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Exclusion of *Melanocortin-1 Receptor* (*Mc1r*) and *Agouti* as Candidates for Dominant Black in Dogs

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Abstract

The domestic dog exhibits a variety of coat colors that encompass a wide range of variation among different breeds. Very little is known about the molecular biology of dog pigmentation; current understanding is based mostly on traditional breeding experiments, which in some cases have suggested genetic interactions that are different from those reported in other mammals. We have examined the molecular genetics of dominant black, a uniform coat color characteristic of black Labrador retrievers or Newfoundlands that has been proposed to be caused by either variation in the *melanocortin-1 receptor* gene (*Mc1r*) or by variation in the *Agouti* gene (*A*). We identified several coding polymorphisms within *Mc1r* and several simple sequence repeat polymorphisms closely linked to *A*, and examined their inheritance in a Labrador retriever × greyhound cross that segregates dominant black. No single *Mc1r* allele was found consistently in animals carrying dominant black, and neither *Mc1r* nor *A* cosegregated with dominant black. These results refine our understanding of mammalian coat color inheritance and suggest that dominant black coat color in dogs is caused by a gene not previously implicated in pigment type switching.

The study of coat color variation in mammals has resulted in the identification of many genes required for melanocyte development, migration, and regulation. A variety of pigmentation patterns are created by differential production of two types of pigment: pheomelanin, which is usually yellow or red, and eumelanin, which is usually black or brown. Individual melanocytes can switch between the synthesis of eumelanin and pheomelanin in response to a paracrine signaling molecule, Agouti protein, and a seven transmembrane domain receptor expressed by hair follicle melanocytes, melanocortin-1 receptor (*Mc1r*).

Mc1r is coupled to adenylate cyclase and its activation causes accumulation of cAMP and promotes eumelanin instead of pheomelanin synthesis. Agouti protein inhibits *Mc1r* activation, and production of Agouti protein therefore causes hair follicle melanocytes to produce pheomelanin instead of eumelanin (Lu et al. 1994; Ollmann et al. 1998). In mice, gain-of-function *Agouti* mutations or loss-of-function *Mc1r* mutations give rise to animals with pheomelanic coats, whereas loss-of-function *Agouti* mutations or gain-of-function *Mc1r* mutations give rise to animals with eumelanic coats. *Mc1r* mutations are epistatic to *Agouti* mutations; thus mice carrying gain-of-function mutations in both *Agouti* and *Mc1r* produce eumelanic coats, while those carrying loss-of-function mutations in both *Agouti* and *Mc1r* produce pheomelanic coats (reviewed in Silvers 1979).

Gain-of-function *Mc1r* mutations have also been described in cattle, sheep, foxes, and pigs, and produce a uniform black coat color transmitted in a dominant manner (Kijas et al. 1998; Klungland et al. 1995; Våge et al. 1997, 1999). Dominant black also exists in the domestic dog, but its genetic basis is unclear. Based on pedigree and segregation studies carried out several decades before *Agouti* and *Mc1r* were identified at the molecular level, C. C. Little proposed that dominant black was one of a series of *Agouti* alleles, named A^s, along with a^y (sable/tan) and a^t (tan points), with a dominance hierarchy of $A^s > a^y > a^t$. Little (1957) also proposed that production of different pigment types was controlled by allelic variation at the *Extension* (*E*) locus, in which four alleles, E^m (mask), E (wild type), e^{br} (brindle), and e

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(red-yellow), exhibited a dominance hierarchy of $E^m > E > e^{br} > e$, and in general were epistatic to *Agouti* alleles (an exception was the proposal that A^S would be epistatic to e^{br}) (Little 1957).

Previous work from our laboratory demonstrated that *Mc1r* is the *Extension* gene and that a nonsense mutation, R306ter, was responsible for the *e* mutation in a number of red or pale-colored dogs including yellow Labrador retrievers, Irish setters, golden retrievers, and Samoyeds. We also identified an *Mc1r* polymorphism that was a candidate for dominant black, S90G, because it was found in Newfoundlands, flat-coated retrievers, and black Labrador retrievers, but not in golden retrievers or Irish setters; however, the absence of pedigree analysis prevented a more direct test for allelism (Newton et al. 2000).

Here we report a more extensive study of the genetics of pigment type switching in dogs using multiple molecular markers for both *Mc1r* and *Agouti* to carry out a haplotype analysis of a large Labrador retriever × greyhound cross. No single *Mc1r* haplotype or variant was found consistently in animals carrying dominant black, and neither *Mc1r* nor *A* cosegregated with dominant black. These results refine our understanding of mammalian coat color inheritance and suggest that dominant black coat color in dogs is caused by a gene not previously implicated in pigment type switching.

Results and Discussion

In our initial study of *Mc1r* variation in dogs, we determined the complete coding sequence in black and yellow Labrador retrievers, Newfoundlands, golden retrievers, Irish setters, flat-coated retrievers, and Doberman pinschers. Each of these breeds is thought to be fixed for dominant black except for the Doberman. A pale or pheomelanin-colored coat in, for example, yellow Labrador retrievers, golden retrievers, and Irish setters, is caused by homozygosity for the Mc1r R306ter allele, with a genotype referred to as $A^S/A^S\ e/e$ in the original notation used by Little. Thus the presence of dominant black in pedigrees becomes apparent when an animal of genotype $A^S/A^S\ E/E$ (black Labrador retrievers, Newfoundlands, flat-coated retrievers) or $A^S/A^S\ e/e$ (yellow Labrador retrievers, golden retrievers, or Irish setters) is crossed to an animal of genotype $a^y/a^y\ E/E$ (e.g., a tan Basenji) or $a^t/a^t\ E/E$ (e.g., a black-and-tan Doberman), yielding litters where all progeny are completely black.

These criteria fit those of a cross established at Cornell by Lust and colleagues to study hip dysplasia, in which black or yellow Labrador retrievers were crossed to greyhounds and their progeny backcrossed to a greyhound parent (Todhunter et al. 1999). Greyhounds can be fawn, yellow, red, brindle, or masked; Little (1957) suggested that the uniform pheomelanic appearance (fawn, yellow, or red) could be caused by either *Agouti* (a^y/a^y) or *Extension*, that is, *Mc1r* (*e*/*e*); however, as described below, we did not find the Mc1r R306ter allele in any of the greyhound samples we examined.

Informative kindreds from this cross are depicted in Figure 1, in which a total of 18 F_1 progeny were produced from three sets of Labrador retriever × greyhound matings; all progeny are completely black. In two of the crosses, the Labrador retriever parent was yellow and homozygous for the Mc1r R306ter allele, yielding F_1 black offspring whose genotype in the original notation is $A^S/a^y\ E/e$ (Figure 1B,C). A single black F_1 animal from each mating was then backcrossed to a greyhound (sometimes from a different pedigree), yielding a total of 21 BC_1 progeny, of which 10 were completely black and 11 were nonblack. These observations conform to Mendelian expectations for a locus that controls uniform black coloration in which the Labrador retriever parents are homozygous for a "black" allele and the greyhound parents are homozygous for a "nonblack" allele, with the Labrador retriever allele dominant to the greyhound allele, and the Labrador retriever allele hypostatic to the Mc1r R306ter allele.

Coat color phenotypes of nonblack progeny in the pedigrees depicted in Figure 1 include yellow, red, reddish-brown, and brindle. We cannot exclude the possibility that some of these animals carried a dominant black allele whose effects are not apparent. For example, the gene or genes responsible for brindling may be epistatic to those responsible for dominant black; also, mutations that impair but do not abolish eumelanin synthesis, such as *Tyrp1*, yield a brown coat color that may be difficult to distinguish from a reddish-brown pheomelanic coat color (Schmutz et al. 2002). Thus, for purposes of evaluating specific loci as candidates, we treated the dominant black allele inherited from Labrador retrievers as potentially nonpenetrant in greyhound backcross progeny, and considered genotypes of the backcross progeny only for black animals.

Like most seven transmembrane receptors, the *Mc1r* coding sequence is contained within a single exon. We polymerase chain reaction (PCR) amplified a fragment of genomic DNA that contained this exon and determined the complete *Mc1r* coding sequence for all black animals and all parental animals except one of the greyhound parents (Isis, Figure 1B). We identified four single-nucleotide variants that alter protein coding sequence, S90G (AGC→GGC), A105T (GCC→ACC), M264V (ATG→GTG), and R306ter (CGA→TGA); all but the M264V variant were identified in our previous survey. As described above, we had previously considered S90G as a candidate for dominant black because it was found in Newfoundlands, flat-coated retrievers, and Labrador retrievers, but not in golden retrievers or Irish setters. However, of the 16 black animals whose genotypes we determined, 10 did not carry S90G, including 1 black Labrador retriever (B53, Figure 1A) and 1 yellow Labrador retriever (A14, Figure 1C), therefore the S90G variant probably has no functional significance.

As a more rigorous approach to determine if a specific *Mc1r* allele might cause dominant black, we first used pedigree information to determine *Mc1r* haplotypes, then asked whether black backcross progeny had inherited a consistent *Mc1r* allele from their Labrador retriever grandparent. The four coding sequence variants were found in seven different haplotype combinations (Table 1). In each kindred we found one or more animals that failed to inherit any *Mc1r* allele from

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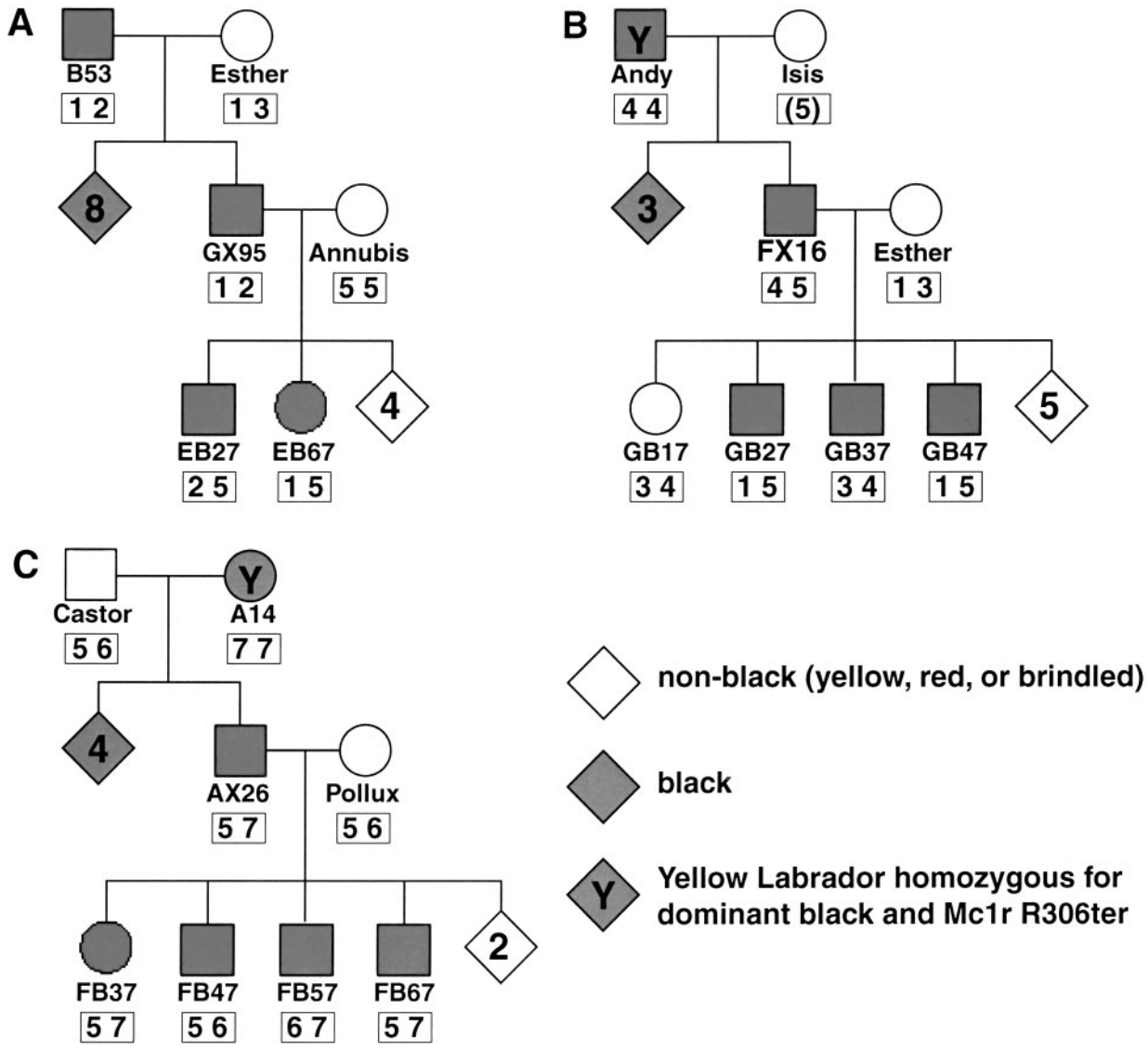

Figure 1. Segregation of coat color phenotypes and *Mc1r* sequence variants. The haplotypes 1–7 (Table 1) are numbered arbitrarily and are indicated below each animal in a box. Haplotypes shown in parentheses were inferred from pedigree analysis.

their Labrador retriever grandparent: EB67 (Figure 1A), GB27 and GB47 (Figure 1B), and FB47 (Figure 1C). Thus dominant transmission of a uniform black coat color cannot be explained by an *Mc1r* allele in any of the kindreds.

We also determined the complete coding sequence for *Agouti* in 14 animals (Figure 2); however, no sequence variants were identified (data not shown). To analyze inheritance of different *Agouti* alleles, we identified three microsatellite markers contained on the same bacterial artificial chromosome clone as the dog *Agouti* gene, genotyped a large series of animals (Figure 2), then, as for *Mc1r*, asked whether black backcross progeny had inherited a consistent *Agouti* allele from their Labrador retriever grandparent. Alleles for the three microsatellite markers were found in five different haplotypes (Table 2). In one of the kindreds, FX16 × Ester, none of the four black backcross

Table 1. *Mc1r* haplotypes

Haplotype[a]	S90G[b]	A105T	M264V	R306ter
1	S	A	M	R
2	S	T	M	R
3	G	A	V	R
4	G	A	M	ter
5	S	A	V	R
6	G	A	M	R
7	S	A	M	ter

[a] Haplotypes are numbered arbitrarily and correspond to the symbols listed in Figure 1. Haplotypes were determined by considering all genotypes independently for the pedigrees depicted in Figure 1 and assuming an absence of crossing over.

[b] *Mc1r* coding sequence variants are referred to by their effect on coding sequence; nucleotide sequences are given in the text.

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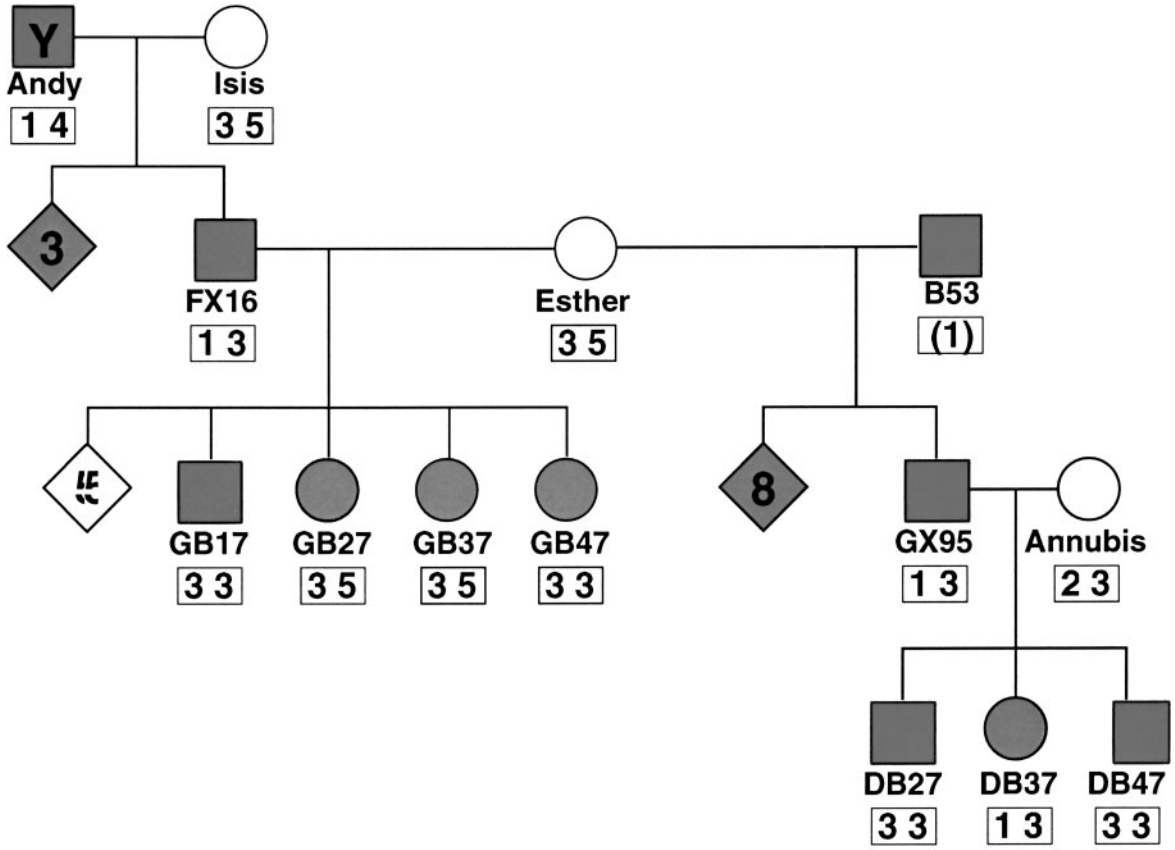

Figure 2. Segregation of coat color phenotypes and *Agouti* sequence variants. The haplotypes 1–5 (Table 2) are numbered arbitrarily; symbols are the same as in Figure 1.

progeny inherited an *Agouti* allele from their Labrador retriever grandparent, Andy (Figure 2). In the second kindred, Ester × B53, two of three black backcross progeny, DB27 and DB47, failed to inherit an *Agouti* allele from their Labrador retriever grandparent, B53 (Figure 2). Thus dominant transmission of a uniform black coat color cannot be explained by an *Agouti* allele.

Our findings provide compelling evidence to exclude *Agouti* and *Mc1r* as the genes responsible for dominant black in the Cornell Labrador retriever × greyhound cross. In mice, three additional classic mutations affect pigment type switching: *Ragged*, *mahogany*, and *mahoganoid*; *Ragged* and *mahogany* have been identified at the molecular level (and are now known as *Sox18* and *Attractin*, respectively) (Gunn et al. 1999; Pennisi et al. 2000). However, each of these genes has nonpigmentary effects, and therefore is unlikely to be responsible for dominant black. Assuming that dominant black is caused by a single locus, the Cornell cross should provide sufficient resolution to identify that locus by linkage using a genomewide panel of microsatellite markers. Molecular identification of dog dominant black will bring unique insight to the biochemistry of pigment type switching and Agouti-melanocortin signaling, since the same gene is apparently not represented in other model organisms.

Table 2. *Agouti* haplotypes

Haplotype[a]	DBar1[b]	DBar2	DBar3
1	4	1	2
2	3	3	1
3	1	2	1
4	2	4	2
5	3	2	1

[a] Haplotypes are numbered arbitrarily and correspond to the symbols listed in Figure 2. Haplotypes were determined by considering all genotypes independently for the pedigrees depicted in Figure 2 and assuming an absence of crossing over.

[b] *Agouti* sequence variants are described in Materials and Methods; different alleles are numbered arbitrarily.

Materials and Methods

DNA Samples

Frozen leukocytes from a pedigree involving a cross between Labrador retrievers and greyhounds were kindly provided by George Lust and Michael Olivier from the Cornell Veterinary School, Ithaca, NY. DNA was isolated using a standard low-salt/phenol/chloroform DNA extraction protocol. Pedigree structures depicted in Figures 1 and 2

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were verified by demonstrating that multiple unlinked microsatellite markers in addition to *Agouti* and *Mc1r* exhibited Mendelian segregation in accordance with expectations.

Mc1r Sequence Analysis

The entire coding sequence of *Mc1r* was amplified using the following primers: (F: 5′-GGTCATTGCTGAGCTGACAC-3′) and (R: 5′-GAGATGCTGTCCAGTAGTCTCCC-3′) and the following conditions: 40 cycles of 30 sec denaturing at 94°C, 1 min annealing at 60°C, and a 2-min extension at 72°C (Newton et al. 2000). The 1.3 kb amplicon was purified using Qiagen column purification and directly sequenced using ABI technology. The sequences were analyzed using Polyphred to detect polymorphisms.

Agouti Sequence Analysis

A bacterial artificial chromosome clone that contains the dog *Agouti* locus was isolated and sequenced; details will be presented elsewhere. We identified three microsatellites within the BAC sequence as follows:

D*Bar*1 (CTTTT)n (231bp): forward: 5′[5HEX]CATGATCCTGGTCCCACGTA; reverse: 5′-TGTTTCAATGTGCTATGCACTG-3′.

D*Bar*2 (CA)n (150bp): forward: 5′[5Hex]-TCCGGATACTGTTATCTCCCAT; reverse: 5′-CCAATTGGCTGTGTACCTCA-3′.

D*Bar*3 (CTT)n . . . (CA)n (300bp): forward: 5′-GCATCCCCTTCCTCATCTAA; reverse: 5′-TTCCCCGTCCCTTTAATAAT-3′.

The PCR conditions for each marker were 35 cycles at 94°C for 30 sec, 55°C for 30 min, and 72°C for 30 min.

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