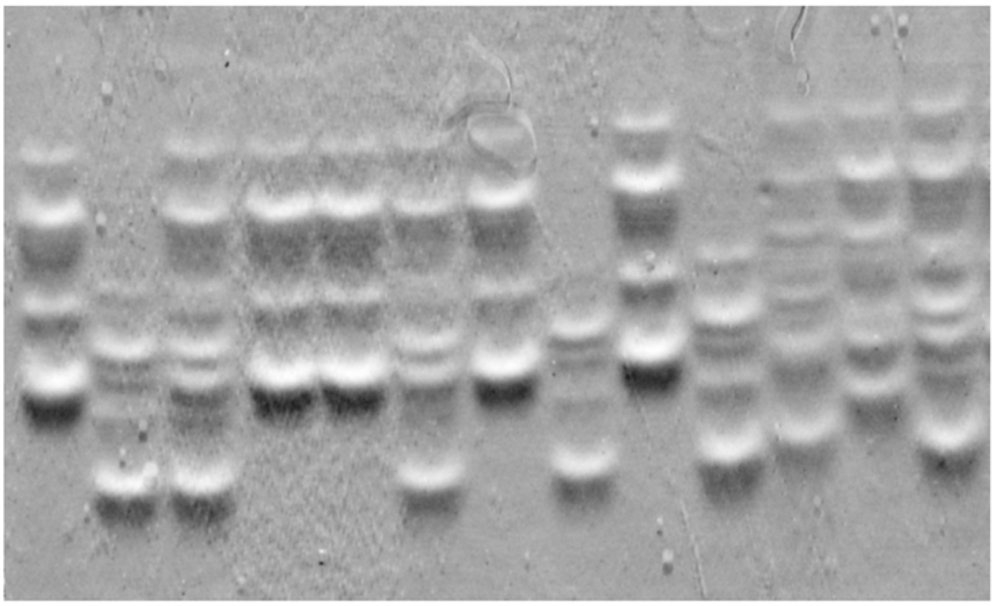
Lamb identity	Ewe identity	Lamb genotype
1027	86	AB
1028	162	AA
1029	57	BB
1030	114	AA
1031	59	AB
1032	59	AA
1033	105	AA
1034	89	AA
1035	51	AB
1036	49	AD
1037	120	AA
1038	56	AB
1039	65	AC
1040	47	AB
1041	119	BB
1045	77	BB
1046	155	AA
1048	14	BB
1049	130	AA
1050	161	AA
1051	84	BB
1052	150	BB
1053	17	BB
1054	NT	AA
1055	113	AB
1056	140	BB
1057	21	AB
1058	87	AA
1059	135	AB
1060	137	AA
1061	117	AC
1062	38	AB
1063	148	AB
1064	68	AB
1065	.	?
1067	116	AA
1068	58	?
1069	64	AA

[?]The genotype of the sheep could not be ascertained

Table 7. Genotype of KAP8 SL2 progeny

3.1. KAP8 (Polymorphic and informative in SL2)

Four banding patterns were identified for the KAP8 microsatellite amplimer using PCR-SSCP typing methods, and these were named A, B, C and D (Figure 3.1). The alleles were not sequenced. Mendelian inheritance was observed in SL2 half-sib family for KAP8 (Table 3.2). A Chi-square goodness of fit analysis to test whether the segregation of the sire alleles differed from a 1:1 ratio confirmed normal Mendelian segregation (Table 3.3).



AA BB **AB** *AA AA AB AA BB AA BB AC AD AB*

Figure 3. PCR-SSCP of the 124 bp amplimer of the KAP8 microsatellite showing the four alleles identified. Amplimers were electrophoresed on a 10% non-denaturing acrylamide/bis-acrylamide gel for 4 hours, 200 V at room temperature (20 °C). Genotype of an individual animal is shown below each lane. SL2 genotype (AB) is bolded, and his randomly selected half-sib progeny are shown in italics.

SL2 Genotype	AB
Number of progeny inheriting allele A	17
Number of progeny inheriting allele B	12
Number of progeny genotyped same as the sire	5
Total number (n)	34
$\chi 2$	0.8621
P-value1	0.3532

Table 8. Segregation of sire alleles within SL2 sire-line. Chi-square goodness of fit was used for to ascertain whether the sire alleles inherited by the progeny occurred in a 1:1 ratio within the population. Probability values (*P* values) are given. [1]A *P*-value > 0.05 means that the allele segregation did not differ significantly from a 1:1 ratio.

3.1.1. Genotype

SL1 was homozygous at the KAP8 locus based on SSCP gel patterns, and hence uninformative. SL2 was heterozygous at the KAP8 locus, having the genotype AB. Eleven out of 36 progeny had the genotype AB (Table 3.2), which was the same as that of the sire. The genotype of the ewes for these lambs was subsequently determined. Five of the ewes genotyped as AB, and the progeny of these ewes were excluded from further statistical analysis as the allelic contribution from the sire could not be determined.

3.1.2. Association between segregating sire alleles and wool traits

The sire alleles at the KAP8 locus showed a Mendelian pattern of inheritance and segregated in a 1:1 ratio in the progeny of each half sib (Table 3.3). Statistical analyses within sire SL2 half-sib family showed that there were no association between the sire alleles (or gender) and variation of wool traits.

3.1.3. Power analyses

The number of differences between alleles within sire-lines which were not statistically significant suggested the possibility of Type II errors (failing to detect a difference when in fact there is one). To address this issue, a power analysis was conducted for each trait within each of the sire-lines to determine whether the sample sizes available were adequate to detect at least 10% differences between alleles, within each sire-line, at $P<0.05$ with 80% power, i.e. $n_{\text{per allele}} = (8 \times 2 \times \text{ERROR MEAN SQUARE}_{\text{estimate}})/(0.1 \times \text{TRAIT AVERAGE}_{\text{across sire-lines}})^2$.

This equation was then rearranged to allowed the actual detectable difference to be calculated for each sire-line, i.e. % detectable difference = $[\sqrt{(8'2'\text{EMS}/n_{\text{per allele}})}\ /\text{TRAIT AVERAGE}_{\text{across sire-lines}}] \times 100$. A power analysis was performed for the KAP8 data. Wool trait measurements were only taken at 12 months for the SL2 half-sib family. There were inadequate SL2 progeny numbers (n=29) to detect a 10% difference between sire allele groups for yield, curvature, CVD, FDSD, staple length, brightness and yellowness (CFW and GFW were not measured). A comparison of the smallest detectable difference between sire-allele groups with the progeny numbers used with the observed difference between the sire-allele groups is shown in Table 3.4.

3.2. KAP3.2 and BfMS (Polymorphic, but uninformative)

KAP3.2 and BfMS were found to be polymorphic in the progeny used in this study, although they appeared to be homozygous for both sires used (Figures 3.2 and 3.3, respectively). This was confirmed with cloning and sequencing amplimers derived from sire SL1.

Sire-line	N_{lambs}	Trait[1]	Trait average[2]	EMS[3]	$N_{per\ allele}$ to detect at least a 10% difference	N_{lambs} required to detect a 10% difference
SL2	29	Prickle factor	1.61	1.75	1080	2159
	29	MFD	19.07	10.57	46	93
	29	FDSD	3.89	67.23	7103	14207
	29	CVD	20.46	91.34	349	698
	29	Curvature	94.58	0.45	0	0
	29	Yield	71.84	9.79	3	6
	29	Staple length	73.33	2.04	1	1
	29	Staple strength	31.30	12.58	21	41
	29	Brightness	69.92	0.27	0	0
	29	Yellowness	-2.83	135.71	27195	54389

[1]MFD: mean fibre diameter; FDSD: fibre diameter standard deviation; CVD: coefficient of variation of fibre diameter; GFW: greasy fleece weight; CFW: clean fleece weight. [2]Across all progeny measured. [3]Error Mean Square-taken from the ANOVA for each individual trait.

Table 9. Sample size required to detect at least a 10% difference between KAP8 sire allele groups in the wool traits list for each sire-line, at P<0.05 with 80% power.

Sire-line	N_{lambs}	Age (months)	Trait[1]	Trait average[2]	Smallest detectable difference (%)[3]	Difference observed between alleles (%)
SL2	29	12	Prickle factor	1.77	86.3	29.5
	29	12	MFD	19.21	17.9	3.2
	29	12	FDSD	3.91	221.3	1.9
	29	12	CVD	20.45	49.1	-0.4
	29	12	Curvature	97.04	0.7	6.4
	29	12	Yield	72.69	4.6	1.1
	29	12	Staple length	33.56	2.0	4.3
	29	12	Staple strength	69.06	11.9	22.6
	29	12	Brightness	-2.97	0.8	-2.1
	29	12	Yellowness	72.60	-433.1	7.1

[1]MFD: mean fibre diameter; FDSD: fibre diameter standard deviation; CVD: coefficient of variation of fibre diameter. [2]Across all progeny measured. [3]At 80%.

Table 10. A comparison of the smallest detectable difference between KAP8 sire-allele groups with the progeny numbers used and the observed difference between the sire-allele groups means for each wool trait measured.

3.3. KAP6.1, KAP7 and KRT2.10 (Non-polymorphic in SL1 and SL2)

Polymorphism could not be detected at the KAP6.1, KAP7 and KRT2.10 loci in any of the animals used in this study. KAP 7 was sequenced, and nucleotide sequences from SL1 KAP7 amplimer (GenBank accession number AY791846) was aligned with the published KAP7 gene by Kuczek and Rogers (1987); GenBank accession number X05638) which shows two unique sequences (Figure 3.4).

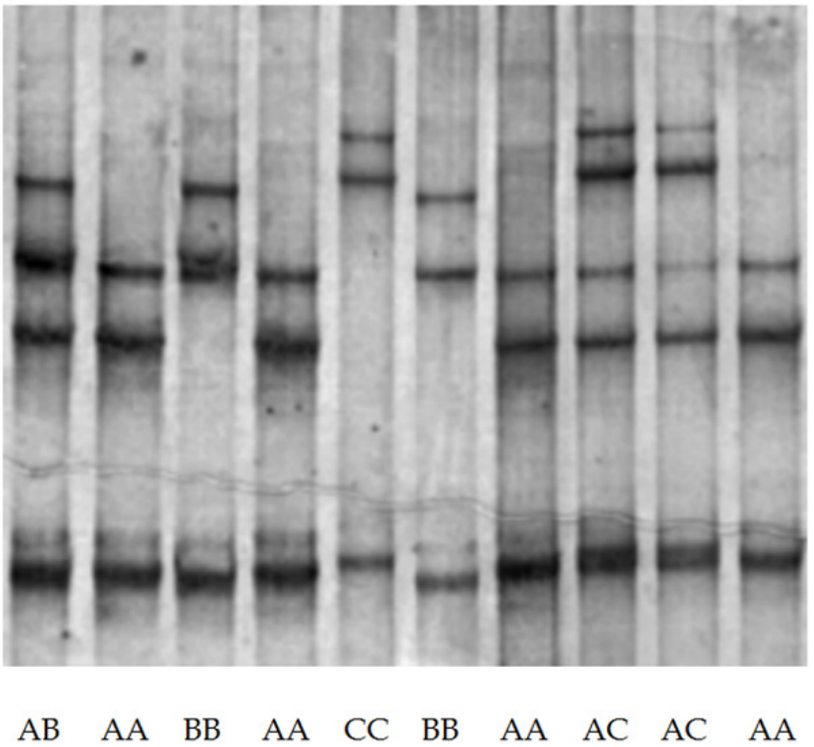


Figure 4. PCR-SSCP analysis of the 424 bp amplimer of the KAP3.2 gene showing the three alleles identified (A, B and C). The genotype of an individual animal is shown below each lane.

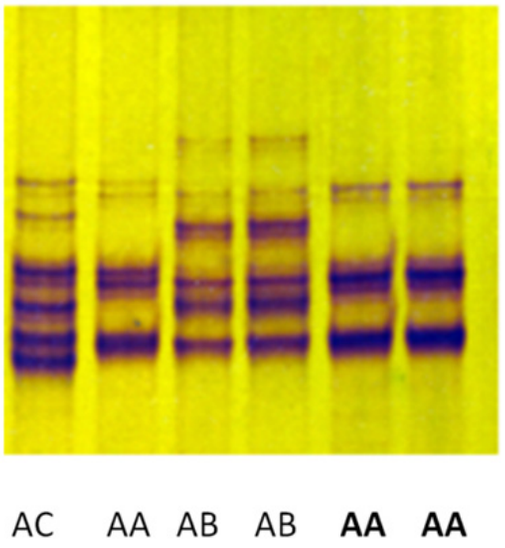


Figure 5. PCR-SSCP analysis of the 200 bp amplimer of the BfMS microsatellite showing the three alleles identified (A, B and C) using a half-sib test family. The genotype of an individual animal is shown below each lane. Sires SL1 and SL2 genotypes are bolded.

```
KAP7 (AY791846) CATCGGACAGCTTGAGGTATAAAAGG.TCCCGTGCAGGAC     39
KAP7 (X05638)   -------------------------t---------tt--     40

KAP7 (AY791846) GAGAACTTCATTCCTTCTTGGGTAACTTGCTCTTCACATT     79
KAP7 (X05638)   ----------------------------------------     80

KAP7 (AY791846) CTATCCAAATCCTTCCCACTCCTGCCACAATGACTCGTTT    119
KAP7 (X05638)   ----------------------------------------    120

KAP7 (AY791846) CTTTTGCTGCGGAAGCTACTTCCCAGGCTATCCTTCCTAT    159
KAP7 (X05638)   ----------------------------------------    160

KAP7 (AY791846) GGAACCAATTTCCACAGGACCTTCAGAGCCACCCCCCTGA    199
KAP7 (X05638)   ----------------------------------------    200

KAP7 (AY791846) ACTGCGTTGTGCCCCTTGGCTCTCCCCTTGGTTATGGATG    239
KAP7 (X05638)   ----------------------------------------    240

KAP7 (AY791846) CAATGGCTACAGCTCCCTGGGCTACGGTTTCGGTGGAAGC    279
KAP7 (X05638)   ----------------------------------------    280

KAP7 (AY791846) AGCTTTAGCAACCTGGGCTGTGGCTATGGGGGCAGCTTTT    319
KAP7 (X05638)   ----------------------------------------    320

KAP7 (AY791846) ATAGGCCATGGGGCTCTGGCTCTGGCTTTGGCTACAGCAC    359
KAP7 (X05638)   ----------------------------------------    360

KAP7 (AY791846) CTACTGATGGACCATGGCTCCAGATGACTACGGG.ACCCG    398
KAP7 (X05638)   ---------------------------------a-----    400

KAP7 (AY791846) CCCTCAATTCTCTGT                           413
KAP7 (X05638)   ---------------                           415
```

Figure 6. Alignment of the KAP7 gene sequence cloned from sire MV144-58-00 (Accession number AY791846) with Kuczek and Rogers (1987) published KAP7 gene (Accession number X05638). Upstream and downstream primers are underlined and the start and stop codons are bolded). Dashes represent same nucleotides to the nucleotide above and dots represent nucleotides missing in the other sequence.

Sex Average (cM)	Female (cM)	Male (cM)	Locus code	Marker	Marker description or associated gene
101.9	84.3	119.3	\BM4129	BM4129	Sequence – tagged site
104.1	86.6	122.0	\UCDO31	UCD031	RAPD Marker
107.1	89.0	125.2	\MCM58	MCM58	Microsatellite
111.3	91.7	130.5	\BL41	BL41	VANGL1
111.3	91.7	132.2	\BM723	BM723	STS
111.3	91.7	132.2	\BM723	BM723A	STS
113.8	92.9	133.9	\OARAE57	AE57	Microsatellite
123.4	105.2	142.2	\MCMA6	MCMA6AH	?
124.5	106.8	142.2	\MCMA6L	MCMA6AL	?
124.5	106.8	142.2	\BMS482	BMS482	Sequence – tagged site
124.5	106.8	142.2	\CSSM054	CSSM54	Phosphoglycerate dehydrogenase
126.0	107.9	143.2	PRPF3	BMS963	PRPF3 protein
126.0	108.9	143.2	ARNT	RME23	Aryl hydrocarbon receptor nuclear translocator
126.0	108.9	143.2	THH	TRHY	Trichohyalin
127.3	111.1	144.3	\RM065	RM65	Dinucleotide repeat
132.0	115.3	149.1	~CSAP033E	CSAP33E	Microsatellite
134.9	120.0	150.4	IGSF9	KIA1355	Immunoglobulin superfamily 9
135.7	121.5	150.4	ATP1A2	INRA6	ATPase
137.0	121.5	152.9	ADAMTS4	ADAMST4	ADAM metallopeptidase
139.8	122.7	156.8	\URB006	URB006	Sequence – tagged site
143.6	127.6	160.4	\BM6438	BM6438	Sequence – tagged site
143.6	127.6	160.4	OLIG2	OLIG2	Oligodendrocyte transcription factor 2
144.8	127.6	162.2	\SRCRS23H	SRCR23H	?
144.8	127.6	162.2	\TGLA49	TGLA49	Microsatellite
144.8	127.6	162.2	\DVEPC88	DVEPC88	Neu associated kinase
145.3	127.6	163.1	KRTAP7-1	KAP7HAP	Keratin associated protein 7.1
145.3	127.6	163.1	KRTAP7-1	KAP7_B	Keratin associated protein 7.1
145.3	127.6	163.1	KRTAP8-1	KAP8	Keratin associated protein 8.1
145.3	127.6	163.1	KRTAP7-1	KAP7_M	Keratin associated protein 7.1
145.3	127.6	163.1	KRTAP11-1	1-105	Keratin associated protein 11.1
145.4	127.6	163.2	KRTAP6-1	KAP6	Keratin associated protein 6.1
145.8	127.6	164.0	GRIK1	GRIK1	Glutamate receptor, ionotropic, kainite 1
149.6	131.5	168.1	APP	APPO10	Amyloid beta (A4) precursor protein
150.5	131.5	169.5	\BMS574	BMS574	Sequence – tagged site
150.5	131.5	170.2	\DVEPC117	DVEP117	Sequence – tagged site
150.5	131.5	170.2	\DVEPC117	DVEPC96	Sequence – tagged site
152.1	132.8	171.3	\BMS2321	BMS2321	Sequence – tagged site
153.2	132.8	173.1	\DVEPC128	DVEP128	Neural cell adhesion molecule 2
157.1	138.0	176.7	\RM095	RM095	Dinucleotide repeat
158.1	138.0	177.6	\MAF64	MAF64	Dinucleotide repeat
169.2	150.1	188.2	\ILSTS004	ILSTS04	Sequence – tagged site
171.1	152.6	188.2	\DVEPC54	DVEPC54	Microsatellite
174.4	154.8	194.2	\MCMA8	MCMA8	Sequence – tagged site
176.0	154.8	197.2	\MNS94	MNS94A	Microsatellite
193.1	169.9	216.0	\CSSM004	CSSM04	Microsatellite
195.3	171.1	219.6	\BMS4000	BMS4000	Sequence – tagged site
200.0	177.1	223.6	\UCDO46	UCD046	?

Figure 7. Linkage map for part of ovine chromosome 1 (modified from http://rubens.its.unimelb.edu.au/~jillm/jill.htm). The bolded genetic markers were investigated in this study.

Sex Average (cM)	Female (cM)	Male (cM)	Locus Code	Marker	Marker description or associated gene
149.6	151.4	148.8	\BMS695	BMS695	Sequence – tagged site
149.6	151.4	148.8	\BM827	BM827	Microsatellite
152.5	151.4	153.4	\MCM141	MCM141A	?
153.1	151.4	154.3	\OARSHP2	SHP2	Microsatellite
153.1	151.4	154.3	\ILSTS042	ILSTS42	Sequence – tagged site
154.1	151.4	156.3	\BMS424	BMS424	Sequence – tagged site
163.1	155.2	170.4	\BP1	BP1	Blood pressure QTL1
163.1	155.2	170.4	\DU469297	DU469297	?
165.6	160.5	170.4	\EPCDV025	EPCDV25	?
167.1	166.2	170.4	KITLG	SCF	KIT Ligand
168.7	166.6	172.4	\UCDO13	UCD013	?
170.7	166.6	174.3	KERA	KERA	Keratocan
170.7	166.6	174.3	LUM	CSAP19E	Lumican
177.8	172.1	182.4	\AGLA293	AGLA293	Microsatellite
179.4	174.9	183.5	~CSAP017E	CSAP17E	Microsatellite
179.4	174.9	183.5	\OARFCB5	FCB5	Dinucleotide repeat
179.4	174.9	183.5	GLYCAM1	GLYCAM1	Glycosylation dependant cell adhesion molecule
179.4	174.9	183.5	\OARHH38	HH38	Microsatellite
180.0	176.2	183.5	\ILSTS022	ILSTS22	Sequence – tagged site
180.0	176.2	183.5	RARG	RARG	Retinoic acid receptor 8
182.9	178.7	186.5	KRTHB*	KRT2.10	Keratin
183.9	181.2	186.5	KRTHB*	KRT2.13	Keratin
183.9	181.2	186.5	\BMC1009	BMC1009	Similar to intermediate filament type II keratin
186.3	181.2	190.9	\CABB011	CABB11	Genomic survey sequence
188.2	185.3	190.9	\CSSM034	CSSM34	Microsatellite
188.2	185.3	190.9	HDAC7A	KD103	Histone deacetylase 7A
188.2	185.3	190.9	\UCDO52	UCD052	?
195.5	188.6	201.0	\BL4	BL4	Bell-like homeodomain protein 4
197.0	190.4	202.8	LYZ	LYZ	Lysozyme
198.6	191.8	204.6	\CSRD2125	CSRD125	?
199.1	191.8	205.5	IFNG	KP6	Interferon gamma
199.1	191.8	205.5	IFNG	IFNG	Interferon gamma
199.1	191.8	205.5	IFNG	IFNGHAP	Interferon gamma
202.2	195.4	207.8	\BMS1617	BMS1617	STS
204.1	196.7	210.6	\OARVH34	VH34	Microsatellite
206.2	197.7	213.7	\BR2936	BR2936	Sequence – tagged site
207.0	197.7	215.5	\OARVH130	VH130	Microsatellite
207.0	197.7	215.5	\MAF23	MAF23	Microsatellite
209.0	199.0	218.1	\OARCP43	CP43	Microsatellite
214.7	208.6	219.8	\RM154	RM154	Tandem repeat region
218.5	211.3	223.7	IGF1	IGF1	Insulin like growth factor
218.5	211.3	223.7	IGF1	IGF1.B	Insulin like growth factor
218.5	211.3	223.7	IGF1	IGF1HAP	Insulin like growth factor
218.5	211.3	223.7	IGF1	CSAP40E	Insulin like growth factor
223.9	215.4	231.6	\CSRD2111	CSRD111	?
224.4	215.4	232.5	~CSAP009E	CSAP09E	?

Figure 8. Linkage map for part of ovine chromosome 3 (modified from http://rubens.its.unimelb.edu.au/~jillm/jill.htm). The bolded genetic markers were investigated in this study.

4. Discussion

Four alleles, designated A, B, C and D were identified at the KAP8 (CA)n repeat microsatellite locus using PCR-SSCP in this study. The microsatellite at the KAP8 locus was included in the study because this region is highly polymorphic, with 15 alleles previously reported (Wood *et al*., 1992) using denaturing polyacrylamide gel electrophoresis, while Parsons *et al*. (1994a) detected four allelic fragments (123, 125, 133 and 139 bp) at the same locus using the methods by Wood *et al*. (1992) in a Merino half-sib family. Only SL2 was heterozygous at the KAP8 microsatellite in this study. SL1 was homozygous, despite the reported highly polymorphism in this locus (Wood *et al*., 1992). The method used to detect polymorphism in this study differed to that of (Wood *et al*., 1992), which used denaturing polyacrylamide gel electrophoresis. In this study, PCR-SSCP was used because this technique is simple, sensitive, relatively inexpensive and routinely used in the laboratory where the research was carried out. It is possible that if the original technique was employed, more alleles may have been observed at this locus.

Neither of the SL2 alleles were associated with variation in the wool traits that were measured (data not shown). The possibility of this locus having an affect on wool traits cannot be ruled out however, because only two alleles (that were the genotype of SL2) were analyzed, and that the sample numbers used in the study were relatively small (n = 29). Power analysis results (Table 9.5) showed that the observed differences between the sire allele groups were smaller than the smallest detectable difference for MFD, FDSD, CVD, curvature, yield, staple length, brightness and yellowness and therefore the possibility of making a Type II error (i.e. not detecting an association when there was one) is likely. Variation in MFD has previously been significantly associated with alleles at the KAP8 locus (Parson *et al*., 1994a). The authors did not describe the alleles associated, and no sequence data was presented. Though alleles at the KAP8 microsatellite locus were not sequenced in this study, it is possible that SL2's alleles were different from those associated with differences in average MFD by Parsons *et al*., (1994a).

Three alleles, designated A, B and C were identified at the KAP3.2 locus. However, both sire lines were homozygous, and thus uninformative. McLaren *et al*. (1997) identified two alleles at the KAP3.2 locus using PCR-SSCP methods. KAP3.2 (together with KAP1.1, KAP1.3 and K33) have been mapped to ovine chromosome 1 (Figure 3.5). Variations in all of the three genes (KAP1.1, KAP1.3 and K33) have been previously associated with variation in wool traits (Itenge *et al*., 2009; Itenge *et al*., 2010; Rogers *et al*., 1994b). It is therefore suggested that sires that are heterozygous get investigated in further studies. Three alleles, designated A, B and C were identified at the BfMS microsatellite. Bot *et al*. (2003) reported eight alleles at the BfMS locus. Two of these alleles were significantly associated with CFW and GFW. However, both sire lines were homozygous, and thus uninformative at the BfMS locus.

Polymorphism could not be detected at the KAP6.1, KAP7 and KRT2.10 loci in this study, although all of these genes have been reported to be polymorphic in the literature (Parsons

et al., 1993; McLaren *et al*., 1997). The reported polymorphism in KRT2.10 (two alleles) and KAP7 (four alleles) was identified using PCR-RFLP (McLaren *et al*., 1997) whereas the polymorphism within KAP6.1 (two alleles) was revealed with PCR-SSCP of *Alu*I-digested PCR amplimers (McLaren *et al*., 1997). Parsons *et al*. (1993) reported a diallelic polymorphism using *Bam*HI PCR-RFLP to give alleles designated A1 (24.5 kb) and A2 (14.1 kb). However, no sequence data was presented. Since only two KAP6.1 alleles have previously been reported, thus it was accepted that SL1 was homozygous at this locus without further sequencing although this locus could still be polymorphic which only sequencing would reveal. The KAP6.1 amplimers were also subjected to a variety of PCR-SSCP conditions in an effort to detect sequence variation. Digestion of the amplimer with *Alu*I or *Bam*HI as per McLaren *et al*. (1997) was not performed, however, and it is possible that this may have revealed variation at KAP6.1. KRT2.10 has been mapped to ovine chromosome three and two alleles have been reported at the KRT2.10 locus using a *Bsr*DI PCR-RFLP (McLaren *et al*, 1997). Genes coding for the KRT proteins are highly conserved during evolution (Powell, 1996; Marshall and Gillespie, 1982), and do not have much variation within them. Therefore, it was easy to accept that the KRT2.10 locus (with only two alleles) was likely to be homozygous. The fact that the KRT proteins are highly conserved during evolution (Powell, 1996; Marshall and Gillespie, 1982) suggests that genes coding for these proteins are intolerant to major changes and that they are very important to the integrity of the wool fibre.

5. Conclusion

Loci that were polymorphic, but uninformative in this study (KAP3.2, BFMS))need to be investigated further. Sires that are heterozygous at these loci need to be identified and used in half-sib analysis. Other loci that map to the same chromosome regions as the keratin genes investigated in this study are also worth of investigating in the future as potential gene markers for wool quality traits. On chromosome 1, future genes of interest include KAP11.1 and genes coding for trichohyalin (a very important wool follicle protein) (refer to Figure 3.5). On chromosome 3, loci of interest include KRT2.13, BMC1009 (Similar to intermediate filament type II keratin), RARG (Retinoic acid receptor 8) and IGF1 (insulin like growth factor) (refer to Figure 3.6). It is worth noting that previous studies by Damak *et al*. (1996) have shown positive effects of IGF1 on wool traits. Transgenic sheep produced by pronuclear microinjection with a mouse ultra-high-sulphur keratin promoter linked to an ovine IGF1 resulted in significant increase of CFW and bulk in transgenic sheep compared to non-transgenics, although MFD did not show significant differences (Damak *et al*.,1996).

There are other genes that have not been positioned on the linkage map that may be potential gene markers for wool quality traits. Some of these have already been associated with wool quality traits. These include the retinoic acid receptor (RARα) (Nadeau *et al*., 1992), homeobox proteins (HOX2) (Nadeau *et al*., 1992) and growth hormone (Hediger *et al*.,

1990). Retinoic acid induces expression of genes such as homeobox and KRTs and there is a possibility that retinoic acid is involved in the regulation of KAPs, given its genomic position on chromosome 11 (Parsons *et al*, 1994c). Growth hormone has been positioned on chromosome 11 through *in situ* hybridization (Hediger *et al*., 1992). Furthermore, there have been numerous reports with variable effects of growth hormone on wool characteristics. For example, Ferguson (1954) and Johnson *et al*. (1985) observed significant increase in GFW during the injections of growth hormone. In contrast, no effect of recombinant growth hormone on CFW was found in a study by Zainur *et al*. (1989). Wheatley *et al*. (1966) found that growth hormone suppressed wool growth and that there was accelerated wool growth after withdrawal of growth hormone. Polymorphism at the genes encoding growth hormone have been reported (Valinsky *et al*., 1990; Wallis *et al*., 1998; Sami *et al*., 1999), and different alleles of growth hormone may affect wool growth in different ways.

5.1. Genetic markers versus genetic engineering

The search for genetic markers affecting wool quality traits is very different to genetic engineering (GE) and transgenesis. While GE involves the manipulation or modification of genetic composition of an organism, and transgenesis requires the development and use of transgenic animals, the former detects changes within the genetic make-up of an organism, but does not alter it. Marker-assisted selection may therefore be better preferred within the wider "non-scientific" community, than the use of transgenic sheep to produce superior wool traits. Transgenesis in sheep is also still in its infancy, and successful transgenesis rates are very low (less than 13%) (Powell *et al*., 1994). This makes marker-assisted-selection a more efficient, relatively cheaper and easier technique to improve wool quality traits than sheep transgenesis. The debate on GE will most likely continue and intensify especially where animals are involved. However, marker-assisted technology in livestock offers a powerful "green" alternative to gene manipulation.

5.2. Advantages of marker-assisted selection

Genetic markers are not affected by environmental noise and would allow sheep breeders to select animals with improved wool characteristics at an early age and cull the non-desirable lambs. This would speed up the process of genetic selection and decrease the generation interval. There is therefore a potential to select superior animals very early in life and not have to wait for an animal to reach its adult life to demonstrate that it has superior wool quality. This has the advantage of overcoming the limitation of "blind" selection, and increase the accuracy and efficiency of selection and result in a more profitable wool industry with direct benefits of cost to the consumer.

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