MITF and White Spotting in Dogs: A Population Study

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Abstract

This study was designed to determine if one of the variants found in our laboratory, or previously reported in microphthalmia-associated transcription factor (MITF), was associated with one or more spotting patterns in dogs. None of the rare variants found in the coding sequence consistently occurred in dogs of any particular spotting pattern. However, an insertion of a short interspersed nucleotide element (SINE) over 3000 bp 5’ of the MITF-M start codon (Karlsson et al. 2007) did fit with random spotting in many dog breeds. Most (319/324) dogs of 45 breeds fit 1 of 2 inheritance patterns. All dogs that were homozygous for the SINE had white markings that either covered at least the ventral surface (mantle pattern) or most of the body (piebald or extreme white spotting). In most breeds, dogs heterozygous for the SINE insertion were solid colored or had minimal white, such as on the toes, but in some others, heterozygotes had white undersides, often with a white collar in the pattern called pseudo-Irish by Little (1957). However, none of the 15 dogs of 5 breeds in which all individuals have markings known as Irish spotting had the SINE insertion. Finally, we studied RNA expression in skin. The 2 MITF-M forms, M+ that contains an extra 18 bp that adds 6 amino acids between exons 5 and 6 and the M− form, were present. MITF-M is considered to be specific to melanocytes but was found in skin from a white Samoyed. A putative pseudogene containing exon 1M was also identified.

Key words: CFA20, coat color, Irish spotting, microphthalmia-associated transcription factor, piebald, white spotting

There are several patterns involving white markings in dogs. Little (1957) postulated that there was a single locus for white spotting in dogs, which he designated the S locus. He further suggested that 4 alleles explained the major patterns (Figure 1). S is the top dominant allele in the dominance hierarchy and causes solid or self-colored dogs. The next allele is s or “Irish spotting” that is a pattern in which there are white undersides, often a white neck collar, and sometimes white facial markings. The third allele is s′ or piebald spotting in which random white spots occur on the body of the dog. The fourth allele is s″ or extreme white in which the dog is almost entirely white but usually has at least some color on the head. Furthermore Little (1957) described a “pseudo-Irish” pattern that looks similar to the Irish spotting pattern but occurs in dogs that are S/s′ heterozygotes (Figure 2, middle dog).

Little (1957) also discussed that plus and minus modifiers may explain extreme white as an extreme case of piebald and that on the other hand dogs with minimal white markings on the toes, for example, could still have an S/S genotype with minus modifiers. Pape (1990) also invoked plus and minus modifiers to explain the variation in piebald spotting in Newfoundlands.

Recently, 3 groups independently published that at least some forms of white spotting in dogs cosegregated with the gene microphthalmia-associated transcription factor (MITF) (Karlsson et al. 2007; Leegwater et al. 2007; Rothschild et al. 2006). Karlsson et al. (2007) and Leegwater et al. (2007) used a genome scan to map boxer patterns of white markings to CFA20, near MITF; Karlsson et al. (2007) then expanded their study to bull terriers to refine the chromosomal interval. They used haplotype analysis to suggest sets of variants present in some of the white spotting patterns. Rothschild et al. (2006) used a candidate gene approach and showed that families of Newfoundlands and crossbred dogs cosegregated for single nucleotide polymorphisms (SNPs) found in MITF and the presence or absence of piebald spotting.

MITF is a gene involved in multiple developmental processes. It regulates the differentiation of neural crest–derived melanocytes, optic cup–derived retinal pigment epithelium, and bone marrow–derived mast cells and osteoclasts (Hodgkinson et al. 1993; Steingrímsson et al. 1994; Moore 1995). MITF affects coat color in mice, with many mutations leading to coat color dilutions, white spotting, or complete loss of pigmentation (Moore 1995; Yasumoto et al. 1998). Other mouse mutations cause reduced eye size, failure of secondary bone resorption, and early onset of deafness (Hodgkinson et al. 1993; Hughes et al. 1993).
The basic MITF gene structure has 9 exons (Supplementary Figure 1) and contains a basic helix-loop-helix leucine zipper protein structure (Hodgkinson et al. 1993). MITF is a gene with at least 6 alternate transcripts expressed in different tissues (Amae et al. 1998). All isoforms are identical from exons 2–5 and 6–9. MITF-M, contained in melanocyte lineage–specific cells, has an alternatively spliced exon 6A that consists of an insertion of 6 amino acids, ACIFPT (Amae et al. 1998; Yasumoto et al. 1998). There are 2 types of MITF-M, and both are suggested to be identical except for the presence or absence of this insertion (Yasumoto et al. 1998). The notation used to differentiate between these 2 forms is MITF-M+ for the form with the insert and MITF-M− for the form without the insert, as used in Goding (2000).

In this study, we primarily focused on one variant in the 5′ untranslated region (UTR) reported by Karlsson et al. (2007). This variant is a short interspersed nucleotide element (SINE) insertion 3167 bp before the start codon of exon 1M (Supplementary Figure 1). We especially wanted to determine if this SINE insertion could be used as a marker to test for carriers of piebald spotting, which is considered to be recessive to solid color. We also tested its presence or absence in dogs with other spotting patterns. In addition, we studied litters of dogs that included either individuals that were or were not white spotted or that had different white spotting patterns to confirm that MITF variants cosegregated with these patterns.

**Materials and Methods**

**Dogs and Families**

We used cDNA prepared from skin biopsies, tail dockings, and surgeries collected from previous coat color studies (Schmutz et al. 2002) and placed in liquid nitrogen or RNAlater (Ambion) within 20 min of collection to obtain RNA sequence of MITF-M.

Cheek brush DNA samples (Epicentre, Madison, WI) from 12 dog families that segregated for white spotting patterns were obtained from dog breeders and owners and used for genotyping to confirm cosegregation with MITF variants. These included Chinese Shar-Pei (1), Collie (2), French Bulldog (2), “German Shepherd” (1), Great Dane (1), Icelandic Sheepdog (1), Italian Greyhound (2), and Newfoundland (2) families. Photographs were also supplied to document the pattern of white markings or lack thereof. In total, 324 individual dogs of 45 breeds were genotyped for the SINE insertion.

**RNA Isolation and cDNA Preparation**

mRNA was extracted from skin biopsies as previously described (Berryere et al. 2003) using the TRIzol Reagent Isolation of Total RNA method (Gibco, Burlington, ON, Canada). The RNA was stored at −80 °C until cDNA preparation. RNA samples were digested with DNase I
(Gibco), and then cDNA was prepared as previously described (Berryere et al. 2003) using Superscript Preamplification System for First Strand cDNA Synthesis (Gibco). The samples were stored at −80 °C.

DNA Sequencing

Polymerase chain reaction (PCR) products greater than 1000 bp were resolved on a 1% agarose gel, whereas products less than 1000 bp were resolved on a 2% agarose gel, and the amplified bands were excised. Products were isolated using the QIAquick method (Qiagen, Mississauga, ON, Canada) and quantified on a 1% agarose gel using a DNA mass ladder. PCR fragments were 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 computer software (Gene Codes Corporation, Ann Arbor, MI).

Genotyping

Various primers were designed for the purposes of sequencing MITF, as well as for detecting and isolating both MITF-M forms (Supplementary Table 1). Some of these primers were designed off of cattle MITF genomic sequence, prior to the release of the dog sequence. Others were designed off of dog sequence obtained in early sequence attempts using such primers or from later dog sequence in GenBank.

The PCR of 15 ml contained 50–100 ng of DNA template, 1.5 ml of 10× PCR buffer (Invitrogen, Carlsbad, CA), 1–3 mM MgCl₂ (Invitrogen), 0.2 mM dNTP (Invitrogen), 0.5 U of Tag DNA polymerase (Invitrogen), 0.66 pmol of each primer, and 9.6 μl of ddH₂O. The PCR parameters consisted of an initial denaturation at 94 °C for 4 min, followed by 28 cycles of 50 s at 94 °C, 50 s at the appropriate annealing temperature for the primer pair (Table 1), and 50 s at 72 °C, followed by a final 4-min extension at 72 °C. To determine their relative amounts of both MITF-M forms, the products were resolved on a 4% DNA agar gel.

The microsatellite in the 5’ UTR was detected by using the appropriate primers in Supplementary Table 1 at 61 °C. A product of approximately 242 bp was run on a polyacrylamide gel to determine the alleles.

Karlsson et al. (2007) also discuss a length polymorphism in the 5’ UTR. We attempted to detect this by sequencing but were unable to reliably repeat our determination of the number of base pairs in the same individual. We also tried to run an amplified product that

Table 1. Genotypes of 79 dogs from breeds that appear to have codominant inheritance of white markings

<table>
<thead>
<tr>
<th>Breed or “type” spotting</th>
<th>del/del, Minimal torso white</th>
<th>ins/del, Pseudo-Irish spotting</th>
<th>ins/ins, Extreme white</th>
</tr>
</thead>
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<tr>
<td>Collie</td>
<td>4</td>
<td>12</td>
<td>3</td>
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<tr>
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<td>13°</td>
<td>7°</td>
<td>7</td>
</tr>
<tr>
<td>Italian Greyhound</td>
<td>2</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>“Shetland Sheepdog”</td>
<td>13°</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

° One additional Great Dane with a del/del genotype, relative to the SINE, had Pseudo-Irish phenotype and one additional one with an ins/del genotype shows extreme white spotting.

Figure 2. Italian Greyhound littermates (top row) and Collie parents and pup (bottom row) representing the codominant inheritance of spotting and their genotype for the SINE insertion 5’ of MITF: Left: solid or minimal white markings, center: pseudo-Irish pattern, right: extreme white with color head and minimal pigmented areas.
contained this region on a polyacrylamide gel and could not reliably show inheritance of it in families.

Results

**MITF-M Sequence**

The complete mRNA sequence from the MITF-M+ isoform was obtained (GenBank NM_001003337). Skin from an Australian Shepherd was used to prepare the cDNA, and this breed is fixed for white undersides, typically known as Irish spotting.

MITF-M+ contains an additional 18 bp or 6 amino acids known as exon 6A (GenBank AY240952). We obtained both MITF-M+ and MITF-M− from cDNA prepared from pigmented dog skin from 2 Australian Shepherds and unpigmented skin of 1 Samoyed, in approximately equal proportions (Figure 3). It was assumed that the primers used would bind to both forms with equal opportunity, and therefore, the relative abundance of each form would be subject to relative abundance within the sample.

Although we identified 2 variants in exonic sequence, neither affected the amino acid sequence. There is a c.771C>T variant in exon 8 and a c.*72A>G that is 72 bp past the stop codon in exon 9. None were consistently associated with any pattern of white markings.

Two microsatellites were detected in GenBank AAEX01006754 that contains MITF genomic sequence. One was a GAAA repeat 627 bp after the start of exon 1D and over 58,000 bp before exon 1M. It was heterozygous in some parents and therefore useful in those families to follow cosegregation. The other was a GT repeat detected in intron 6 that was not very polymorphic in the parents and hence did not yield further data.

**Putative Pseudogene**

An additional variant was discovered in a PCR product from genomic DNA that is very similar to MITF exon 1M (GenBank EU726637). However, it includes an SNP in the codon that would normally be the start codon (Supplementary Figure 2). Exon 1M is 158 bp, and there is a different nucleotide at 11 positions, and 4 codons are missing in the putative pseudogene. However, the exon 1M coding sequence is identical, except for a variant in the start codon. The sequence after exon 1M is much less homologous. There are different nucleotides at 15 of the first 80 bp, and then the homology is minimal (Supplementary Figure 2).

Because these 2 sequences could both be obtained from the same dogs, it was concluded that one is a pseudogene. The putative pseudogene sequence was obtained from 21 different dogs of 10 breeds, including dogs with and without white markings. Comparison of sequence obtained from mRNA from skin and genomic DNA from some of the same dogs made it possible to confirm which sequence was the actual exon 1M.

The location of this putative pseudogene has not been determined. Using basic local alignment search tool (BLAST) to the dog Build 2.1 sequence in the National Center for Biotechnology Information database, this segment matches 2 bacterial artificial chromosome sequences in GenBank (AC191512 and AC191629) well, but not perfectly.

**Family Studies**

Two Italian Greyhound families of 7 and 5 pups and 2 Collie families of 10 and 3 pups were studied, and both fit a codominant inheritance pattern as illustrated in Figure 2. Cosegregation with alleles at the microsatellite identified in the 5’ UTR and the SINE insertion and phenotype were found in the Italian Greyhounds (θ = 0.01, logarithm of the odds [LOD] = 2.96). Although all Collies have Irish spotting or white undersides, there were 2 families that showed additional white markings, also inherited in a codominant pattern that cosegregated with the SINE insertion. The Collie parents were homozygous for the microsatellites. The dogs heterozygous for the SINE had a much larger white neck collar, and the ins/ins homozygotes were mostly white, with pigmented heads and 1 or 2 pigmented body areas, a pattern called color headed in this breed (Figure 2) (θ = 0.01, LOD = 3.25).

One family of 8 Chinese Shar-Pei pups was solid or piebald spotted and cosegregated with the SINE insertion and the 5’ microsatellite. ins/ins Homozygotes were piebald, but both heterozygotes and the dogs without the SINE insertion were solid colored. Two families of 4 and 5 Newfoundland pups cosegregated for the solid and piebald spotting called Landseer in this breed. In this breed, some but not all heterozygotes had white hair on the toes and/or a white chest spot. All Landseer Newfoundlands in these families were homozygous for the SINE insertion.

Although German Shepherd Dogs do not typically have any white markings, there are some lines in which piebald markings occur. Both piebald, but none of the 4 pups nor

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**Figure 3.** Agarose gel showing a PCR product of MITF-M that spans exons 5 and 6 of mRNA prepared from dog skin. All 3 samples from different dogs (2 Australian Shepherds and 1 Samoyed) include the 18 bp known as exon 6A that distinguishes the M+ form, as well as the M− form in which it is absent.
2 parents without white were SINE homozygotes, in one such family of 6 pups.

Two families of 5 French Bulldog pups cosegregated for solid and piebald spotting. Again all the piebald individuals were SINE homozygotes. One dog had a pattern known as mantle in some breeds (Figure 4), which is somewhat similar to Irish spotting, although the white markings cover the undersides and more of the thigh and torso. This dog was also a SINE homozygote.

Mantle markings also occur commonly in Great Danes. Contrary to the French bulldog and 1 Large Munsterlander with markings that might be called mantle (Figure 4), 3 similarly marked Great Danes in a large family of 15 pups from 1 sire and 2 dams were heterozygous for the SINE, but another did not have a SINE.

Although most of the families cosegregated “perfectly” for a variant in MITF or a microsatellite near MITF and forms of white spotting, in one family of Icelandic Sheepdogs, the white marking phenotype did not cosegregate perfectly with the genotype at the SINE insertion for either dominant or codominant inheritance (Supplementary Figure 3). Note that the dam and sire of these 8 Icelandic Sheepdog pups were verified by parentage testing, even though one pup’s genotype of no SINE does not seem possible given the sire’s homozygous SINE insertion genotype.

SINE Insertion Studies in Individual Dogs

Dogs of some breeds are fixed for the Irish spotting pattern of white undersides, a white band around all or most of the neck and a white muzzle and/or blaze. None of the 15 dogs fixed for this phenotype had a SINE insertion. These included 9 Australian Shepherds, 1 Boston Terrier, 2 Caucasian Mountain Dogs, 1 Corgi, and 2 Nova Scotia Duck Tolling Retrievers. Old English Sheepdogs and Bearded Collies have a different pattern in which the whole head and front leg across the shoulder region is white with white undersides. The 3 dogs of these breeds also did not have the SINE insertion. Note that some breeds such as collie are also fixed for Irish spotting but can have additional white markings (Figure 2), and those dogs with more than typical Irish spotting did have the SINE insertion.

As discussed in the section on families, in 2 breeds (Collie and Italian Greyhound), the presence of the SINE insertion fit a codominant pattern of inheritance. The dogs homozygous for the SINE were mostly white, often with some pigmentation on the head. The heterozygotes had a pattern with white undersides and a white neck band, often with a blaze on the head. The dogs without the SINE insertion were solid colored (Table 1). Although the heterozygotes had a phenotype similar to Irish spotting, this pattern is known as pseudo-Irish by Little (1957) because the inheritance pattern is codominant. It also seems that the Shetland Sheepdog and Great Dane fit this codominant pattern. If so, the Great Dane in Figure 4 with the mantle pattern could also be called pseudo-Irish.

Random spotting occurred in 17 of the breeds we studied. All ins/ins homozygous dogs in these breeds had random white spots that covered some or most of the torso (Table 2). This pattern is often called piebald spotting, but this term is not used in all these breeds (Table 2).

In some breeds, all individuals have random white spotting. Seventy individuals of 13 such breeds were homozygous for the SINE insertion. As mentioned earlier, most of these dogs would be classified as “piebald” in phenotype but a few were called “mantle,” which more closely resembles Irish spotting or pseudo-Irish spotting (Figure 4).

A few dogs (1 Akita, 1 Jack Russell Terrier, 2 Norrbottenspets, 2 Great Danes, in addition to the 3 Icelandic Sheepdogs) had a SINE insertion genotype that did not fit with their expected spotting phenotype in comparison to all 315 other dogs tested (Supplementary Figure 4).

Discussion

Bismuth et al. (2007) have recently suggested that MITF-M+ and MITF-M− have different effects. MITF-M+ appears to inhibit DNA synthesis or be antiproliferative, whereas MITF-M− has little or no such effect. MITF-M+ binds to E box regions of promoters with higher affinity than does MITF-M− (Hemesath et al. 1994). It is not clear

Figure 4. Three dogs with a pattern called mantle and their genotype for the SINE insertion 5’ of MITF. Left: French Bulldog, middle: Large Munsterlander, right: Great Dane.
why both seem to be present in relatively equal ratios in skin of dogs, as shown in this study (Figure 3), and in mice (Bismuth et al. 2007). The mouse mutant (MITF-M\textsuperscript{bw}) has no production of MITF-M\textsuperscript{+} and therefore only produces MITF-M\textsuperscript{−} when homozygous. Mice that have one copy of this MITF-M\textsuperscript{bw} allele and one null allele are white spotted, not pure white (Bismuth et al. 2007). MITF-M\textsuperscript{bw} was a spontaneous mutation in which one C residue is inserted near the 3’ end of intron 5 that appears to cause a splicing problem that eliminates exon 6A (Steingrimsson et al. 1994).

One dog studied was a Samoyed, and this breed always has a white coat. Because equal proportions of MITF-\textsuperscript{M+} and MITF-\textsuperscript{M−} were also present in this dog (Figure 3), it would appear that this dog has melanocytes in the skin that are not producing pigment. Although white fur is often believed to occur where no melanocytes are present, this suggests that there are other explanations as well. Four Samoyed were genotyped for the SINE insertion, and although 3 were ins/ins homozygotes, 1 was heterozygous. In a previous study, Samoyed were found to have a melanocortin 1 receptor (MC1R) genotype of e/c and an agouti signal peptide (ASIP) genotype of a/a which may result in production of neither eumelanin nor phaeomelanin by their melanocytes (Schmutz and Berryere 2007). MC1R e/c genotype has been shown to be epistatic to the production of black pigment from dogs with ASIP a/a or DEFB103 K\textsuperscript{B}, but whether the ASIP a/a genotype is epistatic to phaeomelanin production or not is less clear because most breeds with an ASIP a allele do not have the MC1R e allele.

SINE insertions have been reported to be important mutations in some phenotypes in dogs. Clark et al. (2006) reported that a SINE followed by a run of thymine in the last intron of the \textit{SILV} gene was responsible for the merle phenotype in dogs. However, the SINE insertion alone did not lead to this phenotype. When both the SINE and run of thymine residues were present, the last exon was not transcribed properly (Clark et al. 2008). A SINE insertion in intron 3 of \textit{HCRTR2} that caused exon 4 to be skipped was associated with narcolepsy in Doberman Pinschers (Lin et al. 1999). A SINE insertion in exon 2 of \textit{PTPLA} is associated with centronuclear myopathy of Labrador Retrievers (Pelé et al. 2005). Wang and Kirkness (2005) report that the SINE-CF is exceedingly common throughout the canine genome. They report that when the SINE is inserted in the reverse orientation, as the SINE in \textit{SILV} is, a splice acceptor site occurs that can lead to splice-junction errors.

The SINE insertion studied here is 3167 bp before the start codon of \textit{MITF-M} (Karlsson et al. 2007). The SINE is in the typical orientation with several adenine nucleotides following it, interrupted by thymine nucleotides. Exon 1B is the next most 5’ exon, and it begins 55 710 bp before exon 1M. Therefore, this SINE could affect the melanocyte form, and not the other forms, allowing for none of the eye development, hearing, etc., problems that occur in some mutant mice (Bharti et al. 2006). Therefore, it would appear that the SINE insertion in the 5’ UTR could be a mutation that causes white markings or that it is a marker for some other mutation or set of mutations that are actually the causative mutation(s).

In mice, the black-eyed, white-furred mutant (\textit{Mitf}\textsuperscript{mi-bw}), which is also deaf, has an insertion of a long interspersed nucleotide element (LINE) (Yajima et al. 2003). The particular LINE L1bw that the authors show reduced the amount of one of the eye forms (MITF-A) and one of the forms for the pigmented epithelium (MITF-H) and resulted in the absence of the melanocyte form (MITF-M) through altered splice patterns.

Although 315 of the 324 dogs genotyped for the SINE fit a codominant or dominant/recessive inheritance pattern, there were 6 exceptions among the individual dogs and 3 in the Icelandic Sheepdog family. As such, can it be accurately used in DNA testing to predict one or more spotting phenotypes in at least some breeds? We would suggest that it could be useful in the 19 breeds in which the inheritance pattern of random spotting appears to be recessive (Table 2). Heterozygous ins/del dogs could be detected and bred, or not, depending on the desire of the owner to obtain pups with random spotting.

At first glance, the SINE insertion seems to be involved in 2 different forms of inheritance of white markings. However, Little (1957) suggested that there was a pseudo-Irish pattern that occurred in dogs heterozygous for a solid allele \textit{S} and a piebald allele \textit{s}. If one accepts that pseudo-Irish pattern occurs in only some breeds (Table 1), as he postulated, and that most breeds have a dominant–recessive relationship between these 2 alleles (Table 2), then the SINE insertion may be a marker for the \textit{s} allele with greater than 95% accuracy. There is clearly a large range of spotting patterns in dogs, and dog owners of different breeds used

### Table 2

<table>
<thead>
<tr>
<th>Breed or “type”</th>
<th>del/del</th>
<th>ins/del</th>
<th>ins/ins</th>
<th>Term for spotted</th>
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<tbody>
<tr>
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<td>6</td>
<td>7</td>
<td>Particolor</td>
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<tr>
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<td>1</td>
<td>2</td>
<td>Piebald</td>
</tr>
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<td>2</td>
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<tr>
<td>Whippet</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>Spotted</td>
</tr>
</tbody>
</table>

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**Table 2.** Genotypes of 151 dogs from breeds in which individuals with random spotting were homozygous for the SINE insertion (ins/ins). Dogs without the SINE and heterozygotes had minimal or no white markings.
different terms for the pattern of random spotting (Table 2). To add to the complexity there are similarities among spotting patterns in dogs with different genotypes. Dogs with pseudo-Irish pattern had a $S^/$ or ins/del genotype. Dogs with Irish spotting did not have a SINE insertion. Additionally, there are the dogs with a mantle pattern (Figure 4), in which dogs of at least some breeds are ins/ins homozygotes. Dogs of the phenotype of white undersides and minimal to no other white markings was the most difficult to predict genotype at the SINE insertion, especially in dogs of mixed breed ancestry.

Little (1957) postulated an $s^r$ allele that he believed to cause extreme white spotting. On the other hand, he suggested that some dogs with an extreme white phenotype may simply be of $s^p/s^p$ genotype with modifiers. All dogs in this study with an extreme white spotting phenotype, such as the Japanese Chin in Figure 1, were ins/ins homozygotes. The Cocker Spaniel, which represents the piebald allele in Figure 1, is likewise an ins/ins homozygote. The German Longhair representing the $s$ allele was an ins/del heterozygote, and the Shetland Sheepdog representing the $s^r$ allele did not have a SINE insertion. The data from the dogs in this study therefore fit better with the absence of a specific $s^r$ allele at $MITF$.

Although C. C. Little is the most often quoted author for the genetics of dog coat colors, an earlier book was published by Ojvind Winge in 1950. Winge (1950) stated that he believed that there are 2 alleles at the locus for white mottling: $t$ for totally colored or nearly so and $t$ for mottled. Our data would fit well with the SINE insertion $5^{'MITF}$ as the $t$ allele, as he describes it.

We are unable to determine if the $s$ allele exists as an allele of $MITF$. Karlsson et al. (2007) reported that dogs with Irish spotting did not have a SINE insertion but did have a length polymorphism that was of a different size range than solid dogs in their study but overlapped with the size found in the piebald and extreme white spotted dogs. We were unable to replicate this length polymorphism reliably and so can neither support nor refute this length polymorphism in the absence of the SINE insertion as the $s$ allele. Because most breeds that have this Irish spotting phenotype are fixed for it, albeit perhaps with or without additional white, family studies using cosegregation were not possible. Crossbred dogs would be useful to study this pattern. Metallinos and Rine (2000) used crossbred dogs derived from Newfoundlands and Border Collies and were able to exclude 2 good candidate genes, $KIT$ and $EDNRB$, as the genes causing Irish spotting.

Several genes in the pigmentation pathway interact with $MITF$ (reviewed in Lin and Fisher 2007). These include $LEF1$, $PAX3$, $SOX10$, and $CREB$. Both $PAX3$ and $SOX10$ have been associated with white spotting in mice (Vogan et al. 1993; Stanchina et al. 2006) and were chosen as candidate genes for spotting in dogs by Rothschild et al. (2006). Although they found variants in both these genes, there was no cosegregation with the piebald spotting phenotype they studied. Similarly, Brenig et al. (2003) studied $PAX3$ in relation to Dalmatian deafness, often thought to be related to their spotting pattern, and did not find an association.

How could a mutation such as the SINE insertion in $MITF$ cause the absence of pigmentation in only some hair patches and not in others? Barsh (2007) suggests that the spotting allele of $MITF$ is likely a regulatory mutation that alters expression of $MITF$ and potentially melanocyte survival during embryogenesis and fetal development. Although no Dalmatians were included in this study, Karlsson et al. (2007) included this breed that develops their characteristic small spots or ticks in the first few weeks after birth and found them to be ins/ins homozygotes. Their Dalmatian data would suggest that at least some melanocytes in the white areas survived into adulthood but were temporarily prevented from, or delayed in, expressing pigmentation. Other breeds, including some in this study (most listed in Table 3), which also have ticking, do not show these small pigmentation areas at birth and were homozygous for the SINE insertion.

If the variant altering the start codon of exon 1M had occurred in the actual gene and not a pseudogene, then it would have fit well as a possible explanation for random spotting. If the SINE somehow reroutes transcription to that alternate exon 1M, then when the chromosome bearing $ATA$ instead of ATG is used, no $MITF$-M would be expressed, and the hair formed would presumably be white. This would also explain how there could occasionally be dogs that do not have the expected white spotting pattern for the genotype at the SINE insertion. Such dogs might not have the $ATA$ variant at all and not be as white as their $MITF$ genotype might predict, or the chromosome bearing the $ATA$ variant was transcribed more or less than the average half of the time and lead to dogs with more or less white than predicted. However, for this hypothesis to explain white spotting, including the discrepant dogs, the effects would have to occur at an early stage of coat color development because dogs do not change their pattern substantially from a few months of age onward through adulthood. However, based on the flanking sequence of this alternate exon 1M in comparison to the sequence obtained

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borzoi</td>
<td>10</td>
</tr>
<tr>
<td>Brittany Spaniel</td>
<td>2</td>
</tr>
<tr>
<td>Clumber Spaniel</td>
<td>1</td>
</tr>
<tr>
<td>English Pointer</td>
<td>2</td>
</tr>
<tr>
<td>English Setter</td>
<td>2</td>
</tr>
<tr>
<td>Jack Russell Terrier</td>
<td>5</td>
</tr>
<tr>
<td>Japanese Chin</td>
<td>12</td>
</tr>
<tr>
<td>Large Munsterlander</td>
<td>17</td>
</tr>
<tr>
<td>Papillon</td>
<td>2</td>
</tr>
<tr>
<td>Polish Lowland Sheepdogs</td>
<td>3</td>
</tr>
<tr>
<td>Small Munsterlander</td>
<td>2</td>
</tr>
<tr>
<td>Spinone</td>
<td>2</td>
</tr>
<tr>
<td>Stabyhoun</td>
<td>10</td>
</tr>
</tbody>
</table>

* One additional Jack Russell Terrier had a heterozygous genotype (ins/del).
from mRNA prepared from skin, it is more likely a pseudogene. This pseudogene does not appear to be in the current 2.1 assembly, and so its location was not determined using BLAST search.

Tsuchida et al. (2008) reported that they had located the MITF distal enhancer (MDE) of the canine MITF-M gene about 14 kb 5¢ of exon 1M by comparison with the human sequence. It is approximately 9 kb before the SINE insertion. Watanabe et al. (2002) identified the MDE, and they define the promoter region of MITF-M as beginning with this MDE, which in turn would suggest that the SINE insertion in some dogs lies within the promoter region. The MDE binds to SOX10. They showed that MITF-M and SOX10 were expressed in melanoblasts migrating to the otic vesicles and epidermis by in situ staining of 11.5-day mouse embryos. However, by day 13.5, SOX10 was no longer expressed. They further suggest that SOX10 binding to the MDE caused conformational changes, or DNA bending, that might facilitate interaction with transcription factors closer to exon 1M. If their theory is correct, then the insertion of a SINE would alter this distance and may change the resulting proximity to the more proximal transcription factors. We suggest that polymorphisms in SOX10 may alter the binding to this MDE region and/or the more proximal region, and hence, studies on the variation in canine SOX10 are currently underway.

The SINE insertion 5¢ of MITF-M first described by Karlsson et al. (2007) was associated with white markings in many and diverse breeds in this study, suggesting that it is an "old" mutation. There is considerable debate about the age of particular breeds. However, the Chinese Shar-Pei, Akita, and other Asian dogs are typically considered to be among the oldest breeds (Parker et al. 2004). The SINE insertion has been found in individuals with white markings in these breeds.

Supplementary Material
Supplementary Figures 1–4 and Table 1 can be found at http://www.jhered.oxfordjournals.org/.

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