PIGMENT CELL RES 16: 693–697. 2003
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Short Communication

Mapping and Characterization of the Dominant Black Colour Locus in Sheep

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Received 3 April 2003; in final form 4 July 2003

Dominant black pigment synthesis in sheep is caused by alterations of the melanocortin-1 receptor (MC1-R) coding sequence. Using five bovine microsatellite markers we have mapped the sheep *MC1-R* gene to chromosome 14, corresponding to the location in other mammalian species. The existence of two independent mutations, both causing an amino acid substitution, in distantly related breeds of sheep, support the hypothesis that the observed black pigment synthesis is caused by a mutual effect of the two mutations. As similar mutations are found separately at both locations in dominant black variants of other animal species, it is also possible that any of the two mutations could be sufficient for a partial pigment switch.

Key words: Sheep, Coat colour, Melanocortin-1 receptor, Melanocyte-stimulating hormone receptor, Mapping, Evolution

In a number of mammalian species, the α-melanocyte-stimulating hormone (α-MSH) and the melanocortin-1 receptor (MC1-R; MSH-receptor), which is encoded by the extension locus (1, 2), is a major system regulating the synthesis of eumelanin (black–brown pigment) and phaeomelanin (red–yellow pigment) (3). We have previously shown that the dominant black pigment synthesis observed in the Norwegian Dala breed of sheep is caused by mutation(s) in the coding sequence of the *MC1-R* (4). In sheep, this allele is referred to as allele E^D at the extension locus (5). One of the two identified mutations that alters the amino acid sequence (Met → Lys mutation in position 73) was shown to cause constitutive activation of the mouse *MC1-R* in a cell assay. However, the second mutation (Asp → Asn at position 121) is required for high affinity ligand binding (6). Both these mutations were present in individuals of a family of Dala sheep that presented a dominant black phenotype.

The Dala breed was developed between 1860 and 1920 from Cheviot, Leicester and Old Norwegian breeds. Although these animals are normally white there are families in which black individuals are maintained. It is not clear from which breed the dominant black originated, but both Cheviot and Leicester sheep are usually selected for white wool. Dominant black has previously been recognized to exist in a wide range of old European breeds (e.g. Jacob, Black Welsh Mountain, Pialdo Merino, Bizet, Dutch and Zwartbles sheep) and Asiatic breeds including Russian, Mongolian and Karakul sheep (5, 7).

To further characterize the dominant-acting *MC1-R* variant in sheep, other families were studied, in which dominant black appeared to be involved. These include relatively distantly related breeds, the fat tailed Damara, Black Merino and Black Corriedale (Fig. 1).

A research project involving crossbreeding of Merino ewes to two Damara rams (8) produced a family in which half of the progeny were black or black with white spots. This family involved a black ram and the progeny phenotypes indicated heterozygosity for a form of dominant black. Although the Merino ewes were separately mated with each of the Damara rams they gave birth as one group, but lambs were ear-tagged and matched with ewe side brand numbers on a daily basis. In addition to the two Damara rams, 55 Merino ewes that were single-sire mated with the black ram, or that were recorded as producing a black lamb, and their 88 Damara × Merino lambs were examined for mapping the gene.

The fat tailed Damara breed originated from ancient sheep in Egypt (9) and was introduced from South Africa to

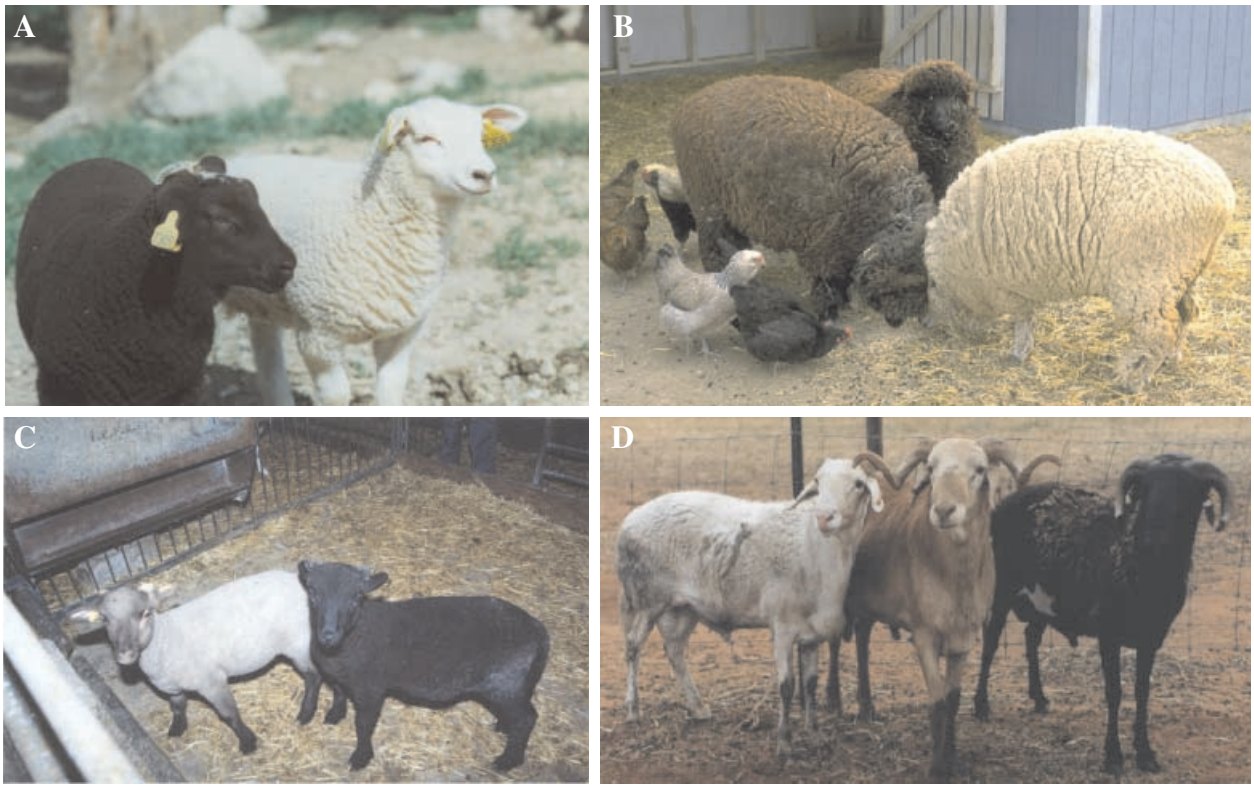

Fig. 1. Illustrations show dominant black and white individuals of: (**A**) Dala, (**B**) Corriedale, (**C**) Merino and (**D**) Damara breeds.

Western Australia in 1996. This breed has a mostly short hair coat that shows a wide range of colours (9) of which the dominant black is studied here. According to Ryder (10), the first evidence of divergence from a hair coat to primitive wool fleeces dates to 1500 BC in Europe and 3000–5000 BC in the Middle East. Around 1000 BC, the advent of dye processes in the Middle East brought about the demand for white wool that prevails to the present.

Black Merino is an ancient breed still remaining in remote areas in central and western Spain and Portugal, where it is preserved in small families. It is supposed to carry the original coat colour of Iberian sheep that has been replaced by white animals as required by wool markets. The Corriedale breed was first developed in New Zealand and Australia during the late nineteenth-century from crosses of Lincoln or other English Longwool breeds with Merino and later established as an important breed in several other countries. In Uruguay, Corriedale is the major sheep breed whereas in Australia the Merino breed prevails.

Apart from the Damara breed, where coloration of the hair coat is unselected or encouraged as part of the breed description, it is usual for individuals of the Dala, Merino and Corriedale breeds that show pigmented wool to be culled from commercial flocks. Therefore, it was relevant to determine whether these various occurrences of dominant black pigment arise from the same mutation(s) that could suggest preservation of the ancient variant rather than independent recent mutation, which makes a comparative study of great interest.

The bovine *MC1-R* has been mapped to chromosome 18 where close linkage to microsatellites TGLA357 and INRA121 was shown (11). Five microsatellites were selected for mapping the sheep homologous region (BMS1355, TGLA357, BMS2355, BMS2213 and INRA121; Table 1). These microsatellites cover a region of approximately 25 cM that spans the *MC1-R* region in cattle (see http://sol.marc.usda.gov/). Although four microsatellites showed polymorphism in the material (all except INRA121), thereby verifying the highly conserved nature of microsatellites in cattle and sheep (12–14), only a single marker, BMS2355, was heterozygous for the black Damara ram used in this study. Information from a second highly polymorphic marker, TGLA357, was used for verifying the paternity. Nine alleles were observed for BMS2355, and among 74 informative offspring, recombination between BMS2355 and black coat colour/*MC1-R* was observed in 13 samples, indicating a distance of approximately 17.6 cM with 7.34 in lod score (15). This result places the locus encoding the *MC1-R* at homologous regions in sheep (chromosome 14) and cattle, where the gene is located to chromosome 18 (11), as well as in other mammalian species (16).

In most of the mammalian species where the *MC1-R* has been studied at the molecular level, a single mutation (causing an amino acid substitution) explains the dominant production of eumelanin or black pigment (16). In this study, we found the same mutations in the Damara breed that have previously been found in Norwegian Dala sheep (4), thereby supporting the proposed preservation of the two separate

Table 1. Primers and annealing temperatures used in the study

Markers	Primer sequences 5′ → 3′	Temperature (°C)	References
BMS1355	F: TAA AAC CCC AAA AAG AAC CC R: ATA TTT GCG ACA TTG GAT GAA	56	24
BMS2213	F: ATG GGC AGC TTA GGG ATT G R: CTT CAA GAG CCT TCA GTG GG	66	24
BMS2355	F: TAT GAA GAG GAA TGA AGG GAG A R: CAT TTT CAA TGT GAG AGT GTC AA	55	25
INRA121	F: GGA AAC CCA TTG GAG GAT TTG R: CTT CAC TAT TCC CCA CAA AGC	62	26
TGLA357	F: CGA GAG TCT GAG TTT AAA CTT CTC TAA CAC C R: GAG GGC AAA AAG GTT TGG GGT GTA TGG	69	27
MAF18	F: GTA TAC TGC CTC TCT GCG TGA TGG G R: TTT TCC TTC AGA GCC AAG AGG ACA AG	63	28
MAF50	F: GTA GAC TAC TCA TGA AAA TCA GGT CTT AGG R: GGG ACA TGC AGC TAT ACA CTT GAG	61	29
MCM527	F: GTC CAT TGC CTC AAA TCA ATT C R: AAA CCA CTT GAC TAC TCC CCA A	60	30
OARCP125	F: GCA AAT AGC CTC TTG TAT GAT C R: ACC AAA ACA AGA CCT TTA TTT TTC ATG G	63	17
OARFCB20	F: GGA AAA CCC CCA TAT ATA CCT ATA CG R: AAA TGT GTT TAA GAT TCC ATA CAT GTG	60	31
Oar JMP8	F: CGG GAT GAT CTT CTG TCC AAA TAT GC R: CAT TTG CTT TGG CTT CAG AAC CAG AG	63	17
E3 (MC1-R)	F: GTG CCT GGA GGT GTC CAT C		4
E4 (MC1-R)	R: AAG CAG AGG CTG GAC ACC AT	60	
E8 (MC1-R)	R: GGC CAG GAA GAG GTT GAA G	59	

Amplification of sheep microsatellites was based on bovine sequences following standard protocols. Amplification of melanocortin 1-receptor (MC1-R) coding sequence was performed as previously described by Våge et al. (4). Briefly, the Met-73-Lys RFLP were performed with primers E3–E4 followed by *Nla*III digestion, whereas the Asp-121-Asn RFLP were analysed with primers E3–E8 followed by digestion with *Mse*I.

mutations in a wide variety of sheep breeds expressing the dominant black coat colour. The Met → Lys mutation at position 73 and the Asp → Asn mutation at position 121 were present in the black Damara ram and all of the black lambs sired by that ram (36 black lambs were analysed), while the 55 white Merino ewes all contained the wild-type sequence (Fig. 2A). The mutation at amino acid codon 73 is located on the inside boundary of transmembrane domain 2 of the *MC1-R*, whereas the 121 mutation is located on the outside boundary of transmembrane domain 3.

Identical results were shown for the two other distant breeds, Black Merino in Spain and Black Corriedale in Uruguay. The studied group of Black Merino sheep, consisting of two rams and 11 ewes, all of which were completely black, was sent to the Faculty of Veterinary of Zaragoza (Spain) in 2000, where they were isolated before mating. The two rams were successfully mated with five ewes (Fig. 2C). Unexpectedly, during the January 2001 lambing season, two of the five newborns were white. Paternity of the mentioned lambs was verified by means of OarFCB20,

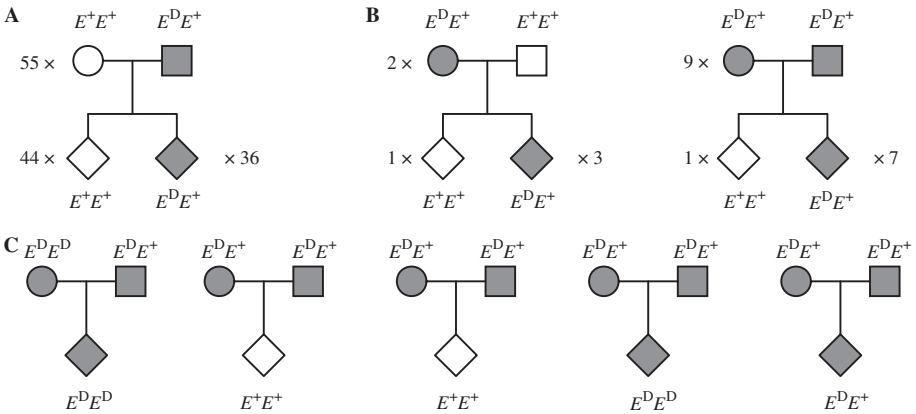

Fig. 2. Pedigree schemes for breeds included in the study. Sex is not specified among the lambs. (**A**) Damara: 55 white Merino ewes produced the lambs from the black Damara ram segregating dominant black. All 36 black Damara × Merino lambs was identified to have the E^DE^+ genotype, whereas 44 white lambs were E^+E^+. (**B**) Corriedale: two flocks were used in the study. One white ram was mated with two E^DE^+ black ewes (first pedigree) giving birth to one white (E^+E^+) and three black E^DE^+ lambs. In the second pedigree, a black E^DE^+ ram was mated with nine black E^DE^+ ewes. In these matings, eight lambs were produced. One lamb was white (E^+E^+) and the rest were E^DE^+ black. (**C**) Merino: two black E^DE^+ rams were mated with five black ewes, one homozygous and four heterozygous. Two white lambs were produced and three black, of which two were homozygous.

MCM527, MAF18 and MAF50 and the existence of the above-mentioned mutations for *MC1-R* was then investigated in the complete flock (including newborns). The PCR-RFLP results were completely consistent with the findings of Våge et al. (4), and both mutations appeared to be in complete linkage with the dominant black phenotype. Nucleotide sequence analysis of *MC1-R* confirmed the RFLP results. The two rams and 13 of the ewes (including those producing the white lambs) were found to carry the *MC1-R* mutations in heterozygous conditions.

For the third breed involved in the study, two Corriedale rams (one white and one black) were mated with 11 black Corriedale ewes at the Experimental Station of the Veterinary Faculty in Uruguay, producing 12 lambs. Two microsatellites (Oar JMP8 and Oar CP125) were used to identify paternity (17). Moreover, for these families (Fig. 2B), the results are in complete agreement with those previously described. The 11 black individuals were all heterozygous for the *MC1-R* mutations, whereas for the ram and nine lambs with white colour these mutations were absent.

Black coat colour can be independently determined in a recessive manner in various mammalian species (16). Individuals expected to be recessive black from the Spanish Rasa Aragonesa breed, the synthetic Salz breed (from crosses between Romanov and Rasa Aragonesa 30 yr ago) and the Norwegian Pelt Sheep were also tested in this study. For these individuals that express the recessive black phenotype alterations in the *MC1-R* gene were not observed.

In flocks composed entirely of white sheep, black lambs continue to arise because of the present inability of breeders to distinguish carriers of the recessive variation involved except from the production of a black lamb. This limitation represents an important opportunity for the development of DNA diagnostic technologies of commercial importance to the white sheep and wool industries. In Merino sheep, the recessive black phenotype was mapped to ovine chromosome 13 (18). While the *Agouti* gene is the primary candidate for recessive black in sheep, the sequence and limited hybridization studies that were undertaken did not reveal the DNA variation responsible (19).

Both mutations that are found in breeds of sheep that express a dominant black coat colour are located within amino acid codons that are completely conserved in mammalian species that do not express a dominant black phenotype (6), except for a neutral mutation in the reindeer (20). Therefore, these two positions could be essential for normal receptor activity. Interestingly, both mutations occur at positions that are involved in altered pigment synthesis in other species. The pig mutation causing an Asp → Asn substitution at position 121 is identical to one of the sheep mutations, and is sufficient for black pigment production in the pig (21). At position 73, a Met → Thr substitution is found in the black chicken (22), compared with the Met → Lys substitution in sheep. Both threonine (aliphatic hydroxyl side chain) and lysine (basic side chain) have altered features relative to the sulphur-containing methionine found in the wild-type receptor at this position. Therefore, we conclude that the Sheep E^D variant of the *MC1-R*, containing two amino acid substitutions, is probably a strong dominant acting variant of the receptor producing black pigment, exclusively. The existence of these two mutations in a number of breeds that are all distantly related could be explained by the relatively short history of domestication in evolutionary terms. As mutations at both locations are found in dominant black variants of other animal species, any of the two mutations could be sufficient for black pigment synthesis. Alternatively, each of these mutations could produce a weaker activation of the receptor similar to the one found in Alaska silver fox. In this case, the constitutively active E^A mutation does not produce entirely black animals unless functional agouti signal protein is lacking or the E^A mutation is found in both alleles (23).

Acknowledgements – Dr Bob Holloway and Mark Bennie at SARDI's Minnipa Agricultural Centre are thanked for resources and management of the Damara crossbreeding project. Dr I. Sierra at the Veterinary Faculty of Zaragoza is thanked for access to Salz and Black Merino flocks. Dr Aldo Calliari and Dr R. Kremer at the Department of Sheep of Uruguayan Veterinary Faculty are acknowledged for providing samples for the first pedigree and for the management of the Corriedale animals. Picture of the black and white Corriedale sheep (Fig. 1B) was kindly provided by Joanna and Keith Gleason.

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