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Structure and Variation of Three Canine Genes Involved in Serotonin Binding and Transport: The Serotonin Receptor 1A Gene (*htr1A*), Serotonin Receptor 2A Gene (*htr2A*), and Serotonin Transporter Gene (*slc6A4*)

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Abstract

Aggressive behavior is the most frequently encountered behavioral problem in dogs. Abnormalities in brain serotonin metabolism have been described in aggressive dogs. We studied canine serotonergic genes to investigate genetic factors underlying canine aggression. Here, we describe the characterization of three genes of the canine serotonergic system: the serotonin receptor 1A and 2A gene (*htr1A* and *htr2A*) and the serotonin transporter gene (*slc6A4*). We isolated canine bacterial artificial chromosome clones containing these genes and designed oligonucleotides for genomic sequencing of coding regions and intron-exon boundaries. Golden retrievers were analyzed for DNA sequence variations. We found two nonsynonymous single nucleotide polymorphisms (SNPs) in the coding sequence of *htr1A*; one SNP close to a splice site in *htr2A*; and two SNPs in *slc6A4*, one in the coding sequence and one close to a splice site. In addition, we identified a polymorphic microsatellite marker for each gene. *Htr1A* is a strong candidate for involvement in the domestication of the dog. We genotyped the *htr1A* SNPs in 41 dogs of seven breeds with diverse behavioral characteristics. At least three SNP haplotypes were found. Our results do not support involvement of the gene in domestication.

Aggressive behavior is by far the most frequently encountered behavioral problem in dogs, resulting in bite injuries reaching epidemic proportions (Beaver 1994; Lockwood 1995; Mikkelsen and Lund 2000; Wright and Nesselrote 1987). Aggression is influenced by both genetic and environmental factors. The nature, relative importance, and interaction of these factors are still poorly understood. To approach these questions, we have embarked on a study of the genetic factors underlying aggressive behavior in dogs.

Abnormalities in human serotonin (5-HT) metabolism have been found in a variety of mental disorders, including pathological aggression and anxiety (Gingrich and Hen 2001). There is evidence for a modulatory role of the serotonergic system in behavioral traits in dogs as well. For instance, Reisner et al. (1996) reported decreased concentrations of 5-hydroxyindoleacetic acid (the major metabolite of 5-HT) in cerebrospinal fluid of dominant aggressive dogs. Badino et al. (2004) found modifications of serotonergic receptor concentrations in the brains of aggressive dogs.

We aim to study the association of genes of the canine serotonergic system with aggression and fear in golden retriever dogs (van den Berg et al. 2003a). The first step in these candidate gene studies is the analysis of the gene structure and variation. We have described the isolation and characterization of the canine serotonin receptor 1A and 1B genes (*htr1A* and *htr1B*; van den Berg et al. 2003b, 2004). Here,

we describe the characterization of two additional genes of the canine serotonergic system: the genes encoding the serotonin receptor 2A (*htr2A*) and the serotonin transporter (solute carrier family member 6A4, *slc6A4*). Moreover, we have studied the canine *htr1A* in more detail.

The serotonin receptors 1A and 2A are G-protein-coupled receptors with seven transmembrane domains. Several studies have suggested that the 1A receptor is associated with anxiety, depression, aggression, and stress response. For instance, *htr1A* knockout mice have shown increased anxiety in a number of experimental paradigms (Heisler et al. 1998; Parks et al. 1998; Ramboz et al. 1998). The association of polymorphisms in human *HTR2A* with neuropsychiatric disorders has been studied frequently. A silent mutation in human *HTR2A* (*T102C*) has been shown to be associated with altered 5-HT binding, which has been implicated in schizophrenia, suicidal behavior, impaired impulse control, and aggression history (Abdolmaleky et al. 2004; Bjork et al. 2002; Khait et al. 2005). In addition, Peremans et al. (2003) measured a higher density of serotonin 2A receptors in cortical brain regions of impulsive aggressive dogs.

The serotonin transporter encoded by *slc6A4* belongs to the family of sodium- and chloride-dependent transporters, and it contains 12 transmembrane domains. The serotonin transporter is localized on the presynaptic membrane of serotonergic neurons and is responsible for the reuptake of 5-HT from brain synapses. The protein is a target for antidepressants and psycho stimulants (Barker and Blakely 1996; Feldman et al. 1997). A polymorphism in its promoter region influences serotonin transporter density in the brain and is associated with mental disorders in humans (Anguelova et al. 2003; Hariri et al. 2002; Katsuragi et al. 1999; Lesch et al. 1996, 1999). *Slc6A4* knockout mice show reduced aggression and reduced home-cage activity (Holmes et al. 2003).

The structure of these genes has been elucidated in humans and several other organisms. Human *HTR1A* is an intronless gene that maps to HSA5q11.2–q13 (Kobilka et al. 1987). *HTR2A* consists of three exons in humans and is located on HSA13q14–q21 (Chen et al. 1992; Hsieh et al. 1990; Saltzman et al. 1991; Sparkes et al. 1991; Stam et al. 1992). Human *SLC6A4* maps to HSA17q11.1–q12 and consists of 15 exons (Gelernter et al. 1995; Ramamoorthy et al. 1993). Exon 1 and 2 are noncoding (Lesch et al. 1994). Canine *htr1A* and *htr2A* have been cloned and sequenced previously (Masuda et al. 2004; van den Berg et al. 2003b).

We studied canine *htr1A* in more detail because the single nucleotide polymorphisms (SNPs) in this gene are nonsynonymous. *Htr1A* is a strong candidate for involvement in the process of domestication of wolves because indications suggest that it plays a role in fearful behavior. It could be hypothesized that specific variants of genes that modulate anxiety were selected for in the ancestors of the present-day domestic dogs. In that case, we would expect to find little variation in this region of the canine genome. We have therefore analyzed the presence of *htr1A* SNP haplotypes in dogs from seven breeds with diverse behavioral characteristics.

Materials and Methods

Dogs and DNA Isolation

All dogs included in this study were privately owned. The golden retrievers participated in our research project involving canine fear and aggression. The owners of these dogs considered them to be neither anxious nor aggressive. Beagles, boxers, Cairn terriers, Dobermans, Norwegian elkhounds, and Shetland sheepdogs were recruited from our clinical DNA bank. These dogs were suffering from a somatic disease; their behavior characteristics were not recorded. The breeds were selected because they have been reported to have diverse behavioral characteristics (Hart and Hart 1988). We selected dogs that had no known common grandparents. Dog and mouse (DBA/2) genomic DNA was isolated from whole blood lymphocytes using the salt extraction method of Miller et al. (1988).

Isolation of Canine Bacterial Artificial Chromosome Clones Containing *htr2A* or *slc6A4*

We designed two oligonucleotides (5′-TGC CAA TCC CAG TCT TCG GG -3′ and 5′-CAT GGA GCA GTC ATT AGC TGT CGG C-3′) based on exon 3 of murine *htr2A* (GenBank accession number: NM_172812). With these oligonucleotides, a 713 bp labeled probe (*htr2A*-713) was produced as described by van den Berg et al. (2004). For *slc6A4*, we used OVERGO MAKER (http://genome.wustl.edu/) to design pairs of overlapping oligonucleotides in exon 3 (5′-CTG AGC TTC ATC AAG GGG AAC GGG-3′ and 5′-TTC TTG CCC CAG GTC TCC CGT TCC-3′) and exon 13 (5′-AGG ATC TGC TGG GTG GCC ATC AGC-3′ and 5′-ACA GGA GAA ACA GAG GGC TGA TGG-3′) based on the human *SLC6A4* sequence NM_001045. The two 40 bp overgo probes (*slc6A4*-40.3 and *slc6A4*-40.13) were synthesized and labeled as described by Stabej et al. (2004). Screening of the canine genomic bacterial artificial chromosome (BAC) library RPCI-81 with these probes and BAC DNA isolation were performed as described previously (Li et al. 1999; van den Berg et al. 2004). To confirm the presence of the genes in the BAC clones, BAC DNA was subcloned and sequenced as described by van den Berg et al. (2004; we have described the isolation of BAC clone 160O12 for canine *htr1A*; see van den Berg et al. 2003b).

In Silico Characterization of Gene Sequences

We performed a BLAST (basic local alignment search tool) search for *htr1A*, *htr2A*, and *slc6A4* sequences in dog-specific databases at the NCBI website (National Center for Biotechnology Information, http://www.ncbi.nih.gov/genome/seq/CfaBlast.html) using DNA sequences with accession numbers AY134445 (Dobermann *htr1A)*, NM_001005869 (beagle *htr2A*), and NM_001045 (human *SLC6A4*) as queries. In addition, we blasted individual exons of the boxer *slc6A4* sequence (ensembl gene ID ENSCAFG00000018990; http://www.ensembl.org/) against these databases. BLASTn was used to detect similarities between flanking regions of

the genes in dogs and other mammals. We searched for CpG islands in the 5′ flanking regions with the WEBGENE CpG islands prediction tool (http://www.itba.mi.cnr.it/webgene/).

DNA sequences were imported into SeqMan (DNA Star Software) and assembled. Regions with overlapping reads were inspected to find SNPs. Functional effects of polymorphisms were predicted with POLYPHEN (http://genetics.bwh.harvard.edu/cgi-bin/pph/polyphen.cgi). Furthermore, we searched for simple sequence repeats in flanking regions and mate pairs of traces (DNA sequence chromatograms) containing coding regions of the genes.

Similarities between *htr2A* or *slc6A4* and their orthologues in humans, mice, rats, pigs, cows, and chickens were calculated with MegAlign (DNA Star Software, clustal W method). These protein sequences have the following GenBank accession numbers: NP_000612 (human *HTR2A*), NP_766400 (murine *htr2A*), NP_058950 (rat *htr2A*), NP_999382 (porcine *htr2A*), NP_001036 (human *SLC6A4*), NP_034614 (murine *slc6A4*), CAA71909 (rat *slc6A4*), NP_777034 (bovine *slc6A4*), and NP_998737 (chicken *slc6A4*). Predicted positions of the transmembrane regions and key amino acid residues in the protein were derived from the SWISSPROT website (http://us.expasy.org/sprot/; Swissprot accession number P28223 and P31645) and from several publications referred to in the relevant sections (we have described the homologies between canine *htr1A* and its human and murine orthologue; see van den Berg et al. 2003b).

DNA Sequence Analysis in Golden Retrievers

We developed oligonucleotides to amplify the coding nucleotide sequences of the genes with polymerase chain reactions (PCRs; see Table 1). The coding sequence of *htr1A*, with the exception of the first 14 nucleotides, was amplified by four overlapping PCRs with primers 1–8. Twenty-five μl PCR reactions contained 0.7 mM Tris-HCl, 0.67 mM $MgCl_2$, 1.0 mM mercapto-ethanol, 0.67 mM EDTA (pH = 8.0), 1.66 mM $(NH_4)_2SO_4$, 0.5 U AmpliTaq DNA polymerase, 100 ng of both primers, 0.15 mg/ml BSA, 2.5 μl DMSO, 1.5 mM dNTPs, and 800 ng genomic DNA. The thermocycler profile was as follows: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at T_A, and 1 min at 72°C, concluded with a final extension step of 10 min at 72°C. SNP genotyping in the dogs of seven breeds was performed by DNA sequencing of PCR products that were generated with primer pairs 1–2 and 5–6.

We used 7 M13-tailed oligonucleotide pairs to amplify the three exons of canine *htr2A* (primers 9–22 in Table 1) and 16 M13-tailed oligonucleotide pairs to amplify the coding exons of canine *slc6A4* (primers 23–54 in Table 1). Parts of the flanking regions were also amplified. The PCR reactions contained 12.5 picomoles of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U DNA polymerase, and 25 ng genomic DNA in a 25 μl reaction with Gibco-BRL buffer. The thermocycler profile was as follows: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at T_A, and 30 s at 72°C, concluded with a final extension step of 4 min at 72°C.

PCR products were purified with a QIAquick PCR purification kit, and the DNA sequence was analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) as described by van den Berg et al. (2004). *Htr1A* PCR products were sequenced with 3.2 picomoles of one of the primers (1–8). All *htr2A* and *slc6A4* PCR products contained M13 tails and were sequenced with 3.2 picomoles of HPLC-purified M13 forward (5′-GTT TTC CCA GTC ACG AC-3′) or reverse (5′-CAG GAA ACA GCT ATG AC-3′) primer (Eurogentec). The chromatograms were inspected by eye to detect heterozygotes.

Microsatellite Marker Genotyping

We analyzed one polymorphic CA dinucleotide repeat for each gene with oligonucleotides 55–56 (*htr1A*), 57–58 (*htr2A*), and 59–60 (*slc6A4*). The 5′ end of the forward primer was labeled with 6-FAM fluorescent dye (Eurogentec). PCRs and automated analysis of the PCR products were performed as described by van den Berg et al. (2004). We used either 500-LIZ or TAMRA-GS500 as size standard (Applied Biosystems). GENESCAN 3.7 software was used for genotype assessment. The oligonucleotides for the *htr1A* microsatellite marker were also tested on BAC160O12 DNA because this canine BAC clone contains *htr1A*. This PCR product was sequenced to confirm the identity of the fragment.

Results

Isolation of Canine BAC Clones Containing *htr2A* or *slc6A4*

BAC library screening with probe *htr2A*-713 resulted in two positive BAC clones: 351G1 and 422M22. A 0.63 kb DNA sequence read from one of the 351G1 subclones displayed 98% homology to position 890 to *106 of the canine gene (*106 means 106 bp 3′ of the stop codon). Comparison of this DNA sequence with the dog genome assembly resulted in only one hit: a chromosome 22 genomic contig (accession number NW_139892). Further analysis showed that this contig contains the complete canine *htr2A*. This result confirms the presence of exon 3 of *htr2A* in BAC clone 351G1 and implies only one copy of the 0.63 kb fragment in the canine genome.

BAC library screening with the overgo probes *slc6A4*-40.3 and *slc6A4*-40.13 resulted in 13 positive BAC clones: 16P8, 45A9, 59N4, 76G6, 85K12, 202E23, 273H2, 281C3, 287M9, 307D21, 342D19, 356B20, and 326I14. The inserts of clones 76G6 and 287M9 were subcloned and randomly sequenced. One of the 76G6 subclones contained a 0.8 kb DNA sequence containing a CA repeat. This repeat is located at position IVS4-216 in intron 4 of canine *slc6A4*, and we named the marker UU76G6. Comparison of the 0.8 kb DNA sequence containing the marker against the dog genome database retrieved a single hit with high similarity on CFA09 (accession number NW_139866). Further analysis showed that this contig contains the complete canine *slc6A4*, as shown in the section concerning the in silico characterization of *slc6A4*. UU76G6 was demonstrated by PCR to be present

Table 1. Primer pairs used in this study

Primer numbers	Sequence (5′ – 3′)[a]	Position[b]	Length of product (bp)[c]	T_A (°C)[d]	DNA polymerase used[e]
1	GCA GGC ATG GAG GGG CTC AG	−6 to 14			
2	CTT GTT CAC GTA GTC GAT GG	422 to 441	447	60	Ampli
3	TGT GCT GCA CCT CGT CCA TC	353 to 372			
4	TGC ACT TCA ATC ACC TCC AG	868 to 887	535	60	Ampli
5	ACA GTC AAG AAG GCG GAG AG	685 to 704			
6	GCC AGC AGA GGA TGA ACG TG	1056 to 1075	391	60	Ampli
7	GAT TGA AGT GCA CCG CGT GG	876 to 895			
8	ACC GGG CGG GCC TTC TCG TC	*14 to *33	430	60	Ampli
9	CCA ACA AGA CTC CAC TAA CG	−219 to −200			
10	GCT GCT GCC ACT CAC CAT A	409 to IVS1+15	680	57	*Taq*
11	CCT GAT GTC ACT TGC CAT AG	336 to 355			
12	CAC TCT CCG GTG ATA ATA GG	IVS1+181 to +200	311	57	*Taq*
13	CCT CAC TGA CGC TAA CCT TC	IVS1-30 to −11			
14	TAC CTT GCG GCA ATG ACC AC	IVS2+115 to +134	399	57	*Taq*
15	ACC GTT GTG TAG CAG TGC TC	IVS2-71 to −52			
16	CAT AGT CCT CCT GCC GTA G	915 to 933	425	57	*Taq*
17	CCA ACG ATC AAT CCA CAG G	885 to 903			
18	GTA GGA GCG TCT TCT GAA TG	1351 to 1370	520	57	*Taq*
19	AAC ACT ATA CCG GCC TTG G	1231 to 1249			
20	GCT ATG GCA ACT GGT CTA TC	1412 to *18	235	57	*Taq*
21	AGA AGA CGC TCC TAC AGA CA	1356 to 1375			
22	AAG ACC ACG CTG GAG ATT G	*744 to *762	854	57	*Taq*
23	TGC GTA ACT CTG TTC TCC	−154 to −137			
24	GCC AGA CTC CAC CTT ATC	106 to 123	311	57	*Taq*
25	GGA GCT ATC AGC ATG TAA GG	30 to 49			
26	AGA CAT GAT CAC TGC TCT GG	IVS3+61 to +80	428	60	*Taq*
27	GTG AGG TCA TTC AAC ACA GG	IVS3-147 to −128			
28	CTG ATT CCA GAA GAA GGT CC	IVS4+78 to +97	413	58	Plat
29	TTA CCA CAT TGC CAC CTG	IVS4-146 to −129			
30	TTC CTC GGA AGC CAA GTC	IVS5+44 to +61	461	62	Plat
31	AGG AGT TCC TAA GGC TGG TC	IVS5-130 to −111			
32	TCT GTG GCT GTC CAG GAT AC	IVS6+69 to +88	391	57	*Taq*
33	CCT GCC TCC TAT AGT TAC	IVS6-123 to −106			
34	GAC AGA CAG GTG CAC ATC	IVS7+20 to +37	329	57	*Taq*
35	TTG CAC TTG GTA TGT GGC TG	IVS7-127 to −108			
36	TCA ATC TCT GAA TGG CCT GG	IVS8+172 to +191	456	62	Plat
37	CAG TTC ACA ACA GGA CCA TC	IVS8-242 to −223			
38	AGC AAC TCA GTG AGA GCA AG	IVS9+28 to +47	451	57	*Taq*
39	TCA TTG TTG GTG TGG CTG AG	IVS9-90 to −71			
40	TCA AGA GCA CCA CAG TGA GG	IVS10+152 to +171	408	57	*Taq*
41	CTA CTC ATG ACC AGC AAC	IVS10-98 to −81			
42	CCA GAT ACT CTG TCA AGC	IVS11+252 to +269	533	57	*Taq*
43	AGT GCT CCA TAG GAC AGG	IVS11-202 to −185			
44	TTG TGG TAG AGC GTG AAG	IVS12+176 to +193	529	60	Plat
45	CGT CTC AAC TTC AGA GCA G	IVS12-141 to −123			
46	GAT GTG ACA CAT GCA GCA G	IVS13+293 to +311	587	57	*Taq*
47	TCA GAA CTG TCT GCC AGG	IVS13-61 to −44			
48	CCA CTG CAT CTA AGG CTC	IVS14+176 to +193	456	56	Plat
49	GTC ACA TTG TCC AAC TGA GC	IVS14-224 to −205			
50	GTC ATT GGA GGC CAT AAG AG	*163 to *182	515	57	*Taq*
51	AGT CAT GCC TCA CCT TCA CC	*56 to *75			
52	TCC TGA CTC CAC AGC AGC AC	*514 to *533	512	60	Plat
53	ATG TGT GAG GCT GTG TAT GG	*254 to *273			
54	GGC AGA GCA TGT TGT AGT AG	*704 to *723	504	60	Plat
55	CCT CTA TCT CAG CAC TTG				
56	GCT AAC ACC AGA GGA ACC	Downstream of *htr1A*	±297	53	Gold
57	ACT GTT GAC TGA CCG CCT AC	IVS2+1397 to +1416			
58	GCT TCA TTC TCT CGC TCC TAC	IVS2+1510 to +1530	±134	57	*Taq*
59	TGT GGT GAC CGA TGA CAG	IVS4-392 to −375			
60	CAG GTG GCA ATG TGG TAA	IVS4-146 to −129	±264	52	*Taq*

[a] Primers 9–54 had M13 sequence primer tails (not shown in the table).

[b] We used the nomenclature recommended by den Dunnen and Antonarakis (2001): the A of the ATG start codon is designated number 1, the nucleotide 5′ to this A is numbered −1, and the nucleotide 3′ of the translation termination codon is *1. Positions in introns refer to the nearest exon. The nomenclature of the introns is based on the human gene structure.

[c] Product length includes M13 tail for primers 9–54.

[d] T_A = annealing temperature.

[e] Ampli = AmpliTaq DNA polymerase (Applied Biosystems); *Taq* = *Taq* DNA polymerase (Invitrogen); Plat = Platinum *Taq* DNA polymerase (Invitrogen); Gold = AmpliTaq Gold DNA polymerase (Applied Biosystems).

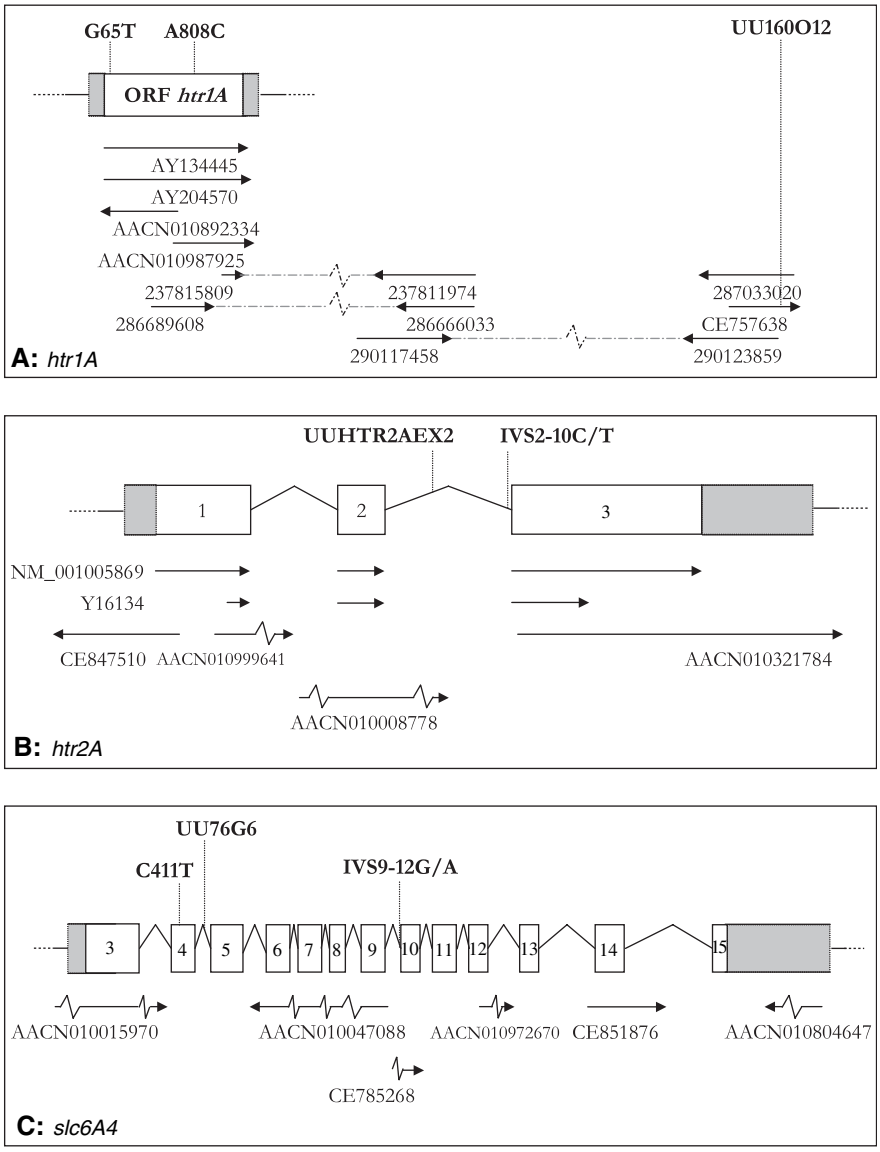


Figure 1. Position of retrieved canine DNA sequences relative to each other and to the exons of three serotonergic genes. The directions of the arrows represent the orientation of the sequences. Boxes mark the position of the exons. White areas represent coding sequence; gray areas represent flanking untranslated regions. The size of the untranslated regions and the numbers of the exons are based on the structure of the human genes, which does not necessarily correspond to the structure of the canine orthologues of the genes. Dotted vertical lines indicate the position of the polymorphic microsatellite markers and SNPs that were analyzed in golden retrievers in this study. Introns and flanking regions are not drawn to scale. (**A**) *Htr1A* sequences: AY134445 is a Doberman *htr1A* sequence; AY204570 is derived from a collie; AACN010892334 and AACN010987925 are derived from a poodle; and the sequences with numbers are traces retrieved from the boxer genome project. Trace 237815809 and 237811974 form a mate pair containing sequences from the ends of a genomic DNA clone. The same applies to trace 286689608–286666033 and 290117458–290123859. Trace 290123859 and 287033020 contain a CA repeat, which is also present in the poodle sequence CE757638. Thirteen additional traces that overlap with the *htr1A* open reading frame (ORF) are omitted in this figure. The 45.4 Mb boxer chromosome 2 genomic contig NW_139841 spans the entire region and is not shown here. (**B**) *Htr2A* sequences: NM_001005869 is a cDNA sequence from a beagle; Y16134 is a partial cDNA sequence of a dog of unspecified breed; CE847510, AACN010999641, AACN010008778, and AACN010321784 are poodle DNA sequences. Thirty-two boxer traces that overlap with the *htr2A* coding sequence are not shown. The 55.6 Mb boxer chromosome 22 contig NW_139892 spans the entire region (not shown). A CpG-enriched island is located about the start codon at position −1019 to 307 (not shown). (**C**) *Slc6A4* sequences: AACN010015970, AACN010047088, CE785268, AACN010972670, CE851876, and AACN010804647 are poodle DNA sequences. More than 100 boxer traces that overlap with the *slc6A4* coding sequence are not depicted. The 37.3 Mb boxer contig NW_139866 spans the entire region (not shown). The noncoding exons 1 and 2 are not shown in this figure.

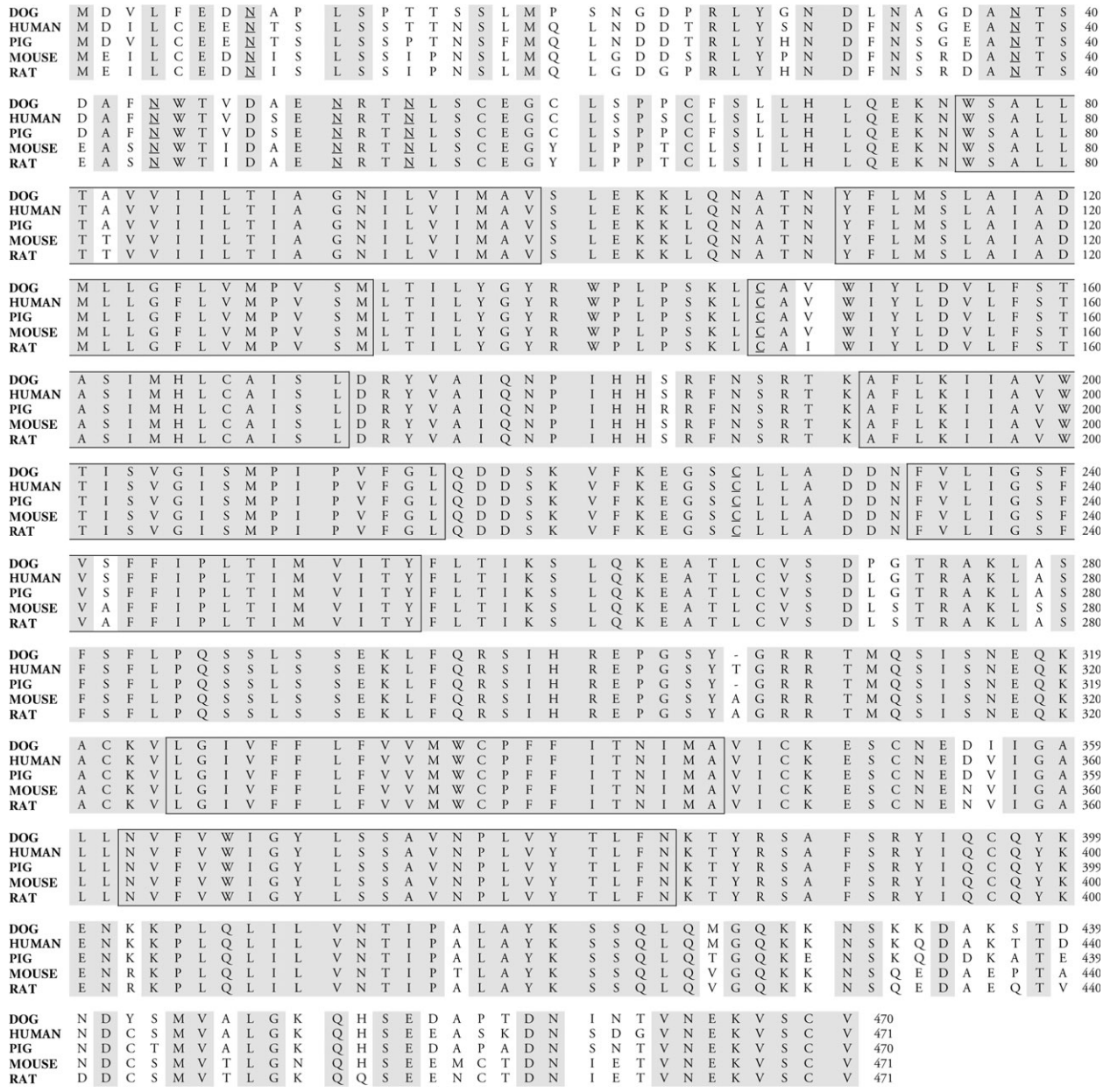
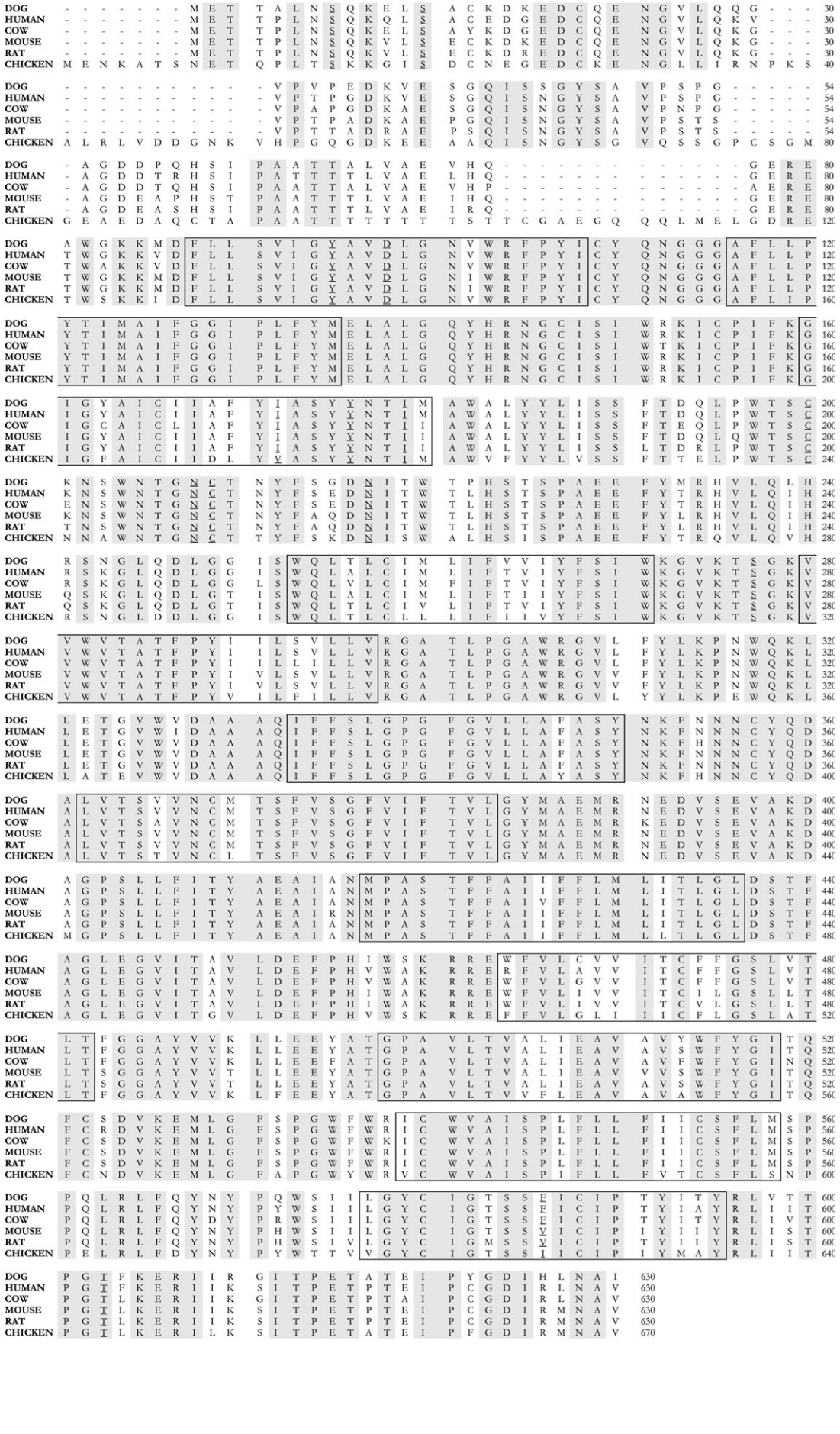


Figure 2. Alignment of the amino acid sequence of the canine serotonin receptor 2A with human (accession number NP_000612), porcine (NP_999382), murine (NP_766400), and rat (NP_058950) sequences. Amino acids are presented in groups of 10. Residues that are conserved in the five species are shown in gray background and predicted transmembrane regions according to SWISSPROT (http://us.expasy.org/cgi-bin/niceprot.pl?P28223) are boxed. The seven transmembrane domains are predicted to form a pocket where 5-HT can bind. Residues 1–75 form the amino terminus; residues 100–110 form the first intracellular loop, and so on. Key residues that are highlighted in the results section are underlined: 8N, 38N, 44N, 51N, 54N are N-linked glycosylation sites, and 148C and 227C form a disulfide bridge according to SWISSPROT. All positions of key residues refer to the canine protein.

in clone 287M9 (data not shown). These results confirm the presence of intron 4 of *slc6A4* in the two BAC clones.

In Silico Characterization of *htr1A*

The BLAST search with Doberman *htr1A* sequence AY134445 retrieved one chromosome 2 genomic contig and 15 traces from the boxer genome project, two sequences from the poodle genome project, and a sequence from a collie dog (Kukekova et al. 2004). The relative position and accession numbers of these sequences are depicted in Figure 1A. We found several segments with high homology to corresponding human and murine regions in the sequence upstream of the *htr1A* start codon. For instance, there is


DOG
HUMAN
COW
MOUSE
RAT
CHICKEN

a 113 bp segment with 85% identity to the murine 5′ region of *htr1A* located at 3.5 kb upstream of the start codon. This sequence is also highly similar to the corresponding human region. In addition, a 298 bp segment with 80% identity to the human 5′ region of *HTR1A* was located 0.4 to 0.1 kb upstream of the start codon.

Two SNPs were identified in the coding sequence of *htr1A* by comparing the canine sequences: G65T and A808C. The latter was reported by van den Berg et al. (2003b). Both SNPs are nonsynonymous. G65T causes an arginine-leucine polymorphism of amino acid 22, and A808C gives rise to a lysine-glutamine polymorphism of amino acid 270. The variations were predicted to be functionally insignificant by POLYPHEN.

A CA microsatellite repeat was found in a trace located downstream of *htr1A* (Figure 1A). A PCR indicated that this repeat was present in BAC clone 160O12, which has been shown to contain *htr1A* (data not shown; van den Berg et al. 2003b). The presence of the CA repeat in this clone confirms its proximity to the gene. We have named the marker UU160O12.

In Silico Characterization of *htr2A*

Using the beagle *htr2A* sequence NM_001005869 as a BLAST query, we retrieved the earlier mentioned chromosome 22 genomic contig NW_139892 and 32 traces from the boxer genome project, four sequences from the poodle genome project, and a partial mRNA sequence of canine *htr2A* (Figure 1B). We detected a fragment of 38 bp with at least 90% identity to corresponding regions in humans, hamsters, cows, mice, and rats at 0.9 kb upstream of the start codon. One kb 5′ sequence and the first 307 bp of the coding sequence are part of a CpG island, according to the definition of Milanesi and Rogozin (1998).

We searched for polymorphisms within or close to *htr2A* in the retrieved DNA sequences. No variation was observed in the coding sequence. We found a G/A SNP at position IVS1+405, a T/C SNP at position IVS2+444, and a G/A SNP at IVS2+450. Several polymorphic simple sequence repeats were identified, including two CA repeats and an ATTT repeat in intron 2, as well as two A repeats downstream of the stop codon. One of the CA repeats was analyzed in golden retrievers in this study (discussed later). This marker is located at position IVS2+1439, and we have named it UUHTR2AEX2.

The serotonin receptor 2A protein consists of 470 amino acids in dogs and pigs and 471 amino acids in humans, mice, and rats. An alignment of these amino acid sequences is shown in Figure 2. Canine amino acid sequence identity is 94% with humans, 90% with mice and rats, and 94% with pigs. *HTR2A* protein sequence similarity is especially high in the seven transmembrane domains. This has been observed in other G-protein-coupled receptors (Stam et al. 1992). Identification of key residues in the amino acid chain is important for future studies on the association of mutations or polymorphisms in the genes with behavioral traits in dogs. The key ligand binding areas of G-protein-coupled receptors are located mainly in the distal one-third of transmembrane domains 3–7 (Hartig 1997). In Figure 2, several specific key amino acid residues in the serotonin receptor 2A protein can be recognized: residues involved in N-linked glycosylation (8Asn, 38Asn, 44Asn, 51Asn, and 54Asn) and in a disulfide bridge (148Cys and 227Cys; SWISSPROT website, http://expasy.org/sprot/; accession number P28223). These residues are conserved between the five species.

In Silico Characterization of *slc6A4*

We retrieved the earlier mentioned chromosome 9 genomic contig, more than 100 traces, and six poodle sequences by blasting the human *SLC6A4* sequence NM_001045 (Figure 1C). A region with 78% identity to the noncoding exon 1 of human *SLC6A4* was found at 12.5 kb upstream of the start codon. This region is part of a CpG island, according to the definition of Milanesi and Rogozin (1998). The region is therefore likely to represent the first exon of the canine gene. We detected a region with 52% identity to the noncoding exon 2 of human *SLC6A4* at 0.1 kb upstream of the start codon. This region is flanked by a putative splice acceptor AG and donor GT site. We have adopted the human nomenclature of the exons in this article: the canine exon containing the ATG start codon is referred to as exon 3.

We found a single SNP at position −2200 by comparison of the poodle and boxer sequences, but we could not confirm this variation with traces. The serotonin transporter protein consists of 630 amino acids in dogs, humans, mice, rats, and cows and 670 amino acids in chickens (Figure 3). The canine amino acid homology is 94% with humans, 91% with mice and cows, 90% with rats, and 79% with chickens. In Figure 3, several specific key amino acid residues of *slc6A4* can be recognized: residues involved in N-linked glycosylation (208Asn

←

Figure 3. Alignment of the amino acid sequence of the canine serotonin transporter with human (accession number NP_001036), bovine (NP_777034), murine (NP_034614), rat (CAA71909), and chicken (NP_998737) sequences. Amino acids are presented in groups of 10. Residues that are conserved in the six species are shown in gray background, and predicted transmembrane regions according to SWISSPROT (http://us.expasy.org/cgi-bin/niceprot.pl?P31645) are boxed. Residues 1–87 form the amino terminus; residues 109–115 form the first extracellular loop, and so on. The following key residues are underlined: 95Y and 586F are antagonist binding sites; 98D is a coordination site for 5-HT; 172I and 176Y are binding sites for 5-HT; 179I is part of an external gate; 200C and 209C form a disulfide bridge; 208N and 217N are N-linked glycosylation sites; and 8S, 13S, 277S, and 603T are potential sites of protein kinase A and C phosphorylation. All positions of key residues refer to the canine protein.

and 217Asn; SWISSPROT website http://expasy.org/sprot/; accession number P31645), in a disulfide bridge (200Cys and 209Cys; Chen et al. 1997), in binding of 5-HT (172Ile and 176Tyr; Chen and Rudnick 2000), and in the interaction with antagonists (95Tyr and 586Phe; Barker and Blakely 1996; Barker et al. 1998). Amino acids 8Ser, 13Ser, 277Ser, and 603Thr were marked as potential sites of protein kinase A and C phosphorylation by Ramamoorthy et al. (1993) and Chang et al. (1996). Chen and Rudnick (2000) suggested that 179Ile acts as part of an external gate. Most of the amino acid residues described here are conserved between the six species, except for 172Ile and 586Phe.

Genotyping of Golden Retrievers

DNA sequence analysis of position 15 to *13 of *htr1A* was performed in three golden retrievers. This fragment did not display variation in the retrievers. The dogs were homozygous for a T-residue at position 65 and homozygous for a C at position 808. We found three alleles of the marker UU160O12 in these dogs, with PCR product lengths of 297, 303, and 305 bp. Each of the three dogs was heterozygous.

Htr2A DNA sequence analysis and marker genotyping were performed in eight golden retrievers. There was no variation in the coding sequence of *htr2A* in these dogs. However, we did find a C/T SNP at position IVS2-10. This polymorphism was not detected in the canine sequences that we retrieved from the NCBI website. Five dogs were heterozygous, and three dogs were homozygous for the C allele. We found two alleles for the marker UUHTR2AEX2 with lengths of 130 and 132 bp. Three combinations of the SNP and microsatellite marker alleles were observed in the golden retrievers (130-C, 132-C, and 132-T).

DNA sequence analysis of *slc6A4* was performed in eight golden retrievers. We identified a synonymous SNP in the coding sequence: C411T. In addition, we found a G/A SNP at position IVS9-12. Both SNPs were not detected in the canine sequences that we retrieved from the NCBI website. Four dogs were homozygous at both loci; these dogs had two copies of haplotype C-G of the two SNPs combined. The other four dogs were heterozygous at both positions; their haplotypes are likely to be C-G and T-A. We tested the marker UU76G6 in seven golden retrievers and found two alleles: one dog was heterozygous, with product lengths of 262 and 264 bp, and six dogs were homozygous for the 262 bp fragment.

Htr1A Genotyping in Seven Dog Breeds

We determined the genotypes of the *htr1A* SNPs in eleven golden retrievers, five beagles, seven boxers, three cairn terriers, six Dobermans, five Norwegian elkhounds, and four Shetland sheepdogs (Table 2). At least three SNP haplotypes were detected. Twenty-five dogs were homozygous for SNP haplotype T-C, and five boxers were homozygous for the haplotype G-A. A third SNP haplotype (G-C) was identified in three Norwegian elkhounds, which were heterozygous at position 65 and homozygous C at position 808. Eight dogs (one golden retriever, two beagles, one Cairn terrier, and four Dobermans) were heterozygous at both loci. Their haplotypes could therefore not be determined with certainty. We also genotyped the marker UU160O12 in these dogs and found six alleles, with product lengths of 293, 295, 297, 299, 303, and 305 bp.

Table 2. Genotype frequencies of two single nucleotide polymorphisms (SNPs) in *htr1A* in 41 dogs of diverse breeds

		SNP 65 genotype			SNP 808 genotype		
	n	TT	TG	GG	CC	CA	AA
Golden	11	10	1	0	10	1	0
Beagle	5	3	2	0	3	2	0
Boxer	7	2	0	5	2	0	5
Cairn terrier	3	2	1	0	2	1	0
Doberman	6	2	4	0	2	4	0
Elkhound	5	2	3	0	5	0	0
Sheltie	4	4	0	0	4	0	0

Discussion

We characterized three canine serotonergic genes, isolated canine BAC clones containing them, developed oligonucleotides for genomic sequencing of their coding regions and intron-exon boundaries, and identified both single nucleotide polymorphisms and polymorphic microsatellite markers in or about the vicinity of the genes. This work provides a starting point for mutation scans and association studies on canine behavioral traits.

The release of the 7.8× redundant boxer genome sequence and the 1.5× poodle sequence has enabled a rapid elucidation of the structure of candidate genes in the dog (Kirkness et al. 2003; Sutter and Ostrander 2004). We retrieved canine DNA sequence contigs from chromosomes CFA02, CFA22, and CFA09 for *htr1A*, *htr2A*, and *slc6A4*, respectively. The chromosomal location of these contigs is in accordance with the position of the genes on the human genome and with our previous work. We predicted *htr1A* to be located on CFA02 by radiation hybrid mapping (van den Berg et al. 2003b). *HTR2A* maps to human chromosome 13q14–21, which displays synteny with *CFA22*. A large section of *CFA09* corresponds to *HSA17*, which contains human *SLC6A4* (Guyon et al. 2003).

Genotyping in Golden Retrievers

This study is embedded in a research project involving aggressive behavior in golden retrievers, and we analyzed the coding sequence of the three genes in dogs of this breed. The golden retriever breed has been shown to be relatively heterogeneous (Nielen et al. 2001; Sutter et al. 2004), which is in agreement with the large number of haplotypes that we describe here. The SNPs that were identified in *htr2A* and the intron of *slc6A4* in this study are close to splice sites and may affect splicing (Pagani and Baralle 2004). Alternative splicing of the serotonin receptor 2A has been demonstrated in human brain cDNA (Guest et al. 2000).

Htr1A Genotyping in Seven Dog Breeds

The domestication of dogs is likely to have involved genetic selection for less fearful and aggressive behavior in wolf ancestors. *Htr1A* is a strong candidate for involvement in this process because indications suggest that it plays a role in anxious behavior. If *htr1A* were indeed involved in domestication, we would expect to find little variation in this region of the canine genome. We have analyzed the genotypes of *htr1A* in dogs from seven breeds and have identified at least three SNP haplotypes. This result does not support involvement of the gene in the domestication of the wolf. However, this conclusion has to be treated with caution because patterns of polymorphism cannot always be used in the search for domestication genes. The amount by which variation is reduced by strong artificial selection depends on the initial frequency of the beneficial allele (Innan and Kim 2004).

Although the SNPs in canine *htr1A* are not likely to affect the function of the receptor, it is possible that they are in linkage disequilibrium with functional polymorphisms in a regulatory region of the gene. Differences in *htr1A* haplotype frequencies between dog breeds with diverse behavioral characteristics might point to an influence of *htr1A* on canine temperament. The *htr1A* haplotype frequencies in the group of boxers seemed to differ from those in other breeds. However, our study group was too small to draw firm conclusions on this topic. It will be very interesting to study breed differences in *htr1A* haplotype frequencies in more detail, as was already done for the gene encoding the serotonin receptor 1B (*htr1B*) by Masuda et al. (2004). These researchers detected interbreed variations in genotype and allele frequencies of *htr1B* SNPs.

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