

Detection of MLPH gene polymorphism in population of the Belgian Shepherd variety Malinois

Detekcia polymorfizmu génu MLPH v populácii belgického ovčiaka variety malinois

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ABSTRACT

Melanophilin (MLPH) gene affects dilution of coat colour. It focuses on D locus alleles and in c.-22G>A polymorphism 2 alleles were described. Dominant *D* allele controls regular colour whereas recessive *d* allele causes dilution. Diluted colouration in Belgian Shepherd variety Malinois is undesirable and excluded from breeding. Dilution of the coat colour can sometimes be accompanied by skin disease (CDA or BHFD). Methods of molecular genetics including PCR-RFLP method were used to identify c.-22G>A polymorphism in MLPH gene. Within the analysed population of 56 dogs following genotypes were discovered: 50 dogs with homozygous *DD* genotype, i.e. 89.29% frequency of occurrence and 6 dogs with heterozygous *Dd* genotype, i.e. 10.71% frequency of occurrence.

Keywords: melanophilin, dilution, dog, colouration, polymorphism

ABSTRAKT

Gén melanofilín (MLPH) je zodpovedný za zriedenie farby srsti. Jedná sa o sériu alel D lokusu, pričom pre polymorfizmus c.-22G>A boli popísané 2 alely. Dominantná alela *D* kontroluje normálne sfarbenie srsti a recesívna alela *d* je zodpovedná za zriedenie. Zriedené sfarbenie je u belgického ovčiaka variety Malinois nežiaduce a vyradujúce z chovu. Zriedenie farby srsti môže byť niekedy sprevádzané ochorením kože (CDA alebo BHFD). Pomocou molekulárno-genetických metód s využitím PCR-RFLP metódy prebehla identifikácia polymorfizmu c.-22G>A v géne MLPH. V sledovanej populácii 56 psov boli zistené tieto genotypy: 50 psov s homozygotným genotypom *DD*, čo predstavuje frekvenciu výskytu 89,29 % a 6 psov s heterozygotným genotypom *Dd*, čo zodpovedá frekvencii výskytu 10,71 %.

Kľúčové slová: melanofilín, zriedenie, pes, sfarbenie, polymorfizmus

INTRODUCION

Belgian Shepherd is considered interesting breed since it includes four closely connected varieties: Malinois, Groenendael, Tervueren and Laekenois. Given varieties are distinguished by fur coat colour and texture. In some countries including the USA, which are not FCI members, some varieties are considered independent dog breeds. The most common variety in Slovakia is Malinois.

The standard colouring for Malinois is fawn with black overlay and black mask. Disqualifying faults are: any colours which do not correspond with those of the described varieties (FCI, 2002).

Coat colour dilution in dogs is a specific pigmentation phenotype caused by a defective transport of melanosomes leading to large clumps of pigment (Drögemüller et al., 2007).

The MLPH gene encodes melanophilin, protein involved in melanocyte transport. A dog's MLPH gene is located in locus D (dilution) (Charon and Lipka, 2015).

Coat colour dilution (*d*) is inherited as a Mendelian autosomal recessive trait in various dog breeds (Schmutz et al., 1998). In the study by Drögemüller et al. (2007) the c.-22G>A transition at the last nucleotide of the 5'-untranslated first exon of the MLPH gene has been identified as a candidate causative mutation for the dilute phenotype. This variant is termed as *d* or *d*¹ allele. Bauer et al. (2018) identified MLPH: c.705G>C as a variant explaining a second dilution of the coat colour in dogs, which they termed as allele *d*². Although relatively rare overall, this *d*² allele is segregating in at least three dog breeds (Chow Chows, Sloughis and Thai Ridgebacks). Van Buren et al. (2020) described third MLPH variant termed as *d*³ caused by mutation c.667_668insC in dogs whose coat colour dilution could not be explained by known variants c.-22G>A (*d*¹) or c.705G>C (*d*²). The *d*³ allele is found at low frequency in multiple dog breeds (Hungarian pumi, Hungarian mudi, Chihuahua, Pekingese, Italian greyhound, Shih Tzu, Tibetan mastiff, Yorkshire terrier, and Shetland sheepdog) as well as indigenous dogs, wolves, and wolf-dog hybrids.



Figure 1. Phenotypic expression of the MLPH gene in the Malinois variety. The puppy on the left has a diluted colour and the puppy on the right has a standard fawn colour with a black mask

In some dogs the coat colour dilution is sometimes accompanied by hair loss and recurrent skin inflammation, the so called colour dilution alopecia (CDA) or black hair follicular dysplasia (BHFD) (Philipp et al., 2005a). Hereditary alopecia occurs as an autosomal recessive trait (Šťastná and Šťastný, 2018). Colour-dilution alopecia is a relatively uncommon hereditary skin disease in dogs, characterized by loss of coat in the colour-diluted areas of the skin. It is observed in „Blue“ colouration which is a dilution of normal black colour (Figure 1) (Kim et al., 2005; Perego et al., 2009; Palumbo et al., 2012). This syndrome is associated with a colour-dilution gene. Disease is characterized by a progressive loss of hair, initial clinical signs are the gradual onset of a dry, dull and poor hair coat quality which is sensitive to sunburn or extreme cold. Hair shafts and hair regrowth are poor, and follicular papules may develop and progress to frank comedones. Hair follicles are characterized by atrophy and distortion. Heavily clumped melanin is present in the epidermis, dermis and hair follicles. Disease may be accompanied by recurrent bacterial infections of the hair follicles (folliculitis) (Kim et al., 2005; Philipp et al., 2005b). Canine black hair follicular dysplasia (BHFD) is a rare disorder confined to black coat regions affecting bicolor or tricolor animals within the first few weeks of life (von Bomhard et al., 2006).

The aim of this study was to analyse polymorphism c.-22G>A of MLPH gene associated with coat colour dilution in population of Belgian Shepherd Malinois dogs.

MATERIAL AND METHODS

Animals

Biological material was obtained from 56 Belgian Shepherd Malinois dogs. All individuals are purebred and have had a pedigree. The samples come from individuals from several kennels mostly Slovakia further the Czech Republic, Hungary and Slovenia. These individuals work in police, military, professional dog sports but also as pet dogs. Most of them have intervened, intervenes and will interfere in breeding in Slovakia and abroad.

Genotyping

Genomic DNA was isolated from buccal cell swabs using a REDEExtract-N-Amp™ Tissue PCR Kit (Sigma Aldrich). The polymorphism of c.-22G>A of MLPH gene was determined by the PCR-RFLP method. To amplify a specific region of the c.-22G>A MLPH gene was used oligonucleotide primers according to the methodology of Drögemüller et al. (2007).

PCR amplifications were performed in a total volume of 25 µl containing 20 ng of genomic DNA, 1.5 mM MgCl₂ (FERMENTAS), 0.2 mM of each dNTP (INVITROGEN), 1 µM of each primers (GeneriBiotech), 1U of Taq DNA polymerase (FERMENTAS) and 1x PCR reaction buffer (FERMENTAS). PCR amplification was performed as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final extension step of 72 °C for 5 min. PCR products were digested 1 hour at 37 °C with 5U of *Bse*FI restriction enzyme (NEB). The amplified MLPH gene PCR products were visualized on a 3% agarose gel with the addition of the intercalating agent GelRed™(Biotium).

Statistical Analysis

Based on molecular genetics analyses was established genotype structure of following population and calculated allelic frequencies for polymorphism c.-22G>A of MLPH

gene. Significance of differences between experimental and theoretically expected frequencies of genotypes was verified with χ^2 -test.

RESULTS

In population of 56 Malinois variety were found 50 dogs with homozygous genotype *DD* and 6 dogs with heterozygous genotype *Dd*. The homozygous genotype *dd* was not observed in test population. The allele *D* consists of fragments of 238 bp and 74 bp in length, and the allele *d* constitutes a 312 bp fragment (Figure 2).

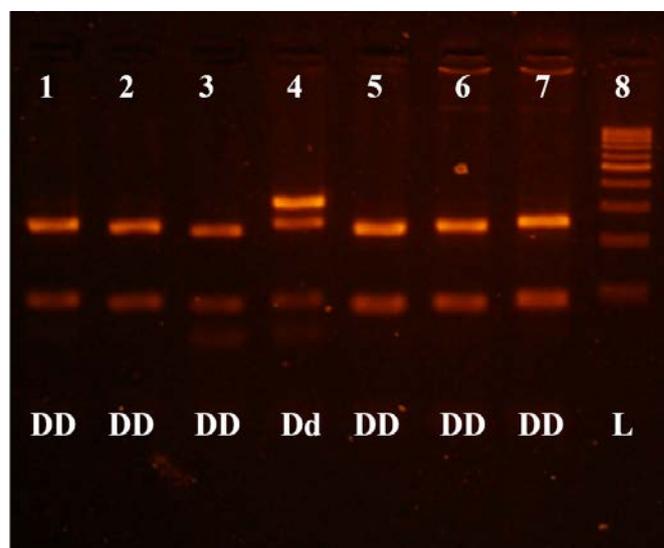


Figure 2. Representative results of PCR-RFLP analyses SNP c.-22G>A MLPH gene on 3% agarose gel. 1, 2, 3, 5, 6, 7 – genotype *DD*; 4 – genotype *Dd*; 8 – DNA ladder 100 bp (Fermentas)

The highest representation was of the homozygous genotype *DD* with a frequency of 89.29%. The heterozygous *Dd* genotype occurred at a lower frequency of 10.71%. The homozygous genotype *dd* was not observed in the Malinois variety population. Based on the results, it can be seen that the allele *D* has a significant predominance with a frequency of 94.64%, over the *d* allele with a frequency of 5.36% in test population (Table 1.).

Based on the χ^2 test, was found that the difference between the expected and observed frequencies of genotypes is not statistically significant, which means that an equilibrium state was found in the population under review according to Hardy-Weinberg law.

Table 1. Genotypes and allelic frequencies of SNP c.-22G>A of MLPH gene

	Genotype Frequencies			Allelic Frequencies		χ^2	P
	<i>DD</i>	<i>Dd</i>	<i>dd</i>	<i>D</i>	<i>d</i>		
MLPH c.-22G>A	0.8929	0.1071	0	0.9464	0.0536	0.181	0.9134

DISCUSSION

In study were analysed 56 dogs of the Belgian Shepherd variety Malinois to identify polymorphism c.-22G>A in the MLPH gene, which causes the appearance of diluted colouration, which is also sometimes called breeders "Blue" colouration in the Belgian Shepherd. Welle et al. (2009) found a demonstrable association of c.-22G>A polymorphism in the MLPH gene with diluted hair colour in more than 900 dogs supporting the hypothesis that this polymorphism is indeed the cause of mutation. Also Santos et al. (2017) in their study pointed that a mutation G>A at 5'-UTR region of exon 1 of the MLPH gene is responsible for the coat colour dilution phenotype in dogs.

In total of 56 tested dogs, 50 dogs had the dominant homozygous genotype *DD*. Dogs with this genotype are not carriers of the diluted "Blue" colour nor are they transmitted to the offspring. The remaining 6 dogs have heterozygous genotype *Dd*, i. e. they carry one dominant allele "*D*" and the other recessive allele "*d*", which is responsible for the diluted phenotype. These individuals do not have a dilute coat colour, but they are carriers. Thus, puppies with diluted colouration may occur in the offspring of two heterozygotes. When comparing the pedigrees of these 6 dogs, it was found that 3 individuals have the same ancestor in the pedigree, which is considered to be a dilute colour carrier, since in its offspring puppies with a diluted phenotype were recorded in the past.

In tested population, all individuals have had a standard colour (fawn with black overlay and black mask) that was consistent with the genotypes obtained. In population were observed only two genotypes, dominant homozygous *DD* and heterozygous *Dd*.

Recessive homozygous genotype *dd* has not been found in population because individuals exhibit *dd* genotype as a dilute stain. In population, the individual with diluted colouration was not found, which is confirmed by results.

Welle et al. (2009) in their study genotyped 935 dogs with recorded coat colour from 20 different dog breeds at this SNP marker. In 9 of the 20 dog breeds, informative 2-generation families were available, and in these families the "*d*" allele showed perfect cosegregation with the dilute phenotype. Furthermore, they observed perfect association of the dilute phenotype with the c.-22G>A polymorphism in all 20 different breeds, and each of the 112 dilute coloured dogs in their study was homozygous for the "*d*" allele at the c.-22G>A polymorphism.

Honkanen (2018) states in her study that the "*d*" allele is a rare allele in the Belgian Shepherd.

The Slovak Club of Belgian Shepherds cooperates with the database of Belgian and Australian Shepherds of the Club KCHBO, which provides information on individuals registered in the Slovak and Czech herd books. In 2004 one dog of variety Tervueren with diluted colouring was registered, which came from parents of variety Malinois. From 2010 to 2020 there were 17 Malinois dogs with diluted colouration registered. All these dogs come from Czech breeds. In Slovakia, no individual with diluted colouring has been registered yet. However, some of the dogs whose offspring were registered in the database with diluted colour, were used as stud dogs in the Slovak breeding.

The dilution of the coat colour is inherited mendelistically as an autosomal recessive trait. When comparing the pedigrees of the tested subjects with those of the diluted colouration registered in the Belgian and

Australian Shepherd database of the KCHBO Club, was found that heterozygous dogs from tested population carrying the recessive allele *d* have common ancestors with the individuals with diluted colouration in the database. Based on these findings, it could be identified some dilute colour carrier dogs. Although no individuals with diluted colouring were registered in Slovakia, results confirm the presence of the recessive allele *d* in the Slovak population of Belgian Shepherd variety Malinois and this is confirmed by the information obtained from the comparison of pedigrees.

Dilute coloured dogs are assumed to be predisposed to develop hair loss in the form of CDA or BHFD (Welle et al., 2009). MLPH gene mutations may cause skin and hair pigmentation disorders. In dogs MLPH gene mutations cause coat colour dilution as well as a disorder called colour dilution alopecia (CDA) (Charon and Lipka, 2015). Although data clearly indicate that the MLPH mutation increases the risk for CDA/BHFD, there seem to be additional modifying factors, however it is currently not fully clear which additional genetic and / or environmental risk factors are involved in CDA (Welle et al., 2009, Bauer et al., 2018). CDA seems to be a dog specific phenomenon, other known animals with dilute coloured phenotypes, such as e.g. cats, rabbits or mice, apparently have no pathological alterations of their hair or skin (Welle et al., 2009, Bauer et al., 2018).

CONCLUSION

Diluted colouration by breeders, also called "Blue", is a rare colouration in Belgian Shepherd and is considered non-standard and excluded from breeding for all Belgian Shepherd varieties including the Malinois variety, which is the most widespread bred variety in Slovakia.

In analysed population were found two genotypes *DD* (dominant homozygote) and *Dd* (heterozygote). The genotype *dd* (recessive homozygote) was not detected in population, because this genotype is manifested as a dilute stain and there was no dilute stained in population.

Although dilute staining is undesirable because it is excluded from breeding, genetic testing of DNA can

easily identify heterozygotes that are carriers of diluted staining. Testing and keeping records of individuals can give breeders the possibility to choose the most suitable parent pairs for further breeding in order to prevent the birth of diluted puppies. Also, to avoid the necessity of automatically decommissioning heterozygous individuals from the breeding, without considering their nature and working abilities that could be beneficial to the breed.

Diluted dogs are expected to increase the risk of developing CDA and BHFD. In population there was no dog with diluted colouration or manifestations of these diseases, so this theory cannot be confirmed or refuted.

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REFERENCES

- Bauer, A., Kehl, A., Jagannathan, V., Leeb, T. (2018) A novel MLPH variant in dogs with coat color dilution. *Animal genetics*, 49 (1), 94-97.
DOI: <https://doi.org/10.1111/age.12632>
- Drögemüller, C., Philipp, U., Haase, B., Günzel-Apel, A. R., Leeb, T. (2007) A noncoding melanophilin gene (MLPH) SNP at the splice donor of exon 1 represents a candidate causal mutation for coat color dilution in dogs. *Journal of heredity*, 98 (5), 468-473.
DOI: <https://doi.org/10.1093/jhered/esm021>
- FCI (2002) BELGIAN SHEPHERD DOG (Chien de Berger Belge). [Online] Available at: <http://www.fci.be/Nomenclature/Standards/015g01-en.pdf> [Accessed 26.03.2020].
- Honkanen L. (2018) Genetic testing of Belgian Shepherd Dogs – what have we learned so far? [online] Available at: <https://mydogdna.com/blog/genetic-testing-belgian-shepherd-dogs-what-have-we-learned-so-far/> [Accessed 28. 03. 2020]
- Charon, K. M., Lipka, K. R. (2015) The effect of a coat colour-associated genes polymorphism on animal health—a review. *Annals of Animal Science*, 15 (1), 3-17.
DOI: <https://doi.org/10.2478/aoas-2014-0066>
- Kim, J. H., Kang, K. I., Sohn, H. J., Woo, G. H., Jean, Y. H., Hwang, E. K. (2005) Color-dilution alopecia in dogs. *Journal of veterinary science*, 6 (3), 259-261.
- Palumbo, M. I. P., Fabris, V. E., Machado, L. H. A. (2012) Carcinoma de células escamosas em um cão com alopecia por diluição de cor. *Vet. Zootec.*, 19, 507-512.
- Perego, R., Proverbio, D., Roccabianca, P., Spada, E. (2009) Color dilution alopecia in a blue Doberman pinscher crossbreed. *The Canadian Veterinary Journal*, 50 (5), 511.

- Philipp, U., Hamann, H., Mecklenburg, L., Nishino, S., Mignot, E., Günzel-Apel, A. R., Schmutz, S. H., Leeb, T. (2005a). Polymorphisms within the canine MLPH gene are associated with dilute coat color in dogs. *Bmc Genetics*, 6 (34), 1-15.
DOI: <https://doi.org/10.1186/1471-2156-6-34>
- Philipp, U., Quignon, P., Scott, A., André, C., Breen, M., Leeb, T. (2005b) Chromosomal assignment of the canine melanophilin gene (MLPH): a candidate gene for coat color dilution in Pinschers. *Journal of heredity*, 96 (7), 774-776.
DOI: <https://doi.org/10.1093/jhered/esi079>
- Santos, L. M., Messas, N. B., Palumbo, M. I. P., Miyasato, L. A., Leal, P. V., Martins, T. B., Ramos, C. A. N. (2017) Identification of SNP c.-22G>A in the melanophilin gene from a dog with color dilution alopecia: case report. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 69 (6), 1503-1507. DOI: <https://doi.org/10.1590/1678-4162-9710>
- Schmutz, S. M., Moker, J. S., Clark, E. G., Shewfelt, R. (1998) Black hair follicular dysplasia, an autosomal recessive condition in dogs. *Can Vet J.*, 39 (10) 644-646.
- Šťastná, D., Šťastný, P. (2018) Chov a choroby psov. Nitra: Slovenská poľnohospodárska univerzita v Nitre.
- Van Buren, S. L., Minor, K. M., Grahn, R. A., Mickelson, J. R., Grahn, J. C., Malvick, J., Colangelo, J. R., Mueller, E., Kuehnlein, P., Kehl, A. (2020) A Third MLPH Variant Causing Coat Color Dilution in Dogs. *Genes*, 11 (6), 639. DOI: <https://doi.org/10.3390/genes11060639>
- Von Bomhard, W., Mauldin, E. A., Schmutz, S. M., Leeb, T., Casal, M. L. (2006) Black hair follicular dysplasia in Large Münsterländer dogs: clinical, histological and ultrastructural features. *Veterinary dermatology*, 17 (3), 182-188.
DOI: <https://doi.org/10.1111/j.1365-3164.2006.00517.x>
- Welle, M., Philipp, U., Rüfenacht, S., Roosje, P., Scharfenstein, M., Schütz, E., Brenig, B., Linek, M., Mecklenburg, L., GREST, P., Drögemüller, M., Haase, B., Leeb, T., Drögemüller, C. (2009) MLPH Genotype–Melanin Phenotype Correlation in Dilute Dogs. *Journal of heredity*, 100(suppl_1), S75-S79.
DOI: <https://doi.org/10.1093/jhered/esp010>