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Research article

IDENTIFICATION OF CRYPTIC ALLELE FOR MERLE PATTERNING IN DOGS BY MOLECULAR GENETICS METHODS

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Merle patterning in dogs, caused by the insertion of a short interspersed element (SINE) in the genetic structure of SILV gene, is characterized by patches of diluted pigment intermingled with normal melanin. Sequencing analyses of SINE element localized in the canine SILV gene discovered a variability of the poly (A)-tail length which is responsible for the different expression of merle pattern. The SINE element with the length of poly(A)-tail between 91 - 101 nucleotides is responsible for the merle phenotype with all characters of merle pattern. On the contrary the dogs which have SINE element with the shorter length of poly(A) tail between 54-65 nucleotides are referred as cryptic merles without expresion of Merle pattern. The aim of this study was to improve molecular genetics method for the detection of cryptic allele for merle patterning in dogs. A total of 40 dogs of four breeds - Border collie, Shetland sheepdog, Australian Shepherd dog, and Chihuahua were used in this study. Canine genomic DNA was isolated from samples of whole blood and buccal cells by commercial column kit. Detection of merle (*M*), cryptic merle (*Mc*) and non-merle (*m*) alleles was done using M13-tailed primer protocol and two different allele-sizing methods for the verification of the electrophoresis result. In the analyzed population of dogs were detected 20 dogs with non-merle genotype *mm*, 17 dogs with merle genotype *Mm*, 2 dogs with double merle genotype *MM* and one dog with merle phenotype but with the presence of cryptic merle allele *Mc* with the consequential genotype *MMc.*

Key words: dog, SILV gene, SINE element, Merle pattern, cryptic allele, DNA

INTRODUCTION

Coat color in mammals depends on the skin and hair pigment synthesis. Melanocytes manufacture two types of melanin: the black/brown photo-protective eumelanin pigment, and the red-yellow cytotoxic phaeomelanin pigment [1,2].

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Merle is a coat pattern in the domestic dog characterized by patches of diluted pigment intermingled with normal melanin. The coat color pattern is seen in the Collie, Australian Shepherd, Shetland Sheepdog, Catahoula Leopard Dog, Cardigan Welsh Corgi, Dachshund, and Great Dane breeds, and less commonly in the Chihuahua, American Pit Bull Terrier, American Staffordshire Terrier, Beauceron, Border Collie, Coolie, and others [3,4].

Merle is inherited in an autosomal, incompletely dominant fashion. Merle patterning in several breeds of dogs is caused by the insertion of a short interspersed element (SINE) in the genetic structure of SILV gene. One of the most striking aspects of merle is that it is genetically unstable. Whitney and Lamoreux [5] presented the hypothesis that the merle locus contains a transposable element. Sequencing analysis revealed that the variability in the SINE is found in the poly(A)-tail, which is an important factor in retrotransposition [6]. The poly(A)-tail is evolutionarily unstable and subject to mutation and degradation over time. This phenomenon may exist in part because poly(A)-tails are subject to strand slippage during replication and unequal crossing over [7]. During development, melanoblasts migrate from the neural crest and differentiate into the pigment producing melanocytes [8]. Melanocytes having a larger SINE insertion in the SILV gene would produce diluted pigment, while those with a truncated A-tail would produce full pigment [6].

Instability of the poly(A)-tail during this migration could result in cell populations with varying tail lengths. There are three groups of alleles, the ancestral allele (*m*) that lacks the SINE insertion, the derivative allele (*M*) with the SINE insertion containing long poly(A)-tail (91–101 nucleotides long) that disrupts SILV function and causes merle phenotype and a cryptic allele (*Mc*) which carries the SINE insertion with a shorter poly(A)-tail of 54–65 nucleotides [6]. An important implication of this idea is that both the *M* and the *Mc* alleles would exhibit instability, the former for tail shortening to a 'normal' phenotype, and the latter for tail expansion to an abnormal phenotype, in which case the phenomenon of merle reversion might more accurately be referred to as pseudoreversion. Indeed, the idea of an unstable *Mc* allele is likely to underlie what has been described as a cryptic or phantom merle, in which a dog with little or no pigmentary abnormalities gives typical merle offspring. Additionally, expansion of the oligo(dA)-rich tail in the germ line of a nonmerle dog having the smaller insertion may result in merle offspring and may be the mechanism behind the cryptic merle phenotype [6].

Two mutant copies *(M)* of SILV may develop problems. Double Merle dogs (MM) may be deaf or have impaired hearing to some degree. Double Merle dogs also have eye defects. These defects can occur in any color of eye. The size and shape of the eye may be affected. Some dogs may have eyes covered by the third eyelid permanently. The defects can vary from minor vision and hearing loss to complete deafness and blindness [4,6].

The aim of this work is the improvement and optimization of molecular genetics methods for the identification of dogs that carry the SILV SINE element with different size of poly (A)-tail and may produce offspring with various degrees of merle patterning.

MATERIAL AND METHODS

Animals

The material involved 40 dogs from 4 different breeds (Border collie, Shetland sheepdog, Australian Shepherd dog, Chihuahua). Canine genomic DNA was isolated from samples of buccal cells and whole blood by using QIAamp DNA Mini kit (Qiagen) according to the manufacturer's protocol.

Genotyping

The amplification of the specific SINE element localized in exon 11 was done by DNA primers described by Strain et al. [4] with a modification of the reverse primer. The identification of Merle allele was done using a locus-specific reverse primer (5´CCTCGGCAAATCACAGCA 3´) designed with an added universal M13 tag (5´CACACAGGAAACAGCTATGACCAT 3´) at the 5´ end, a locus-specific forward primer (5´CAGTTTCTCCTTTATTCTCCCA 3´) and a M13 homologous primer labeled with D3 WellRED dye (Sigma-Aldrich). Amplifications were conducted on a C1000-Touch thermocycler (Bio-Rad). The PCR reaction mixture contained, 1U MyTaq Hot-Start polymerase (Bioline), 1X MyTaq PCR buffer, 5 mM $MgCl_2$, 0.5 μM regular untagged forward primer, 0.5 μM universal fluorescent M13 primer, 0.05 regular tagged reverse primer and 50 ng DNA in the final 25 μl volume. The following amplification parameters were applied: an initial denaturation step at 95°C for 3 min followed by 30 cycles of denaturation (95°C for 10 sec), annealing (63°C for 10 sec) and DNA extension (72°C for 30 sec). The reaction was completed by the final extension step at 72 °C for 10 min. The amplified PCR products were first separated by electrophoresis on 2 % agarose gel (Serva) containing GelRed dye (Biotium) at 180 V in 1 x sodium borate buffer [9] for 20 min for the verification of DNA bands intensity. After separation the gel was analyzed by UV transilluminator and photographed with a documentation system Olympus C7070. Subsequently, 1 μl of the fluorescently labeled PCR product was added to 40 μl sample loading solution and 0.5 μl DNA size standard – 600 (Beckman Coulter) and run on the GeXP Genetic Analyzer (Beckman Coulter). The evaluation and data processing were performed using GenomeLab System software, version 10.2, which is part of the automatic genetic analyzer GenomeLab GeXP Genetic Analysis System package (Beckman Coulter, USA).

RESULTS

A total of 40 dogs of four breeds were included in this study for the detection of SINE element in the canine SILV gene. Identification of merle allele *M* (the full length of SINE element), cryptic merle allele *Mc* (the shorter variant of SINE element) and non-merle allele *m* (absence of SINE element) was performed using M13-tailed reverse primer and two different allele sizing methods. Fragments of PCR reaction were amplified with M13 primer end-labeled with WellRED fluorescent dye D3 (Sigma-Aldrich), M13-tailed specific reverse primer and specific forward primer. On agarose gel the allele *M* responsible for merle coloration produced a fragment slightly smaller than 500 bp, and allele *m* produced a fragment with an approximate size of 200 bp. The non-merle dogs were homozygote *mm* and merle dogs were heterozygote *Mm* or homozygote *MM*. However one sample of dog had a unique pattern of DNA fragments. This dog had homozygous genotype *MM* (approximately one fragment of size 500 bp) after the first 20 minutes of separation but a longer separation on 2 % agarose gel for 35 minutes allowed the identification two fragments with sizes between 450 – 500 bp (Figure 1).

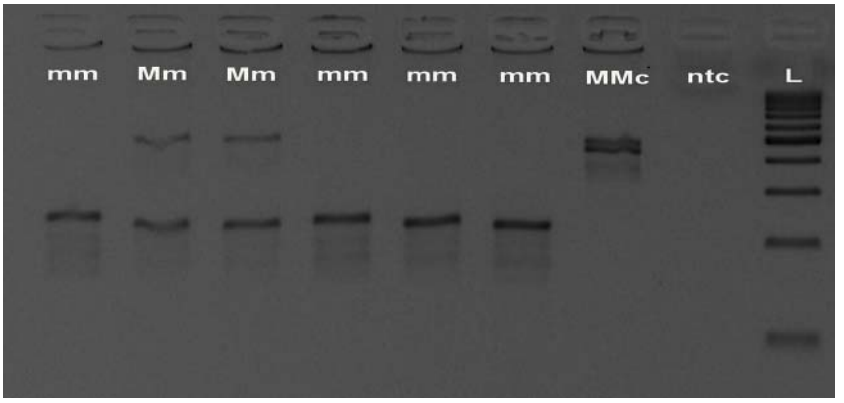

Figure 1. Representative results of PCR analysis for the detection of merle allele amplified by using M13-tailed reverse primer separated on 2 % agarose gel. Genotype *mm* (230 bp); genotype *Mm* (230 bp, 495 bp); genotype *MMc* (458 bp, 495 bp); *ntc* – no template control, *L* – GeneRuler Ladder 100 bp (ThermoScientific). The size of fragments was determined by fragment analysis on a genetic analyzer GeXP with a combination of D3 labelled M13 primer (24-mer)

The verification of all results obtained by agarose electrophoresis was done by fragment analysis on an automatic capillary genetic analyzer GeXP (Figure 2).

In the populations of Border collies, Shetland sheepdogs and Chihuahuas heterozygote genotype *Mm* was detected in merle colored dogs with a frequency of 0.14, 0.45, 0.8 and homozygote genotype *mm* in non-merle colored dogs with a frequency of 0.86, 0.55 and 0.2, respectively. In the Australian Shepherd dog all *MM* genotypes (included *MMc* genotype), *Mm* and *mm* with frequencies 0.3, 0.6 and 0.1 were observed. The *M*

allele (included cryptic merle allele *Mc*) was distributed with an allele frequency ranging from 0.07 to 0.6. The *m* allele was distributed with an allele frequency ranging from 0.4 to 0.93. The detailed genotype and gene frequencies per breed are presented in Table 1.

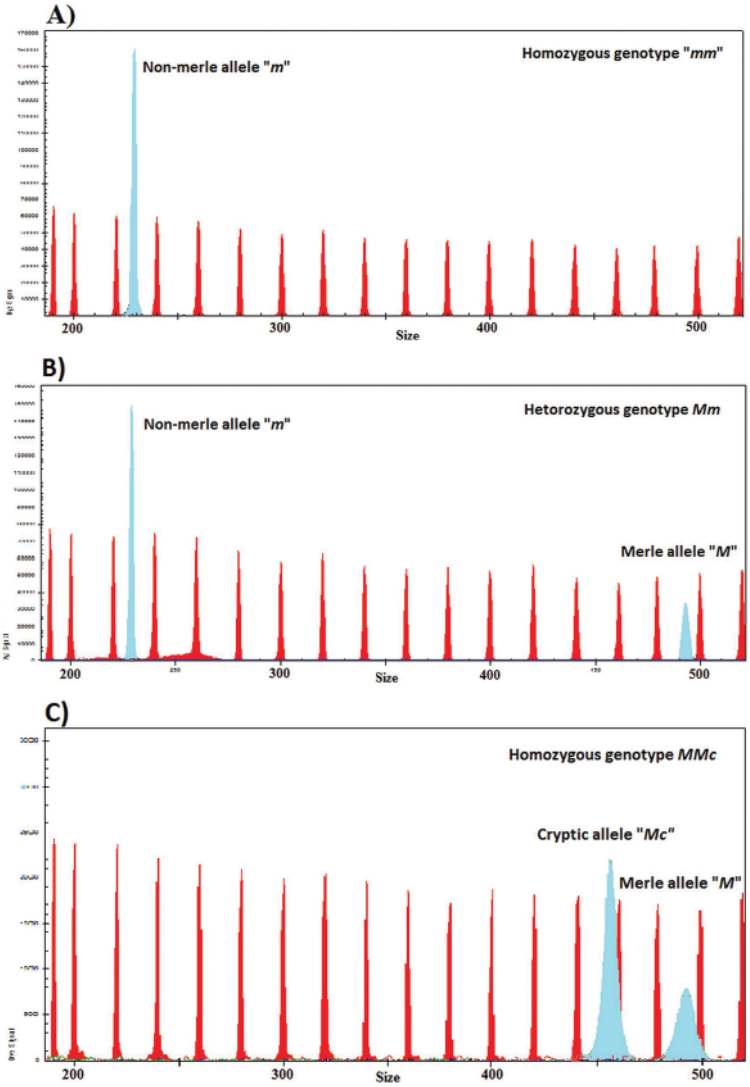

Figure 2. Comparison of GeXP electropherograms from three PCR reactions for detection of SINE insertion in canine SILV gene with M13-tailed reverse primer labeled with WellRED dye D3. Panel **A** shows the result of non-merle homozygous genotype *mm*. The size of peak for non-merle allele *m* was 230 bp. Panel **B** shows the result of heterozygote genotype *Mm* with the size of peaks 230 bp (allele *m*) and 495 bp (allele *M*). Panel **C** shows the result of Merle homozygous genotype *MMc* with the size of peaks 458 bp (allele *Mc* – shorter variant of SINE insertion called as cryptic Merle allele) and 495 bp (allele *M* – variant of SINE insertion associated with Merle coat pattern in dog). The sizes of peaks 230 bp, 458 bp and 495 bp are included with M13 tail (24-mer)

Table 1. Frequency of genotypes and alleles of *SILV gene* in the population of dog breeds

Breed	Dogs	Genotype frequencies			Allele frequencies	
		MM	Mm	mm	M	m
Border collie	14	0	0.14	0.86	0.07	0.93
Shetland sheepdog	11	0	0.45	0.55	0.23	0.77
Australian Shepherd dog	10	0.3	0.6	0.1	0.6	0.4
Chihuahua	5	0	0.8	0.2	0.4	0.6
Total	40	0.075 (3)	0.425 (17)	0.5 (20)	0.29	0.71

DISCUSSION

The merle phenotype of the dog is a pattern pigmentation which is extremely popular in some breeds like Australian Shepherd dog or Catahoula Leopard dog. Dogs homozygous for merle (*MM*) are known as Double Merles and are predominantly white. Hetorozygous *Mm* animals have mild-to-moderate dilution of eumelanic areas. Animals homozygous for the presumptive ancestral or wild type allele *m* are normally pigmented. Characteristically, small patches of normal color appear within areas of diluted pigmentation in both *MM* and *Mm* dogs [10]. Dogs having the merle genotype but not expressing the merle phenotype are known as cryptic merles (represented here by *Mcm*) [4]. Unfortunately, the Double Merle phenotype is associated with a wide range of developmental defects like deafness, blindness, skeletal defects and sterility [3,6,11]. In the present study non-merle homozygous genotypes *mm* and merle heterozygous genotype *Mm* were detected in all analyzed breeds (Australian Shepherd dog, Shetland sheepdog, Border collie, Chihuahua). The double merles genotypes *MM* were detected only in three Australian shepherd dogs but one of these dogs carried a merle allele *M* and a cryptic merle allele *Mc* so the genotype for this dog was *MMc*. The results of merle genotyping for this dog by molecular genetics methods confirmed a hypothesis that the cryptic merle allele *Mc* was inherited from one of the parents. The sire of this dog had a merle phenotype with genotype *Mm* and the dam was described by the owner as a tricolor without any merle pattern (not genotype tested). Because the tested dog has the genotype *MMc* instead of the expected genotype *Mm*, its dam must be the cryptic merle with genotype *Mcm*. This tested dog with genotype *MMc* can produce merle puppies but also non-merle puppies (*Mcm*) which inherited the cryptic allele from him. Moreover, Strain et al. [4] described that some of the genotyped double merles could carry one cryptic merle allele. Strain et al. [4] also confirmed the association between merle coloration with deafness and found out a significant association between hearing status and heterozygous versus homozygous merle genotype. In their study, 2.7% were unilaterally deaf and 0.9% bilaterally deaf for single merles (*Mm*). For double merles (*MM*), 10% were unilaterally deaf and 15% were bilaterally deaf. Similarly, Reetz et al. [12] studied the auditory capacity of Dachshunds and found that 54.6% of MM and 36.8% of Mm dogs had auditory

dysfunction, ranging from mild to severe deafness. All control dogs (*mm*) in the study had normal hearing. Double merle dogs may also have a variety of eye defects that can occur in any eye color and most of them may have loss of vision, sometimes to the point of blindness [4].

In this study we detected double merle only in the Australian Shepherd dog population, in which one of the three genotyped double merles dogs has one cryptic merle allele (*Mc*). This suggests that our improvement of the genetic test for the SILV locus, which is responsible for merle coloration in dogs allows us to classify merle dogs as single or double merle, and to identify cryptic merles. The genetic testing can help breeders of merle dogs to prevent undesirable double merle progeny which often exhibit a wide range of auditory and ophthalmic abnormalities.

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IDENTIFIKACIJA SKRIVENOG ALELA ZA MRAMORIRANU ŠARU PASA POMOĆU METODA MOLEKULARNE GENETIKE

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Mramorirana šara pasa, nastala umetanjem kratkih ponovaca elemenata (SINE) u genetsku strukturu SILV gena, karakteriše se prisustvom slabije pigmentisanih polja pomešanih sa poljima normalne pigmentacije. Analizom SINE elementa lokalizovanog u SILV genu pasa sekvencioniranjem otkrila je varijabilnost poli(A) repa koji je odgovoran za različitu ekspresiju mramorirane šare. SINE element sa poli(A) repom dužine od 91 do 101 nukleotida je odgovoran za mramorirani fenotip sa svim karakterima mramorirane šare. Nasuprot tome, psi koji poseduju SINE element sa kratkim poli(A) repom dužine od 54 do 65 nukleotida su opisani kao prikriveni mramorirani bez ekspresije mramorirane šare. Cilj ove studije je poboljšanje metoda molekularne genetike za detekciju skrivenog alela za mramoriranu šaru pasa. Ukupno 40 pasa četiri rase: border koli, šetlandski ovčar, australijski ovčar i čivava bilo je uključeno u studiju. Genom DNK pasa bio je izolovan iz uzoraka pune krvi i ćelija sluznice usne duplje bio je izolovan pomoću komercijalnih kitova. Detekcija mramoriranog (M), prikriveno mramoriranog (Mc) i ne-mramoriranog (m) alela je izvršena pomoću „M13-tailed" prajmera protokola i dve različite metode određivanja veličine alela za verifikaciju rezultata elektroforeze. U analiziranoj populaciji pasa otkriveno je 20 pasa sa ne - mramoriranim genotipom mm, 17 pasa sa mramoriranim genotipom Mm, 2 psa sa duplim mramoriranim genotipom MM i jedan pas sa mramoriranim fenotipom, ali sa prisustvom prikrivenog mramoriranog alela Mc sa posledičnim genotiom MMC.