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A SINE Insertion Causes the Black-and-Tan and Saddle Tan Phenotypes in Domestic Dogs

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Abstract

Agouti Signaling Protein (ASIP) controls the localized expression of red and black pigment in the domestic dog through interaction with other genes, such as *Melanocortin 1 Receptor* and *Beta-Defensin 103*. Specific *ASIP* alleles are necessary for many of the coat color patterns, such as black-and-tan and saddle tan. Mutations in 2 *ASIP* alleles, a^y and *a*, have previously been identified. Here, we characterize a mutation consisting of a short interspersed nuclear element (SINE) insertion in intron 1 of *ASIP* that allows for the differentiation of the a^w wolf sable and a^t black-and-tan alleles. The SINE insertion is present in dogs with the a^t and *a* alleles but absent from dogs with the a^w and a^y alleles. Dogs with the saddle tan phenotype were all a^t/a^t. Schnauzers were all a^w/a^w. Genotypes of 201 dogs of 35 breeds suggest that there are only 4 *ASIP* alleles, as opposed to the 5 or 6 predicted in previous literature. These data demonstrate that the dominance hierarchy of *ASIP* is $a^y > a^w > a^t > a$.

Key words: *agouti, black and tan, canid, coat color, pigmentation, saddle tan, wolf sable*

Agouti Signaling Protein (ASIP) has been implicated in coat color production in a variety of domestic animals including horses (Rieder et al. 2001), cattle (Girardot et al. 2005), pigs (Drögemüller et al. 2006), and dogs (Kerns et al. 2004; Berryere et al. 2005). *ASIP* alleles can act as an antagonist to α-Melanocyte Stimulating Hormone (α-MSH), preventing the binding of α-MSH to Melanocortin 1 Receptor (MC1R) and resulting in the production of phaeomelanin over some or all of the body (Lu et al. 1994). The function of *ASIP*, and in turn the expression of phaeomelanin and eumelanin, is dependent on mutations in *ASIP*, as demonstrated by the nonagouti alleles causing solid black in sheep (Royo et al. 2008), dog (Kerns et al. 2004), and horse (Rieder et al. 2001). As demonstrated in mice (Vrieling et al. 1994), there can be multiple transcripts of *ASIP* resulting from alternate first exons.

Over the past 60 years, many authors have attempted to predict the various alleles of *ASIP*, or the *A* locus, in dogs. Winge (1950) predicted 6 alleles at what he termed the *C* locus and called them: solid color (C^s), wolf sable (*C*), black and tan (c^{bi}), brindle (c^{br}), saddle tan (c^{sa}), and melanistic mask (c^{ma}). Little (1957) postulated 4 *A* locus alleles producing solid color (A^s), fawn (a^y), wolf sable (a^w), and black and tan (a^t). Finally, Willis (1989) predicted 6 *A* locus alleles causing solid color (*A*), fawn (a^y), wolf sable (a^s), saddle tan (a^s), black and tan (a^t), and recessive solid black (*a*). Modern nomenclature for dog coat color alleles is taken largely from the work of Little (1957). Recent advances in coat color genetic research have shown that some of the earlier predicted alleles are alleles at other genes. Brindle (k^{br}) and solid color (K^B) have been mapped to *Canine Beta-Defensin 103 (CBD103)* (Kerns et al. 2007; Candille et al. 2007). Melanistic mask (E^M) is caused by a M264V mutation of *Melanocortin 1 Receptor* (Schmutz et al. 2003). The allele causing fawn (a^y), predicted as part of the *A* locus series by Little (1957) and Willis (1989) is caused by 2 point mutations in adjacent amino acids, A82S R83H, in exon 4 of *ASIP* (Berryere et al. 2005). The recessive solid black allele is caused by a R96C mutation in exon 4 of *ASIP* (Kerns et al. 2004), although this allele is rare. The predicted *ASIP* alleles that cannot currently be distinguished are wolf sable (a^w), black and tan (a^t), and saddle tan.

The a^w wolf sable allele causes a pattern of hairs that are banded in alternating sections of eumelanin and phaeomelanin along the hair shaft. Dogs that are a^w wolf sable have a predominantly phaeomelanin ventral surface, with banded hairs appearing on the dorsal surface and head (Figure 1A). The Keeshond pictured in Figure 1A has diluted phaeomelanin that appears cream, whereas in other breeds this pigment can be a deeper red/orange color. The black-and-tan phenotype (Figure 1B) is a very distinct pattern of phaeomelanin points on a eumelanin background. The "tan points" that are phaeomelanin in color are traditionally the distal portions of the legs, along the sides of the muzzle,

Figure 1. Coat color variation attributed to the *ASIP* locus. (**A**) a wolf sable (a^w) Keeshond, (**B**) a black-and-tan (a^t) Doberman Pinscher, (**C**) a fawn (a^y) Shetland Sheepdog, (**D**) a recessive black (a) German Shepherd Dog, and (**E**) a saddle tan Basset Hound.

small dots above each eye, on the caudal surface of the chest, and around the anus and ventral surface of the tail. The saddle tan phenotype (Figure 1E) is very similar to the black-and-tan phenotype, except the phaeomelanin points are larger in size, expanding completely up the legs onto the shoulder and hip, and over the entire face and head. Eumelanin pigmentation is restricted to a "saddle" shaped area on the dorsal surface.

Materials and Methods

Dogs and Families

Two hundred one dogs from 35 breeds were selected to investigate the interaction of the *ASIP* alleles and their associated phenotypes. The coat color patterns expressed include wolf sable ($n = 57$), fawn ($n = 48$), black-and-tan ($n = 30$), saddle tan ($n = 8$), solid eumelanin ($n = 34$), solid phaeomelanin ($n = 11$), and miscellaneous patterns ($n = 13$).

Five breeds, Siberian Husky, Alaskan Malamute, Keeshond, Swedish Vallhund, and Norwegian Elkhound, were selected for the study because they were suspected of being fixed at a^w/a^w, the wolf sable pattern. Ten breeds, Cardigan Welsh Corgi, Pembroke Welsh Corgi, Collie, Shetland Sheepdog, Eurasier, Dachshund, Saluki, German Shepherd Dog, Finnish Lapphund, and Jack Russell Terrier, were chosen because they exhibit phenotypes attributed to multiple *ASIP* alleles.

Five breeds that exhibit the saddle tan phenotype: Airedale Terrier, German Shepherd Dog, Beagle, Basset Hound, and Pembroke Welsh Corgi were included in the study. Thirty-six dogs from 12 hunting breeds, including Brittany Spaniel, French Brittany Spaniel, Chesapeake Bay

Retriever, German Longhair Pointer, German Shorthair Pointer, German Wirehair Pointer, English Springer Spaniel, Golden Retriever, Large Munsterlander, Labrador Retriever, Weimaraner, and Vizsla, were included because these breeds commonly have solid coat colors caused by the K^B allele of *CBD103* (Candille et al. 2007) or the *e/e* genotype of *MC1R* (Newton et al. 2000). These alleles are epistatic to the *ASIP* alleles, so the *ASIP* genotypes of many hunting breeds were largely unknown. Thirteen additional dogs of 6 breeds were selected because they exhibit unusual color patterns for their breed. These include 2 fawn Akitas with more than the usual amount of eumelanin pigment, a Border Collie with banded hairs, a wild boar Dachshund, a "patterned sable" Shar Pei, a salt-and-pepper Standard Schnauzer, and 7 Miniature Schnauzers, 3 of which are salt and pepper, 2 black and silver, and 2 solid black.

DNA samples were gathered using cheek brushes (Epicentre, Madison, WI) and were collected widely from Canada and the United States. The dogs utilized in this research are owned by private individuals and, in accordance with the Canadian Animal Care guidelines, signed consent for use of the DNA in coat color studies was obtained from the owners.

Primer Design and PCR

A 35-kb region between the predicted exon 1 (Kerns et al. 2004) and the exon 2 start codon of *ASIP* was compared between 2 publicly available BAC sequences from a black-and-tan (a^t) Doberman Pinscher (GenBank AC092250) and a fawn (a^y) Boxer (GenBank NW_876277). Primers were subsequently designed for this region (Supplementary Table 1).

Segments were amplified by polymerase chain reaction (PCR) in 15 μl reactions consisting of 1.5 μl 10× PCR buffer (Fermentas), 0.3 μl of 10 mM dNTP, 1.5 mM $MgCl_2$, 10 pmol/μl each of forward and reverse primers, 0.1 μl of 5 u/μl *Taq* polymerase (Fermentas), 9.2 μl dH_2O, and 1 μl of roughly 50 ng/μl DNA template. PCR was carried out in Stratagene Robocycler Gradient40 machines, with 4 min initial denaturation at 94 °C, followed by 35–37 cycles of 50 s 94 °C denaturation, 50 s annealing at primer specific temperatures, and 50 s 72 °C extension. This was followed by a final 4 min 72 °C extension period. Product bands were excised from 2% agarose gel and isolated using the QIAquick gel extraction kit (Qiagen, Mississauga, ON) and sequenced at the National Research Council of Canada Plant Biotechnology Institute, using an ABI Prism 373 Sequencer (Perkin Elmer Corporation) and the Big Dye Terminator kit (Perkin Elmer Corporation). Sequences were aligned using the Sequencher 4.8 software program (Gene Codes Corporation, Ann Arbor, MI).

Genotyping

Dogs were genotyped for the a^y allele of *ASIP* as previously described (Berryere et al. 2005). A portion of the dogs were also genotyped for the *a* allele of *ASIP* (Kerns et al. 2004). Because the *a* allele is rare outside of herding breeds, only individuals suspected of carrying *a* were genotyped. Dogs were also genotyped for the presence of a newly discovered short interspersed nuclear element (SINE) insertion, described below.

Results

Sequence Analysis

A polymorphism consisting of a 239-bp SINE insertion was found in the Doberman Pinscher but not in the Boxer. A second SINE is located in both Boxer and Doberman Pinscher 23.5 kb upstream of the exon 2 start codon and approximately 215 bp downstream of the variable SINE (Figure 2).

The fixed SINE, found in the forward orientation, is present in dogs of all *ASIP* genotypes. The more 5′ variable SINE is in the reverse orientation and is present only in dogs with a^t or *a ASIP* alleles. The 215-bp region between these 2 SINEs is found in dogs of all *ASIP* genotypes, as are flanking regions 3′ of the fixed SINE and 5′ of the variable SINE (Figure 2) (GenBank HQ910236, HQ910237, HQ910238, HQ910239).

The 2 SINE insertions are 95% similar to each other on average, excluding variability in the length of the poly-A tail. The fixed SINE is 94.8% similar to the family of canine SINEs (SINEC_Cf) described by Wang and Kirkness (2005), whereas the variable SINE is 92.3% similar to the Wang and Kirkness SINEC_Cf sequence. Variants within the SINEs, and between the fixed and variable SINEs, included single nucleotide polymorphisms and single nucleotide insertions/deletions. None of these polymorphisms appeared to affect the pigmentation phenotypes.

Individual Genotypes

Two hundred one dogs were genotyped for the *ASIP* alleles (Table 1), including the variable SINE insertion. All a^t black-and-tan ($n = 30$) and *a* recessive black ($n = 9$) dogs had only the larger fragment which included the variable SINE insertion. The dogs that are a^y fawn ($n = 48$) and a^w wolf sable ($n = 57$) all had at least one copy of the smaller fragment which lacks the variable SINE insertion. The subset of 46 heterozygous dogs with both the larger and the smaller fragments were either fawn or wolf sable in color (Table 1), with the exception of 4 hunting breed dogs which were solid black due to the K^B allele of *CBD103* or solid red due to an *e/e* genotype at *MC1R*.

All 8 dogs described as saddle tan were determined to be a^t/a^t by genotyping for the SINE insertion (Table 1). This genotype therefore occurs in dogs that are traditional black and tan and also in dogs that are saddle tan.

All 8 Schnauzers, whether their phenotype was salt and pepper, black and silver, or black were genotyped for the SINE insertion and found to be a^w/a^w wolf sable (Table 1).

Dogs of many sporting breeds exhibit coat colors of either solid phaeomelanin or solid eumelanin, with or without white spotting. These phenotypes are caused by an *e/e* genotype at *MC1R* (Newton et al. 2000) or the K^B allele

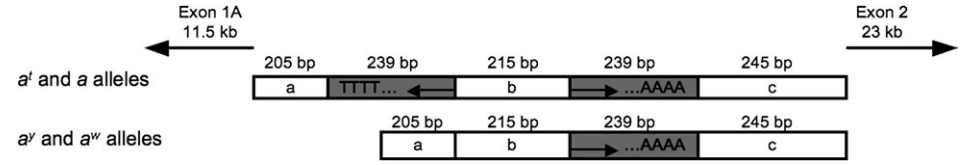

Figure 2. An illustration of an 1143-bp fragment, from 5′ of the *ASIP* start codon in exon 2, obtained from genomic DNA from a black-and-tan (a^t) Shetland Sheepdog and a wolf sable (a^w) Siberian Husky. Shaded boxes represent SINEs, with arrows denoting orientation. Segments a, b, and c of this fragment are present in all dogs.

of *CBD103* (Candille et al. 2007). These genotypes are epistatic to the *ASIP* alleles. Thirty-six dogs belonging to 12 sporting breeds were genotyped for the variable SINE insertion and the fawn a^y allele. Because the *a* allele is not expected to occur in sporting breeds, it was not genotyped in these dogs. Three alleles, a^t, a^y, and a^w, were present in these breeds (Supplementary Table 2).

Of the 35 breeds genotyped, the 5 that were predicted to be fixed for a^w: Siberian Husky, Alaskan Malamute, Swedish Vallhund, Norwegian Elkhound, and Keeshond were indeed found to be homozygous for absence of the variable SINE (Table 1). After excluding dogs that genotyped as having the a^y allele, 12 additional breeds were found to have one allele that did not have the variable SINE. Only one breed, the Eurasier, was found to have all 4 *ASIP* alleles.

Dominance Hierarchy of *ASIP* Alleles

The dominance hierarchy of the 4 *ASIP* alleles can be determined by observing the genotype results, relative to the phenotypes observed. A number of heterozygous dogs were genotyped, including 4 a^y/a^w, 28 a^y/a^t, 4 a^y/a, 11 a^w/a^t, 3 a^w/a, and 3 a^t/a dogs (Table 1 and Supplementary Table 1). Forty-seven of these dogs do not have the $K^B/\_$ or e/e genotypes that are epistatic to the *ASIP* alleles so were used to determine *ASIP* allele inheritance. All dogs with the a^y allele expressed a fawn phenotype, regardless of the second allele present, indicating that this is the top dominant allele of the hierarchy. Dogs with an a^w/a^w, a^w/a^t, or a^w/a genotype, all exhibited a wolf sable coat pattern, suggesting that a^w is dominant to a^t and *a*. Dogs with either an a^t/a^t or a^t/a genotype were black and tan. Only dogs with an a/a genotype expressed the recessive solid black phenotype. Therefore, the *ASIP* allele hierarchy was determined to be $a^y > a^w > a^t > a$.

Discussion

A black-and-tan phenotype is seen in mice and is caused by a 6-kb insertion in intron 1a of *ASIP* (Bultman et al. 1994). Similarly, in the dogs in this study with a black-and-tan phenotype or saddle tan phenotype, an insertion of approximately 239 bp was found in intron 1a. In dogs, the insertion is a SINE.

Previous research has demonstrated that some dog coat color patterns are caused by SINE insertions. A family of similar SINES, termed SINEC_Cf, is widely found throughout the canine genome (Wang and Kirkness 2005). A SINE insertion at the intron 10/exon 11 boundary of *SILV* causes the merle phenotype in dogs (Clark et al. 2006). The *SILV* SINE insertion, present in the reverse orientation, is predicted to alter exon splicing or lariat branching within intron 10 (Clark et al. 2006), as presence of a SINEC_Cf in the reverse orientation can introduce a 3′ splice acceptor site (Wang and Kirkness 2005). A SINE insertion in the forward orientation, located 3.5 kb upstream of the *MITF* 1M promoter, causes the solid white coloring in breeds such as Boxers and Bull Terriers (Karlsson et al. 2007). This same insertion causes piebald spotting and pseudo-Irish spotting in numerous other breeds (Schmutz et al. 2009). Schmutz et al. (2009) predicted that the *MITF* SINE, present in the forward orientation, may alter transcription or regulatory elements required for expression of *MITF*.

Multiple alternate first exons are responsible for variable transcripts of *ASIP* in mouse. Vrieling et al. (1994) report 4 alternate first exons in mice, 2 of which, 1A and 1A′ are associated with ventral specific production of phaeomelanin. The remaining 2, 1B and 1C, are associated with hair cycle specific production of phaeomelanin, resulting in banded hairs. To date only one first exon has been described in dogs (Kerns et al. 2004). This first exon is orthologous to exon 1A of mouse. It was previously described in fox (Vage et al. 1997).

Individual hairs of dogs that have an a^w or a^y allele have alternating bands of phaeomelanin and eumelanin along the hair shaft. Individual hairs of dogs that have only the a^t or *a* alleles do not alternate between pigment types. Both have only solid eumelanin hairs on the dorsal area of the torso. Both *ASIP* SINEs are located 3′ of the dog exon 1A, between exon 1A and the exon 2 start codon (Figure 2). Because the forward orientation fixed SINE is present with all *ASIP* phenotypes, it probably does not affect pigmentation. The location of the reverse orientation variable SINE, present in only the a^t and *a* alleles, may function to disrupt splicing of *ASIP* transcripts due to the 3′ splice acceptor site described by Wang and Kirkness (2005). Considering the mouse first exons described by Vrieling et al. (1994), the orthologous dog exon 1A is located upstream of the SINE insertions. The dog exon 1A, expected to affect ventral expression of *ASIP* in mice (Vrieling et al. 1994), is not impacted by the SINE insertions as dogs with the a^w, a^y, and a^t alleles express tan points. The mouse exons 1B and 1C, located between 1A and exon 2 and not yet identified in the dog, are responsible for hair cycle specific expression of *ASIP* (Vrieling et al. 1994),

Table 1 *ASIP* genotypes of 166 dogs of 23 breeds illustrating that the variable SINE is present in both the a^t and the a allele

Breed	Coat color	*n*	*ASIP* genotype	Presence of variable SINE	A82[a]	R96C[b]
a^w/a^w expected						
Keeshond	Wolf sable	11	a^w/a^w	No	A/A	R/R
Alaskan Malamute	Wolf sable	12	a^w/a^w	No	A/A	R/R
Norwegian Elkhound	Wolf sable	11	a^w/a^w	No	A/A	R/R
Siberian Husky	Wolf sable	7	a^w/a^w	No	A/A	R/R
Swedish Vallhund	Wolf sable	5	a^w/a^w	No	A/A	R/R
Multiple ASIP alleles expected						
Basset Hound	Solid red	1	a^t/a^t	Yes	A/A	R/R
	Fawn	3	a^y/a^t	Yes	S/A	R/R
Cardigan Welsh Corgi	Black and tan	2	a^t/a^t	Yes	A/A	R/R
	Fawn	2	a^y/a^y	No	S/S	R/R
	Fawn	1	a^y/a^t	Yes	S/A	R/R
Collie	Black and tan	3	a^t/a^t	Yes	A/A	R/R
	Fawn	2	a^y/a^y	No	S/S	R/R
	Fawn	5	a^y/a^t	Yes	S/A	R/R
Dachshund	Black and tan	4	a^t/a^t	Yes	A/A	R/R
	Fawn	1	a^y/a^y	No	S/S	R/R
	Fawn	2	a^y/a^t	Yes	S/A	R/R
Eurasier	Black	5	a/a	Yes	A/A	C/C
	Wolf sable	1	a^w/a^w	No	A/A	R/R
	Wolf sable	1	a^w/a^t	Yes	A/A	R/R
	Wolf sable	1	a^w/a	Yes	A/A	C/R
	Fawn	1	a^y/a^w	No	S/A	R/R
	Fawn	3	a^y/a^t	Yes	S/A	R/R
	Fawn	1	a^y/a	Yes	S/A	C/R
Finnish Lapphund	Black and tan	2	a^t/a^t	Yes	A/A	R/R
	Fawn	3	a^y/a^t	Yes	S/A	R/R
German Shepherd Dog	Black	3	a/a	Yes	A/A	C/C
	Black and tan	7	a^t/a^t	Yes	A/A	R/R
	Black and tan	2	a^t/a	Yes	A/A	C/R
	Wolf sable	6	a^w/a^t	Yes	A/A	R/R
	Wolf sable	2	a^w/a	Yes	A/A	C/R
Jack Russell Terrier	Black and tan	2	a^t/a^t	Yes	A/A	R/R
	Fawn	2	a^y/a^t	Yes	S/A	R/R
Pembroke Welsh Corgi	Fawn	1	a^y/a^t	Yes	S/A	R/R
Saluki	Black and tan	2	a^t/a^t	Yes	A/A	R/R
	Brown and tan	1	a^t/a^t	Yes	A/A	R/R
	Fawn	2	a^y/a^y	No	S/S	R/R
	Fawn	6	a^y/a^t	Yes	S/A	R/R
Shetland Sheepdog	Black	1	a/a	Yes	A/A	C/C
	Black and tan	3	a^t/a^t	Yes	A/A	R/R
	Black and tan	1	a^t/a	Yes	A/A	C/R
	Fawn	2	a^y/a	Yes	S/A	C/R
	Fawn	2	a^y/a^t	Yes	S/A	R/R
	Fawn	9	a^y/a^y	No	S/S	R/R
Dogs of miscellaneous colors						
Akita	Dark Fawn	1	a^y/a^w	No	S/A	R/R
	Dark Fawn	1	a^y/a^y	No	S/S	R/R
Border Collie	Banded hairs	1	a^w/a^w	No	A/A	R/R
Dachshund	Wild boar	1	a^w/a^t	Yes	A/A	R/R
Miniature Schnauzer	Black	2	a^w/a^w	No	A/A	R/R
	Black and silver	2	a^w/a^w	No	A/A	R/R
	Salt and pepper	3	a^w/a^w	No	A/A	R/R
Standard Schnauzer	Salt and pepper	1	a^w/a^w	No	A/A	R/R
Shar Pei	Patterned sable	1	a^w/a^w	No	A/A	R/R
Saddle tan pattern						
Airedale Terrier	Saddle tan	2	a^t/a^t	Yes	A/A	R/R
Basset Hound	Saddle tan	1	a^t/a^t	Yes	A/A	R/R
Beagle	Saddle tan	2	a^t/a^t	Yes	A/A	R/R
German Shepherd Dog	Saddle tan	1	a^t/a^t	Yes	A/A	R/R
Pembroke Welsh Corgi	Saddle tan	2	a^t/a^t	Yes	A/A	R/R

[a] 82S is indicative of the a^y allele.
[b] 96C is indicative of the a allele.

Table 2 *ASIP* alleles present in 35 dog breeds

Breed	FCI type	*ASIP* alleles a^y	a^w	a^t	a
Eurasier	Spitz	✓	✓	✓	✓
Border Collie	Herder		✓	✓	✓
German Shepherd Dog	Herder		✓	✓	✓
Dachshund	Dachshund	✓	✓	✓	
Golden Retriever	Retriever	✓	✓	✓	
Vizsla	Pointer	✓	✓	✓	
German Shorthair Pointer	Pointer		✓	✓	
German Wirehair Pointer	Pointer		✓	✓	
Brittany Spaniel	Pointer		✓	✓	
Akita	Spitz	✓	✓		
Shar Pei	Pinscher/Molossoid	✓	✓		
Keeshond	Spitz		✓		
Alaskan Malamute	Spitz		✓		
Norwegian Elkhound	Spitz		✓		
Siberian Husky	Spitz		✓		
Swedish Vallhund	Spitz		✓		
Standard Schnauzer	Pinscher/Molossoid		✓		
Miniature Schnauzer	Pinscher/Molossoid		✓		
Shetland Sheepdog	Herder	✓		✓	✓
Collie	Herder	✓		✓	
Cardigan Welsh Corgi	Herder	✓		✓	
Pembroke Welsh Corgi	Herder	✓		✓	
Finnish Lapphund	Spitz	✓		✓	
Jack Russell Terrier	Terrier	✓		✓	
Airedale Terrier	Terrier			✓	
Saluki	Sighthound	✓		✓	
Basset Hound	Scenthound	✓		✓	
French Brittany Spaniel	Pointer	✓		✓	
Large Munsterlander	Pointer	✓		✓	
Chesapeake Bay Retriever	Retriever	✓			
Labrador Retriever	Retriever			✓	
Beagle	Scenthound			✓	
English Springer Spaniel	Retriever			✓	
German Longhair Pointer	Pointer			✓	
Weimaraner	Pointer			✓	

Breed type is classified based on the Fédération Cynologique Internationale standards.

causing banding on the individual hairs. If regulatory exons orthologous to either mouse 1B or 1C exist in dogs, then the variable SINE insertion may disrupt those exons, preventing hair banding in dogs with the a^t and a alleles. A reverse variable SINE inserted prior to a regulatory exon, such as 1B or 1C, may cause alternate splicing of the exon, incorporating some of the SINE sequence into the regulatory exon. A disruption such as this in the regulatory exon responsible for producing phaeomelanin banding on individual hairs may prevent the *ASIP* expression required for such banding. This would be consistent with the a^t and a phenotypes seen in dogs. This suspected mode of action suggests that a regulatory exon may be located in close proximity to the variable SINE insertion. This provides information that may be valuable in identifying alternate first exons for dog *ASIP*. Retrotransposons have been shown to affect coat color phenotypes in dogs (Clark et al. 2006; Karlsson et al. 2007; Schmutz et al. 2009), but it is also possible that the *ASIP* SINE is in linkage disequilibrium with another polymorphism that is causative for the black-and-tan phenotype.

Utilizing formulas and methods suggested previously (Li et al. 1981; Yasue and Wada 1996; Chou et al. 2002), the divergence between the *ASIP* fixed SINE and the variable SINE is too great to suggest that the variable SINE is a direct duplication and inversion of the fixed SINE. We suggest that the variable SINE, present with the a^t and a alleles, resulted from a separate insertion event of a related SINE not identical to the fixed SINE already present in this region in all dogs.

Different breeds have different sets of *ASIP* alleles. Utilizing data collected in the course of this research, as well as previously published a^y data (Berryere et al. 2005), and breed study data (Schmutz, unpublished), the *ASIP* alleles present in the 35 breeds examined in this study could be ascertained (Table 2). Despite the presumption that it is the ancestral *ASIP* allele of dogs, only 7 breeds appear to be fixed for the a^w allele. This suggests that there has been a high level of divergence through mutation and selection at the *ASIP* locus in domestic dog breeds. The a^w allele was found in only an additional 11 breeds, as one of multiple *ASIP* alleles present. The a^t allele was found in 25 of the breeds studied. The a^y allele was found in 17 breeds in this study, in addition to another 16 breeds discussed by Berryere et al. (2005) but not utilized here.

The Fédération Cynologique Internationale (FCI) (2010) is the primary world federation of purebred dog registries. It was formed in 1911 and governs purebred standards for 84 member countries, recognizing 339 dog breeds. The FCI recognizes 10 breed types: herders, pinschers/molossoid breeds, terriers, dachshunds, spitz, scent hounds, pointers, retrievers, companions, and sight hounds. Based on these breed type categories, the a^w allele was found in spitz, herder, pincher/molossoid, dachshund, retriever, and pointer groups (Table 2). The a^y allele was found in a wider selection of groups, including the pointer, retriever, sight hound, scent hound, pinscher/molossoid, herder, dachshund, terrier, and spitz groups. The a^t allele was also found in a wide range of breed types, including the pointer, retriever, sight hound, scent hound, terrier, herder, dachshund, and spitz groups. The a allele was found only in herding breeds and one spitz breed in this study, though it has been documented in Samoyeds (Schmutz, unpublished data), another spitz breed, and more herding breeds not listed here (Kerns et al. 2004; Berryere et al. 2005). The narrow range of breed types in which the a allele is found in may suggest that it is a relatively recent mutation. The spitz breeds have been shown to be some of the earliest developed breeds (Parker et al. 2004). The high frequency of the a^w allele in many of the spitz breeds supports that the a^w allele is the ancestral *ASIP* allele in domestic dogs, despite the relatively small number of breeds with the allele.

Although the a^w allele can be considered the ancestral *ASIP* allele, we suggest that the a^t allele is the result of a relatively old mutation as well. The a^t allele is present in a wide range of breed types, indicating its occurrence prior to modern breed development.

Only one breed, the Eurasier, was found to have all 4 *ASIP* alleles (Table 2). The Eurasier is a relatively recent breed developed in the 1960s from the Wolfspitz, commonly known as the Keeshond, the Chow Chow, and the Samoyed. This accounts for the presence of the a^w allele from the Keeshond, the a^y allele from the Chow Chow, and the *a* allele from the Samoyed. The presence of the a^t allele in Eurasiers is less certain, though it may have arisen from either the Wolfspitz or Chow Chow, prior to current breed standards that prohibit the black-and-tan pattern in those breeds. The breeding history of the Eurasier is reasonably well documented (Schneider and Schneider 2010) and publicly accessible online pedigrees (http://www.berndschneider100845.de/) show the emergence of black-and-tan individuals from reported matings between Chow Chows and Wolfspitz. Unfortunately, coat color is not documented for all individuals, so we are unable to determine whether the black-and-tan coloration was introduced by the Chow Chow or the Wolfspitz.

Based on the discovery of the variable SINE in this study, which can be used to identify the a^t allele, all 4 of the alleles at *ASIP* can now be discriminated with DNA testing. The present data (Table 1) demonstrate that dogs with the saddle tan phenotype are a^t/a^t at *ASIP*. The difference between the traditional black-and-tan pattern and the saddle tan pattern is likely caused by a modifier gene that allows for the expansion of the phaeomelanin points. A similar phenomenon involving expansion of phaeomelanin regions in black-and-tan mice has been observed and shown to be caused by a deletion of 216 kb, spanning from intron 1 of *TBX15* to 148 kb downstream of the adjacent *mannose-6-phosphate receptor* pseudogene (Candille et al. 2004). The saddle tan phenotype in mice, referred to as droopy ear, occurs in conjunction with craniofacial abnormalities and decreased body size (Candille et al. 2004). These conditions are not present with the saddle tan phenotype in dogs, suggesting that a more minor mutation within this region may only affect pigmentation, without the additional skeletal malformations.

Somewhat surprisingly, the 3 phenotypes present in Miniature and Standard Schnauzers: salt and pepper, black and silver, and solid black were all found to be a^w/a^w at *ASIP*. Little (1957) predicted that the salt-and-pepper phenotype of Schnauzers may be caused by the a^w allele, though this suggestion has been discounted by many Schnauzer breeders. The solid black Schnauzers also have a K^B allele of *CBD103* (unpublished data), which is epistatic to the *ASIP* alleles. Considering the relatively rare occurrence of the a^w allele in dogs, this finding may play a role in understanding Schnauzer breed development and history.

The limited occurrence of the a^w allele in breeds such as Shar Pei, Border Collie, and Akita (Table 2), where it was unexpected, may be due to lack of selection against this color pattern or the high frequency of the dominant a^y allele in the Shar Pei and Akita. Most Shar Peis and Akitas have only the dominant a^y allele at *ASIP* (unpublished data), so the rare occurrence of the recessive a^w allele may remain hidden for generations. The rare phenotype of a^w/a^w dogs in these breeds may be misclassified as fawn due to the similarity of these phenotypes in breeds with shorter coat length. The presence of the a^w allele in sporting breeds, such as pointers and retrievers, was unexpected. Though because the K^B allele, fixed in most of these breeds, is epistatic to the *ASIP* alleles, it is likely that the a^w allele has persisted in the sporting breeds due to lack of selection against it.

The presence of the variable SINE insertion in both the a^t and the *a* alleles provides some insight into the evolution of the *ASIP* alleles (Supplementary Figure 1). The a^t allele does not have any coding region polymorphisms when compared with the a^w allele. The only genetic difference between the a^w and a^t alleles is the presence of the variable SINE insertion in a noncoding region of a^t. This suggests that the a^t allele arose from the a^w allele and not from a^y. Additionally, the *a* allele does not have the a^y polymorphisms, but it does have the variable SINE insertion of a^t. This indicates that the *a* allele arose from the a^t allele and differs from it solely by the point mutation in exon 4 (Kerns et al. 2004). The a^y allele arose independently from the a^w allele, through 2 point mutations in exon 4 (Berryere et al. 2004).

The characterization of the mutation responsible for the black-and-tan phenotype in dogs can lead to a better understanding of *ASIP* and how it interacts with *MC1R* and other modifier genes to produce the phenotypic variation in color seen in domestic dogs This research will continue with investigation of the SINE region of *ASIP* and its role in alternate transcript production. Exploration of potential modifier genes of *ASIP* may explain the variation seen within *ASIP* alleles, such as a^t/a^t saddle tan and the grizzle/domino phenotype in Afghan Hounds and Salukis that results from an interaction of *MC1R* and the a^t/a^t *ASIP* genotype (Dreger and Schmutz 2010).

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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