Comprehensive genetic testing combined with citizen science reveals a recently characterized ancient MC1R mutation is associated with partial recessive red phenotypes in dog

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Abstract

Background

The Melanocortin 1 Receptor (MC1R) plays a central role in regulation of coat color determination in dogs and is commonly referred to as the “E (extension) Locus”. Allelic variation of the *MC1R* gene is associated with coat color phenotypes E^M (melanistic mask), E^G (grizzle/domino) and e^{1-3} (recessive red) in dogs. In addition, a previous study of archeological dog specimens over 10,000 years of age identified a variant p.R301C in the *MC1R* gene that may have influenced coat color of early dogs.

Results

Commercial genotyping of 11,726 dog samples showed the R301C variant of the *MC1R* gene was present in 34 breeds or breed varieties, at an allele frequency of 1.48% in the tested population. We detected no linkage disequilibrium between R301C and other tested alleles of the E locus. Based on current convention we propose that R301C should be considered a novel allele of the E locus, which we have termed e^A for “e ancient”. Phenotype analysis of owner-provided dog pictures reveals e^A allele has an impact on coat color and is recessive to wild type *E* and dominant to the *e* alleles. In dominant black ($K^B/*$) dogs it can prevent the expression of the K locus, and the expressed coat color is solely determined by the A locus. In the absence of dominant black, e^A/e^A and e^A/e genotypes result in the coat color patterns referred to in their respective breed communities as domino in Alaskan Malamute and other Spitz breeds, grizzle in Chihuahua, and pied in Beagle.

Conclusions

This study demonstrates a large genotype screening effort to identify the frequency and distribution of the *MC1R* R301C variant, one of the earliest mutations captured by canine domestication, and citizen science empowered characterization of its impact on coat color.

Background

Coat color in dogs is determined by expression of two melanin pigments, eumelanin (black/brown) and pheomelanin (yellow/red) and by spatial and temporal regulation of these pigments’ expression in the body and in the individual hair shaft. *Melanocortin 1 Receptor* (*MC1R*), known as the E locus, represents the key signaling molecule on melanocytes inducing expression of enzymes responsible for eumelanin synthesis. The alleles in order of dominance at the E locus are; E^M (melanistic mask) > E^G (grizzle/domino) > *E* (wild type) > e^{1-3} (recessive red) (1–5). The e^{1-3} variants result in loss of gene function and consequently for dogs with the genotype *e*/*e* only pheomelanin (yellow/red) pigment is present. The allelic variant e^1 is common and found in a wide variety of dog breeds (4, 6), while the e^2

and e^3 alleles represent rare additional *e* variants found in Australian Cattle Dogs and white Alaskan and Siberian Huskies (1), respectively. The E^G allele is one of the rarest trait-associated alleles present in dogs, requiring specific genotype combinations at more than one locus in order to produce a domino or grizzle phenotype. This phenotype has been characterized in Afghan Hounds and Salukis only (3, 6). *Agouti Signaling Protein* (*ASIP*) gene, known as the A locus, is an antagonist of MC1R inhibiting eumelanogenesis and promoting pheomelanogenesis. Four known alleles in order of dominance are a^y (fawn) > a^w (wolf sable) > a^t (tan point) > *a* (recessive black) (7–9); of these a^w is considered the wild type *ASIP* allele. The most dominant allele a^y represents a gain-of-function mutation causing a yellow/red phenotype, whereas the alleles recessive to wild type show reduced-function and increased expression of eumelanin. In addition, the *canine beta-Defensin 103* (*CBD103*) gene, known as the K locus, is another ligand of *MC1R*. The dominant K^B (dominant black) allele prevents ASIP inhibition enabling high levels of basal receptor activity resulting in solid eumelanin coat color. Similarly, a phenotypically intermediate k^{br} allele (brindle) of the K locus produces dominantly overlaying eumelanin stripes on A locus determined background (10, 11). In the presence of k^y (wild type) allele of the K locus, the A locus is expressed normally. The expression of the A and K loci is dependent on the presence of at least one functional *MC1R* allele, and are not expressed in the presence of an *e*/*e* genotype.

Coat color variation in dogs and various other domesticated species is a result of domestication and subsequent selective pressure to enrich desired phenotypes (12). In dogs the R301C variant of *MC1R* and the dominant black K^B allele of the *CBD103* gene were both found in the DNA of over 10,000 years old Siberian and South-Eastern European dogs (13). Only dog-like samples and no wolf-like samples were found to carry R301C or the K^B allele suggesting that these variants could represent some of the first coat color variants present at the time of early dog domestication (13). While the R301C variant has been found in two modern day breeds the Alaskan Malamute and the Siberian Husky, its phenotypic impact could not be determined (13). Potential reduced-function was postulated based on functional characterization of a mutation at the same codon position (301) together with two other polymorphisms found in 43,000 years old woolly mammoth that resulted in nearly complete loss of basal activity and ~ 65% reduction in efficacy to agonists *alpha*-Melanocyte Stimulating Hormone (α-MSH) (14).

The aim of this study was to utilize the potential of commercial genetic panel screening to genotype large numbers of dogs for the presence or absence of the R301C variant of *MC1R* found in prehistoric dogs to better understand its frequency and distribution in modern dog breeds. To further unravel the potential influence of R301C, coat color variant genotypes were correlated with the dog's actual coat color phenotype from photos provided by dog owners.

Results

Ancient R301C variant of the MC1R gene is present in various breeds of today

To screen for the presence and frequency of the ancient R301C variant of *MC1R* in today's canine population, 11,726 dog samples were genotyped as a part of a custom-designed microarray panel test commercially available as MyDogDNA™/Optimal Selection™ Canine Genetic Breeding Analysis. The R301C variant was present in a total of 262 tested dogs representing 34 different breeds and breed varieties as well as mixed breed dogs. The allele frequency for R301C in all dogs representing 302 different breeds and mixed breeds was 1.48% (N = 11,726; Table 1, and Table S1). The R301C variant was fixed in the Alaskan Malamute breed. The additional 33 breeds in which the R301C variant was found could be classified into old Nordic Spitzes (East-Siberian Laika, Finnish Lapphund, Finnish Spitz, Karelian Bear Dog, Lapponian Herder, Nordic Spitz, Siberian Husky, West-Siberian Laika), other Primitive Spitz Type dogs (Basenji, Cirneco Dell'Etna, Kritikos Lagonikos, Peruvian Hairless Dog – Large, Medium and Miniature), Scent Hounds (Basset Fauve de Bretagne, Beagle, Drever, English Foxhound, Finnish Hound, Hungarian Hound, Plott, Serbian Hound), one gundog breed (Chesapeake Bay Retriever), one guardian dog breed (Pyrenean Mastiff), three Companion and Toy Dogs (Chihuahua, Chinese Crested Dog, Phalene) and some recently created breeds (Alaskan Husky, Alaskan Klee Kai, Chinook, Northern Inuit, Tamaskan Dog, Saarlooswolfdog). In this study sample the R301C variant was not found in dog breeds with Eastern Asian origin (Akita, Chow Chow, Hokkaido, Kai, Kishu, Shar Pei, Shiba, Shikoku, Korean Jindo Dog) or Middle Eastern/Central Asian origin (Afghan Hound, Saluki, Tibetan Mastiff, Tibetan Spaniel, Tibetan Terrier, Lhasa Apso, Shih-Tzu, Central Asian Ovcharka).

Due to technical limitations, Table 1 is provided in the Supplementary Files section.

R301C is a novel alternative allele of the E locus

To elucidate the relationship of R301C and other known E locus variants, genotypes were obtained for E^M (melanistic mask), E^G (grizzle/domino) and e^1 (recessive red) alleles of the *MC1R* gene. Two rare additional recently characterized *e* allelic variants(1); e^2 discovered in Australian Cattle Dog and e^3 discovered in Siberian Husky were not genotyped as a part of this study. The R301C variant and the tested E locus variants showed no linkage disequilibrium. The R301C variant was not present in dogs with two copies of the tested E locus variants; E^M, E^G or e^1, while in dogs with two copies of the R301C variant no E^M, E^G or e^1 variants were present. Also, no more than one copy of E^M or e^1 variants was present when one copy of R301C was found. The rarest *MC1R* coat color variant, the E^G allele, is only found in one of the dog breeds, Kritikos Lagonikos, in which R301C was identified. However, in this study sample no individuals carrying both E^G and R301C variants were identified.

Notably, using current conventional practices for calling of E locus genotypes at commercial genotyping laboratories, dogs carrying R301C would have been interpreted as carrying *E*. As our findings suggested that R301C rather represents an independent alternative allele at the E locus, we refer to it as e^A (for ancient *e*) for clarity hereafter.

e ^{A} allele of MC1R is associated with partial recessive red phenotypes

To interpret the phenotypic impact of the R301C variant on the dog's coat color, also genotypes for *Canine Beta-Defensin 103* (*CBD103*) and *Agouti Signaling Protein* (*ASIP*) were obtained for the phenotype

analysis. Color phenotypes were available for 118 (45%) dogs of the 262 dogs identified with one or two copies of the e^A allele in this study.

The coat color phenotype was altered in all 64 dogs with the e^A allele present in homozygous form (N = 29) or in heterozygous form paired with the recessive red e^1 allele (N = 35). Phenotyping using dog owner-provided photos revealed that the e^A allele was associated with partial recessive red coat color patterning. These phenotypes manifested in dogs with e^A/e^A and e^A/e^1 genotypes as follows. All six dogs with e^A/e^1 and dominant black on the K locus express a non-solid eumelanin shade phenotype. In four out of six dogs, three Cirneco dell'Etna's and one Drever, the phenotype is clear fawn and virtually indistinguishable from typical recessive red e^1/e^1 (Table 2 and Fig. 1; A, B and C). Of the remaining two K^B dogs, one Siberian Husky is wolf sable and one mixed breed dog is tan point (modified into saddle tan) (Table 2). Given that the four clear fawn dogs have a^y/a^y fawn genotype, the wolf sable dog has a^w/a^t genotype and the mixed breed has a^t/a genotype on the A locus, we conclude that these dogs express the coat color pattern of their A locus despite the presence of one copy of dominant K^B variant. Moreover, all 58 dogs with e^A/e^1 or e^A/e^A genotype expressing A locus a^y fawn, a^w wild sable, a^t tan point or a recessive black in the absence of dominant black, have increased pheomelanin expression in the hair root and abundant blend of pheomelanin hairs in areas of head, legs and body from which the coat color pattern known as "domino" or "grizzle" is formed (Table 2 and Fig. 1; D-K). The produced color pattern has high phenotypic similarity to previously characterized E^G domino in Afghan Hound and E^G grizzle in Saluki which have been shown to be dependent on the A locus a^t/a^t genotype (3). Here, the domino pattern is observed independently from E^G on divergent breed backgrounds. Domino phenotype encompasses pale facial markings with receded eumelanin line forming a widow's peak in the forehead, and often also white markings expressed up the centerline of the face including reduced pigment in the centerline of the nose referred to as a dudley nose. The latter phenotypic feature, white markings and a dudley nose, is also common in recessive red dogs, but not typical in grizzle/domino coat color pattern caused by the E^G genotype. Of the two a^y fawn dogs, one Finnish Lapphund is a domino on shaded fawn coat (Fig. 1; D) and in one Nordic Spitz the homozygosity for another coat color modifying White Spotting gene (15) obscures effective observation of domino pattern on the body, while the clear fawn colored spots on the head and a dudley nose match with domino phenotype (Table 2). The phenotype in 40 a^w wolf sable dogs is typical domino on wolf sable, a common pattern in breeds such as the Alaskan Malamute and Siberian Husky (Table 2 and Fig. 1; F). The phenotype in 8 out of 12 a^t tan point dogs is classic domino on tan point (Table 2). Moreover, in one Beagle in which tan point coat color modifier Saddle Tan (16) is present, the widow's peak is not visible. The resulting coat color pattern is called as "pied" in this breed which we now demonstrate to have the same genetic background with e^A domino dogs. There is also variation in the level of pheomelanin expression in 3 out of 12 a^t tan point dogs. One Drever homozygous for the e^A allele is without visible increase in the pheomelanin expression on the coat color, the dog expresses normal tan points, but also the white markings on the centerline of the face and a dudley nose. On contrary, almost no eumelanin pigment is present in two Hungarian Hounds with e^A/e^1

genotype manifesting rich red coat color. Also, the white markings on the centerline of the face and a dudley nose are present in these dogs. And lastly, the phenotype in four *a* allele homozygote recessive black dogs is domino on recessive black (Table 2, Fig. 1; J).

Table 2

Phenotype analysis of 118 dogs with known genotype for *MC1R*, *CBD103* and *ASIP* loci.

Number of phenotyped dogs (Total 118)	genotype E locus K locus A locus	Breed	Phenotype
6 (e^A/e^1 with $K^B/*$)	e^A/e^1 K^B/k^y a^y/a^y	Cirneco dell' Etna	clear fawn
	e^A/e^1 K^B/k^y a^y/a^y	Cirneco dell' Etna	clear fawn
	e^A/e^1 K^B/k^y a^y/a^y	Cirneco dell' Etna	clear fawn
	e^A/e^1 K^B/k^y a^y/a^y	Drever	clear fawn
	e^A/e^1 K^B/k^y a^t/a	Mixed breed	saddle tan
	e^A/e^1 K^B/k^y a^w/a^t	Siberian Husky	wolf sable
58 (e^A/e^A or e^A/e^1 without K^B)	e^A/e^1 k^y/k^y a^y/a^t	Finnish Lapphund	domino
	e^A/e^1 k^y/k^y a^y/a^y	Nordic Spitz	domino (with white spotting)
	e^A/e^1 k^y/k^y a^w/a^w	Alaskan Klee Kai	domino
	e^A/e^1 k^y/k^y a^w/a^w	Alaskan Klee Kai	domino
	e^A/e^1 k^y/k^y a^w/a^t	Alaskan Klee Kai	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^A k^y/k^y a^w/a^w	Alaskan Malamute	domino
	e^A/e^1 k^y/k^y a^w/a^t	Chinook	domino
	e^A/e^1 k^y/k^y a^w/a^t	Finnish Lapphund	domino
	e^A/e^1 k^y/k^y a^w/a^t	Mixed breed	domino
	e^A/e^1 k^y/k^y a^w/a^w	Northern Inuit Dog	domino
	e^A/e^A k^y/k^y a^w/a	Northern Inuit Dog	domino
	e^A/e^A k^y/k^y a^w/a^w	Siberian Husky	domino
	e^A/e^A k^y/k^y a^w/a^w	Siberian Husky	domino
	e^A/e^A k^y/k^y a^w/a^w	Siberian Husky	domino
	e^A/e^A k^y/k^y a^w/a^w	Siberian Husky	domino
	e^A/e^A k^y/k^y a^w/a^t	Siberian Husky	domino
	e^A/e^A k^y/k^y a^w/a^t	Siberian Husky	domino
	e^A/e^A k^y/k^y a^w/a^w	Tamaskan Dog	domino
	e^A/e^A k^y/k^y a^w/a^w	Tamaskan Dog	domino
	e^A/e^A k^y/k^y a^w/a^w	Tamaskan Dog	domino
	e^A/e^A k^y/k^y a^w/a^w	Tamaskan Dog	domino
	e^A/e^1 k^y/k^y a^w/a^w	Tamaskan Dog	domino

Number of phenotyped dogs (Total 118)	genotype E locus K locus A locus	Breed	Phenotype
	$e^A/e^1\ k^y/k^y\ a^w/a^w$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a^w$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a^w$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a^w$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a^w$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a^t$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a^t$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a^w/a^t$	Tamaskan Dog	domino
	$e^A/e^A\ k^y/k^y\ a^t/a^t$	Alaskan Malamute	domino
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Beagle	pied
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Chihuahua	grizzle
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Chihuahua Smooth-haired	grizzle
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Drever	tan point[a]
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Finnish Lapphund	domino
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Hungarian Hound	red[b]
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Hungarian Hound	red[b]
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Lapponian Herder	domino
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Mixed breed	domino
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Siberian Husky	domino
	$e^A/e^1\ k^y/k^y\ a^t/a^t$	Tamaskan Dog	domino
	$e^A/e^A\ k^y/k^y\ a/a$	Northern Inuit Dog	domino
	$e^A/e^A\ k^y/k^y\ a/a$	Northern Inuit Dog	domino
	$e^A/e^1\ k^y/k^y\ a/a$	Tamaskan Dog	domino
	$e^A/e^1\ k^y/k^y\ a/a$	Tamaskan Dog	domino
39 (E/e^A *with/without* K^B)	$E/e^A\ K^B/k^y\ a^y/a^w$	Pyrenean Mastiff	black
	$E/e^A\ k^y/k^y\ a^y/a$	Chesapeake Bay Retriever	fawn
	$E/e^A\ k^y/k^y\ a^y/a^t$	Nordic Spitz	fawn
	$E/e^A\ k^y/k^y\ a^w/a^t$	Alaskan Husky/Mixed breed	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^t$	Mixed breed	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^w$	Mixed breed	wolf sable
	$E/e^A\ K^{B(r)}/k^y\ a^w/a^w$	Pyrenean Mastiff	wolf sable (with brindle)
	$E/e^A\ k^y/k^y\ a^w/a^w$	Saarlooswolfdog	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^t$	Siberian Husky	domino[c]
	$E/e^A\ k^y/k^y\ a^w/a^t$	Siberian Husky	domino[c]
	$E/e^A\ k^y/k^y\ a^w/a^t$	Siberian Husky	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^w$	Siberian Husky	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^t$	Tamaskan Dog	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^t$	Tamaskan Dog	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^t$	Tamaskan Dog	wolf sable
	$E/e^A\ k^y/k^y\ a^w/a^t$	Tamaskan Dog	wolf sable

Number of phenotyped dogs (Total 118)	genotype E locus K locus A locus	Breed	Phenotype
	E/e^A k^y/k^y a^w/a^t	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^t	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^t	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^w/a^w	Tamaskan Dog	wolf sable
	E/e^A k^y/k^y a^t/a^t	Basenji	tan point
	E/e^A k^y/k^y a^t/a^t	Hungarian Hound	tan point
	E/e^A k^y/k^y a^t/a^t	Tamaskan Dog	tan point
	E/e^A k^y/k^y a^t/a^t	Tamaskan Dog	tan point
15 (E^M/e^A)	E^M/e^A K^B/k^y a^w/a^w	Pyrenean Mastiff	black
	E^M/e^A K^B/k^y a^w/a^w	Pyrenean Mastiff	black
	E^M/e^A K^B/k^y a^y/a^t	Drever	dark masked fawn
	E^M/e^A k^y/k^y a^y/a^y	Nordic Spitz	dark masked fawn
	E^M/e^A k^y/k^y a^y/a^y	Nordic Spitz	dark masked fawn
	E^M/e^A k^y/k^y a^y/a^y	Phalene	dark masked fawn
	E^M/e^A k^y/k^y a^w/a^t	Alaskan Husky	dark masked wolf sable
	E^M/e^A $K^{B(r)}/k^y$ a^w/a^t	Mixed breed	dark masked wolf sable (with brindle)
	E^M/e^A k^y/k^y a^w/a^t	Mixed breed	dark masked wolf sable
	E^M/e^A k^y/k^y a^w/a^t	Pyrenean Mastiff	dark masked wolf sable (with brindle)
	E^M/e^A k^y/k^y a^w/a^w	Pyrenean Mastiff	dark masked wolf sable (with brindle)
	E^M/e^A k^y/k^y a^w/a^w	Tamaskan Dog	dark masked wolf sable
	E^M/e^A k^y/k^y a^t/a^t	Chinook	dark masked tan point
	E^M/e^A k^y/k^y a^t/a^t	Lapponian Herder	dark masked tan point
	E^M/e^A $K^{B(r)}/k^y$ a^t/a^t	Pyrenean Mastiff	dark masked tan point (with brindle)

[a]This dog has normal tan points without blend of pheomelanin hairs manifesting domino pattern only as white markings on the centerline of dog's head and reduced color on the dog's nose while there is more eumelanin expressed than in other dogs with this genotype.

Number of phenotyped dogs (Total 118)	genotype E locus K locus A locus	Breed	Phenotype
[b]This dogs has almost all eumelanin expression removed manifesting domino pattern only as white markings on the centerline of dog's head and reduced color on the dog's nose.			
[c]This dog manifests domino despite only carrying one copy of the e^A and no other tested E locus variants. However, we suspect this dog may also carry one copy of e^3 allele recently identified in Siberian Husky that was not tested as a part of this study.			

We observed no phenotype change in 52 dogs genotyped E^M/e^A (N = 15) or E/e^A (N = 37) strongly proposing that the allele's dominance hierarchy at E locus is recessive to E^M and E and dominant to e, while further information on phenotypes produced by E^G/e^A genotype remains to be collected. In two Siberian Huskies with one copy of e^A and no other tested E alleles present the phenotype was altered to domino as if no wild type E was present. We did not have DNA availability to test for the presence of a rare e^3 variant discovered in Huskies (1), but we hypothesize that the actual genotype of these dogs is e^A/e^3 based on the observed phenotype. Phenotypic impact of e^A allele as recessive to wild type E and dominant to e allele is further demonstrated in a litter of Tamaskan Dogs (Fig. 2).

Taken together, phenotype data available in 14 different breeds consistently shows that e^A results in two phenotypes that we interpret to be partial recessive red coat colors. In dogs with K^B dominant black genotype, the K locus is overridden and A locus is expressed instead, while in dogs expressing the A locus (in the absence of K^B variant) the ability to produce eumelanin is reduced resulting in coat color patterns known by the names "domino", "grizzle" and "pied" depending on the breed background.

Discussion

The goal of this study was to screen for the presence of an ancient R301C variant in today's dog population and determine if it has any effect on coat color phenotypes in dogs. The R301C variant of *MC1R* was first identified in over 10,000-year-old prehistoric dog DNA samples (13). Our study sample consisted of nearly 12,000 samples representing over 300 modern dog breeds and breed varieties which were genotyped for the presence of R301C and known coat color alleles as a part of a commercial genetic testing service (MyDogDNA™/Optimal Selection™). We confirm presence of the R301C variant in 34 dog breeds with a variant frequency of 1.48% in all dogs genotyped. A frequency of 50% or higher for R301C was observed in Spitz breeds (Alaskan Klee Kai, Northern Inuit Dog, Siberian Husky), and in Hound breeds (Drever, English Foxhound, Plott and Serbian Hound).

Phenotype analysis combined known coat color variant genotypes for *MC1R*, *CBD103* and *ASIP* genes with phenotype information from photos provided by the owners of the tested dogs. R301C was not found to be linked with any of the tested E locus variants (E^M, E^G or e^1). Phenotype analysis further verified that the R301C variant is a novel reduced-function allele at the E locus, recessive to E^M and wild type E, but dominant to the e allele. We demonstrate that the phenotypic impact of the newly characterized allele, which we have termed e^A, is a loss of solid eumelanin shade in K^B dogs and receding eumelanin expression in any color pattern produced by the A locus. This pattern manifests as E^G-independent "domino" or "grizzle" coat color phenotypes when e^A is present in homozygous form or in

heterozygous form with *e* as the second allele. Although E^G was associated with similar grizzle/domino color patterning in dogs such as tan point Saluki and Afghan Hound, e^A has a much broader impact spectrum on coat color phenotypes. Unlike E^G grizzle, the altered phenotypes associated with e^A are expressed in the absence of wild type *E*. We observed two Siberian Huskies manifesting a domino phenotype while carrying one copy of e^A and no other tested E locus variant, which according to current conventions would be interpreted as wild type *E* being the second allele at the locus in these dogs. Although we did not have DNA sample availability to test for the presence of the recently discovered additional *e* alleles, e^2 and especially e^3 discovered in Huskies (1), we suggest that an e^A/e^3 genotype is the most likely explanation for the domino phenotype in these dogs.

We propose that the newly characterized reduced-function variant R301C is designated e^A, where "*e*" is chosen for its *partial* recessive red identity and "A" is denoting "ancient". We propose an updated dominance hierarchy at the E locus; $E^M > E^G > E > e^A > e^{1-3}$, while acknowledging that we could not identify any dog with the E^G/e^A genotype and thus, the phenotype impact of this specific rare genotype combination remains unexplored. Interestingly, the previously identified domino variant E^G is almost exclusively observed in breeds in which e^A is not detected (6). In this study, we found both E^G and e^A allele present in only one breed, Kritikos Lagonikos; a primitive hunting dog originating from the Greek island of Crete. Phenotype analysis of this rare breed might help to resolve the phenotypes presented by rare allele combinations, for which conclusions could not be made through this study.

Based on our phenotype analysis, common to e^A associated patterning is an increase in the level of pheomelanin expression and decrease in the level of eumelanin expression, indicating that e^A represents a reduced-function variant of MC1R as postulated (13). It is plausible that reduced-function of MC1R manifests as the partial recessive red phenotypes observed by us, while other previously characterized recessive red alleles e^{1-3} represent loss-of-function variants of MC1R enabling expression of pheomelanin only when present in two copies. Consequently, *e/e* dogs always only express pheomelanin pigment regardless of variants present at the K locus and A locus, whereas the R301C variant e^A results in reduced MC1R function preventing expression of the K locus in dominant black dogs, while pigmentation phenotypes correlated with the expression pattern of the A locus. Logically on the other hand, increased pheomelanin expression is observed in dogs expressing the A locus due to the effects of e^A.

While partial recessive red phenotypes are produced by e^A/e^A or e^A/e^1 genotypes, two copies of the reduced- function allele e^A could allow a bit more eumelanin to be expressed than when the e^A allele is present with a loss of function allele e^1. We observed a Drever with e^A/e^A genotype expressing normal tan points with only reduced pigment in the centerline of the head (white star) and nose (dudley nose), respectively. Outside of this study sample, we have further observed full domino pattern present in this dog's offspring with e^A/e^1 genotype. The most depleted eumelanin expression was observed in two Hungarian Hounds with e^A/e^1 genotype and a^t/a^t genotype for tan points in the A locus, these two dogs were essentially indistinguishable from recessive red individuals of the same breed. In addition, it should

be noted that the domino pattern in all a^y fawn dogs is not clearly visible and their phenotype is similar to recessive red.

MC1R is not only central to determination of pigment phenotype. Besides its role in stimulation of eumelanin synthesis to protect skin from UV radiation and DNA damage, MC1R has a physiological role in vascular homeostasis and cell migration (17), erythroblast differentiation (18), prevention of cartilage degradation (19), and dopaminergic neuron survival (20). MC1R signaling activates antioxidant, DNA repair and anti-inflammatory pathways (21–23). MC1R genotype affects the probability of developing malignant melanoma (24), nonmelanoma skin cancer (24–26), risk for developing complicated sepsis after trauma (27) and development of Parkinson's disease (20, 28, 29) in humans. Loss- or reduced-function variants in human MC1R have also been investigated in the response to pain, analgesia and anesthetics (30–33). Moreover, in Standard Poodle the e^1/e^1 genotype has been shown to prevent clinical signs of disease in dogs carrying causal variant causing Squamous Cell Carcinoma of the Digit (SCCD) (34). Further work is needed to establish the molecular effect of the reduced-function variant R301C, and to understand its potential effects beyond determining coat color pigmentation.

In summary, genotype to phenotype correlation characterizes a novel allele of the E locus, caused by an old polymorphism in the *MC1R* gene associated with reduced eumelanin pigment that potentially represents one of the earliest mutations enriched by canine domestication still present in the dog population. This ancient E locus variant (MC1R p. R301C), which we have designated as e^A, is recessive to E^M and E alleles of the E locus and dominant to the e allele. The genotypes e^A/e and e^A/e^A result in phenotypically reduced expression of eumelanin, and these genotypes exhibit partial dominance over the A locus expression pattern and dominance over the K locus.

Conclusions

This study represents a large genotype screening effort of pet dogs, aiming to identify the presence of and understand the potential effect of one of the earliest mutations captured by canine domestication. It underscores the crucial role of dog owners in citizen science and more specifically in supporting studies aiming to elucidate the genetic background of trait phenotypes. The present discoveries could only have been made by comprehensive screening of coat color variants across a large number of breeds and individuals, in combination with the openness of dog owners to submit pictures of their dogs for research purposes. In conclusion, our findings explain the non-eumelanin coat color phenotypes observed in some dogs despite presence of the dominant black allele on the K locus, and identify that the same molecular cause explains the coat color phenotype commonly referred to as "domino" in Alaskan Malamute and other Spitz breeds, "grizzle" in Chihuahua, and "pied" in Beagle.

Methods

Study sample. The study sample (N = 11,727) consisted of non-invasive cheek swab samples collected by dog owners, and either blood or cheek swab samples collected at certified veterinary clinics in

accordance with international standards for animal care and research as a part of voluntary submission of samples to commercial DNA testing. In addition, the dog owners provided consent for the use of their dog's DNA information for research purposes. The samples were submitted for MyDogDNA / Optimal Selection analysis at Genoscoper Laboratories (Helsinki, Finland) and Wisdom Health (formerly Mars Veterinary) between April 3rd, 2015 and April 10th, 2018. Most of the tested dogs were from Finland (N = 5005, 42.7%) and the United States (N = 3192, 27.2%). The other major subgroups were formed by dogs from the Netherlands (N = 774, 6.6%), Denmark (N = 598, 5.1%), Austria (N = 413, 3.5%), UK, (N = 290, 2.5%), France (N = 286, 2.4%), Sweden (N = 164, 1.4%) and Australia (N = 121, 1.0%). Most of the tested dogs were from breeds recognized by Fédération Cynologique Internationale (FCI) or American Kennel Club (AKC), and the breed of the dog was reported by its owner with accompanying registration information. A few additional breeds not yet recognized by any major breed registry but with an established number of breed hobbyists, and mixed breed dogs, were also included in the study sample. Altogether, it amounted to 303 breeds and breed varieties, and 391 dogs representing the mixed breed population.

Genotyping. Genotyping of coat color gene variants of *MC1R* (2–5), *CBD103* (10, 11) and *ASIP* (7–9) loci, and the R301C variant of the *MC1R* gene was carried out according to manufacturer-recommended standard protocols on a custom-designed Illumina Infinium technology bead chip ((35, 36), Illumina, San Diego, Ca, USA). The genotyping quality control measures for this platform were previously described in (35, 36). For the purposes of this study, the R301C variant assay findings were additionally validated with a second genetic technology by Sanger sequencing in representatives of the breed Finnish Lapphund and Cirneco dell'Etna on a ABI3730xl DNA Analyzer platform (Thermo Fisher Scientific, Waltham, MA, USA) at the Finnish Institute of Molecular Medicine (FIMM) Sequencing Unit as described earlier in (35). Primers used for sequencing of the R301C locus were: 5- ACACTCACTATCCTGCTGGG – 3 (forward) and 5-TATTCCTTTCTCTGGCCCCA-3 (reverse).

Phenotypic Association. Coat color phenotype analysis utilized customer provided photos of dogs where the evaluator of the dog's phenotype was blind to the genotype. The phenotypic impact of the R301C variant was evaluated by considering the genotypes for R301C in conjunction with genotypes at the interacting coat color loci *MC1R*, *ASIP* and *CBD103.*

Declarations

Ethics approval and consent to participate

The study used DNA collected through non-invasive buccal swabbing or blood collected at certified veterinary clinics in accordance with international standards for animal care and research. The samples were voluntarily submitted for commercial genotyping, and no ethical permissions for dog handling or sampling were required.

Consent for publication

Not applicable.

Availability of data and materials

All relevant data is provided with the paper and its Supporting Information files.

Competing interests

HA, LH, PR, JM and JD are employees of Wisdom Health that offers canine DNA testing as a commercial service.

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Authors' contributions

Conceptualization: HA, LH, PR, JM and JD. Data curation: HA, LH, PR and JD. Formal analysis and validation: HA, LH, JM and JD. Methodology: JD, HA, LH and JM. Project administration: HA and JD. Supervision: HA and JD. Visualization: HA, JM and PR. Writing – original draft: HA, LH, JM and JD. Writing – review & editing: HA, LH, JM, PR and JD.

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References

1. Dürig N, Letko A, Lepori V, Hadji Rasouliha S, Loechel R, Kehl A, et al. Two MC1R loss-of-function alleles in cream-coloured Australian Cattle Dogs and white Huskies. Anim Genet. 2018;49(4):284–90.
2. Newton JM, Wilkie AL, He L, Jordan SA, Metallinos DL, Holmes NG, et al. Melanocortin 1 receptor variation in the domestic dog. Mamm Genome. 2000;11(1):24–30.
3. Dreger DL, Schmutz SM. A new mutation in MC1R explains a coat color phenotype in 2 “old” breeds: Saluki and Afghan Hound. J Hered. 2010;101(5):644–9.

4. Schmutz SM, Berryere TG, Ellinwood NM, Kerns JA, Barsh GS. MCIR Studies in Dogs with Melanistic Mask or Brindle Patterns. J Hered. 2003;94(1):69–73.
5. Everts RE, Rothuizen J, Van Oost BA. Identification of a premature stop codon in the melanocyte-stimulating hormone receptor gene (MC1R) in Labrador and Golden retrievers with yellow coat colour. Anim Genet. 2000;31(3):194–9.
6. Dreger DL, Hooser BN, Hughes AM, Ganesan B, Donner J, Anderson H, et al. True Colors: Commercially-acquired morphological genotypes reveal hidden allele variation among dog breeds, informing both trait ancestry and breed potential. PLoS One. 2019;14(10):1–24.
7. Kerns JA, Newton J, Berryere TG, Rubin EM, Cheng JF, Schmutz SM, et al. Characterization of the dog Agouti gene and a nonagouti mutation in German Shepherd Dogs. Mamm Genome. 2004;15(10):798–808.
8. Berryere TG, Kerns JA, Barsh GS, Schmutz SM. Association of an Agouti allele with fawn or sable coat color in domestic dogs. Mamm Genome. 2005;16(4):262–72.
9. Dreger DL, Schmutz SM. A SINE Insertion Causes the Black-and-Tan and Saddle Tan Phenotypes in Domestic Dogs. J Hered. 2011;102(Suppl 1):11–8.
10. Candille SI, Kaelin CB, Cattanach BM, Yu B, Thompson DA, Nix MA, et al. A β-defensin mutation causes black coat color in domestic dogs. Science. 2007;318(5855):1418–23.
11. Kerns JA, Cargill EJ, Clark LA, Candille SI, Berryere TG, Olivier M, et al. Linkage and segregation analysis of black and brindle coat color in domestic dogs. Genetics. 2007;176(3):1679–89.
12. Linderholm A, Larson G. The role of humans in facilitating and sustaining coat colour variation in domestic animals. Semin Cell Dev Biol. 2013;24(6–7):587–93.
13. Ollivier M, Tresset A, Hitte C, Petit C, Hughes S, Gillet B, et al. Evidence of Coat Color Variation Sheds New Light on Ancient Canids. PLoS One. 2013;8(10).
14. Römpler H, Rohland N, Lalueza-Fox C, Willerslev E, Kuznetsova T, Rebeder G, Bertranpetit J, Schöneberg T, Hofreiter M. Nuclear Gene Indicates Coat-Color Polymorphism in Mammoths. Science. 2006;313(5783):62.
15. Karlsson EK, Baranowska I, Wade CM, Salmon Hillbertz NHC, Zody MC, Anderson N, et al. Efficient mapping of mendelian traits in dogs through genome-wide association. Nat Genet. 2007;39(11):1321–8.
16. Dreger DL, Parker HG, Ostrander EA, Schmutz SM. Identification of a mutation that is associated with the saddle tan and black-and-tan phenotypes in Basset Hounds and Pembroke Welsh Corgis. J Hered. 2013;104(3):399–406.
17. Saporiti F, Piacentini L, Alfieri V, Bono E, Ferrari F, Chiesa M, et al. Melanocortin-1 receptor positively regulates human artery endothelial cell migration. Cell Physiol Biochem. 2019;52(6):1339–60.
18. Simamura E, Arikawa T, Ikeda T, Shimada H, Shoji H, Masuta H, et al. Melanocortins contribute to sequential differentiation and enucleation of human erythroblasts via melanocortin receptors 1, 2 and 5. PLoS One. 2015;10(4):1–17.

19. Lorenz J, Seebach E, Hackmayer G, Greth C, Bauer RJ, Kleinschmidt K, et al. Melanocortin 1 receptor-signaling deficiency results in an articular cartilage phenotype and accelerates pathogenesis of surgically induced murine osteoarthritis. PLoS One. 2014;9(9).
20. Chen X, Chen H, Cai W, Maguire M, Ya B, Zuo F, Logan R, Li H, Robinson K, VanderBurg CR, Yu Y, Wang Y, Fisher, David E, Schwartzchild MA. The Melanoma-Linked "Redhead" MC1R Influences Dopaminergic Neuron Survival. Ann Neorol. 2017;81(3):395–406.
21. Mountjoy KJ, Robbins LS, Mortrud MT, Cone RD. The Cloning of a Family of Genes That Encode the Melanocortin Receptors. Science. 1992;275(5074):1248–51.
22. Swope V, Alexander C, Starner R, Schwemberger S, Babcock G, Abdel-Malek ZA. Significance of the Melanocortin 1 Receptor in the DNA Damage Response of Human Melanocytes to Ultraviolet Radiation. Pigment Cell Melanoma Res. 2014;4:601–10.
23. Kadekaro AL, Leachman S, Kavanagh RJ, Swope V, Cassidy P, Supp D, Santor M, Schwemberger S, Babcock G, Wakamatsu K, Ito S, Koshoffer A, Boissy RE, Manga. Prashiela; Sturm, Ric ZA. Melanocortin 1 receptor genotype: an important determinant of the damage response of melanocytes to ultraviolet radiation. FASEB J. 2010;10:3850–60.
24. Tagliabue E, Gandini S, Bellocco R, Maisonneuve P, Newton-Bishop J, Polsky D, et al. MC1R variants as melanoma risk factors independent of at-risk phenotypic characteristics: A pooled analysis from the M-SKIP project. Cancer Manag Res. 2018;10:1143–54.
25. Box NF, Duffy DL, Irving RE, Russell A, Chen W, Griffyths LR, et al. Melanocortin-1 receptor genotype is a risk factor for basal and squamous cell carcinoma. J Invest Dermatol. 2001;116(2):224–9.
26. Binstock M, Hafeez F, Metchinikoff C, Arron ST. Signle-nucleotide polymorhphisms in pigment genes and nonmelanoma skin cancer predisposition: a systematic review. BJD. 2014;171(4):713–21.
27. Seaton ME, Parent BA, Sood RF, Wurfel MM, Muffley LA, Gibran NS. Risk of Complicated Sepsis After Trauma. 2018;47(1):79–85.
28. Chen X, Feng D, Schwarzschild MA, Gao X. Red hair, MC1R variants, and risk for Parkinson's disease - a meta-analysis. Ann Clin Transl Neurol. 2017;4(3).
29. Gao X, Simon, Kelly C, HanmJiali; Schwarzschild MA, Ascherio A. Genetic determinants of hair color and Parkinson's disease risk Xiang. Ann Neorol. 2009;65(1):76–82.
30. Liem EB, Lin CM, Suleman MI, Doufas AG, Gregg RG, Veauthier JM, et al. Anesthetic requirement is increased in redheads. Anesthesiology. 2004;101(2):279–83.
31. Liem EB, Joiner TV, Tsueda K, Sessler DI. Increased sensitivity to thermal pain and reduced subcutaneous lidocaine efficacy in redheads. Anesthesiology. 2005;102(3):509–14.
32. Mogil JS, Ritchie J, Smith SB, Strasburg K, Kaplan L, Wallace MR, et al. Melanocortin-1 receptor gene variants affect pain and μ-opioid analgesia in mice and humans. J Med Genet. 2005;42(7):583–7.
33. Delaney A, Keighren M, Fleetwood-Walker SM, Jackson IJ. Involvement of the melanocortin-1 receptor in acute pain and pain of inflammatory but not neuropathic origin. PLoS One. 2010;5(9):1–10.

34. Karyadi DM, Karlins E, Decker B, vonHoldt BM, Carpintero-Ramirez G, Parker HG, et al. A Copy Number Variant at the KITLG Locus Likely Confers Risk for Canine Squamous Cell Carcinoma of the Digit. PLoS Genet. 2013;9(3).
35. Donner J, Kaukonen M, Anderson H, Möller F, Kyöstilä K, Sankari S, et al. Genetic panel screening of nearly 100 mutations reveals new insights into the breed distribution of risk variants for canine hereditary disorders. PLoS One. 2016;11(8):1–18.
36. Donner J, Anderson H, Davison S, Hughes AM, Bouirmane J, Lindqvist J, et al. Frequency and distribution of 152 genetic disease variants in over 100,000 mixed breed and purebred dogs. PLoS Genet. 2018;14(4):1–20.

Figures

Figure 1

Photos representing the phenotypic impact of the eA allele. In the presence of KB at the K locus, eA/e1 genotype produces a partial recessive red phenotype representing the dogs A locus genotype (fawn) A) which can be seemingly indistinguishable from recessive red typical in the breed Cirneco dell'Etna B) differing from the rare KB solid eumelanin shade in Cirneco dell'Etna (in which the eumelanin shade is brown due to mutation in the TYRP1 gene). In the dogs expressing A locus color patterns (in absence of K

locus) eA/e1 and eA/eA genotypes produce domino pattern on fawn D) instead of heavily shaded fawn observed E) in Finnish Lapphund; domino pattern on wolf sable F) instead of typical wolf sable in Siberian Husky G); domino pattern on tan points H) compared to normal tan points I-J) in Chihuahua; and domino pattern on recessive black K) instead of normal recessive black L) in Tamaskan Dog.

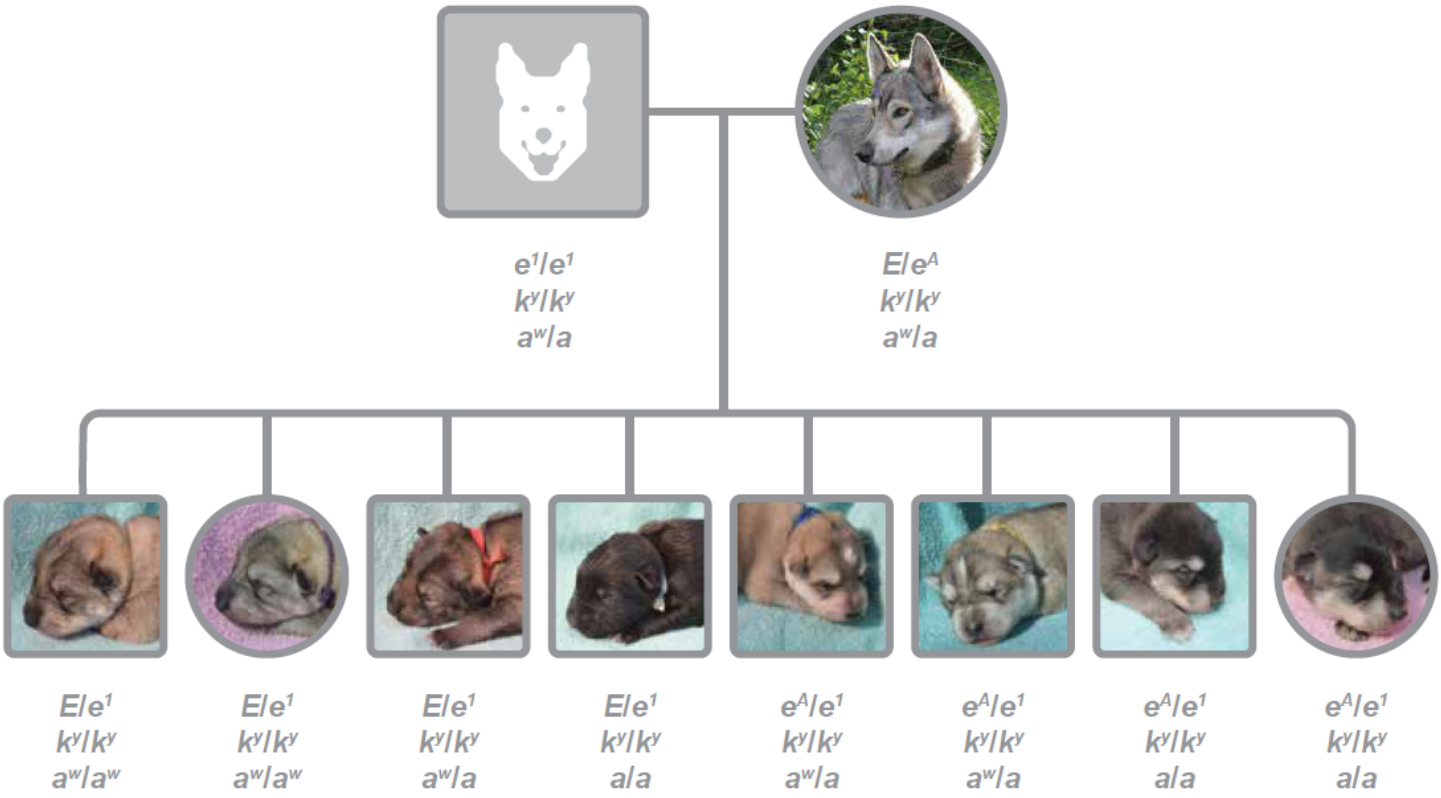

Figure 2

The eA allele associated domino pattern is recessive to the wild type E allele. Mating of recessive red (cream colored) e1/e1 sire to E/eA wolf sable dam in the Tamaskan Dog breed resulting in four normal wolf sable puppies with E/e1 genotype and four puppies with domino pattern, of which two express domino on wolf sable and two express domino on recessive black. In newborns, domino pattern is visible as large pheomelanin colored areas on both sides of the muzzle, pheomelanin areas around the eyes and overall as a lighter coat color in the body with dark bar of hair left on the back side around the vertebra.

Supplementary Files

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- Table1Andersonetal.png
- TableS1Andersonetal.xlsx