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Polymorphism of Dopamine Receptor D4 Exon I Corresponding Region in Chicken

Akinori Sugiyama¹, Miho Inoue-Murayama^{1*}, Mitsuru Miwa¹, Riyako Ohashi¹,
Boniface Baboreka Kayang^{1†}, Makoto Mizutani², Keijiro Nirasawa³,
Masaharu Odai³, Mitsuru Minezawa⁴, Shigeru Watanabe⁵
and Shin'ichi Ito¹

¹Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan

²Laboratory Animal Research Station, Nippon Institute of Biological Science,
Kobuchizawa 408-0041, Japan

³National Institute of Livestock and Grassland Science, Tsukuba 305-0901, Japan

⁴National Institute of Agrobiological Sciences, Tsukuba 305-8602, Japan

⁵Department of Psychology, Keio University, Tokyo 108-8345, Japan

ABSTRACT—In stockbreeding, there are indications that behavioral traits of livestock have an effect on breeding and production. If the variation in individual behavior is related to that in neurotransmitter-related genes such as in humans, it would be possible to breed pedigrees composed of individuals having behavioral traits that are useful to production and breeding using selection based on genotypes. In this study, we investigated the exon I region of dopamine receptor D4 (*DRD4*), in which variation is related to psychiatric disorder in humans, in major poultry species namely Japanese quail (*Coturnix japonica*), chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*) and helmeted guinea fowl (*Numida meleagris*). Furthermore, we investigated Japanese cormorant (*Phalacrocorax capillatus*) and Japanese jungle crow (*Corvus macrorhynchos*) as an out-group. In these species of birds, the repeat of proline was identified in the region corresponding to the human polymorphic region. The repeat number was 9 in Japanese quail, ring-necked pheasant and Japanese cormorant; 12 in helmeted guinea fowl; and 3 in Japanese jungle crow. However, no polymorphism was found in these species. In contrast, polymorphism was observed in chicken and two alleles with 8 and 9 repeats were identified. Although 9 repeats (allele 9) were predominant in most chicken breeds, Black Minorca had only 8 repeats (allele 8). Intra-breed polymorphism was found in 6 out of 12 breeds, and two alleles (alleles 8 and 9) were detected in these breeds. This polymorphism, which is the first to be reported on a neurotransmitter-related gene in birds, would contribute significant information for elucidation of differences in behavioral traits in chicken breeds.

Key words: dopamine receptor D4 gene, polymorphism, poultry, chicken, proline repeat

INTRODUCTION

In stockbreeding, there are indications that behavioral traits of livestock have an effect on breeding and production. It may be possible to reduce the burden imposed on farming by selection of tame individuals in livestock and poultry.

There are many reports about the relation of behavioral traits to production in bovine and swine, which are known as

major livestock. In bovine, Simmental was more difficult to handle than German Angus (Gauly *et al.*, 2001). In swine, it was reported that there were positive relations between backtest scores and lean meat percentage that reflect a possibly heritable behavioral trait, which might be influenced by the environment and by the previous experiences (van Erp-van der Kooij *et al.*, 2000). Additionally, pigs that walked less in the open field pen seemed to have grown faster for a certain period than did pigs with high open field scores (Beilharz *et al.*, 1967).

In poultry, fearfulness and growth have been reported to be negatively associated in Japanese quail (Jones *et al.*, 1997), and feather pecking is a major welfare problem in

* Corresponding author: Tel. +81-58-293-2874;

FAX. +81-58-293-2874.

E-mail: miho-i@cc.gifu-u.ac.jp

† Present address: Faculty of Agriculture, University of Ghana Legon, Ghana

egg production of chicken (Savory, 1995). Besides, feather loss has an economic impact on egg production because it increases the bird's food requirement (Emmans and Charles, 1976; Tauson and Svensson, 1980; Tullett *et al.*, 1980) and feather pecking birds with feather damage are more fearful than non-feather pecking birds (Vestergaard *et al.*, 1993).

In a study using full-sibling families in cattle, it has been suggested that there is inheritance of variability in the vocal responses of cattle, which is independent of previous experience and learning (Watts *et al.*, 2001). Thus, it is possible to breed pedigrees composed of individuals having behavioral traits that are useful to production and breeding through artificial selection.

In humans, several candidate genes coding proteins concerning regulation of neurotransmitter such as dopamine or serotonin in the brain have been reported to have association between their polymorphisms and particular personality (Benjamin *et al.*, 1996; Lesch *et al.*, 1996; Sabol *et al.*, 1999). In human dopamine receptor, 5 subtypes are known and are classified into 2 major groups, D1 like receptors (D1 and D5) and D2 like receptors (D2, D3 and D4) by their ability to promote/inhibit adenylate cyclase activity (Seeman and Van Tol, 1994). Among them, dopamine receptor D4 gene (*DRD4*), one of the 5 dopamine receptors known in humans, has received considerable attention because it is distributed mainly in the limbic system and is expressed in neural cells having an effect upon cognitive and emotional behaviors (Van Tol *et al.*, 1991) and a possible target of antipsychotic drug (Seeman and Van Tol, 1994). *DRD4* is polymorphic at several positions of the gene and some of them are related to expression (Okuyama *et al.*, 1999), ligand binding (Van Tol *et al.*, 1992), personality (Ebstein *et al.*, 1996) and several psychiatric diseases. For example, the relation has been reported between the number of 12 bp repeats in the exon I region of *DRD4* and delusional disorder (Catalano *et al.*, 1993). We have subsequently found two alleles in the corresponding region of dog (Ito *et al.*, 2004).

In poultry, reports of dopamine receptors are few. Chicken *DRD1*, which consists of 3 subtypes (D1A, D1B and D1D) (Demchyshyn *et al.*, 1995), and turkey *DRD2* (Schnell *et al.*, 1999) have been reported. However, there has been no report about *DRD4* homologue in birds. In the present study, to help elucidate the genetics of behavior in birds, we investigated the exon I region of *DRD4* in major poultry species, namely Japanese quail (*Coturnix japonica*), chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*) and helmeted guinea fowl (*Numida meleagris*). In addition, we investigated Japanese cormorant (*Phalacrocorax capillatus*) and Japanese jungle crow (*Corvus macrorhynchos*) as an out-group. Japanese cormorants are used for a type of fishing called "u-kai", a traditional method of catching sweetfish (Inoue-Murayama *et al.*, 2002). Japanese jungle crows are regarded as one of the injurious birds and, hence, an understanding of their behavior would be

useful to reduce damages (Yamamoto *et al.*, 2001).

MATERIALS AND METHODS

We investigated the corresponding regions in avian *DRD4* employing the following species: Japanese quail ($n=96$), chicken (12 breeds; $n=492$), ring-necked pheasant ($n=20$), helmeted guinea fowl ($n=200$), Japanese cormorant ($n=23$) and Japanese jungle crow ($n=16$). Japanese quail were sampled from a colony maintained at Gifu University. Among the 12 breeds of chicken, White Leghorn ($n=145$) and Fayoumi ($n=115$) were obtained from both Nippon Institute of Biological Science (NIBS) (Kobuchizawa, Yamana-shi Prefecture, Japan) and National Institute of Livestock and Grassland Science (NILGS) (Tsukuba, Japan); Black Minorca ($n=22$) were from NIBS. Three Japanese native fowls, Tsushimajidori ($n=40$), Tosajidori ($n=10$) and Hinaidori ($n=40$), and Hamburg ($n=10$), Brabanter ($n=10$), Araucana ($n=10$), Silky ($n=10$), Rhode Island Red ($n=40$) and Barred Plymouth Rock ($n=40$) were all sampled from NILGS. Helmeted guinea fowls and ring-necked pheasants were obtained from JAFRA TRADING CO. LTD (Kasumigaura, Ibaragi Prefecture, Japan) and a pheasant farm (Kiwa, Mie Prefecture, Japan) respectively, while Japanese cormorants were sampled from a stock kept by a master of cormorant fishing on the Nagara River in the city of Gifu. Japanese jungle crows were wild-derived and kept in Keio University (Tokyo, Japan).

Genomic DNA was extracted from blood samples using the QIAamp blood kit (Qiagen, CA, USA). The genomic DNA of quail was amplified using the F (5'-CGCCATGGGGAACCGCAG-3') and R (5'-CGGCTCACCTCGGAGTAGA-3') primers of exon I polymorphic region of *DRD4* reported in humans (Catalano *et al.*, 1993). Ten μ l of reaction mixtures containing 0.5 U *LA Taq* polymerase, GC buffer I (TaKaRa, Shiga Prefecture, Japan), 20–50 ng of template DNA, 0.5 μ M of each primer, and 400 μ M of each dNTP were used. After an initial incubation at 95°C for 2 min, PCR amplification was performed for 35 cycles consisting of 95°C for 30 sec, 65°C for 1 min, and 74°C for 2 min, followed by a final extension at 74°C for 10 min. PCR products were cloned into TA cloning vector pCR2.1 (Invitrogen Corp., CA, USA) and sequenced by the dye termination method using an ABI 3100 DNA Sequencer (Applied Biosystems, CA, USA).

Fluorescent-labeled F2 (5'-CCCCTGCAACGGCACCG-3'), F3 (5'-GGCCGCCCCGTGCAACGGCA-3') and R2 (5'-AGTAGACGTAGAGGGGCGAG-3'), R3 (5'-GAGGGGCGAGGACGAGGAG-3'), R4 (5'-CGGCGACGGCGAGGCTGACGATGAA-3') primers were then designed on the basis of the quail sequence, and these primers were used for the subsequent PCR and sequencing of the other species. For genotyping, fluorescent-labeled forward and reverse primers were employed and product size was detected by GeneScan systems using an ABI 3100 DNA Sequencer.

RESULTS

PCR amplification of *DRD4* exon I corresponding region and determination of sequence

By using just the original pair of F and R primers initially reported in humans, the DNA of Japanese quail could be successfully amplified. The nucleotide sequences of the remaining 5 species (chicken, ring-necked pheasant, helmeted guinea fowl, Japanese cormorant, and Japanese jungle crow) were determined using new pairs of primers (F2+R3 for chicken, F3+R3 for ring-necked pheasant and helmeted guinea fowl, F2+R4 for Japanese cormorant, and F2+R2 for Japanese jungle crow) designed from the Japa-

A

Humans	1: ATGGGGAACCGCAGCACCCGCGGACGCGGACGGGCTGCTGGCTGGGCGCGG-GCCGGCCCGG	GG-GGCATCTGCC	72
Quail ¹	1: .G...G..C...CCCCT.CAACGGCA.C	-CTC..CC...T--C.TCCTCCTCCTCCTCCG	58
Chicken 9 ²	1: F	CTC..CC...C.GC.TCCTCCTCCTCCTCCA	29
Chicken 8 ³	1: F2	CTC..CC...C.G--CCTCCTCCTCCTCCA	26
Pheasant ⁴	1: F3	...-CTC..CC...C.TC.TCCTCCTCCTCCTCCA	32
Guinea fowl ⁵	1: F3	...-CTC..CC...TC.TC.TCCTCCTCCTCCTCCACCTC	39
Cormorant ⁶	1: F3	CGCT.CC...C.GC..CCGCCGCC.CCGCCG	32
Crow ⁷	1: F3	C..GACCC...C...C.-CAC.C	23
Humans	73: ----GGGCTGGCTGGGCAGGGCGCGGGCGCTGGTGGGGGGC	---GTG-----CTGCTCATCGGGCGGGTGTCTCGCG	138
Quail ¹	59: ----ACCGGCCACAACATC	----.C.C.....CT...ATC..CCTCATC.....T---.G.	118
Chicken 9 ²	30: ----CCGGCCACAACATC	----.C.C.....CT...ATC..CCTCATC.....C---.G.	89
Chicken 8 ³	27: ----CCGGCCACAACATC	----.C.C.....CT...ATC..CCTCATC.....C---.G.	86
Pheasant ⁴	33: ----CCGGCCACAACATC	----.C.C.....CT...ATC..TCTCATC.....C---.G.	92
Guinea fowl ⁵	40: CCGCC---GGCCACAACATC	----.C.C.....CT...ATC..CCTCATC.....C---.G.	101
Cormorant ⁶	33: --GCC.CCGGCCACAACGTC	----.C.C.....CT...ATC..CCTCATC.....G.-----G.	95
Crow ⁷	24: ----GGCCACAGCATC	----.C.C.....CTC...ATCC.CCTCATC..C.....C---.GC	80
Humans	139: GGGAACTCGCTCGTGTGCGTGAGCGTGGCCACCGAGCGCGCCTGCAGACGCCACCAACTCCTTCATCGTGAGCCTGGC	218	
Quail ¹	119: .C...GG..G...TC.C...TG..G...G..T.CA...CA...A.TT...C...C...	198	
Chicken 9 ²	90: .C...GG..G...C.C...TG..G...G..G.CA...CA...A.TTT...C...C...	169	
Chicken 8 ³	87: .C...GG..G...C.C...TG..G...G..G.CA...CA...A.TTT...C...C...	166	
Pheasant ⁴	93: .C...GG..G...C.C...TG..G...G..G.CA...AA...A.TTT...C...C...	172	
Guinea fowl ⁵	103: .C...GG..G...C.C...TG..G...G..G.CA...CA...A.TTT...C...C...	181	
Cormorant ⁶	96: .C...GG..G...C.C...CTG..G...G..G.A...CA...A	158	
Crow ⁷	81: .C...GG...C..TC...CTG..G...G..G.CA...CA...A	160	
Humans	219: GGCCGCCACCTCCTCCTCGCTCTCCTGGTGTGCCGCTCTTCGTTACTACTCCGAGGT	275	
Quail ¹	199: C.T.....G..G...C...C...C...A..	242	
Chicken 9 ²	170: C.T.....T..G...C	191	
Chicken 8 ³	167: C.T.....T..G...C	188	
Pheasant ⁴	173: C.T.....G..A...C	194	
Guinea fowl ⁵	182: C.T.....G..G...C	203	
Cormorant ⁶	159: R4	158	
Crow ⁷	161: C.TG.....G..G...C...C..C	191	

B

Humans	1: MGNRSTADADGLLAGRGPAA	GASA	GLAQGAAALVGG-V--LLIGAVLAGNSLVCVSVATERALQTPINSFIVSLA	73
Quail ¹	1: F	AGA.PCNGTAPP.PPPPPP	TGHNI...L.I.LI...-G..G...L.C...K.T.Y...	66
Chicken 9 ²	1: F2	PP.PPPPPP	AGHNI...L.I.LI...-G..G...L.C...K.T.Y...	56
Chicken 8 ³	1: F2	PP.PPPPPP	AGHNI...L.I.LI...-G..G...L.C...K.T.Y...	55
Pheasant ⁴	1: F3	APP.PPPPPP	AGHNI...L.I.LI...-G..G...L.C...K.T.Y...	57
Guinea fowl ⁵	1: F3	APP.PPPPPPP	PAGHNI...L.I.LI...-G..G...L.C...K.T.Y...	60
Cormorant ⁶	1: F3	LP.PPPPPP	AAGHNV...L.I.LI.V--G..G...L.C...K.T.Y...	53
Crow ⁷	1: F3	GP.P.AP	-----GHSI...L.I.LLI...-G..G...L.C...K.T.Y...	53
Humans	74: AADLLLALLVLPFLVYSE			91
Quail ¹	67: V.....Y			80
Chicken 9 ²	57: V.....			63
Chicken 8 ³	56: V.....			62
Pheasant ⁴	58: V.....			64
Guinea fowl ⁵	61: V.....			67
Cormorant ⁶	59: R4			52
Crow ⁷	54: V.....			63

Fig. 1. Multiple alignments of the nucleotide (A) and deduced amino acid (B) sequences of the *DRD4* exon I corresponding region. The human sequence was previously reported (Accession No. L12398). Boxes indicate repeated regions in the human longer allele. Primer combinations for amplification were: F2+R3 in chicken, F3+R3 in ring-necked pheasant and helmeted guinea fowl, F2+R4 in Japanese cormorant, and F2+R2 in Japanese jungle crow. The chicken sequence was derived from White Leghorn with 9 repeats of proline. In Black Minorca, only 8 repeats of proline were observed. The flanking sequences of the 2 breeds were identical. Dots indicate sequence identity with the human sequence, while dashes represent gaps introduced to optimize the alignment. Arrows indicate coding sequences of primers and underlined parts correspond to repeat of CCT/G or proline. Transmembrane regions 1 and 2 are indicated above amino acids alignment. 1: Japanese quail, 2: White Leghorn, 3: Black Minorca, 4: Ring-necked pheasant, 5: Helmeted guinea fowl, 6: Japanese cormorant, 7: Japanese jungle crow. These sequences can be obtained from the DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers, AB125362, AB125363, AB125364, AB166867, AB166868, AB166869, AB125365, respectively.

Table 1. Allele frequency distribution of *DRD4* exon I corresponding region in 12 chicken breeds.

Breed	<i>n</i>	Genotype ^a			Allele frequency		<i>He</i> ^b
		8/8	8/9	9/9	8	9	
Black Minorca	22	22	0	0	1.000	0.000	0.000
Barred Plymouth Rock	40	21	17	2	0.738	0.263	0.392
Rhode Island Red	40	17	19	4	0.663	0.338	0.453
Tsushimajidori	40	15	19	6	0.613	0.388	0.481
Hinaidori	40	5	19	16	0.363	0.638	0.468
Fayoumi	115	1	0	114	0.009	0.991	0.017
White Leghorn	145	1	0	144	0.007	0.993	0.014
Tosajidori	10	0	0	10	0.000	1.000	0.000
Silky	10	0	0	10	0.000	1.000	0.000
Araucana	10	0	0	10	0.000	1.000	0.000
Hamburg	10	0	0	10	0.000	1.000	0.000
Brabanter	10	0	0	10	0.000	1.000	0.000

^a Allele name indicates the number of prolines.

^b Expected heterozygosity $He=2n(1-\sum q_i^2)/(2n-1)$. Nei and Roychoudhury, 1974

nese quail sequence (Fig. 1).

In the upstream of transmembrane 1 (29th–56th amino acid residue of the human sequence), humans and poultry (Japanese quail, chicken, ring-necked pheasant, helmeted guinea fowl) showed low sequence similarity. The human sequence includes a polymorphic region based on insertion/deletion of 4 amino acids. In contrast, the poultry sequences had a repeat of proline at the corresponding region. In the downstream of transmembrane 1, they showed higher similarity with the human sequence. In this region, the homology of amino acid sequence was 71.8% between humans and Japanese quail, and 69.4% between humans and chicken.

Poultry species of the order Galliformes were similar to each other in the whole sequence. Sequences of Japanese quail and chicken were similar except for the substitution of 8 nucleotides (1 amino acid). Among the Galliformes investigated, chicken and helmeted guinea fowl had the highest sequence similarity, showing only 3 nucleotide substitutions. Hydrophobic amino acids included in the transmembrane region were not replaced with hydrophilic amino acid by substitution.

By comparing the Japanese quail sequence with the five human dopamine receptor subtypes, human D4 showed the highest homology (58.9%; 73 amino acids) while human D1 had the lowest homology (52.1%; 48 amino acids). When compared with the three subtypes of chicken dopamine receptor, D1A, D1B and D1D (Demchyshyn *et al.*, 1995), D1A showed the highest homology (52.1%; 48 amino acids) while D1B had the lowest (40.8%; 76 amino acids). Finally, comparison with the turkey D2 (Schnell *et al.*, 1999) showed a homology of 66.7% (51 amino acids).

Polymorphism of the number of proline repeats

The repeat of proline was identified in the region corresponding to the human polymorphic region. The repeat

number of proline was 9 in Japanese quail, ring-necked pheasant and Japanese cormorant; 12 in helmeted guinea fowl; and 3 in Japanese jungle crow. No polymorphism was found in these species. In chicken, however, polymorphism was found in the repeat number of proline (Table 1). Black Minorca had only 8 repeats (allele 8), whereas Tosajidori, Silky, Araucana, Hamburg and Brabanter had only 9 repeats (allele 9). Intra-breed polymorphism was observed in White Leghorn, Barred Plymouth Rock, Rhode Island Red, Tsushimajidori, Hinaidori and Fayoumi, and two alleles (alleles 8 and 9) were detected in these breeds. The sequences of both alleles were identical except for the proline repeat. By the analyses of families including heterozygous individuals, Mendelian inheritance was confirmed (data not shown).

DISCUSSION

Among five human dopamine receptor subtypes, the sequence amplified in the Japanese quail showed the highest homology with that of the D4 dopamine receptor suggesting amplification of orthologous region. The sequence that we found showed low homologies (40.8–66.7%) with dopamine receptors reported in birds, chicken D1 like D1A, D1B and D1D receptors (Demchyshyn *et al.*, 1995) and turkey D2 receptor (Schnell *et al.*, 1999).

The *DRD4* exon I sequences of the avian species that we studied showed low homologies with that in humans. This may be due to the evolutionary distance between humans and Aves. The human polymorphic region exists in an extra cellular region and is not a ligand binding site. Thus, the effect of polymorphism on signal transduction is not defined. This region may have another function or may be linked to an unknown functional site in exons. In humans, recombinant proteins of both alleles in exon I are reported to have a minor effect with respect to antipsychotic, quin-

pirole and clozapine *in vitro*. However, no functional differences were detected for receptor activation by dopamine (Zenner *et al.*, 1998).

Polymorphism of avian *DRD4* exon I was due to variation in the number of proline repeats, in contrast to the insertion/deletion of 4 amino acid residues found in humans. It has been reported that the proline-rich motif has a WW domain binding region (Chen and Sudol, 1995) and that WW domain-containing proteins participate in signal transduction (Lu *et al.*, 1999), transcriptional regulation (Sudol *et al.*, 2001) and pre-mRNA splicing (Bedford *et al.*, 1998). Further investigation would thus be required concerning the effect of polymorphism in this region.

Allele frequency distribution indicated that intra-breed polymorphism was present in 6 chicken breeds. No relationship was found between the allele frequency and phylogeny of breeds. For example, Tosajidori and White Leghorn had allele 9 predominantly, although both are remotely related by the principal component analysis based on polymorphism of 7 blood protein loci among 25 chicken breeds (Tanabe *et al.*, 1991). Also, Tsushimajidori and Rhode Island Red had the same predominant allele 8 although they are remotely related.

Black Minorca had only the allele 8, implying that this may be a consequence of selection for decorative use of this breed. Other breeds with frequent allele 8, Barred Plymouth Rock, Rhode Island Red, Tsushimajidori and Hinajidori, are mainly used as the meat chicken. However, Tosajidori with only allele 9, is also used as the meat chicken and the relation with use of the breed is not clear. A survey of more chicken breeds would thus be necessary to ascertain the relation. Since Black Minorcas are noted to be acutely sensitive for sound (Mizutani, 2000), this behavioral trait may be associated with the allele 8. An attempt by the authors to analyze the relation between alleles and behavioral traits in chicken is ongoing.

The repeat numbers of proline were different between species. Species of the order Galliformes and Japanese cormorant (order Pelecaniformes) had higher (8 and more) repeat number, while Japanese jungle crow (order Passeriformes) had only 3. The comparison of wide range avian species will provide a history of expansion of the repeat numbers in avian species. Among species in the order Galliformes, if the relation between behavioral traits and genetic polymorphism is found, it would be of great use for stock improvement. Recently, a lot of reports on behavior in crows have been published. In the Northwestern crow (*Corvus caurinus*), their prey characteristics were investigated (Ha and Ha, 2003) while in the Spanish carrion crow (*Corvus corone corone*), social behavior was variable between populations and individuals (Baglione *et al.*, 2002). Although we could not find polymorphism among the 16 jungle crows studied, a further survey of more individuals/species could derive some information about the genetic background of such behaviors.

Polymorphism of *DRD4* exon I is related to delusional

disorder in humans. In this study, polymorphism in chicken was found in the corresponding region of humans. It possibly causes an individual difference in behavioral trait and is possibly related to psychotic disorder in poultry as well as in humans. However, since sequence of polymorphic region is greatly different between humans and birds, the function of the polymorphism cannot be predicted.

Human *DRD4* is polymorphic in terms of the repeat number of the 48-bp sequence in exon III (Van Tol *et al.*, 1992) and it has been demonstrated that the *DRD4* genotype is possibly related to personality known as novelty seeking (Benjamin *et al.*, 1996; Ebstein *et al.*, 1996). We previously found that the *DRD4* exon III region is also polymorphic in the dog (*Canis familiaris*) (Niimi *et al.*, 1999; Niimi *et al.*, 2001) and we reported polymorphisms between inter- and intra-species. In this study, we investigated the exon III in poultry but no polymorphism was found.

This is the first report on variation in a neurotransmitter-related gene in birds. This polymorphism would contribute significant information for elucidation of differences in behavioral traits in chicken breeds. To figure out the whole dopamine function at synapsis, studies of genetic variation of other dopamine-related proteins, dopamine transporter for reuptaking (Sabol *et al.*, 1999) and catechol-O-methyltransferase for resolution (Rujescu *et al.*, 2003), will be necessary. To clarify the genetic background of behavior, it would be also necessary to analyze other genes that have been suggested to play some role in human personality, such as serotonin transporter (Lesch *et al.*, 1996).

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