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Master's thesis

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Dandy-Walker-like malformation in the Danish Eurasier population

An estimation of the allele frequency of the mutation causing Dandy-Walker-like malformation

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Abstract

Dandy-Walker-like malformation (DWLM) is an autosomal recessive inherited disease recently described in Eurasiers. The main clinical sign is non-progressive cerebellar ataxia, observed when the puppies begin to practice walking. The ataxia is usually combined with additional other cerebellar signs of varying degrees. The causing mutation is VLDLR:c1713delC, a single nucleotide deletion in the very low density lipoprotein (*VLDLR)* locus at chromosome 1, which causes a premature stop codon and thereby affects the cerebellar development, since the VLDLR plays an important role in neuroblast migration.

The aim of the present study was to investigate the allele frequency of the DWLM-causing mutation in the Danish Eurasier population. This prevalence study consisted of 54 Eurasiers, which were genotyped through polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).

It was estimated that the mutant allele frequency in the Danish Eurasier population is 6.48 ± 4.70%. The selection of candidates for the study population was not fully randomised and pedigree analyses showed several grades of kinship between the dogs. This influences the interpretation of the results, which cannot readily be extrapolated to the Danish Eurasier population. To make a more accurate estimation of the true prevalence and a to get at narrower confidence interval the study requires random sampling and a larger study population.

Resumé

Dandy-Walker-like malformation (DWLM) är en autosomal recessiv ärftlig sjukdom som nyligen beskrivits hos eurasier. Det huvudsakliga kliniska tecknet är icke-progressiv cerebellär ataxi som yttrar sig när valparna börjar lära sig att gå. Ofta ses ataxin i kombination med olika grader av andra tecken på cerebellär störning. Den orsakande mutationen är VLDLR:c1713delC – en deletion av en nukleotid i very low density lipoprotein receptor (*VLDLR)* lokuset på kromosom 1. Denna orsakar ett prematurt stoppkodon som påverkar utvecklingen av cerebellum, eftersom VLDLR spelar en viktig roll i neuroblasters migration.

Syftet med denna studie var att undersöka allelfrekvensen av den DWLM-orskande mutationen hos danska eurasier. Denna prevalensstudie bestod av gentypning av 54 eurasier genom polymerase chain reaction och restriction fragment length polymorphism (PCR-RFLP).

Det estimerades att frekvensen av den mutanta allelen i den danska eurasierpopulationen är 6,48 ± 4,70%. Eftersom kandidatselektionen till studiepopulationen inte var helt slumpmässig och att stamtavleundersökningarna visar på förstagradssläktskap mellan flera av hundarna, kan resultaten inte extrapoleras till den danska eurasierpopulationen utan vidare. För att kunna göra en säkrare uppskattning av den sanna populationsprevalensen och för att få ett smalare konfidensintervall krävs slumpmässigt urval och en större studiepopulation.

Foreword and acknowledgements

This study is the result of our veterinary thesis project, conducted at the Department of Animal Genetics, Bioinformatics and Breeding, at the Faculty of Health and Medical Sciences, University of Copenhagen. The study was initiated upon request from Eurasier Club Denmark, and the results are intended to provide a basis for the Danish Kennel Club's (DKK) Health Committee, when deciding about future breeding recommendations and/or restrictions concerning the disease investigated. Hence, this study targets academics, DKK, and breeders with special interest in the subject.

We would like to thank our supervisor Merete Fredholm for guidance and help through the project process. We would also like to thank Torsten Toksvad and Christina Friis at Eurasier Club Denmark's Breeding and Health Committee for their help, commitment and administrative work. A special thanks to the laboratory technicians Tina Neergaard Mahler, Jennifer Mari Jacobsen, Charlotte Bjørner Larsen and Mai-Britt Jørgensen at the Department of Animal Genetics, Bioinformatics and Breeding. Last but not least, we would like to express our appreciation to DKK, which enabled this study through financial support, and also thanks to the dog owners and their 54 Eurasiers for volunteering for this study.

List of abbreviations

bp	Base pair
CI	Confidence interval
CNS	Central nervous system
CT	Computer tomography
DKK	Danish Kennel Club
DNA	Deoxyribonucleic acid
DWM	Dandy-Walker malformation
DWS	Dandy-Walker syndrome
DWLM	Dandy-Walker-like malformation
FCI	Federation Cynologique Internationale
GWAS	Genome wide association study
LDL	Low density lipoprotein
MRI	Magnetic resonance imaging
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
VLDLR	Very low density lipoprotein receptor
WT	Wild-type

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1. Introduction

The Eurasier is a fairly recently developed breed, and the intention to create a new breed with the desired characteristics resulted in a history of inbreeding. The foundation of the breed evolved in Germany in 1960, as four Wolfspitz females and three Chow-Chow males were systematically mated to breed a first generation called "Wolf-Chow" (Müller 2003). The German name "Eurasier" arose in 1972 as Samoyed was introduced to the breeding programme, and the Eurasier was recognised by the Federation Cynologique Internationale (FCI) in 1973 (FCI 1999). In spite of a small initial gene pool, today's Eurasier is an overall healthy breed with few described heritable and congenital disorders, including hypoadrenocorticism, lymphocytic thyroiditis, thyroiditis, hypothyroidism and glaucoma (Dodds & Laverdure 2011; Boillot et al. 2014). The breed also seems to have an inherited variant of exocrine pancreatic insufficiency, but the genetics behind this is not fully elucidated (Proschowsky & Fredholm 2007; Ackerman 2011). Some conditions, like hip dysplasia, elbow dysplasia, patellar luxation and osteochondrosis, are controlled through breeding restrictions (DKK n.d. a).

In 2015 cerebellar hypoplasia resembling Dandy-Walker-like malformation (DWLM) was added to the list of inheritable diseases among Eurasiers, as the clinical signs and radiological findings were described and the molecular genetics of the phenotype was investigated (Gerber et al. 2015; Bernardino et al. 2015).

1.1 Clinical signs and radiological findings

The recently published study by Bernardino et al. (2015) is a retrospective and prospective clinical cohort study describing clinical phenotype and radiological findings in purebred Eurasiers with cerebellar hypoplasia resembling a DWLM. In 14 out of 23 neurological abnormal dogs in both the retrospective and the prospective cohorts, cerebellar changes were confirmed by computer tomography (CT), magnetic resonance imaging (MRI) or necropsy. The main finding, seen in all 14 dogs with cerebellar abnormalities, was inferior cerebellar hypoplasia, characterised by absence of caudal portions of the cerebellar vermis as well as symmetrical absence of the caudal aspects of the cerebellar hemispheres, and an enlarged fourth ventricle. Enlargement of the posterior fossa, which is consistent with classic Dandy-Walker malformation (DWM) (Encha-Razavi 2003), was found in 21% of the dogs. Furthermore 29% of the dogs were diagnosed with hydrocephalus (Bernardino et al. 2015).

The main clinical sign reported was non-progressive ataxia, which was observed as the puppies began to walk, improved in some of the adult dogs. All but one dog presented with mild to moderate generalised cerebellar ataxia with additional various degrees of other cerebellar signs. The neurological examinations performed revealed hypermetria or dysmetria, nystagmus, absent menace reaction above 12 weeks of age, episodic falling and/or rolling, head tremors, delayed initiation of hopping and wheelbarrowing reactions, and other abnormalities, as visualised in Table 1.

Clinical and neurological findings	Retrospective cohort (n = 8)	Prospective cohort (n = 6)
Smaller size for breed standards	-	17.0%
Mild to moderate generalized ataxia	87.5%	100.0%
Severe generalized ataxia	12.5%	-
Dysmetric/hypermetric gait	50.0%	83.0%
Truncal sway	25.0%	17.0%
Circling	25.0%	-
Episodic falling and/or rolling	25.0%	50.0%
Leaning	-	17.0%
Head tilt	-	17.0%
Head tremors	37.5%	50.0%
Absent menace reaction >12 weeks of age	N/A	67.0%
Nystagmus	N/A	67.0%
Slow medial eye movements	N/A	17.0%
Proprioceptive deficits	37.5%	33.0%
Delayed initiation of hopping and wheelbarrowing reaction	N/A	50.0%
Hypermetric wheelbarrowing	N/A	33.0%
Seizures	37.5%	17.0%

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Table 1. Frequencies of the main clinical features in the two patient cohorts as presented by Bernhardino et al. (Bernhardino, F. et al., 2015. Inferior cerebellar hypoplasia resembling a Dandy-Walker-like malformation in purebred Eurasier dogs with familial non-progressive ataxia: a retrospective and prospective clinical cohort study. *PloS one*, 10(2), p.e0117670).

1.2 Genetics

Pedigree analyses indicated autosomal recessive inheritance of the disease, originating from one common ancestor (Bernardino et al. 2015). Based on data provided by Bernhardino et al. (2015), Gerber et al. (2015) aimed at describing the molecular genetics of the DWLM phenotype, and below is a short summary of their study.

The group of affected Eurasiers was characterised as expressing the DWLM phenotype, whereas clinical and radiological normal dogs were considered non-affected. EDTA stabilised blood was collected from 9 affected and 25 non-affected Eurasiers, and another 546 dogs of various other breeds added to the complete cohort. Genomic deoxyribonucleic acid (DNA) was isolated and genotyped and a genome-wide association study (GWAS) was performed. After exclusion of individuals with call rates <90%, minor allele frequencies <5%, and deviations from Hardy-Weinberg equilibrium at $p < 10^{-5}$, 9 affected and 11 non-affected Eurasiers remained, and 110 848 markers continued on to further analysis.

After an allelic association study, the 7 best associated single nucleotide polymorphisms (SNP) were found in the critical interval 92.5-93.8 Mb at chromosome 1, with a corrected p-value after 100 000 permutations of 0.00045. Homozygosity mapping showed that all 9 affected dogs were homozygous and with simultaneously shared alleles, while dogs in the non-affected group were not. As there are 39 genes within the critical interval, the genomes of one affected Eurasier and one non-affected Boxer were sequenced. 150 homozygous variants were found within the critical interval, of which 20 were predicted to be non-synonymous. It was hypothesised that the causative mutation would be absent in all other breeds, and after comparison with genomes from 47 dogs of various breeds, only 4 non-synonymous variants remained. As 3 of these could be excluded as several non-affected Eurasiers carried these variants, only one variant was left, VLDLR:c.1713delC, which is a single nucleotide deletion in the very low density lipoprotein receptor (*VLDLR*) locus, causing a premature stop codon, and thus a defect VLDLR. This mutant allele was perfectly associated with DWLM in the 34 Eurasiers, and was absent in the more than 500 dogs of other breeds. 96 randomly selected Eurasiers were genotyped, and it was found that 15 dogs (16%) were heterozygous carriers of the mutant variant, while 0% were homozygous carriers.

1.3 The role of VLDLR in CNS development

The transmembrane receptors VLDLR and apolipoprotein E receptor 2 (ApoER2) are members of the low density lipoprotein (LDL) receptor family, and both receptors are essential components of the Reelin signalling pathway (Trommsdorff et al. 1999). During the development of the central nervous system (CNS), Purkinje cells developed in the ventricular zone migrate radially and form an architectonic pattern at the end of their migration (Yuasa et al. 1991; Sidman & Rakic 1973; Lambert de Rouvroit & Goffinet 2001). The neuroblast migration and positioning is regulated by the extracellular protein Reelin, that calcium-dependently binds to ectodomains of VLDLR or ApoER2 on the cell membrane in the Reelin signalling pathway (D'Arcangelo et al. 1999; Hiesberger et al. 1999). The signal is transmitted through these receptors, inducing binding of intracellular Dab1 to the cytoplasmatic tails of VLDLR or ApoER2 (Trommsdorff et al. 1998). Dab1 is tyrosine phosphorylated (Trommsdorff et al. 1998; D'Arcangelo et al. 1999; Hiesberger et al. 1999; Howell et al. 1999), enabling cell migration through the microtubule stabilising protein tau. VLDLR/ApoER2 double knockout mice exhibit hyperphosphorylation of tau (Hiesberger et al. 1999), which causes microtubule dissociation (Merrick et al. 1997). In VLDLR/ApoER2 double knockout mice Purkinje cell migration cannot occur, and the cells remain in the cerebellar nuclei (Trommsdorff et al. 1999).

VLDLR and ApoER2 are both required for normal radial neuronal migration in the developing CNS, and their functions have been suggested to partially overlap, since VLDLR and ApoER2 knockouts exhibit milder phenotypes than double knockouts (Trommsdorff et al. 1999), which correlates well with single knockouts only show mildly increased hyperphosphorylation of tau (Hiesberger et al. 1999). ApoER2 defect predominantly manifests in the neocortex, while VLDLR defect mainly affects the cerebellum, which gets smaller and less foliated than wild-type (WT) (Trommsdorff et al. 1999).

1.4 Terminology

Dandy-Walker syndrome (DWS) in humans is a congenital anomaly, which is usually defined as total or partial agenesis of the vermis and fourth ventricle cystic dilatation, leading to elevation of the cerebellar tentorium and enlargement of the posterior fossa size (Encha-Razavi 2003). Several forms of DWMs have been described based on type and severity of the abnormalities but as there is no clear demarcation between the various degrees of malformation, differentiation between these can be difficult (Schmidt et al. 2008). The term DWLM describes a milder form of the disease and is mainly characterised by fourth ventricle dilatation and without partial or total vermis aplasia (Encha-Razavi 2003).

1.5 Cerebellar hypoplasia in canines and other species

Several publications in the veterinary literature concern cerebellar malformations in canines and other species, but few of these regard inherited cerebellar hypoplasia or cerebellar malformations comparable to DWS in humans. DWLM was recently described in Eurasiers (Gerber et al. 2015), and several single cases of dogs suggested to have DWLM have been reported in Chow Chows, Golden Retrievers, Beagles, Silkie Terriers, Cocker Spaniels, Miniature Schnauzers, Wire-haired Miniature Dachshunds, Boston Terriers, Tervurens, Briards, Labrador Retrievers, Bull Terriers, Weimaraners, Dachshunds and mixed breeds (Knecht et al. 1979; Pass et al. 1981; Kornegay 1986; Schmid et al. 1992; Noureddine et al. 2004; Choi et al. 2007; Lim et al. 2008; Schmidt et al. 2008; Kobatake et al. 2013). Congenital cerebellar abnormalities resembling DWLM have been described in cows, horses, sheep, and a cat (Pass et al. 1981; Jeffrey et al. 1990; Madarame et al. 1990; Regnier et al. 1993; Wong et al. 2007), in addition to humans and dogs.

Most primary cerebellar diseases in domestic animals are considered as congenital, as a result of in utero viral infection or a developmental disorder, or as inherited cerebellar cortical abiotrophy (de Lahunta et al. 2009). In utero infections with several different vira resulting in cerebellar hypoplasia have been described in cats, sheep, pigs, cows, and chickens (Barlow 1980;

MacLachlan et al. 1985; Kitano et al. 1996; Schatzberg et al. 2003). Although no agent primarily affecting the developing cerebellum in dogs has been recognised (de Lahunta et al. 2009), in utero canine parvovirus infection has been a suggested etiology for cerebellar malformation (Schatzberg et al. 2003). Cerebellar cortical abiothrophy is a more common cause of cerebellar disease in dogs and has been described in a number of breeds (Deforest et al. 1978; van Tongern et al. 2000; Sandy et al. 2002; Berry & Blas-Machado 2003; Jokinen et al. 2007, de Lahunta et al. 2009). It is regarded an inherited disorder, usually involving an autosomal recessive gene, causing intrinsic degeneration of neurons which leads to premature death of neurons in the cerebellar cortex (de Lahunta et al. 2009). Unlike cerebellar hypoplasia as seen in dogs with DWLM, where the hypoplasia is present since birth and ataxia is non-progressive, an animal with cerebellar cortical abiotrophy is normal at birth and develop a slowly progressive cerebellar ataxia (de Lahunta et al. 2009, Nelson & Couto, 2009).

1.6 Purpose

The aim of the present study was to investigate the allele frequency of the DWLM-causing mutation in the Danish Eurasier population, in order to provide the Danish Kennel Club (DKK) with data to establish future recommendations and/or restrictions for breeding.

2. Materials and methods

2.1 Study design

The study was performed as a cross sectional survey, aiming at estimating the prevalence of the mutant allele among Danish Eurasiers used for breeding. The study population mainly consisted of dogs currently or previously active for breeding, but also dogs planned to be used for breeding were included.

2.2 Animals

2.2.1 Dogs

All dogs included were purebreds registered in DKK. 76 dogs nationwide were selected as candidates for the study population by the project's initiative takers from Eurasier Club Denmark. The owners were contacted by Eurasier Club Denmark and offered to participate voluntarily and free of charge, by contributing with material for DNA analysis. Samples from 52 out of the total 76 dogs were received, plus 2 additional samples that were sent spontaneously.

The two additional samples were added to the study, as they were collected from dogs matching the study population criteria.

2.2.2 Pedigree analyses

Kinship between dogs in the study population was analysed manually using data from DKK's register (URL: https://www.hundeweb.dk).

2.3 Samples

Sampling was conducted from January through March 2016 and total of 9 blood samples and 45 hair samples was collected. Hair samples were stored in a refrigerator and non-stabilised or EDTA stabilised blood were stored in a –20°C freezer until the day of analysis.

2.4 DNA extraction and purification

2.4.1 Hair samples

DNA was extracted and purified using QIAamp DNA Investigator Kit according to the manufacturer's instructions with small adjustments of minor significance.

2.4.2 Blood samples

DNA was extracted using the salting out method according to the protocol described in Appendix A.

2.5 DNA analysis

2.5.1 PCR

The DWLM-causing mutation, VLDLR:c.1713delC, changes the WT allele sequence TACTGG to TATGGT (Gerber et al. 2015) (Figure 1). Polymerase chain reaction (PCR) was run to amplify a 414 base pair (bp) fragment including the mutation site, and the PCR product size was checked by gel electrophoresis on a 2% agarose gel by comparison to a 100 bp ladder.

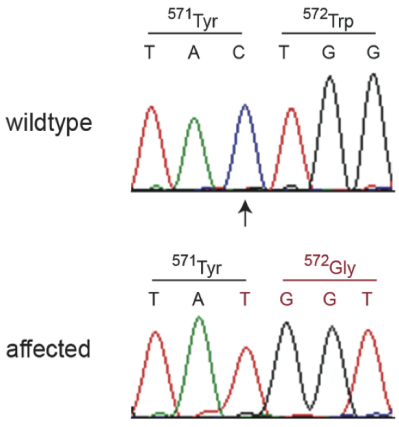

Figure 1. WT and mutant sequences. *Note*: reprinted from Gerber et al. 2015 (Gerber, M. et al., 2015. A deletion in the VLDLR gene in Eurasier dogs with cerebellar hypoplasia resembling a Dandy-Walker-like malformation (DWLM). *PloS one*, 10(2), p.e0108917).

2.5.2 Digestion

The restriction enzyme Tat1 was used for digestion of the PCR product as it digests in the motif, which was used to differentiate between mutant and WT alleles. The digested PCR products were analysed by gel electrophoresis on a 2% agarose gel and fragment sizes were compared to a 100 bp ladder.

2.5.3 Interpretation

Digestion of the 414 bp PCR product by Tat1 is possible at 2 sites. Digestion of WT alleles result in fragments 140, 260 and 10 bp, while mutant alleles result in 400 and 10 bp fragments when digested (Figure 2). As the 10 bp fragments would not form a proper band on the gel used, interpretation of the gels was as follows:

- 3 bands: heterozygous carrier of the mutant allele, carrier.
- 2 bands: homozygous carrier of the WT allele, clear.
- 1 band: homozygous carrier of the mutant allele, sick.

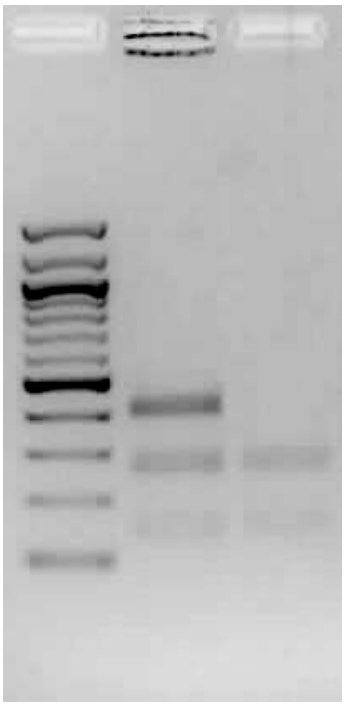

Figure 2: Interpretation of digestion. Left lane: 100 bp ladder, middle lane: heterozygote (carrier), right lane: WT homozygote (clear).

3. Results

Genotyping by PCR revealed 47 WT homozygotes, 7 heterozygotes and 0 mutant homozygotes (Table 2), hence 7 out of 108 alleles were mutant. In the following calculations, p and q are the population allele frequencies, whereas $\hat{p}$ and $\hat{q}$ are the allele frequencies in the study population, and n is the number of mutant alleles out of N in total.

3.1 Mutant allele frequency

$$\hat{q} = \frac{n}{N} \rightarrow \hat{q} = \frac{7}{108} \approx 0.0648$$

Confidence interval (CI) for the mutant allele frequency is given by the formula:

$$\hat{q} \pm Z_{1-\frac{\alpha}{2}} SE_{\hat{q}} = \left\{ SE_{\hat{q}} = \sqrt{\frac{\hat{q}(1-\hat{q})}{N}} \; ; \; \hat{q} = \frac{n}{N} \right\} = \frac{n}{N} \pm Z_{1-\frac{\alpha}{2}} \sqrt{\frac{\frac{n}{N}\left(1-\frac{n}{N}\right)}{N}}$$

A 95% CI was given by α=0.05 and hence $Z_{1-\frac{\alpha}{2}} \approx 1.9824$ at 107 degrees of freedom. Insertion of values yielded a 95% CI for q:

$$0.0648 \pm 0.0470\ ;\ 0.0179 < q < 0.1118$$

3.2 Genotype distribution

As there are two possible alleles at the *VLDLR* locus, $p + q = 1$.

$$\hat{p} = 1 - \hat{q}\ \approx 0.9352$$

When assuming that the population is in Hardy-Weinberg equilibrium, then the genotypes are $p^2 + 2pq + q^2 = 1$. The expected genotypes were calculated:

- WT homozygote ($\hat{p}^2$):

$$\hat{p}^2 = \left(\frac{101}{108}\right)^2 \approx 0.8746$$

- Heterozygote ($2\hat{p}\hat{q}$):

$$2\hat{p}\hat{q} = 2\times\frac{7\times101}{108^2} \approx 0.1212$$

- Mutant homozygote ($\hat{q}^2$):

$$\hat{q}^2 = \left(\frac{7}{108}\right)^2 \approx 0.0042$$

Chi-square (χ^2) statistics between the observed and calculated genotypes yielded $\chi^2 \approx 0.2594$, with a p-value of 0.8784. At 2 degrees of freedom, ${\chi^2}_{0.05} \approx 5.99$ and ${\chi^2}_{0.20} \approx 3.22$, therefore a null-hypothesis about the genotypes in the study population being in Hardy-Weinberg equilibrium cannot be rejected even at a significance level of 0.2.

3.3 Pedigree analyses

Pedigree analyses could not conclude about one sole common ancestor and origin of the mutation. Relationships between study units are listed in Table 2.

3.4 Result summary

The statistics presented suggest that with 95% certainty the population mutant allele frequency is within the interval 1.79-11.12%, provided that the study units are randomly selected and the study population is representative for the target population. Also, the genotypes appear to be in Hardy-Weinberg equilibrium. Pedigree analyses did not reveal one common ancestor that could be the origin of the mutation.

Dog ID	Genotype	Relationship between study units	Year
DWLM_001	N/DWLM	daughter of DWLM_015; littermate to DWLM_027 and DWLM_032	2013
DWLM_002	N/N	daughter of DWLM_047 and DWLM_039	2011
DWLM_003	N/N	littermate to DWLM_004 and DWLM_005	2012
DWLM_004	N/N	littermate to DWLM_003 and DWLM_005	2012
DWLM_005	N/N	father of DWLM_006; littermate to DWLM_003 and DWLM_004	2012
DWLM_006	N/N	son of DWLM_005	2015
DWLM_007	N/N		2013
DWLM_008	N/N	son of DWLM_0042 and DWLM_043	2013
DWLM_009	N/DWLM		2012
DWLM_010	N/DWLM	son of DWLM_0029; littermate to DWLM_030 and DWLM_051	2015
DWLM_011	N/N		2015
DWLM_012	N/N		2014
DWLM_013	N/N	daughter of DWLM_049; littermate to DWLM_037 and DWLM_50	2014
DWLM_014	N/N		2009
DWLM_015	N/DWLM	mother of DWLM_001 and DWLM_032; littermate to DWLM_017	2010
DWLM_016	N/N	daughter of DWLM_018	2014
DWLM_017	N/DWLM	littermate to DWLM_015	2010
DWLM_018	N/N	father of DWLM_016; littermate to DWLM_049	2011
DWLM_019	N/N		2009
DWLM_020	N/N		2014
DWLM_021	N/N		2014
DWLM_022	N/N		2009
DWLM_023	N/N		2008
DWLM_024	N/N		2010
DWLM_025	N/N	littermate to DWLM_035	2012
DWLM_026	N/N	mother of DWLM_033 and DWLM_046	2007
DWLM_027	N/N	daughter of DWLM_015; littermate to DWLM_001 and DWLM_032	2013
DWLM_028	N/N	littermate to DWLM_041	2015
DWLM_029	N/DWLM	father of DWLM_010, DWLM_030 and DWLM_051	2006
DWLM_030	N/N	daughter of DWLM_029; littermate to DWLM_010 and DWLM_051	2015
DWLM_031	N/N	daughter od DWLM_032	2015
DWLM_032	N/N	mother of DWLM_031; daughter of DWLM_015; littermate to DWLM_001 and DWLM_027	2013
DWLM_033	N/N	father of DWLM_045; son of DWLM_026	2011
DWLM_034	N/N		2014
DWLM_035	N/N	littermate to DWLM_025	2012
DWLM_036	N/N		2011
DWLM_037	N/N	daughter of DWLM_049	2014
DWLM_038	N/N		2013
DWLM_039	N/N	mother of DWLM_002 and DWLM_044	2010
DWLM_040	N/N		2014
DWLM_041	N/N	littermate to DWLM_028	2014
DWLM_042	N/N	mother of DWLM_008	2007
DWLM_043	N/N	father of DWLM_008	2009
DWLM_044	N/N	daughter of DWLM_39	2013
DWLM_045	N/N	son of DWLM_033	2014
DWLM_046	N/N	son of DWLM_026	2012
DWLM_047	N/N	father of DWLM_002	2009
DWLM_048	N/N		2010
DWLM_049	N/N	mother of DWLM_013, DWLM_37 and DWLM_050; littermate to DWLM_018	2011
DWLM_050	N/N	son of DWLM_049, littermate to DWLM_013 and DWLM_037	2014
DWLM_051	N/DWLM	son of DWLM_029; littermate to DWLM_010 and DWLM_030	2015
DWLM_052	N/N		2012
DWLM_053	N/N		2011
DWLM_054	N/N		2012

Table 2. Genotyping results. N/N = WT homozygote, N/DWLM = heterozygote. Shaded fields in relationship column = no first-degree relationship within the study. Year column = year of birth, as registered by DKK.

4. Discussion

4.1 Results

The mutant allele frequency in the Danish Eurasier population is calculated with a 95% CI to be 6.48 ± 4.70%. The reason for this wide CI is the relatively small study population, and a narrower interval could be acquired by using a larger study population. The observed heterozygote frequency was 13.0% (7 out of 54 dogs), which is close to the by Bernhardino et al. (2015) previously presented frequency of 15.6% (15 out of 96 dogs). This is as expected, when presuming that the two studies are approximately equally representative for the population(s), the genes of the dog in which the mutation first occurred has been widely spread among Eurasiers nation wide, and also that no selection for the WT *VLDLR* allele has occurred. The latter is confirmed, as the genotypes in the study population appear to be in Hardy-Weinberg equilibrium, which also means that the study population does not appear to be non-representative for the Danish Eurasier population. Analyses of the heterozygous dogs' pedigrees could not reveal one common ancestor, which could be explained by the mutation originating a long time ago, and the mutation has widespread throughout the population ever since.

4.2 Study design and animals

The aim was to select a study population representative for the entire Danish Eurasier population, but an occurrence of selection bias cannot be excluded in the study design. The people responsible for selecting dogs for the study population might have contributed to the selection bias due to personal relations, where dogs of certain owners might have been more likely to get recruited before others. Also, some owners got the opportunity to participate with a larger amount of dogs. Although the intention was to exclude dogs closely related to another dog included in the present study, or related to a dog that previously had been genotyped for DWLM, pedigree analyses of the 54 dogs showed that there were several different grades of kinship between the dogs (Table 2). The reason for this remains unclear, but it is possible that there was some miscommunication in this matter. Selection of candidates for the study population was not fully randomised, which influences the interpretation of the results presented in this study and may aggravate extrapolation of the results to the Danish Eurasier population. Furthermore it is unknown how well the study population corresponds to the target population. As there is a high level of consanguinity between several study units, there is a risk of observing a false high or

false low number of carriers, as the parents' genotypes directly affect the outcome of the offspring's genotypes. This could have been avoided by limiting the study population to one defined age group, and hence limit the risk of sampling first-degree relatives. One impediment to this is the limited population size (as outlined in the next paragraph), and the risk of not being able to achieve a sufficiently high sample size in order to calculate a narrow frequency interval. This would require a redefinition of the study population not only to include animals used for breeding, but also to extend the criterion to include all dogs in a certain age group. The present criteria were thought to be a good estimate of the population, but with hindsight it seems like the suggested widened study population could be as good an estimate, if not better, as it would increase the validity of the study and the results more readily could be extrapolated to the population.

The Danish Eurasier population is dynamic, due to breeding, import, export, death, cross-national mating, etc., which contributes to lack of a registry of the precise population size. An estimate based on the life length expectancy of 12 years, and the number of new registrations in DKK (DKK n.d. b) over the last 12 years, yields a population size of approximately 1350 dogs. Even though this estimate presupposes that most Danish Eurasiers are registered at DKK, and does not consider factors as export and premature death, it was thought to be the best possible estimate available.

4.3 Material and method

The genotyping method (PCR-RFLP) used in the present study is well suited for the cause, as it has both high reliability and validity. The restriction enzyme used digests at the motif in the 414 bp fragment from PCR, as described in Appendix B, which is a very exact way of differentiating mutant from WT alleles. There is a very low risk of misinterpretation of genotypes after gel electrophoresis of the digested fragments, as the largest fragment (i.e. 405 or 265 bp) always would be the most fluorescent. Hence, not fully completed restrictions would result in diffuse bands of larger fragments, but they would not turn out most fluorescent.

4.4 Limitations

It can be discussed whether the study population is representative for the target population or not. The validity degree is uncertain and the results should be interpreted carefully, as the performed study is not a true random sampling cross-sectional study and it is based on relatively few individuals of which many are closely related. Confident extrapolation of the results is

limited to a specific part of the population, namely the one included in the present study, and the conclusions stated in the present study is not convincing for the Danish Eurasier population.

One attempt to aid the interpretation of the genotyping results is described in Appendix C, as the algorithm outlined therein removes closely related dogs from the subsequent calculations. Calculations conducted in Appendix C yielded a mutant allele frequency, with a 95% CI, of 5.56 ± 5.38%, and the refined study population was in Hardy-Weinberg equilibrium. The allele frequency calculated in Appendix C does not deviate much from the previously presented frequency. The interpretation of this could be that the various degrees of relatedness among the dogs in the study population do not largely affect the allele frequency calculated.

5. Conclusion

The aim of the present study was to investigate the allele frequency of the DWLM-causing mutation in the Danish Eurasier population, in order to provide DKK with data to establish future breeding recommendations and/or restrictions on. The present study suggests a population mutant allele frequency of 6.48 ± 4.70% (95% CI), but it is uncertain whether the study population provided for the study is representative for the Danish Eurasier population. This together with the fact that the dogs genotyped were not randomly selected, complicates extrapolation of the results to the population.

6. Perspective

The somewhat inconclusive outcome of this study might not be insignificant after all, when concerning future breeding recommendations from DKK. The mutant allele frequency does not seem to be critically high, which means that there is quite a low risk of mating two heterozygotes by chance, and hence an even lower risk of breeding DWLM puppies. However, to eradicate the mutation from the Danish Eurasier population, some breeding limitations seem to be in order. Because of the limited population size it is important to remain focused on genetic diversity in order to establish future generations of healthy Eurasiers. To achieve this the breeders should not only base their breeding decisions on the DWLM genotypes of the dam and sire, and carriers of the mutation should continue to be utilised for breeding. It is reasonable to suggest that dogs affected by DWLM should not be used for breeding, and that mating between two heterozygotes should be prohibited, since both crosses probably will increase the mutant allele frequency. The distribution of the mutation can be minimised over time, if breeding of carriers and affected dogs

are being controlled. An important point of this suggestion is that heterozygotes should continue to be bred, in order to not limit the genetic diversity.

Follow-up studies for investigation of the eradication progress might be difficult to compare with the results of the present study, because of the limited applicability of the results presented. For future studies covering this area, we highly recommend sampling from a random selection of dogs, and to exclude close relatives, in order to get a wider estimate and results that are easier to extrapolate to the population. As the Eurasiers to some extent are mated across national borders (Friis & Toksvad 2016, personal communication), a future international study could be of interest. One could then compare countries, to see how extended the mutation actually is over national borders.

Working with the present study has generated some new questions. The study by Bernhardino et al. (2015) reports on various degrees of clinical manifestations of the disease among dogs that are homozygous for the mutant allele, and it can be asked how this is possible. Firstly, is the VLDLR:c.1713delC mutation the only cause of the development of DWLM? Secondly, to what extent is CNS development individual, and is variable receptor gene expression the cause of this? Is neuronal migration through the Reelin signalling pathway possible by up-regulation of ApoER2 in VLDLR defect individuals? It was also reported that the cerebellar ataxia improved in some dogs. It remains unanswered whether the disease actually regressed, or if the dogs just learned to compensate for the ataxia, even though the latter one seems more probable.

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Appendix A: Protocols

Salting out

Day 1:

1. A refrigerated centrifuge was put on 4 °C and blood samples were thawed.
2. The contents of the blood tubes were poured into 50 mL tubes.
3. Triton lysis buffer was added to the 50 mL tubes.

   10 mL blood = 40 mL triton lysis buffer

   5 mL blood = 20 mL triton lysis buffer

   2.5 mL blood = 10 mL triton lysis buffer

   1.25 mL blood = 5 mL triton lysis buffer
4. The contents were mixed by turning the tubes, before incubation in refrigerator for 30 minutes. The tubes were turned every 10 minutes during the incubation.
5. The tubes were centrifuged for 20 minutes at 2500 rpm and 4 °C.
6. The supernatant was discarded, and 0.9% NaCl was added to the tubes.

   10 mL blood = 2 mL 0.9% NaCl

   5 mL blood = 1 mL 0.9% NaCl

   2.5 mL blood = 0.5 mL 0.9% NaCl

   1.25 mL blood = 0.25 mL 0.9% NaCl
7. The tubes were thoroughly vortexed until the pellets were dissolved.
8. The tubes were centrifuged for 10 minutes at 2500 rpm and 4 °C.
9. Pronase mixture was prepared. One unit was calculated for 10 mL blood and consisted of:

   896 µL nuclease free H_2O

   50 µL pronase (20 mg/mL)

   150 µL 20% SDS

   4 µL 0.5 M Na_2EDTA, pH 8.0
10. The supernatant was discarded, and nuclear lysis buffer was added to the tubes.

    10 mL blood = 3 mL

    5 mL blood = 1.5 mL

    2.5 mL blood = 0.75 mL

    1.25 mL blood = 0.375 mL

11. Pronase mixture was added to the tubes.

    10 mL blood = 1.1 mL

    5 mL blood = 0.55 mL

    2.5 mL blood = 0.275 mL

    1.25 mL blood = 0.1375 mL

12. The tubes were incubated at room temperature on a shaking table overnight.

Day 2:

1. A refrigerated centrifuge was put on 4 °C
2. Dissolution of pellets was checked before continuing to step 3. Additional pronase mixture was added to non-dissolved samples, which were further incubated a few more hours on shaking table.
3. 6 M NaCl was added to the tubes.

    10 mL blood = 1.0 mL

    5 mL blood = 0.5 mL

    2.5 mL blood = 0.25 mL

    1.25 mL blood = 0.125 mL

4. The tubes were vortexed at full power for 15 seconds.
5. The tubes were centrifuged for 15 minutes at 2500 rpm and 4 °C.
6. The supernatants were transferred into new 50 mL tubes.
7. The supernatant was blended with 1 volume of isopropanol precipitated DNA, which was collected using a closed Pasteur pipette.
8. The DNA was transferred into a 1.5 mL tube containing 100 µL TE buffer.
9. The tubes were incubated overnight at 4 °C on a turning machine.

Day 3:

1. As DNA was dissolved, optical density of the samples was determined.
2. Samples were diluted to a concentration of 25 ng/mL, and stored in freezer until analysis.

PCR

Master mix

$18.75 \times (n + 1)$ µL nuclease free H_2O

$3.0 \times (n + 1)$ µL 10x Ammonium Buffer (15 mM $MgCl_2$)

$1.5 \times (n + 1)$ µL dNTP (4 mM)

$1.2 \times (n + 1)$ µL Primer F (10 pmol/µL)

$1.2 \times (n + 1)$ µL Primer R (10 pmol/µL)

$0.15 \times (n + 1)$ µL TEMPase Hot Start Polymerase (5 units/µL)

$1.2 \times (n + 1)$ µL $MgCl_2$ (25 mM)

$n =$ number of samples run. For PCR 3 µL DNA was added to 27 µL Master mix.

PCR program – fixed annealing

The following program was used, together with the settings "calculated temperature" and "heated lid".

1. 95 °C for 15 minutes.
2. 95 °C for 30 seconds.
3. 62 °C for 30 seconds.
4. 72 °C for 60 seconds.
5. Go to 2., 34 times.
6. 72 °C for 10 minutes.
7. 8 °C forever.

Restriction

Restriction enzyme mix

$8.5 \times (n + 1)$ µL nuclease free H_2O

$2.0 \times (n + 1)$ µL 10x Buffer Tango

$0.5 \times (n + 1)$ µL Tat1 (5 units/µL)

$n =$ number of samples run. For restriction reaction 11 µL Restriction enzyme mix and 18 µL PCR product was added to a 1.5 mL Safe-Lock micro centrifuge tube.

Restriction

The tubes were covered with aluminium foil and incubated at 65 °C for 16 hours. Thereafter Tat1 was inactivated by adding 1.16 µL 0.5 M EDTA 8.0 pH to each tube.

Appendix B: Establishment of primers and restriction enzyme

Restriction enzyme design

The gene transcript was found using the NCBI Reference Sequence Database (URL: http://www.ncbi.nlm.nih.gov/refseq/). The TACTGG sequence was found at two locations but only one could be the motif, as a deletion of C should give the sequence TATGGT. The motif is underscored in the sequences printed below.

Canis lupus familiaris very low density lipoprotein receptor (VLDLR), mRNA:

NCBI Reference Sequence: NM_001286978.1

>gi|558757342|ref|NM_001286978.1| Canis lupus familiaris very low density lipoprotein receptor (VLDLR), mRNA

GCGGGCACCATGGGCACGTCCGCGCGCTGGGCGCTCTGGCTGCTGCTCGCGCTGTGCTGGGCGCCCCGGG
AGAGCCGCGCCACCGGAGCGGGAAGAAAAGCCAAATGTGAACCCTCCCAATTCCAGTGCACAAATGGCCG
CTGTATTACATTGCTGTGGAAATGCGACGGGGATGAAGACTGCGCTGATGGCAGTGACGAAAAGAACTGT
GTAAAGAAGACGTGTGCAGAGTCTGACTTTGTGTGCAACAATGGCCAGTGCGTCCCCAACCGGTGGCAGT
GTGACGGGGATCCTGACTGTGAAGATGGTTCTGATGAAAACCCAGAACAGTGCCATATGAGAACATGCCG
CATAAATGAAATCAGCTGTGGCGCCCGTTCTACTCAGTGTATCCCAGTGTCCTGGAGGTGTGATGGTGAA
AATGATTGTGACAGTGGAGAAGATGAAGAAAACTGTGGCAACATCACATGCAGCCCGGACGAGTTCACCT
GCTCCAGCGGCCGTTGCATCTCCAGGAACTTCGTCTGCAATGGCCAGGACGACTGCAGTGACGGCAGCGA
CGAGCTGGACTGCGCGCCCCCCACGTGCGGCGAGCATGAGTTCCAGTGCAGCACCTCTTCCTGCATCCCC
CTGAGCTGGGTATGCGACGATGACGCCGACTGCTCCGACCAGTCAGATGAGTCCCTGGAGCAGTGTGGCC
GCCAGCCCGTGATGCACACCAAGTGCCCAGCCAGCGAGACCCAGTGCGGCTCAGGCGAGTGCATCCACAA
GAAGTGGCGCTGCGATGGGGACCCCGACTGCAAAGACGGCAGCGACGAGGTCAACTGTCCTTCGAGAACC
TGCCGGCCTGACCAGTTTGAGTGTGAGGACGGGAGCTGCATCCATGGCAGCAGGCAGTGCAACGGTATCA
GGGACTGTGTGGACGGCTCGGATGAAGTCAACTGCAAGAACGTCAATCAGTGCTTGGGCCCTGGAAAGTT
CAAGTGCAGAAGTGGGGAGTGCATAGATATCGGTAAAGTATGTAACCAGGAGCAGGACTGCAGGGACTGG
AGTGACGAACCCCTGAAAGAATGTCACGTAAATGAATGCTTGGTTAATAATGGTGGCTGTTCGCACATCT
GCAAAGACCTAGTTATAGGCTATGAATGTGACTGCGCAGCTGGGTTTGAACTGATAGATAAGAAAATTTG
TGGAGATATTGACGAATGCCAAAATCCAGGAATCTGCAGCCAAATTTGTATCAACTTAAAAGGCGGTTAC
AAGTGTGAATGTAGTCGGGGCTATCAGATGGATCTTGCCACCGGCGTGTGCAAGGCAGTAGGCAAAGAGC
CAAGTCTGATCTTCACAAATCGAAGAGACATCCGGAAGATTGGCTTGGAGAGGAAAGAATATATTCAACT
AGTTGAACAGCTAAGAAACACTGTAGCTCTTGATGCAGACATTGCAGCTCAGAAACTGTTCTGGGCTGAT
CTGAGCCAAAAGGCCATCTTCAGTGCCTCAATTGATGACAAGGTTGGTAGACATGTTAAAATGATCGACA
ATGTCTATAATCCTGCAGCCATTGCTGTTGATTGGGTGTACAAGACCATCTACTGGACTGATGTGGCTTC
TAAGACTATTTCAGTAGCTACCCTAGATGGGACCAGAAGGAAGTTCCTGTTTAACTCTGACTTGCGGGAG
CCTGCCTCCATAGCTGTGGATCCACTCTCTGGGTTTGTGTACTGGTCAGACTGGGGAGAACCAGCTAAAA
TAGAAAAAGCAGGAATGAATGGATTTGATAGGCGGCCACTTGTGACAGTGGACATCCAATGGCCTAATGG
AATTACACTTGACCTTATAAAGAGTCGCCTCTACTGGCTTGACTCCAAGTTGCACATGTTGTCCAGCGTG
GACTTAAATGGCCAAGACCGTAGGATTGTCCTTAAATCTCTGGAGTTCCTAGCTCATCCTCTGGCGCTAA
CCATATTTGAGGATCGTGTCTACTGGATAGATGGGGAAAATGAAGCAGTCTACGGTGCCAATAAATTCAC
TGGATCAGAGCTGGCCACGCTAGTAAACAACCTTAATGATGCCCAAGACATCATTGTCTATCACGAACTC

GTCCAGCCGTCAGGTAAAAACTGGTGTGAAGAGGACATGCAGAATGGAGGCTGTGAATATCTGTGCCTGC
CAGCACCACAAATCAACGATCACTCTCCAAAATACACGTGTTCCTGTCCCAATGGGTACACTCTAGAGGC
AAACGGCCGGGAGTGCCACAGTACTGCAACTACTCTGACTTACAGTGAGACAAAAGATATCCACACAACA
GAAATTCCTCCAACTAGTGGACTAGTTCCCAGAGGGATCAATGTGACCACAGCAGTATCGGAGGCCACTG
TTCCCCCAAAAGGCACTTCTGCTGCCTGGGCCATCCTTCCTCTCTTGCTCTTAGCAATGGCAGCGGTAGG
TGGCTACTTGATGTGGCGGAATTGGCAGCACAAGAACATGAAAAGCATGAACTTTGACAATCCTGTGTAC
TTGAAGACTACTGAAGAGGACCTGTCCATAGACATTGGTAGACACAGTGCTTCTGTTGGACACACGTACC
CCGCAATATCAGTTGTAAGCACAGATGATGACCTAGCTTGACTTCAGAGACAAGCCTTGACCTTTGAGGT
CTAAACCAATAACCCTCCACTTCGAAATGGTAACCGAGCCAGCGCCTGAAGTCTTTCTTCCTTCAGTCTG
GAAGAACATCAAGATATCTTTACTTGGATCAAGCTTGTATACTTGATCATTTTTATATTACTTTTGTAAA
TATTCCTATCCACATTCTACTTCAGCTTTGGATGTGGTTACCAAGTATCTAATCCTTGAGTTTCTAGACA
GTATTGCCACATCTGGCCAAATATGCACTTTCCCTAGAAAGCCATATTCCAGCAATGAAACTTGTGCTAT
AGTGTATACCACCTGTACATACATTGTATAGGCCATCTGTAAATATCCCAGAGAACAATCACTATTCTTA
AGCACTTTGAAAATATTTCTATGTAAATTATTGTAAACTTTTTCAATGGTTGGGACAATGGCAATAGGAT
AAAACGGGTTACTAAGATGAAATTGCCAAAAAAATTTGTAAACTAATTTTGTACGTATGAATGAAATCTT
TGACCTCAGTGGAGGTTTGCAAAGACTGAGTATTCAAACTACTGTACATTTTTTTTTCAAGTGCTAAAAC
AATTAAACCAAGCAGCTTAA

The genomic sequence of the motif was found using the Ensambl database (URL: http://www.ensembl.org/index.html):

>chromosome:CanFam3.1:1:91265835:91266553:1

91265835 TAACACATATTCACCTTTCAGTTCTCTGAAAGAATTGGCCTTTTGTAAAGAAGTCTATAA 91265894
91265895 CCTGTGACTTGGGCTTGAAATATAATTATCACACTTAGATAGATTAATATAGCTCATAAT 91265954
91265955 GAGCTTGTATATTCTTAGTGTTAACTTCTGTTTACTCAGGACATAATTTGGACCTGTTCC 91266014
91266015 TGTTACTGTTGGTCTCTAGACTTTTGTAGTCATTCTTTAAATGTGTGTCTCAAATTACAC 91266074
91266075 ATTTATCCCTTCTAAACCACTGAGGCTTTTTTTTTTTTTTTTTCATTTAATTCTTCCCAG 91266134
91266135 GTTTGTGTACTGGTCAGACTGGGGAGAACCAGCTAAAATAGAAAAAGCAGGAATGAATGG 91266194
91266195 ATTTGATAGGCGGCCACTTGTGACAGTGGACATCCAATGGCCTAATGGAATTACACTTGG 91266254
91266255 TATGTCTGTCCTTCCTTCCTTGGCCACCAACTCAATGGTCTCTGCTGCTTTCGCTTCCCT 91266314
91266315 CCATAGTTTATTCTGGACTACAGCAGACAGCTCCCATGGTTCCTTTAGTAGCAAGAATTT 91266374
91266375 TGGATGAGAGTAACTGACCAATGCAAGTCGAGTACTGGCCTTAACGAATTACATGCTCCA 91266434
91266435 TCATTACTAGCTTTTTGGTCCATATGGACCTAATATGTGACATGAATTCAGTGCATCTTG 91266494
91266495 GGCCTTGGCAACTCTCATCCATCAGGGGAGAGCAATATGCTAGCTTCCTTTAACACTTC 91266553

RestrictionMapper (URL: http://www.restrictionmapper.org/) was used to search for restriction enzymes. Tat1, W^GTACW, was selected as it would digest in the motif and at one more location in the *VLDLR* gene.

5' ... **W** ↓ **G T A C** **W** ... 3'
3' ... **W** **C A T G** ↑ **W** ... 5'

Hence a WT 480 bp fragment would result in digestion at positions 186 bp and 451 bp, while only at 450 bp in a mutant delC:

```
91265955 GAGCTTGTATATTCTTAGTGTTAACTTCTGTTTACTCAGGACATAATTTGGACCTGTTCC 91266014
91266015 TGTTACTGTTGGTCTCTAGACTTTTGTAGTCATTCTTTAAATGTGTGTCTCAAATTACAC 91266074
91266075 ATTTATCCCTTCTAAACCACTGAGGCTTTTTTTTTTTTTTTTTCATTTAATTCTTCCCAG 91266134
91266135 GTTTGT//GTACTGGTCAGACTGGGGAGAACCAGCTAAAATAGAAAAAGCAGGAATGAATGG 91266194
91266195 ATTTGATAGGCGGCCACTTGTGACAGTGGACATCCAATGGCCTAATGGAATTACACTTGG 91266254
91266255 TATGTCTGTCCTTCCTTCCTTGGCCACCAACTCAATGGTCTCTGCTGCTTTCGCTTCCCT 91266314
91266315 CCATAGTTTATTCTGGACTACAGCAGACAGCTCCCATGGTTCCTTTAGTAGCAAGAATTT 91266374
91266375 TGGATGAGAGTAACTGACCAATGCAAGTCGA//GTACTGGCCTTAACGAATTACATGCTCCA 91266434
```

Primer design

Primers were designed using the Primer 3 database (URL: http://bioinfo.ut.ee/primer3-0.4.0/). VLDLR_F: TTTGGACCTGTTCCTGTTACTG and VLDLR_R: GGCCAGTACTCGACTTGCAT results in a 414 bp fragment that includes the motif.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any    3' seq
LEFT PRIMER        107   22   59.14   45.45  5.00  3.00 TTTGGACCTGTTCCTGTTACTG
RIGHT PRIMER       520   20   60.29   55.00  6.00  2.00 GGCCAGTACTCGACTTGCAT
SEQUENCE SIZE: 540
INCLUDED REGION SIZE: 540

PRODUCT SIZE: 414, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00

    1 TGTTACTGTTGGTCTCTAGACTTTTGTAGTCATTCTTTAAATGTGTGTCTCAAATTACAC

   61 GAGCTTGTATATTCTTAGTGTTAACTTCTGTTTACTCAGGACATAATTTGGACCTGTTCC
                                                >>>>>>>>>>>>>>>
  121 TGTTACTGTTGGTCTCTAGACTTTTGTAGTCATTCTTTAAATGTGTGTCTCAAATTACAC
      >>>>>>>>
  181 ATTTATCCCTTCTAAACCACTGAGGCTTTTTTTTTTTTTTTTCATTTAATTCTTCCCAG

  241 GTTTGTGTACTGGTCAGACTGGGGAGAACCAGCTAAAATAGAAAAAGCAGGAATGAATGG

  301 ATTTGATAGGCGGCCACTTGTGACAGTGGACATCCAATGGCCTAATGGAATTACACTTGG

  361 TATGTCTGTCCTTCCTTCCTTGGCCACCAACTCAATGGTCTCTGCTGCTTTCGCTTCCCT

  421 CCATAGTTTATTCTGGACTACAGCAGACAGCTCCCATGGTTCCTTTAGTAGCAAGAATTT

  481 TGGATGAGAGTAACTGACCAATGCAAGTCGAGTACTGGCCTTAACGAATTACATGCTCCA
                           <<<<<<<<<<<<<<<<<<<
VLDLR_F: TTTGGACCTGTTCCTGTTACTG
VLDLR_R: GGCCAGTACTCGACTTGCAT
```

Digestion of the PCR product

The combination of the primers mentioned and Tat1 would result in the following fragments.

- WT allele: 140, 265 and 9 bp.
- Mutant delC allele: 405 and 9 bp.

Appendix C: Additional calculations

In order to remove closely related individuals (Table 2), the following algorithm was performed:

1. Inclusion of all non-related dogs.
2. Inclusion of parent dogs.
3. Inclusion of randomly selected parent littermates.
4. Inclusion of randomly selected offspring littermates whose parents were excluded in previous steps.

Four out of the 36 remaining dogs were heterozygous, and hence 4 out of 72 alleles were mutant. In the following calculations, p and q are the population allele frequencies, whereas $\hat{p}$ and $\hat{q}$ are the allele frequencies in the study population, and n is the number of mutant alleles out of N in total.

Mutant allele frequency

$$\hat{q} = \frac{n}{N} \rightarrow \hat{q} = \frac{4}{72} \approx 0.0556$$

Confidence interval (CI) for the mutant allele frequency is given by the formula:

$$\hat{q} \pm Z_{1-\frac{\alpha}{2}} SE_{\hat{q}} = \left\{ SE_{\hat{q}} = \sqrt{\frac{\hat{q}(1-\hat{q})}{N}} \; ; \; \hat{q} = \frac{n}{N} \right\} = \frac{n}{N} \pm Z_{1-\frac{\alpha}{2}} \sqrt{\frac{\frac{n}{N}\left(1-\frac{n}{N}\right)}{N}}$$

A 95% CI was given by α=0.05 and hence $Z_{1-\frac{\alpha}{2}} \approx 1.9939$ at 71 degrees of freedom. Insertion of values yielded a 95% CI for q:

$$0.0556 \pm 0.0538 \; ; \; 0.0017 < q < 0.1094$$

Genotype distribution

As there are two possible alleles at the *VLDLR* locus, $p + q = 1$.

$$\hat{p} = 1 - \hat{q} \approx 0.9444$$

When assuming that the population is in Hardy-Weinberg equilibrium, then the genotypes are $p^2 + 2pq + q^2 = 1$. The expected genotypes were calculated:

- WT homozygote ($\hat{p}^2$):

$$\hat{p}^2 = \left(\frac{68}{72}\right)^2 \approx 0.8920$$

- Heterozygote ($2\hat{p}\hat{q}$):

$$2\hat{p}\hat{q} = 2\times\frac{4\times68}{72^2} \approx 0.1049$$

- Mutant homozygote ($\hat{q}^2$):

$$\hat{q}^2 = \left(\frac{7}{108}\right)^2 \approx 0.0031$$

Chi-square (χ^2) statistics between the observed and calculated genotypes yielded $\chi^2 \approx 0.1244$, with a p-value of 0.9397. At 2 degrees of freedom, ${\chi^2}_{0.05} \approx 5.99$ and ${\chi^2}_{0.20} \approx 3.22$, therefore a null-hypothesis about the genotypes in the study population being in Hardy-Weinberg equilibrium cannot be rejected even at a significance level of 0.2.