

# Agouti Sequence Polymorphisms in Coyotes, Wolves and Dogs Suggest Hybridization

SHEILA M. SCHMUTZ, THOMAS G. BERRYERE, JODI L. BARTA, KIMBERLEY D. REDDICK, AND JOSEF K. SCHMUTZ

From the Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Canada S7N 5A8 (Schmutz, Berryere, and Reddick); the Department of Anthropology, McMaster University, Hamilton, Ontario, Canada L8S 4L9 (Barta); and the Centre for Studies in Agriculture, Law and the Environment, University of Saskatchewan, Saskatoon, Canada S7N 5A8 (Schmutz).

Address correspondence to S. M. Schmutz at the address above, or e-mail: sheila.schmutz@usask.ca.

Domestic dogs have been shown to have multiple alleles of the Agouti Signal Peptide (*ASIP*) in exon 4 and we wished to determine the level of polymorphism in the common wild canids of Canada, wolves and coyotes, in comparison. All Canadian coyotes and most wolves have banded hairs. The *ASIP* coding sequence of the wolf did not vary from the domestic dog but one variant was detected in exon 4 of coyotes that did not alter the arginine at this position. Two other differences were found in the sequence flanking exon 4 of coyotes compared with the 45 dogs and 1 wolf. The coyotes also demonstrated a relatively common polymorphism in the 3' UTR sequence that could be used for population studies. One of the *ASIP* alleles (R96C) in domestic dogs causes a solid black coat color in homozygotes. Although some wolves are melanistic, this phenotype does not appear to be caused by this same mutation. However, one wolf, potentially a dog–wolf hybrid or descendant thereof, was heterozygous for this allele. Likewise 2 coyotes, potentially dog–coyote or wolf–coyote hybrid descendants, were heterozygous for the several polymorphisms in and flanking exon 4. We could conclude that these were coyote–dog hybrids because both were heterozygous for 2 mutations causing fawn coat color in dogs.

Two of the most common wild canids in Canada are the wolf (*Canis lupus*) and the coyote (*Canis latrans*). Both share similar coat coloration of banded hairs, considered therefore to be the wild-type coat color of the *Agouti* locus. Some breeds of domestic dogs such as German Shepherd dogs and Siberian Huskies also share this coat coloration, typically called sable in those breeds, but many other breeds of dogs have diverged from this ancestral pattern. Recently we have presented evidence that the fawn coat coloration common to many dog breeds is caused by mutations in 2 adjacent amino acids in exon 4 of the *Agouti Signal Peptide* (*ASIP*) gene, A82S and R83H (Berryere et al. 2005). Another allele of *ASIP* is commonly known as “recessive black” or “nonagouti.” Such solid

black dogs are homozygous for a R96C mutation in exon 4 (Kerns et al. 2004; Berryere et al. 2005). This genotype occurs in all the black dogs of a few breeds such as the German Shepherd dog and the Shetland Sheepdog and in only some of the black dogs of a few breeds such as the Groenendael, the Schipperke, and the Puli. This represents a rare form of black in the entire domestic dog population, however.

Some dark wolves are referred to as “black.” Closer examination of photographs, live animals, or pelts reveals that such wolves are not uniformly black, however, but only “melanistic” in the sense that they show more black and much less reddish undercoat than other wolves. We wished to document whether the R96C mutation occurred in melanistic wolves and was therefore likely an “old” mutation or whether it arose later, after domestication. If it did indeed arise later, then this variant may provide a marker for dog–wolf hybridization. Because it occurs in relatively few dog breeds (Berryere et al. 2005), we predicted that it arose relatively recently.

In order to document the agouti genetic sequence most correctly considered “wild type” in the domestic dog, we sequenced exon 4 in the coyote (GenBank AY691404), and the complete *agouti* coding region of exons 2–4 in the wolf (GenBank DQ288670, DQ288672, AY691405) and domestic dog (GenBank DQ288669, DQ288671, AY691406) (Berryere et al. 2005), as well as most of the coding sequence of a melanistic wolf using cDNA (GenBank EF439842). We focused this study primarily on exon 4 because the 2 alleles causing altered coat color identified thus far in domestic dogs are both the result of mutations in this exon. We discuss some of the sequence differences found among these 3 canid species and their possible implications.

## Materials and Methods

### Animals

We obtained DNA via cheek brush samples from 45 domestic dogs during our previous study (Berryere et al. 2005).

**Table 1.** Polymorphisms and/or sequence differences detected among the coyote, wolf, and dog samples analyzed. For reference, exon 4 begins at bp 4481 of the genomic sequence from the start codon of the dog.

Location (bp)	Amino acid change	Coyote			Canid species wolf			Dog		
4505 & 4509	A82S & R83H	6 G&G	0 T&A	2 G&G/T&A <sup>a</sup>	2 G&G	0 T&A	0 G&G/T&A	24 G&G	12 T&A	9 G&G/T&A
4547	R96C	11 C/C	0 T/T	0 C/T	10 C/C	0 T/T	1 C/T <sup>b</sup>	32 C/C	9 T/T	4 C/T
4662	not applicable	10 C/C	3 G/G	3 C/G	3 C/C	0 GG	0 C/G	45 C/C	0 G/G	0 C/G
4451	not applicable	7 A/A	0 G/G	2 A/G <sup>a</sup>	0 A/A	1 G/G	0 A/G	0 A/A	45 G/G	0 A/G
4636	remains R	11 G/G	0 C/C	2 C/G <sup>a</sup>	0 G/G	8 C/C	0 C/G	0 G/G	45 C/C	0 G/C
4857	not applicable	13 +TT insertion	2 hetero <sup>a</sup>			1 -TT			45 -TT	

<sup>a</sup> Two coyotes from Ontario were heterozygous at several nucleotides and were presumably dog–coyote hybrids.

<sup>b</sup> One traditionally colored wolf from Alberta was a C/T heterozygote and was presumably a dog–wolf hybrid.

Previously we had DNA from a single coyote and a single wolf, but have expanded these samples in this study. DNA samples from 16 additional coyotes and 11 additional wolves were obtained from pelts, hairs, or tissue samples. Skin from a melanistic wolf was used for cDNA preparation. The additional coyotes were collected in southern Saskatchewan (Prairie ecozone) and southern Ontario (Mixedwood Plains ecozone) and the original coyote was from east-central Saskatchewan (Boreal Plains ecozone) (Ecological Stratification Working Group 1996). The original wolf and an additional melanistic wolf were from east-central Saskatchewan and 9 additional wolves were from west-central Alberta (Montane Cordillera and Boreal Plains transition) and 2 from Baffin Island (Northern Arctic ecozone). The Alberta wolves were photographed to record coat color variation.

### Primers and PCR Protocols

Using the same PCR primers and conditions as reported previously, we amplified a product that contained exon 4 (Kerns et al. 2004) or a portion thereof (Berryere et al. 2005) for sequencing. PCR products were obtained and sequenced at the National Research Council Plant Biotechnology Lab, using an ABI sequencer. A forward primer in exon 2 and a reverse primer in exon 4 (Kerns et al. 2004) after the stop codon were used to obtain the cDNA sequence from a melanistic wolf. The sequence results were aligned and analyzed using Sequencher (Version 4.1, Gene Codes Corporation, Ann Arbor, MI).

Primers used to detect the R96C allele causing “recessive black” when homozygous in dogs: Forward A: 5′- GATGTCTGGTCTGGAGCCCTC-3′; Reverse B: 5′- CGCCGGG-GACTTGCAGCTGTTTC-3′.

A 199-bp fragment containing part of exon 4 was amplified as follows. The PCR reaction of 15 µl contained 1 µl of DNA (50–100 ng), 1× PCR buffer (Gibco, Gaithersburg, MD), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.5 units *Taq* polymerase (Gibco), 0.33 µM of the forward primer, 0.66 µM of the reverse primer and 10.15 µl of dH<sub>2</sub>O. The cycling protocol was 4 min at 94 °C, followed by 33 cycles of 50 s at 94 °C, 50 s at 64 °C, 40 s at 72 °C and finished with a 4-min extension at 72 °C on a Robocycler. Digestion with *TaqI* yields a constant fragment of 121 bp. The remaining 78-bp fragment is cut into

23- and 55-bp fragments in canids with a cytosine or wild-type allele encoding a cysteine amino acid.

Primers were designed to detect a specific fragment of exon 4 coding sequence including bp 4636: Forward C: 5′- GGCCACTCGCAACAGCTGCAAG-3′; Reverse D: 5′- CTCAGCATCTGGGACTGAGACC-3′.

This PCR product was a 119-bp fragment and was amplified as described above although the annealing temperature was 57 °C. Digestion with *MspI* yields a constant fragment of 26 bp. The remaining 93-bp fragment is cut into 25- and 68-bp fragments in coyotes with a G allele but not in the other canids with a C allele.

Primers were also designed to detect a specific fragment of the coyote 3′ UTR sequence including bp 4662 (GenBank DQ238596): Forward E: 5′- CTCAGTCCCAGATGCTG-AGCGG-3′; Reverse F: 5′- CCCTGGGCCAAAAGCAG-GCGTC-3′.

This PCR product was 120 bp and was amplified as described above, although the annealing temperature was 64 °C. The G allele will cut into 25- and 95-bp fragments with *SdaI* but the C allele remains uncut.

### Results

Coat color varied considerably among the Alberta wolves with some clearly falling into the category that is often termed “melanistic.” Coat color varied minimally among the coyotes with none having a pelt one might describe as black. One coyote was a bit paler than average.

We had included a sample from a single coyote and a single wolf when sequencing exon 2, 3, and 4 previously (Berryere et al. 2005). The original amplified PCR fragment including all of exon 4 also included portions of intron 3 and 3′ UTR sequence. We noted several sequence variants between the original coyote, original wolf, and all 45 dogs. However, in the current study because amplification products of differing size were obtained from the additional coyote and wolf samples, the sample size for specific sequence variants is not consistent (Table 1). Because long PCR products are often not obtainable from pelts and hairs collected many months after death, several sets of primers were used to detect smaller regions of interest. Direct sequencing of these small fragments is not possible and so PCR-restriction length polymorphism

(RFLP) tests were designed to detect specific polymorphisms. Some specimens did not amplify with any of the primer sets and these individuals were therefore excluded from this analysis and discussion.

### Polymorphisms

Sequence was obtained from 8 coyotes and 2 wolves for the portion of exon 4 including the A82S and R83H changes characteristic of the  $a^y$  allele associated with fawn coat color in domestic dogs (Berryere et al. 2005). Two of the coyotes were heterozygous for these amino acid changes (Table 1). These 2 coyotes were from Ontario and were also heterozygous at several other nucleotides providing a strong suggestion that they were both the result of hybridization between a coyote and fawn-colored domestic dog.

Although 5 of the 9 Alberta wolves and 1 Saskatchewan wolf were melanistic in coat coloration, none were homozygous for the R96C allele characteristic of recessive black in domestic dogs (Kerns et al. 2004). However, one more traditionally colored wolf from Alberta was heterozygous for this mutation. The cDNA sequence from the melanistic Saskatchewan wolf showed no evidence of splicing problems between exons 2 and 3 or 3 and 4. Because there are no melanistic coyotes in Canada, it was not very surprising that none of the 9 coyotes had a cysteine at this residue (Table 1).

The coyotes had a G/C polymorphism (GenBank DQ238596) 5 bp after the stop codon in the 3' UTR at bp 4662. Although the coyote genotype varied, the dogs and wolves were all homozygous C/C at this nucleotide (Table 1).

### Species-Specific Sequence Differences

Three sequence differences in or flanking exon 4 were detected between coyotes and the dog and wolf. All 7 Saskatchewan coyotes had an A at bp 4451 (based on the dog start as bp 1) in intron 3, whereas all 45 dogs and 1 wolf had a G (Table 1). The 2 presumptive coyote–dog hybrids from Ontario were both heterozygous C/G at bp 4451. In exon 4 at bp 4636, all 11 coyotes from Saskatchewan were homozygous G/G, the 2 presumptive coyote–dog hybrids from Ontario were heterozygous G/C, whereas the 45 dogs and the 5 western Canadian wolves and 2 Baffin Island wolves were homozygous C/C (Table 1). Note that the arginine residue remained unchanged with this bp 4646 polymorphism. All 13 Saskatchewan coyotes also had a TT insertion after bp 4857 in the 3' UTR compared with the 45 dog and 1 wolf sequence (Table 1). The 2 presumptive coyote–dog hybrids from Ontario were both heterozygous for this insertion.

### Discussion

The coding region of exon 4 of *ASIP* appears to be highly conserved between the coyote and the wolf showing only a single base-pair difference that did not alter an amino acid (Berryere et al. 2005). On the other hand, the domestic dog has been selected on the basis of coat color in several breeds and has at least 3 amino acid changes that result in coat color differences (Berryere et al. 2005) compared with the

wild-type sequence exhibited by the wolf and some dogs with banded hairs (Figure 1). The melanistic wolves were not black because of the R96C variant in *ASIP*. Variation in wolf shades may be attributable to dietary or hormonal differences, or more likely to variation at another gene.

The coyote polymorphism in the 3' UTR at bp 4662 is one of the few polymorphisms described within a coyote gene thus far, outside the Major Histocompatibility Complex (MHC) (Hedrick et al. 2002). Bardeleben et al. (2005) suggest that 2 loci of the 6 they studied varied among the coyote, wolf, domestic dog, and golden jackal. They do not list any intraspecific indels found in the coyote, wolf, or dog in the 6 genes they studied (Bardeleben et al. 2005, Supplementary Table S3). This *ASIP* SNP was found among the coyotes of southern Saskatchewan and hence represents a variant within a population that likely interbreeds. This sample does not allow us to determine how widespread this polymorphism is in coyotes in general.

Coyotes, wolves, and dogs have cohabited much of northern Saskatchewan (Cummins 2002) and there has been considerable speculation about interbreeding among these canids (Vila et al. 1997, 2003; Adams et al. 2003). These *agouti* DNA data suggest that the coyote and wolf and dog may not have interbred often in Canada, but that some interbreeding occurs as evidenced by heterozygosity for polymorphisms in exon 4 in the 2 Ontario coyote samples and 1 Alberta wolf sample. The 2 heterozygous Ontario coyotes originate from an area previously identified as a hybrid zone of wolves and coyotes (Lehman et al. 1991). The A82S and R83H polymorphisms are unique to the dog and therefore the 2 heterozygous coyotes in this study must have had a dog ancestor. Fawn is a relatively common color in many breeds of dogs and so one could not postulate the type of dog. The R96C SNP allele is also unique to the dog that suggests that the heterozygous wolf has a dog in its ancestry. This R96C SNP is rare even in dogs, occurring only in black dogs of herding breeds and therefore is likely to underestimate the level of wolf/dog hybridization but provides an additional means of detecting this.

Many previous studies have documented differences in the mitochondrial DNA that can be used in studies of evolution and hybridization (Vila et al. 1997; Leonard et al. 2002, 2005). These same studies often suggest that these mtDNA differences detect coyote hybrids (or descendants thereof) that result from matings between a female wolf or dog and male coyote but not the descendants of reciprocal matings. Thus, nuclear DNA differences such as those in *ASIP* may be more useful to detect descendants of matings between 2 canid species. Other nuclear differences have been reported previously in the MHC (Hedrick et al. 2000; Vila et al. 2005) and various microsatellites (Vila et al. 2003) and indels or SNPs in introns of coding genes (Bardeleben et al. 2005). Evidence that a juvenile male who was found as a roadkill in Norway was a wolf–dog hybrid was based on microsatellite data for its dog lineage and mtDNA for its wolf lineage (Vila et al. 2003).

Evidence that coyotes and dogs interbred in the southeastern United States of America was based on mtDNA

```

                                @
Dog      cgagacagacgtgaggacaggtggggtggacgtggccGgcttgggcagccctggcgtttc
Wolf     cgagacagacgtgaggacaggtggggtggacgtggccGgcttgggcagccctggcgtttc
Coyote   cgagacagacgtgaggacaggtggggtggacgtggccAgcttgggcagccctggcgtttc

          4                *   *
          K K A S M K N V S H P R P P P P T P
Dog      cctgcagaaaaaggcttcgatgaagaacgtgtctcatccccggccccccgccaccacccc
Wolf     cctgcagaaaaaggcttcgatgaagaacgtggctcgtccccggccccccgccaccacccc
Coyote   cctgcagaaaaaggcttcgatgaagaacgtggctcgtccccggccccccgccaccacccc

          *
          C V A T R N S C K S P A P A C C D P C
A
Dog      ctgcggtggccactcgcaacagctgcaagtccccggcgcccgctgctgtgaccctgcgc
Wolf     ctgcggtggccactcgcaacagctgcaagtccccggcgcccgctgctgtgaccctgcgc
Coyote   ctgcggtggccactcgcaacagctgcaagtccccggcgcccgctgctgtgaccctgcgc

          #
          S C Q C R F F R S A C T C R V L S P R
C
Dog      ctctctgccagtgcgcttcttccgcagcgcctgcacctgccgCgttctcagtcaccagatg
Wolf     ctctctgccagtgcgcttcttccgcagcgcctgcacctgccgCgttctcagtcaccagatg
Coyote   ctctctgccagtgcgcttcttccgcagcgcctgcacctgccgGgttctcagtcaccagatg

          X   @
Dog      ctgagcgcGcccagcggcctccagggggttgctgattatctaagaagtacggctttggg
Wolf     ctgagcgcGcccagcggcctccagggggttgctgattatctaagaagtacggctttggg
Coyote   ctgagcgcCcccagcggcctccagggggttgctgattatctaagaagtacggctttggg

Dog      gatccctgggtggctcagcggtttgacgcctgcttttggcccaggggtgcgacctggagt
Wolf     gatccctgggtggctcagcggtttgacgcctgcttttggcccaggggtgcgacctggagt
Coyote   gatccctgggtggctcagcggtttgacgcctgcttttggcccaggggtgcgacctggagt

Dog      gccgggatcgagtcaccatgtcaggctccggcgtggagcctgcttctccctctgcctgtgt
Wolf     gccgggatcgagtcaccatgtcaggctccggcgtggagcctgcttctccctctgcctgtgt
Coyote   gccgggatcgagtcaccatgtcaggctccggcgtggagcctgcttctccctctgcctgtgt

                                @@
Dog      ctctgctccctctctctttctct--ctatgtctatcatgaataaataaataaaatcttt
Wolf     ctctgctccctctctctttctct--ctatgtctatcatgaataaataaataaaatcttt
Coyote   ctctgctccctctctctttctctTTctatgtctatcatgaataaataaataaaatcttt

Dog      aaaaaa
Wolf     aaaaaaaaaaaaaaaaaa
Coyote   aaaaaaaaa

* = Polymorphic in domestic dogs (affects coat color)
@ = Different in Coyotes than dog and wolf
# = Polymorphic in Coyotes

```

**Figure 1.** The nucleotide sequence of the coding portion of exon 4 of ASIP and the flanking sequence for the dog, wolf, and coyote. Polymorphic nucleotides are indicated with symbols above. The amino acid sequence is also shown above.

studies (Adams et al. 2003). Evidence that coyotes and wolves interbred was based on MHC alleles (Hedrick et al. 2002). Our study suggests that polymorphisms in *ASIP* could also be used to determine interspecific canid ancestry.

## Funding

Natural Science and Engineering Research Council-Industry Oriented Research Program to coat color studies in dogs; Social Sciences and Humanities Research Council to J.L.B.

## Supplementary Material

Supplementary Table S3 can be found at <http://www.jhered.oxfordjournals.org/>.

## Acknowledgments

We thank Rick Espie, Ron Chesney, and Zeb Chesney for providing additional coyote samples and Robert W. Park, Douglas R. Stenton, Kyle Knopff, Nathan F. Webb, Ray Collingwood, and Solomon Carrière for providing additional wolf samples.

## References

- Adams JR, Leonard JA, Waits LP. 2003. Widespread occurrence of a domestic dog mitochondrial DNA haplotype in southeastern US coyotes. *Mol Ecol*. 12:2175–2186.
- Bardeleben C, Moore RL, Wayne RK. 2005. A molecular phylogeny of the Canidae based on six nuclear loci. *Mol Phylogenet Evol*. 37:815–831.
- Berryere TG, Kerns JA, Barsh GS, Schmutz SM. 2005. Association of an agouti allele with fawn or sable coat color in domestic dogs. *Mamm Genome*. 16:262–272.

- Cummins BD. 2002. First nations, first dogs. Calgary (AB): Detselig Enterprises Limited.
- Ecological Stratification Working Group. 1996. A National Ecological Framework for Canada. Ottawa/Hull (Canada): Agriculture and Agri-Food Canada, Research Branch, and Environment Canada, Ecozone Analysis Branch.
- Hedrick PW, Lee RN, Garrigan D. 2002. Major histocompatibility complex variation in red wolves: evidence for common ancestry with coyotes and balancing selection. *Mol Ecol.* 11:1905–1913.
- Hedrick PW, Lee RN, Parker KM. 2000. Major histocompatibility complex (MHC) variation in the endangered Mexican wolf and related canids. *Heredity.* 85:617–624.
- Kerns JA, Newton J, Berryere TG, Rubin EM, Cheng J-F, Schmutz SM, Barsh GS. 2004. Characterization of the dog *Agouti* gene and identification of a *nonagouti* mutation in German Shepherd dogs. *Mamm Genome.* 15: 798–808.
- Lehman N, Eisenhaver A, Hansen K, Mech LD, Peterson RO, Wayne RK. 1991. Introgression of coyote mitochondrial DNA into sympatric North American Gray Wolf populations. *Evolution.* 45:104–119.
- Leonard JA, Vila C, Wayne RK. 2005. Legacy lost: genetic variability and population size of extirpated US grey wolves (*Canis lupus*). *Mol Ecol.* 14:9–17.
- Leonard JA, Wayne RK, Wheeler J, Valadez R, Guillen S, Vila C. 2002. Ancient DNA evidence for Old World origin of New World dogs. *Science.* 298:1613–1616.
- Vila C, Savolainen P, Maldonado JE, Amorim IR, Rice JE, Honeycutt RL, Crandall KA, Lundeberg J, Wayne RK. 1997. Multiple and ancient origins of the domestic dog. *Science.* 276:1687–1689.
- Vila C, Seddon J, Ellegren H. 2005. Genes of domestic mammals augmented by backcrossing with wild ancestors. *Trends Genet.* 21:214–218.
- Vila C, Walker C, Sundqvist AK, Flagstad O, Andersone Z, Casulli A, Kojola I, Valdmann H, Halverson J, Ellegren H. 2003. Combined use of maternal, paternal and bi-parental genetic markers for the identification of wolf-dog hybrids. *Heredity.* 90:17–24.

Received April 10, 2006

Accepted March 9, 2007

Corresponding Editor: Stephen J. O'Brien