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(54) Title: MARKERS FOR PIGMENTATION

(57) Abstract: The present invention relates to a genetic marker for distinguishing animals that have a trait of pigmentation, wherein said marker is a polymorphism in the tyrosinase- related protein gene, and use thereof in prediction, selection, breeding and identification of pigmentation.

Markers for Pigmentation

Technical Field

The present invention relates to genetic markers for pigmentation in skin and fleece areas of animals. In particular, the present invention relates to methods, systems and kits for predicting pigmentation and pigmented fibre contamination in animals using genetic markers, to associated methods, systems and kits for selecting animals using marker assisted selection, and to associated methods and systems for animal breeding and prediction of lifetime performance.

Background Art

The presence of pigmented wool fibres grown in white wool fleece for use in apparel presents a serious quality assurance problem in wool production and processing. Risk of such pigment contamination, especially non-visual contamination, can arise when apparently white wool animals grow isolated pigmented fibres, or when white wool and coloured wool sheep are mixed in grazing or in shearing sheds. The former risk is the most serious since isolated pigmented fibres are not easily detected, whereas in relation to the latter risk good management practices can ensure separation of coloured sheep from white sheep. In addition the prediction of coloured animals may not be evident from breeding apparently white animals, since some fleece colouration characteristics –either full, partial or in isolated areas, maybe inherited in a recessive manner.

Diagnostic tests that are currently available for determining dark pigmented fibre contamination in wool are based on evidence of pigmented spots on key locations of the body, or evidence of pigmented hair (as distinct from wool) on eye lashes and horn sites. Scoring animals for these traits is laborious and often cannot be done until later in the life of the animals. Hence the accurate assessment of animals which are either potentially affected by or free from problematic pigmentation is difficult to achieve. Furthermore, in the absence of extensive progeny testing, there is no easy or reliable means by which to predict the breeding quality of sires and dams, so as to predict the quality of possible progeny. Currently there exists no DNA test which is capable of predicting the genetic profile for pigmentation.

The present invention is predicated on the development of a genetic marker shown to be associated with pigmentation and pigmented fibre contamination in animals.

Summary of the Invention

According to a first aspect of the present invention, there is provided a genetic marker for distinguishing animals that have a trait of pigmentation, wherein said marker is a polymorphism in the tyrosinase-related protein gene.

The tyrosinase-related protein gene includes associated intronic and regulatory sequences.

According to a second aspect of the present invention, there is provided a method for predicting pigmentation in an animal, said method comprising analysing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal.

The pigmentation may include pigmented skin or dark fibre contamination.

The fibre may be selected from the group comprising wool or hair. The wool may be wool or cashmere. The hair fibre may be mohair.

The animal may be an artiodactyl species. The animal may be sheep, alpaca, lama or goat.

The tyrosinase-related protein (TYRP) may be TYRP-1.

The presence or absence of the polymorphism may be analysed through the use of nucleic acid sequences flanking the polymorphism, such as PCR primers. For example, reference is made to the nucleic acid sequences set forth in SEQ ID NOS: 1-33 and 48-71.

According to a third aspect of the present invention, there is provided a method for selecting an animal using marker assisted selection, wherein said method comprises :

(a) analysing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in an animal; and

(b) selecting an animal based on the presence or absence of said genetic marker.

According to a fourth aspect of the present invention, there is provided a method for breeding an animal using marker assisted selection, wherein said method comprises:

(a) analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal;

(b) breeding from said animal based on the presence or absence of said genetic marker; and

(c) selecting progeny of said animal based on the presence or absence of said genetic marker.

According to a fifth aspect of the present invention, there is provided a system for predicting pigmentation in an animal, wherein said system comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal.

According to a sixth aspect of the present invention, there is provided a system for selecting an animal using marker assisted selection, wherein said system comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal; and

(b) means for selecting said animal based on the presence or absence of the genetic marker.

According to a seventh aspect of the present invention, there is provided a system for breeding an animal using marker assisted selection, wherein said system comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal; and

(b) means for breeding said animal based on the presence or absence of the genetic marker, and

(c) means for selecting progeny of said animal based on the presence or absence of the genetic marker.

According to an eighth aspect of the present invention, there is provided a kit for predicting pigmentation in an animal, wherein said kit comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal.

According to a ninth aspect of the present invention, there is provided a kit for selecting an animal using marker assisted selection, wherein said kit comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal; and

(b) means for selecting said animal based on the presence or absence of the genetic marker.

According to a tenth aspect of the present invention, there is provided a kit for breeding an animal using marker assisted selection, wherein said kit comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal; and

(b) means for breeding said animal based on the presence or absence of the genetic marker, and

(c) means for selecting progeny of said animal based on the presence or absence of the genetic marker.

According to an eleventh aspect of the present invention, there is provided a polypeptide comprising a tyrosinase-related protein (TYRP).

The TYRP may be TYRP-1. The TYRP-1 may comprise the amino acid sequence set forth in SEQ ID NOS: 36, 39, 42, 45, 47, 74, 77 and 80.

According to a twelfth aspect of the present invention, there is provided a polynucleotide encoding the polypeptide of the eleventh aspect.

According to a thirteenth aspect of the present invention, there is provided a vector comprising the polynucleotide of the twelfth aspect.

According to a fourteenth aspect of the present invention, there is provided a host cell transformed with the vector of the thirteenth aspect.

According to a fifteenth aspect of the present invention, there is provided an animal selected by the method of the third aspect, the system of the sixth aspect or using the kit of the ninth aspect.

According to a sixteenth aspect, there is provided an animal bred by the method of the fourth aspect, the system of the seventh aspect or using the kit of the tenth aspect.

According to a seventeenth aspect of the present invention, there is provided an animal product derived from the animal of the fifteenth aspect.

The animal product may be selected from the group comprising hair, wool, skin, cashmere, and mohair. The animal product may be wool.

According to an eighteenth aspect of the present invention, there is provided a method of sampling fibres to determine non-visual pigmentation, wherein said method comprises

(a) genotyping a plurality of animals based on the presence or absence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal;

(b) grouping said animals according to genotype;

(c) sampling fibres from animals grouped as having said genetic marker(s); and

(d) determining a likelihood of contamination of said fibre samples with pigmented fibres.

Tests for sampling dark or pigmented fibres are well known in the art. For example, Hansford, Literature review for managing the risk of dark/and or medullated fibre contamination. Australian Wool Innovation project EC573, describes a range of methods.

According to a nineteenth aspect of the present invention, there is provided a method for identifying pigmentation in an animal, wherein said method comprises:

(a) analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene wherein the presence of the genetic marker is indicative of pigmentation in said animal.

In an embodiment of the nineteenth aspect of the present invention, the pigmentation is present in the form of pigmented fibres. Further, the pigmented fibres may be in the form of pigmented wool, hair, mohair or cashmere.

In a further embodiment of the present invention, the pigmented fibres are rare and/or isolated. For example, the proportion of pigmented fibres may comprise less than 10% of total fibre count, less than 5% of total fibre count, less than 1% of total fibre count, less than 0.1% of total fibre count, less than 0.01% of total fibre count, less than 0.001% of total fibre count, less than 0.0001% of total fibre count, less than 0.00001% of total fibre count, less than 0.000001% of total fibre count, less than 0.0000001% of total fibre count, less than 0.00000001% of total fibre count, or less than 0.0000000001% of total fibre count.

In a further embodiment of all aspects of the present invention, the genetic marker for distinguishing animals that have a trait of pigmentation includes polymorphisms in the tyrosinase-related protein gene together with one or more genetic markers in linkage disequilibrium thereto. For example, genetic markers in linkage disequilibrium to the tyrosinase-related protein gene capable of distinguishing animals that have a trait of pigmentation may be TGLA10, bracketed by the markers CSAP16E or MCM252.

Brief Description of the Drawings

The present invention will now be described, by way of example only, with reference to the following drawings.

Figure 1. QTL location for Binary Colour Score on Chromosome 2.

Figure 2. A. QTL location for Pigmented Skin on Chromosome 2; **B.** QTL location for Pigmented Skin on Chromosome 19; **C.** QTL location for Arc Sine transformed NoseLipsPigmented skin on Chromosome 2; **D.** QTL location for Arc Sine transformed NoseLipsPigmented skin on Chromosome 3; **E.** QTL location for Arc Sine transformed NoseLipsPigmented skin on Chromosome 19; **F.** QTL location for Arc Sine transformed NoseLipsPigmented skin on Chromosome 25; **G.** QTL location for Pigmented Fibre on Chromosome 2; **H.** QTL location for Pigmented Fibre on Chromosome 26; **I.** QTL location for Hoof Pigmentation on Chromosome 2; **J.** QTL location for Hoof Pigmentation on Chromosome 18; **K.** QTL location for Binary score hornsite and leg Fibre score on Chromosome 2; **L.** QTL location for Binary score hornsite and leg fibre score on Chromosome 8; **M.** QTL location for Binary score hornsite and leg fibre score on Chromosome 19.

Figure 3. Schematic of TYRP1 gene layout

Figure 4. Black and white (top), and negative picture (bottom) of PCR products for TYRP-1 exons 2, 3, 4 and 5 electrophoresed on a 2% agarose gel. Symbols, M = marker, - = negative control, C = cattle, H = human, M = mouse, S = sheep. Number in brackets indicates the ~ size of the PCR product in relation to the molecular weight of the marker.

Figure 5. Black and white (top), and negative photograph (bottom) of PCR products for TYRP-1 exons 6, 7, 8 and 8-1 (using R2 primers described below) electrophoresed on a 2% agarose gel.

Figure 6. Black and white photograph of PCR products of TYRP-1 exons 1 and upstream region electrophoresed on a 2% agarose gel. Primers are indicated by number according to Tables 7-9

Figure 7. Association between genotype and phenotype at Marker TGLA10. Genotype '1' is significantly more associated with a higher Fibre Pigmentation Score (PigFibre) than genotype '2'.

Figure 8. Parental haplotypes inherited from the Merino granddam (C7_M), and the Awassi grandsire (C7_AW), and other (partial) haplotypes. In relation thereto, animals with haplotype "34" also had a confirmed high isolated pigmented fibre count using the AWTA test.

Figure 9. Histogram figure of Pigmented Fibre score out of 500 for pigmented fibre, against haplotypes predictive of HIGH and LOW risk of pigmentation respectively from the 1997 mapping family

Definitions

In the context of this specification, the term "comprising" means "including principally, but not necessarily solely". Furthermore, variations of the word "comprising", such as "comprise" and "comprises", have correspondingly varied meanings.

The term "primer" as used herein means a single-stranded oligonucleotide capable of acting as a point of initiation of template-directed DNA synthesis. An "oligonucleotide" is a single-stranded nucleic acid typically ranging in length from 2 to about 500 bases. The precise length of a primer will vary according to the particular application, but typically ranges from 15 to 30 nucleotides. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize to the template.

The term "genotype" as used herein means the genetic constitution of an organism. This may be considered in total, or with respect to the alleles of a gene(s) or genetic marker(s).

The term "homozygote" refers to an organism that has identical alleles at a given locus on homologous chromosomes.

The term "heterozygote" refers to an organism in which different alleles are found on homologous alleles for a given locus.

The term "linkage disequilibrium" describes a situation in which some combinations of alleles of two or more different loci (haplotypes) occur more or less frequently within a population than would be expected by random chance alone.

The term "pigmentation" as used herein includes skin and fibre pigmentation, and in particular includes skin and fibre pigment contamination. In this context, skin may include nose, lips, hoofs, eyelids, udder, and fibre may include hair, wool, fleece, and eyelashes. The term "pigmentation" may include isolated or partial fleece colouration, also known as "white spotting", piebald, or any other form of discolouration or full-fleece colouration. In this context, the term "pigmentation" may include fibres containing a degree of colouration that is not or substantially not visible to the eye. Also, the term "dark fibre" and "pigmentation" are synonymous. The terms "pigment" and "pigmented" have corresponding meanings.

As used herein, the term "genetic marker" refers to a variant or polymorphism at DNA sequence level linked to a specific chromosomal location unique to an individual's genotype, inherited in a predictable manner, and measured as a direct DNA sequence variant or polymorphism, such as at least one Single Nucleotide Polymorphism (SNP), Restriction Fragment Length Polymorphism (RFLP), or Short Tandem Repeat (STR), or as measured indirectly as a DNA sequence variant (eg. Single-strand conformation polymorphism (SSCP), Denaturing Detergent Gradient Gel Electrophoresis (DDGE). A marker can also be a variant at the level of a DNA derived product such as RNA polymorphism/abundance, protein polymorphism or cell metabolite polymorphism, or any other biological characteristics which have a direct relationship with the underlying DNA variants or gene product.

As used herein, the term "single nucleotide polymorphisms" or "SNP" or "SNPs", as used herein, refers to common DNA sequence variations among subjects. The DNA sequence variation is typically a single base change or point mutation resulting in genetic variation between individuals. The single base change can be an insertion or deletion of a base.

The term "base pair" as used herein means a pair of nitrogenous bases, each in a separate nucleotide, in which each base is present on a separate strand of DNA and the bonding of these bases joins the component DNA strands. Typically a DNA molecule contains four bases; A (adenine), G (guanine), C (cytosine), and T (thymidine). A and G are purine bases, typically designated by the letter "R", whereas C and T are pyrimidine bases, typically designated by the letter "Y". Where A or T may occupy a single position it is typically designated by the letter W. Where G or C may occupy a single position it is typically designated by the letter S. Where A or C may occupy a single position it is typically designated by the letter M. Where G or T may occupy a single position it is typically designated by the letter K. Where A, T or C may occupy a single position it is typically designated by the letter H. Where G, C or T may occupy a single position it is typically designated by the letter B. Where G, A or T may occupy a single position it is typically designated by the letter D. Where G, C or A may occupy a single position it is typically designated by the letter V. Where G, C, A or T may occupy a single position it is typically designated by the letter N. The term "base pair" is abbreviated to "bp", and the term "kilobase pair" is abbreviated to kb.

It will be understood that the term "animal" as used herein refers to an individual at any stage of life, or after death, including an entity prior to birth such as a fertilised ovum,

either before fusion of the male and female pro-nucleus or after the pro-nuclei have fused to form a zygote, an embryo (created by any means including somatic cell nuclear transfer) or an individual cell (N, 2N or greater); for the avoidance of doubt, this also includes a cell or a cluster of cells including stem cells and stem cell-like cells, cell line, haploid gametes and their progenitor cell lines, as well as products resulting from the gametes, including embryos.

Best Mode of Performing the Invention

A comprehensive gene mapping experiment identified chromosomes which contained genes contributing to skin and fibre pigmentation in sheep, detailed in Table 6. One of the major regions with strongest significance was found on chromosome 2, and was significant for 6 skin and fibre traits.

Association between the closest linked marker TGLA10, and 177 mapping progeny was sufficient to account for over 50% of the differences in pigmentation, and indicated that the colour phenotype originated from the Awassi allele (Figure 7). This region was shown to contain a very strong candidate gene, namely tyrosine-related protein-1 (TYRP-1 – Figure 3). TYRP-1 was sequenced by alignment of TYRP-1 sequence from all known species, with conserved sequence sites used to design DNA primers for ovine sequence. A predicted ovine gene structure was then identified from comparative mapping, with ovine sequence confirming close alignment to bovine TYRP-1.

Novel DNA sequence was generated for TYRP in sheep and DNA variants were discovered based on single nucleotide polymorphisms (SNPs) or short tandem repeat sequences, insertion deletion mutations or similar. These DNA variants were discovered in the mapping sire known to be heterozygous for a major gene linked to skin and fibre pigmentation (Sire C7). A screen of additional advanced cross progeny with 7 selected phase known markers (Figure 8) confirmed an association between the parental C7_AW parental/individual TYRP1 haplotype and pigmentation (Figure 9), thereby suggesting that markers linked to variants located in and around the TYRP1 gene represent a powerful means for predicting the genetic pigmentation status of animals, both for their own performance and as breeding candidates. To test this hypothesis, surviving animals from the initial backcross strategy, and advanced repeat cross animals were shorn, and their fleece allocated to bins according to two broad parental haplotypes (C7_M, C7_AW) to be tested for fleece colour.

Other QTL regions of significance for pigmentation were found to be located on chromosome 19 (LOD>5), possibly harbouring a second major gene for pigmentation which may account for the remainder of the variation seen in pigmentation. QTLs on remaining chromosomes appeared to be of lesser significance.

Detection of genetic markers

Genetic assay-assisted selections for animal breeding are important in that they allow selections to be made without the need for raising and phenotypic testing of progeny. In particular, such tests allow selections to occur among related individuals that do not necessarily exhibit the trait in question and that can be used in introgression strategies to select both for the trait to be introgressed and against undesirable background traits.

It is readily apparent that, having identified a polymorphism for a particular associated trait, there are an essentially infinite number of ways to genotype animals for this polymorphism. The design of such alternative tests merely represents a variation of the techniques provided herein and is thus within the scope of this invention as fully described herein.

Non-limiting examples of methods for identifying markers corresponding to genetic polymorphisms between members of a population include: restriction-fragment-length polymorphism (RFLP) Bostein et al (1980) Am J Hum Genet 32:314-331; single-strand conformation polymorphism (SSCP) Fischer et al. (1983) Proc Natl Acad Sci USA 80:1579-1583, Orita et al. (1989) Genomics 5:874-879; amplified fragment-length polymorphism (AFLP) Vos et al. (1995) Nucleic Acids Res 23:4407-4414; microsatellite or single-sequence repeat (SSR) Weber J L and May P E (1989) Am J Hum Genet 44:388-396; rapid-amplified polymorphic DNA (RAPD) Williams et al (1990) Nucleic Acids Res 18:6531-6535; sequence tagged site (STS) Olson et al. (1989) Science 245:1434-1435; genetic-bit analysis (GBA) Nikiforov et al (1994) Nucleic Acids Res 22:4167-4175; allele-specific polymerase chain reaction (ASPCR) Gibbs et al. (1989) Nucleic Acids Res 17:2437-2448, Newton et al. (1989) Nucleic Acids Res 17:2503-2516; nick-translation PCR (e.g., TAQMAN.TM.) Lee et al. (1993) Nucleic Acids Res 21:3761-3766; and allele-specific hybridization (ASH) Wallace et al. (1979) Nucleic Acids Res 6:3543-3557, (Sheldon et al. (1993) Clinical Chemistry 39(4):718-719) and gene chip analysis such as the Affymetrix or ParAllele systems, among others. Each

technology has its own particular basis for detecting polymorphisms in DNA sequence but all are applicable to the detection of SNP's.

In particular, techniques employing PCR. detection are advantageous in that detection is more rapid, less labour intensive and requires smaller sample sizes. Primers that may be used in this regard may, for example, comprise sequences set out in Tables 7, 8 and 9 and complements thereof.

Once an assay format has been selected, selections may be unambiguously made based on genotypes assayed at any time after a nucleic acid sample can be collected from an individual, such as an infant animal, or even earlier in the case of testing of embryos in vitro, or testing of foetal offspring. Any source of nuclear DNA may be analyzed for scoring of genotype.

In one embodiment of the invention, nucleic acids are screened that have been isolated from the blood or semen of the animal analyzed. DNA from the animal to be assessed may be extracted by a number of suitable methods known to those skilled in the art. Most typically, DNA is extracted from a blood or semen sample, and in particular from peripheral blood leucocytes. A sufficient amount of cells are obtained to provide a sufficient amount of DNA for analysis, although only a minimal sample size will be needed where scoring is by amplification of nucleic acids. The DNA can be isolated from the blood cells by standard nucleic acid isolation techniques known to those skilled in the art.

Amplification of nucleic acids

Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies. Such embodiments may find particular use with the invention, for example, in the detection of repeat length polymorphisms, such as microsatellite markers. In certain embodiments of the invention, amplification analysis is performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid.

Pairs of primers designed to selectively hybridize to nucleic acids are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids containing one or more mismatches with the

primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles", are conducted until a sufficient amount of amplification product is produced.

The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical and/or thermal impulse signals. Typically, scoring of repeat length polymorphisms will be done based on the size of the resulting amplification product.

A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (PCR) which is described in detail in U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in their entirety.

Genetic markers

The genetic marker(s) in the tyrosinase related protein gene useful in the invention may be selected from, but is not limited to, the group of polymorphisms shown in Table 1 below.

Table 1: Selection of markers within and upstream of the TYRP1 gene

Genetic Marker	Postn	Polymorph	C7 Genotype (Sire)	-C4 Genotype (Grand sire)	C17 Genotype (Grand dam)
TYRP-1 Upstream 32	32	A/G	A/A	A/A	A/G
TYRP-1 Upstream 288	288	Indel'A'	-/-	A/-	A/-
TYRP-1 Upstream 357	357	Indel'GA'	4GA/5GA	5GA/5GA	4GA/4GA
TYRP-1 Upstream 386	386	G/A	G/G	G/A	G/A
TYRP-1 Upstream 388	388	C/G	C/G	C/C	C/G
TYRP-1 Upstream 415	415	Indel CA/AGG	CA/CA	CA/AGG	CA/AGG
TYRP-1 Upstream 604	604	T/C	T/C	T/T	T/C
TYRP-1 Upstream 634	634	T/C	T/C	T/C	T/T
TYRP-1 Upstream 917	917	Indel'A'	A/-	A/A	A/-
TYRP-1 Upstream 951	951	C/T	C/T	C/C	C/T
TYRP-1 Upstream 955	955	G/A	G/G	G/A	G/A
TYRP-1 Upstream 983	983	G/T	G/G	G/T	G/T
TYRP-1 Upstream 997	997	A/T	A/T	A/T	T/T

TYRP-1 Upstream 1085	1085	A/G	A/A	A/G	A/G
TYRP-1 Upstream 1092	1092	G/T	G/T	G/G	G/T
TYRP-1 Upstream 1145	1145	C/T	T/T	C/T	C/T
TYRP-1 Upstream 1244	1244	A/C	C/C	A/C	A/C
TYRP-1 Upstream 1320	1320	G/C	G/C	G/G	G/C
TYRP-1 Upstream 1570	1570	A/G	A/A	A/G	A/G
TYRP-1 Upstream 1621	1621	Indel'TCA'	TCA/TCA	TCA/-	TCA/-
TYRP-1 intron 1 1830	1830	C/T	C/T	C/T	T/T
TYRP-1 intron 1 1910	1910	C/T	T/T	T/T	C/T
TYRP-1 intron 1 2041	2041	G/T	T/T	T/T	G/T
TYRP-1 exon 2 2131	2131	Indel'A'	-/-	-/-	-/A
TYRP-1 exon 2 2144	2144	C/G	C/C	C/C	C/G
TYRP-1 exon 2 2145	2145	G/T	T/T	T/T	G/T
TYRP-1 exon 2 2224	2224	C/G	C/G	C/G	C/C
TYRP-1 exon 2 2291	2291	C/T	C/C	C/T	C/T
TYRP-1 exon 2 2318	2318	C/T	C/T	C/C	C/T
TYRP-1 exon 2 2404	2404	C/T	C/T	C/T	C/C
TYRP-1 intron 5	Intron	SSR 300/302	300/302	295/300	302/302
TYRP-1 exon 6	30	C/T	T/C	T/T	C/C
TYRP-1 exon 8 50	50	T/C	T/C	T/C	C/C
TYRP-1 exon 8 63	63	C/A	C/A	C/C	A/A
TYRP-1 exon 8 109	109	C/A	C/A	C/C	A/A

The means for analyzing the nucleic acid sample may be selected from the group of oligonucleotides set forth in SEQ ID NOS 1-33 and 48 - 71.

Table 2: Sequence Identification Number

DNA sequence name	**Sequence ID No.**
Ovis aries TYRP-1 5`, exon1 and exon 2 DNA sequence	SEQ ID NO: 72
Ovis *aries* TYRP-1 exon 3 DNA sequence	SEQ ID NO:34
Ovis aries TYRP-1 exon 4 DNA sequence	SEQ ID NO:7
Ovis aries TYRP-1 exon 5 DNA sequence	SEQ ID NO:40
Ovis aries TYRP-1 exon 6 DNA sequence	SEQ ID NO:75
Ovis aries TYRP-1 exon 7 DNA sequence	SEQ ID NO:43
Ovis aries TYRP-1 exon 8 DNA sequence	SEQ ID NO:78
Ovis aries TYRP-1 encoded exonic DNA sequence	SEQ ID NO:46

Detection of polymorphsim

After amplification, it may be desirable to separate the amplification product from the template and/or the excess primer and other unused reagents. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. Amplification products isolated in this way may be eluted from an excised portion of the gel for further manipulation. The nucleic acid may be removed from the excised portion of an agarose gel by heating the gel in a chaotropic solution, followed by extraction of the nucleic acid.

Separation and isolation of nucleic acids may also be performed by chromatographic techniques known in art. Numerous kinds of chromatography may be used in the present invention, including adsorption, partition, ion-exchange, hydroxylapatite, gel-filtration (molecular sieve), and reverse-phase. These may be performed in a number of formats including, column, paper, thin-layer, and gas chromatography as well as by batch HPLC or FPLC.

In certain embodiments, the amplification products are visualized. A typical visualization method involves staining of a gel with ethidium bromide and visualization of bands under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the separated amplification products can be exposed to x-ray film or visualized under light of the appropriate excitatory wavelength.

In one embodiment, following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled or otherwise labeled to facilitate detection. In another embodiment, the probe is conjugated to a molecule, such as an antibody or biotin, or another molecule carrying a moiety to facilitate detection.

In particular embodiments, detection is by nucleic acid blotting and hybridization with a labeled probe. The techniques involved in nucleic acid blotting are well known to those skilled in the art. For example, the nucleic acid blotting and hybridization may be performed under stringent conditions.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50

nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1.times. to 2 x SSC (20xSSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5x to 1xSSC at 55 to 50°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1xSSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984): T_m =81.5°C +16.6 (log M)+0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with 90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-

Hybridization with Nucleic Acids Probes, Part I, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995).

Kits

All the essential materials and/or reagents required for screening animals for genetic marker genotype in accordance with the invention may be assembled together in a kit. This generally will comprise a probe or primers designed to hybridize specifically to individual nucleic acids of interest in the practice of the present invention, for example, primer sequences such as those for amplifying TYRP-1. Also included may be enzymes suitable for amplifying nucleic acids, including various polymerases, deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such kits also may include enzymes and other reagents suitable for detection of specific nucleic acids or amplification products. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or enzyme as well as for each probe or primer pair.

In certain embodiments, the invention also can provide for a kit which can be used to determine the TYRP-1 genotype of genetic material, for example the kit may include a set of primers used for amplifying the genetic material. A kit can contain a primer including a nucleotide sequence for amplifying a region of the genetic material containing one of the polymorphisms described herein.

The present invention will now be further described in greater detail by reference to the following specific examples, which should not be construed as in any way limiting the scope of the invention.

Examples

Example 1: QTL mapping of pigmentation traits

Six pigmentation traits, being (1) tail wool colour (ColourScore), (2) face skin colouration (PigmSkin), (3) nose and lip skin pigmentation (NoseLPig), (4) face, legs and hooves fibre colouration (PigFibre), (5) hoof pigmentation alone (HoofPigm), and (6) horn and leg fibre (LHSFibre), as set out in Table 3, were assessed in a quantitative trait loci (QTL) gene mapping experiment with n = 177 individuals. The 177 offspring were male backcrossed progeny from mating between a single Awassi/Merino F1 sire (C7/A95.0453) and unselected Merino ewes, giving a progeny of 75% Merino and 25% Awassi. Animals were born from May 1997, and separated into two management cohorts according to birth dates.

Table 3: Pigmentation traits and trait description

Colour Score Trait:	Trait description
ColourScore	Tail scored based on % coloured fibre along tail 0, 5,10,20,30,40,50,60,70,80,90,95,100%
Pigmentation Traits:	**Trait description**
PigmSkin	Skin pigmentation on nose/lips, face skin under fibres, bare skin around eyes and the skin on ears under fibre (Score/400)
NoseLPig	Skin pigmentation on lips and nose (Score/100)
PigFibre	Fibre pigmentation on ears, horns, legs, hooves and face excluding eyelashes (Score/500)
HoofPigm	Fibre pigmentation on hooves (Score/100)
LHSFibre	Fibre pigmentation on horns and legs (Score/200)

For all traits detailed in Table 3 except ColourScore, the fixed effect of management cohort was significant, and traits were adjusted accordingly. The fixed effect of sex was not significant. Traits were transformed as follows: ColourScore transformed to a binary trait; NoseLPig was arcsin transformed, and LHSFibre was transformed to a binary trait. A full genome scan was conducted with 204 microsatellite markers. A framework map was constructed using the open source mapping programs Carthagene and Mapmaker, which was used to inform marker positions. A full genome scan was conducted using a maximum likelihood analysis written in R, developed by the inventors (QTL-MLE).

The QTL-MLE algorithm was developed to match the particular experimental design and genotyping characteristics of the study. These included (1) incorporation of known linkage phase information resulting from the backcross design, (2) utilisation of all the relevant genotypic information, and (3) valid modelling the marker-QTL associations by means of a finite mixture model. Item (2) involved development of an algorithm to utilise information from heterozygous marker genotypes, using estimates of marker allele frequencies in the merino population to assist this. The QTL model is fitted using a

maximum likelihood routine at each putative QTL position along the length of the target chromosome (interval mapping). In addition to the estimated QTL location and effects, the probability of each backcross offspring inheriting either allele from the F1 sire (Q or q) is provided, in terms of a posterior probability. This allows animals with a high probability of receiving the "favourable" QTL genotype to be identified. In addition, a 1-LOD support interval is provided to give an indication of the precision of estimating the location of the QTL. The procedure (QTL-MLE) has been coded using the open source statistical program R (also compatible with the commercial version, S=PLUS).

The above analysis identified QTL regions with log of odds (LOD) scores >2 (P<0.05) on chromosomes 2, 3, 8, 18, 19, 25 and 26. The most significant effect was seen on chromosome 2, with a QTL LOD >20 for all 6 traits, as shown below in Tables 4 and 5 and Figures 1 and 2.

Table 4: Colour Score Trait

Trait	Chr	QTL position (support)	LOD
ColourScore[2]	2	150 (145,155)	20.49

Table 5: Pigment Traits

Trait	Chr	QTL position (support)	LOD
PigmSkin	2	148 (144,154)	21.38
	19	34 (6,48)	5.58*
NoseLPig[1]	2	158 (146,167)	12.08*
	3	275 (258,291)	2.56
	19	37 (24,51)	5.82*
	25	25 (8,49)	3.94
PigFibre	2	146 (143,148)	28.56
	26	30 (16,47)	2.45
HoofPigm	2	163 (159,166)	33.82
	18	135.5 (122,135.5)	2.25
LHSFibre[2]	2	143 (120,19)	10.04
	8	101 (72,109)	2.23
	19	39 (27,51)	5.24

*: from interaction model; [1]: ArcSine transformation; [2]: Binary trait

Shown below in Table 6 are the summarized QTL positions on each chromosome for the different traits based on the data shown above in Tables 4 and 5. As can be seen from Table 6, one of the major regions with strongest significance was on chromosome 2.

Table 6: QTL positions for each pigmentation trait

	Chromosome						
	2	**3**	**8**	**18**	**19**	**25**	**26**
ColourScore[2]	150						
PigmSkin	148				34*		
NoseLPig[1]	158*	275			37*	25	
PigFibre	146						30
HoofPigm	163			135.5			
LHSFibre[2]	143		101		39		

*: from interaction model; [1]: ASin transformation; [2]: Binary trait

Based on the results of this QTL mapping experiment, TGLA10 was found to be the closest linked mapped genetic marker and tyrosinase-related protein-1 (TYRP-1) was identified as the most significant positional candidate gene by comparative mapping to human, cattle and rat using the Comparative Predictions of Orthologues software at the Oxford Grid Project (http://oxgrid.angis.org.au).

In particular, a GenBank database search was performed for the *Ovis aries* TYRP-1 DNA sequences generated in this study using the Basic Local Alignment Search Tool (BLAST) on human, sheep and mouse genomes at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). This search identified that orthologous sequences of the *Ovis aries* TYRP-1 DNA map to chromosomes 8, 9, and 5 in cattle, human and rat respectively. For comparative predictions of orthologues between sheep versus cattle, human and rat, the OXGRID program (http://oxgrid.angis.org.au) was used. OXGRID results indicate that the orthologous position of cattle, human and rat TYRP-1 gene corresponds to the chromosome number 2 in sheep (*Ovis aries*), which is consistent with the QTL mapping described above.

Example 2: Characterisation of sheep TYRP-1

2.1 Primer Design for sheep TYRP-1

At the time of designing primers for exonic regions, the human TYRP-1 gene was the only available full length DNA sequence for the gene that included all exonic and intronic regions. The human sequence, together with the available exonic sequences from cattle and goat, was used as a sequence model to design primers and estimate the expected size of TYRP-1 exons and introns in sheep. Primers for certain of the upstream regions were designed based on the *Bos taurus* chromosome 8 genomic contig, whole genome shotgun sequence NW_932015.1.

Primers as shown below in Tables 7-9 were designed based on the entire Genbank human sequence as well as partial and mRNA bovine, ovine, horse and dog sequences (Figure 4). Sequence line ups showing primer design and individual species sequence are shown in Figures 5 – 13. One primer pair was positioned at the beginning and end of each exon. In certain cases, other primers were chosen to flank the exon from intronic regions, all of which is detailed in Figure 3.

Table 7. Primer pairs used to amplify exonic regions 2-8. Forward and reverse primer sequences start at the first and last position respectively of each exon.

Exon	F/R	Primer sequence 5'- 3'	Predicted exonic position
Exon 2	F	CTTGATTTTTATCTACGTGCCTCAGTC (SEQ ID NO: 1)	5` 1bp
	R	CTGTGAGAACCCTCTGGTCACAG (SEQ ID NO: 2)	5` 470bp
Exon 3	F	TCAGGAGAAACCTTCTGGACTTAAG (SEQ ID NO: 3)	5` 1bp
	R	CTGCATGTCTCTCTCCAGCT (SEQ ID NO: 4)	5` 323bp
Exon 4	F	GAAATGTTGCAGGATCCTTCTTTC (SEQ ID NO: 5)	5` 1bp
	R	ACTGTTGCAAAGGGTTCCCAGG (SEQ ID NO: 6)	5` 207bp
Exon 5	F	GCACTGAGGGTGGGCCAATTAAG (SEQ ID NO: 7)	5` 1bp
	R	CTTCCACTGTGTTTCGGAAGCTG (SEQ ID NO: 8)	5` 168bp
Exon 6	F	GGTTACAGTGATCCCACAGGAAG (SEQ ID NO: 9)	5` 1bp
	R	CAGCATTGTATCGCCTCAGCCA (SEQ ID NO: 10)	5` 180bp
Exon 7	F	AGATATATCCACATTTCCACTGGA (SEQ ID NO: 11)	5` 1bp
	R	CTCACCTGGCCATTGAACTT (SEQ ID NO: 12)	5` 152bp

Exon 8	F	GGTCGGAGTTTTAGTATTCCTGA (SEQ ID NO: 13)	5` 1bp
	R1	*TGATTCAAATGCATATGAGAATTTAC (SEQ ID NO: 14)	5` 240bp
	R2	ATTCACTTATGACCACCTTATACAGTTCT (SID NO: 15)	5` 1251bp

* M13(-29) sequence added for the purposes of fluorescent dye incorporation

Table 8. Additional primers used to amplify and sequence upstream region

Region	F/R	Primer sequence 5'- 3'	Position
Exon 1, intron 1	TYRP1F TYRP1R	AAAGGGTAGAAAAGAGGACTGG (SEQ ID NO: 16) GGCACGTAGATAAAAATCAAGC (SEQ ID NO: 17)	5` 1520bp 5` 2141bp
Exon 1, intron 1, exon 2 partial	TYRP1F E2AR	AAAGGGTAGAAAAGAGGACTGG (SEQ ID NO: 16) GTTCCACAGTTGTGTCCTGAGAAAT (SEQ ID NO: 18)	5` 1520bp 5` 2536bp
Intron 1, exon 2 partial	Intron1F E2R3	*GTGGATTCCCACCAAAACTG (SEQ ID NO: 19) ACACGCCATTCCTCAAAGCC (SEQ ID NO: 20)	5` 1722bp 5` 2322bp
Upstrea m, exon 1, intron 1	UP5AF TYRP1R	AGATGGAGATGAACACCCACCTATT (SEQ ID NO: 21) GGCACGTAGATAAAAATCAAGC (SEQ ID NO: 17)	5` 1063bp 5` 2141bp
Upstrea m	UP6AF UP6AR	*AACAGAAGGGATCAAATCACAAG (SEQ ID NO: 22) TGTGTCCAGAGAGACTAACTG (SEQ ID NO: 23)	5` -29bp 5` 1198bp

* M13(-29) sequence added for the purposes of fluorescent dye incorporation

Table 9. Additional primers used to amplify genotyping markers

Region	F/R	Primer sequence 5'- 3'	Position
TYRP1 Upstrea m 357	Indel357F Indel357R	*TAAAAATCCAAGAAAATAATCCAAATC (SEQ ID NO: 24) GGTCAACTTGATATGTCATTGGTAAG (SEQ ID NO: 25)	5` 290bp 5` 397bp
TYRP1 Upstrea	SNP_UPi F	*TGACAGCATTTTGCATCATTTAG (SEQ ID NO: 26) ATTTGACCCTCAGTCATAAAATTGTC (SEQ ID NO:	5` 494bp 5` 802bp

m 604	SNP_UPi R	27)	
TYRP1 Upstrea m 917	Indel_UPc F UP6AR	*CTGAGGGTCAAATAGGACAGC (SEQ ID NO: 28) TGTGTCCAGAGAGACTAACTG (SEQ ID NO: 23)	5` 790bp 5` 1198bp
TYRP1 Upstrea m 1621	TYRP1F Indel1621 R	AAAGGGTAGAAAAGAGGACTGG (SEQ ID NO: 16) *AAGGCATTCTCTGCTGAACC (SEQ ID NO: 29)	5` 1520bp 5` 1680bp
TYRP1 Intron1 1830	Intron1F Intron1R	*GTGGATTCCCACCAAAACTG (SEQ ID NO: 19) CAACCCACCTTCAAAATGC (SEQ ID NO: 30)	5` 1722bp 5` 2049bp
TYRP1 Exon 2 2131 and 2404	Indel2131 F E2AR	*TCATTTTGCTATTTTCTTTTCAGC (SEQ ID NO: 31) GTTCCACAGTTGTGTCCTGAGAAAT (SEQ ID NO: 18)	5` 2097bp 5` 2536bp
TYRP1 Intron 5	Intron5SS R_F Intron5SS R_R	*AGAAAACAGCAACCCACTCC (SEQ ID NO: 32) TCTGAAAAGACTGCTGTCAAAAC (SEQ ID NO: 33)	

* M13(-29) sequence added for the purposes of fluorescent dye incorporation

2.2 PCR amplification of sheep TYRP-1

PCR cycle and reaction conditions are described in Tables 10, 11 and 12, 13 respectively.

Table 10. PCR cycle conditions for sequencing product amplification

Temperature (°C)	Time (min)	Special conditions	Cycles
94	3.30		1
94 62 72	0.45 0.30 1.30	-2°C per cycle	4
94 54 72	0.45 0.30 1.30		30
72	5.00		1

Table 11. PCR cycle conditions for marker amplification

Temperature (°C)	Time (min)	Special conditions	Cycles
94	3.30		1
94 62 72	0.40 0.20 0.30	 -2°C per cycle for 1st 4 cycles	10
92 50 72	0.30 0.20 0.30	 -	35
72	12.00		1

Table 12. PCR reaction components – 25ul volume

Volume and name of component	Concentration in the stock solution
1 ul DNA template	100 ng/ul
2 ul forward primer	20 pmol/ul
2 ul reverse primer	20 pmol/ul
1.0 ul $MgCl_2$	25 mM
1.5 ul dNTPs	2 mM
0.2 ul Taq DNA polymersae	10 unit/ul
2.5 ul PCR buffer	10X
14.8 ul H_20	
Total volume of 25 ul reaction	

Table 13. PCR reaction components – 10ul volume

Volume and name of component	Concentration in the stock solution
3 ul DNA template	20 ng/ul
0.01 ul forward primer	20 pmol/ul
0.05 ul reverse primer	20 pmol/ul
0.05 ul M13 tail with dye	20 pmol/ul
0.5 ul $MgCl_2$	50 mM
0.5 ul dNTPs	20 mM
0.06 ul Taq DNA polymersae	1 unit/ul
1 ul PCR buffer	10X
4.84 ul H_20	
Total volume of 10 ul reaction	

DNA samples from grandparents (C4 and C17) and the sire (C7) from the Awassi sheep family were used to test the TYRP-1 exonic and intronic primers. In addition, negative controls and other DNA species such as cattle, human and mouse were used. Results of the PCR are shown in Figures 4, 5 and 6.

2.3 PCR of sire C7 for direct sequencing

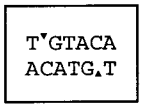
TYRP-1 exons from sample C7 were amplified by PCR using the conditions described in Tables 10 and 12 using *Tli* DNA polymerase (Promega) or 11 and 13 using Invitrogen Taq DNA polymerase. PCR products were gel-purified using UltraClean DNA purification kits (Mo Bio Laboratories, Inc.), or were enzymatically cleaned using ExoSAP-IT (USB). PCR products were directly sequenced using the SUPAMAC (Sydney University Prince Alfred Macromolecular Analysis Centre) commercial sequencing service on an ABI PRISM® 3700 platform (Applied Biosystems, Foster City), or were sequenced using an ABI BigDyeV3 kit, and run in-house on an ABI PRISM® 3100 platform. The DNA and amino acid (based on nuclear code) TRYP-1 sequences obtained from the upstream region, and exons 2 to 8 (as per Table 2) .

For all variant sites the chromatograms showed ambiguous sites (N), with two collocated peaks of significant height, thereby indicating potential SNP sites. Insertion deletion mutations were recognised from a sudden and repeatable loss of sequencing readability. These ambiguities were corroborated with the alternate sequencing experiments of the other two individuals, and by sequencing from the other end of the PCR fragment.

2.4 PCR-RFLP approach

Six of the potential SNPs were tested by restriction fragment length polymorphism (RFLP) analysis to determine whether they were consistent with restriction enzyme sites.

For marker TYRP1 Upstream 604, the C/T variation was tested using restriction enzyme *BsrGI* (NEB) which recognises:



The variant TGTA(C)A is recognised and digested by the *BsrGI* while the variant TGTA(T)A is not recognised by the enzyme. For example, BsrGI PCR-RFLP of TYRP-1 Upstream 604 using the restriction enzyme *BsrGI* for C7 was shown to be heterozygous containing the cut product (123bp) and the uncut product (326bp). C4 is a homozygote

for the uncut product (326bp), while C17 is a heterozygote for the cut product (123bp) and the uncut product (326bp).

For marker TYRP1 Upstream 917, the A/- variation was tested using restriction enzyme *Xmn I* (NEB) which recognises:

```
GAANN'NNTTC
CTTNN.NNAAG
```

The variant G(A)AAAAATTC is recognised and digested by the *Xmn I* while the variant G(-)AAAAATTC is not recognised by the enzyme. For example, TYRP1 Upstream 917, insertion deletion mutation identified via Xmn I PCR-RFLP of TYRP-1 Upstream 604 product for C7 was shown to be heterozygous for the cut product (426bp) and the uncut product (149bp). C4 is a homozygote for the cut product (149bp), while C17 is a heterozygote for the cut product (149bp) and the uncut product (426bp).

For marker TYRP1 Intron 1 1830, the C/T variation was tested using restriction enzyme *Ase I* (NEB) which recognises:

```
AT'TAAT
TAAT.TA
```

The variant A(T)TAAT is recognised and digested by the *Ase I* while the variant A(C)TAAT is not recognised by the enzyme. For example,, Ase I PCR-RFLP of TYRP-1 Intron 1 1830 for C7 was shown to be heterozygous for the cut product (347bp) and the uncut product (127bp). C4 is a heterozygote for the cut product (127bp) and the uncut product (347bp), while C17 is a homozygote for the cut product (127bp).

For marker TYRP1 Exon 2 2404, the C/T variation was tested using restriction enzyme *Hph I* (NEB) which recognises:

```
GGTGANNNNNNNN'
CCACTNNNNNNN.
```

The variant GG(T)GATAGCCGAC is recognised and digested by the *Hph I* while the variant GG(C)GATAGCCGAC is not recognised by the enzyme. For example, , Hph I PCR-RFLP of TYRP-1 Exon 2 2404 after electrophoresis on a 4% agarose gel reflects. C7 as heterozygous for the cut product (339bp) and the uncut product (453bp). C4 is a heterozygote for the cut product (339bp) and the uncut product (453bp), while C17 is a homozygote for the uncut product (453bp).

For Exon 6, the C/T variation in position 30 of this exon was tested using restriction enzyme *Rsa I* (Promega) which recognises.

GT'AC
CA▲TG

The variant GTA(C)G is recognised and digested by the *Rsa I* while the variant GTA(T)G is not recognised by the enzyme. For example, PCR-RFLP of TYRP1 exon 6 using restriction enzyme *Rsa I* on a 4% agarose gel indicates C7 as heterozygous, containing both the cut allele (158bp) and the uncut allele (186bp). C4 is homozygous for the uncut allele (186bp), while C17 is homozygous for the cut allele (158bp). PCR = PCR product without digestion, DIG = PCR product after incubation with the enzyme restriction.

For TYRP1 Exon 8 63, the C/A variation was tested using restriction enzyme *Acc II* (Amersham Biosciences) or *BstU I* (NEB) which recognises:

CG'CG
GC▲GC

The variant (C)GCG is recognised and digested by the *Acc II* while the variant (A)GCG is not recognised by the enzyme. For example, PCR-RFLP of TYRP-1 exon 8 63 using the restriction enzyme *Acc II* on a 4% agarose gel indicates C7 to be heterozygous, containing both the cut allele (244bp) and the uncut allele (180bp). C4 is homozygous for the cut allele (180bp), while C17 is homozygous for the uncut allele (244bp).

Samples C7, C4 and C17 were amplified using PCR conditions described above, with 10 ul of PCR products mixed with 0.5 - 2 units of restriction enzyme, 3 ul of Enzyme Buffer and 7 ul of H_2O and incubated for 3 hours at 37°C (BstUI at 65 °C).

Similarly, for marker TYRP1 Upstream 357, microsatellite marker for C7 was shown to be heterozygous at position 357, with both 4x 'GA' (111bp) and 5x 'GA' repeats (113bp). C4 is a homozygote for 5x 'GA' (113bp) repeat, while C17 is a homozygote for 4x 'GA' (111bp) repeat.

Also, for marker TYRP1 Upstream 1621, insertion deletion mutation marker for C7 was shown to be homozygous for a TCA insertion (179bp). C4 and C17 are heterozygotes, with both the insertion (179bp) and deletion (176bp) fragment lengths.

Further, for marker TYRP1 Exon 2 2131, insertion deletion mutation marker for C7 was shown to be homozygous for a 1bp deletion product size (453bp). C4 is a homozygote for the deletion product size (453bp) while C17 is a heterozygote, with both the 'A' insertion (454bp) and deletion (453bp) fragment lengths. Note: A second indel fragment length (455bp) is also identified by this PCR.

Also, with respect to TYRP1 exon 6, using restriction enzyme *Rsa I* on a 4% agarose gel C7 was shown to be heterozygous, containing both the cut allele (158bp) and the uncut allele (186bp). C4 is homozygous for the uncut allele (186bp), while C17 is homozygous for the cut allele (158bp). PCR = PCR product without digestion, DIG = PCR product after incubation with the enzyme restriction.

Still further for marker TYRP1 Intron 5, microsatellite marker for C7 was shown to be heterozygous, with product size 300bp and 302bp. C4 is a heterozygote, with product size 295bp and 300bp, while C17 is a heterozygote, with product size 302bp and 302bp.

2.5 Genotype analysis of Awassi sheep families

PCR (10 ul) and restriction enzyme digestion conditions as described above were then used to genotype all available Awassi sheep progeny, including the backross mapping population, complex crosses, and other sire families, for seven loci.

The families include the following mating designs. In addition to the 177 males of the mapping population, a further 336 animals were generated using the same mating design (backcrossed progeny from mating between a single Awassi/Merino F1 sire (C7) and unselected Merino ewes). Advanced intercrosses were made by crossing C7 to the progeny of three independent Awassi/merino F1 sires (sires A96.0463, A96.0473, A96.0474) mated to merino ewes (n=64). Finally C7/merino backcross progeny were double backcrossed to the C7 sire (n=119), and likewise, sire A96.0463 and sire A96.0474 were double backcrossed to their own backcrossed progeny, or to the backcrossed progeny of A96.0473 (n=88 and 53, respectively).

The data in Table 14 show summarised genotyping results with the seven loci genotyped, although some loci are currently incompletely genotyped, and secondary (gap filling) genotyping needs to be completed.

Table 14. Summary of genotyping results

	Position in TYRP1 sequence													
	357		917		1621		1830		Intron_5		Exon_6		Exon_8_63	
	Allele	**N**	**Allele**	**N**	**Allele**	**N**	**Allele**	**N**	**Allele**	**N**	**Allele**	**N**	**Allele**	**N**
Awassi origin	113	319	146	1188			347	334	300	380	186	393	181	831
Merino origin	111	803	424	426			127	1123	302	723	158	517	239	669
Unknown origin					176	431			295	495				
					179	621			298	6				

Example 3: Detection of QTL

Following QTL analysis, the TGLA 10 locus was identified as being closest to the estimated QTL location. Male sheep from the mapping population (Sire C7 backcrossed

to merino sheep) with genotypic information at the TGLA10 locus, and phenotypic information for PigFibre (Table 3) were selected (n=177). Progeny were allocated a genotypic score of "1" if the allele passed on by the sire originated from the grandsire, and a genotypic score of "2" if the sire donated an allele originating from the granddam. Phase unknown inheritance was indicated as a "12". In the mapping population, the "1" allele was of Awassi origin, and the "2" allele was of Merino origin. Animals were binned according to genotypic score, and "12" animals were discarded as having unclear inheritance. A histogram of the PigFibre trait for animals in the "1" bin (n=70) and animals in the "2" bin (n=68), showed distinct differentiation in the trait range, in that animals with an Awassi inheritance had no animals without pigmentation, while 16.17% of animals with the Merino allele were scored as 0 (colourless) (Figure 7). A two sample t test assuming unequal variances revealed significant support for phenotypic differentiation between the two classes (p(two tailed)<5.95x10E23) as shown in Table 15.

Table 15: T test- two sample assuming unequal variances

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	279.70	74.03
Variance	12224.27	8493.97
Observations	71.00	69.00
Hypothesized Mean Difference	0.00	
df	135.00	
t Stat	11.97	
P(T<=t) one-tail	0.00	
t Critical one-tail	1.66	
P(T<=t) two-tail	0.00	
t Critical two-tail	1.98	

Example 4: Statistical association analysis of TYRP-1 markers and pigmentation

Following genotyping of all of the described Awassi families for the seven loci, analysis was conducted to test for an association between known TYRP1 haplotypes and PigFibre or ColourScore (1-9). Initially, the same individuals used in the previous analysis (Example 3) were used, with ANOVA results confirming that TYRP1 haplotypes ("Combined") explained a large proportion of the variance of the PigFibre trait (Figure 9, Table 16).

Table 16. ANOVA results for PigFibre Trait, with sex, management group and haplotype ("Combined") as fixed effects

Factor	Type	Levels	Values
Sex	fixed	2	ewe, wet
Group	fixed	2	1, 2
Combined	fixed	21	10_34, 11_34, 15_33, 15_34, 18_34, 29_34, 3_10, 3_15, 3_18, 3_20, 3_23, 3_26, 3_29, 3_3, 3_34, 3_4, 3_9, 4_11, 4_29, 6_11, 9_34

Analysis of Variance for PigFibre, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Sex	1	26855	6531	6531	0.65	0.420
Group	1	23867	11074	11074	1.11	0.294
Combined	20	1735625	1735625	86781	8.68	0.000
Error	138	1379497	1379497	9996		
Total	160	3165845				

S = 99.9818 R-Sq = 56.43% R-Sq(adj) = 49.48%

Subsequently, animals from the complex crossing strategies detailed in section 2.5 were used in an ANOVA analysis, confirming that TYRP1 haplotypes ("Combined") explained a large proportion of the variance of the ColourScore trait (Table 17).

Table 17. ANOVA results for ColourScore Trait, with year of birth, sex and haplotype ("Combined") as fixed effects

General Linear Model: ColourScore versus Year, Sex, Combined

Factor	Type	Levels	Values
Year	fixed	5	1999, 2000, 2002, 2004, 2005
Sex	fixed	2	ewe, wet
Combined	fixed	55	*, 10_15, 10_23, 10_34, 11_13, 11_15, 11_18, 11_34, 15_15, 15_17, 15_18, 15_20, 15_21, 15_23, 15_29, 15_33, 15_34, 18_18, 18_22, 18_29, 18_34, 1_15, 26_34, 29_34, 2_18, 30_34, 34_34, 34_36, 3_10, 3_11, 3_15, 3_18, 3_20, 3_22, 3_29, 3_3, 3_34, 3_37, 3_4, 3_9, 4_10, 4_11, 4_34, 4_35, 5_22, 6_15, 6_34, 7_15, 7_18, 7_20, 7_28, 7_29, 8_34, 9_18, 9_34

Analysis of Variance for ColourNumberOnly, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	4	153.184	34.461	8.615	1.37	0.246
Sex	1	21.742	0.823	0.823	0.13	0.718
Combined	54	603.921	603.921	11.184	1.77	0.002
Error	242	1525.527	1525.527	6.304		
Total	301	2304.374				

S = 2.51074 R-Sq = 33.80% R-Sq(adj) = 17.66%

CLAIMS

1. A genetic marker for distinguishing animals that have a trait of pigmentation, wherein said marker is a polymorphism in the tyrosinase-related protein gene.

2. A method for predicting pigmentation in an animal, said method comprising analysing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal.

3. The method of claim 1 or 2, wherein the pigmentation comprises pigmented skin, pigmented fibre or partial fleece colouration or full fleece colouration.

4. The method of claim 3, wherein the fibre is selected from the group comprising wool, hair or cashmere.

5. The method of claim 4, wherein the fibre is hair or mohair.

6. The method of claim 5, wherein the fibre is wool or cashmere.

7. The method of any one of claims 1-6, wherein the animal is an artiodactyl species.

8. The method of claim 7, wherein the animal is selected from the group comprising sheep, alpaca, lama and goat.

9. The method of claim 7, wherein the animal is a sheep.

10. The method of any one of claims 1-9, wherein the tyrosinase-related protein is tyrosinase-related protein – 1.

11. A method for selecting an animal using marker assisted selection, wherein said method comprises

(a) analysing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in an animal; and

(b) selecting an animal based on the presence or absence of said genetic marker.

12. A method for breeding an animal using marker assisted selection, wherein said method comprises:

(a) analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal;

(b) breeding from said animal based on the presence or absence of said genetic marker; and

(c) selecting progeny of said animal based on the presence or absence of said genetic marker.

13. A system for predicting pigmentation in an animal, wherein said system comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal.

14. A system for selecting an animal using marker assisted selection, wherein said system comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal; and

(b) means for selecting said animal based on the presence or absence of the genetic marker.

15. A system for breeding an animal using marker assisted selection, wherein said system comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal; and

(b) means for breeding said animal based on the presence or absence of the genetic marker, and

(c) means for selecting progeny of said animal based on the presence or absence of the genetic marker.

16. A kit for predicting pigmentation in an animal, wherein said kit comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal.

17. A kit for selecting an animal using marker assisted selection, wherein said kit comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal; and

(b) means for selecting said animal based on the presence or absence of the genetic marker.

18. A kit for breeding an animal using marker assisted selection, wherein said kit comprises:

(a) means for analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein said genetic marker is predictive of pigmentation in said animal; and

(b) means for breeding said animal based on the presence or absence of the genetic marker, and

(c) means for selecting progeny of said animal based on the presence or absence of the genetic marker.

19. A polypeptide comprising a sheep tyrosinase-related protein (TYRP).

20. The polypeptide of claim 19, wherein said polypeptide is sheep tyrosinase-related protein-1 (TYRP-1).

21. The polypeptide of claim 19, wherein TYRP-1 may comprise the amino acid sequence set forth in SEQ ID NO: 36, 39, 42, 45, 47, 74, 77 and 80.

22. A polynucleotide encoding the polypeptide of any one of claims 19 to 21.

23. A vector comprising the polynucleotide of claim 22.

24. A host cell transformed with the vector of claim 23.

25. An animal selected by the method of claim 11, or the system of claim 14 or the kit of claim or 17.

26. An animal bred by the method of claim 12, or the system of claim 15 or the kit of claim or 18.

27. An animal product derived from the animal of claim 25 or 26.

28. A method of sampling fibres to determine non-visual pigmentation, wherein said method comprises

(a) genotyping a plurality of animals based on the presence or absence of at least one genetic marker in the tyrosinase-related protein gene, wherein the genetic marker is predictive of pigmentation in said animal;

(b) grouping said animals according to genotype;

(c) sampling fibres from animals grouped as having said genetic marker(s); and

(d) determining a likelihood of contamination of said fibre samples with pigmented fibres.

29. A method for identifying pigmentation in an animal, wherein said method comprises:

(a) analyzing a nucleic acid sample from said animal for the presence of at least one genetic marker in the tyrosinase-related protein gene, wherein the presence of the genetic marker is indicative of pigmentation in said animal.

30. The method of claim 29, wherein the pigmentation is present in the form of pigmented fibres.

31. The method of claim 29, wherein the pigmented fibres are in the form of pigmented wool, hair, mohair or cashmere.

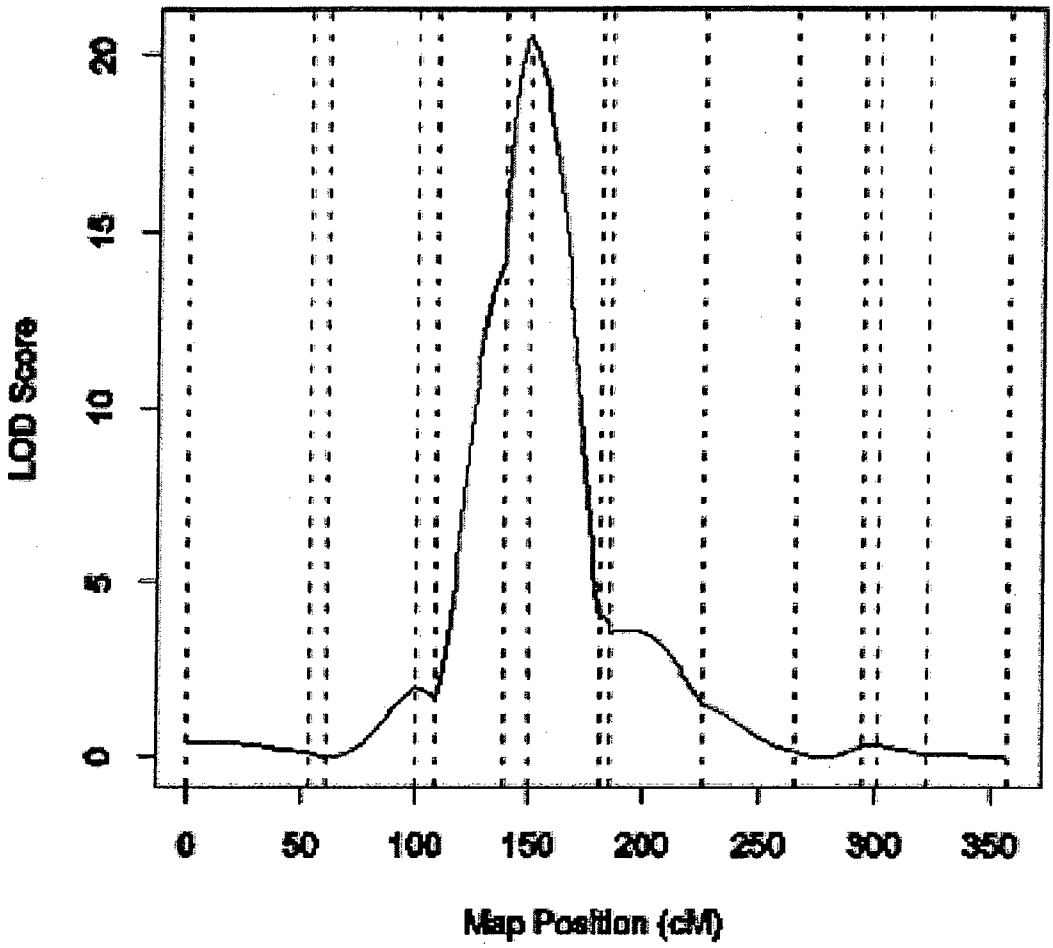

LOD Score
0
5
10
15
20
0
50
100
150
200
250
300
350
Map Position (cM)

Figure 1

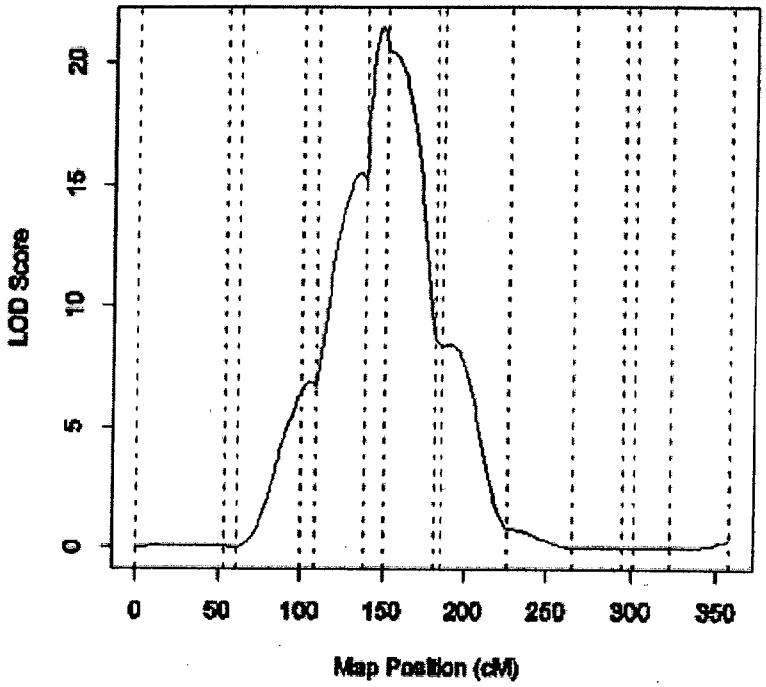


Figure 2A

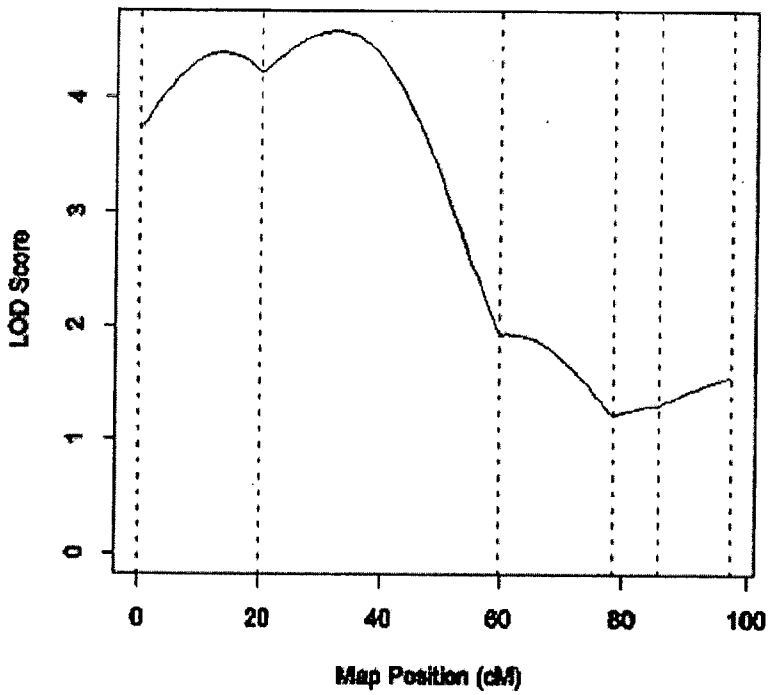


Figure 2B

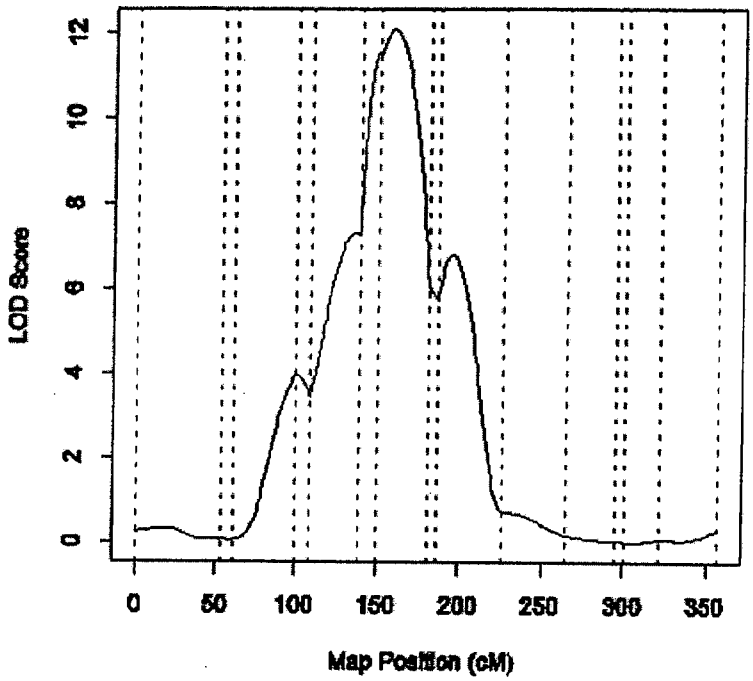


Figure 2C

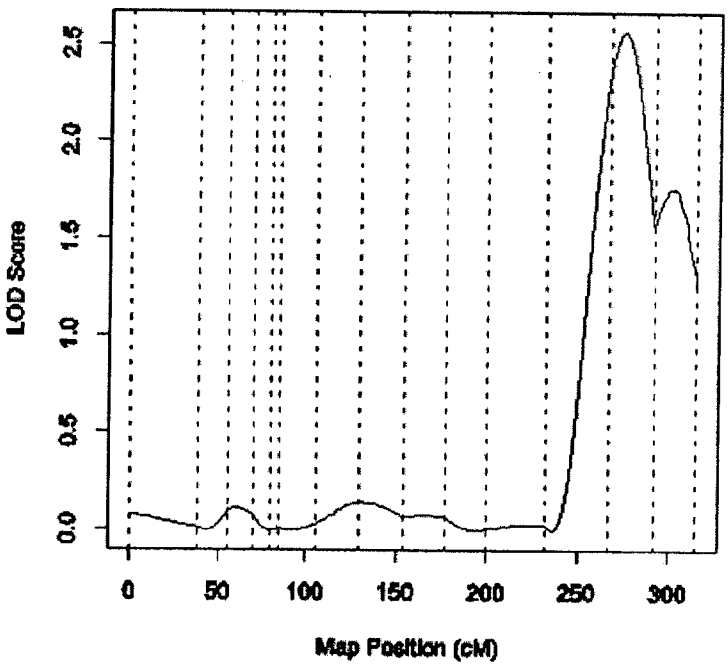


Figure 2D

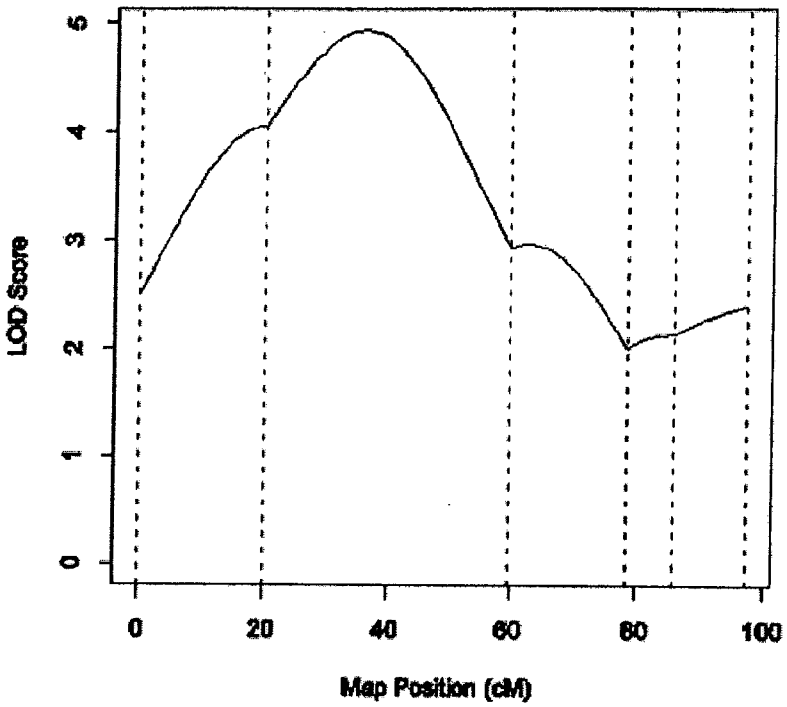


Figure 2E

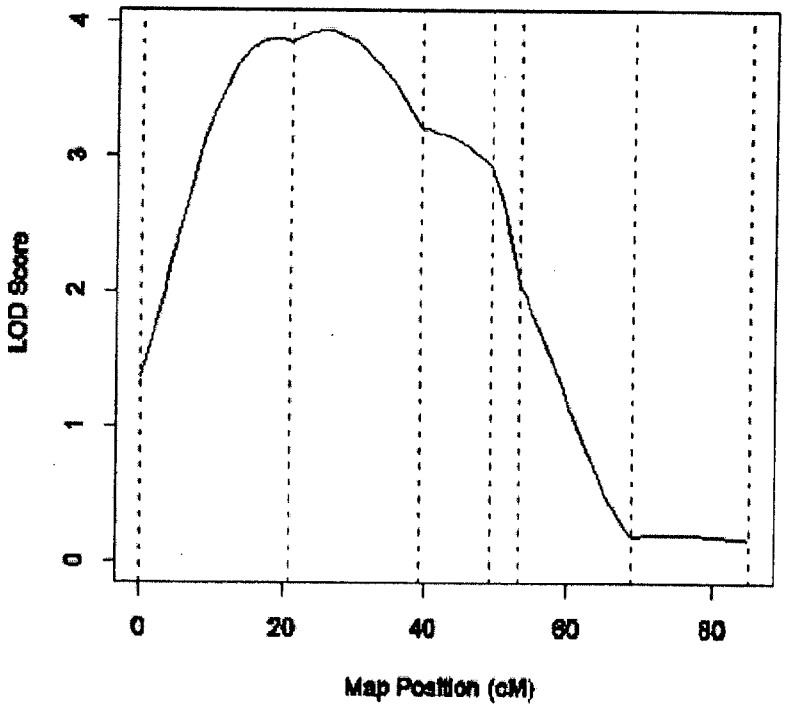


Figure 2F

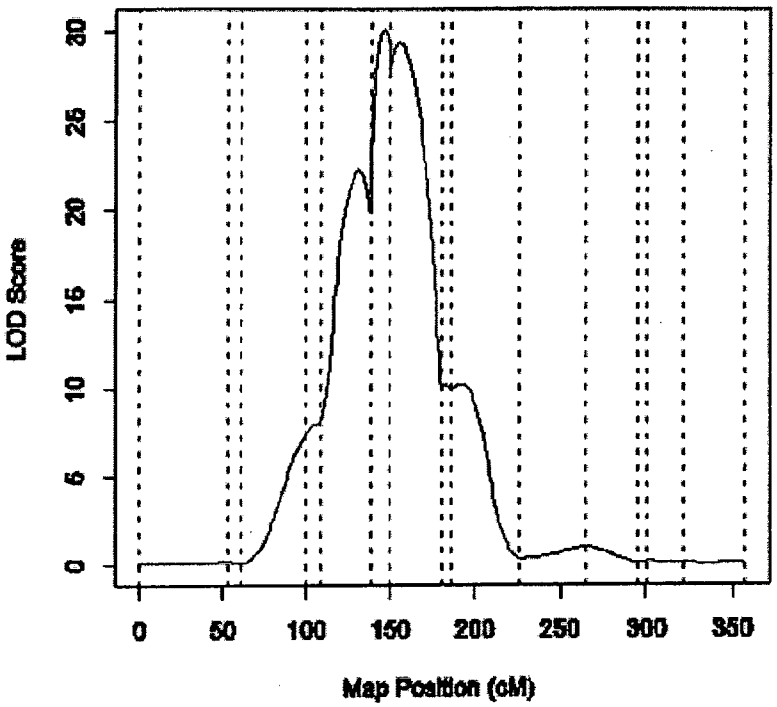


Figure 2G

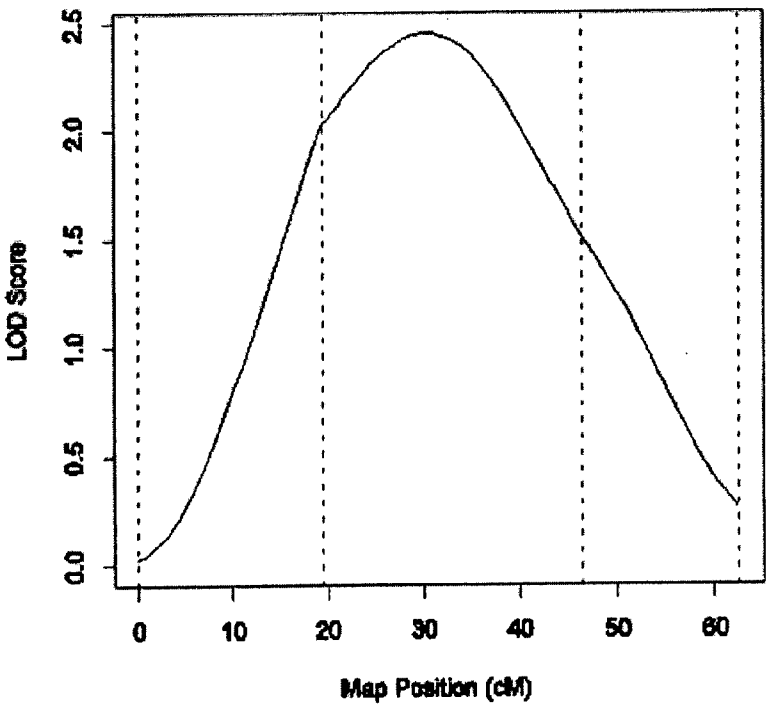


Figure 2H

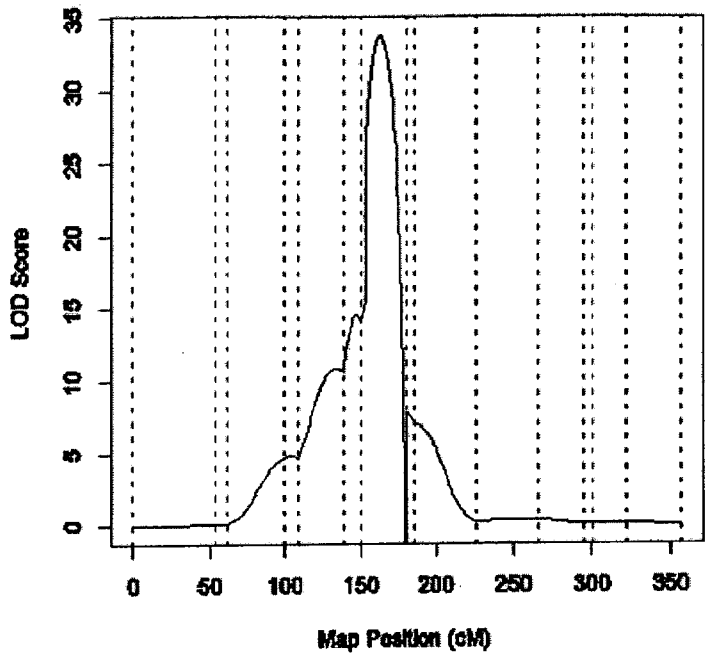


Figure 2I

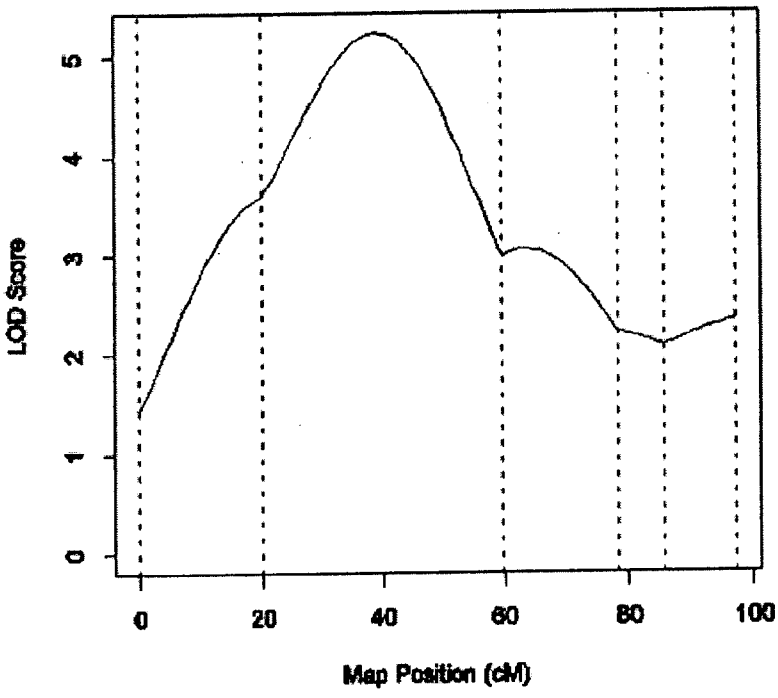


Figure 2J

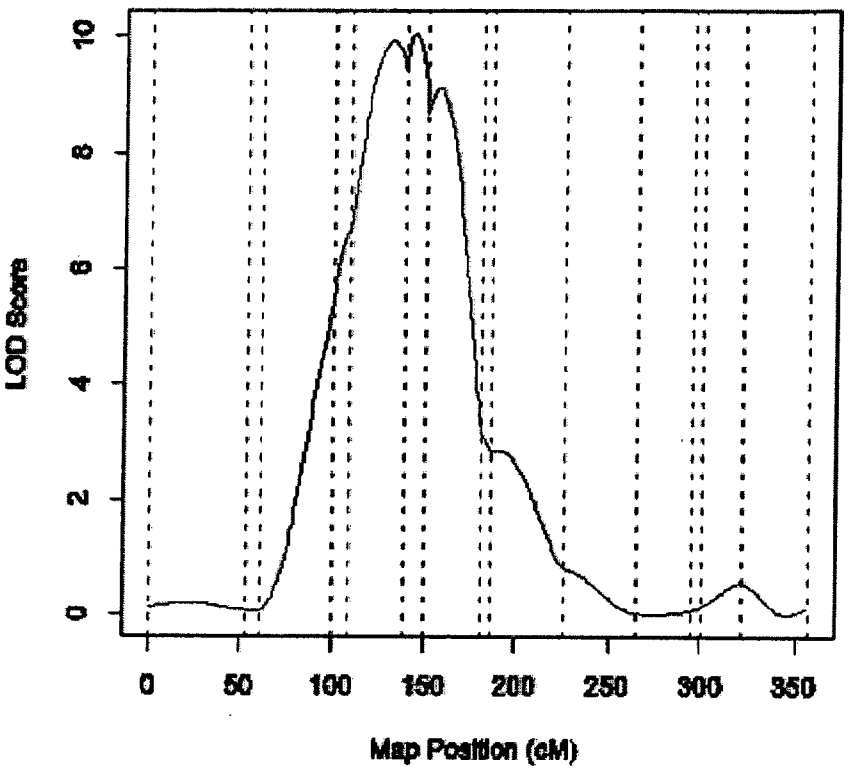


Figure 2K

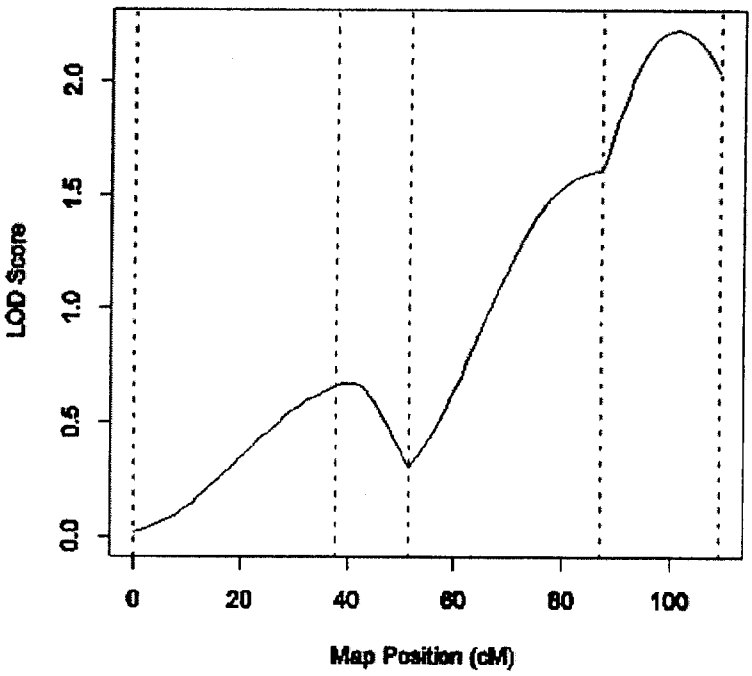


Figure 2L

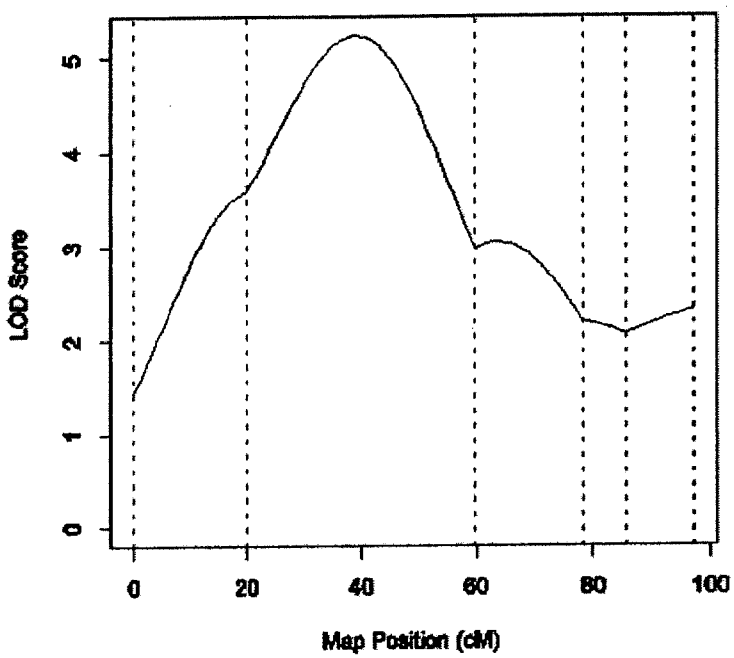


Figure 2M

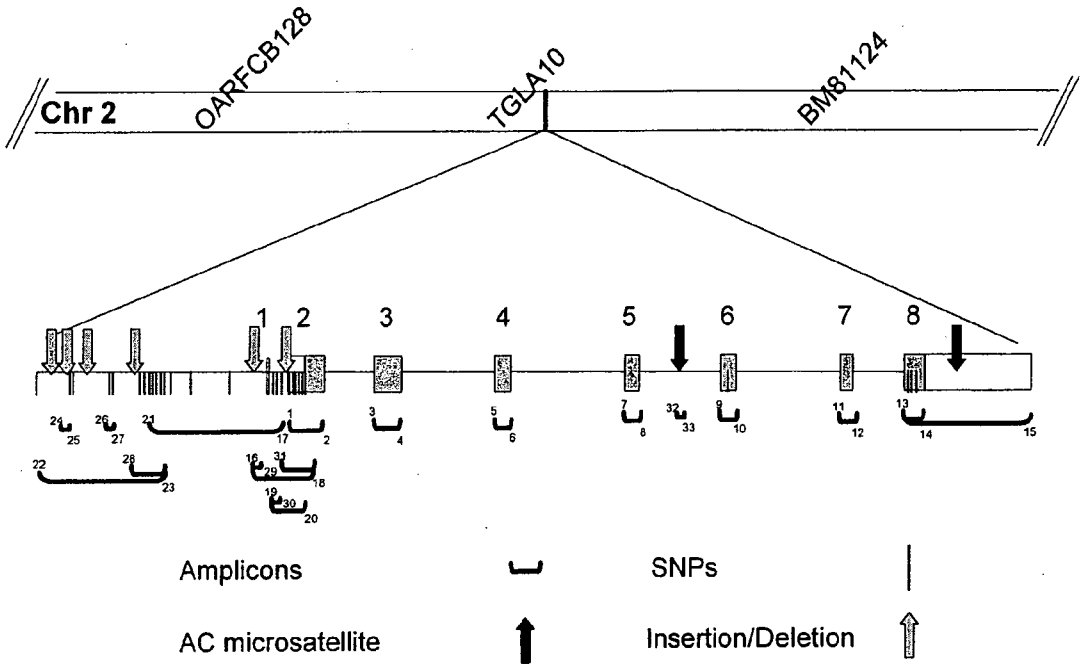

Chr 2
OARFCB128
TGLA10
BM81124
1
2
3
4
5
6
7
8
Amplicons
SNPs
AC microsatellite
Insertion/Deletion

Figure 3

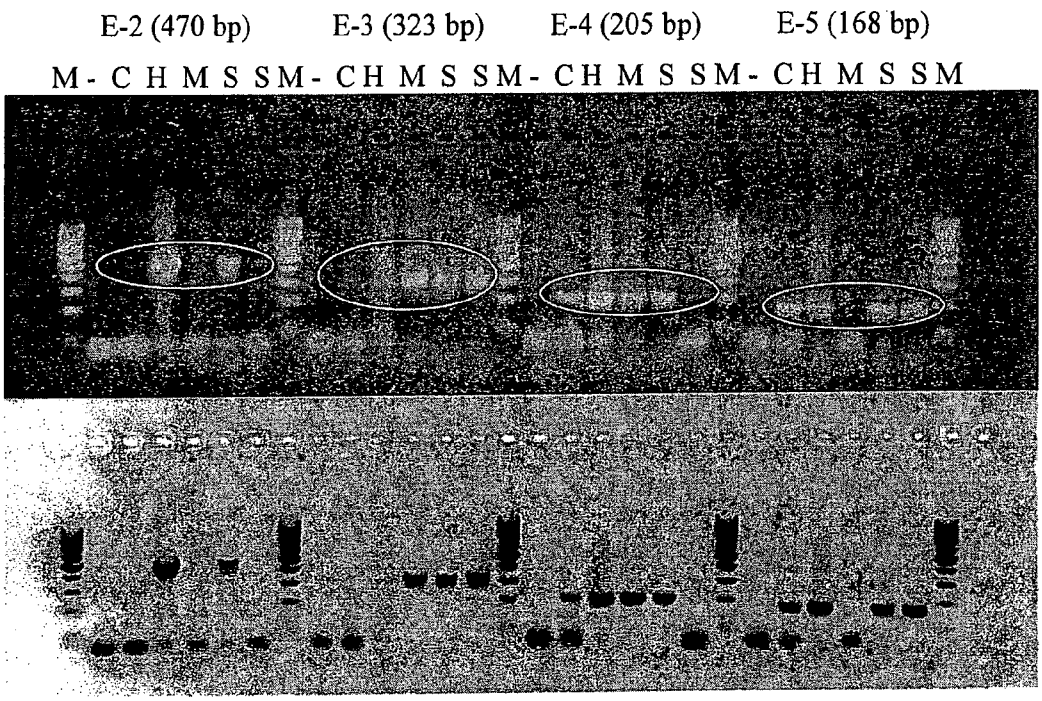

E-2 (470 bp)
E-3 (323 bp)
E-4 (205 bp)
E-5 (168 bp)
M - C H M S S M - C H M S S M - C H M S S M - C H M S S M

Figure 4

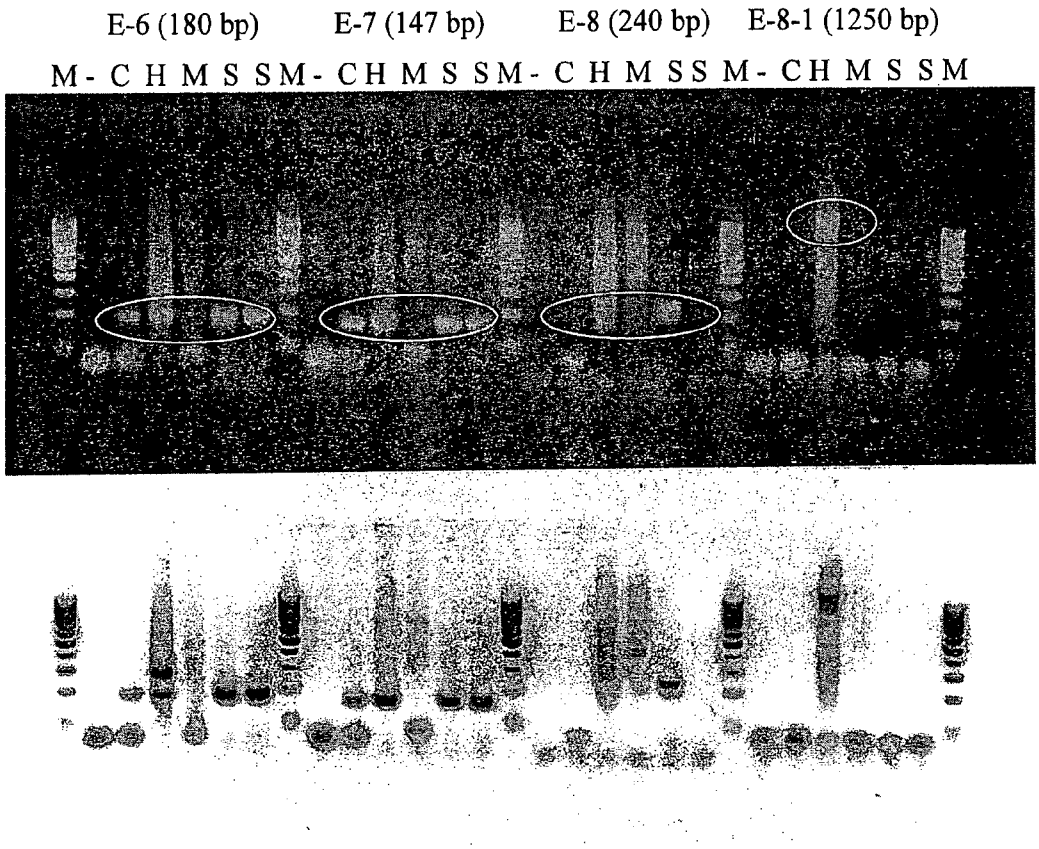
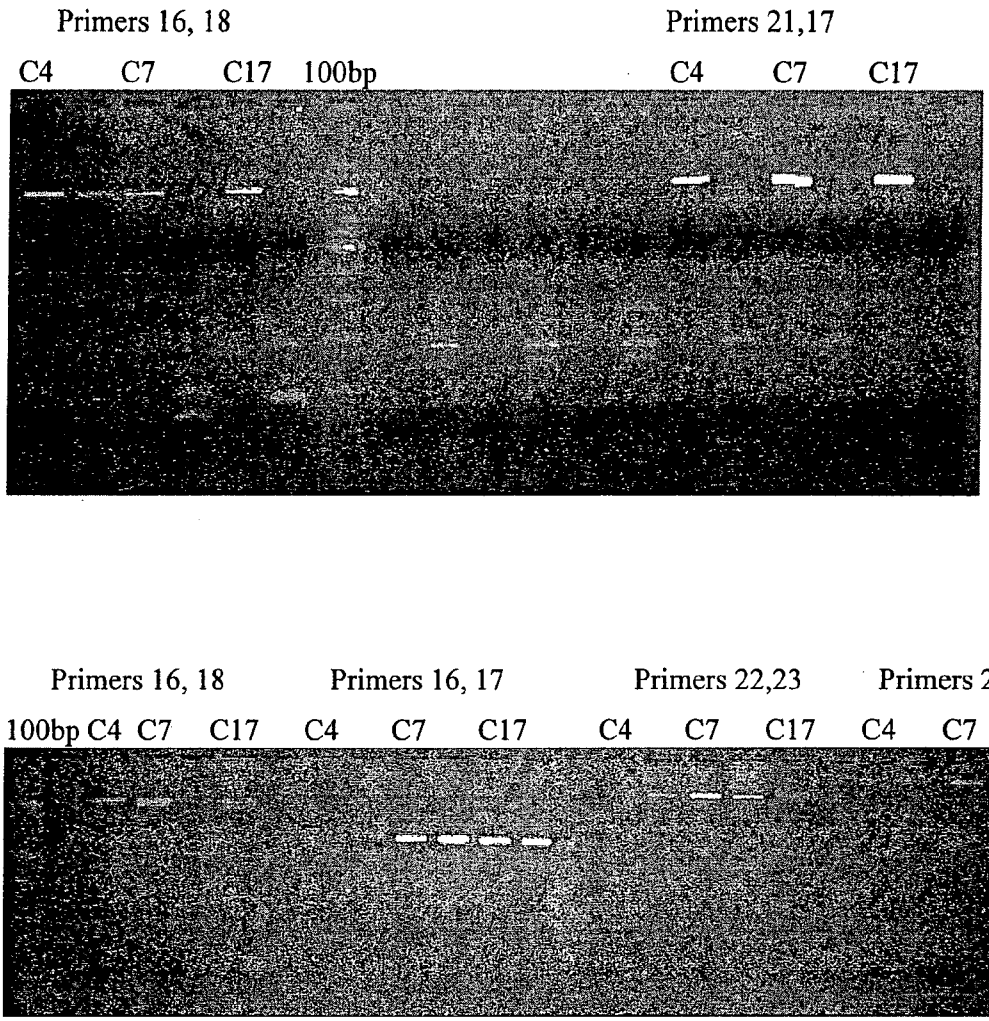

E-6 (180 bp)
E-7 (147 bp)
E-8 (240 bp)
E-8-1 (1250 bp)
M - C H M S S M - C H M S S M - C H M S S M - C H M S S M

Figure 5


Primers 16, 18
Primers 21,17
C4 C7 C17 100bp
C4 C7 C17
Primers 16, 18
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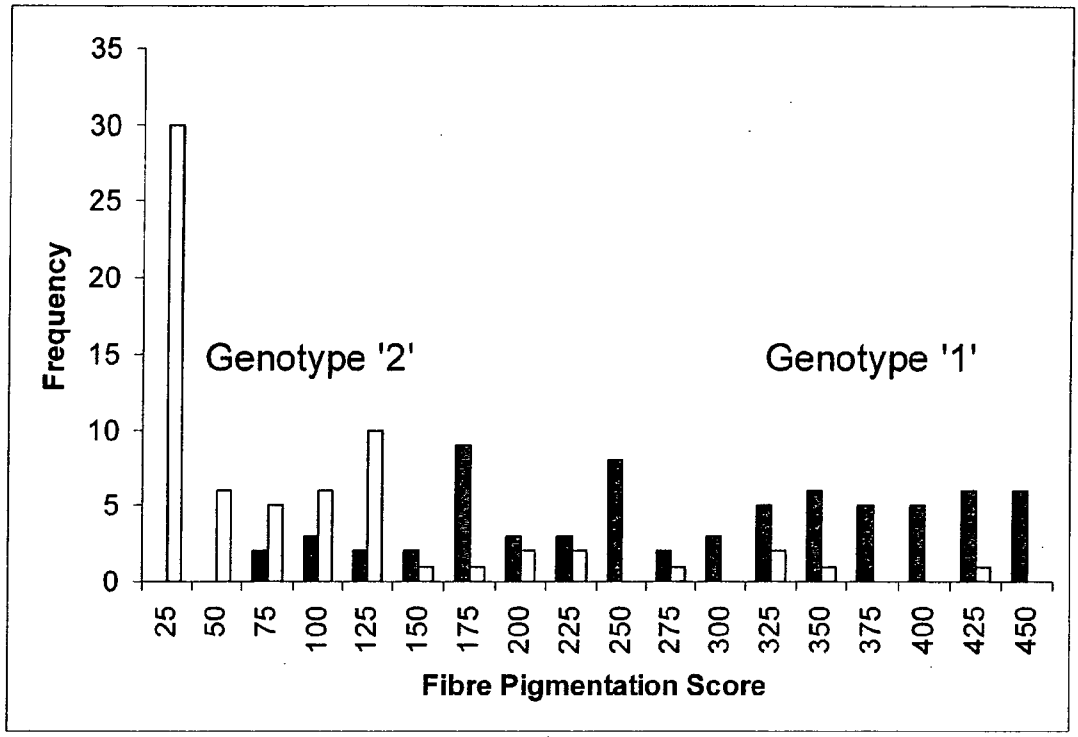
Primers 22,23
Primers 22,17
100bp C4 C7 C17 C4 C7 C17 C4 C7 C17 C4 C7 C17

Figure 6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30
25
20
15
10
5
0
Frequency
Genotype '2'
Genotype '1'
25
50
75
100
125
150
175
200
225
250
275
300
325
350
375
400
425
450
Fibre Pigmentation Score

Figure 7

		Position								
	HapCode	357	604	917	1621	1830	2131	2404	Intron 5	Exon 6	Exon 8 63
C7 haplotypes											
Haplotype C7_M	3	111	123	424	179	127	454	454	302	158	244
Haplotype C7_AW	34	113	326	146	179	347	453	339	300	186	180
Other known haplotypes											
	1	111		424	179	127			295	158	244
	2	111		424	179	127			295	186	180
	4	111		424	179	127			302	158	180
	5	111		424	179	347			302	158	244
	6	111		424	176	127			295	186	180
	7	111		424	176	127			302	158	244
	8	111		424	176	127			302	158	180
	9	111		146	179	127			295	158	244
	10	111		146	179	127			295	158	180
	11	111		146	179	127			302	158	244
	12	111		146	179	347			300	186	180
	13	111		146	176	127			295	158	180
	14	111		146	176	127			295	186	244
	15	111		146	176	127			295	186	180
	16	111		146	176	127			300	158	180
	17	111		146	176	127			300	186	180
	18	111		146	176	127			302	158	244
	19	111		146	176	127			302	186	180
	20	111		146	176	347			295	186	180
	21	111		146	176	347			302	158	244
	22	111		146	176	347			302	186	244
	23	113		424	179	347			295	186	180
	24	113		146	179	127			295	158	180
	25	113		146	179	127			297	186	180
	26	113		146	179	127			298	186	180
	27	113		146	179	127			300	186	244
	28	113		146	179	127			300	186	180
	29	113		146	179	127			302	158	244
	30	113		146	179	127			302	186	180
	31	113		146	179	347			295	186	180
	32	113		146	179	347			300	158	180
	33	113		146	179	347			300	186	244
	35	113		146	179	347			302	158	244
	36	113		146	176	127			295	186	180
	37	113		146	176	127			300	186	180

Figure 8

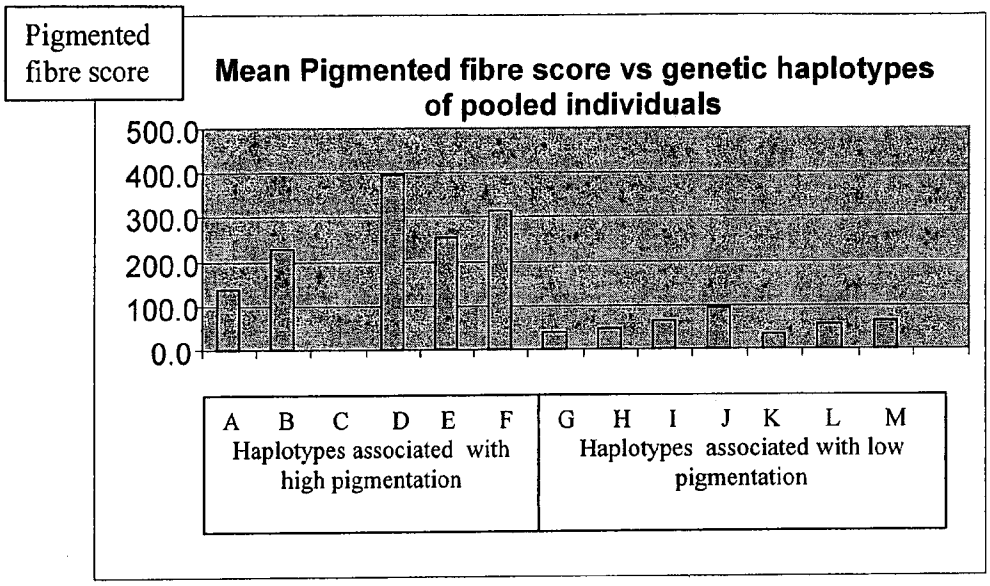
Pigmented fibre score
Mean Pigmented fibre score vs genetic haplotypes of pooled individuals
500.0
400.0
300.0
200.0
100.0
0.0
A B C D E F
Haplotypes associated with high pigmentation
G H I J K L M
Haplotypes associated with low pigmentation

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001070

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 15/11 (2007.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS MEDLINE CAPLUS BIOSIS AGRICOLA (tyrosinase related protein-1, polymorph, SNP, sheep, pigment and other like terms)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Gratten J. *et al.* Compelling evidence that a single nucleotide substitution in TYRP1 is responsible for coat-colour polymorphism in a free-living population of Soay sheep. Proc. R. Soc. B. 2007, vol 274, pages 619-626 See whole document	1-31
P,X	Deng W.D. *et al.* Physiological and genetic characteristics of black-boned sheep (*Ovis aries*). Animal Genetics. 2006, vol 37, pages 586-588 See whole document	1-31
X	Beraldi D. *et al.* Development of a Linkage Map and Mapping of Phenotypic Polymorphisms in a Free-living Population of Soay Sheep (*Ovis aries*). Genetics. 2006, vol 173, pages 1521-1537 See p. 1528, col. 1, ln. 8-11	1-31

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2007	0 3 SEP 2007
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **ANDREW LEECH** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6283 3656

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001070

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Smyth I.M. *et al.* Genomic anatomy of the Tyrp1 (brown) deletion complex. Proc. Natl. Acad. Sci. 2006, vol 103(10), pages 3704-3709 See: p. 3704, Introduction para.; p. 3708, 1st para; and p. 3704, Results and Discussion, 1st para.	1-31
X	Castrinano F. *et al.* SUPREME-Project: Sequence of tyrosinase related protein-1 (TRP-1) in alpaca. EAAP Publication. 2001, vol 105, pages 199-206 See: Abstract; p. 202, 1st para.; and Fig 4	1-31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001070

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 13-18, 25-27 (partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The term "means for" is so broad that a search could not be reasonably conducted, therefore the claims have to be limited to use with a particular marker – partial search

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See Supplemental Box

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. [X] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: III

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

In assessing whether there is more than one invention claimed, I have given consideration to those features which can be considered to potentially distinguish the claimed combination of features from the prior art. Where different claims have different distinguishing features they define different inventions.

This International Searching Authority has found that there are different inventions as follows:

- Claims 1-18 and 25-31 are directed to TYRP1 polymorphisms and associated methods and kits. It is considered that the specified claims comprise a first distinguishing feature.

- Claims 19-24 are directed to TYRP1 polypeptides and polynucleotides *per se*. It is considered that the specified claims comprise a second distinguishing feature.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

The only feature common to all of the claims is TYRP1. However this concept is not novel in the light of:

Beraldi D. *et al.* Genetics. 2006, vol 173, pages 1521-1537

This means that the common feature can not constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since it makes no contribution over the prior art.

Because the common feature does not satisfy the requirement for being a special technical feature it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore the claims do not satisfy the requirement of unity of invention *a posteriori*.