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Detection of C295G mutation *T* gene in Polish Lowland Sheepdog

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The aim of the study was to identify C295G mutation in *T* gene in the Polish population of Polish Lowland Sheepdog and to analyze the consequences of matchmaking the carriers of the mentioned mutation of *T* gene conditioning shortened tail and tailessness. The samples were collected from 61 Polish Lowland Sheepdogs. Polymerase chain reaction was performed using the same primers such Indrebø et al. [6] had used. To identify mutations in exon 1 C295G *T* gene, there was used *Bst*EII restriction enzyme which cuts the sequence with a length of 702 bp at nucleotide position 191. The mutation created an additional restriction site at position 160. Among the 61 tested dogs there were 18 recessive homozygotes and 43 heterozygotes identified. There was no dominant homozygote, because, as the authors of other studies indicate, mutation of the gene C295G *T* is lethal in that system and causes embryos' mortality at an early stage of embryonic development. We observed decreasing litter size with short-tail x short-tail crosses, compared with the litter size of long-tail x long tail crosses, which confirms a major role of *T* gene during embryogenesis. However, the recorded differences were not statistically significant. The results of these studies show that the presented molecular test is an excellent tool for a clear statement of the Polish Lowland Sheepdog breed test: whether a short tail is genetically determined, or it is the result of a surgical procedure.

KEY WORDS: C295G mutation / *T* gene / Polish Lowland Sheepdog

The Polish Lowland Sheepdog is one out of five Polish dog breeds officially registered by the Fédération Cynologique Internationale (http://www.fci.be/). The first information on dogs of this type, indicating also a characteristic trait of this breed, i.e. appearance of animals with a shortened or rudimentary tail, date back as far as the 18th century [9, 10]. In the past the breed standards required the tail in that breed to be naturally short or docked very short [7, 12]. The presently binding standard of the Polish Lowland Sheepdog admits any tail length, in acceptance of legal regulations found in many countries and concerning ear cropping and tail docking in dogs (http://www.fci.be/uploaded_files/251GB98_en.doc).

In the countries which ratified the European Convention for the Protection of Pet Animals a complete ban on ear cropping and tail docking in dogs is in force (http://conventions.coe.int/Treaty/en/Treaties/Html/125.htm). This ban is also binding in Poland, as it was incorporated in the Act on Animal Protection, even though Poland is presently not a party to the Convention [13]. For this reason many cynological associations have introduced a ban on presenting dogs with cropped ears and/or docked tails at exhibitions and official shows (http://www.zkwp.pl/zg/komunikat_ZG_ciecie_uszu.pdf). In order to participate in a show dogs of the breeds, in which natural bobtails are found, have to be officially certified, confirming that the tail shortening was not a result of docking. In Polish Lowland Sheepdogs and in 17 other dog breeds a genetic cause was found to be responsible for tail shortening, i.e. a mutation within the *T* gene [5]. The *T* gene belongs to a large family of transcription factors, the so-called T-box. In dogs a non-synonymous C295G mutation in exon 1 of this gene results in the Ile63Met amino acid substitution [3]. Apart from dogs, its homologues are also found in other animal species. The *T* gene influences development of hind body structures, including the tail, and mutations in its region may lead not only to tail shortening, but also to serious developmental defects [1, 3, 5, 6].

The aim of this study was to identify carriers of the C295G mutation in the *T* gene, responsible for tail shortening in the Polish Lowland Sheepdog and to analyse consequences of mating with carriers of this mutation.

Material and methods

Biological material for analyses conducted in this study comprised peripheral blood collected from 61 dogs of the Polish Lowland Sheepdog breed, coming from Polish kennels (permit 9/2013 of the 3rd Local Ethical Committee in Warszawa of 27 March 2013). Blood was collected from the cephalic vein to 4ml test tubes containing K_3EDTA. Among dogs, which blood was used in the analyses, 14 had phenotypically long tails and 41 had bobtails or were tailless, some of them also as a result of docking. Moreover, analyses were also performed on blood collected from 6 dogs, for which no information on their tail length was available.

Genomic DNA from whole blood was isolated using the phenol-chloroform extraction and next it was kept in cold storage at 4°C for further analyses. In order to assess the quality of isolated genomic DNA its electrophoretic separation was performed in 1% agarose gel and the electrophoretic gel images were analysed by spectrophotometry (NanoDrop 2000, Thermo Scientific).

A fragment (702 bp) of the *T* gene [6] was amplified using the Polymerase Chain Reaction (PCR) in 200µl test tubes in a Trioblock Thermocycler (Biometra) applying respective primers (Table) and under temperature conditions and the composition of the PCR reaction mixture identical to those presented in a study by Gruszczyńska and Czapla [2]. In order to perform qualitative evaluation of the PCR products their electrophoresis was run in

Table
Sequences of primers used in PCR

Primer	Sequence (5'-3')
Primer 1 (Forward)	GAAGAGCCTGCAGTACCGAGT
Primer 2 (Reverse)	CACTCTCCGTTCACGTACTTCC

2% high resolution agarose gel with an addition of ethidium bromide (0.5 μg/ml). The image visualisation under UV light was performed in an ImageMaster® VDS apparatus (PharmaciaBiotech).

In order to identify the C295G mutation the amplified fragment of the *T* gene was additionally digested with the *Bst*EII restriction enzyme (BioLabs) following the manufacturer's protocol. Digestion products were subjected to electrophoretic separation in 12% native polyacrylamide gel; subsequently, the gels were stained with silver nitrate, dried in a vacuum drier (Biometra) and photographs were taken for documentation purposes.

Information on the size of litters coming from respective mating types was obtained from litter records. The following types of matings were analysed: 23 − bobtail x bobtail; 7 − long tail x long tail; 25 − long tail x bobtail. The significance of differences in the mean size of the litter depending on the type of mating, from which litters originated, was verified using the χ^2 test.

Results and discussion

Amplification of all the analysed samples provided a product of 702 bp. Digestion of the PCR product with the *Bst*EII restriction enzyme made it possible to identify the genotype of the investigated dogs in terms of the *T* gene, since this enzyme cleaves the unmutated sequence only at the position of 191 bp, whereas the presence of the mutation leads to the formation of an additional restriction site at 160 bp. In the case of the wild type allele two restriction fragments of 511 and 191 bp, respectively, are formed, while the mutated allele produces three restriction fragments of 511, 160 and 31 bp, respectively (Fig. 1). In the case of recessive homozygotes 2 bands of 511 and 191 bp are visible on the stained polyacrylamide gel. In the case of heterozygotes, although in reality four restriction fragments of 511, 191, 160 and 31 bp are formed, only three bands of 511, 191 and 160 bp are visible, while that of 31 bp remains invisible (Fig. 2). In their studies Haworth et al. [3] and Hytönen et al. [5] also used the *Bst*EII restriction enzyme to identify the investigated mutation. Due to the fact that they used other primers to amplify the *T* gene, the length of the amplified sequences as well as the size of obtained restriction fragments differed from those we obtained in this study.

The length of the restriction fragments (bp)	Recessive homozygote	Heterozygote	Dominant homozygote
511	———	———	———
191	———	———	
160		———	———
31		———	———
Restriction pattern	A	B	C

Fig. 1. Possible results of the diagnostic test, to detect the C295G mutation of *T* gene in dogs

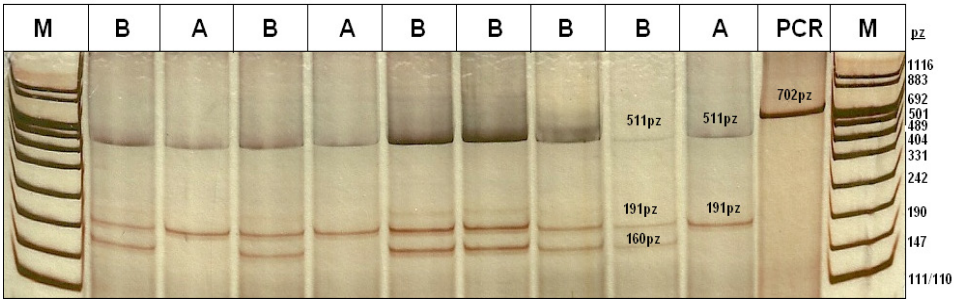

Fig. 2. Electrophoresis of the products of digestion with enzyme *Bst*EII in 12% native polyacrylamide gel. M – standard size pUC Mix Marker 8; A – recessive homozygote (visible restrictive fragments of the length of 511 bp and 191 bp); B – heterozygote (restrictive fragments of the length of 511 bp, 191 bp, 160 bp are visible, restrictive fragment of 31 bp is not visible); PCR – amplification product with a length of 702 bp

In the course of this study the genotype was determined for 61 Polish Lowland Sheepdogs. A total of 18 recessive homozygotes and 43 heterozygotes were identified. No dominant homozygote was identified. Similarly as it was reported by other authors [3, 5, 6], it was found that all recessive homozygotes had tails of normal length, while heterozygotes were either tailless or were bobtails. The length of shorter tails varied depending on

the number of caudal vertebrae. Based on the testing results it was shown that 6 animals, in which the short tail phenotype was observed, genetically were animals with long tails. All recessive homozygotes were born with long tails; however, docking was performed in 4 animals, which was confirmed by the results of the performed genetic tests. All the dogs originating from mating parents with normal length tails also had tails of normal length and they were recessive homozygotes. No heterozygote came from mating animals of normal length tails. In the case of matings, in which both animals had shortened tails, or one of them had a shortened tail and the other had a tail of normal length, litters were composed of both heterozygous animals (with a shortened tail or tailless) and recessive homozygotes (tails of normal length).

After analysing the mean size of the litter depending on the type of mating, a decrease was observed for the mean number of puppies in the litters coming from mating two animals with shorter tails (bobtails) in comparison to the mean size of the litter coming from the mating of two animals with long tails, or mating an animal with the long tail with an animal with a short tail (Fig. 3). However, these differences were statistically non-significant. The observed reduction of the size of litters produced by heterozygous parents was comparable to that reported in a study by Hytönen et al. [5]. Those authors found a statistically highly significant 29% decrease in the size of the litter coming from mating of dogs with short tails (bobtails) in the Swedish Vallhund breed. In that breed the C295G mutation in the T gene is also responsible for a shortened tail (bobtail) or taillessness.

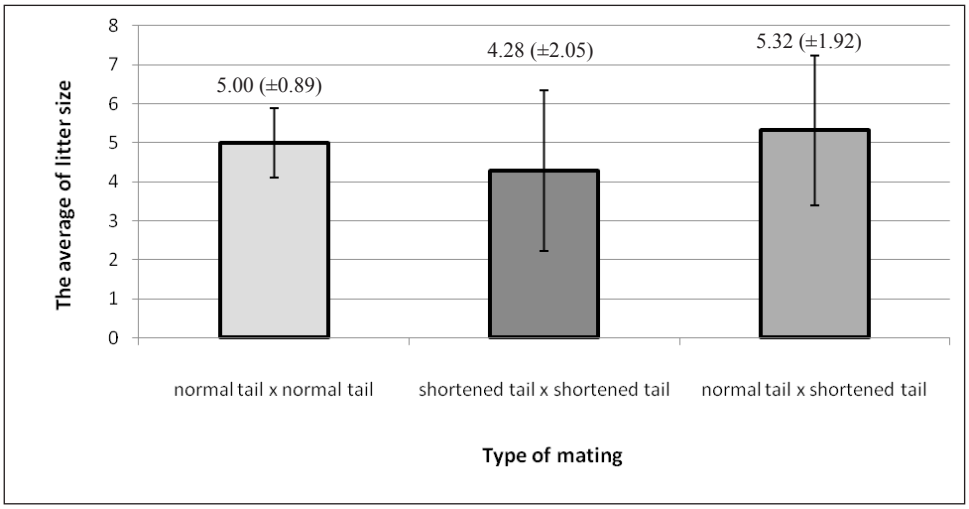

Fig. 3. The average litter size (along with standard deviation) depending on the type of mating in the population of Polish Lowland Sheepdog breed

These results were consistent with the expected 25% decrease in the size of the litter obtained after heterozygous parents, due to the mortality during embryonic development of embryos being dominant homozygotes. The lethality of the mutation in the homozygous system during embryonic development is confirmed by the fact that no dominant homozygote was found in this study. Similar results were recorded by Haworth et al. [3] and Hytönen et al. [5]. Only Indrebø et al. [6] reported a case of a birth of two puppies being dominant homozygotes. These puppies, apart from taillessness and rectal atresia, showed many other lethal developmental defects. To date no other reports have been published on the birth of puppies being dominant homozygotes. Also in other species it was found that mutations of the T gene homologues in the homozygous system are lethal. Mice being dominant homozygotes exhibit many systemic disorders and die at day 10 of embryonic development [14]. Similar irregularities were also reported in the zerbafish [11].

The fact that all dogs with the bobtail (shortened tail) phenotype were heterozygotes and no dogs with normal length tails were carriers of the C295G mutation, indicates a complete gene penetrance. In dogs being heterozygotes, apart from tail shortening or taillessness, no other disorders were found in their anatomical structure [6]. A similar situation is observed in the case of zebrafish, in which carriers of the mutation show tail shortening as the only effect of this mutation [11]. In contrast to dogs or zebrafish, heterozygous mice, apart from having a shortened tail, also exhibit other irregularities in their skeletal structure [4]. The fact that studies did not show any other anatomical defects – apart from tail shortening – does not suggest in any of the heterozygotes that the only effect of the C295G mutation in the *T* gene in heterozygotes is connected with tail shortening (the bobtail phenotype). Mating of heterozygous animals with recessive homozygotes makes it possible to obtain approx. 50% progeny with a shortened tail and 50% progeny with normal length tails. Mating of two heterozygotes also provides approx. 50% progeny with a shortened tail (bobtails); however, only approx. 25% will have long tails. The other 25% embryos will be dominant homozygotes, which as a rule die in the course of their embryonic development. Since to date only one case was reported, presenting live births of two puppies being dominant homozygotes, the probability of having puppies with the defects resulting from this type of mating should be considered as extremely low. Breeding dogs with the genotypes, which may be the underlying cause of defects, should be avoided. If future studies show that births of puppies suffering from serious defects, being dominant homozygotes, are not exceptional or rare cases, mating of heterozygous animals should not be recommended [6].

To date, the effect on tail shortening found for the C295G mutation in exon 1 of the *T* gene in dogs was reported in 18 breeds [3, 5]. This mutation is found in shepherd dogs and herding dogs, which suggests its origin from a common ancestor [5]. Hytönen et al. [5] in terms of the C295G mutation analysed 23 dog breeds, in which tail shortening or taillessness are found. It was found that in 6 breeds neither this nor any other mutation is found within the *T* gene. This indicates that apart from the *T* gene other genetic factors affecting tail length in dogs have to occur. Further studies are needed to identify them and determine the inheritance model. Moreover, in breeds, in which the C295G mutation is

found, we observe natural variation in tail length in heterozygotes. This may be caused by the variability in alleles of genes directly interacting with the *T* gene or the occurrence of more complex interactions between the T-box genes [6, 8].

Owners of dogs with the bobtail phenotype have to present certificates confirming the natural (genetic) origin of the short tail (bobtail). In the case of breeds, in which it was found that the C295G mutation in the *T* gene is responsible for tail shortening, subjective veterinary evaluation may be replaced with a simple, objective molecular test, definitely identifying the source of the short tail phenotype. The presented molecular test may serve as an objective tool to diagnose the C295G mutation of the *T* gene in Polish Lowland Sheepdogs in order to issue documents confirming the genetic genesis of the short tail (bobtail) or taillessness in dogs.

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