Annals of Warsaw University of Life Sciences – SGGW
Animal Science No 49, 2011: 35–43
(Ann. Warsaw Univ. of Life Sc. – SGGW, Anim. Sci. 49, 2011)

A molecular test for the detection of the C295G mutation in the T gene responsible for shortened tail and taillessness in the Pembroke Welsh Corgi

JOANNA GRUSZCZYŃSKA, AGATA CZAPLA
Department of Genetics and Animal Breeding, Warsaw University of Life Sciences – SGGW

Abstract: *A molecular test for the detection of the C295G mutation in the T gene responsible for shortened tail and taillessness in the Pembroke Welsh Corgi*. A ban on cutting tail in dogs has been introduced in many countries. In order to exhibit on an official pedigree show a tailless or short-tailed dog, it's owner must provide a statement confirming that the short tail is natural characteristic of his dog. The aim of this paper was to design a test to diagnose a C295G dominant mutation in T gene which is responsible for short-tail phenotype in 17 dog breeds. This mutation creates a *Bst*E-II restriction site. The molecular test designed is based on genomic DNA analysis. A fragment of T gene is amplified in polymerase chain reaction (PCR). Then, the PCR product is treated with restriction enzyme *Bst*EII, and the analysis of restriction fragments is carried out in polyacrylamide gel. A genotype of 30 Polish Pembroke Welsh Corgi regarding T gene has been determined. Within the studied group of dogs, 10 heterozygotes and 20 recessive homozygotes have been found. No dominant homozygotes have been present.

Key words: *Canis familiaris*, Pembroke Welsh Corgi, short-tail, taillessness, T-box gene.

INTRODUCTION

The T gene belongs to the family of T-box genes and occurs in many species of animals, both vertebrates and invertebrates. This is a large family of genes encoding transcription factors expressed during the embryonic development. Most of the T proteins act as transcription activators, only few of them are repressors. The T protein activates genes specific to the mesoderm and is involved in the development of structures derived from the posterior mesoderm, including notochord. The first discovered gene belonging to a T-box gene family is known as Brachyury or T (for tail) gene, which is responsible for the short tail in mice (Kavka and Green, 1997; Papaioannou and Silver, 1998).

In many species the short tail can be conditioned by the presence of mutations in the T gene. T/Brachyury gene at the stage of the embryonic development is involved in the process of differentiation of axial structures, such as notochord and neural tube. Therefore, taillessness and short-tail phenotype induced by the mutation of this gene is associated with the presence of a number of other defects revealing mainly in homozygotes during the embryonic development. Abnormalities in the embryonic development are usually the cause of necrobiosis of these embryos. So in homozygotes the T gene mutation is lethal (DeForest and Basrur,

1979; Adalsteinsson, 1980; Robinson, 1993). Heterozygotes for the T gene either do not demonstrate any genetic defects, or have a shortened or lack of tail. For example, heterozygous mice for the T gene mutation, are short tailed or taillessness, and in addition they have spinal congenital defects. However, the dominant homozygotes decay around the 11th day of pregnancy, and foetuses have no tail and the posterior mesoderm derived structures (Showell et al., 2004). In humans, the T gene mutation is not yet associated with any disease. Contrary, in cats, shortened tail, or tailless is linked to the existence of dominant gene M (Manx), which yet have not been sequenced. The M gene mutation, responsible for the lack of tail in cats also determines other defects. In heterozygotes an urinay and faecal incontinence and abnormal hindlimb action are observed (Robinson, 1993).

The T gene mutation causing taillessness or shortened tail occurs in 17 breeds belonging to two groups of dogs: sheepdogs and hunting dogs (Hytönen et al., 2009). This mutation was also found in the Pembroke Welsh Corgi. In this breed it is inherited in a dominant manner and in heterozygotes it causes bobtail phenotype, while, dominant homozygotes die during embryonic life or shortly after birth (Indrebø et al., 2008).

Knowledge of the genotype for the T gene is essential in the case of purebred, pedigree dogs that will be used for breeding, because an increasing number of countries is prohibiting cutting tails. Molecular test based on the analysis of the genomic DNA allows unambiguous determination of the origin of the short tail.

MATERIAL AND METHODS

Biological material and the isolation of the genomic DNA

The whole blood was collected into K_3EDTA tubes from 30 Pembroke Welsh Corgi born in 1998–2007 and bred in Poland. Out of the 30 dogs from which the blood samples were collected: 20 had a normal tail length, or docked, 6 – short tail, and 4 – no tail. The full pedigree data for each dog were also available, as well as description of parents' tail length. The genomic DNA was isolated from blood using standard phenol-chloroform extraction. The presence and the purity of isolated DNA was checked on a Nanodrop 2000 spectrophotometer (THERMO SCIENTIFIC) and in a 1% agarose gel.

PCR reaction conditions

Polymerase chain reaction (PCR) was performed using the following pairs of primers: 5'-GAAGAGCCTGCAGTAC-CGAGT-3 'in exon 1 and 5'-CACTCTC-CGTTCACGTACTTCC-3' in exon 2 of the T gene (Indrebø et al., 2008). The reaction mixture entered: 1 ng DNA, reaction buffer (SIGMA-ALDRICH, USA) 1 × concentrated, 2.5 mM $MgCl_2$, 200 µM/µl dNTP mix (POLGEN, Poland), 5 pmol primers, 1 U/µl Taq DNA polymerase (SIGMA-ALDRICH, USA). The reaction was carried out under the following conditions: initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 45 s, final synthesis at 72°C for 10 min. The PCR product was analysed by the electrophoresis in native 12% polyacrylamide gel. The electrophoresis was carried out in a vertical electrophoresis

apparatus (BIOMETRA, Germany) in 1 × TBE buffer (10 × TBA: 89 mM Tris, 2 mm EDTA pH 8.0, 89 mM boric acid) for 1 h at a voltage of 180 V. After the electrophoresis, the gel was stained with silver, dried and then scanned for further analyses.

Diagnostic test

The purpose of this study was to develop a diagnostic test based on the amplification of a part of the T gene with mutation site, and the differentiation of normal and mutant alleles with differences resulting from restriction enzyme digestion of the PCR product. The substitution of the cytosine for guanine at nucleotide position 189 T gene creates a place recognized by the restriction enzyme *Bst*EII from *Bacillus stearothermophilus*. As a result of the PCR reaction, the 702 base pairs fragment is amplified. Through *Bst*EII digestion of the PCR product, the 511 bp fragment is obtained, regardless of whether the DNA came from an individual who has the C295G mutation in his genotype or not, and a 191 bp fragment from the wild allele, and 160 and 31 bp from the mutant allele. The additional *Bst*EII restriction site for the both types of allele acts as an internal positive control for *Bst*EII digestion.

PCR product restriction enzyme digestion

The PCR product was digested using 5 U *Bst*EII (BIOLABS, UK) restriction enzyme in the presence of the recommended buffer, and 100 mg/ml bovine serum albumin (BSA) (BIOLABS, UK) for 1 h at 60°C.

Analysis of digested PCR products

Restriction enzyme digestion products were analysed by the vertical electrophoresis in 12% native polyacrylamide gel in 1 × TBE buffer for 90 min at a voltage of 180 V. After the electrophoresis, the gel was stained with silver method, dried and then scanned.

RESULTS AND DISCUSSION

The whole sequence of T-box gene is availabl in the GenBank (gi:74027283). We amplifed 702 bp fragment of T-box gene. This fragment consist of: exon 1 – 173 bp, intron 2 – 406 bp and exon 2 – 123 bp. Sample results of the designed diagnostic test for the T gene mutation in Pembroke Welsh Corgi are shown in Figure 1. Restriction enzyme digestion PCR product indicates the genotype of studied dogs. Recessive homozygous animals have fragment of 511 and 191 bp, and the carriers (heterozygotes) have 511, 191, 160 and 31 bp fragments. Figure 1 does not include the fragment of 31 base pairs, but this fragment is shown on Figure 2.

The presence of the additional restriction site for *Bst*EII in all PCR products functions as a positive control ensuring that the appropriate reaction conditions for *Bst*EII restriction digestion were provided. Using the diagnostic test developed in this study the genotype of 30 dogs was identified: 20 recessive homozygotes, 10 heterozygotes and no dominant homozygotes were found. Twenty dogs that were recessive homozygous had phenotypically normal tail. Heterozygous dogs for the T gene had either a short tail (6 dogs) or no tail at all (4 dogs). After examining

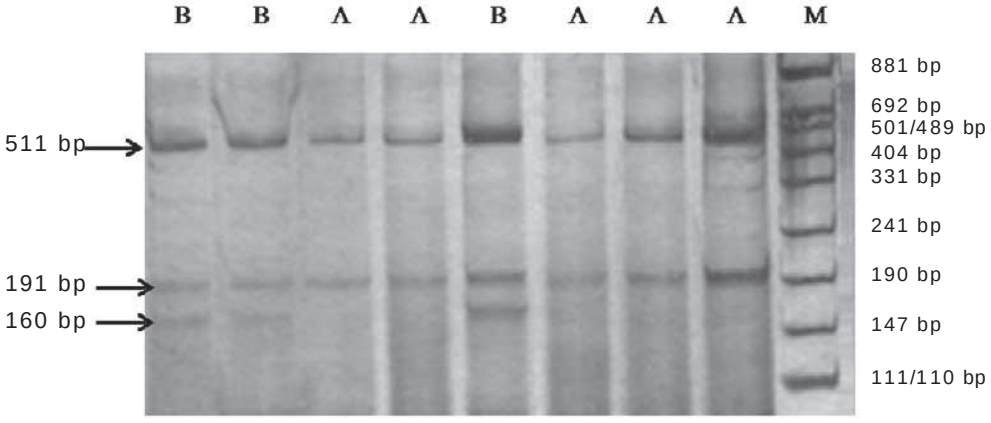

FIGURE 1. Electrophoretic separation of *Bst*EII restriction enzyme digestion fragments in native 12 % polyacrylamide gel. M – pUC Mix Marker 8; A – recessive homozygote (511 and 191 bp fragments are visible); B – heterozygote (511, 191 and 160 bp fragments are visible, 31 bp fragment not shown). Arrows marks restriction fragment lengths

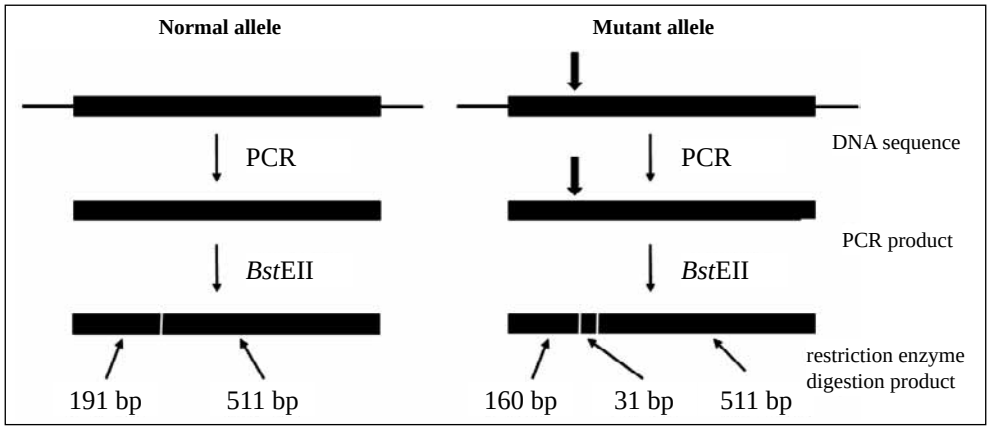

FIGURE 2. Principle of the diagnostic test for detection the C295G mutation of the T gene in Pembroke Welsh Corgi. The length of restriction fragments are given in the last row. Thick arrows showing the site of mutation

the pedigrees of tested dogs, it was found that for all individuals that were heterozygous one of the parents had phenotype of short tail.

British breed standard for the Pembroke Welsh Corgi of April 2004 (http://www.fci.be/uploaded_files/039A2003_en.doc) defines the tail of this breed as a short, and therefore the preferred tail is a naturally short tail. It was a common practice in many European countries, to cut off tails of dogs that were born with long tails.

Only after the European Convention for the Protection of Pet Animals came into the force, (http://conventions.coe.int/Treaty/EN/Treaties/Html/125.htm, Strasbourg, 1987) most countries

introduced a ban on cutting tails. Dogs with their tails docked can not participate in official shows. The owner of a dog with bobtail (short tail or no tail) needs to present a certificate from a veterinarian to confirm the natural origin of a short tail in his dog. In breeds in which a mutation of the T gene was identified, a veterinary observation can be now replaced by a simple genetic test that unambiguously identifies the origin of taillessness (Hytönen et al., 2009).

Canine T-box gene has been cloned by Haworth et al. (Haworth et al., 2001). It is located in the chromosome 1q23. As a result of the analysis of DNA sequences of the T gene C295G mutation was found in exon 1. Substitution of cytosine for guanine (C > G) leads to changes in ATC codon for isoleucine at codon ATG for methionine. After the transcription the mRNA is the codon AUG, which is recognized by the ribosome, as the translation start site (Haworth et al., 2001).

C295G mutation is also known as Ile63Met or C189G mutation (Hytönen et al., 2009). Substitution of cytosine for guanine creates a place recognized by the *Bst*EII restriction enzyme from *Bacillus stearothermophilus*.

Haworth et al. compared the canine T gene sequence to the T gene in other species (Haworth et al., 2001). It was found that the isoleucine at 63 amino acid position is conservative in the T protein from Drosophila to tunicates to man. The location of this isoleucine in the highly conserved region of T domain may indicate that the substitution of isoleucine for methionine at this position changes the ability of mutant T protein to DNA binding (Papapetrou et al., 1997).

In this paper, primers published by Indrebø et al. were used for the polymerase chain reaction (Indrebø et al., 2008). As a result of the PCR reaction, DNA segment with a length of 702 bp was obtained, which consists of a fragment of exon 1, intron 2 and a part of exon 2.

Haworth et al. (2001) applied two starters – both in exon 1 and an amplified fragment length of 317 bp to the PCR reaction exon 1 of the T gene with a mutation site (Haworth et al., 2001). Also Hytönen et al. (Hytönen et al., 2009) applied the same pair of primers that Haworth et al. (Haworth et al., 2001), and primers designed by themselves within exon 1 of the T gene.

C295G mutation in the designed DNA test was identified by the analysis of the length of restriction fragments generated in *Bst*EII digestion of the PCR product. After the electrophoresis of restriction fragments in polyakrylamide gel, the gel was stained with silver method. Polyacrylamide gels are characterized by a higher resolution than agarose gels. By contrast, the method of silver staining of DNA fragments is more sensitive to ethidium bromide staining. Indrebø et al. analysed the sequenced PCR product (Indrebø et al. 2008). By contrast, Haworth et al. and Hytönen et al. subjected the PCR product to both *Bst*EII enzyme digestion and sequencing (Haworth et al., 2001; Hytönen et al., 2009).

Among the dogs studied by Haworth et al. in order to analyse T gene mutations, there was no dominant homozygote even in the litter from mating two short tailed dogs (Haworth et al., 2001). All the dogs with a shortened tail were carriers of the C295G mutation. This may mean that the T gene mutation is lethal

in homozygous animals (Haworth et al., 2001). Also mice heterozygous for the T gene mutation have short tail or no tail and it is associated with skeletal defects. Moreover, mice homozygous for the T gene mutation exhibit severe malformations, and fetuses survive to the 10.5 day of the gestation (Meisler, 1997). While in the fish *Danio rerio* carrying the mutation in the ntl gene, homologous to the mouse T gene, there were no visible phenotypic changes that would be associated with shortening of the tail. The ntl gene phenotypic effect in homozygous fish is similar to the phenotypic effect of gene Brachyury in homozygous mice (Schulte-Merker et al., 1992, Schulte--Merker et al., 1994).

Indrebø et al. examined 19 naturally short tailed Pembroke Welsh Corgi heterozygous for the T gene mutation in order to determine their suitability for congenital spine (Indrebø et al., 2008). All dogs were subjected to a radiological examination. The results showed no spinal congenital defects connected with the T gene mutation, which means that the only phenotypic effect of the C295G mutation observed in the carriers of the Pembroke Welsh Corgi is short tail or tailless.

Indrebø et al. reported also two Pembroke Welsh Corgi puppies, which were dominant homozygous for the T gene mutations (Indrebø et al., 2008). One of them survived only a few seconds after birth, and the second was euthanized. Both puppies were radiographically and genetically tested and submitted to an autopsy. In addition to the lack of tail and anorectal atresia, they showed a series of spinal defects, for example: shortened cervical, thoracic and lumbar vertebral bodies, and no sacral or caudal vertebrae, scoliosis of thoracic and lumbar spine and kyphosis of the thoracic spine (Indrebø et al., 2008).

There are no other reports of homozygous puppies born or stillborn fetuses, which suggests that in most cases, the homozygotes for the C295G mutation is lethal even in an early fetal development (Haworth et al., 2001; Indrebø et al., 2008). Until now there was only one report of homozygous puppies born alive. Thus, it appears that there is a possibility for these puppies to be born and be able to survive for a short period of time. The reasons for such phenotypic differences in dogs with the same genotype regarding T gene, have not been explained yet. Natural variability in the length of the tail in dogs of Pembroke Welsh Corgi with short tails indicates that there may be differences in the sequences of other genes directly interacting with the T gene (Indrebø et al., 2008). It was demonstrated that the genes belonging to a family of genes with a T-box and their cofactors may have complex interactions (Packham and Brook, 2003).

After crossing a short tailed with a long tailed dog it can be expected that about 50% of litter are short tailed (heterozygous). In the same time, when crossing two short tailed dogs there is a possibility of getting homozygous dominant pupies for the C295G mutation. However, the probability of obtaining homozygotes in the progeny is relatively low. Indeed, in the past, such crossing was conducted in many countries, but so far there has been no information about puppies with congenital defects indicating the presence of the T gene mutations in homozygous genotype. Nevertheless, mating of dogs

with genotypes that could cause an increased presence of hereditary defects should be prohibited by law (Indrebø et al., 2008).

Hytönen et al. examined 23 breeds of dogs and demonstrated the presence of the C295G mutation in all short tailed dogs belonging to 17 breeds (Hytönen et al., 2009) . In the remaining 6 breeds, neither mutation Ile63Met, nor any other T gene mutations were detected. 9 breeds of dogs, which phenotypes do not exhibit natural taillessness were also studied. The results show that the T gene mutation is not present in all breeds of dogs, in which short tailed phenotype is observed. Thus, there must be some other genetic factor that determines the length of the tail in dogs (Hytönen et al., 2009). The possible second mutation affecting this trait might explain while some of T gene heterozygous dogs had short tail while others had no tail at all. All tested dogs with short tails were heterozygous for the C295G mutation, and all dogs with long tails were not carriers of this mutation. This indicates full penetration of the T gene and its lethality in the homozygous configuration. The T gene lethality was further confirmed by statistical analysis, based on comparison of the litter size of two mating groups (long tailed × long tailed and short tailed × short tailed) of Swedish Vallhund. When two parents were short tailed a 29 percentage decrease in the litter size was observed. This litter size reduction is likely due to the lethality of the embryos in the uterus, which is consistent with the expected 25-percent decrease in the litter size when crossing two heterozygous parents As expected 1/4 of all puppies should inherit a mutation from both parents and became lethal homozygous (Hytönen et al., 2009).

Seventeen breeds of dogs, in which the presence of the Ile63Met mutation was detected, belong to two groups of dogs (sheepdog and hunting dogs). This may suggest an ancestral origin of the T gene mutation. To determine the pattern of inheritance and the reasons for taillessness in other breeds of dogs further research are needed (Hytönen et al., 2009).

In the present study the genotype of 30 dogs for the T gene C295G mutation in Pembroke Welsh Corgi was determined. Ten dogs proved to be the carriers of the mutation and the other 20 showed no presence of the T gene mutation. As in the previous studies conducted by other authors (Haworth et al., 2001; Indrebø et al., 2008; Hytönen et al., 2009), all heterozygous dogs were short tailed (6 dogs) or taillessness (4). In turn, all dogs without the C295G mutation were born with a long tail. There were no dominant homozygotes for this mutation, because they died during embryonic or perinatal period, as shown by the work of other authors (Haworth et al., 2001; Indrebø et al., 2008; Hytönen et al., 2009).

Using the designed diagnostic test allows to determine the T gene mutation carriers that are responsible for taillessness or short tailed phenotype in Pembroke Welsh Corgi. This is important because tail docking is prohibited in many countries. If a dog is to take part in pedigree shows a certificate to ascertain a natural short tail will be required; such certificate can be based now on objective molecular analysis.

Acknowledgements

We would like to thank all breeders from Welsh Corgi Club in Lublin of Polish Kennel Club for permission to collect blood samples.

REFERENCES

ADALSTEINSSON S., 1980: Establishment of Equilibrium for the Dominant Lethal Gene for Manx Taillessness in Cats. Theoretical and Applied Genetics 58 (2): 49–53.

DEFOREST M.E., BASRUR P.K., 1979 – Malformations and the Manx Syndrome in Cats. The Canadian Veterinary Journal 20, 304–314.

HAWORTH K., PUTT W., CATTANACH B., BREEN M., BINNS M., LINGAAS F., EDWARDS Y.H., 2001: Canine homolog of the T-box transcription factor T; failure of the protein to bind to its DNA target leads to a short-tail phenotype. Mammalian Genome 12: 212–218.

HYTÖNEN M.K., GRALL A., HÉDAN B., DRÉANO S., SEGUIN S.J., DELATTRE D., THOMAS A., GALIBERT F., PAULIN L., LOHI H., SAINIO K., ANDRÉ C., 2009: Ancestral T-box Mutation Is Present in Many, but Not All, Short-Tailed Dog Breeds. Journal of Heredity 100 (2): 236–240.

INDREBØ A., LANGELAND M., JUUL H..M, SKOGMO H.K., RENGMARK A.H., LINGAAS F., 2008: A study of inherited short tail and taillessness In Pembroke Welsh corgi. Journal of Small Animal Practice 49 (5): 220–224.

KAVKA A.I., GREEN J.B.A., 1997: Tales of tails: Brachyury and the T-box genes. Biochimica et Biophysica Acta 1333 (2): F73–F84.

MEISLER M.H., 1997: Mutation watch: Mouse brachyury (T), the T-box gene family, and human disease. Mammalian Genome 8 (11): 799–800.

PACKHAM E.A., BROOK J.D., 2003: T-box gene in human disorders. Human Molecular Genetics 12 (1): R37–R44.

PAPAIOANNAOU V.E., SILVER L.M., 1998: The T-box gene family. BioEssays 20 (1): 9––19.

PAPAPETROU C., EDWARDS Y.H., SOWDEN J.C., 1997: The T transcription factor functions as a dimer and exhibits a common human polymorphism Gly-177-Asp in the conserved DNA-binding domain. The Federation of European Biochemical Societies Letters 409 (2): 201–206.

ROBINSON R., 1993: Expressivity of the Manx Gene in Cats. Journal of Heredity 84(3): 170––172.

SCHULTE-MERKER S., EEDEN VAN F.J.M., HALPERN M.E., KIMMEL C.B., NÜSSLEIN-VOLHARD C., 1994: No tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. Development 120: 1009–1015.

SCHULTE-MERKER S., HO R.K., HERRMANN B.G., NÜSSLEIN-VOLHARD C., 1992: The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development 116: 1021–1032.

SHOWELL C., BINDER O., CONLON F.L., 2004: T-box Genes in Early Embryogenesis Developmental Dynamics 229 (1): 201–218.

European Convention for the Protection of Pet Animals Strasbourg, 13 XI 1987, http://conventions.coe.int/Treaty/EN/Treaties/Html/125.htm

FCI – Standard Welsh Corgi (Pembroke) No39/28.11.2003/GB, http://www.fci.be/uploaded_files/039A2003_en.doc

Streszczenie: *Molekularny test wykrywania mutacji C295G w genie T odpowiedzialnego za krótkoogoniastość i bezogoniastość u pembroke welsh corgi.* Gen T należy do dużej rodziny genów z motywem T, która koduje czynniki transkrypcyjne, ulegające ekspresji podczas rozwoju zarodkowego. Białko T aktywuje geny specyficzne dla mezodermy i jest zaangażowane w proces rozwoju struktur wywodzących się z tylnej części mezodermy, m.in. struny grzbietowej. Mutację genu T warunkującą krótkoogoniastość i bezogoniastość zidentyfikowano u 17 ras psów (Hytönen i wsp., 2008). U psów rasy pembroke welsh corgi jest ona dziedziczona w sposób autosomalny dominujący i u heterozygot warunkuje fenotyp o skróconym lub szczątkowym ogonie. Homozygoty dominujące giną w okresie płodowym lub zaraz po urodzeniu (Indrebø i wsp., 2007). Obecnie w wielu krajach prawnie zabroniono cięcia ogonów u psów. Aby pies bez ogona lub też z ogonem szczątkowym mógł uczestniczyć w oficjalnych pokazach lub

wystawie psów rasowych, jego właściciel musi mieć zaświadczenie, potwierdzające „naturalność" krótkiego ogona u psa. Celem niniejszych badań było zaproponowanie testu diagnostycznego, identyfikującego mutację C295G w genie T oraz identyfikacja w polskiej populacji psów rasy pembroke welsh corgi heterozygot mutacji genu T warunkującego krótkoogoniastość i bezogoniastość. W tym celu wykorzystano enzym restrykcyjny *Bst*EII, który tnie sekwencję w 191 pozycji nukleotydowej w eksonie 1 genu T. W wyniku mutacji powstaje dodatkowe miejsce restrykcyjne w pozycji 160. Spośród 30 przebadanych psów zidentyfikowano 20 homozygot recesywnych i 10 heterozygot. Psy heterozygotyczne pod względem genu T miały fenotypowo ogon krótki (6 psów) lub szczątkowy (4 psy). Zmienność długości ogona u krótkoogoniastych psów rasy pembroke welsh corgi wskazuje na istnienie być może różnic w sekwencjach innych genów, bezpośrednio oddziałujących z genem T.

MS. received August 2011

Authors' address:
Katedra Genetyki i Ogólnej Hodowli Zwierząt – SGGW
ul. Ciszewskiego 8
02-786 Warszawa
Poland
e-mail: joanna_gruszczynska@sggw.pl