FULL PAPER *Ethology*

Allele Frequency Distribution of the Canine Dopamine Receptor D4 Gene Exon III and I in 23 Breeds

Hideyuki ITO[1], Hidetoshi NARA[1], Miho INOUE-MURAYAMA[1]*, Makoto K. SHIMADA[1], Akiko KOSHIMURA[1], Yuko UEDA[1], Hitoshi KITAGAWA[2], Yukari TAKEUCHI[3], Yuji MORI[3], Yuichi MURAYAMA[4], Mitsuo MORITA[5], Toshiroh IWASAKI[6], Katuaki ÔTA[7], Yuichi TANABE[8] and Shin'ichi ITO[1]

[1]Laboratories of Animal Genetics, [2]Veterinary Internal Medicine, Gifu University, Gifu 501–1193, [3]Laboratory of Veterinary Ethology, The University of Tokyo, Tokyo 113–8657, [4]National Institute of Animal Health, Tsukuba 305–0856, [5]Livestock Improvement Association of Japan, Inc., Maebashi 371–0121, [6]Department of Veterinary Internal Medicine, Tokyo University of Agriculture and Technology, Tokyo 183–8509, [7]Faculty of Agriculture, Shinshu University, Nagano 399-4598 and [8]School of Veterinary Medicine, Azabu University, Kanagawa 229–8501, Japan

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ABSTRACT. Various canine breeds are remarkably different from each other not only in their sizes and shapes but also in behavioral traits, suggesting that some of them are under genetic control. Although dopaminergic neurotransmission system is considered to affect animal behavior, little is known about related genes in canine. Relations between specific alleles in polymorphic regions of the dopamine receptor D4 gene (*DRD4*) and personality or psychiatric disorders have been reported in humans, and we first found polymorphism in exon III region of the gene in 4 canine breeds. In this study we surveyed allele frequency distribution in 23 breeds including a total of 1,535 unrelated individuals. In exon III, 8 alleles including a novel allele were identified. A group of breeds in which the alleles *447b*, *498* and *549* were frequent tended toward high scores in aggression-related behavioral traits than that with frequent alleles *435* and *447a*. Moreover, a polymorphism based on 24 bp insertion/deletion was found in exon I region for the first time in dogs. This information may be of use for candidate gene studies of behavioral variation in dogs.

KEY WORDS: canine, dopamine receptor D4 gene, exons III and I, polymorphism, variation among breeds.

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The dog (*Canis familiaris*) is one of the oldest domestic species [6]. Repeated selection for the various purposes, such as hunting, herding and guarding, has made dogs vary in size, shape and behavior. Consequently, more than 400 canine breeds currently exist, and behavioral traits as well as external morphology are different among breeds [2, 9, 19], suggesting that some of them are under genetic control.

In humans, several candidate genes have been reported to have association between their polymorphisms and particular personality [1, 8, 12, 17]. Dopamine receptor D4 gene (*DRD4*), which is one of the genes relevant to neurotransmitters, includes polymorphisms in several regions. Associations have been reported between these polymorphisms and personality or psychiatric disorders, such as 'Novelty Seeking' [1], attention deficit hyperactivity disorder (ADHD) [11] and delusional disorder [4], although a considerable number of reports found no association [3, 21]. The association between *DRD4* and 'Novelty Seeking' is further supported by the study that *DRD4* knock-out (*DRD4–/–*) mice are significantly less behaviorally responsive to novelty than are *DRD4+/+* wild-type mice [7], suggesting that this association may be applied to other mammalian species.

We previously investigated exon III region of *DRD4* in dogs, demonstrated polymorphism as observed in humans, identified 7 alleles (*396*, *435*, *447a*, *447b*, *486*, *498* and *549*) based on the number and order of the 12 and 39 bp units, and found that allele frequencies significantly varied among 4 breeds (Beagle, Golden Retriever, Shetland Sheepdog and Shiba) [14, 15]. Moreover, we also found, among species in Carnivora, only Canidae (dog, wolf, and raccoon dog) had repeat structure and/or polymorphism in exon III of *DRD4* [10], indicating that this polymorphism is not widely spread in Carnivora.

In the present study we found a new polymorphism in exon I region of *DRD4* and investigated the allele frequency distribution in both regions of exons III and I among 23 breeds including a total of 1,535 individuals. Breeds were divided into two groups by their allele frequency distribution, and the difference in the scores of behavioral traits for breeds [19] were examined.

MATERIALS AND METHODS

A total of 23 breeds were selected to cover a wide range of the place of origin (the place of breed establishment), both Occident (Europe and North America) and Orient (East Asia) [22]. Genomic DNA was extracted from blood or buccal mucous membrane obtained from a total of 1,535 unrelated individuals (close-kin, parent-child and siblings were excluded) using QIAamp blood kit (Qiagen, Valencia, CA). A list of breeds and number of individuals genotyped for each breed are presented in Table 1.

The genotyping method of *DRD4* exon III polymorphic region was described in the previous study [14]. Briefly, the *DRD4* exon III polymorphic region was amplified by PCR.

* CORRESPONDENCE TO: INOUE-MURAYAMA, M., Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193, Japan.

One hundred nanograms of DNA were used in 20 μl PCR solution containing 0.5 μM of fluorescent-labeled forward D4F (5'-TTCTTCCTACCCTGCCCGCTCATG-3') and reverse D4dogR (5'-CCGCGGGGGCTCTGCAGGGTCG-3') primers, 250 μM of dATP, dCTP, and dTTP, 125 μM of dGTP, 125 μM of 7-deaza-dGTP (Boehringer-Mannheim, Germany), 5% dimethyl sulfoxide, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 2 μg BSA, and 2 U *Pfu* Turbo DNA Polymerase (STRATAGENE, U.S.A.). After initial incubation at 98°C for 2 min, PCR amplification was performed for 35 cycles composed of denaturation at 98°C for 30 sec, annealing at 65°C for 1 min, and extension at 74°C for 1 min. This was followed by a final extension at 74°C for 10 min. The sizes of PCR products were estimated employing an ABI 3100 DNA Sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA) and GENESCAN software (Perkin-Elmer). To facilitate the identification of genotypes of the same length, D4dogBR (5'-TGGGCTGGGGGTGCCGTCC-3') was designed and employed in combination with the D4F primer. For genotyping of exon I polymorphic region, fluorescent-labeled forward (5'-CGCCATGGGGAACCGCAG-3') and reverse (5'-CGGCTCACCTCGGAGTAGA-3') primers [4] were used in the same PCR conditions as described above, except for modifying the annealing temperature to 60°C. Also, PCR products were separated by electrophoresis on 2.5% agarose gel and directly sequenced by the dye termination method using an ABI 3100 DNA Sequencer.

To illustrate the resemblance of allelic composition, a tree was constructed with respect to breeds using neighbor joining (NJ) method based on genetic distances calculated from allele frequencies of exon III [16]. For 23 breeds selected for this study, 12 behavioral traits were graded on the basis of 5 points (1; 'very low' to 5; 'very high') by the questionnaire to 191 dog specialists, such as veterinarians, trainers and groomers, and the average scores were obtained for each breed [19]. Average number of specialists answered was 85.8 per breed. The traits were 'adaptability to new owner', 'friendliness to extra family members', 'the degree to which a dog is pleased when its demand is met (degree of satisfaction)', 'the degree to which a dog is bored when its demand is not met (degree of dissatisfaction)', 'the degree to which a dog wants to be together with family members (intimacy)', 'obedience training', 'aggression to owner', 'tendency to consider itself dominant over owner when its demand is met (dominance over owner)', 'territorial defense', 'aggression to dogs', 'fear' and 'housebreaking ease'. In this study we assigned these 12 behavioral traits into fewer non-correlated components by principal component analysis (PCA) based on variance-covariance matrix of scores [18]. These traits were assigned into any of the components according to the weight of their factor loadings. Twenty-three breeds were divided into two groups by NJ method and the average scores of behavioral traits were compared between groups by one way ANOVA.

RESULTS

Eight alleles (*396*, *435*, *447a*, *447b*, *486*, *498*, *549* and *576*) were detected in exon III (Fig. 1a), 7 of which except the allele *576* have been reported in the previous study [15]. Hence, the present study has added a new allele (*576*) in dogs. By employing D4F and D4dogR primers, we determined the nucleotide sequences of *576* of exon III to be 576 bp in length. Figure 1a shows the alignment of the repetitive regions of the 8 alleles of exon III identified so far. The allele *576* shared the same sequences as the allele *549* except for insertion at the 10th and 11th units (serial number of units) and deletion at the 2nd and 6th units of the allele *549*. Also, nucleotide substitutions at 4 positions (A55G, A60G, A157G and A162G) and amino acid substitutions at 2 positions (S19G and S53G) were observed between the alleles *549* and *576*.

We found novel polymorphism in canine exon I (Fig. 1b). Alleles *S* and *L* shared common sequences, except for a 24 bp (8 amino acids) deletion in the allele *S* at the 61st nucleotide of the allele *L*. After excluding the insertion/deletion sequence from the 61st to 102nd nucleotide positions in Fig. 1b, the sequence and deduced amino acid homology between humans and dogs were 0.91 and 0.89, respectively. In humans, polymorphic region of exon I consists of the repeat structure of 4 amino acids, GASA. On the other hand, the repeat structure was not observed in dogs. Therefore, the polymorphic region was less homologous between humans and dogs compared to the other region of exon I. The low homology between humans and dogs was also observed in polymorphic region of exon III [14].

The allele frequencies and expected heterozygosities (H_e) [13] of exons III and I in the individual breeds are presented in Table 1. Exon III was polymorphic in all of the 23 breeds and 2 to 7 alleles for each breed were observed, although there was no breed which had all of the 8 alleles. On the other hand, for exon I, most of the breeds had both alleles *S* and *L*, except Hokkaido Dog and Shikoku Dog, which had only the *L* allele. Although the allele *L* was predominant in most breeds, the allele *S* was especially predominant in Pug (0.781) and relatively high frequency (0.500) were observed in Toy Poodle.

In a neighbor joining tree based on the allele frequencies of exon III, the 23 breeds were divided into 2 main groups (Fig. 2). Group A comprised 15 breeds in which alleles *435* and *447a* were frequent. On the other hand, 8 breeds in group B had *447b*, *498* and *549* frequently. The group A were composed of breeds of Occidental origin except Pug, while in the group B, breeds of Oriental origin were prevalent except Siberian Husky, Welsh Corgi Pembroke and West Highland White Terrier. In exon I, since the allele *L* was predominant in most breeds and grouping by allele frequencies was difficult, further analysis on relation with behavioral trait scores was not made.

The average scores for 12 behavioral traits of 23 breeds in the groups A and B were compared (Table 2). By PCA of 12 behavioral traits, the percentage of contribution of the

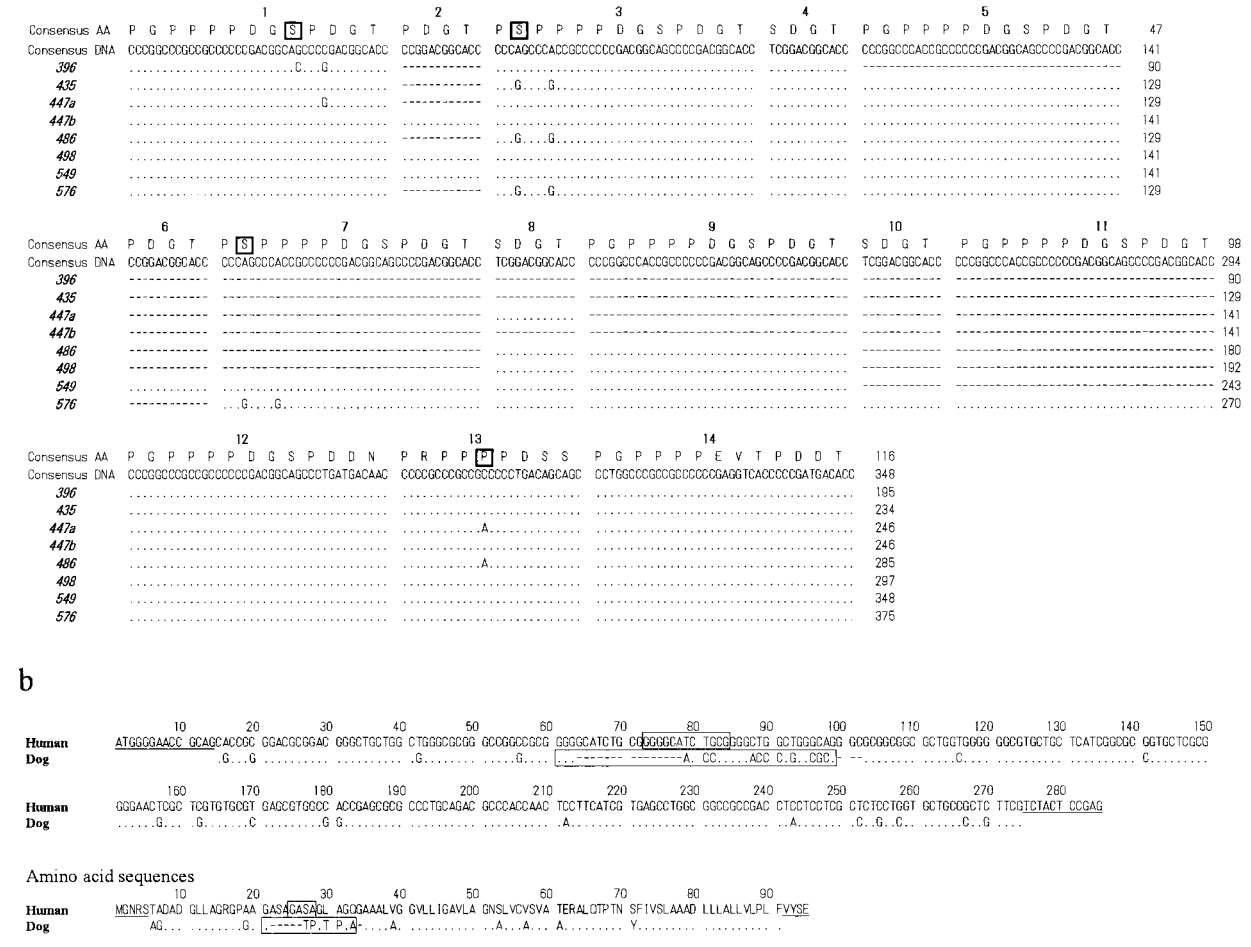

Fig. 1. (a) Multiple alignment of *DRD4* exon III repeat sequences in the dog. The consensus nucleotide sequence and amino acid (AA) sequence are also shown in the figure. Full points indicate identity with the consensus DNA sequences. Amino acid substitutions occurred at four positions (boxed, SSSP→TGGT). Dashes indicate deletions. (b) Comparison of nucleotide and amino acid sequences between human and dog *DRD4* exon I. Full points and dashes in the dog sequence indicate identity with and deletions from the human sequence, respectively. Boxes indicate inserted regions compared with the shorter allele. Underlined parts indicate coding sequences of primers. Full sequences of primers are indicated in text. Dog sequences of the allele *576* in exon III, and short (*S*) and long (*L*) alleles in exon I can be obtained from the DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers, AB105052, AB105053, and AB105054, respectively.

first and the second principal components were 69.2% and 17.3%, respectively. The nine traits, 'friendliness to extra family members' (0.985), 'adaptability to new owner' (0.978), 'degree of satisfaction' (0.850), 'intimacy' (0.663), 'degree of dissatisfaction' (0.637), 'dominance over owner' (–0.805) 'aggression to owner' (–0.908), 'territorial defense' (–0.942) and 'aggression to dogs' (–0.975) made very high contribution to the first principal component. Numbers in parenthesis indicate factor loadings. The former 5 traits, expressed as a category 'reactivity' positively, and the latter 4, expressed as 'aggressiveness', negatively contributed. Among the remaining 3 traits, 'housebreaking ease' (0.808) and 'obedience training' (0.792) positively, and 'fear' (–0.779) negatively contributed to the second principal component. The former two traits were categorized as 'trainability'. In Table 2, breeds in the group A showed higher scores in 'reactivity' and lower scores in 'aggressiveness' than those in the group B ($P<0.01$).

DISCUSSION

In this study, an additional new allele was identified in exon III polymorphic region of canine *DRD4*, and in exon I a novel polymorphism was found based on 24 bp insertion/deletion. Allele frequencies of both exons III and I showed

Table 1. Allele frequencies and expected heterozygosities of exon III and exon I in 23 dog breeds

Breed	exon III											exon I				
	Number of individuals genotyped (*n*)	Allele frequency (*qi*)								Number of alleles found	H_e[a)]	Number of individuals genotyped (*n*)	Allele frequency (q_i)		Number of alleles found	H_e[a)]
		396	*435*	*447a*	*447b*	*486*	*498*	*549*	*576*				*S*	*L*		
Akita	19	0.000	0.000	0.079	0.553	0.132	0.211	0.026	0.000	5	0.643	15	0.067	0.933	2	0.129
Beagle	142	0.011	0.606	0.345	0.035	0.000	0.000	0.004	0.000	5	0.515	102	0.113	0.887	2	0.201
Cavalier King Charles Spaniel	35	0.014	0.229	0.714	0.014	0.000	0.000	0.029	0.000	5	0.443	10	0.450	0.550	2	0.521
Chihuahua	32	0.016	0.313	0.594	0.047	0.000	0.016	0.000	0.016	6	0.556	14	0.107	0.893	2	0.198
German Shepherd Dog	25	0.000	0.580	0.360	0.020	0.000	0.040	0.000	0.000	4	0.543	17	0.118	0.882	2	0.214
Golden Retriever	174	0.000	0.741	0.227	0.032	0.000	0.000	0.000	0.000	3	0.399	149	0.198	0.802	2	0.319
Great Pyrenees	16	0.000	0.719	0.250	0.031	0.000	0.000	0.000	0.000	3	0.433	10	0.300	0.700	2	0.442
Hokkaido Dog	37	0.000	0.054	0.014	0.189	0.000	0.446	0.297	0.000	5	0.683	33	0.000	1.000	1	0.000
Labrador Retriever	134	0.000	0.246	0.720	0.034	0.000	0.000	0.000	0.000	3	0.421	136	0.379	0.621	2	0.472
Maltese	126	0.000	0.194	0.694	0.087	0.004	0.008	0.008	0.004	7	0.474	86	0.192	0.808	2	0.312
Miniature Shunauzer	53	0.000	0.000	0.972	0.019	0.000	0.009	0.000	0.000	3	0.056	46	0.163	0.837	2	0.276
Papillon	15	0.000	0.400	0.600	0.000	0.000	0.000	0.000	0.000	2	0.497	10	0.100	0.900	2	0.189
Pomeranian	57	0.000	0.544	0.219	0.070	0.000	0.158	0.009	0.000	5	0.632	21	0.095	0.905	2	0.177
Pug	46	0.000	0.130	0.685	0.185	0.000	0.000	0.000	0.000	3	0.485	16	0.781	0.219	2	0.353
Shetland Sheepdog	107	0.000	0.164	0.813	0.005	0.000	0.005	0.000	0.014	5	0.313	52	0.106	0.894	2	0.191
Shiba	192	0.000	0.008	0.096	0.531	0.047	0.055	0.260	0.003	7	0.637	138	0.022	0.978	2	0.043
Shih Tzu	88	0.000	0.028	0.278	0.688	0.006	0.000	0.000	0.000	4	0.452	35	0.071	0.929	2	0.135
Shikoku Dog	63	0.000	0.000	0.008	0.921	0.000	0.008	0.063	0.000	4	0.149	66	0.000	1.000	1	0.000
Siberian Husky	47	0.000	0.011	0.330	0.128	0.011	0.511	0.011	0.000	6	0.620	19	0.237	0.763	2	0.371
Toy Poodle	30	0.000	0.150	0.750	0.100	0.000	0.000	0.000	0.000	3	0.412	13	0.500	0.500	2	0.520
Welsh Corgi Pembroke	13	0.038	0.077	0.346	0.385	0.038	0.077	0.000	0.038	7	0.745	13	0.154	0.846	2	0.271
West Highland White Terrier	35	0.000	0.000	0.429	0.029	0.000	0.543	0.000	0.000	3	0.528	33	0.136	0.864	2	0.239
Yorkshire Terrier	49	0.000	0.388	0.490	0.061	0.020	0.010	0.000	0.031	6	0.611	20	0.200	0.800	2	0.328
Total	1535											1054				

a) Expected heterozygosity: $H_e=2n(1-\sum q_i^2)/(2n-1)$ [13].

a difference among 23 breeds.

Exon I corresponds to extra-cellular region of the receptor and it is not expected that polymorphism in this region results in altered ligand-binding properties of the receptor. However, there might be a possibility of linkage with some functional difference of the receptor. In humans, recombinant proteins of both alleles in exon I are reported to have a minor effect with respect to antipsychotic to quinpirole and clozapine *in vitro*, however, no functional differences were detected for receptor activation by dopamine [23]. The analysis of the effect of canine alleles on the difference in functions should be needed in the next step.

The 23 breeds were divided into 2 main groups based on the allele frequencies of exon III, the group A in which alleles *435* and *447a* were frequent and the group B with frequent *447b*, *498* and *549*. In the group B, the average score of 'aggressiveness' was higher and that of 'reactivity' was lower compared to those in the group A. Therefore, the alleles *447b*, *498* and *549* might have relation with 'aggressiveness' in dogs. In humans, longer alleles of exon III are reported to have relation with higher score of 'Novelty Seeking' [1], however, we could not compare scores of 'Novelty Seeking' between canine breeds in this study. In dogs, the alleles *498* and *549* are 63 bp (21 amino acids) and 114 bp (38 amino acids) longer than the allele *435*, respectively, and this difference in length may affect the function of the receptor. On the other hand, *447a* and *447b* share the same length and are different each other in the position of 12 bp sequence insertion and a base substitution [14]. Since the sequence of polymorphic region is less homologous between dogs and humans compared to the other regions [14], sequence difference as well as length difference may affect the function of receptor in dog.

Another possible course for this difference is geographical regional origin of breeds. The allele frequencies in Occidental and Oriental breeds greatly differed, suggesting that the gene constitution differed among ancestors of breeds domesticated in different regions of the world. In humans, the difference between Asian and European populations in the allele frequencies in *DRD4* exon III has also been reported [5]. To elucidate the contribution of geographical effect and behavioral effect to the allele frequency distribution in breeds, a comparison of behavioral scores of breeds with different allele construction originated in the same geographical region will be necessary. In this study although the number of breeds originated in the same region was not enough to obtain significant difference (in groups A and B, breeds of Occidental origin were 14 and 3, and those of Oriental origin was 1 and 5, respectively), the tendency of the average scores within each geographical region was the same as the result of whole 23 breeds. Further analysis using larger number of breeds will be necessary to elucidate the effect of genetic difference on canine behavior.

This is the first report suggesting the relation between genetic variation and behavioral variation in animals except humans. More detailed analysis concerning correlation between individual behavioral traits and individual genotypes within a breed is underway by authors. Furthermore, it will be necessary to analyze in dogs the other genes, which were suggested to play some roles in human personality, such as those concerning dopamine transporter [17] and serotonin transporter [12]. Indeed, if some genes modulate the behavioral traits of dogs, they may become useful markers for selecting working dogs, such as guide dogs for the visually-impaired or the hearing-impaired, drug sniffing dogs and rescue dogs by aptitude for various purposes. In

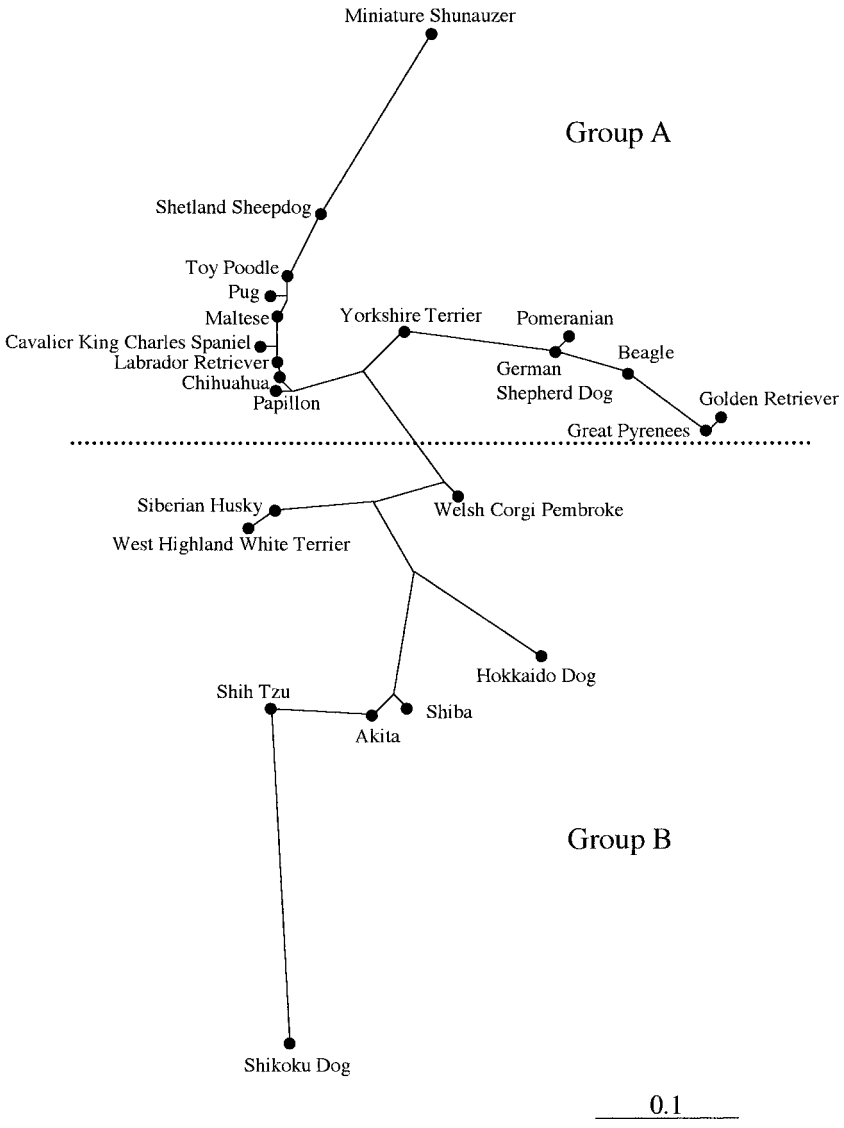

Fig. 2. A neighbor joining tree indicating the resemblance of allele composition of exon III made by the NJ method using genetic distance [16]. Breeds were divided into 2 main groups, A and B.

Table 2. Average behavioral trait scores in two groups of breeds sorted by exon III allele frequencies

	1st principal component (69.2%)		2nd principal component (17.3%)	
	+ (5 traits)	– (4 traits)	+ (2 traits)	– (1 trait)
	Reactivity[a)]	Aggressiveness[b)]	Trainability[c)]	Fear[d)]
Group A (*n*=15)	3.33 ± 0.27	2.83 ± 0.34	3.23 ± 0.39	3.01 ± 0.50
Group B (*n*=8)	2.90 ± 0.43	3.38 ± 0.43	3.17 ± 0.17	2.77 ± 0.44

a) F=8.57, *P*=0.008.
b) F=10.97, *P*=0.003.
c) F=0.18, *P*=0.675.
d) F=1.28, *P*=0.271.

the same context of searching genetic background of canine behavioral traits or psychiatric problems, canine serotonin receptor 1A gene was recently isolated and a nucleotide substitution between breeds was reported [20]. The difference in allele frequency distribution among breeds showing different behavioral traits reported in this paper will provide a basic information for these further analysis.

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