GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Polymorphisms associated with egg number at 300 days of age in chickens

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Genet. Mol. Res. 10 (4): 2279-2289 (2011)
Received November 19, 2010
Accepted February 2, 2011
Published October 3, 2011
DOI http://dx.doi.org/10.4238/2011.October.3.5

ABSTRACT. We looked for variations that could be associated with chicken egg number at 300 days of age (EN300) in seven genes of the hypothalamic-pituitary-gonadal axis, including gonadotrophin-releasing hormone-I (*GnRH-I*), GnRH receptor (*GnRHR*), neuropeptide Y (*NPY*), dopamine D2 receptor (*DRD2*), vasoactive intestinal polypeptide (*VIP*), VIP receptor-1 (*VIPR-1*), prolactin (*PRL*), and the QTL region between 87 and 105 cM of the Z chromosome. Ten mutations in the seven genes were chosen to do marker-trait association analyses in a population comprising 1310 chickens, which were obtained from a company located in Guangdong Province of China. The C1704887T of *VIPR-1* was found to have a highly significant association with EN300. The T5841629C of *DRD2* and the C1715301T of *VIPR-1* were significantly associated with EN300. A highly significant association was also

found between the C1704887T-C1715301T haplotypes of *VIPR-1* and EN300. H1H3 had the highest EN300. Four PCR-RFLP variations in the candidate QTL region were selected to investigate their genetic effects on EN300. The haplotypes of T32742468C-G32742603A in this region showed a highly significant association with EN300. Bioinformatics analyses showed that both T32742468C and G32742603A were located in intron 1 of the SH3-domain GRB2-like 2 (*SH3GL2*) gene. We conclude that five SNPs, including C1704887T and C1715301T of *VIPR-1*, T5841629C of *DRD2*, and T32742468C and G32742603A of *SH3GL2*, would be useful as markers for breeding to increase chicken EN300.

Key words: Association analysis; Chicken; Polymorphism; QTL; Candidate gene; Egg number at 300 days of age

INTRODUCTION

Egg production is an important economic trait in poultry. Endocrine factor (Kim et al., 2004) and many environment factors such as the length of photoperiod and different feeding allowances could influence egg production (Liu et al., 2004; Lewis and Gous, 2006). Nevertheless, the genetic factor is the prerequisite. Egg production is a polygenic inheritance trait with low to moderate heritability, which depends on the period involved (Emsley, 1997; Luo et al., 2007).

In poultry breeding programs, egg number at 300 days of age (EN300) is usually used as a valuable indicator for total egg production. The avian egg-production process is strictly controlled by the hypothalamic-pituitary-gonadal axis (Kuo et al., 2005). Gonadotropin-releasing hormone (GnRH) is a key hormone located at the pinnacle of this axis (Shacham et al., 2001). GnRH, binding with its receptor GnRHR, stimulates gonadotrophin secretion from the pituitary gland and then evokes steroidogenesis in the gonads, resulting in egg production in hens (Shacham et al., 2001; Proudman et al., 2006; Sonez et al., 2010). Neuropeptide Y (NPY) is known to be involved in the regulation of reproductive function at the hypothalamic level through the control of GnRH secretion (Dhillon et al., 2009; Klenke et al., 2010). Beside this GnRH-gonadotrophin pathway, avian seasonal reproduction is also controlled by the vasoactive intestinal peptide (VIP) - prolactin (PRL) neuroendocrine pathway (Sharp, 2005; Leska and Dusza, 2007). Plasma PRL level had a negative effect on the chicken egg production (Reddy et al., 2007). VIP is a well-known hypothalamic PRL-releasing factor in avian (El Halawani et al., 1996) and its physiological effects are mediated through activating its specific receptor (Chaiseha et al., 2004). Furthermore, dopamine was demonstrated to play a dual role in PRL release by affecting VIP secretion, exerting its inhibitory effect via dopamine D2 receptor (DRD2) (Al Kahtane et al., 2003). Therefore, *GnRH-I*, *GnRHR*, *NPY*, *PRL*, *VIP* and one of its receptors, *VIPR-1*, as well as *DRD2* were regarded as candidates of chicken EN300 in this study.

Many studies have been performed to identify QTL regulating chicken egg number (Hansen et al., 2005; Schreiweis et al., 2006; Chatterjee et al., 2010). QTL influencing the egg number from 16 to 25 weeks of age was mapped to chromosome 4, whereas QTL related

to the egg number between 18 and 40 weeks of age were mapped to chromosome 8, Z and 11 (Tuiskula-Haavisto et al., 2002, 2004; Schreiweis et al., 2006). Additionally, the QTL region between 87 and 105 cM of the Z chromosome was found to affect the egg number from 18 to 40 weeks of age and from 41 to 60 weeks of age, the average egg weight of these two periods, as well as the age of first egg (AFE) (Tuiskula-Haavisto et al., 2002; Sasaki et al., 2004). In the present study, this area was taken as the candidate QTL region for EN300.

Although there have been increasing studies on the relationship of genes with egg production (Dunn et al., 2004; Xu et al., 2010a), few genetic markers applied in MAS have been obtained. The objective of this study was to identify variations associated with EN300 in the candidate genes and QTL region. In this study, SNPs in the candidate QTL region were identified by direct sequencing. Subsequently, 10 mutations in the candidate genes and 4 PCR-RFLP variations in the QTL region were chosen to test their association with EN300 in a Chinese native chicken population.

MATERIAL AND METHODS

Chicken populations

A population comprising 1310 Ningdu Sanhuang (NDH) female individuals from one hatch were used for association analyses in the present study. All chickens were obtained from a female line of the Guangdong Wens Foodstuff Company Ltd., Guangdong, China. NDH was a Chinese native chicken breed originating from the Jiangxi Province, China, and its total egg production for 500 days of age was about 113 eggs. Chickens had been kept in closed breeding for five generations, and the fifth generation population was used in the present study. All birds were fed *ad libitum* to 77 days of age with a diet of 2837 kcal ME/kg and then changed to restrict feed with a diet of 2907 kcal ME/kg. During the first 2 days post-hatch, all individuals were exposed to a continuous 24-h photoperiod and then transferred to a 16-h photoperiod. All females were housed in individual laying cages after 91 days of age and their egg production was observed and recorded at 16:00 h every day. Finally, the number of eggs produced from 91 to 300 days of age was calculated individually. The average value of EN300 in this population was 94.0 eggs. Blood samples were collected from the vein under the wing after 300 days of age and genomic DNA was extracted.

In addition, 10 randomly selected NDH chickens were used for polymorphism identification in the candidate QTL region (32.17 ~ 34.26 Mb of the Z chromosome).

SNP identification and selection

Based on the dbSNP database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp) and previous reports (Cui et al., 2006; Zhou et al., 2008b, 2010; Xu et al., 2010b), 10 variations in 7 candidate genes, including *GnRH-I*, *GnRHR*, *NPY*, *DRD2*, *VIP*, *VIPR-I*, and *PRL*, were used to analyze their effects on EN300. A total of 10 pairs of primers were designed by the Genetool software (http://www.biologysoft.com/; BioTools, Alberta, Canada) to genotype the above variations (Table 1).

Table 1. Detail information for primers of the candidate genes of EN300.

No.	Site	Chr	Gene	Primer sequence (5'→3')	Length (bp)	AT (°C)	Restriction enzyme
M1	G840327C	Chr22	*GnRH-I*	F:tgtcacacccaggatctcaa R:gctgttcagaggcacgtgag	310	59.0	*Mnl*I
M2	A19960831G	Chr10	*GnRHR*	F:ggtgtctgaggctcatttca R:tagcaatcgcttgcccaga	417	58.0	*Bpu*1102I
M3	I31391359D	Chr2	*NPY*	F:tctcagagctccaacgtatga R:atatttctgtgcctgaacaaca	248/252	58.0	*Dra*I
M4	C31394761T	Chr2	*NPY*	F:cgtggctgctttgcttcctttc R:ggggtacgaggcaaggacatg	324	60.0	*Kpn*I
M5	T5841629C	Chr24	*DRD2*	F:tgcacataaaagcccactcactg R:gcctgagctggtgggggg	248	60.0	*Bse*GI
M6	G51389822T	Chr3	*VIP*	F:gcttggactgatgcgtactt R:gtatcactgcaaatgctctgc	520	58.0	*Apo*I
M7	A1661691G	Chr2	*VIPR-1*	F:tgaaagcccccaggatct R:agcaaaacaaaacccaaatca	364	58.2	*Tai*I
M8	C1704887T	Chr2	*VIPR-1*	F:ccccgttaaactcagcagac R:cccaaagtcccacaaggtaa	434	58.2	*Hha*I
M9	C1715301T	Chr2	*VIPR-1*	F:ctcctcaggcagaccatcatg R:cttgcacgtatccttgggtagc	486	58.2	*Taq*I
M10	I59724210D	Chr2	*PRL*	F:tttaatattggtgggtgaagagaca R:atgccactgatcctcgaaaactc	130/154	54.0	PCR

The sites are based on the chicken genome sequences released in May 2006 (http://genome.ucsc.edu/cgi-bin/hgGateway). Chr refers to chromosome. Length indicates the length of PCR products. AT refers to annealing temperature.

Based on the genomic sequence of the 87 ~ 105 cM (about 32.17 ~ 34.26 Mb) region on the Z chromosome (http://www.ensembl.org/Gallus_gallus/Index.html), 5 pairs of primers (P1 to P5) were designed to scan polymorphisms of the QTL region, and the average distance between 2 adjacent pairs of primers was 500 kb (Table 2). PCR products were then subcloned into the pMD18-T vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) and sequenced by a commercial company (Biosune, Shanghai, China). The sequences were analyzed by the DNASTAR software (http://www.dnastar.com/).

Among the identified variations, only 4 markers could be easily genotyped by PCR-RFLP. Therefore, 3 pairs of primers (M11, M13 and M14 in Table 2) were designed and synthesized for genotyping these 4 markers in association analyses.

PCR amplification and genotyping of polymorphisms

PCR amplification was carried out in a final volume of 25 µL: 50 ng genomic DNA, 1 µM of each primer, 200 µM dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, and 1.0 U Taq DNA polymerase (Sangon Biological Engineering Technology Company, Shanghai, China), using an Eppendorf Mastercycler (Eppendorf Limited, Hamburg, Germany). The following reaction conditions were used: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30 s, n°C (n was the annealing temperature shown in Tables 1 and 2) for 35 s, 72°C for 35 s, and a final elongation at 72°C for 7 min. Genotyping assay of M10 was directly observed by 3.5% agarose gel electrophoresis after PCR amplification. Genotypes of the other polymorphisms were determined by the PCR-RFLP method. According to the manufacturer protocol, 8 µL PCR products was further digested at 37°C (55°C for *Bse*GI, 65°C for *Tai*I and *Taq*I) overnight. After digestion, the products were subjected to 2.5% agarose gel electrophoresis, and the genotypes were determined with a TFM-40 Ultraviolet Transilluminator (UVP Company, Cambridge, UK) by ethidium bromide staining.

Table 2. Detailed information of primers used for marker screening and association analyses on the QTL region of chicken Z chromosome.

Primer	Primers sequences (5'→3')	Location (nt)/site	Length (bp)	AT (°C)	Restriction enzyme
P1	F:acagctaaagcagacaagtgac R:cagggaaaaagagagcattatc	32173030 to 32173816	787	58	/
P2	F:aggagctgggtgacattgtg R:tggggtaaggacagcacagt	32742159 to 32742879	721	58	/
P3	F:gagggaaatgggaagcaaagtag R:gccaaaagctgaaagtaggtctg	33379518 to 33380294	777	62	/
P4	F:ttgcctactcacctggatgt R:ggagggaagtgggaaggatt	33824372 to 33825087	716	58	/
P5	F:accgcttcctgtgttcttaatc R:tgcaaggatgtcaaaaattatg	34263732 to 34264499	768	60	/
M11	F:aggagctgggtgacattgtg R:tggggtaaggacagcacagt	T32742394C	721	58	*Msp*I
M12	F:aggagctgggtgacattgtg R:tggggtaaggacagcacagt	T32742468C	721	58	*Pau*I
M13	F:tgcaagcccaggaatcatcactc R:taaaactcttctttccttctaca	G32742603A	294	58	*Alu*I
M14	F:tcttcgaacacattactcactga R:ggcgttttgtgttttcttggcat	C33379782T	400	57	*Alu*I

Location is based on chicken Z chromosome. Length indicates PCR product length. AT refers to annealing temperature.

Statistical analyses of haplotypes

The haplotype structure was analyzed by the Haploview version 3.32 software (http://www.broad.mit.edu/mpg/haploview/). Haplotypes were constructed based on the genotype data by the PHASE 2.0 software (http://en.wikipedia.org/wiki/Phase).

Marker-trait association analyses

Association analyses of polymorphisms or haplotypes with the chicken EN300 were carried out by the SAS GLM procedure (SAS Institute Inc., Cary, NC, USA), and the genetic effects were evaluated by the mixed model as follows:

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

where Y is a trait observation, μ is the overall population mean, G is the fixed effect of genotype or haplotype, F is the random effect of family, and e is the residual error. Multiple comparisons were conducted with least square means. A $P \leq 0.05$ was considered to be significant in all analyses.

Bioinformatics analyses in the QTL region

Gene mapping and function analyses of variations associated with EN300 in the candidate QTL region were performed on bioinformatic websites of www.ncbi.nih.gov/mapview/ and www.ensembl.org/Gallus_gallus/index.html.

RESULTS

Genotype and haplotype of the 7 candidate genes

Three genotypes were found in each of M1-M10 in the candidate genes of EN300. For the *NPY* gene, 4 distinct haplotypes, E1, E2, E3, and E4, with each frequency higher than 1%, were present in the haplotype block composed of I31391359D and C31394761T (Block 1). Among the four haplotypes, E1 (47.51%) and E4 (25.71%) were the predominant ones, whereas E2 (17.19%) and E3 (9.59%) were minor. On the other hand, haplotype structure analyses showed that there was a haplotype block for the 3 SNPs of the *VIPR-1* gene, and it was composed of M8 and M9 (Block 2). Four haplotypes were observed, and 3 of these, namely H1 (CT, 18.91%), H2 (CC, 77.69%) and H3 (TC, 3.04%), had a frequency higher than 1%.

Association of variations in the 7 candidate genes with EN300

As shown in Table 3, a highly significant association was found between the C1704887T of the chicken *VIPR-1* gene and the EN300 ($P < 0.01$), and chickens with the TT genotype had a lower EN300 than those with the TC and CC genotypes ($P < 0.01$, highly significant). Furthermore, the C1715301T of the *VIPR-1* gene was significantly associated with EN300 ($P < 0.05$), and allele T was advantageous for chicken EN300 (Table 4). The T5841629C of the *DRD2* gene also showed a significant association with EN300 ($P < 0.05$). In addition, the EN300 values of individuals with the TT genotype were higher than those with the CC and TC genotypes ($P < 0.01$, highly significant). No significant association was found in the other variation sites (G840327C of *GnRH-I*, A19960831G of *GnRHR*, I31391359D and C31394761T of *NPY*, G51389822T of *VIP*, A1661691G of *VIPR-1*, and I59724210D of *PRL*) with EN300 ($P > 0.05$).

Association of the haplotypes in the 7 candidate genes with chicken EN300

A total of 1204 NDH individuals with 9 diplotypes (246 of E1E1, 216 of E1E2, 131 of E1E3, 305 of E1E4, 41 of E2E2, 116 of E2E4, 12 of E3E3, 76 of E3E4, and 61 of E4E4) were used in association analyses in the haplotype block composed of I31391359D-C31394761T in the *NPY* gene. No significant association was found in this haplotype block with chicken EN300 ($P > 0.05$).

A total of 1295 NDH individuals with 6 diplotypes, including 114 of H1H1, 249 of H1H2, 14 of H1H3, 853 of H2H2, 60 of H2H3, and 5 of H3H3, were used in association analyses in the haplotype composed of C1704887T-C1715301T in the *VIPR-1* gene. A highly significant association ($P < 0.01$) was found between the haplotypes and chicken EN300 (Table 4). H3H3 had a lower value of EN300 than other diplotypes ($P < 0.01$, highly significant). Nevertheless, H1H3 had much higher EN300 values and was significantly different from H1H2 and H2H2 ($P < 0.05$). H2H2 was also shown to be significantly different from H1H1 ($P < 0.05$).

Polymorphisms in the QTL region and their association with EN300

Sixteen variations were found in a total of 3769 bp in the 32.17 to 34.26 Mb region of the chicken Z chromosome (shown in Table 5). Four PCR-RFLP markers, including

T32742394C, T32742468C, G32742603A, and C33379782T, were selected to perform marker-trait association analyses. The results are shown in Table 6, and no significant association was detected between any of the 4 sites and EN300 ($P > 0.05$).

Table 3. Association of variations in the candidate genes with chicken EN300.

No.	Candidate gene	Chr	Site	Genotype	N	EN300	P value
M1	*GnRH-I*	Chr22	G840327C	CC	26	93.31 ± 5.46	0.592
				GC	208	96.28 ± 1.97	
				GG	972	94.12 ± 0.86	
M2	*GnRHR*	Chr10	A19960831G	GG	30	96.40 ± 4.91	0.531
				GA	420	92.99 ± 1.29	
				AA	823	94.60 ± 0.95	
M3	*NPY*	Chr2	I31391359D	II	156	93.18 ± 2.11	0.622
				ID	578	93.59 ± 1.10	
				DD	522	95.00 ± 1.18	
M4	*NPY*	Chr2	C31394761T	CC	392	94.55 ± 1.35	0.923
				TC	600	94.20 ± 1.08	
				TT	214	95.04 ± 1.85	
M5	*DRD2*	Chr24	T5841629C	CC	1060	94.03 ± 0.83^{A}	0.025*
				TC	217	92.83 ± 1.94^{A}	
				TT	5	124.64 ± 11.66^{B}	
M6	*VIP*	Chr3	G51389822T	GG	74	95.52 ± 3.16	0.179
				GT	400	96.50 ± 1.37	
				TT	732	93.26 ± 1.01	
M7	*VIPR-1*	Chr2	A1661691G	GG	1028	94.27 ± 0.87	0.204
				GA	95	98.81 ± 2.91	
				AA	83	92.12 ± 3.93	
M8	*VIPR-1*	Chr2	C1704887T	CC	1128	94.38 ± 0.79^{A}	0.007**
				TC	72	98.70 ± 3.19^{A}	
				TT	6	65.08 ± 10.70^{B}	
M9	*VIPR-1*	Chr2	C1715301T	CC	842	93.57 ± 0.91^{a}	0.050*
				TC	252	95.32 ± 1.68^{ab}	
				TT	112	100.10 ± 2.55^{b}	
M10	*PRL*	Chr2	I59724210D	II	10	85.01 ± 8.34	0.154
				ID	187	97.29 ± 1.95	
				DD	1009	93.99 ± 0.85	

Chr indicates chromosome. Site refers to the corresponding chromosomal location of chicken genome sequences (http://genome.ucsc.edu/cgi-bin/hgGateway; released in May 2006). N indicates the number of chickens tested for each genotype. EN300 = total egg number at 300 days of age; the values are shown with least-square means ± standard errors (SE). ^{a,b} or ^{A,B} values with no common superscripts within a column for each site differ significantly (*$P < 0.05$) or highly significantly (**$P < 0.01$).

Table 4. Association of the haplotype blocks with EN300.

Block 1	EN300	Block 2	EN300	Block 3	EN300
E1E1 (246)	95.29 ± 1.71	H1H1 (114)	99.33 ± 2.56^{c}	I1I1 (429)	94.60 ± 1.38^{A}
E1E2 (216)	96.06 ± 1.82	H1H2 (249)	93.86 ± 1.71^{bc}	I1I3 (5)	22.75 ± 11.65^{B}
E1E3 (131)	94.53 ± 2.31	H1H3 (14)	109.49 ± 7.26^{ac}	I2I2 (29)	101.81 ± 5.06^{A}
E1E4 (305)	93.03 ± 1.53	H2H2 (853)	93.28 ± 0.93^{b}	I3I3 (399)	92.27 ± 1.48^{A}
E2E2 (41)	92.55 ± 4.06	H2H3 (60)	96.23 ± 3.55^{abc}	I3I4 (62)	95.15 ± 3.54^{A}
E2E4 (116)	95.18 ± 2.47	H3H3 (5)	60.49 ± 11.92^{B}	I4I4 (372)	95.02 ± 1.50^{A}
E3E3 (12)	86.67 ± 7.43				
E3E4 (76)	92.80 ± 3.05				
E4E4 (61)	97.17 ± 3.35				
P value	0.827		0.003**		0.0001**

Blocks 1, 2 and 3 refer to the haplotype of I31391359D-C31394761T of *NPY*, the haplotype of C1704887T-C1715301T of *VIPR-1* and the haplotype of T32742468C-G32742603A in the QTL region, respectively. Data are reported as least-square means ± standard errors (SE); numbers in parentheses indicate the number of chickens tested for each diplotype. EN300 = total egg number at 300 days of age. ^{a,b,c}Means within a column with no common superscripts differ significantly ($P < 0.05$ or $P < 0.01$). **Indicates $P < 0.01$.

Table 5. Polymorphisms identified in the 32.17 to 34.26 Mb region of Z chromosome.

No.	SNP	Restriction enzyme	No.	SNP	Restriction enzyme
1	A32173202C	/	9	T32742468C	*Pau*I/*Bsh*1236I
2	T32173392A	/	10	A32742557G	/
3	A32173403T	*Alu*I	11	G32742603A	*Bau*I/*Alu*I
4	C32173455T	/	12	T32742747G	/
5	T32173517C	/	13	C32742849T	/
6	G32742256A	/	14	C33379782T	*Alu*I
7	C32742374G	/	15	G33380110A	/
8	T32742394C	*Msp*I	16	G34263878A	*Ssi*I

SNP location is the location on the Z chromosome referred to the released chicken genomic sequence (http://genome.ucsc.edu/; released in May 2006).

Table 6. Association of the 4 SNPs with chicken EN300.

No.	Location	Genotype	N	EN300	P value
M11	T32742394C	C	415	92.90 ± 1.44	0.4080
		T	797	94.43 ± 0.98	
M12	T32742468C	C	443	94.40 ± 1.33	0.6563
		T	769	93.63 ± 0.99	
M13	G32742603A	A	400	95.91 ± 1.43	0.1022
		G	812	92.91 ± 0.97	
M14	C33379782T	C	102	97.45 ± 2.74	0.1793
		T	1110	93.60 ± 0.79	

The sites refer to the location on the Z chromosome of the chicken genome sequences (http://genome.ucsc.edu/cgi-bin/hgGateway; released in May 2006). N indicates the number of chickens tested for each genotype. EN300 = total egg number at 300 days of age; the values are reported as least-square means ± standard errors (SE).

Haplotype structure of the 4 sites in the QTL region and their association with EN300

For the 4 variations of the Z chromosome, a haplotype block composed of T32742468C and G32742603A was detected in the NDH population (Block 3). In this block, 4 haplotypes, including I1 (CG, 33.24%), I2 (CA, 2.31%), I3 (TG, 33.36%), and I4 (TA, 31.08%), were observed. A total of 1296 chickens with 6 diplotypes (429 of I1I1, 5 of I1I3, 29 of I2I2, 399 of I3I3, 62 of I3I4, and 372 of I4I4) were used for the following association analyses, and the results are shown in Table 4. The haplotype of T32742468C and G32742603A showed a highly significant association ($P < 0.01$) with chicken EN300. Compared to other diplotypes, I1I3 had lower value of EN300, which was highly significant ($P < 0.01$).

Bioinformatics analyses

Bioinformatics analyses of the QTL region were conducted on the basis of Ensembl database (http://www.ensembl.org/Gallus_gallus/index.html). Both T32742468C and G32742603A, which constructed the haplotype block related to EN300, were located in intron 1 of the chicken SH3-domain GRB2-like 2 (*SH3GL2*) gene.

DISCUSSION

As a valuable index for overall egg production, EN300 has been widely studied, and

the identification of molecular markers for it has been well performed (Dunn et al., 2004; Xu et al., 2010a; Zhou et al., 2010). In a commercial broiler breeder hen population, Dunn et al. (2004) detected polymorphisms of the *GnRHR* and *NPY* genes and discovered an SNP in the intron 1 of the *GnRHR* gene and a 4-bp indel located 700 bp upstream of the *NPY* transcription start site. Subsequently, marker-trait association analyses were carried out, but no association of these two variations with total egg production was found. Similarly, in this study, the same sites including the site I31391359D of the *NPY* gene and the A19960831G of the *GnRHR* gene were selected to analyze their effects on EN300, and the results were compatible with findings of Dunn et al. (2004). Previous reports confirmed that active immunization with VIP could increase egg production in turkeys (El Halawani et al., 1995; Caldwell et al., 1999). Also, another study discovered that an AGG indel in the 5' regulatory region of the *VIP* gene was related to EN300 (Zhou et al., 2010). However, another polymorphism in the intron 2 of the *VIP* gene, G51389822T, was not significantly associated with EN300, which was in agreement with the report by Zhou et al. (2010). On the other hand, it has been demonstrated that the *PRL* expression is correlated with the egg number (Shiue et al., 2006; Chen et al., 2007). In an F_2 population produced from Nongdahe × Taihe Silkies chicken, Cui et al. (2006) proved that a 24-bp indel at the site of -358 of the *PRL* gene was associated with egg production. However, the results of this study did not find any association between this 24-bp indel and EN300.

The dopaminergic system has been shown to play an important role in the regulation of the avian reproductive system and these physiological effects are exerted by activating its receptors (Sartsoongnoen et al., 2008; Xu et al., 2010b). Although Xu et al. (2010a) did not find any association between the polymorphisms in the coding region of *DRD1* and EN300, variation of the *DRD2* gene exhibited a significant association with chicken EN300 in this study. As indicated by the present study, C1704887T, which is located in intron 6 of the *VIPR-1* gene, was demonstrated to have a highly significant effect on chicken EN300. The haplotype analysis based on C1704887T and C1715301T of the *VIPR-1* gene also validated this result. It was interesting that these findings about the *VIPR-1* gene were in accordance with the previous investigation, which was performed in a different population (Zhou et al., 2008b). On the other hand, other studies proved that the C1715301T of the *VIPR-1* gene has significant effects on both duration of broodiness and AFE, in which chickens with the CC genotype have longer duration of broodiness and earlier AFE than those with the TT genotype (Zhou et al., 2008a,b). In this study, we found that individuals with the CC genotype of C1715301T have lower EN300 than those with the TT genotype. These results suggest that EN300 in this site might be negatively affected by chicken broodiness. Thus, C1704887T and C1715301T in the *VIPR-1* gene, as well as T5841629C of the *DRD2* gene, may serve as important molecular markers of high egg production.

A QTL region close to the centromere of the Z chromosome was found to have effects on sexual maturity, egg weight, and number of eggs in both 18-40 weeks of age and 40-60 weeks of age (Tuiskula-Haavisto et al., 2002). In the present study, the haplotype of T32742468C - G32742603A in this region was also shown to be related to chicken EN300, even though no association was found between any of these single sites and the trait. These results confirmed previous findings that the association analysis of haplotype with traits was more predominant and reliable (Zhang et al., 2002; Rodriguez et al., 2006). Meanwhile, through bioinformatics analysis, we found that these two SNPs were located in the intron region of the *SH3GL2* gene. SH3GL2, also known as endophilin I, SH3P4, CNSA2,

and SH3D2A, is a member of the endophilin family and it contains 353 amino acids. Previous studies in mammals proved that SH3GL2 was essential for synaptic vesicle endocytosis (Schmidt et al., 1999; Reutens and Begley, 2002). Moreover, Hirayama et al. (2003) found that the endophilin family members differentially contributed to chicken oocyte endocytosis and development. Therefore, it seemed that the *SH3GL2* gene may be one of the causative genes responsible for chicken egg production and that T32742394C and G32742603A may play a crucial role in EN300. However, the confirmation of their genetic effects on EN300 still requires further study.

In conclusion, a total of 5 polymorphisms of the *DRD2*, *VIPR-1*, and *SH3GL2* genes were demonstrated to be associated with EN300. The information derived from this study is useful to enhance the breeding process with regard to chicken egg production.

ACKNOWLEDGMENTS

Research supported by the Major State Basic Research Development Program of China (#2006CB102107) and China National Broiler Industrialized Technology Program (#nycytx-42-G1-04), as well as the China Postdoctoral Science Foundation (project #20100470933).

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