

## Characterization of a Canine Tetranucleotide Microsatellite Marker Located in the First Intron of the Tumor Necrosis Factor Alpha Gene

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**ABSTRACT.** A polymorphic tetranucleotide (GAAT)<sub>n</sub> microsatellite in the first intron of the canine tumor necrosis factor alpha (*TNFA*) gene was characterized in this study; 139 dogs were analyzed: 22 Beagles, 26 Chihuahuas, 20 Miniature Dachshunds, 24 Miniature Poodles, 22 Pembroke Welsh Corgis and 25 Shiba Inus. We detected the presence of the 4 alleles (GAAT)<sub>5</sub>, (GAAT)<sub>6</sub>, (GAAT)<sub>7</sub> and (GAAT)<sub>8</sub>, including 9 of the 10 expected genotypes. The expected heterozygosity (*He*) and the polymorphic information content (*PIC*) value of this microsatellite locus varied from 0.389 to 0.749 and from 0.333 to 0.682, respectively, among the 6 breeds. The allelic frequency differed greatly among breeds, but this microsatellite marker was highly polymorphic and could be a useful marker for the canine *TNFA* gene.

**KEY WORDS:** canine *TNFA* gene, tetranucleotide microsatellite.

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Tumor necrosis factor alpha (TNFA) is a multifunctional pro-inflammatory cytokine, which is secreted mainly by monocytes and macrophages (Online Mendelian Inheritance in Man (*OMIM*, 191160)). In humans, genetic variations in the *TNFA* gene are associated with various disorders, e.g., septic shock [14], rheumatoid arthritis [15], cystic fibrosis [3], inflammatory bowel diseases [11] and insulin resistance syndrome [8].

In the veterinary field, TNFA is also considered to be one of the most important cytokines involved with many inflammatory diseases, including bacterial and protozoan infections and sepsis [6, 13, 17]. For example, increased expression of *TNFA* mRNA in colonic mucosa has been reported on dogs with idiopathic lymphocytic-plasmacytic colitis [16].

The canine *TNFA* gene is located on dog chromosome 12 (CanFam3.1, dog genome assembly *NW\_003726081*), and it comprises 4 exons and 3 introns. However, the genetic polymorphisms in the canine *TNFA* gene have not been clarified.

Microsatellites are tandem repeated sequences, usually di-, tri- and tetranucleotide motifs, which display high levels

of polymorphism, making them ideal genetic markers [7, 18]. Microsatellites were thought to be evolutionarily neutral and to have no generalized function [18]. In recent years, however, many reports have indicated that microsatellites in the promoter regions and introns of functional genes exert genetic effects in many cases [1, 5, 9, 10, 12, 19]. Thus, microsatellites in known functional genes are good targets for analyzing genetic polymorphisms.

In this study, we focused on a (GAAT)<sub>n</sub> repeat motif located in the first intron of the canine *TNFA* gene and demonstrated that this microsatellite locus was highly polymorphic, so it could be a useful marker for genetic analysis.

Whole blood, swabs of the oral mucosa or nail samples were collected from dogs at veterinary hospitals and dog grooming shops in Japan. The 139 samples obtained were from 22 Beagles, 26 Chihuahuas, 20 Miniature Dachshunds, 24 Miniature Poodles, 22 Pembroke Welsh Corgis and 25 Shiba Inus. This study was approved by the Institutional Animal Care and Use Committee (Permission number: 1306094) and carried out according to the Azabu University Animal Experimentation Regulations. DNA was extracted from these samples using a Quick Gene DNA tissue kit (Fuji Film, Tokyo, Japan) or a NucleoSpin Blood Quick Pure kit (Macherey-Nagel, Duren, Germany), according to the manufacturer's protocols.

The primers used for polymerase chain reaction (PCR) analysis were designed using CanFam3.1. A 1.1-kb DNA fragment containing about 400 bp upper stream from the putative transcription start site, a 5'- untranslated region (5'- UTR), the first exon and about 240 bp of the first intron of the canine *TNFA* gene was amplified by PCR using the forward primer dog-*TNFA*-F1 (5'-AAGCCCCACCCCTTG-

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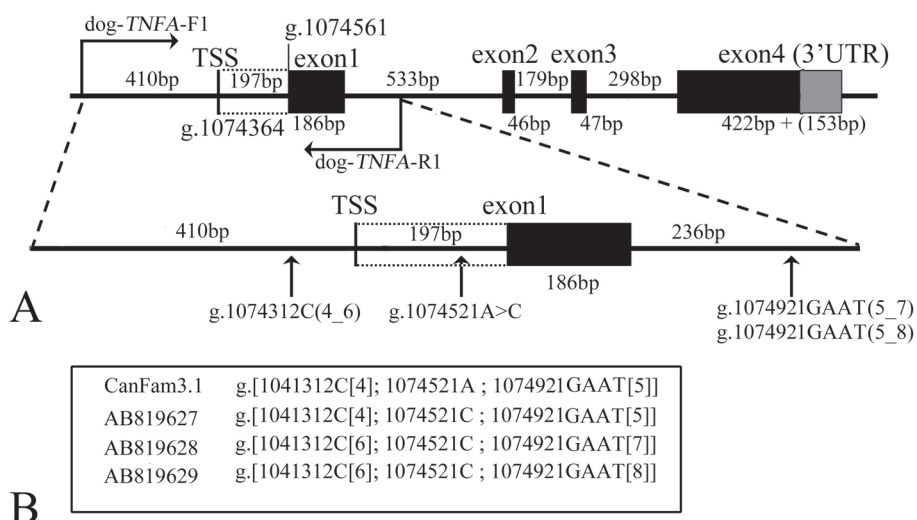


Fig. 1. Scheme of the canine *TNFA* gene showing the region sequenced and the polymorphisms detected. (A) Structure of the canine *TNFA* gene (above) and its sequenced region (below). The protein-coding sequences are indicated by dark boxes. The numbering of base positions was based on the reference sequence, NW\_003726081 (CanFam3.1; chromosome 12). In the reference sequence, g.1074364 and g.1074561 were the putative transcription start site (TSS) and the head of the translation initiation codon of the *TNFA* gene, respectively. (B) The variations detected in the partial *TNFA* gene in 7 Beagles.

CACCTT-3') and the reverse primer dog-*TNFA*-R1 (5'-CCA-CACCCACATCTCTCCACACA-3') (Fig. 1A). PCR was performed using 30  $\mu$ l reaction volumes, according to the manufacturer's instructions for GoTaq Green Master Mix (Promega, Fitchburg, WI, U.S.A.). The program comprised initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 20 sec, annealing at 59°C for 25 sec and extension at 72°C for 60 sec with a final extension at 72°C for 7 min. The amplified products were purified using a QIAquick PCR Purification kit (Qiagen, Hilden, Germany). The purified DNA was sequenced directly using a Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) with dog-*TNFA*-F1 and dog-*TNFA*-R1 primers. First, we analyzed the DNA sequences of 22 Beagles. However, 15 of the 22 individuals produced poor results, because of widespread double peak from the dog-*TNFA*-R1 primer side. As a result, we determined the target sequences successfully in 7 individuals only.

After multiple alignments of the partial *TNFA* sequences of the 7 Beagles with the canine reference sequence, we found 3 polymorphic sites: a cytosine direct repeat variation in the 5' gene flanking region (g.1074312C (4\_6)), an adenine to cytosine substitution in the 5' UTR (g.1074521A>C) and GAAT microsatellite variations in the first intron (g.1074922GAAT (5\_7) and g.1074922GAAT (5\_8)) (Fig. 1A). Next, we identified 3 *TNFA* variations: TNFA-Beagle1 (1 dog), TNFA-Beagle2 (2 dogs) and TNFA-Beagle3 (4 dogs) in the 7 individuals (Fig. 1B). These nucleotide sequences were deposited in the DDBJ, EMBL and GenBank nucleotide databases under accession numbers AB819627–AB819629.

Based on their electropherograms, we also found that the suboptimal sequencing results for the 15 Beagles resulted from the heterozygosity (*He*) in different numbers of (GAAT) repeats in the [(GAAT)<sub>5-8</sub> (GAT) (GAAT)<sub>2</sub>] microsatellite motif in the first intron (Fig. 1). To genotype this microsatellite locus, we designed the following primer set: CFA12-*TNFA*-STR1-F (5'-GGAAGATGCTCATGGATTGCT-3') and CFA12-*TNFA*-STR1-R (5'-TACCCACACCCACATCTCT-3'). PCR was performed using 25  $\mu$ l reaction volumes, according to the manufacturer's instructions for GoTaq Master Mix (Promega). The program comprised initial denaturation at 94°C for 3 min, followed by either 30 or 40 cycles of denaturation at 94°C for 20 sec, annealing at 55°C for 15 sec and extension at 72°C for 30 sec with a final extension at 72°C for 7 min. The PCR products were electrophoresed on a 3.0% agarose gel and visualized by ethidium bromide staining (Fig. 2). Four alleles, i.e., (GAAT)<sub>5</sub>, (GAAT)<sub>6</sub>, (GAAT)<sub>7</sub> and (GAAT)<sub>8</sub>, were detected in the CFA12-*TNFA*-STR1 tetranucleotide microsatellite locus, and the lengths of their PCR products were 114, 118, 122 and 126 bp, respectively. Subsequently, we surveyed the allele distribution of this microsatellite marker in 6 dog breeds. We used the FAM-labeled CFA12-*TNFA*-STR1-F primer, and the fragment lengths of the PCR products were determined automatically using an ABI 3130 genetic analyzer with GeneMapper Ver. 4.0 (Applied Biosystems). Table 1 shows the genotypic and allele distribution in 6 dog breeds. Four alleles were present in 22 Beagles, whereas only 3 alleles were detected in 117 dogs from the other 5 breeds. This is because the (GAAT)<sub>8</sub> allele was absent from Chihuahuas, Miniature Dachshunds, Miniature Poodles,

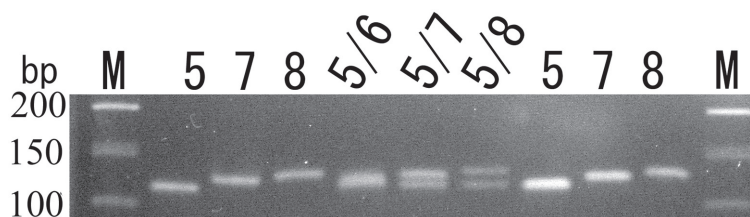


Fig. 2. Genotyped patterns of the tetranucleotide microsatellite polymorphism in the first intron of the canine *TNFA* gene on a 3.0% agarose gel. The genotypes relate to the upper lanes of the gel. M is a 50-bp DNA ladder molecular size marker (Toyobo, Osaka, Japan).

Table 1. Genotypic and allelic frequency of (GAAT)<sub>n</sub> tetranucleotide microsatellite in canine *TNFA* gene among dog breeds

Breeds (number of animals)	Genotype										Allelic frequency				<i>He</i> <sup>a)</sup>	<i>PIC</i> <sup>b)</sup>	Deviations of HWE <sup>c)</sup>
	5	6	7	8	5/6	5/7	5/8	6/7	6/8	7/8	5	6	7	8			
Beagle (22)	1	0	2	4	5	5	1	1	0	3	0.295	0.136	0.295	0.273	0.749	0.682	<i>P</i> <0.05
Miniature Dachshund <sup>d)</sup> (20)	8	0	2	0	2	7	0	1	0	0	0.625	0.075	0.300	0.000	0.527	0.438	NS <sup>e)</sup>
Chihuahua <sup>d)</sup> (26)	12	2	0	0	9	3	0	0	0	0	0.692	0.250	0.058	0.000	0.464	0.391	NS
Miniature Poodle (24)	6	1	0	0	6	11	0	0	0	0	0.604	0.167	0.229	0.000	0.566	0.493	<i>P</i> <0.05
Pembroke Welsh Corgi (22)	8	1	0	0	5	4	0	4	0	0	0.568	0.250	0.182	0.000	0.595	0.516	NS
Shiba Inu (25)	13	0	0	0	2	10	0	0	0	0	0.760	0.040	0.200	0.000	0.389	0.333	NS

a) Expected heterozygosity, b) Polymorphic information content, c) Deviations of Hardy-Weinberg Equilibrium, d) All the individuals had long-coate. e) Not significant.

Pembroke Welsh Corgis and Shiba Inus. (GAAT)<sub>5</sub> was the most common allele in the 6 dog breeds. An exact test of the Hardy-Weinberg equilibrium using a Markov chain and calculation of the expected *He* were performed using Arlequin ver. 3.1 [4]. The polymorphic information content (*PIC*) value for this microsatellite locus was estimated using a formula described previously [2]. The deviations from the Hardy-Weinberg equilibrium were statistically significant (*P*<0.05) for Beagles and Miniature Poodles. *He* was deficient in Beagles, whereas an excess of *He* was observed in Miniature Poodles. The expected *He* and *PIC* value for this microsatellite locus varied from 0.389 (Shiba Inu) to 0.749 (Beagle) and from 0.333 (Shiba Inu) to 0.682 (Beagles), respectively, in the 6 breeds (Table 1). The allelic frequency differed greatly among breeds, but this microsatellite marker was highly polymorphic and could be a useful marker for the canine *TNFA* gene. Given the important roles of *TNFA* in many inflammatory reactions, the tetranucleotide microsatellite alleles could be associated with susceptibility to inflammatory disorders during the lifetime of the domestic dog due to a direct gene effect or linkage disequilibrium with adjacent nucleotide variations. Further studies are needed to test for possible correlations between this microsatellite variation and canine diseases.

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