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Association of Dopamine D2 Receptor Gene Polymorphisms with Reproduction Traits in Domestic Pigeons (*Columba livia*)

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Dopamine inhibited prolactin secretion via dopamine D2 receptor (DRD2) at the pituitary level, but its effects on reproduction in pigeons are unclear. In this study, Single Nucleotide Polymorphisms (SNPs) in the exons of DRD2 gene were identified and analyzed by using DNA sequencing methods in 60 female domestic pigeons (*Columba livia*), and the association between DRD2 polymorphisms and reproduction traits was also analyzed. Sequencing results showed that 7 nucleotide mutations were detected in the exon 1, 4, and 6 regions of DRD2 gene. The analysis revealed three genotypes (AA, AB, and BB) in exon 4 and two genotypes (AA, AB) in exon 6, in which the AA genotype was consistently dominant, and the A allele showed a dominant advantage. The C4532T genotypes located in exon 6 of DRD2 gene were significantly ($P<0.05$) associated with reproductive traits of pigeon. Moreover, the individuals with AB genotype had significantly higher fertility rate and total hatching number within 500 days of age than those with AA genotype ($P<0.05$). These findings suggested that the DRD2 gene should be included in future genetic studies of pigeon reproduction and the SNP of C4532T might be a potential candidate genetic marker for Marker-aid breeding in pigeon.

Key words: dopamine D2 receptor, pigeon, reproduction traits, single nucleotide polymorphisms

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Introduction

Poultry reproductive traits, such as egg production, are economically important in modern poultry production. Many environment factors such as the length of photoperiod and different feeding allowances are critical for egg production (Liu *et al*., 2004; Lewis and Gous, 2006). However, recent studies demonstrated that reproductive traits are also dependent on genetic factors (King`Ori, 2011; Miazi *et al*., 2012). Using a candidate gene approach to identify and isolate quantitative trait loci (QTL) responsible for genetic variation in economic traits provides an excellent opportunity for marker-aid breeding (Khatib *et al*., 2007). To identify QTL regulating reproductive traits, many studies have been carried out in chicken (Hansen *et al*., 2005; Schreiweis *et al*., 2006; Chatterjee *et al*., 2010), but the study on pigeon is still limited. In southern China, domestic pigeons are reared as a kind of poultry and have a wide market in Southeast Asia for their abundant nutrients. Moreover, in pigeon, broodiness is a crucial reproductive behavior and can lead to poor egg production.

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Dopamine, an abundant neurotransmitter in the central nervous system, has received a large amount of attention because of its important roles in cognition, emotion, endocrine function, and hyperprolactinemia in mammals (Missale *et al*., 1998; Nieoullon and Coquerel, 2003; Hansen *et al*., 2005; Puls *et al*., 2008). In avian, dopamine was demonstrated to play a critical role in prolactin (PRL) secretion by binding to its specific receptor such as dopamine D1 receptor (DRD1) and dopamine D2 receptor (DRD2) (Youngren *et al*., 1995, 1996). Plasma PRL level had a negative effect on the chicken egg production because of its role in maintaining broodiness (Reddy *et al*., 2007). Previous studies showed that dopamine inhibited PRL secretion via DRD2 at the pituitary level by operating through vasoactive intestinal peptide (Youngren *et al*., 1996, 1998, 2002; Al Kahtane *et al*., 2003). In addition, the DRD2 has been well studied recently as a candidate gene of broodiness in chicken and turkey (Schnell *et al*., 1999; Xu *et al*., 2010). However, the information of pigeon DRD2 gene is quite limited. Hence, the DRD2 gene was chosen as a candidate gene to analyze the genetic effect on pigeon reproduction traits in this study. We detected the single nucleotide polymorphisms (SNPs) in DRD2 gene, and then investigated associations of the SNPs with reproduction traits to provide a theoretical basis for the molecular-assist breeding of superior pigeons.

Materials and Methods

The experiment was conducted in accordance with Chinese guidelines for animal welfare and was approved by the animal welfare committee of the Animal Science College, Zhejiang University.

Birds and Reproduction Traits

Sixty domestic pigeons (*Columba livia*) were used for the detection of SNPs in DRD2 gene and the analysis of the relationship between the SNPs and the reproduction traits. All birds (Taishen king pigeon) were obtained from Xingliang commercial pigeon farm (Wenzhou, China), hatched on the same day, male-female matched artificially based on pedigree data, and reared in a stair-step cage under the same nutritional and environmental conditions.

Four reproduction traits were measured according to The Poultry Production Performance Terms and Measurement Statistics Method (NY/T823-2004). These traits include egg production (total number of eggs; EP), total number of fertile eggs (FE), fertility rate (the ration of FE to EP; FR), and total hatching number (HN) within 500 days of age. Venous blood samples were collected from all sixty birds by venipuncture and stored at −20℃. The genomic DNA was extracted using Genome DNA Extraction Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The quality of DNA was checked by both native DNA electrophoresis on 1.0% agarose gel and the ultraviolet spectrophotometer.

PCR Amplification and Sequencing

Previous work in our lab demonstrated that no nucleotide mutations were found in the exon 2, 3, 5, and 7 regions of DRD2 gene (unpublished data). Thus, we just focused on the exon 1, 4, and 6 regions of DRD2 gene in this study. Based on the published pigeon gene sequence (GenBank accession no. NW_004973237.1), three primer pairs (Table 1) were designed to amplify those studied exon regions of the DRD2 gene using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Primers were synthesized by Tsingke Biotech Co., Ltd (Hangzhou, China). The PCR system was carried out in a 50 μL total volume, including 25 μL of 2×Taq PCR MasterMix (including Mg^{2+}, dNTP, and Taq DNA Polymerse), 2 μL of each primer (10 μmol/L), 2 μL DNA template, and double-distilled water. The PCR amplifications were carried out as follows: initial denaturation at 94℃ for 5 min; 30 cycles of 94℃ for 30 sec, 45 s at the annealing temperature and extension at 72℃ for 1 minute; and finally 7 min of extension at 72℃. Each product was verified by 1.5% agarose gel electrophoresis, and sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). Sequences were analyzed with ClustalX 2.0 and DNAman software (DNAMAN Application 4.0.1.1, Lynnon BioSoft, CA, USA).

Statistical Analysis

Genotype and allelic frequencies were calculated and checked for deviations from Hardy Weinberg equilibrium. Single factor and multiple comparisons were used to measure the effects of different genotypes on reproductive traits (means±standard deviation). Associations between genotype and reproductive traits were assessed using the general linear model (GLM procedure) in the JMP statistical software (SAS Institute, Cary, NC, USA). The linear model used was as follows:

$$Yij = \mu + Gj + eij$$

Where Yij is phenotypic values, μ is the population mean, G is genotype effect of DRD2, and eij represents the random error.

Results

Genetic Polymorphisms of Pigeon DRD2 Gene

Sequencing results showed that 7 nucleotide mutations were found in the exon regions of DRD2 gene. Briefly, two SNPs (C103A and G108A) were detected in exon 1, three SNPs (A3008C, G3085A, and C3169T) were detected in exon 4, and two SNPs (C4523T and C4532T) were detected in exon 6, respectively. Referring to amino acid mutations, G108A in exon 1, and C4523T and C4532T in exon 6 were synonymous, since they had no effects on the amino acid sequences, whereas the others SNPs were nonsynonymous in the DRD2 protein (Table 2).

Genotypic and Allelic Frequencies

Genotype and allelic frequencies were calculated and checked for deviations from Hardy Weinberg equilibrium.

Table 1. **Primers sequences of PCR amplification of pigeon DRD2 gene**

Region	(5′-3′) Sequence	Product size (bp)	Annealing temperature (℃)
exon 1	F: AATGGCAGAAGGCACAATCC R: GTCTCGGAACTGCTGGTGTC	517	58.81
exon 4	F: AAGGGGAGTTTGCTTTTCTG R: AAACCATTGCCATTGAACCC	442	56.13
exon 6	F: GGAGGGAGAAGGGGGATTGA R: TACCGGCAGCCTCTTGTCTA	615	60.3

DRD2=dopamine D2 receptor.
F=forward; R=reverse.
Genome accession No.: NW_004973237.1.

Table 2. **SNPs in the exon of DRD2 gene and the amino acid mutations in pigeon**

SNPs	Mutation sites	Exon region	Amino acid mutations
C103A	103	exon1	Gln/Lys
G108A	108	exon1	Lys
A3008C	3008	exon4	Arg /Ser
G3085A	3085	exon4	Arg/His
C3169T	3169	exon4	Thr/Ile
C4523T	4523	exon6	Gln
C4532T	4532	exon6	Arg

SNP=single nucleotide polymorphisms; DRD2=dopamine D2 receptor.
mRNA accession No.: XM_005500718.1.

Table 3. **Genotypic and allelic frequency of SNPs in the DRD2 gene of pigeon**

SNPs	Location	N	Genotypic			Allele		P value
			AA	AB	BB	A	B	
A3008C	Exon 4	60	0.96 (58)	0.02 (1)	0.02 (1)	0.97	0.03	3.44E-07
G3085A	Exon 4	60	0.95 (57)	0.02 (1)	0.03 (2)	0.96	0.04	8.82E-10
C3169T	Exon 4	60	0.58 (35)	0.05 (3)	0.37 (22)	0.61	0.39	4.11E-12
C4523T	Exon 6	60	0.98 (59)	0.02 (1)	0.00 (0)	0.99	0.01	0.95
C4532T	Exon 6	60	0.75 (45)	0.25 (15)	0.00 (0)	0.88	0.12	0.27

SNP=single nucleotide polymorphisms; DRD2=dopamine D2 receptor.
N=the number of tested birds of each genotype.
AA genotype is a homozygous genotype consisted of two bases located before the number of a SNP site, while BB is a homozygous genotype composed of two bases situated after the number of a SNP site. For example, AA, AB and BB genotypes of the C103Asite mean genetic composition of CC, CA and AA.
The test of Hardy-Weinberg Equilibrium $P>0.05$ suggested the population conforms to Hardy-Weinberg Equilibrium.

Table 4. **Reproductive traits and their variable coefficients (CV) in pigeon**

Traits	N	Min	Max	Average	Std.	CV (%)
EP	60	8	28	17.43	3.48	19.97
FE	60	8	18	13.93	2.33	16.73
FR	60	50	100	81.21	11.67	14.37
HN	60	8	18	13.93	2.33	16.73

These traits include egg production (total number of eggs; EP), total number of fertile eggs (FE), fertility rate (the ration of FE to EP; FR), and total hatching number (HN) within 500 days of age.
N=the number of tested birds of each genotype.
Std.=standard deviation.

As the variants (C103A and G108A) in exon 1 were rare in the samples, they were excluded from further analysis. The genotypic and allelic frequencies of the other identified SNPs in the DRD2 gene were shown in Table 3. Three genotypes (AA, AB, and BB) were detected in SNPS of exon 4, and the AA genotype was the most frequent one, whereas the AB and BB genotype frequency were relative low. There were two genotypes (AA, AB) identified in SNPs of exon 6, in which genotype AA was found at a higher frequency, while genotype BB at a lower frequency. The results showed that allele A was consistently the dominant allele in all nucleotide mutations of exon 4 and 6 regions in DRD2 gene. The genotype distributions for C4523T and C4532T fit the Hardy-Weinberg equilibrium with the P-value higher than 0.05.

Reproductive Traits of Pigeon

The Reproductive traits of pigeon were shown in Table 4. Variable coefficients (CV) for EP, FE, FR, and HN were 19.97%, 16.73%, 14.37%, and 16.73%, respectively. The correlation among those 4 reproductive traits in pigeon was shown in Table 5. There was a positive correlation between EP and FE ($P<0.01$), a negative correlation between EP and FR ($P<0.01$), and a positive correlation between HN and

Table 5. **The correlation among the 4 reproductive traits in pigeon**

Traits	EP	FE	FR	HN
EP	1			
FE	0.682**	1		
FR	−0.561**	0.200	1	
HN	0.682**	1**	0.200	1

These traits include egg production (total number of eggs; EP), total number of fertile eggs (FE), fertility rate (the ration of FE to EP; FR), and total hatching number (HN) within 500 days of age.
Data with ** indicate very significant correlation ($P<0.01$).

Table 6. **Association of the C4532T SNP genotypes of DRD2 gene with reproductive traits in pigeon**

SNPs	Traits	Phenotypic value of different genotypes	
		AA	AB
C4532T	EP	17.24±0.52	18.00±0.90
	FE	13.56±0.34[a]	15.07±0.58[b]
	FR	80.04±1.72	85.20±2.98
	HN	13.56±0.34[a]	15.07±0.58[b]

SNP=single nucleotide polymorphisms; DRD2=dopamine D2 receptor.
These traits include egg production (total number of eggs; EP), total number of fertile eggs (FE), fertility rate (the ration of FE to EP; FR), and total hatching number (HN) within 500 days of age.
Within a row, means with different superscripts differ at $P<0.05$; $n=60$.

EP, FE ($P<0.01$).

Association of Gene Polymorphisms with Reproductive Traits

Association studies between each SNP genotypes and reproduction traits were performed by using the general linear model. Among all the nucleotide mutations detected in this study, only C4532T SNP genotypes in exon 6 of DRD2 gene was significantly ($P<0.05$) associated with reproductive traits of pigeon (Table 6, the data of other SNP genotypes were not shown). The individuals with AB genotype had significantly higher FE and HN than those with AA genotype ($P<0.05$).

Discussion

The link between DRD2 and PRL secretion makes this gene a good candidate for containing a polymorphism to serve as a marker assisted selection for breeding. DRD2 is a member of the G protein-coupled receptor with seven transmembrane domains and was first isolated from a brain cDNA library in rat (Bunzow *et al*., 1988). Subsequently, the DRD2 gene had been studied in many species, including human, rat, canine, pig, turkey, and chicken (Gandelman *et al*., 1991; Montmayeur *et al*., 1991; Itokawa *et al*., 1993; Schnell *et al*., 1999; Myeong *et al*., 2000; Xu *et al*., 2010, 2011a, b, c). Despite identifying DRD2 biological functions in mammals and other animals, its biological functions in pigeon are still unclear. Also, nothing is known about its genetic mutations in pigeon. In this study, the directly DNA sequencing method was used to detect pigeon DRD2 gene polymorphisms. A total of 7 SNPs in exon 1, 4, and 6 of DRD2 were detected. Referring to amino acid mutations, four nonsynonymous mutations including three synonymous mutations were also identified in the studied region of pigeon DRD2 protein. All the above results reveal that DRD2 gene is rich in polymorphisms and the sequence of DRD2 amino acid seems to be conserved at a certain extent. In addition, three genotypes (AA, AB, and BB) were detected in SNPs of exon 4, and two genotypes (AA, AB) were identified in SNPs of exon 6. The results showed that AA genotype was dominant, and the A allele showed a dominant advantage in SNPs of pigeon DRD2.

Poultry reproductive traits, including the age laying first egg, egg production, egg fertility, hatchability and so on, are economically important in modern poultry production. In the present study, there was a relative high genetic variation for reproductive traits, such as EP, FE, FR, and HN, indicating that this population of pigeon is suit for analyzing markers correlated with such traits. Recently, polymorphisms within the DRD2 gene have been reported to be associated with reproduction traits in poultry. In turkeys, two spliced isoforms were isolated and some studies suggested that the expression of the DRD2 mRNA was correlated with broodiness (Schnell *et al*., 1999). In chickens, studies indicated that the polymorphisms of DRD2 gene exhibited a significant association with chicken egg number at 300 days of age (Xu *et al*., 2011b), the age at first egg (Xu *et al*., 2011c), and broody frequency and duration of broodiness (Xu *et al*., 2010). Both of these studies suggested that DRD2 gene played a critical role on regulating the reproductive traits of avians. Consistent with those studies, our study showed that C4532T, which is located in exon 6 of the DRD2 gene, was demonstrated to have a highly significant effect on pigeon reproductive traits, such as FE and HN. C4532T was a synonymous mutation, indicating that its amino acid sequences were highly conserved in different individuals of the same species. It has been reported that even synonymous mutations may affect gene function by influencing translation with codon bias, changing the stability of the mRNA or controlling of transcription of the gene (Duan, 2003). Meanwhile, the mutations located in the intron region, could also affect the mRNA splicing or protein modification and were related with diseases or traits (Xu *et al*., 2011a). In this study, we found that individuals with the AB genotype of C4532T have higher FE and HN than those with the AA genotype. The results indicated that C4532T of DRD2 gene could be used as a molecular marker for screening high value reproductive traits in domestic pigeons. Therefore, further research should be performed in order to validate the causative functions of pigeon DRD2 gene on reproduction.

In conclusion, the present study provided valuable information that the DRD2 gene had important effects on

pigeon reproduction. The SNP of C4532T located in exon 6 of the DRD2 gene could be a potential candidate genetic marker for Marker-aid breeding in pigeon, but a larger sample size is needed to validate this hypothesis.

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