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Novel repeat polymorphisms of the dopaminergic neurotransmitter genes among dogs and wolves

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Abstract Genetic polymorphisms of the neurotransmission systems are intensively studied in the human because of a possible influence on personality traits and the risk of psychiatric disorders. The investigation of genetic variations of the dog genome has recently been a promising approach, as a considerable similarity can be observed between dogs and humans, in both genetic and social aspects, suggesting that the dog could become an appropriate animal model of human behavioral genetic studies. The aim of our study was the identification and analysis of variable number of tandem repeats polymorphisms (VNTRs) in the genes of the dopaminergic neurotransmitter system of dogs. The *in silico* search was followed by the development of PCR-based techniques for the analysis of the putative VNTRs. Highly variable repetitive sequence regions were found in the tyrosine hydroxylase (*TH*), dopamine transporter (*DAT*), and dopamine β-hydroxylase (*DBH*) genes. Allele frequency and genotype distribution of these novel polymorphisms together with the exon 3 and exon 1 VNTR of the dopamine D4 receptor gene were determined in a large sample involving four dog breeds (German Shepherd, Belgian Tervueren, Groenandael, and Malinois) and European Grey Wolves. A significant difference of allele and genotype frequencies was demonstrated among the analyzed breeds; therefore, an association analysis was also carried out between the activity–impulsivity phenotype and the described VNTRs. Preliminary findings are presented that polymorphisms of the *DRD4*, *DBH*, and *DAT* genes can be associated with attention deficit among Belgian Tervuerens.

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Introduction

The investigation of genetic variations has recently been the focus of interest because they play a significant role in the development of inherited features (i.e., psychological traits) and they are supposed to code for risk or protective factors of complex diseases. One of the most often used approaches for the identification of genetic components of multifactorial traits is the analysis of candidate genes in association studies; this methodology has been used so far in studies of dogs. Single nucleotide polymorphisms of the cytokine genes were investigated in canine malignant histiocytosis (Soller et al. 2006) and in rupture of the cranial cruciate ligament (Wilke et al. 2005). Microsatellite markers were employed for the analysis of dog breed phylogeny, and significant divergence was shown in each of the seven tested American Kennel Club groups (Irion et al. 2003).

The genetic variations of the human dopaminergic neurotransmitter system are major targets in human psychiatric genetic studies, involving the investigation of polymorphisms in the dopamine D4 receptor, dopamine transporter, and dopamine-β-hydroxylase genes. The dopamine D4 receptor (*DRD4*) gene has recently been the focus of interest, as this protein is highly abundant in the limbic

system, which is responsible for emotions and cognitive functions, and it is the target of atypical antipsychotic drugs (e.g., clozapine) used in the therapy of several psychiatric illnesses (Andreasen 1995). Moreover, both the 5′ region and the coding sequence of the *DRD4* gene are highly polymorphic in humans (Szantai 2004). The most thoroughly investigated polymorphism of the gene is located in exon 3, where the repeat number of a 48-base pair (bp)-long segment varies from 2 to 10. A large number of studies deal with this variable number of tandem repeats (VNTR) as a possible risk factor for several psychiatric disorders (for recent reviews see Eisenberg et al. 2000; Jonsson et al. 2003; Lopez Leon et al. 2005; Lusher et al. 2001). Interestingly, an analog of this polymorphism was described in dogs (*Canis familiaris*) as well (Niimi et al. 1999). Moreover, it is notable that no similar genetic variation can be found in rodent models such as mice or rats (O'Malley et al. 1992). In contrast to the human VNTR, the repetitive segment of the dog *DRD4* exon 3 polymorphism consists of modules of 12 and 39 bp, respectively. The distribution of the different alleles was analyzed in several dog breeds (Niimi et al. 2001). Our group recently investigated the effect of this polymorphism on the behavior of German Shepherds (Hejjas et al. 2007), as the *DRD4* exon 3 VNTR was demonstrated to be associated with the novelty-seeking personality trait in human (Ebstein 2006) and in a nonhuman primate's behavior (Bailey et al. 2007).

Several further repeat variants have also been described in the coding region of the human *DRD4* gene. A 12-bp duplication and a 13-bp deletion were demonstrated and analyzed simultaneously (Chang and Kidd 1997) in exon 1, and it was shown that the latter results in a nonfunctional truncated protein (Nothen et al. 1994). Polymorphisms are abundant in the 5′ noncoding region of the *DRD4* gene as well. A 120-bp duplication was investigated as a possible risk factor for attention deficit hyperactivity disorder (Kereszturi et al. 2006b; McCracken et al. 2000), and the impact of this polymorphism on gene expression was also analyzed (D'Souza et al. 2004; Kereszturi et al. 2006b).

Besides the *DRD4* exon 3 VNTR of dogs, an insertion/deletion polymorphism was described in exon 1 of the canine gene. This variation is supposed to be the analog of the human 12-bp duplication based on its location in the gene (Ito et al. 2004). Although there is a considerably high variance between allele frequencies of these polymorphisms in different canine breeds (Ito et al. 2004), little is known about their effect on either receptor function or behavior.

Another major element of the dopaminergic neurotransmission is the dopamine transporter (DAT) playing a role in the reuptake of dopamine. This protein is the target of amphetamine-like prescription stimulants (e.g., methylphenidate). The human *DAT* gene also contains polymorphic VNTRs, which were analyzed as possible genetic components of disruptive behavior disorders (Lee et al. 2006), alcoholism (Kohnke et al. 2005), and attention deficit hyperactivity disorder (Purper-Ouakil et al. 2005). Moreover, polymorphisms in the tyrosine hydroxylase (*TH*) and dopamine-β-hydroxylase (*DBH*) genes have also been studied in genetic association analyses. These genes code for enzymes that play a role in the metabolism of monoaminergic neurotransmitters. Numerous groups investigated the putative association between *DBH* polymorphisms and attention deficit hyperactivity (Tang et al. 2006; Wigg et al. 2002), and polymorphisms of the *TH* gene were also suggested to be genetic risk factors for various disorders such as schizophrenia (Kurumaji et al. 2001), dependent smoking (Olsson et al. 2004), and mood disorders (Serretti et al. 2003).

Although researchers have been accumulating a significant body of knowledge about the *DRD4* polymorphisms among dogs, nothing is known about the canine genetic variations related to the other dopaminergic genes. Here we report the identification and analysis of several novel repeat polymorphisms in the *DAT*, *DBH*, and *TH* genes and a detailed study of the known *DRD4* polymorphisms among four dog breeds as well as in European Grey Wolves. Because dysfunction of the dopaminergic system is known to be in the background of attention deficit hyperactivity disorder (ADHD) (e.g., Faraone et al. 2005), behavioral data of Belgian Tervuerens were also collected and investigated in a genetic association study.

Materials and methods

Animals

Four dog breeds (Belgian Tervueren, $N = 102$; Belgian Groenandael, $N = 105$; Belgian Malinois $N = 50$; and German Shepherd, $N = 240$) and 22 European Grey Wolves (*Canis lupus*) were involved in our study. For the most common characteristics of the investigated breeds, see Supplementary Table 1. DNA sampling was performed by applying a noninvasive approach; buccal smear was collected with cotton swabs from the inner surface of the cheek, and DNA purification was carried out using the Gentra DNA Purification Kit (Qiagen, Valencia, CA).

In silico analysis

The sequences of the investigated genes (except that of *DRD4*) were downloaded from the GenBank (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/) databases. GenBank accession numbers were as follows:

Table 1 The primers used for the amplification of the investigated repetitive regions

Gene		Primer	Sequence (5′-3′)	T_A (°C)[a]
DAT	(intron 5)	dat_5F	GCA CCT CTC CCG CCC TCT	56
		dat_5R	TTA ACG CCA TCT ACC CTG TGA	56
	(intron 9)	dat_9F	CTC CTG TGT CCC CGC TGT CTT	65
		dat_9R	GAC AGA GCA GGG CAG GGA GG	65
DBH	(intron 4)	dbh_4F	CCC CTC ACC TCC AAG CAG	56
		dbh_4R	AGG GTG ATG TGG GCA GGA T	56
	(exon 11)	dbh_11F	GTG AAG CCG ACC AGC CAT TG	58
		dbh_11R	CTG ATT TCT CCA GCC GTG TTG	58
TH	(intron 4)	th_F	GTC TGT CTG CTG TCT GGC TCC C	65
		th_R	TGG AGA GGC TTC CTG ACA CCC	65
DRD4	(exon 3)	D1c	CGC GCG TCG GGC CAA GCT G	67
		D2c	GCG GGG GGC AGG GGG CG	67
		D4dogBR	TGG GCT GGG GGT GCC GTC C	67
	(exon 1)	24d_F	CGC CAT GGG GAA CCG CAG	60
		24d_R	CGG CTC ACC TCG GAG TAG A	60

DAT = dopamine transporter; DBH = dopamine-β-hydroxylase; TH = tyrosine hydroxylase; DRD4 = dopamine receptor D4

[a] T_A shows the applied annealing temperature

tyrosine hydroxylase: NM_001002966, AB097058, and ENSCAFG00000010099; dopamine-β-hydroxylase: NM_001005263, AB097057, and ENSCAFG00000019783; and dopamine transporter: ENSCAFG00000010574. Exon-intron boundaries were determined based on the data from Ensembl. The sequence of the *DRD4* gene was obtained from the publication of Niimi et al. (1999). The investigated genes were *in silico* searched for repetitive sequences applying the Web-based Tandem Repeats Finder search tool (Benson 1999).

PCR amplification

Polymerase chain reaction (PCR) primers were designed by Oligo 5.0 software (Plymouth, MN). The Qiagen HotStarTaq polymerase kit was used for PCR amplification in each analysis except the investigation of exon 1 of the *DRD4* gene. The reaction mixture contained 1 μM of each primer (Table 1), approximately 5 ng of DNA template, 200 μM dATP, dCTP, dTTP, and 100 μM of dGTP and dITP, 0.025 U HotStarTaq DNA polymerase, 1× buffer, and 1× Q-solution supplied together with the enzyme. The total volume of the PCR mixture was 10 μl. The PCR cycle consisted of an initial denaturation at 95°C for 15 min, 35 cycles of 1-min denaturation at 95°C, an 1-min annealing at various temperatures (see T_A in Table 1), an 1-min extension at 72°C, and a 10-min final extension at 72°C. A second, independent PCR was performed to identify the "3a" and "3b" alleles of *DRD4* exon 3 according to Niimi et al. (1999), using the forward primer D1C in combination with an allele-specific reverse primer D4dogBR. The PCR analysis of the *DRD4* exon 1 polymorphism was carried out as described by (Ito et al. 2004).

Electrophoretic separation

PCR products were analyzed by conventional submarine agarose gel electrophoresis (Biocenter, Szeged, Hungary), using 1.5% agarose + 2% Metaphore composite gel and visualized by ethidium bromide staining.

Investigation of the activity–impulsivity endophenotype of dogs, association study

The human ADHD Rating Scale (ADHD RS) Parent Version questionnaire (DuPaul 1998) was adapted and validated for dog owners (Vas et al. 2006) and was used here to characterize activity-impulsivity and attention of dogs. Owners filled out the dog ADHD RS Owner Version questionnaire, containing 13 items (7 items form the activity-impulsivity scale and 6 items form the inattention scale). Scale scores were calculated for each dog as the sum of scores given by the owner (range = 0–3). Fifty-nine Belgian Tervueren (24 males, 35 females, age mean ± SD = 5.5 ± 3.6 years) were characterized.

SPSS for Windows (SPSS, Inc., Chicago, IL) was used for all statistical analyses. Associations of the ADHD test scores with the investigated polymorphisms were assessed by one-way ANOVA and independent-samples *t* test.

Results

In silico search for dog analogs of human VNTRs in the dopaminergic genes

As a first step, the selected dopaminergic genes were *in silico* searched for repetitive sequences in the dog genome. Both 5′ (1000-bp long), intronic and exonic segments were included in our study because exon variations may result in alteration of the protein structure, whereas polymorphisms in introns or in the 5′ region may influence the activity of gene expression. Regions were chosen for further analysis if (1) the length of the repeated module was larger than 6 bp (i.e., not microsatellites) and (2) the sequence similarity of the repeated units was at least 85%. Based on the above considerations, the following regions were involved in the remaining parts of our study: 36-bp duplication in intron 4 of the *TH* gene; a 17-bp duplication in intron 4 and a 24-bp duplication in exon 11 of the *DBH* gene; a 59-bp duplication in intron 5 and a 38-bp duplication in intron 9 of the *DAT* gene, besides the known polymorphisms of *DRD4* exon 1 and exon 3.

Pilot studies for selection of polymorphic repeat regions among dog breeds and European Grey Wolves

A subset of samples ($N = 48$) of each of the four investigated breeds (Tervueren, Groenandael, Malinois, German Shepherd) and all wolves ($N = 22$) was used for a pilot analysis to decide which of the *in silico* identified and selected repetitive regions are polymorphic in our dog populations. No polymorphic variations were found in the repeat regions identified *in silico* in exon 11 of the *DBH* gene and in intron 5 of the *DAT* gene. On the other hand, variable repeat numbers were shown in intron 4 of the *TH* and *DBH* genes, in intron 9 of the *DAT* gene, and in exon 1 of the *DRD4* gene in our breeds. These regions, together with the exon 3 polymorphism of the *DRD4* gene described earlier by (Niimi et al. 1999), were subjected to downstream analysis on the whole population ($N = 495$) of various dog breeds. The accurate chromosomal localization of the VNTRs is shown in Table 4.

Analysis of the known VNTRs in the dopamine D4 receptor

The VNTR in exon 3 of the *DRD4* gene was shown to be polymorphic in each of the investigated breeds as well as among European Grey Wolves. Figure 1A, B depicts the electropherogram of the obtained PCR products. In a first reaction, two flanking primers were employed to determine the length (i.e., repeat number) of the alleles (Fig. 1A). A second PCR was necessary to distinguish between variants 3a and 3b because they differ only in the order of the 12- and 39-bp-long modules (Niimi et al. 1999). Figure 1B represents a typical electropherogram of a homozygote for allele 3a, but no allele 3b was found in the studied populations. Comparing the allele frequencies of 2 and 3a, the latter was most frequent in Tervuerens and Malinois, whereas allele 2 was dominant in German Shepherds. The two variants had nearly the same frequencies in Groenandaels. No other described variations were found in these breeds, while allele 5 was the major variant among wolves (Table 2). Moreover, a novel allele (we named/signed allele 8) was demonstrated to be present at a large frequency in this group. This form is longer than any of those described earlier, presumably by the insertion of an extra 39-bp unit, demonstrated in Figure 1C. The χ^2 test showed a significant difference in the genotype distributions among the studied breeds ($\chi^2 = 523.28$; df = 32; $p < 0.0001$).

The 24-bp insertion/deletion polymorphism in exon 1 of the dopamine D4 receptor (*DRD4*) gene described by Ito et al. (2004) was shown to be polymorphic in all dogs; however, wolves possessed only the long variant. Figure 1D shows the electrophoretic analysis of three samples with L/L, S/L, and S/S genotypes, respectively ("L" stands

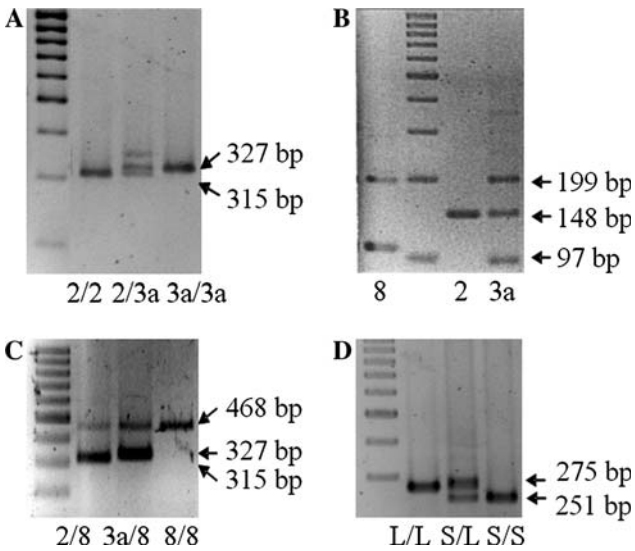

Fig. 1 Analysis of the VNTRs in the dopamine D4 receptor gene. **A** *DRD4* exon 3 amplicons in the "first PCR": Identification of the repeat number (2 or 3). **B** Differentiation between alleles 3a and 3b (allele 2 and 8 are also shown) in the "second PCR." **C** Identification of allele 8 in homo- and heterozygous form **D** Genotyping of 24-bp insertion/deletion polymorphism in exon 1 (L = insertion, S = deletion). Genotypes are shown under the electropherograms, and the estimated lengths of the PCR fragments are depicted on the right. A 100-bp DNA ladder was used as size marker

Table 2 Distribution of *DRD4* exon 1 and exon 3 genotypes among dog breeds and wolves

Genotype	Belgian Tervueren		Belgian Groenandael		Belgian Malinois		German Shepherd		European wolf	
***DRD4* (exon 3)**										
2/2	14	(14.00)	31	(29.52)	9	(18.00)	101	(42.26)	1	(4.50)
2/3a	47	(47.00)	46	(43.81)	18	(36.00)	107	(44.77)	1	(4.50)
3a/3a	39	(39.00)	28	(26.67)	23	(46.00)	31	(12.97)	0	(0.00)
2/5	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	4	(18.20)
5/5	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	7	(31.90)
8/8	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	4	(18.20)
3a/8	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	2	(9.10)
2/8	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	2	(9.10)
5/8	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	1	(4.50)
Total	100	(100.00)	105	(100.00)	50	(100.00)	239	(100.00)	22	(100.00)
***DRD4* (exon 1)**										
S/S	32	(32.32)	26	(25.75)	3	(6.25)	0	(0.00)	0	(0.00)
S/L	50	(50.51)	45	(44.55)	22	(45.83)	8	(3.40)	0	(0.00)
L/L	17	(17.17)	30	(39.70)	23	(47.92)	227	(96.60)	22	(100.00)
Total	99	(100.00)	101	(100.00)	48	(100.00)	235	(100.00)	22	(100.00)

This table shows the number of individuals in each category as well as frequency values (percentage), which are in parentheses

for the insertion allele and "S" for the deletion allele). The L allele was the dominant form in German Shepherds; on the other hand, approximately the same frequencies have been found among Groenandael and Malinois, while the long form was the minor variant in Tervuerens. The genotype frequencies were thus highly variable among the breeds (Table 2), and the difference was statistically significant ($\chi^2 = 280.58$; df = 8; $p < 0.0001$).

Analysis of the novel VNTRs

The 36-bp-long sequence in the intron 4 region of the *TH* gene was demonstrated to be present either as a single copy or in a duplicated form. Figure 2A depicts the result of the PCR analysis of this region. The presence of allele 1 was rare in German Shepherds, Malinois, and Grey Wolves, but this allele was fairly frequent in Tervuerens and Groenandaels (Table 3). Thus, genotype frequencies differed in a fairly wide range in the various populations ($\chi^2 = 218.61$; df = 8; $p < 0.0001$). However, the measured genotype frequencies did not show statistically significant deviation from the Hardy-Weinberg equilibrium.

The 17-bp variation in the fourth intron of the *DBH* gene was demonstrated to be polymorphic in each breed; the copy number was interestingly either 1 or 3, while no duplication could be observed in our samples. The electrophoretic analysis of samples with 1/1, 1/3, and 3/3 genotypes is shown in Figure 2B. The variant containing three repeats was the minor allele in all breeds. This variant occurred most frequently in Malinois (23.96%) and had the lowest frequency among German Shepherds (3.12%). Genotype ($\chi^2 = 77.212$; df = 8; $p < 0.0001$) distribution of this polymorphism also showed a significant difference among the five breeds. The measured genotype frequencies fit the Hardy-Weinberg equilibrium.

The results of the PCR-based investigation of the 38-bp repetitive sequence in intron 9 of the *DAT* gene can be seen in Figure 2C. Interestingly, German Shepherds possessed only the longer variant (allele 2), and this form was the most common allele in the other breeds (82.61–100%), except the Malinois (Table 3). In this latter group, the frequency of the two alleles was almost the same. The difference of the genotype frequencies among the breeds was also statistically significant ($\chi^2 = 248.06$; df = 8; $p < 0.0001$), and the measured genotype frequencies fit the Hardy-Weinberg equilibrium.

Pilot study of associations between the VNTRs and the activity-impulsivity and attention deficit rating scale in Belgian Tervuerens

Belgian Tervuerens were used for the association analyses of the investigated polymorphisms because (1) almost all genetic variants could be identified in this group and (2) a relatively large number of individuals were available for our study.

One-way ANOVA was used to compare the three genotype groups in the case of *TH*, *DRD4* exon 1, and *DRD4* exon 3 polymorphisms. In the case of *DBH* and *DAT* polymorphisms, allele 3 and allele 1 were rather rare,

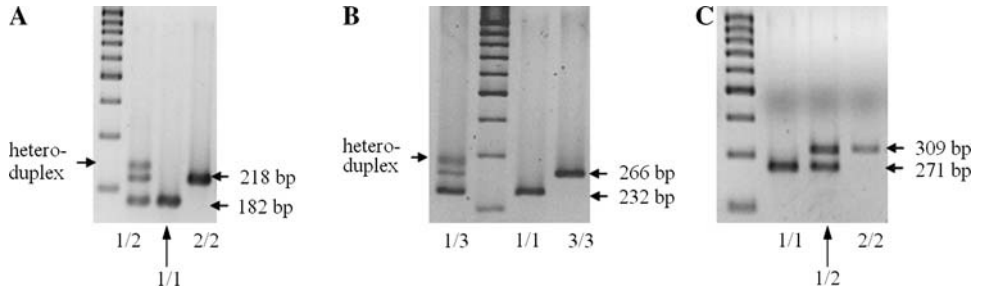

Fig. 2 Genotyping of the novel VNTRs. **A** Tyrosine hydroxylase intron 4 VNTR (1 or 2 repeats). **B** Dopamine-β-hydroxylase intron 4 VNTR (1 or 3 repeats). **C** Dopamine transporter intron 9 VNTR (1 or 2 repeats). Genotypes are shown under the electropherograms, and the estimated lengths of the PCR fragments are depicted on the right. A 100-bp DNA ladder was used as the size marker

Table 3 Genotype frequencies of the polymorphisms in the *TH*, *DBH*, and *DAT* genes among the investigated dog breeds and wolves

Genotype	Belgian Tervueren		Belgian Groenandael		Belgian Malinois		German Shepherd		European wolf	
***TH* (intron 4)**										
1/1	18	(18.00)	48	(46.15)	1	(2.00)	4	(1.67)	2	(9.09)
1/2	44	(44.00)	49	(47.12)	7	(14.00)	47	(19.58)	8	(36.36)
2/2	38	(38.00)	7	(6.73)	42	(84.00)	189	(78.75)	12	(54.55)
Total	100	(100.00)	104	(100.00)	50	(100.00)	240	(100.00)	22	(100.00)
***DBH* (intron 4)**										
1/1	81	(79.41)	63	(60.58)	31	(64.58)	225	(93.75)	18	(82.61)
1/3	19	(18.63)	36	(34.62)	11	(22.92)	15	(6.25)	4	(17.39)
3/3	2	(1.96)	5	(4.81)	6	(12.50)	0	(0.00)	0	(0.00)
Total	102	(100.00)	104	(100.00)	48	(100.00)	240	(100.00)	22	(100.00)
***DAT* (intron 9)**										
1/1	1	(0.99)	3	(2.88)	15	(31.25)	0	(0.00)	0	(0.00)
1/2	10	(9.90)	29	(27.88)	24	(50.00)	0	(0.00)	8	(34.78)
2/2	90	(89.11)	72	(69.23)	9	(18.75)	237	(100.00)	14	(65.22)
Total	101	(100.00)	104	(100.00)	48	(100.00)	237	(100.00)	22	(100.00)

The number of individuals belonging to each category is displayed, and percentage values are given in parentheses

respectively (Tables 2 and 3); consequently, individuals possessing only the minor allele in homozygous form and heterozygote animals were merged into a single group for reliable statistical analysis. Therefore, two groups have been defined in case of both polymorphisms: (1) samples possessing the minor allele in homozygous or heterozygous form (DBH 3+ and DAT 1+ groups, respectively) and (2) dogs lacking this variant (DBH 3– and DAT 1– groups, respectively). These genotype groups were compared by using independent-samples *t* test.

Our results demonstrated that there was no difference between genotype groups in the *activity-impulsivity scale* of the dog ADHD Rating Scale (the three *TH* groups: $F_{(2,56)} = 0.2246$, $p = 0.7996$; the three *DRD4* exon 1 groups: $F_{(2,56)} = 1.3066$, $p = 0.2789$; the three *DRD4* exon3 groups: $F_{(2,56)} = 0.3576$, $p = 0.7010$; the two *DBH* groups: $t_{(57)} = 1.4049$, $p = 0.1655$; and the two *DAT* groups: $t_{(57)} = 1.6171$, $p = 0.1114$).

On the other hand, genotype-dependent differences were found in the case of the *attention-deficit scale* of the dog ADHD Rating Scale. Individuals possessing at least one *DBH* allele 1 ($N = 13$), at least one *DAT* allele 1 ($N = 9$), or dogs carrying the short variant in homozygous form in *DRD4* exon 1 ($N = 10$) were rated to have a higher degree of attention deficit (*DRD4* exon 1: $F_{(2,56)} = 4.1120$, $p = 0.0216$; *DBH*: $t_{(13.8)} = -2.2238$, $p = 0.0434$; and *DAT*: $t_{(57)} = 2.8763$, $p = 0.0057$, respectively). There was, however, no difference in *TH* ($F_{(2,56)} = 0.3037$, $p = 0.7393$) and *DRD4* exon 3 ($F_{(2,56)} = 1.7938$, $p = 0.1758$) variants.

Discussion

Genetic variants presumably responsible for inherited traits and disorders spread throughout the whole genome. The size of these polymorphisms varies in an extremely wide

range from affecting a single nucleotide expanding to large, microscopically visible chromosome alterations with a length of several millions of base pairs (Redon et al. 2006). The former variant is referred to as single nucleotide polymorphism (SNP), and a large number of these variations have already been discovered and investigated (Shianna and Willard 2006), as their high throughput and automated analysis is readily accessible. These variations can be used as markers in linkage (Amos et al. 2006) and genome-wide analyses (John et al. 2004; Middleton et al. 2004). Moreover, they were investigated as functional variants in both coding (Ollerenshaw et al. 2004) and noncoding (Kereszturi et al. 2006a; Okuyama et al. 1999) gene regions. Repeat polymorphisms (i.e., microsatellites, deletions, duplications, and VNTR), on the other hand, are of great practical and functional significance for several reasons. First, the application of multiallelic microsatellites can improve the efficiency of whole-genome scans by increasing the power of the analysis compared with the use of biallelic SNPs (Vignal et al. 2002). Moreover, a large set of repeat polymorphisms have been investigated in association analyses, and their functional effect was also demonstrated (Nakamura et al. 1998). VNTRs in coding regions can result in either frameshift and consequently a truncated protein (e.g., 13-bp deletion in the human *DRD4* gene [Nothen et al. 1994]) or in a repeated amino acid sequence (e.g., human and canine *DRD4* exon 3 polymorphism).

Here we described several novel VNTRs of the dopaminergic genes in the canine genome besides the extended study of the known polymorphisms in the *DRD4* gene (Table 4). Our results show a considerable similarity between human and canine VNTRs only in exonic polymorphisms, while the intronic polymorphic variations have quite a different nature between the dog and the human genome (Table 4). It is obvious that the nonexonic regions of the genome have a lower selection pressure and therefore a higher variability among species. On the other hand, intronic sequences might have an important regulatory role in gene expression, as was shown for a VNTR in intron 2 of the human serotonin transporter gene (Fiskerstrand et al. 1999). Moreover, a considerable homology was demonstrated between the repeat sequences in the noncoding region of the human dopamine and serotonin transporters in spite of their differential localizations (Michelhaugh et al. 2001). Functional significance of the canine repeat regions analyzed in our study is also conceivable, as the binding site of the human Sp1 transcription factor can be demonstrated in the VNTR of tyrosine hydroxylase intron 4, whereas the *DBH* intron 4 VNTR contains an AML-1a transcription factor binding site according to TFSEARCH (Searching Transcription Factor Binding Sites ver 1.3; http://www.cbrc.jp/research/db/TFSEARCH.html)

database. More studies are needed, however, to establish the functional significance of the identified nonexonic polymorphisms of the dopaminergic genes of the dog genome.

Our results also illustrate that the genetic architecture of various dog breeds is quite different. A high divergence was observed even within the Belgian Shepherd breed, between Belgian Malinois and the two other Belgian Shepherd subgroups (Table 2, tyrosine hydroxylase intron 4, dopamine transporter intron 9). Another interesting finding is that the genetic difference among dog breeds was shown to be larger than the variability between dogs and wolves. This observation is in agreement with the findings of Vila et al. (1997), who investigated mitochondrial DNA. They suggested that the genetic difference between dogs and wolves may be not so significant because of the fact that the two species continued to exchange genetic material after their divergence, which happened more than 100,000 years ago.

Our study also suggested that the investigated length polymorphisms could have a functional role in determining behavior in Belgian Tervuerens. Although the sample size in this association study is not too high, these are the first results showing the effect of a few gene polymorphisms in a physiologic system on observable behavior in a relatively homogeneous sample, i.e., in a single breed. Furthermore, in an earlier study we found some effect of *DRD4* exon 3 polymorphism on attention deficit in police German Shepherds but not in pet German Shepherds (Hejjas et al. 2007). It is important to note that genetically homogeneous sample populations are preferred to be analyzed in genotype–phenotype association studies to avoid false-positive correlations (Hamer and Sirota 2000).

These findings can be supported in the future by large-scale studies. Such genetic effects on behavior could have, in principle, an important consequence in dog breeding. Attention skills have significant relevance in trainability and communicative behavior, both of which contribute to the everyday challenges of dog-human interaction. Present results suggest that the process of selecting a dog for a definite purpose such as therapy, sport, police work, or pets can be based on the animal's genetic composition, so a large amount of money and energy can be saved if the individual with appropriate genotype is chosen. Furthermore, dogs can offer an opportunity to model human dopaminergic diseases as a natural animal model (Overall 2000).

In summary, we developed and applied a PCR-based technique for the investigation of *in silico* identified putative repeat polymorphisms and verified the presence of VNTRs in some candidate genes of the dopaminergic neurotransmitter system in four dog breeds (Belgian Tervueren, Belgian Groenandael, Belgian Malinois, and

Table 4 Repeat sequences and their polymorphisms among the genes of dopaminergic neurotransmitter system

Gene		Human		Dog/Wolf		Location
		Length	Allele	Length	Allele	
DRD4	5′ region	120 bp	1–4	?	?	
	exon 1	12 bp	1–2	24 bp	ins/del	chr 18[a]
	exon 3	48 bp	2–10	39 bp	2–8	chr 18: 28722804–28723009
				12 bp		
DAT	intron 5	–	–	59 bp	np	
	intron 8	30 bp	2–4	–	–	
	intron 9	–	–	38 bp	1–2	chr 34: 142 411 027–142 411 102
	3′ region	40 bp	9–11	–	–	
DBH	intron 4	–	–	17 bp	1/3	chr 9: 53 348 625–53 348 675
	exon 11	–	–	24 bp	np	
TH	intron 4	–	–	36 bp	1–2	chr 18: 49 355 111–49 355 075

? = sequence is unknown. Numbers in the allele columns indicate the repeat number except in the case of dog *DRD4* exon 3 polymorphism where alleles are designated according to Niimi et al. (1999); np = nonpolymorphic repeats. The length of the repeated modules is shown in the length column. Chromosomal localization of the polymorphic VNTRs is also shown according to the Ensembl database

[a] The whole sequence of the *DRD4* gene is not available in Ensembl; sequence information was obtained from NCBI, accurate chromosomal localization cannot be obtained however

German Shepherd) and in wolves. We presented some preliminary results that *DRD4* exon 1, *DBH*, and *DAT* polymorphisms can be associated with attentive behavior in Belgian Tervuerens. These novel variants are subjects for further analysis in other populations as well as for behavioral association studies, which can clarify their role in the development of various phenotypic traits in dogs and wolves.

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