The dopamine D2 receptor gene polymorphisms associated with chicken broodiness

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ABSTRACT Chicken broodiness is a polygenic trait controlled by autosomal genes. Prolactin gene is a candidate of great interest in molecular studies of broodiness. However, another candidate dopamine D2 receptor (*DRD2*) gene has not been studied extensively. The objective of this study was to analyze the genetic effects of the *DRD2* gene on chicken broodiness through linkage disequilibrium analyses, tag SNP selection, genetic diversity observation, 2-tailed test, and association analyses. In this study, we assayed 27 variations of this gene in 456 individuals from 6 chicken populations to observe linkage disequilibrium pattern, the tag SNP, and genetic diversity. Among the 6 populations, Taihe Silkies exhibited no characteristic between the square of the correlation coefficient of gene frequencies (r^2) and physical distance. The other populations including Red Jungle Fowls, Xinghua chickens, Ningdu Sanhuang chickens (NDH), Baier Huang chickens, and Leghorn layers exhibited conspicuous characteristic of decreasing r^2 value over physical distance. Linkage disequilibrium decayed more rapidly in Red Jungle Fowls, Xinghua, and NDH than in Baier Huang and Leghorn layers. Allelic frequencies and genotype distributions in the 5 populations showed that A-38600G, I-38463D, T-32751C, A-16105G, A-6543G, C-6539T, and A+2794G were possibly associated with broodiness. Besides the above 7 sites, another 2 sites that might be associated with broodiness were screened by 2-tailed test. All 9 sites were used for association analyses with broodiness in 644 NDH chickens. A significant association ($P < 0.05$) was found between A-16105G and broody frequency (%), and the T+619C in intron 1 was significantly associated with duration of broodiness ($P < 0.05$). These findings suggested that the *DRD2* gene should be included in future genetic studies of chicken broodiness and 2 SNP of A-16105G and T+619C might be markers for breeding against broodiness.

Key words: chicken dopamine D2 receptor gene, broodiness, linkage disequilibrium, genetic diversity

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INTRODUCTION

With the development of artificial incubation technology, broodiness is no longer required in poultry production. Although the incidence of broodiness can be reduced effectively by traditional breeding methods, it is difficult to eradicate broodiness completely. Recently with the advance of molecular biological approaches, the genetic basis of broodiness has been extensively studied at the molecular level.

Broodiness is a polygenic trait (Romanov et al., 2002; Sharp, 2004; Jiang et al., 2005; Zhou et al., 2008). However, the results on the inheritance of broodiness are controvertible. Several studies proposed autosomal inheritance, whereas this was refuted by others. Saeki and Inoue (1979) presented evidence of sex-linked genes contributing to broodiness. Romanov et al. (1999, 2002) found that broodiness was not controlled by major genes on the Z chromosome and proposed that at least 2 dominant autosomal genes were involved.

To find out the QTL of broodiness, several studies have been carried out. Sharp (2004) tried to identify the QTL for the broody trait by genome-wide scan approach, but no QTL was identified. Prolactin (***PRL***) was considered to be the main candidate gene for broodiness due to its critical roles on the onset of broodiness (Youngren et al., 1991; March et al., 1994). For example, 2 studies suggested that a 24-bp indel in the promotor region of the chicken *PRL* gene was a

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genetic marker in breeding against broodiness (Jiang et al., 2005; Liang et al., 2006). In addition, 2 polymorphisms of the vasoactive intestinal peptide receptor-1 (***VIPR-1***) gene were reported to be in association with broodiness (Zhou et al., 2008). Otherwise, very few other candidate genes for broodiness were studied.

In mammals, the dopaminergic system has received a large amount of attention because of its important roles in the regulation of neuroendocrine secretion, cognition, emotion, locomotion, renal function, alcohol disease, and hyperprolactinemia (Missale et al., 1998; Nieoullon and Coquerel, 2003; Hansen et al., 2005; Puls et al., 2008). In avian, previous studies showed that dopamine (**DA**) played a critical role in PRL secretion, stimulating PRL secretion via D1 DA receptors (**DRD1**) at the hypothalamic level and inhibiting PRL secretion via D2 DA receptors (**DRD2**) at the pituitary level by operating through vasoactive intestinal peptide (Youngren et al., 1996, 1998, 2002; Al Kahtane et al., 2003). Sartsoongnoen et al. (2008) also proved the association between DA neurons and the regulation of the reproductive system, acting by affecting the vasoactive intestinal peptide secretion and subsequent PRL release. Therefore, it was suggested that the DA receptor might be associated with broodiness. In this study, the *DRD2* gene was selected as a candidate to analyze its genetic effects on chicken broodiness; DRD2 is a G protein-coupled receptor for the neurotransmitter DA. This transmembrane receptor belonged to the rhodopsin family. Schnell et al. (1999) have cloned the cDNA of the turkey *DRD2* gene, but information about the chicken *DRD2* gene has not been reported so far. Although previous studies suggested that the expression of the *DRD2* gene was correlated with broodiness in turkey (Schnell et al., 1999; Chaiseha et al., 2003), very few studies have evaluated the roles of *DRD2* variants in chicken broodiness.

The aim of this study was to identify the *DRD2* gene variations for chicken broodiness through population genetics study and association analyses. After polymorphisms of the chicken *DRD2* gene were scanned in 43.7-kb region, the mutations used in association analyses were selected based on linkage disequilibrium (**LD**) analyses, tag SNP selection, allelic frequency observation, and genotype distribution investigation in chicken populations with different broody traits. In addition, the selection of the mutations used in association analyses was aided with 2-tailed test. Finally, association analyses were conducted using the above selected mutations.

MATERIALS AND METHODS

Chicken Populations

A total of 456 randomly selected chickens from 6 populations, including Red Jungle Fowls (**RJF**), Taihe Silkies (**TH**), Xinghua chickens (**XH**), Ningdu Sanhuang chickens (**NDH**), Baier Huang chickens (**BEH**), and Leghorn layers (**LH**), were used for LD pattern analyses, tag SNP selection, and genetic diversity observation. The details of the populations are presented in Table 1.

The population used for association analyses comprised 644 NDH female chickens. All individuals maintained under cage conditions were subjected to a 24-h photoperiod for the first 2 d of age and then changed to a 16-h photoperiod. Before 77 d of age, chickens were fed ad libitum with 16.5% CP and 2, 800 kcal of ME/kg. After 77 d of age, all of the chickens were changed to be fed a corn-soybean-based diet with 15.0% CP and 2, 900 kcal of ME/kg. From 90 to 300 d of age, the chickens were shifted into single cages and their incubation behavior was observed and recorded at 1600 h every day. A description of the criteria for broody behaviors has been published elsewhere (Zhou et al., 2008). Briefly, when hens exhibited increased body temperature, nesting, incubating, feather loosening, being more defensive and aggressive, and lost their appetite, they were considered to be broody. Behaviors such as specific clucking, loss of luster throughout the body, and the prominent wrinkle of the vent were also considered to be part of the criterion for broodiness. Two parameters, duration of broodiness and broody frequency (%), were investigated in the association study. Duration of broodiness was identified by the number of days a hen was broody during the observation period. Broody frequency (%) was estimated by the percentage of broody chickens, and here hens with obvious broody behavior for more than 1 d were defined as broody chickens considering enough samples in statistics.

Selection of Mutation Sites

Based on the published mRNA sequences (GenBank accession no. EU313425) of the chicken *DRD2* gene and

Table 1. The details of the chicken populations

Population	Number of samples	Origin	Production performance
Red Jungle Fowl	33	Linshan County, Guangxi, China	Seasonal reproduction and broodiness
Taihe Silkie	87	Taihe County, Jiangxi, China	A 70 to 80% incidence of broodiness
Xinghua chicken	102	Fengkai County, Guangdong, China	A 70 to 80% incidence of broodiness
Ningdu Sanhuang chicken	96	Ningdu County, Jiangxi, China	A 50 to 60% incidence of broodiness
Baier Huang chicken	41	Shangrao County, Jiangxi, China	A 10 to 15% incidence of broodiness
Leghorn layer	97	Commercial layer line derived from Italy	No broodiness

Table 2. Details of 16 primer pairs for genotyping of the chicken dopamine D2 receptor gene

Primer	Primer sequence[1] (5′→3′)	Length (bp)	Marker no.	Location	Site	Genotype method	Annealing temperature (°C)
510	F: tggggaagtttgcagcgtttgat R: cccccagagcagtgcaagtcct	276	M1	5′ regulatory region	C-39090T	*Tru1I*	59
509	F: tgggggggactaaacttcaaattg R: cctggccttgttaaacctcatca	249	M2	5′ regulatory region	A-38600G	*BseMI*	57
508	F: ggatgaggtgagagggtgatgag R: ctgcctcatgtcccgcttctg	166	M3 M4 M5 M6 M7 M8 M9 M10 M11 M12	5′ regulatory region	I-38475D I-38473D T-38472C C-38471T T-38470C I-38468D G-38467A I-38463D T-38460C T-38457C	Sequencing	58
507	F: tgcacataaaagcccactcactg R: gcctgagctggtgggggg	223	M13	5′ regulatory region	T-32751C	*BseGI*	60
506	F: aggccctctgttgctttgcttc R: tcggggcactttactcagattca	213	M14	5′ regulatory region	G-27293C	*Eco32I*	57
505	F: ggctcccggtgctgaatc R: gcgagatccgtccccttcc	145	M15	5′ regulatory region	A-16226G	*PstI*	55
504	F: cccccggcaggcagagcac R: acgcgatctgggagcaaaccttc	233	M16	5′ regulatory region	A-16105G	*BseMII*	64
503	F: tgcacttcaatccttcccagctt R: ttgcgctgcccattgacca	187/165	M17	5′ regulatory region	I-13387D	*PCR*	60
502	F: aaggggacacaagaatgcagcag R: gccagtgctgctcaaatttcaaa	404	M18 M19 M20	5′ regulatory region	G-6563A A-6543G C-6539T	*Van91I* *BseG* *MvaI*	56
501	F: tccctgagctgctgttgg R: ccccattgtgctccagaccc	436	M21	5′ regulatory region	T-4089C	*BseGI*	59
E1–1	F: agcagccatcttcacgtggg R: ttgagaggcttgctccagttcc	204	M22	Exon 1 (Leu4Leu)	G+12A	*HinfI*	57
E1–2	F: caacagtggggacaggaac R: ccacccagggcatgaccaga	239	M23	Exon 1 (Ala25Thr)	G+73A	*TatI*	59
I3	F: cgccgtggtctgggtgctct R: gggtttcgggctcgctgc	206	M24	Intron 3	A+2794G	*Eam1104I*	64
I4	F: cctccgttgtctccttctatgt R: ccggctcataggttacacaag	347	M25	Intron 4	G+3085A	*XhoI*	61
E6	F: ccaggaggcggaaagtcacatag R: ggctgtgtgtaggttttctttgg	196	M26	Exon 6 (Leu312Leu)	T+4137C	*Eco130I*	57.5
I6	F: gggcaaggaagctcatgtg R: ccttctgtttgttgctatgtctc	184	M27	Intron 6	T+4677G	*BsuRI*	58

[1]F = forward; R = reverse.

the released chicken genome sequences (http://mgc.ucsc.edu/cgi-bin/hgBlat), primers were designed to obtain the variations of this gene.

According to Xu et al. (2005) and Zhou et al. (2008), the inclusion criteria for selecting markers were as follows: (1) mutations in coding regions were selected preferably over those from noncoding regions; (2) SNP at a splicing site or near exons; (3) mutations located at the potential regulatory region and predicted to change some transcription factors; (4) it was easy to genotype by PCR-RFLP; (5) the average distance between 2 adjacent markers was less than 5 kb; and (6) these SNP displayed polymorphic information in NDH chickens, which was necessary for subsequent association analyses. A total of 27 markers (M1 to M27) from 119 variations, which we have obtained in the chicken *DRD2* gene (data not shown), were selected (Table 2). The potential transcriptional factor binding sites of the 27 SNP were shown in Supplemental Table 1 (all supplemental tables and figures can be found online at http://ps.fass.org/content/vol89/issue3/index.dtl).

PCR Amplification and DNA Sequencing

Genomic DNA of all chickens was extracted from blood. Primers used for amplification of genotyping sites were designed by GeneTool software (BioTools, Edmonton, Alberta, Canada) based on the published gene sequence and described in Table 2. Polymerase chain reactions were performed in 25-μL reaction volumes. Each reaction included 50 ng of genomic DNA, 1 μ*M* of each primer, 200 μ*M* deoxynucleotide triphosphate, 1.5 m*M* $MgCl_2$, 1 × PCR buffer, and 1 U of Taq DNA polymerase (Sangon Biological Engineering Technology Company, Shanghai, China). The PCR amplifications were carried out in an Eppendorf Mastercycler (Eppendorf Ltd., Hamburg, Germany) as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55 to 64°C for 45 s, and 72°C for 1 min; and 72°C for 10 min. The PCR products were checked on 2% agarose gel and visualized in TFM-40 UV Transilluminator (UVP Company, Cambridge, UK) by staining with ethidium bromide. Sequencing was done on an automated sequencer (ABI

3700, Applied Biosystems, Foster City, CA), and the resulting data were analyzed by the software DNAStar V 3.0 (Steve ShearDown, 1998–2001 version reserved by DNAStar Inc., Madison, WI).

Genotyping of Polymorphisms

The markers M3 to M12 were genotyped with direct sequencing, and genotyping of M17 was directly performed by 3.5% agarose gel electrophoresis after PCR amplification. For the other sites, PCR products were subjected to a digestion for 16 h with restriction enzymes (Table 2) according to the protocol of the manufacturer and were then separated on 3% agarose gel.

Statistical Analyses

LD Analyses. Hardy-Weinberg equilibrium (**HWE**), LD, and the inferred haplotype block for the sites with minor allelic frequency >5% were analyzed using the program Haploview v 3.32 (Barrett et al., 2005). The square of the correlation coefficient (r^2) with a threshold of 0.33 was chosen as useful LD in this study (Du et al., 2007). The definition of the haplotype structure was based on the 4-gamete test (Wang et al., 2002). The decline of LD with distance in a population was estimated on the basis of the following model of the Sved (1971) equation:

$$LD_{ij} = 1/(1 + 4b_j d_{ij}) + e_{ij},$$

where LD_{ij} is the observed LD for marker pair i of breed j, d_{ij} is the distance in base pairs for marker pair i of breed j, b_j is a coefficient that describes the decline of LD with distance for breed j, and e_{ij} is a random residual. Parameter b_j was estimated separately for each breed, using the nonlinear fit command option of JMP (JMP software 5.1.2, 1989–2004; SAS Institute Inc., Cary, NC).

Allelic Frequency and Heterozygosity Calculation. Allelic frequency and heterozygosity were calculated with the Microsatellite Toolkit version 3.1 software (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/). The comparisons of genotypic frequency for each site between the 2 unrelated populations were conducted by Mantel-Haenszel χ^2 (SAS 8.1 FREQ; SAS Institute Inc.).

Tag SNP Selection. Haplotype block partitioning and tag SNP selection were based on the following recursive formula (Zhang et al., 2002a,b, 2004):

$$S_j = \min [S_{i-1} + f(i, \ldots, j), \text{ if block } (i, \ldots, j) = 1](1 \le j < n),$$

where S_j is the minimum number of tag SNP for the optimal block partition of the first j SNP, $S_0 = 0$, f (i, ..., j) is the number of tag SNP in this block, block (i, ..., j) is a Boolean function, and block (i, ..., j) = 1 if and only if SNP (i, ..., j) can form a haplotype block (Zhang et al., 2005; http://www.cmb.usc.edu/msms/HapBlock).

2-Tailed Test. In 644 NDH individuals for association analyses, 35 individuals with high duration of broodiness and 35 individuals with low duration of broodiness were genotyped by directly sequencing for all mutation sites in the interest region (between exon 1 and intron 3; Supplemental Table 2). Comparisons for genotypes between 2 groups were assessed by χ^2 tests. A *P*-value less than 0.05 was considered significant.

Association Analyses. Association analyses of mutation sites with duration of broodiness were carried out by the GLM procedures of the SAS 8.0 software (SAS Institute Inc.) and the genetic effects were analyzed by a mixed procedure according to the following model:

$$Y = \mu + G + F + H + e,$$

where Y is an observation on the traits, μ is the overall population mean, G is the fixed effect of genotype, F is the random effect of family, H is the fixed effect of hatch, and e is the residual error. Multiple comparisons were conducted with least squares means by Fisher's least significance difference test. Levels of significance for all statistical analyses were set to $P \le 0.05$.

Comparisons for the frequency of broodiness and nonbroodiness between groups were assessed by χ^2 tests performed on a 2×3 contingency table. A *P*-value less than 0.05 was considered significant.

RESULTS

LD of the DRD2 Gene in the 6 Chicken Populations

One hundred nineteen variations were identified in the chicken *DRD2* gene (data not shown), among which 27 variation sites were genotyped to perform LD analyses in RJF, TH, XH, NDH, BEH, and LH. Similar patterns of LD, in which most tightly linked SNP pairs had the highest r^2 and the values of r^2 rapidly decreased with the increasing of linkage distance, were observed in RJF, XH, NDH, BEH, and LH (Figure 1). As illustrated in Figure 1C, no characteristic was observed between r^2 value and physical distance in TH. Overall, there were clear differences in the decay of LD among the other 5 populations. Linkage disequilibrium decayed more rapidly in RJF, XH, and NDH than in BEH and LH, indicating that the extents of LD were smaller in these populations than in BEH and LH (Supplemental Figure 1). Ten sites, I-38475D, I-38473D, T-38472C, C-38471T, T-38470C, I-38468D, G-38467A, I-38463D, T-38460C, and T-38457C, were always in complete disequilibrium ($r^2 = 1$; Figure 2). Moreover, possible regions of strong LD were also found between exon 1 and intron 3 (between G+73A and A+2794G) in the NDH.

Haplotype Structure and Tag SNP of the DRD2 Gene in the NDH Chickens

In all 6 populations, I-38475D, I-38473D, T-38472C, C-38471T, T-38470C, I-38468D, G-38467A, I-38463D, T-38460C, and T-38457C were always in the same haplotype block (Figure 2). In our subsequent study, we chose I-38463D as the tag SNP for this block. In total, 13, 15, 12, and 11 tag SNP were identified in the RJF, XH, BEH, and LH, respectively. The total number of blocks in these 4 populations was 4, 5, 4, and 5, respectively. In the NDH chickens, 17 tag SNP, C-39090T, A-38600G, I-38463D, T-32751C, G-27293C, A-16226G, A-16105G, I-13387D, G-6563A, A-6543G, C-6539T, T-4089C, G+73A, A+2794G, G+3085A, T+4137C, and T+4677G, were identified, whereas the site G+12A was not the tag SNP.

Allelic Frequency and Heterozygosity in the 5 Populations

The allelic frequencies of mutation sites in the 5 populations were presented in Table 3. Sites of C-39090T, G-27293C, A-16226G, and T+4137G showed deviation from HWE in some populations. Except for these sites, almost no significant deviations from HWE were detected for the other mutation sites. As can be seen in Table 3, the allelic frequencies of 7 variant sites, A-38600G, I-38463D, T-32751C, A-16105G, A-6543G, C-6539T, and A+2794G, were obviously different in different populations and their distributions were consistent with the broody tendency of the RJF, XH, NDH, and BEH. The results of χ^2 test for genotype distribution of the 7 sites between populations were described in Table 4. Distribution of the genotypes in 5 sites, I-38463D, A-16105G, A-6543G, C-6539T, and A+2794G, were significantly different ($P < 0.05$ or $P < 0.01$) among the populations with higher broodiness such as RJF, XH, and NDH and the populations with lower broodiness such as BEH. In A-38600G and T-32751C, the distribution was significantly different at the $P < 0.01$ level between RJF and BEH. The mean values of expected heterozygosities for the RJF, XH, NDH, BEH, and LH were 0.353 ± 0.031, 0.447 ± 0.022, 0.430 ± 0.028, 0.342 ± 0.031, and 0.432 ± 0.030, respectively. Taken together, all of the above results suggested that A-38600G, I-38463D, T-32751C, A-16105G, A-6543G, C-6539T, and A+2794G might be associated with chicken broodiness.

Association of the 9 Sites with Chicken Broodiness

Besides the 7 sites selected through above analyses, 2 sites between exon 1 and intron 3 of the *DRD2* gene,

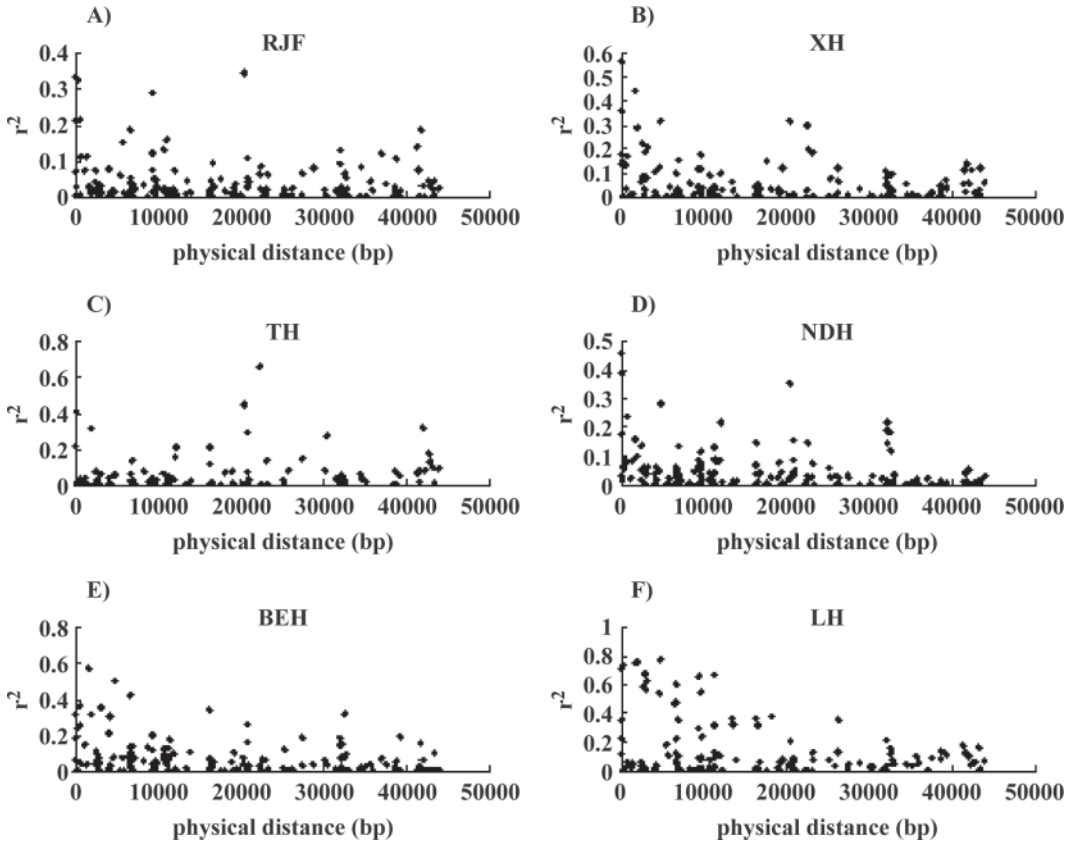

Figure 1. Decline of linkage disequilibrium measured by r^2 against physical distance in the 6 populations. The scatterplot indicated estimates of r^2 for pairs of SNP in each breed. RJF = Red Jungle Fowl; XH = Xinghua chicken; TH = Taihe Silkie; NDH = Ningdu Sanhuang chicken; BEH = Baier Huang chicken; LH = Leghorn layer.

T+619C ($\chi^2 = 7.61$, $0.01 < P < 0.05$) and T+1259C ($\chi^2 = 5.53$, $P > 0.05$) (Table 5), which might be associated with broodiness, were chose by 2-tailed test method. All 9 sites were genotyped in the 644 NDH individuals to evaluate associations of each site with chicken broodiness. Marker-trait association analyses showed that A-16105G of the chicken *DRD2* gene was significantly associated ($P < 0.05$) with broody frequency (Table 6), and the individuals with GG genotype had higher broody frequency than those with AA or AG genotype; a significant association ($P < 0.05$) was observed between T+619C in intron 1 and duration of broodiness, and chickens with the heterozygote genotype of TC had significantly shorter ($P < 0.01$)

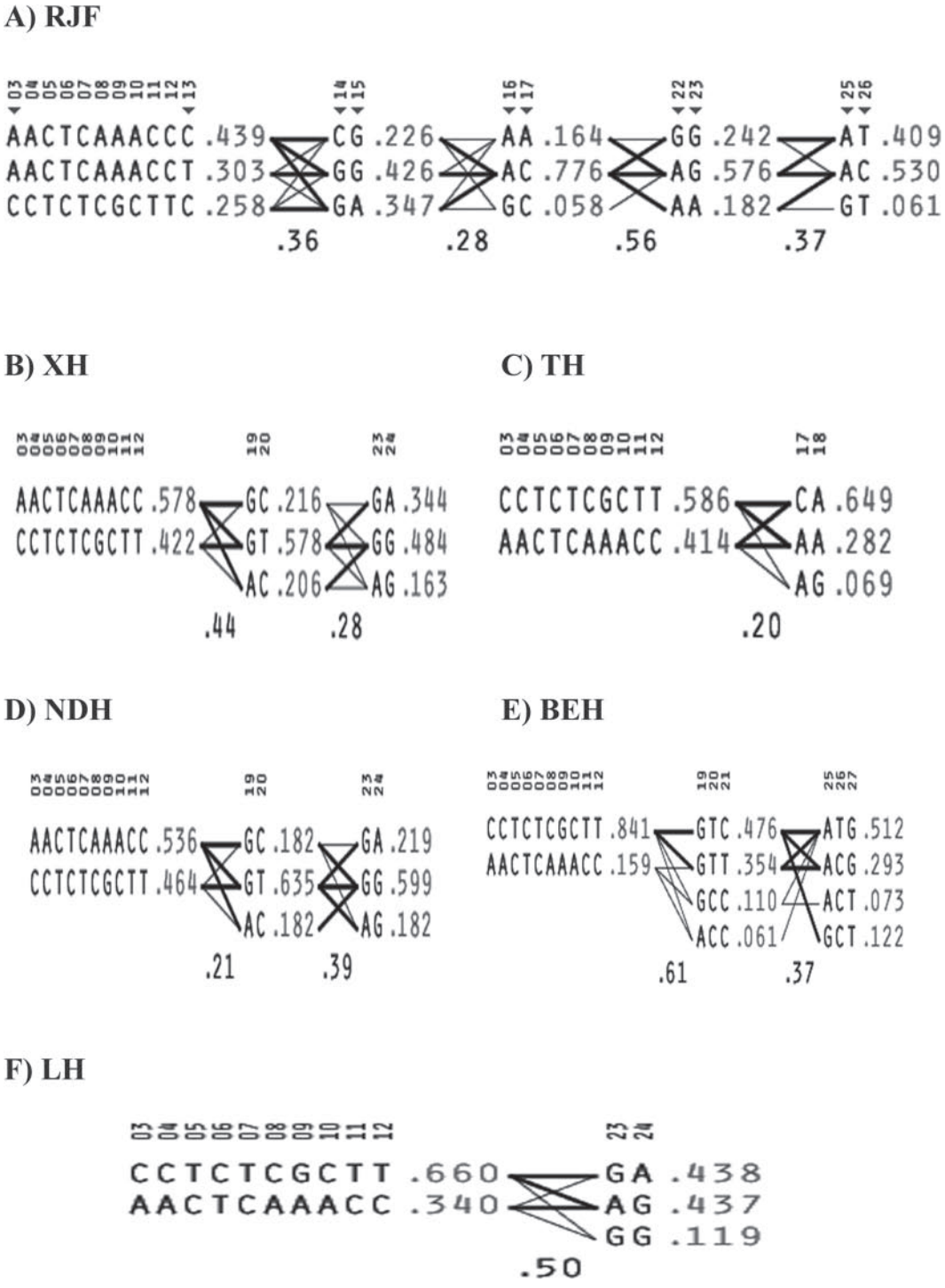

Figure 2. Haplotype structures of the 6 populations. 01 = C-39090T; 02 = A-38600G; 03 = I-38475D; 04 = I-38473D; 05 = T-38472C; 06 = C-38471T; 07 = T-38470C; 08 = I-38468D; 09 = G-38467A; 10 = I-38463D; 11 = T-38460C; 12 = T-38457C; 13 = T-32751C; 14 = G-27293C; 15 = A-16226G; 16 = A-16105G; 17 = I-13387D; 18 = G-6563A; 19 = A-6543G; 20 = C-6539T; 21 = T-4089C; 22 = G+12A; 23 = G+73A; 24 = A+2794G; 25 = G+3085A; 26 = T+4137C; 27 = T+4677G. RJF = Red Jungle Fowl; XH = Xinghua chicken; TH = Taihe Silkie; NDH = Ningdu Sanhuang chicken; BEH = Baier Huang chicken; LH = Leghorn layer.

Table 3. Allelic frequencies of each mutation site in the dopamine D2 receptor (*DRD2*) gene in the 5 chicken populations[1]

Site	Allele	RJF (n = 33)	XH (n = 102)	NDH (n = 96)	BEH (n = 41)	LH (n = 97)
C-39090T	C	0.242	0.436[2]	0.453	0.537	0.312[2]
A-38600G	A	0.136	0.373	0.458	0.524	0.036
I-38463D	D	0.258	0.422	0.464	0.841	0.660
T-32751C	T	0.303	0.103	0.068	0.061	0.340
G-27293C	C	0.227	0.824[2]	0.615[2]	0.707	0.577[2]
A-16226G	A	0.348	0.598[2]	0.568[2]	0.366	0.485
A-16105G	G	0.061	0.314	0.385	0.573	0.141
I-13387D	I	0.167	0.485	0.552	0.427	0.598[2]
G-6563A	A	0.258	0.730	0.693	0.744	0.680
A-6543G	G	0.455	0.794	0.818	0.939	0.758
C-6539T	T	0.424	0.578	0.635	0.829	0.510
T-4089C	C	0.258	0.480	0.380	0.646	0.907
G+12A	G	0.242	0.397	0.125	0.280	0.543
G+73A	A	0.182	0.172	0.182	0.232	0.443
A+2794G	A	0.091	0.353	0.219	0.366	0.453
G+3085A	G	0.061	0.275	0.151	0.122	0.443
T+4137C	T	0.470	0.417[2]	0.383[2]	0.512[2]	0.479[2]
T+4677G	T	0.076	0.377	0.167	0.195	0.490

[1]The first nucleotide of the translation start codon was designated +1, with the next upstream nucleotide being designated −1. RJF = Red Jungle Fowl; XH = Xinghua chicken; NDH = Ningdu Sanhuang chicken; BEH = Baier Huang chicken; LH = Leghorn layer.

[2]Hardy-Weinberg deviation at the level of 0.01.

duration of broodiness than those with the CC or TT genotype (Table 7). As summarized in Table 8, no other sites were found to be associated with broodiness traits significantly ($P > 0.05$).

DISCUSSION

In chicken, the broody trait was related to activity of the dopaminergic system (Al Kahtane et al., 2003). In

Table 4. χ^2 testing for genotype distribution of the 7 sites between populations[1]

		χ^2 value[2]			
Site	Population	XH	NDH	BEH	LH
A-38600G	RJF	13.82**	23.14**	22.01**	9.18**
	XH		3.27	6.21*	67.36**
	NDH			1.23	87.63**
	BEH				73.68**
I-38463D	RJF	6.03*	8.69*	40.07**	31.88**
	XH		0.72	41.46**	22.11**
	NDH			33.17**	14.98**
	BEH				11.02**
T-32751C	RJF	17.67**	24.40**	15.73**	3.14
	XH		1.43	1.44	26.60**
	NDH			0.44	35.44**
	BEH				19.51**
A-16105G	RJF	15.97**	28.07**	37.34**	5.06
	XH		4.90	14.00**	14.51**
	NDH			8.29*	28.12**
	BEH				44.75**
A-6543G	RJF	26.58**	34.36**	37.35**	18.85**
	XH		1.67	9.03*	2.40
	NDH			7.69*	6.73*
	BEH				10.52**
C-6539T	RJF	4.96	10.15**	24.01**	1.74
	XH		2.67	15.35**	1.87
	NDH			11.41**	7.15*
	BEH				23.67**
A+2794G	RJF	12.71**	4.92	15.89**	26.14**
	XH		8.34*	6.53*	6.68*
	NDH			7.81*	20.98**
	BEH				2.91

[1]RJF = Red Jungle Fowl; XH = Xinghua chicken; NDH = Ningdu Sanhuang chicken; BEH = Baier Huang chicken; LH = Leghorn layer.

[2]$\chi^2_{0.05}$ (df = 2) = 5.99; $\chi^2_{0.01}$ (df = 2) = 9.21.

*$P < 0.05$; **$P < 0.01$.

Table 5. Two-tailed test for T+619C and T+1259C in the association analyses population[1]

Site	χ^2 value[2]	TT	TC	CC
T+619C				
High broodiness	7.61*	24	2	4
Low broodiness		23	8	0
T+1259C				
High broodiness	5.53	17	8	6
Low broodiness		11	17	3

[1]Number indicates the number of tested chickens of each genotype.
[2]$\chi^2_{0.05}$ (df = 2) = 5.99, $\chi^2_{0.01}$ (df = 2) = 9.21.
*$P < 0.05$.

the present study, the mutations of the chicken *DRD2* gene were identified for broodiness association analyses in 644 female NDH individuals. In the same NDH population, meanwhile, 2 SNP and a haplotype of the *DRD1* gene were also found to be significantly associated with chicken broody frequency as completed by us (data not shown). The human *DRD2* gene contained 8 exons and spanned 270 kb, in which the large first intron was approximately 250 kb and the start codon was in exon 2, and so did many other species (Eubanks et al., 1992; Luo et al., 2005). Although we have not acquired the exon 1, the cDNA clone of the chicken *DRD2* gene exhibited high homology with previously cloned mammalian *DRD2* receptors (data not shown). Thus, mutations located 40 kb upstream of the start codon were selected in the present study. The 27 variation sites we selected spanned about 45 kb of chicken *DRD2* gene, among which 3 SNP were located in the coding region and 1 SNP was a nonsynonymous mutation (Ala25Thr). The variations were highly polymorphic in almost all populations.

Analyses of the extent and decline of LD could provide information about the number of markers and the sample size required for common diseases or other phenotypic trait mapping (Kruglyak, 1999). Compared with other measures of LD such as the correlation coefficient between pairs of loci (D′), r^2 was the preferred measure for biallelic loci such as SNP because it was less affected by sample size (Devlin and Risch, 1995) and less dependent on levels of minor allelic frequency (Du et al., 2007). We chose r^2 as the measure of LD. In this study, no characteristic level of LD was observed in TH. It was probably because the sample size of TH was not large enough or the density of markers was too low (Ke et al., 2004; Nsengimana et al., 2004; Pe'er et al., 2006). Therefore, the TH population was not chosen to do further analyses about broody traits. Many studies showed high levels of LD over long distances such as in cattle (Vallejo et al., 2003), pigs (Nsengimana et al., 2004; Amaral et al., 2008), and sheep (McRae et al., 2002). But studies in human found that LD extended over very short distances (Kruglyak, 1999; Pritchard and Przeworski, 2001). In chickens, Heifetz et al. (2005) found appreciable LD between microsatellite markers as far as 5 cM apart. However, Andreescu et al. (2007) showed that LD extended over shorter distances in chickens, which was in accordance with the results of the present study. Previous studies demonstrated that different populations showed different patterns of LD (Conrad et al., 2006; Teo et al., 2009) and it could reveal much about domestication and breed history (Pritchard and Przeworski, 2001). During domestication and breeding, selection could increase the LD level of a gene (Przeworski, 2002; Saunders et al., 2005). In this study, the LH, which has undergone intensive artificial selection aiming at the increase of egg production and the decrease of broodiness, is a layer breed with no broodiness. The BEH chicken is a well-known Chinese native layer breed with low incidence of broodiness through long-term selection against broodiness. However, XH and NDH chickens are broiler breeds with more than 40% incidence of broodiness due to the lack of selection for broodiness. We compared the patterns of LD in the 5 populations. Finally, we found that the LD level differed significantly between LH or BEH and the other 3 populations, which increased gradually with the increase of broodiness. In LH, LD decayed moderately with the increasing of linkage distance, whereas in RJF and XH, it was more rapid. These results suggested that the *DRD2* gene might have endured strong selection during breeding against broodiness. Several large-scale studies also revealed that the degree of LD varied greatly in different regions of the gene or genome and modern breeding programs caused these differences (Stephens et al., 2001; Gabriel et al., 2002; Amaral et al., 2008). Generally in the populations of this study, high LD at short distances declined steeply as distance

Table 6. Association of A-16105G with broody traits in Ningdu Sanhuang chickens under cage condition

Trait	*P*-value[1]	AA[2] (201)	AG[2] (341)	GG[2] (102)
Duration of broodiness (d)	0.82	8.00 ± 0.90	7.79 ± 0.74	7.12 ± 1.21
Number of nonbroody chickens	—	104	188	40
Number of broody chickens	—	97	153	62
Broody frequency (%)	—	48.26	44.87	60.78
χ^2 value[3]	<0.05	7.97*		

[1]The *P*-value 0.82 indicates that this site was not significantly associated with duration of broodiness.
[2]Least squares means ± SE; number in parentheses shows the number of tested chickens of each genotype.
[3]$\chi^2_{0.05}$ (df = 2) = 5.99, $\chi^2_{0.01}$ (df = 2) = 9.21.
*$P < 0.05$.

Table 7. Association of T+619C with broody traits in Ningdu Sanhuang chickens under cage condition

Trait	*P*-value[1]	CC[2] (28)	TC[2] (173)	TT[2] (426)
Duration of broodiness (d)	0.04*	8.33 ± 2.19^{AB}	5.95 ± 0.95^{B}	8.57 ± 0.68^{A}
Number of nonbroody chickens	—	14	90	219
Number of broody chickens	—	14	83	207
Broody frequency (%)	—	50.00	47.98	48.59
χ^2 value[3]	>0.05	0.05		

[A,B]Within a row, measurements with no common superscripts are greatly significantly different ($P < 0.01$).

[1]The *P*-value 0.04 indicates that this site was significantly associated with duration of broodiness.

[2]Least squares means ± SE; number in parentheses shows the number of tested chickens of each genotype.

[3]$\chi^2_{0.05}$ (df = 2) = 5.99.

*$P < 0.05$.

increased and this observation was in agreement with previous results (Sved, 1971). Ten sites from I-38475D to T-38457C showed that high LD in all populations was reasonable given the fact that the distances between any 2 sites were less than 10 bp (Kendler et al., 1999). Because the NDH was used to find sites of association with broodiness and the region from exon 1 to intron 3 of the *DRD2* gene had some sites that might be associated with broodiness in this population, 2 other sites, T+619C ($\chi^2 = 7.61$, $0.01 < P < 0.05$) and T+1259C ($\chi^2 = 5.53$, $P > 0.05$), were selected by 2-tailed test method and used in the association analyses.

Previous studies revealed that in some high-LD regions, only a maximally informative set of SNP (tag SNP) were sufficient to capture most of the haplotype structure (Johnson et al., 2001; Patil et al., 2001). In the search for genetic variants that contribute to diseases, the selection of tag SNP for genome-wide association studies has attracted much attention. Thus, genotyping efficiency could be increased greatly. In the haplotype structure and tag SNP analyses, a fixed haplotype block was found in all populations and 17 different tag SNP were found in the NDH.

In the present study, we detected significant difference of the genotype distribution between the LH and the other populations in almost all of the 18 mutation sites, which indirectly proved that the LH was greatly different from the Chinese breeds. Therefore, considering the genetic differences, we thought that the BEH should be a better control population than the LH to do the investigations about allelic frequency, genotype distribution, and genetic diversity to find markers probably associated with broodiness. The results showed that in A-38600G, I-38463D, A-16105G, A-6543G, C-6539T, and A+2794G, the allelic frequency increased progressively with the decreasing of broodiness from the RJF to the XH and NDH, then to the BEH. And in T-32751C, it was decreasing gradually. Further analyses of these 7 sites indicated that there was significant difference of genotype distribution between higher broodiness populations such as RJF, XH, and NDH and lower broodiness populations such as BEH. Departures from HWE in a population usually indicate inbreeding, stratification, and sometimes problems in genotyping (Ebrahimi and Bilgili, 2007). Genotype distribution in the 7 sites was all in HWE, suggesting that the genotypes in these sites were randomly sampled from the general population. In addition, it has to be noted that all 7 sites were included in the 17 tag SNP of the NDH. Therefore, the 7 sites, A-38600G, I-38463D, T-32751C, A-16105G, A-6543G, C-6539T, and A+2794G, were finally selected as part of the candidate markers to do association study with broodiness.

Table 8. Association (*P*-value) of the 9 markers in the chicken dopamine D2 receptor gene with broody traits in Ningdu Sanhuang chickens under cage condition

Variation site	Duration of broodiness (d)	Broody frequency (%)
A-38600G	NS[1]	NS
I-38463D	NS	NS
T-32751C	0.11	NS
A-16105G	NS	$0.01 < P < 0.05$
A-6543G	NS	NS
C-6539T	NS	NS
T+619C	0.04	NS
T+1259C	NS	NS
A+2794G	NS	NS

[1]$P > 0.05$.

For the ascertainment of the candidate markers for association analyses, 2 methods were applied in this study. One method was in terms of LD, allelic frequency, and genotype distribution analyses, aided with tag SNP selection. In this method, several successful studies have been published. Fu et al. (2004) identified polymorphisms of the calsequestrin 1 (*CASQ1*) associated with type 2 diabetes by LD analyses. Zhou et al. (2008) found a significant effect of 2 *VIPR-1* mutations on chicken broodiness through LD, allelic frequency, and genotype distribution analyses. The results of the association study further supported the feasibility of this method. On the other hand, based on the results of LD analyses in this study, the 2-tailed test method was used to select the candidate markers in the region between exon 1 and intron 3. Sutter et al. (2007) proved a single Insulin-like growth factor 1 (*IGF1*) allele to be the major determinant of small size in dogs by this method. The results in the present study showed that the polymorphism T+619C, in which possible effects

on broody traits was indicated with 2-tailed test, was confirmed to be associated with broodiness eventually.

In conclusion, the present study provided valuable information that the *DRD2* gene endured strong selection during chicken domestication and improvement. Two variations, A-16105G and T+619C, associated with chicken broody traits, were identified in the present study. To our knowledge, this report presented the first genetic association study of the *DRD2* gene and broodiness in a single breed population. The information derived from this study could be valuable for understanding the genetics of broodiness and could facilitate further investigation in breeding against broodiness.

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