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Association of an Agouti allele with fawn or sable coat color in domestic dogs

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Abstract

The type of pigment synthesized in mammalian hair, yellow–red pheomelanin or black–brown eumelanin, depends on the interaction between Agouti protein and the Melanocortin 1 receptor. Although the genetics of pigmentation is broadly conserved across most mammalian species, pigment type-switching in domestic dogs is unusual because a yellow–tan coat with variable amounts of dark hair is thought to be caused by an allele of the *Agouti* locus referred to as *fawn* or *sable* (a^y). In a large survey covering thirty seven breeds, we identified an *Agouti* allele with two missense alterations, A82S and R83H, which was present (heterozygous or homozygous) in 41 dogs (22 breeds) with a fawn or sable coat, but was absent from 16 dogs (8 breeds) with a black-and-tan or tricolor phenotype. In an additional 33 dogs (14 breeds) with a eumelanic coat, 8 (German Shepherd Dogs, Groenendaels, Schipperkes, or Shetland Sheepdogs) were homozygous for a previously reported mutation, *non-agouti* R96C; the remainder are likely to have carried dominant black, which is independent of and epistatic to *Agouti*. This work resolves some of the complexity in dog coat color genetics and provides diagnostic opportunities and practical guidelines for breeders.

Introduction

Shades and patterns of coat color are a remarkably diverse trait among domestic dogs, with certain breeds exhibiting a specific phenotype, and other breeds exhibiting one of several phenotypes. For example, Rhodesian Ridgebacks typically occur in various shades of yellow–tan, usually referred to as fawn. By contrast, a phenotype shared across several breeds, including the Doberman Pinscher, the Rottweiler, some Dachshunds, and many Spaniels, is the black-and-tan pattern, in which a black background is marked by discrete and regular areas of reddish yellow around the ears, supraorbital region, lower face, chest, inner aspects of the proximal limbs, and dorsal aspects of the distal limbs. All of these phenotypes are thought to be controlled by variation of the *Agouti* gene.

Agouti encodes a paracrine signaling molecule which causes hair follicle melanocytes to synthesize reddish yellow pheomelanin instead of black or brown eumelanin, so-called pigment type-switching (Bultman et al. 1992; Miller et al. 1993; reviewed in Barsh et al. 2000). In laboratory mice, variation in pigment type-switching is brought about via two sets of *Agouti* mRNA isoforms that differ by virtue of their untranslated first exons and associated promoter activity (Vrieling et al. 1994; Chen et al. 1996). ''Ventral-specific'' isoforms are expressed throughout the anagen phase of hair growth, but only in ventral skin, and are responsible for the characteristic pale yellow appearance of ventral hairs. By contrast, ''hair cycle-specific'' isoforms are expressed throughout the body but only for a brief period during early anagen, such that individual hairs have a subapical band of yellow pigment on a black background. Both isoforms are expressed in the mouse *white-bellied Agouti* (A^W) allele, which causes banded hairs on the dorsum together with pale yellow hairs on the ventrum. Agouti protein is an inhibitory ligand for the Melanocortin 1 receptor (Mc1r), a seven-transmembrane protein expressed on melanocytes whose activation leads to increased production of cAMP (Lu et al. 1994; Ollmann et al. 1998). In

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mice and many other domestic animals, gain-of-function *Mc1r* alleles have an effect similar to decreased expression of Agouti protein, promoting eumelanin instead of pheomelanin synthesis; conversely, loss-of-function *Mc1r* alleles have an effect similar to increased expression of Agouti protein, promoting pheomelanin instead of eumelanin synthesis (Robbins et al. 1993; reviewed in Barsh 1996; Klungland and Vage 2003).

In canids, both Agouti protein and the Mc1r are thought to function like their mouse homologs, but the genetics are more complex. Like rodents, the ancestral canine *Agouti* allele is probably a^w (historically, the dog allele has been referred to as a^w instead of A^W), readily apparent in some wolves, coyotes, and other carnivores, but among domestic dog breeds a phenotype similar to the white-bellied Agouti phenotype of mice with banded dorsal hairs and a pale ventrum is apparent in only a few instances, such as some German Shepherd Dogs or Siberian Huskies (Little 1957; Searle 1968; Willis 1989). Among dogs with a uniform reddish-yellow coat, some, such as Irish Setters, yellow Labrador Retrievers, and Golden Retrievers, have a loss-of-function *Mc1r* allele caused by a nonsense mutation, R306ter (Everts et al. 2000; Newton et al. 2000). However, dogs with a reddish-yellow coat from many other breeds, such as Dachshunds, Great Danes, Chows, and French Bulldogs, have a normal *Mc1r* gene and instead are thought to carry an *Agouti* allele associated with a uniform yellow coat, a^y. Historically, some dog breeders have attempted to distinguish between pheomelanic shades caused by the *Mc1r* R306ter allele (previously referred to as *recessive yellow, e*) and an *Agouti* allele (a^y, as above) in describing the former as red and the latter as fawn or tan. However, there is considerable variation in pheomelanic hue and saturation regardless of whether it is caused by an *Mc1r* or an *Agouti* allele; for example, modifier genes that specifically dilute pheomelanin can give rise to a pale yellow or cream color, as in the yellow Labrador Retriever (*Mc1r* R306ter) or the cream color of the pheomelanic band in hairs from gray wolves (a^w).

An additional complexity in the genetics of pigment type-switching in dogs stems from the sable phenotype in which a yellow coat contains variable numbers of black hairs. The extent of darkening caused by these black hairs varies considerably both among and within breeds, and sable dogs with very little darkening can overlap with those described as yellow, tan, or fawn. In German Shepherd Dogs, the nomenclature is especially complicated, since sable, i.e., completely black hair, can be intermixed with completely yellow hairs or with banded hairs. Various authors have suggested a^y, a^g, a^w, a^s, a^t, and a as potential alleles (Little 1957; Willis 1976; Carver 1984), but in very few instances has it been possible to distinguish allelic variation at *Agouti* from darkening due to modifier genes distinct from *Agouti* and *Mc1r*.

We recently reported the molecular characterization of the dog *Agouti* gene and a missense variant, R96C, which is likely to cause *nonagouti*, a uniform black coat that segregates in a recessive manner relative to *black-and-tan*, a^t, in German Shepherd Dogs (Kerns et al. 2004). We have now carried out a more extensive survey of *Agouti* sequence variation and report a third allele associated with fawn and sable phenotypes. These results resolve some of the complexity in dog coat color genetics, reveal a likely evolutionary history of specific coat color alleles, and provide diagnostic opportunities and practical guidelines for breeders.

Materials and methods

Animals. Most DNA samples were obtained from dog owners who used cheek swab brushes (Epicentre, Madison, WI) and mailed these back to our lab. DNA was also obtained from the frozen hides of a wolf and a coyote trapped in northern Saskatchewan by a licensed trapper. Coat color was recorded by the owner or by us if we took the sample. Photographs of dogs and hair swatches were beneficial for comparison of phenotypes.

Agouti sequencing. PCR primers for exons 2 and 4 were described previously (Kerns et al. 2004). Primers for exon 3 were 5′CTTTACTGCAGCTCGT GGCCTCC3′ and 5′GATTCAGGGCTTGAGGCC AAG3′. Polymerase chain reaction (PCR) products were obtained and sequenced at the National Research Council Plant Biotechnology Lab using an ABI sequencer (Applied Biosystems, Foster City, CA). The sequence results were aligned and analyzed using Sequencher (Version 4.1, Gene Codes Corporation, Ann Arbor, MI).

PCR assay for a^y allele detection. A 285-bp fragment containing part of intron 3 and exon 4 was amplified with 5′GATGTCTGGTCTGGAGCCTC3′ and 5′TCAGCATCTGGGACTGAGAACGC3′ as follows: The PCR reaction of 15 μl contained 1 μl of DNA (50–100 ng), 1.5 μl of 10 × PCR buffer (Gibco, Gaithersburg, MD), 0.45 μl of 50 mM $MgCl_2$, 0.3 μl of 10 mM dNTP, 0.1 μl *Taq* polymerase (Gibco), 0.5 μl (66 pM) of the forward primer, 1 μl of the reverse primer (66 pM) and 10.15 μl of dH_2O. The cycling protocol was 4 min at 94°C, followed by 33 cycles of

50 sec at 94°C, 50 sec at 59°C, 40 sec at 72°C, and finished with a 4-min extension at 72°C on a Robocycler. After digestion with BsmA1 the a^y-allele yields fragments of 42, 90, 153 bp; other alleles yield fragments of 42 and 243 bp.

Results

Detection of new sequence variants. We determined the entire *Agouti* protein-coding sequence in genomic DNA samples from 4 animals: a wolf representing the wild-type coat color, a sable Shetland Sheepdog (Peyton), a black-and-tan Gordon Setter (Rico), and a brindle Afghan Hound (Tiger). The initiation codon is in exon 2 and termination codon is in exon 4. No differences were detected in exon 2 or 3 among these canids.

The sequences were identical to what we previously determined for the Doberman Pinscher (GenBank AY714374), except that the Shetland Sheepdog (GenBank AY691406) carried a G-to-T transversion and a G-to-A transition in exon 4 at positions corresponding to residues 244 and 248 of the cDNA sequence (relative to the translational initiation site) that predicted A82S and R83H substitutions.

Linkage and association studies of a new* Agouti *allele. To explore the potential significance of the A82S and R83H substitutions, we studied Shetland Sheepdogs and Rough/Smooth Collies because some individuals in these breeds also exhibit the sable phenotype as one possible coat color variation and because we had access to pedigrees from these breeds in which different coat colors were segregating. We determined the sequence of *Agouti* exon 4 in Collie (14 individuals, 9 meioses) and Shetland Sheepdog (8 individuals, 6 meioses) families in which some individuals were sable or sable with white, and some were black-and-tan or tricolor. (The presence and extent of white markings are unrelated to, and independent of, variation in pigment type-switching.) In every case (Fig. 1), sable or sable with white appeared dominant to black-and-tan or tricolor, and the A82S R83H variant exhibited complete cosegregation, with A82 R83 associated with the a^t-allele, and S82 H83 associated with the sable allele (LOD = 3.547: $\theta = 0$: $p < 0.001$). In what follows we describe the S82 H83 allele as fawn–sable or a^y and the A82 R83 allele as a^t (Fig. 2: Table 2).

We extended our study to determine whether sable or fawn coat color was associated with a specific *Agouti* allele among individual dogs from other more diverse breeds and a wolf (GenBank AY691404) and coyote (GenBank AY691405). Among 45 unrelated dogs whose exon 4 sequence was determined completely, the A82S substitution was always found together with the R83H substitution, indicating complete linkage disequilibrium. Therefore, we developed a simple assay for the A82S substitution based on the presence of a new *Bsm*AI restriction site; in the data set described below (Table 1), 30 individuals were genotyped for A82S but are inferred to also carry the R83H substitution and 16 individuals were confirmed to carry both A82S and R83H.

We initially excluded from consideration dogs with a black or modified black (black-and-white, brown, or blue merle) coat because these phenotypes can be caused by a gene or genes clearly distinct from *Agouti*. Among 57 dogs from breeds as diverse as the Pug and Great Dane (including only the parents depicted in Fig. 1), 41 fawn, sable, or sable with white colored animals all were heterozygous or homozygous for the S82 H83 allele, and 16 black-and-tan or tricolor individuals were homozygous for the A82 R83 allele (Table 1). A Fisher exact test predicts that the probability of this happening by chance is 0.017^{-12}. Besides breeds that are typically sable, such as the Tervuren, or always black-and-tan, such as the Gordon Setter, this sample includes Dachshunds, Cardigan Welsh Corgi, Staffordshire Bull Terrier, Collies, and Shetland Sheepdogs that are homozygous or heterozygous for S82 H83 and fawn dogs of these breeds that are homozygous for A82 R83 and black-and-tan. Therefore, the association between S82 H83 and fawn or sable occurs separately within at least 5 breeds (Table 1) and is not a breed-specific polymorphism due to population structure.

Agouti *genotypes of black dogs.* We have previously reported that the apparent dominant inheritance of a black coat in a Labrador Retriever × Greyhound cross is unlinked to *Agouti* or *Mc1r* but is epistatic to fawn coat color (Kerns et al. 2003). Dominant black is thought to be present in many breeds, but in German Shepherd Dogs black coat color is likely caused by a missense *Agouti* allele known as *non-agouti* (R96C) and is inherited in a recessive manner. Thus, we hypothesized that a sampling of *Agouti* alleles from black dogs across different breeds would identify a small number carrying the R96C allele (recessive black or nonagouti), with the remainder presumably due to dominant black at a separate locus which we refer to as the K locus until such time that the gene is identified.

Among 32 black or brown dogs from a variety of breeds, we found 8 (from the German Shepherd Dog, Groenendael, Schipperke, Shetland Sheepdog) that were homozygous for the *nonagouti* R96C allele, and 24 (from the Australian Shepherd, Border Collie, Curly Coated Retriever, Flat Coated Retriever,

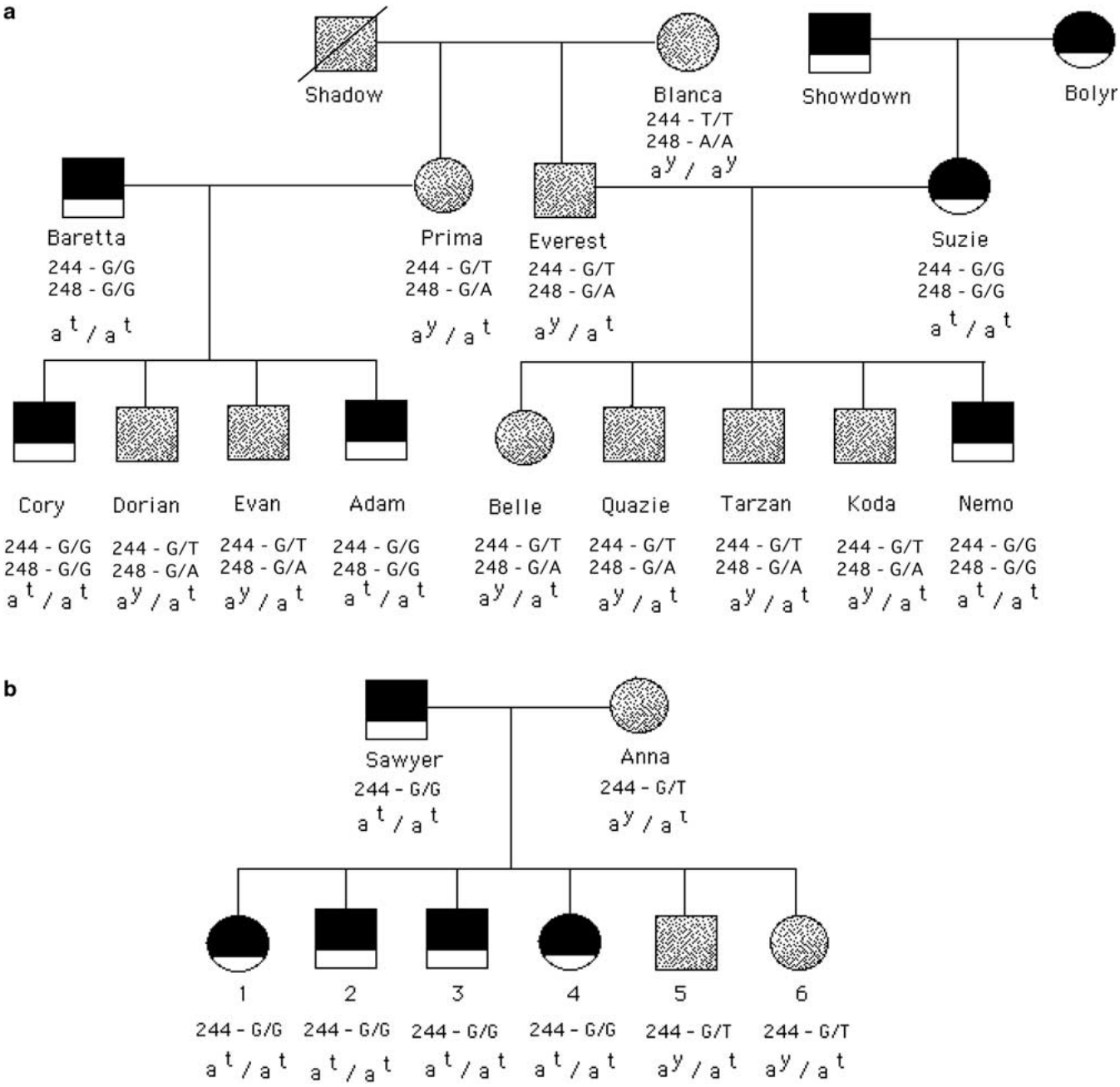

Fig. 1. Collie (**a**) and Shetland Sheepdog (**b**) families illustrating cosegregation of sable coat color with the A82S R83H *Agouti* allele. For each individual, the genotypes are given at residues 244 and 248 (relative to the translational start site). At residue 244, G and T predict alanine and serine, respectively; at residue 248, G and A predict arginine and histidine, respectively. Shaded symbols represent dogs with a sable with white or sable phenotype; two-tone symbols represent dogs with a black-and-tan or tricolor phenotype. Inferred genotypes for a^y and a^t are indicated as described in the text.

Groenendael, Labrador Retriever, Large Munsterlander, Pug, Toy Poodle, and Weimaraner) that were not (Table 1). In every case, the R96C substitution was found together with the A82 R83 rather than the S82 H83 (a^y) allele. In general, these observations agree with breeder reports that a black bicolor coat in Shetland Sheepdogs is recessive to tricolor or sable with white and that a uniform black coat in Groenendaels can be inherited in a dominant or a recessive manner. Although there is little coat color genetic information available for the Schipperke, this breed has a common origin with the larger Belgian Shepherds, and it seems likely that the *non-agouti* R96C allele in the Schipperke, Groenendael,

Fig. 2. Photographs of dogs homozygous for the a^y A82S R83H allele illustrate a variety of shades along the fawn–sable spectrum. Clockwise from top left is Peyton, a female Shetland Sheepdog; Rain, a male Belgian Tervuren; Ginny, a female Akita; Tiara, a female Great Dane; and Bull, a male Mastiff.

and German Shepherd Dog was derived from a common ancestor.

Other coat color phenotypes. We also examined 5 dogs that were brindled (Boxer, Great Dane, Afghan Hound, Scottish Terrier, and Cairn Terrier), a pattern of alternating black and yellow or pale yellow stripes, often in a swirling pattern, as well as one dog (Staffordshire Bull Terrier) who was black with brindled points (brindled areas restricted to the distal limbs and lower face). The latter pattern is very similar to black-and-tan with brindled instead of tan;

Table 1. Sequence results for Agouti variants from unrelated dogs of various breeds and coat colors, a wolf, and a coyote

			Codon[b]		
Individual	*Breed*	*Coat color*[a]	*82*	*83*	*96*
Cumberland	Coyote	a^W-sable	A/A	R/R	R/R
#2[c]	Wolf	a^W-sable	A/A	R/R	R/R
Boone	German Shepherd Dog	a^W-sable	A/A	R/R	R/C
Orryanna	German Shepherd Dog	a^W-sable	A/A	R/R	R/C
Jake	Dachshund	Fawn	A/S	R/H	R/R
Dallee	Dachshund	Fawn	A/S		
Digger	Dachshund	Fawn	A/S		
Dewi	Cardigan Welsh Corgi	Sable	S/S		
Tutti	Cardigan Welsh Corgi	Sable	S/S		
Honey	Cardigan Welsh Corgi	Sable	A/S		
Paris	Cardigan Welsh Corgi	Sable	A/S		
Pepper	Cardigan Welsh Corgi	Sable	A/S		
Tigger	Pembroke Welsh Corgi	Sable	A/S		
Steady	Pekingese	Fawn	S/S		
Tiara	Great Dane	Fawn	S/S	H/H	R/R
Ember	French Bulldog	Fawn	S/S	H/H	R/R
Sadie	French Bulldog	Fawn	S/S	H/H	R/R
Pansy	Pug	Fawn	S/S	H/H	R/R
Mabelline	Pug	Fawn	S/S		
Bella	Staffordshire Bull Terrier	Fawn	A/S		
Lola	Rhodesian Ridgeback	Fawn	S/S		
Loki	Rhodesian Ridgeback	Fawn	S/S		
CJ	Rhodesian Ridgeback	Fawn	S/S		
Peyton[c]	Shetland Sheepdog	Sable with white	S/S	H/H	R/R
Lady	Shetland Sheepdog	Sable with white	A/S		
Anna[d]	Shetland Sheepdog	Sable with white	A/S		
Maria	Belgian Tervuren	Sable	S/S	H/H	R/R
Rain	Belgian Tervuren	Sable	S/S	H/H	R/R
Duff	Belgian Laekenois	Sable	S/S	H/H	R/R
Saline	Belgian Laekenois	Sable	S/S	H/H	R/R
Nick	Belgian Malinois	Sable	S/S	H/H	R/R
Everest[d]	Rough Collie	Sable with white	A/S	R/H	R/R
Claudia	Afghan Hound	Sable	S/S	H/H	R/R
Starzon	Afghan Hound	Sable	A/S	R/H	R/R
Max	Afghan Hound	Sable	A/S		
Chelsey	Chow	Fawn	S/S		
Jasmine	Shar-Pei	Fawn	S/S		
Ginny	Akita	Fawn	S/S		
Talle	Akita	Fawn	A/S		
Chance	Akita	Fawn	S/S		
Diva	Akita	Fawn	S/S		
Bull	Mastiff	Fawn	S/S		
Rufus	Bullmastiff	Fawn	S/S		
Freddie	Bullmastiff	Fawn	S/S		
Benny	Boxer	Fawn	S/S		
Fritz	Boxer	Brindle	S/S		
Banjo	Great Dane	Brindle	S/S	H/H	R/R
Tiger[a]	Afghan Hound	Brindle	S/S	H/H	R/R
Willow	Cairn Terrier	Brindle	S/S		
Gilly	Scottish Terrier	Brindle	S/S		
Taisto	Staffordshire Bull Terrier	Black, brindle points	A/A		
Suzie[d]	Smooth Collie	Black-and-tan	A/A	R/R	R/R
Rico[c]	Gordon Setter	Black-and-tan	A/A	R/R	R/R
Reuben	Dachshund	Black-and-tan	A/A		
Nigel	Dachshund	Black-and-tan	A/A		
Spy	Dachshund	Black-and-tan	A/A		
Sprite	Dachshund	Black-and-tan	A/A		

Continues

Table 1. Continued

Individual	*Breed*	*Coat color*[a]	*Codon*[b] 82	83	96
Drei	Dachshund	Black-and-tan	A/A		
Sammy	Cardigan Welsh Corgi	Black-and-tan	A/A		
Kira	Cardigan Welsh Corgi	Black-and-tan	A/A		
Daisy	Beagle	Black-and-tan	A/A		
Mickey	Beagle	Black-and-tan	A/A		
Sawyer[d]	Shetland Sheepdog	Tricolor	A/A		
Cassidy[e]	Shetland Sheepdog	Tricolor	A/A	R/R	R/R
Tara	Shetland Sheepdog	Tricolor	A/A		
Sharki[e]	Australian Shepherd	Tricolor	A/A	R/R	R/R
Cricket	Australian Shepherd	Black bicolor	A/A	R/R	R/R
Birdie	Australian Shepherd	Red-brown bicolor	A/A	R/R	R/R
Kie	Australian Shepherd	Black bicolor	A/A	R/R	R/R
Bett	Australian Shepherd	Black bicolor	A/A	R/R	R/R
Whistler	Australian Shepherd	Black bicolor	A/A	R/R	R/R
Cinder	Australian Shepherd	Blue Merle	A/A	R/R	R/R
Jag	Australian Shepherd	Blue Merle	A/A	R/R	R/R
Annie	Border Collie	Black and white	A/A	R/R	R/R
Brewster	Border Collie	Black and white	A/A	R/R	R/R
Rajha	Labrador Retriever	Chocolate	A/A	R/R	R/R
Cole	Labrador Retriever	Black	A/A		
Tompei	Labrador Retriever	Black	A/A		
Abrakadabra	Flat Coated Retriever	Black	A/A		
Star	Curly Coated Retriever	Black	A/A		
Ibis	Large Munsterlander	Black and white	A/A		
Tetley	English Cocker Spaniel	Black roan	A/A		
Gin	Weimaraner	Pale brown	A/A		
Lacey	Toy Poodle	Brown Silver	A/A		
Flutey	Toy Poodle	Brown	A/A		
Nubs	Pug	Black	S/S	H/H	R/R
Aviv	Groenendael	Black	S/S	H/H	R/R
Timex	Groenendael	Black	S/S	H/H	R/R
Spot	Groenendael	Black	A/S	R/H	R/C
JD	Groenendael	Black	A/S	R/H	R/C
Rose	Groenendael	Black	A/A	R/R	C/C
Ralph[e]	Groenendael	Black	A/A	R/R	C/C
Raven	Schipperke	Black	A/A	R/R	C/C
C.C.	Schipperke	Black	A/A	R/R	C/C
Chavo[e]	Australian Shepherd	Black	A/A	R/R	R/C
Ozzy[e]	Shetland Sheepdog	Black and white	A/A	R/R	C/C
Tess	German Shepherd Dog	Black	A/A	R/R	C/C
5M[e]	German Shepherd Dog	Black	A/A	R/R	C/C
Carbon	German Shepherd Dog	Black	A/A	R/R	C/C

[a]Coat color designations, mostly according to common usage. For German Shepherd Dogs, we use the designation A^{w}-sable to indicate that there are pale hairs on the ventrum and Agouti banded hairs on the dorsum intermixed with black hairs.
[b]Genotypes are shown at all 3 codons for dogs for whom exon 4 sequence was obtained, whereas only the genotype at codon 82 is shown for dogs tested using the PCR-RFLP test.
[c]Animals for which the entire protein-coding sequence was examined.
[d]Animals also shown in Fig. 1.
[e]Individuals whose genotype was previously described in Kerns et al. (2004).

indeed, this individual was homozygous for the a^{t} A82 R83 allele, whereas animals with brindling over their entire body surface were homozygous for the a^{y} S82 H83 allele. (None of these animals carried the R96C substitution characteristic of *nonagouti* black.)

Finally, we examined 2 German Shepherd Dogs with a pale ventrum and black hairs with a yellow band in the saddle area. Although the terms sable or gray–sable have been used to describe this phenotype, it is distinct from the sable of the Tervuren, for example, since German Shepherd Dogs have dorsal

Table 2. The nucleotides at specific variable positions in exon 4 of the *agouti* gene and the corresponding allele and phenotype

		Sequence variants (codons)		
Allele	*Phenotype*	*82*	*83*	*96*
White-bellied Agouti, a^w (aka a^g)	Banded dorsal hairs and pale Ventral hairs; ± sable markings	A	R	R
Fawn-sable, a^y	Yellow-tan; ± sable markings	S	H	R
Black-and-tan, a^t	Black with yellow/tan points	A	R	R
Nonagouti, a	Black	A	R	C

hairs with alternating eumelanin and pheomelanin banding while the Tervurens do not. The Agouti genotype of both German Shepherd Dogs was A82 R83 R96/A82 R83 C96: corresponding to a^w/a.

Discussion

Many aspects of pigment type-switching are conserved across mammalian phyla, with allelic series of *Agouti* and/or *Mc1r* giving rise to similar effects in carnivores, primates, rodents, and ungulates (Klungland and Vage 2003). In domestic dogs, the situation has been more confusing, in part because banded hairs are present in only a few breeds, and in part because the a^y allele is not apparent in many other species. Our results confirm that a uniform pheomelanic appearance in many breeds of dogs is indeed caused by an *Agouti* allele, suggest that a considerable amount of variation in darkening is due to modifier genes rather than allelic variation, and provide practical opportunities for certain breeding strategies.

The* $\mathbf{a^y}$ *allele. As many as 5 distinct *Agouti* alleles have been postulated in certain breeds, but our survey of *Agouti* sequences among 37 breeds and a multitude of coat color phenotypes identified only 3 variants predicted to alter amino acid sequence, with one, S82 H83 R96, common to all fawn- or sable-colored animals in our sample. We refer to this variant (Table 2) as fawn–sable (a^y) to indicate congruence with both the historical designation and common usage, but we note that a^y in dogs is clearly distinct from the A^y or *lethal yellow* allele known to mouse geneticists. In the latter case, a large deletion causes *Agouti* protein-coding sequences to be expressed abnormally in nearly every tissue in the body (Duhl et al. 1994; Michaud et al. 1994). The A^y mutation is associated with pleiotropic effects including obesity, increased body size, and embryonic lethality when homozygous, and it is found only in laboratory mice. In dogs, however, the a^y allele has no deleterious effects in either the heterozygous or homozygous state. However, the two base pair changes associated with this a^y allele in dogs impart no similar dramatic change to the agouti product. It is somewhat unfortunate that a^y has traditionally been used by both mouse and dog geneticists for such different phenotypes and mutations.

Because the A82S and R83H substitutions are in complete linkage disequilibrium and were found in every fawn- or sable-colored dog we examined, it is impossible to know if one or both substitutions alter Agouti protein function, or if there is an extended haplotype that also carries a regulatory alteration that modulates expression of one or more *Agouti* mRNA isoforms (see below). Residue 82 is conserved in more species than residue 83 (residue 82 is valine in humans and residue 83 is glutamine in cats and pigs); therefore, we designed our PCR–RFLP test to residue 82. These amino acids lie within the 65-residue basic domain, amino-terminal to a proline-rich region, followed by a 40-residue cysteine-rich carboxy-terminal. It is possible that the A82S or R83H substitutions affect the protein interactions between *Agouti* and *Mc1r* (Miltenberger et al. 1999), affecting stability and/or proteolytic cleavage.

Although the a^y allele is very prevalent among domestic dogs, it is likely to be derived from an ancestral a^w allele apparent in many types of mammals and represented today in canids such as wolves, coyotes, and some German Shepherd Dogs. From this perspective, a potential scenario for a mutation of a^w to a^y would be an alteration in *cis*-acting regulatory sequence that either causes hair cycle-specific isoforms to be expressed throughout all of anagen, or causes ventral-specific isoforms to be expressed over the entire body surface and, due to population history, has come to lie in linkage disequilibrium with the A82S and R83H substitutions. For example, evolutionary ancestors of the domestic dog may have carried an a^w allele together with either A82 R83 or S82 H83, with the former haplotype giving rise to the a^t and *a* mutations and the latter giving rise to the a^y mutation (Table 2). Such an event would have to have preceded the derivation of all domestic dogs, since the association between a^y

and A82 R83 extends across distantly related breeds. Recently, Parker et al. (2004) used 96 microsatellites to sort 85 dog breeds into four groupings: ancient Spitz type, giant Mastiff type, herding type, and the majority of other types. The S82 H83 variant was found in representatives of all four of these groups: the Chow, Shar-Pei, Pekingese, Akita, and Afghan Hound in the ancient group; the Mastiff, Bullmastiff, Boxer, and French Bulldog in the giant group; the Belgian Shepherds, Collie, and Shetland Sheepdog in the herding group; and the Dachshund, Great Dane, Pug, and Cairn Terrier in the ''others'' group (Table 2). Thus, from a practical perspective, the *Agouti* coding sequence at residues 82, 83, and 96 should be predictive of a^y, a^t (or a^w, see below), or *a* alleles in all domestic dogs (Table 2).

Sable markings and the saturation of pheomelanin pigment. Here, we have used the term sable markings to describe variable amounts of black hairs on a yellow or tan background; however, the term has been used to describe different phenotypes in various breeds. Carver (1984) uses the term ''gray–sable'' (a^w) and ''tan–sable'' (a^y) for German Shepherd Dogs with and without banded hairs, respectively. To avoid confusion, we use the term *white-bellied Agouti* (and the allele designation a^w) for the banded hair phenotype, with the understanding that sable markings are frequently present. Finally, sable means sand in French, and Belgian Shepherds described as sable have black hairs intermingled in a pale yellow coat (Lee Jiles, personal communication).

The observations that variable amounts of sable markings can be present in dogs carrying either an a^y or an a^w allele suggests that variation is due to modifier genes and is consistent with studies in laboratory mice in which different inbred strains carrying the a^y allele exhibit different degrees of darkening (Lamoreux and Galbraith 1986; Suto and Sekikawa 2003). In addition, two known modifiers of pigment type-switching in mice, *Atrn* and *Mahogunin*, are required for Agouti-induced pheomelanogenesis *in vivo* (reviewed in He et al. 2003a). Null mutations for either of these genes cause neurodegeneration in addition to a dark coat (He et al. 2001, 2003b), but hypomorphic alleles have very mild or no effects on the brain (Gunn et al. 2001; Phan et al. 2002) and, by analogy, could help explain the difference between dogs of a^y/a^y genotype with variable amounts of sable markings (Fig. 2).

Besides variation in the extent of sable markings, the depth of pigmentation in pheomelanin can also vary considerably, as in Carver's description (1984) of a^y dogs that ''range in color from a deep red to a light cream with scattered black hairs in the coat.'' Many dog breeders believe some of these differences are due to gene dosage; in Collies, for example, with a^y/a^y giving rise to light orange ''clear or pure sable'' animals and a^y/a^t giving rise to deep red ''tri-factored sable'' animals. A gene that would alter the depth of pheomelanin expression and not eumelanin expression is another explanation since all of the dogs shown in Fig. 2 were a^y/a^y genotype. Both mechanisms, gene dosage and modifier genes, may pertain to different situations and can now be investigated with the molecular tools described here.

A potential candidate for modifying the extent of sable markings or the depth of pheomelanic pigmentation in dogs carrying a^y is variation in the *Mc1r*. Loss-of-function for *Mc1r* due to a nonsense mutation, R306ter, causes a uniform pheomelanic coat in a variety of breeds including Golden Retrievers, yellow Labrador Retrievers, or Irish Setters. We have previously described an association between the polymorphism M264V and the presence of a melanistic mask (Schmutz et al. 2003). However, the variation in shade among individual fawn or sable dogs in this study (Fig. 2) was not correlated with either the sex of the dog or the presence of the E^M allele (data not shown). The Tervuren, Pug, and Mastiff breeds are fixed as V264, the Shetland Sheepdog is fixed as M264, and Great Danes carry both alleles. Since the R306ter prevents the black pigmentation of any hairs, sabling is eliminated in dogs homozygous for this *e* allele. Sadie, a French Bulldog (Table 1), was homozygous for the *e*/*e* R306ter.

We have examined a few dogs with brindle markings. The genotype at the *Agouti* locus dictates where the brindling will occur on the dog. Dogs that have at least one a^y allele produce pheomelanin over most of their bodies (other than in places with white markings), and thus dark stripes can be seen on these pheomelanin-pigmented areas. Dogs that were a^t/a^t in genotype have pheomelanin pigmentation only ventrally and thus the dark stripes are evident only on these ventral areas.

DNA testing for* Agouti *alleles. The main breeds of dogs for which our observations could be used to plan matings are the Collie, both Rough and Smooth varieties, the Shetland Sheepdog, the Cardigan Welsh Corgi, and the Dachshund. Collie breeders often wish to have both sable with white and tricolor pups in a litter, which is possible if two sable with white parents are a^y/a^t, 25% of the offspring will be tricolor (a^t/a^t). For example, such a test could have been applied to the Collies Prima and Everest (Fig. 1a) or to the Shetland Sheepdog dam Anna (Fig. 1b); in all three cases, their genotype could not have been determined without DNA

Fig. 3. Photographs of Shetland Sheepdogs illustrating the sable pattern (Lady) on the left, the tricolor pattern (Cassidy) in the middle, and the bicolor pattern (Ozzy) on the right.

testing or knowledge of previous offspring. In Shetland Sheepdogs, there are additional opportunities that come from testing for the *nonagouti* R96C substitution, since dogs in this breed may be sable with white (a^y/a^y, a^y/a^t, or a^y/a), tricolor (a^t/a^t or a^t/a), or bicolor (a/a) (Fig. 3).

The* $\mathbf{a^w}$ *and* $\mathbf{a^t}$ *alleles. We found no difference in *Agouti* exon 4 sequences between dogs carrying the a^w and a^t and alleles (Table 2) nor in the coding sequence of a wolf or Gordon Setter. By analogy to black-and-tan (a^t) in laboratory mice, it seems most likely that a^t in dogs represents a regulatory alteration that inactivates expression of hair cycle-specific *Agouti* mRNA isoforms present in an ancestral a^w allele. Large-scale sequencing of flanking regions from genomic DNA of dogs of different *Agouti* phenotypes could provide additional insight.

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