FULL PAPER *Ethology*

Sequencing of Canine 5-Hydroxytriptamine Receptor (5-HTR) 1B, 2A, 2C Genes and Identification of Polymorphisms in the 5-HTR1B Gene

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ABSTRACT. Polymorphisms of human genes encoding 5-hydroxytriptamine (serotonin) receptors (5-HTRs) are thought to be associated with psychiatric disorders and behavioral traits. In the present study, we searched for corresponding polymorphisms in the dog and compared allelic frequencies for the canine 5-HTR1B, 5-HTR2A, and 5-HTR2C genes among five canine breeds. The canine genes consisted of the following: 5-HTR1B, 1170 bp; 5-HTR2A, 1413 bp; and 5-HTR2C, 1377 bp. All of these genes were highly homologous with the human genes. We found six single nucleotide polymorphisms (SNPs) in the 5-HTR1B gene (G57A, A157C, G246A, C660G, T955C, and G1146C). Genotyping of the respective SNPs revealed that there were inter-breed variations in the genotypes and allelic frequencies for four out of the six identified SNPs, suggesting that further analyses of the polymorphisms of the 5-HTR1B gene would be useful in order to gain an understanding of the genetic background underlying the diversified behavioral traits among canine species.
KEY WORDS: behavior, breed difference, canine, 5-hydroxytriptamine receptor, polymorphism.

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A number of studies have been recently conducted, particularly in the field of psychiatry, in the attempt to gain a better understanding of the genetic background of the human temperament [25]. However, as social, cultural, and environmental factors exert such profound effects on the development of human brain functions and on the personality in general, it remains extremely difficult to determine the causal relationships between genes and behavior. Companion animals such as dogs, which have a much simpler social system than human beings, but whose rich individuality can nonetheless be described objectively, have been considered as an advantageous object for this type of research that investigates the genetic components of behavioral traits. We therefore undertook an analysis of the genetic background of the canine temperament in order to create a list of polymorphic regions that might serve as possible candidates for influencing the canine temperament; in this context, we have thus far reported the single nucleotide polymorphisms (SNPs) in the cathechol o-methyltransferase gene [17].

Serotonin (5-hydroxytriptamine: 5-HT) is an important monoaminergic neurotransmitter, and it is thought to play a considerable role in psychiatric disorders such as depression, anxiety, and substance abuse; the polymorphisms of certain 5-HT receptor genes are suspected to be involved in these disorders. Among the 5-HT receptors, the 5-HT 1B receptor (5-HTR1B) is assumed to be associated with antisocial alcoholism [14] and antisocial substance dependencies in humans [13]. In rodents, 5-HTR1B knockout mice have been shown to display more reactive and less anxious behaviors than their wild-type counterparts [34], and they have also been shown to exhibit enhanced aggressive behavior [26]. As regards the 5-HT 2A receptor (5-HTR2A), even a silent mutation of T102C has been indicated as exerting influence on 5-HTR2A binding [31], which has been postulated to be associated with neuroticism and alcohol dependence as well as with other psychopathologies [1, 4, 6, 24, 29]. As regards the 5-HT 2C receptor (5-HTR2C), variations in the Cys 23 Ser have been shown to be related to impulsivity [5], as well as to the personality trait of reward dependence [3] and susceptibility to certain mental disorders [7, 8, 10, 23, 28].

In this study, we sequenced the canine 5-HT receptor (5-HTR1B, 5-HTR2A, and 5-HTR2C) genes and searched for polymorphisms in this species. We then performed an analysis of the genetic diversity of identified polymorphisms among five representative canine breeds of which behavioral traits have been reported to be considerably different [9].

MATERIALS AND METHODS

Complementary DNA (cDNA) from reverse-transcripted brain mRNA was used for the PCR amplifications of the 5-HTR genes. The consensus primers from the respective 5-HTR genes of other species registered in GenBank [accession number; 5-HTR1B: D10995 (human) Z11597 (mouse), 5-HTR2A: NM000621 (human) NM172812 (mouse), 5-HTR2C: NM000868 (human) NM008312 (mouse)] were designed for the full length of the 5-HTR2C gene and fragments of 5-HTR1B, 2A gene (Table 1; 1BF~2CR). Amplification was carried out with TaKaRa ExTaq polymerase (TaKaRa, Japan). The amplified products were direct-sequenced by the dye-termination method using an ABI377 DNA sequencer (Perkin-Elmer, U.S.A.). The 5' region of the 5-HTR2A gene was amplified by the RACE method using the SMART RACE cDNA Amplification Kit (Clontech, U.S.A.). For the 5' region of the 5-HTR1B gene amplified by the inverse PCR method, 2 μg of canine

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Table. 1. Primers designed in this study

Primer	Sequence	Position (human)[a)]
1BF	5'-CGG CTA ACT ACC TGA TCG CC-3'	248 to 267
1BR	5'-CAA CTT GGT CCC CAA AGG TC-3'	1203 to 1222: 3' untranslated region
2AF	5'-CCT GAT GTC ACT TGC CAT AGC-3'	336 to 356
2AR	5'-ACT TGC TCA GTG TGC CTT CC-3'	1456 to 1475: 3' untranslated region
2CF	5'-GAG TCC GTT TCT CGT CTA GC-3'	-212 to -193
2CR	5'-GCA TGA TTG TAA AGT ACA-3'	1531 to 1548: 3' untranslated region
Primer	**Sequence**	**Position (canine)[b)]**
1B1F	5'- AGA GAC ATG GAA GCA GCC GG-3'	-6 to 14
1B1R	5'-TCC GAC GAC AGC CAC AAG TC-3'	364 to 383
1B2F	5'-CGG CTA ACT ACC TGA TCG CC-3'	245 to 264
1B2R	5'-TCG CTC AGG CTA CCC CAT TG-3'	1182 to 1201: 3' untranslated region
2A1F	5'-CTG GAC TTC GTG TGC TAC GG-3'	-35 to -16
2A1R	5'- ACT TGC TCA GTG TGC CTT CC-3'	1452 to 1471: 3' untranslated region
2C1F	5'- AAG GAT GGA ATC ATG AAT CC-3'	-79 to -60
2C1R	5'-CTA CAT TTG GAG CTT TAC CG-3'	1397 to 1416: 3' untranslated region

a) The positions of each consensus primer are based on human gene.
b) The positions of each primer are based on canine gene in this study.

genomic DNA was ligated after being sectioned by the restriction enzyme Apa I (Nippongene, Japan) at the 3' non-coding region of the gene.

Polymorphisms were searched for in the coding regions of the amplified genes using the cDNAs of 10 unrelated Beagles. With the succeeding primer sets [5-HTR1B (divided into two regions): 1B1F and 1B1R; 1B2F and 1B2R. 5-HTR2A: 2A1F and 2A1R. 5-HTR2C: 2C1F and 2C1R (Table 1)], the full lengths of each of the coding regions were successfully amplified before initiating the sequencing step as follows.

5-HTR1B: forward part (5' region to nucleotide 383rd:389-bp): The PCR amplification (95°C for 30 sec, 58°C for 30 sec, 72°C for 40 sec) was carried out for 35 cycles in a 50 μl aliquot of reaction mixture with 15 *n*g cDNA, 15 pM forward 1 and reverse 1 primer, and 1 U Ex Taq polymerase.

5-HTR1B: backward part (3' region from nucleotide 245th:957-bp): The PCR amplification (95°C for 30 sec, 56°C for 30 sec, 72°C for 60 sec) was carried out for 35 cycles in a 50 μl aliquot of reaction mixture with 20 *n*g cDNA, 20 pM forward 2 and reverse 2 primer, and 1 U Ex Taq polymerase.

5-HTR2A: The PCR amplification (95°C for 30 sec, 58°C for 30 sec, 72°C for 90 sec) was carried out for 35 cycles in a 50 μl aliquot of reaction mixture with 20*n*g cDNA, 20 pM forward and reverse primer, and 2.5 U Ex Taq polymerase.

5-HTR2C: The PCR amplification (95°C for 60 sec, 59°C for 60 sec, 72°C for 60 sec) was carried out for 35 cycles in a 50 μl aliquot of reaction mixture with 20*n*g cDNA, 30 pM forward and reverse primer, and 2.5 U Ex Taq polymerase.

For the analysis of the genomic distributions of identified polymorphisms, peripheral blood samples were obtained from 189 individuals (46 Golden retrievers, 40 Labrador retrievers, 40 Malteses, 26 Miniature schnauzers, 37 Shiba Inu) via 11 cooperative veterinary hospitals in the Kansai, Kanto, and Chu-bu areas in Japan [17]. Genomic DNA was extracted with the QIAamp Blood Kit (Qiagen, U.S.A.). The polymorphic regions seen in the 5-HTR1B gene were amplified using the same PCR conditions as those mentioned above, since the 5-HTR1B gene consists of only one exon [12]. The successfully amplified fragments were directly sequenced and genotyped.

The χ^2 tests of independence were used for analyses of inter-breed differences in genotypes and allele frequencies. In cases in which an identified SNP was not found in all 5 breeds, the statistics were carried out using the data from breeds with an identical SNP.

RESULTS

The genes amplified in this study (i.e., 5-HTR1B, 5-HTR2A, and 5-HTR2C) contained an 1167-bp, 1413-bp, and 1377-bp open reading frame, respectively (Figs. 1-a~c). The respective homologies at the nucleotide level with human, mouse, and rat genes were 91%, 89%, and 89% (5-HTR1B); 88%, 86%, 86% (5-HTR2A); and 90%, 88%, 87% (5-HTR2C) and those at predicted amino acid level were 91%, 90%, 89% (5-HTR1B); 89%, 85%, 85% (5-HTR2A); and 83%, 78%, 77% (5-HTR2C). There were six SNPs in the coding region of the 5-HTR1B gene and no polymorphism was found in the two other genes. These six SNPs were as follows: a guanine-to-adenine substitution at the 57th nucleotide (G57A), an adenine-to-cytosine substitution at the 157th nucleotide (A157C), a guanine-to-adenine substitution at the 246th nucleotide (G246A), a cytosine-to-guanine substitution at the 660th nucleotide (C660G), a thymine-to-cytosine substitution at the 955th nucleotide (T955C), and a guanine-to-cytosine substitution at the 1146th nucleotide (G1146C) (Fig. 1-a). In particular, A157C was suspected of causing an amino acid substitution of Isoleucine for Leucine.

Table 2 shows the genotypes and allele frequencies of the 5-HTR1B polymorphic regions in five dog breeds. The

1a 5-HTR1B gene (upper: human, lower: canine)

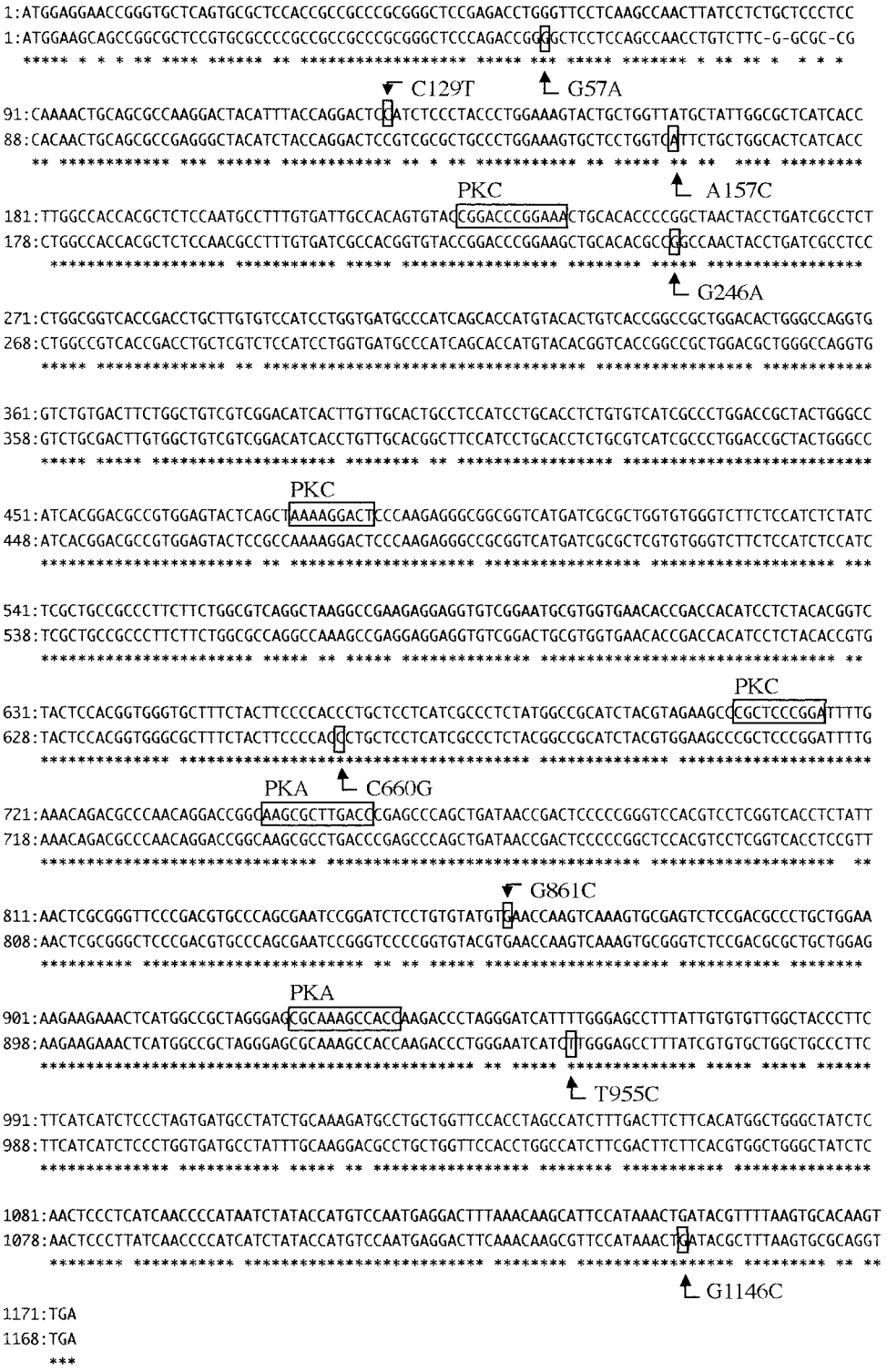
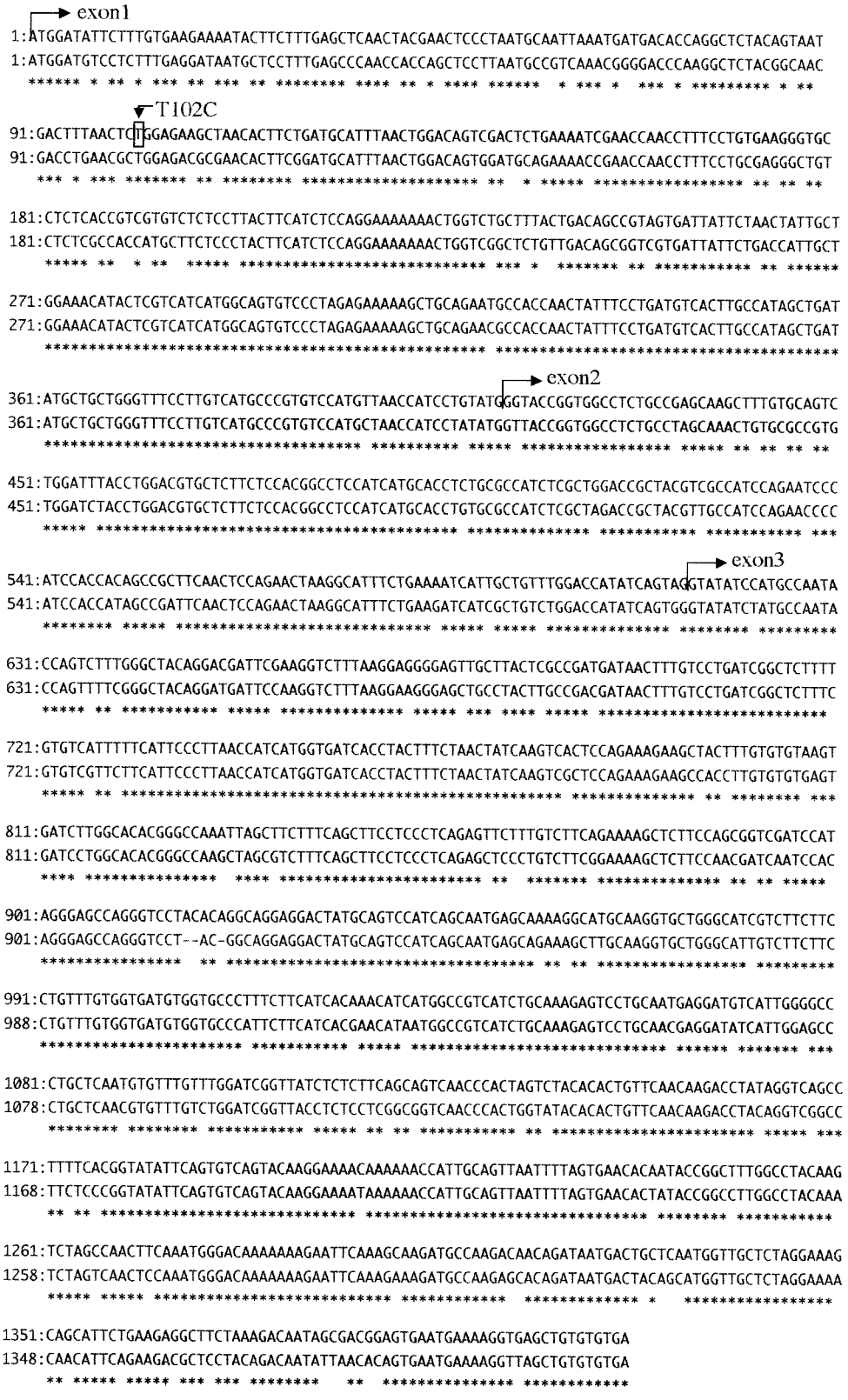


Fig. 1. The canine and human 5-HT receptor genes. (a) Multiple alignment of the human 5-HTR1B gene and the canine 5-HTR1B gene identified in this study. The PKA and PKC binding sites of the human 5-HTR1B gene are shown in boxes. The identified SNPs and SNPs reported in human are shown in boxes with arrows. (b) Multiple alignment of the human 5-HTR2A gene and the canine 5-HTR2A gene. The beginning of each exon in the human gene is indicated by an arrow. The SNP reported in human is shown in box with arrow. (c) Multiple alignment of the human 5-HTR2C gene and the canine 5-HTR2C gene. The beginning of each exon in the human gene is indicated by an arrow. The SNP reported in human is shown in box with arrow.

1b 5-HTR2A gene (upper: human, lower: canine)



1c 5-HTR2C gene (upper: human, lower: canine)

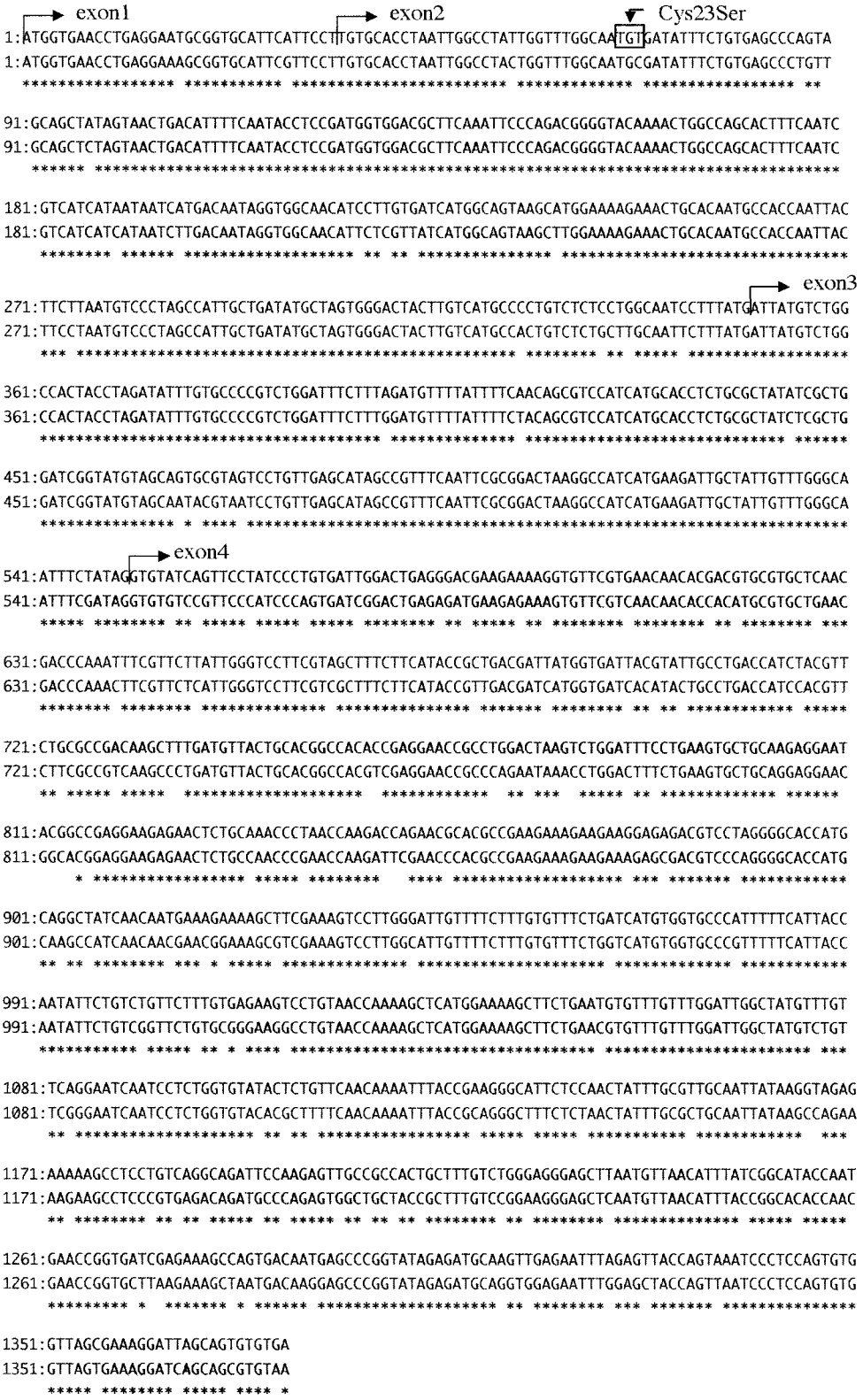


Table. 2. Genotypes, allele frequencies and heterozygosities of six polymorphic regions in five dog breeds

Breed	N	Genotype			Allele		Heterozygosity
G57A		G / G	G / A	A / A	G	A	
Total	189	185 (97.8)	2 (1.1)	2 (1.1)	372 (98.4)	6 (1.6)	0.0313
Golden retriever	46	42 (91.3)	2 (4.3)	2 (4.3)	86 (93.5)	6 (6.5)	0.1233
Labrador retriever	40	40 (100.0)	0 (0.0)	0 (0.0)	80 (100.0)	0 (0.0)	0.0000
Maltese	40	40 (100.0)	0 (0.0)	0 (0.0)	80 (100.0)	0 (0.0)	0.0000
Miniature schnauzer	26	26 (100.0)	0 (0.0)	0 (0.0)	52 (100.0)	0 (0.0)	0.0000
Shiba Inu	37	37 (100.0)	0 (0.0)	0 (0.0)	74 (100.0)	0 (0.0)	0.0000
A157C		A / A	A / C	C / C	A	C	
Total	189	180 (95.2)	4 (2.1)	5 (2.7)	364 (96.3)	14 (3.7)	0.0715
Golden retriever	46	37 (80.4)	4 (8.7)	5 (10.9)	78 (84.8)	14 (15.2)	0.2609
Labrador retriever	40	40 (100.0)	0 (0.0)	0 (0.0)	80 (100.0)	0 (0.0)	0.0000
Maltese	40	40 (100.0)	0 (0.0)	0 (0.0)	80 (100.0)	0 (0.0)	0.0000
Miniature schnauzer	26	26 (100.0)	0 (0.0)	0 (0.0)	52 (100.0)	0 (0.0)	0.0000
Shiba Inu	37	37 (100.0)	0 (0.0)	0 (0.0)	74 (100.0)	0 (0.0)	0.0000
G246		G / G	G / A	A / A	G	A	
Total	189	148 (78.3)	33 (17.5)	8 (4.2)	329 (87.0)	49 (13.0)	0.2263
Golden retriever	46	15 (32.6)	24 (52.2)	7 (15.2)	54 (58.7)	38 (41.3)	0.4902
Labrador retriever	40	38 (95.0)	1 (2.5)	1 (2.5)	77 (96.3)	3 (3.7)	0.0731
Maltese	40	40 (100.0)	0 (0.0)	0 (0.0)	80 (100.0)	0 (0.0)	0.0000
Miniature schnauzer	26	26 (100.0)	0 (0.0)	0 (0.0)	52 (100.0)	0 (0.0)	0.0000
Shiba Inu	37	29 (78.4)	8 (21.6)	0 (0.0)	66 (89.2)	8 (10.8)	0.1955
C660G		C / C	C / G	G / G	C	G	
Total	189	166 (87.8)	17 (9.0)	6 (3.2)	349 (92.3)	29 (7.7)	0.1420
Golden retriever	46	35 (76.1)	9 (19.6)	2 (4.3)	79 (85.9)	13 (14.1)	0.2453
Labrador retriever	40	29 (72.5)	7 (17.5)	4 (10.0)	65 (81.3)	15 (18.7)	0.3085
Maltese	40	40 (100)	0 (0.0)	0 (0.0)	80 (100.0)	0 (0.0)	0.0000
Miniature schnauzer	26	26 (100)	0 (0.0)	0 (0.0)	52 (100.0)	0 (0.0)	0.0000
Shiba Inu	37	36 (97.3)	1 (2.7)	0 (0.0)	73 (98.6)	1 (1.4)	0.0270
T955C		T / T	T / C	C / C	T	C	
Total	189	96 (50.8)	54 (28.6)	39 (20.6)	246 (65.1)	132 (34.9)	0.4557
Golden retriever	46	29 (63.0)	14 (30.5)	3 (6.5)	72 (78.3)	20 (21.7)	0.3440
Labrador retriever	40	17 (42.5)	17 (42.5)	6 (15.0)	51 (63.8)	29 (36.2)	0.4680
Maltese	40	36 (90.0)	3 (7.5)	1 (2.5)	75 (93.8)	5 (6.2)	0.1186
Miniature schnauzer	26	1 (3.8)	4 (15.4)	21 (80.8)	6 (11.5)	46 (88.5)	0.2081
Shiba Inu	37	13 (35.1)	16 (43.3)	8 (21.6)	42 (56.8)	32 (43.2)	0.4975
G1146C		G / G	G / C	C / C	G	C	
Total	189	122 (64.6)	38 (20.1)	29 (15.3)	282 (74.6)	96 (25.4)	0.3799
Golden retriever	46	33 (71.7)	10 (21.8)	3 (6.5)	76 (82.6)	16 (17.4)	0.2904
Labrador retriever	40	30 (75.0)	7 (17.5)	3 (7.5)	67 (83.8)	13 (16.2)	0.2756
Maltese	40	38 (95.0)	2 (5.0)	0 (0.0)	78 (97.5)	2 (2.5)	0.0493
Miniature schnauzer	26	3 (11.5)	4 (15.4)	19 (73.1)	10 (19.2)	42 (80.8)	0.3167
Shiba Inu	37	18 (48.6)	15 (40.6)	4 (10.8)	51 (68.9)	23 (31.1)	0.4342

The percentage in each category is shown in parenthesis.

inter-breed differences as regards the actual number of genotypes and alleles was highly significant in four SNPs, as based on the χ^2 test (G246A; genotype: χ^2=42.556, df=4, p<0.0001 and allele: χ^2=43.338, df=2, p<0.0001. C660G; genotype: χ^2=10.481, df=4, p=0.0331 and allele: χ^2 =11.965, df=2, p=0.0025. T955C; genotype: χ^2=97.443, df=8, p<0.0001 and allele: χ^2=103.879, df=4, p<0.0001. G1146C; genotype: χ^2=99.317, df=8, p<0.0001 and allele: χ^2=114.193, df=4, p<0.0001). The G57A and A157C polymorphisms were seen only in the Golden retrievers. The G246A and C660G polymorphisms were not observed in either the Malteses or the Miniature schnauzers. As regards the T955C and G1146C polymorphisms, the C allele was dominant in the Miniature schnauzers, whereas other alleles were dominant in the other four breeds (Table 2).

DISCUSSION

Since the amplified genes in this study were found to be highly homologous to the 5-HTR genes reported in other species such as humans, mice and rats, the present findings were considered as representative of actual 5-HT receptor genes in the dog. We identified six SNPs in the coding region of the 5HTR1B gene, although no polymorphism was found in either the 5-HTR2A or the 5-HTR2C gene. If a point mutation had occurred by accident and was reserved,

then the disequilibrium of SNPs observed among the 5-HT receptor genes seems to be rather strange at this moment. Further study is therefore needed to clarify whether this disequilibrium was simply the result of an accidental factor dependent on evolutional distance, or if it was the result of a significant factor such as the particular intron-lacking structure of the 5-HT1B gene. Moreover, the number of tandem repeats (VNTR) and SNPs varied; these structures are suspected to be associated with certain personality traits as well as with disorders in the 5'-regulatory region of human 5-HTR2C and 5-HTR2A [7, 24] that have been associated with behavioral abnormalities. It will therefore be necessary in a future study to extend the present search into the upstream regulatory region of these genes.

Most SNPs identified in this study, with the exception of A157C, do not cause amino acid substitution. However, some lines of evidence have suggested that even silent mutations may exert some influence on personality and certain mental disorders [1, 2, 6, 16, 19, 29]. These previous reports presumed that silent mutations might induce genetic linkage to functional variations in a coding or regulatory region, or possibly even in a neighboring gene, although the silent mutations themselves might not be the cause of functional variations. Only A157C was suspected of causing an amino acid substitution of Isoleucine for Leucine in this study. This SNP may lead to functional changes due to a direct structural change in the amino acid product.

In humans, the 5-HT1B receptor has seven transmembrane-spanning regions and four intracellular loops with three PKC and two PKA sites [18]. The G861C polymorphism, which is located between two PKA sites in the longest intracellular third loop, is thought to be related to certain mental disorders, i.e., depression [11] and aggression [14]. The following SNPs in dogs were considered as corresponding to those in the human 5-HTR1B gene: G57A and A157C were located in the first extracellular region, G246A was located in the first intracellular loop, C660G was located in the fifth transmembrane-spanning region, T955C was located in the sixth transmembrane-spanning region, and G1146C was located in the fourth intracellular loop, which also had a palmitoylation site [18]. Although the sites of canine SNPs do not completely coincide with those of the human SNPs, it is likely that the canine SNPs exert similar effects to those observed in humans.

With respect to the genetic diversity caused by polymorphisms in five breeds, it was found that G57A and A157C were present only in the Golden retrievers. The G246A and C660G polymorphisms were not seen in the Malteses or Miniature schnauzers, whereas T955C and G1146C were observed in all of the breeds. It was of note that the T955C and G1146C polymorphisms of Miniature schnauzers showed inverse distributions of genotypes and alleles compared to those of the other four breeds (Table 2). These results let us hypothesize the linkage among these SNPs of this gene; however, complete coincidence in this regard was not observed in the present study. These breed differences were also seen in other canine polymorphic regions [20, 21]. The genetic drift and founder effect (in the process of domestication and selection) may account for this phenomenon [15, 22, 27, 30, 32, 33].

In the present study, we chose to study dogs instead of laboratory mice or rats, although the living environment of the latter can be more easily controlled. It should also be noted in this context that it remains difficult to carry out a complex personality analysis based on behavioral parameters, as there appears to be few individual differences among rodents in terms of personality traits. Dogs appear to have a more simple social system than humans, and yet they seem to express more individuality than rodents; these differences between species rendered dogs a more useful object than rodents for this type of inquiry.

In conclusion, the present study revealed the sequence of three canine 5-HTR genes (i.e., 5-HTR1B, 5-HTR2A, and 5-HTR2C) as well as that of six SNPs in the 5-HTR1B gene; four of these six SNPs were revealed as having inter-breed variations, both in terms of genotype as well as in terms of allelic frequency. Further studies will be needed to clarify the mechanisms by which these SNPs affect the function of the 5-HTR1B gene. Breed-specific as well as individual behavioral traits should also be investigated in more detail in order to gain a better understanding of the genetic background of the canine temperament.

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