Animal Cells and Systems 12: 77-83, 2008

Animal Cells
and
Systems
http://www.AnimalCells.or.kr

The Dopamine D4 Receptor Polymorphism Affects the Canine Fearfulness

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Abstract: The canine fearfulness is a behavioral trait known to have a genetic basis. This research analyzed genetic effects of the dopamine D4 receptor polymorphism on this behavior by postulating a mixed model of inheritance. Genotyping for the three different repeat polymorphism found in the third exon of the receptor gene was carried out for the population of the Korean native dogs. Four hundred fifty eight dogs with known pedigree were genotyped, and 264 individuals were tested for their fear responses to an experimenter, in which four different behavioral paradigms were adopted. Since the results assessed by principal factor analysis revealed a major factor explaining 69% of the total phenotypic variance, the subsequent analyses were conducted for this quantity. Analyses of the factor scores by estimating their posterior means indicated that there is a fixed effect exerted by the three different repeat polymorphism found in the D4 receptor as well as sex, in addition to unidentified polygenic effects. The phenotypic contribution of the D4 genotype was roughly estimated to be about 2%, which is a fraction of the total genetic effects responsible for more than 20% of the total phenotypic variance.

Key words: dopamine receptor, fearfulness, dog, polymorphism, behavior

Dog's response of fear has been observed against various objects, which can be measured as a behavioral phenotype. According to Melzack (1952), four criteria of fear expression were noted; (1) turning its head more than 90 degrees to avoid an object, (2) lowering its head and body as well as flattening ears, (3) hiding in a place away from the object, and (4) sitting at far end of the cage to maintain the furthest distance from the object. The last observation was regarded as an extreme criterion for fearfulness. In this test, two different types of objects were employed, i.e. stationary objects and objects with movable part.

In a subsequent study (Goddard and Beilharz, 1983 and 1985), a large battery of behavioral tests (thirty eight) was developed. A factor analysis of the test scores obtained for the progenies of four different breeds indicated that the phenotypic variation in fear response could be described in terms of 12 factors. A further statistical analysis identified the three discriminant functions, implying three underlying behavioral dimensions labeled as "general fearfulness", "fearfulness of objects", and " inhibited response to fear". A heritability estimate for the subdimensions ranges from 0.47 to 0.80 (Goddard and Beilharz, 1985).

The D4 dopamine receptor has been subjected to behavioral studies in human and mouse. Initially, the repeat polymorphism found in the 3rd exon of the receptor was reported to be associated with the novelty seeking trait in humans (Ebstein et al., 1996; Benjamin et al., 1996), which was supported by an observation with a knock-out mice (Dulawa et al., 1999). The human polymorphism contains variable number of tandem repeats (VNTR) of 48-bp unit, normally occurring in 2-10 copies. Recently, it was reported that a particular allele with seven repeat has been selected in human population (Ding et al, PNAS,2002). A similar repeat polymorphism with variable unit sizes of 27, 39, and 51 bps was found in the canine D4 receptor (Niimi et al., 2001; Inoue-murayama et al., 2002; Jeoung et al., in preparation), which is analogous to the primate counterpart in its location and structural characteristics. They are part of the 3rd cytoplasmic region, exhibiting any notable sequence similarity to that of human except the proline-rich characteristics.

As a study to elucidate functional consequence of genetic polymorphism found in the canine D4 gene, we examined whether the variation at D4 locus, together with other polygenic effects, affect fearfulness of dogs. An analysis

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based on the mixed model revealed that the D4 polymorphism with other unknown genes significantly influences canine fearfulness.

MATERIALS AND METHODS

Animal

The animals tested in our study were chosen from a colony with several hundred dogs raised and maintained in an isolated farm. This Korean breed, *Sapsari* (Kim et al., 1998), tends to recognize humans as large and unfamiliar objects because they have few contacts with humans. Thus, they tend to be cautious and express fear whenever they encounter people except breeders who feed them. In this study, we measured fearfulness behaviors of individual dogs of ages from 6 months to 3 years. The sizes of canine pedigrees used in this analysis were summarized in Table 1. The largest pedigree comprises 268 individuals in seven generations.

Genotyping of the repeat alleles by PCR amplification

Genomic DNAs from *Sapsari* were prepared by the standard method with a minor modification. The canine specific primers, 5'-ggtgg ccgtg ccgct gagtt acaac cgcca-3' (forward) and 5'-cgacc accac gggca gcacc ctcata gcct-3' (reverse), recognizing an immediate upstream region of TM4 and the portion of TM6 were used for PCR amplification of the D4 repeat sequence. The final reaction mixture contains 75 mM Tris-Cl, pH 9.0, 15 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 100 mg/ml BSA, 200 ng of template DNA, 0.4 mM of each primers, 0.2 mM dNTPs, 2 M betain, and 2 units of Taq polymerase in a total volume of 50 ml. PCR reaction was performed with 1 cycle at 95°C (2 min), 35 cycles at 95°C (1 min)/62°C (1 min)/74°C (3 min), followed by an extension reaction at 72°C for 20 min. The PCR products were electrophoresed on 2.0% agarose gel and scored by sizes of 718, 769 and 1218 bp for alleles of small (S), large (L) and ultralong (U).

Fearfulness test

A person never exposed to the dogs was considered a stranger. The home cage was fenced surrounding the area of 6×4 m^2, in which the test was carried out. For an entry of experimenter, a door was installed on one side. The following protocol for behavioral test was initially set up. The experimenter who made observation did not use any fragrances such as perfume and lotions. Also to minimize an environmental disturbance regarding odor, windy days were avoided. Furthermore, the experimenters spent half an hour to get accustomed to odor surrounding the cage prior to an observation. Cages for observation were randomly selected. In order to avoid the influence by the previous test, a careful consideration was given. For example, after monitoring the responses of dogs in the first cage, the experimenter moved to the fifth cage for another testing that was not closely located. Any unusual movement or loud noise was prohibited because they might be perceived as threats by the dogs.

To measure fearfulness, dog's responses to a stranger in four different test situations (O1-O4) were recorded. Our choice of the test objects is comparable to the one used by Melzack (1952) or to the FM6/12 criteria of Goddard *et al.* (1985), which were taken to score the degree of avoidance from the experimenter. Some of the responses were similar to the ones observed by Malzack (1952), but a series of dog's responses to each test object were not exactly matched. Thus, the responses to each test object were ranked independently as in Table 2. The first rank was given to the extreme fearfulness response and the next to the second best, etc.

The actual tests were performed as the followings. First, the experimenter examined the dog's responses when the experimenter was standing outside the cage with the dog inside (the O1). Second, the experimenter walked in and stayed inside the cage while examining the dog's responses (O2). Third, the response was monitored when he or she approached the dog (O3), and when the experimenter touched the dog with his or her hand (O4). The tests were conducted more than five times with an interval of more than a week. The arithmetic means of the observed ranks, excluding the maximum and minimum values, were used as the fearfulness scores (S1-S4) obtained by O1-O4 tests, respectively. All the scores were normalized.

In order to find the relationship among the fearfulness scores for different test objects, correlations between the scores obtained were assessed, followed by principal component factor analysis with varimax rotation (SAS Inst. Inc., Cary, NC, USA). The scores of principal components achieved by each dog were also normalized for easy comparison with the original scores.

Genetic model

The study used a mixed inheritance model as shown below:

$$y = X\beta + Za + e,$$

where y represents a vector of observations for behavior performance, β a vector of sex and fixed genotypic effects, and a a vector of additive polygenic random effects with the assumption of $a \sim N(0, A\sigma_a^2)$, where A is polygenic relationship matrix among animals in the pedigree (Quaas, 1976). σ_a^2 is additive polygenic variance component, and e is a random vector of residuals with the assumption of $e \sim N(0, I\sigma_e^2)$, where I is identity matrix, and σ_a residual variance component. X and Z are known incidence matrices relating the fixed and random effects, respectively, to their corresponding observations.

Parameter estimation

Inferences about unknown variance are based on their marginal posterior distribution components in Bayesian approaches, and the marginalization of the joint posterior distribution can be attained through Gibbs sampling (Tanner, 1993). The joint posterior distribution of all parameters in our model can be expressed by Bayes theorem:

$$f(\beta, a, \sigma_a^2, \sigma_e^2 | y) \propto f(y|\beta, a, \sigma_a^2, \sigma_e^2) f(a|\sigma_a^2)\pi(\beta)\pi(\sigma_a^2)\pi(\sigma_e^2)$$

where $f(y|\beta, a, \sigma_a^2, \sigma_e^2) \sim N(X\beta + Za + I\sigma_e^2$ and $f(a|\sigma_a^2) \sim N(0, A\sigma_a^2)$. The $\pi(\beta)$, $\pi(\sigma_a^2)$, and $\pi(\sigma_e^2)$ are the priors for β, σ_a^2, and σ_e^2, respectively. For the priors, uniform distribution was assumed for the fixed effects, and inverse Gamma distribution was assumed for the variance components, because the application of the Gibbs sampling with flat priors for variance components may yield a theoretically improper posterior distribution (Hobert and Casella, 1996). Furthermore, very small shape parameters for the inverse Gamma distributions were taken due to a lack of prior information, and Gibbs sampling was applied to parameter estimation. It enabled random samples to be drawn with desired posterior distributions.

Then, initial values were randomly assigned for all unknowns. Sampling from the full conditional posterior distributions and updating the distribution based on the sample were repeated to the convergence. Algorithms from Numerical Recipes by Press et al. (1992) were used to generate the samples. Programs from FSPAK by Misztal (1990) were used to deal with sparse matrix inversion. Gibbsit program by Raftery and Lewis (1995) was used to determine warming-up periods and thinning intervals. The Gibbs sampler was run 102,000 rounds, and the first 2000 rounds were discarded as a warming-up period. A conservative thinning interval of 100 rounds was used to retain sampled values that reduced lag correlation among thinned samples. The point estimate used in the current study was the posterior mean estimate, calculated as the mean of the conditional expected values of the parameters in post warming-up rounds.

RESULTS

Allele frequencies of the D4 repeat polymorphism

The repeat region of the canine D4 receptor was found in the 3rd exon of D4 gene, including the 3rd cytoplasmic region of the receptor. This region consists of a novel amino acid sequence preceded by approximately 243 amino acids from the N-terminus that are homologous in the mammalian D4 receptors. The block of repeated sequence is followed by a homologous region of 89 amino acids extended to the C-terminus. To detect various repeat alleles in the D4 receptor gene of the dog population, two primers were designed to amplify the corresponding region of 0.7-1.2 Kb in size. Based on the previous identification of three different alleles (Fig. 1) by DNA sequencing, allelic variations in the *Sapsari* population were genotyped on agarose gel electrophoresis (Materials and Methods). Among 458 animals tested in twelve pedigrees (Table 1), the three alleles of *S*, *L*, and *U* were found at the frequencies of .57, .36, and .07, respectively. The genotypes of SS, LL, SL, SU, and LU among 458 dogs were found at the frequencies of 0.31, 0.106, 0.458, 0.07, and 0.056.

Correlations between the behaviors assessed by factor analysis

Measurements of canine fearfulness were conducted for the test objects or situations (Table 2). Various responses of the dogs to each test were recorded and converted into scores as described in the Materials and Methods section. Thus, each dog has four fearfulness scores, S1 through S4, as measured in Table 2. They were subject to a factor analysis, from which two major components, F1 and F2 presumably associated with fear response, were drawn, each explaining 69 and 17% of the total phenotypic variance, respectively. Figure 2 displays normal distribution of dogs exhibiting different degrees of F1 or F2 fearfulness, shown here as the normalized scores. The F1 factor is almost equally loaded onto the four fearfulness scores (0.86, 0.86, 0.86, and 0.72), while F2 factor in varying degrees (–0.34, –0.32, 0.12, and 0.65). The result indicates that the general fearfulness is represented by F1. An interpretation about F2, however, remains to be determined. This result provides a basis for further analysis of the fearfulness mainly in terms of the F1 scores.

Estimations of fixed effects, variance components, and their correlations

Based on the mixed model of inheritance (Materials and Methods), variance components were inferred from the marginal posterior distributions. Variances due to polygenic and environmental effects were estimated, as shown in Table 3, which are statistically significant ($p<0.05$). The polygenic effect (σ_a^2) on F1 is approximately 20% of the total phenotypic variance, while the environmental effect (σ_e^2) was about 70%. Therefore, about 10 % of the remaining phenotypic variance might be attributed to the fixed effects by sex and D4 genotype.

The posterior mean estimates for the fixed effects due to genotype and sex were obtained (Fig. 3), which are statistically significant ($p<0.05$) for both factors deduced previously. A female is more fearful than a male when the fixed effect was assumed to be caused only by sex difference. The SS and SU groups are the least fearful, while SL and LU groups are the most fearful groups with

```
39A :      cccggcccaccgccc cccgacggcagc cccgacggcacc
            P  G  P  P  P    P  D  G  S    P  D  G  T
39B :      ---a----------- ------------ ------------
              S

51A :      cccggcccaccgccc cccgacggcagc cccgacggcacc tcggacggcacc
            P  G  P  P  P    P  D  G  S    P  D  G  T   S  D  G  T
51B :      ---a----------- ------------ ------------ ------------
              S
51C :      --------g------ ------------ ------------ c-----------
                                                    P
51D :      --------g------ ------------ ------------ ------------

39-27-51:
cccggcccgccgccc cccgacggcagc cctgatgacaac ccccgcccgccgccc cctgacagcagc cctggcccgccgccc cccgaggtcacc cccgatgacacc
cccgacgccaca
  P  G  P  P  P    P  D  G  S    P  D  D  N    P  R  P  P  P    P  D  S  S    P  G  P  P  P    P  E  V  T    P  D  D  T    P  D  A
T

S :  51C-51B-39A-39-27-51
L :  51C-51B-51A-39A-39-27-51
U :  51D-39B-51D-39B-51D-51B-39B-51D-39A-51D-39B-51D-39B-51D-39B-51D-39A-39-27-51
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Fig. 1. Repeat patterns and variations found in the dopamine D4 receptor of *Sapsari*. The basic units with 39, 51, and 39-27-51 base pairs are shown with their nucleotide and amino acid sequences. A variation in their sequences is also designated. In our population of *Sapsari*, three different alleles (S, L, and U) were found with different repeat lengths.

Table 1. Size and genetic complexity of pedigrees used

Maximum generations	Number of pedigrees	Number of dogs
7	1	268
4	3	68
3	8	122
Total	12	458

The number of the dogs included in the principal factor analysis was 264 after omitting untested dogs.

LL group in between, when the dogs were grouped according to their genotypes disregarding their sexes. The differences in fearfulness among genotypes are not caused by sexual bias, since females are predominant in all genotype groups. The contribution of D4 genotypes to the total fixed effect was roughly estimated to be one fifth by analyzing the variances of fixed effects in each sex, which was based on the assumption that D4 genotype and sex effects are independent in fearfulness response.

Table 2. Measurement of canine fearfulness

Rank	Role of experimenter[1]			
	O1	O2	O3	O4
1	Excreting[2]	Excreting[2]	Excreting[2]	Excreting[2]
2	Hiding	Hiding	Hiding	Trembling/rigid
3	Becoming agitated	Becoming agitated	Running away	Becoming rigid
4	Sitting quietly	Sitting quietly	Sitting down plump	Startled
5	Wandering	Wandering	Sitting quietly	Mumbling
6	Approaching	Approaching	Approach/sitting	Sitting quietly
7	Licking hands	Dashing gladly	Dashing gladly	Licking
8	Dashing gladly			Lying on its back

O1: Outside the cage, O2: Standing still inside the cage, O3: Approaching the dog, and O4: Touching the dog.
Includes urination.

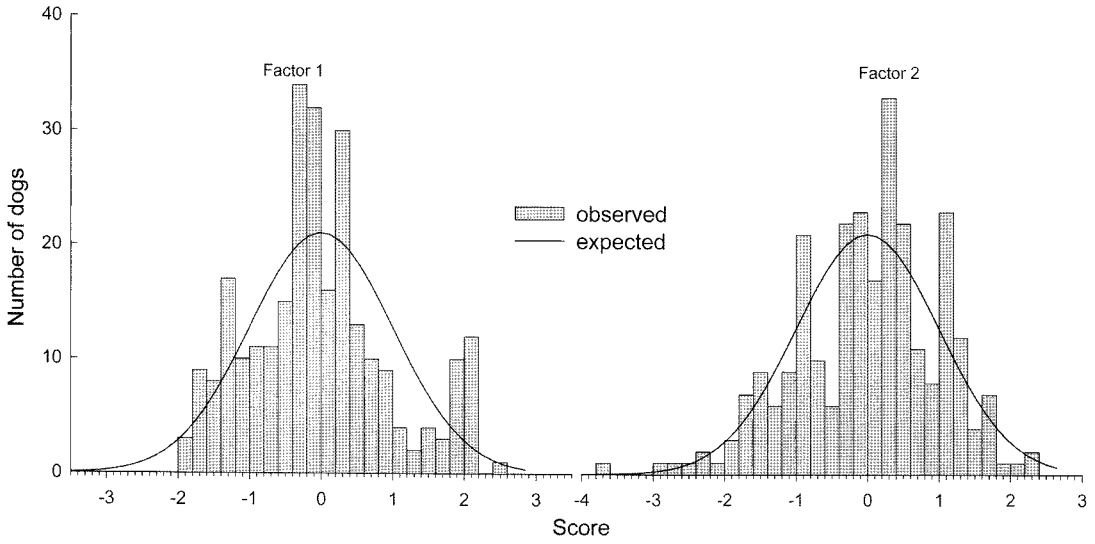

Fig. 2. Score distributions of the first and the second factors. The size of bin is 0.2. The shaded bars represent the number of observed dogs in each bin and the line represents fitted curve with standard normal distribution.

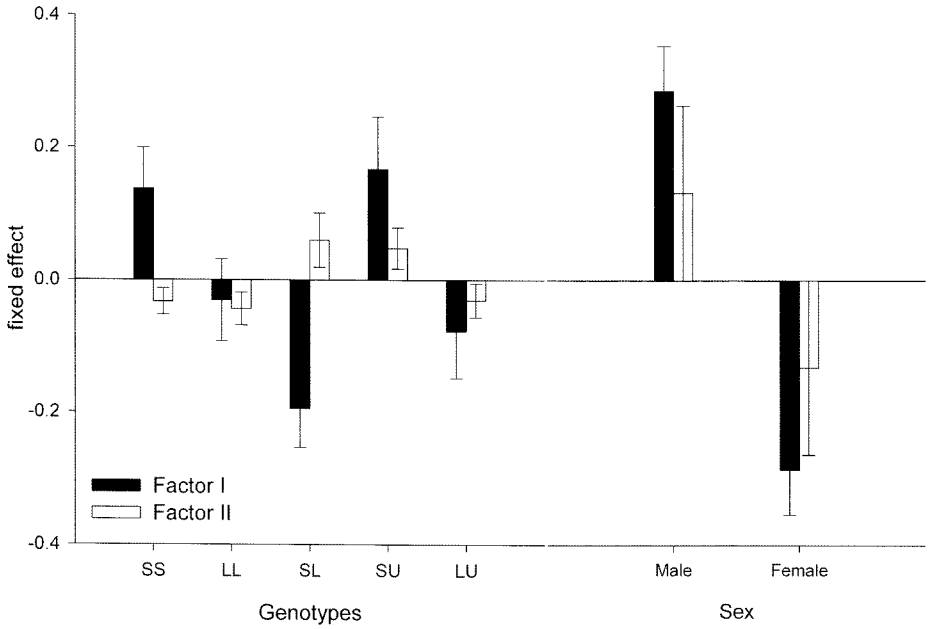

Fig. 3. Fixed effects on F1 and F2 scores of D4 genotypes and sex difference. The filled and empty bars are for the F1 and F2 scores, respectively. Error ranges are indicated by intervals. Although the analyses were primarily focused on the F1 factor score, the F2 results were also included for a comparison. In general, fixed effects of the F2 score are less significant than that of the F1 score. Genotypes of SS/SU, LL, LU, and SL with regard to F1 and those of SS/LL/LU, SL/SU with regard to F2 each represent a group, showing significant ($p<0.05$) differences among them. Sex effects were significant ($p<0.01$) on both F1 and F2.

Additive and dominance effects for the dopamine D4 receptor locus were also assessed (Table 3). The additive effect of the L allele is negative, while the effect of the S allele is positive. Over-dominance observed between the alleles, i.e., that the estimates of dominance effect between the S and L alleles are much larger, suggests that heterozygous individuals (*SL*) are more fearful than homozygous ones (*SS* or *LL*). More analysis on allelic interaction could not be made due to an absence of individuals with *UU* genotype.

Correlations within polygenic and environmental effects were assessed for all pairs of the scores (Table 4). The polygenic and environmental correlations for S1, S2, S3, and S4 are positive and ranging from 0.37 to 0.44 and from 0.42 to 0.67, respectively, indicating that the original variables are better correlated with one another in polygenic effects as well as in fixed effects. Correlations between F1 and F2 are weak, indicating that they are fairly independent even in the extracted components.

Table 3. Additive and dominance effects of dopamine D4 receptor[1]

Factors	Additive		Dominance	Variance	
	S	L	SL	σ_a^2	σ_e^2
F1	0.69	-0.15	-2.49(L)	0.20±0.05	0.70±0.16
F2	-0.17	-0.22	0.99(S)	0.12±0.04	0.82±0.25

[1]Alleles in parentheses are regarded as the dominant ones. The additive and dominance effects of *U* allele were not estimated due to an absence of individual with *UU* genotype. All the numbers were multiplied by ten.

Table 4. Correlations within polygenic (upper off-diagonal elements) and environmental (lower off-diagonal elements) estimates among the six fearfulness traits[1]

	S1	S2	S3	S4	F1	F2
S1		0.44	0.40	0.37	0.58	0.37
S2	0.67		0.38	0.39	0.63	0.33
S3	0.58	0.54		0.41	0.59	0.32
S4	042	0.49	0.51		0.49	0.30
F1	0.65	0.66	0.62	0.60		0.15
F2	0.40	0.37	0.37	0.32	0.17	

[1]Correlations were inferred based on the posterior mean estimates of polygenic effects and residuals.

DISCUSSION

We found that genetic polymorphism at the dopamine D4 receptor influenced fearfulness in an outbred population of a canine breed, *Sapsari*. The same receptor involved in the dopaminergic pathway of human has been implicated in various neurological phenomena including locomotion, emotional stability, and neuroendocrine function (Civelli et al., 1993). Indeed, pharmacological modulation of this pathway resulted in numerous neurological and behavioral consequences. In spite of these pharmaco-medical evidences, recent studies of genetic association in human, primarily focused on the repeat polymorphism (Wong et al., 2000; Lung et al., 2002; Schinka et al., 2002) and the 120-bp duplication in the 5'-UTR (McCracken et al., 2002) lend supports to both positive and negative conclusions. In most cases, the phenotype concerned was either the novelty-seeking personality trait or the attention deficit/hyperactivity disorder (ADHD). In addition, none of the genome-wide screening for related traits including ADHD (Fisher et al., 2002) found a linkage to the D4 locus.

Fearfulness is a personality trait expressing an emotional state of an organism, which is also associated with anxiety. In animal, a fear response can be elicited by various types of stimuli associated with learning, ecological history, and signals from their social group. Apparently, the type of stimulus we employed for testing dogs belongs to a category of novel object, i.e. an unexposed stranger. A similar behavioral paradigm was adopted in the systematic study of canine fear (Melzack, 1952), in which significant difference was noted in various dog breeds. Although we used a single type of object, a modification of the test, e.g. an approaching to an animal, was attempted to incorporate the results of the previous tests (Melzack, 1952).

Although more recent analysis of the canine fear response (Goddard and Beiharz, 1985) included a test of dogs to a human object with an interval of 6 months, scored as the FM6 and FM12 behaviors, they were classified into different categories other than the one named 'fearfulness of object'. It seems odd that the FM12 behavior, the most similar to ours, is in the group called 'general fearfulness' which comprises a variety of behaviors, all involving a sound cue. On the other hand, the object fearfulness defined by their third discriminant function was shown to be higher in females than in males (Goddard and Beiharz, 1985).

Although the linkage analysis searching for major genes involved in the fear and anxiety responses has yet to identify a specific gene in mouse (Flint et al., 1995, Dulawa et al., 1999), the complex nature of these phenotypes may involve a number of genes with minor contributions. As demonstrated in this study, various behaviors expressing canine fearfulness can be explained in part by the genetic diversity found in the D4 locus. In the analytical model, maximum effects of D4 locus was considered because the estimates might be confounded with the effects of other loci that are linked to the D4 locus. Nonetheless, we expected that major contribution would be due to the variation in the dopamine D4 receptor since the estimates in the current study were obtained for the D4 locus regardless of their direct or indirect effects. A possible concern on the analytical model was that the genotypes of D4 might be considered as random effects. In other words, the genotypes could be determined at random, each drawn from infinite population of possible genotypes. Yet in our case, we treated the D4 genotypes as fixed effects since the limited number of the genotypes was observed.

The statistical genetics approach introduced here to reveal an involvement of the D4 polymorphism in canine fearfulness might provide a sensitive tool for finding a genetic association in multi-factorial trait. Indeed, our result obtained with the canine model strongly supports the role of D4 receptor in determining personality trait, which is still controversial in human.

ACKNOWLEDGMENTS

C. Lee and C. Kim contributed equally to this work.
This work was supported by grant from the KOSEF.

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[Received March 10, 2008; accepted May 19, 2008]