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Short Communication

Single nucleotide polymorphisms in the 5'-flanking region of the prolactin gene and the association with reproduction traits in geese

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Abstract

Prolactin (PRL), a polypeptide hormone synthesized and secreted by the animal's anterior pituitary gland, plays an important role in the regulation of mammalian lactation and avian reproduction. Considering the significant association between single nucleotide polymorphisms (SNPs) in the 5'-flanking region of PRL and reproduction traits in the chicken, the objective of this study was to screen for SNP in the 5'-proximal region of PRL in geese and to evaluate the association between SNP and reproduction traits in geese. Chinese Wan-xi White geese (n = 200) and European Rhine geese (n = 80) were used for phenotyping. SNP was screened by comparing sequences of PCR products, and the single-stranded conformational polymorphism (SSCP) protocol was adopted for genotyping. Three SNPs (A-401G, G-268A and T-266A) in the 5'-proximal region of goose PRL were identified in both breeds. Statistical analysis suggested that the genotype AA characterized by A-401, G-268 and T-266 had a positive genetic effect on egg production. Therefore, these polymorphisms have the potential to be utilized in molecular breeding for egg production in geese.

Keywords: Goose, Wan-xi White, Rhine, PRL, egg production, SNP
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Prolactin (PRL) is one of the polypeptide hormones synthesized and secreted by the animal's anterior pituitary gland. It has been demonstrated that PRL is an essential hormone regulating reproduction and lactation in mammals (Byrnes & Bridges, 2005; Bonomo *et al.*, 2007). In domestic chickens and turkeys, PRL has proven to be one of the key hormones in the onset and maintenance of broodiness (Sharp *et al.*, 1988). A sharp rise in plasma PRL level may induce incubation behaviour and thus terminate laying (Sockman *et al.*, 2000), resulting in decreased egg production (Reddy *et al.*, 2002). Shiue *et al.* (2006) showed that mRNA expression levels of PRL were significantly higher in high than in low egg production strains, indicating that egg production was related to a PRL mRNA expression in chickens. In waterfowl species such as ducks and geese, a sharp rise in plasma PRL levels occurred during the formation of the final 10 - 20% of the clutch, during which females markedly increased their nest-box occupancy (Hall, 1987; 1991; Fang *et al.*, 2005). Plasma PRL levels of non-laying ducks were significantly lower than those of laying ducks (Bluhm *et al.*, 1983). Therefore, PRL exhibits similar changing profiles in the reproduction cycle and exerts biological functions in the similar pattern in different domestic avian species. To elucidate the genetics of PRL, genomic structures of PRL in avians have been investigated extensively (Zhou *et al.*, 2001; Kansaku *et al.*, 2005). An abundance of SNP has been reported in the 5'-flanking region of chicken PRL (Liang *et al.*, 2006), and the 24-bp Insert-deletion was significantly associated with broody behaviour and egg production (Jiang *et al.*, 2005; Cui *et al.*, 2006), indicating its usefulness as a molecular marker for egg production. However, whether SNPs exist and are associated with goose reproduction as exhibited in chickens, is still unknown. The purpose of the present study was to identify and characterize the SNP in the 5'-flanking region of PRL and evaluate the genetic effects of the SNP on reproduction traits in geese, thus providing a molecular reference in marker-assisted selection programmes.

The stocks used in the experiment were unrelated female Wan-xi White geese (n = 200), a local Chinese breed from the Anhui Province and unrelated female Rhine geese (n = 80) that have been imported to China previously. Up to 25 wk of age the geese were reared on a floor system in open-sided houses. At 25 wk of age the birds were transferred to pens (3 × 3 m) in groups of four per pen. Each pen contained on one

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side a 3 × 3 m outside ground area with a water bath. During the laying period the experimental geese were fed a commercial maize-soyabean-based diet containing 155 g CP/kg feed with a metabolisable energy content of 10.87 MJ/kg feed, and sufficient forage. The birds had free access to feed and water.

Reproductive traits recorded in the experiment included body weight at first egg (BW), the first egg weight (FEW) and total egg production (EP). The EP for Wan-xi White was measured up to 60 wks of age, i.e. the total first laying cycle, while for Rhine it was up to 45 wks of age, the early stage of the first laying cycle. To accurately record individual EP, each pen was equipped with four separated nest-boxes that prevented escaping. The four geese in a pen were each put into a nest-box for the night and were taken out in the morning after checking whether they had laid or not.

The repeats of broodiness (RB) which were defined as the number of broody cycles of the Wan-xi White, were recorded during the total first laying cycle. A goose was defined as broody when she persistently nested for more than three consecutive days, exhibited nesting defence, and reduced food intake and activities. The criterion for the end of broodiness was that the bird did not exhibit broody behaviour for more than one week.

Genomic DNA was isolated from geese blood samples by the phenol-chloroform method (Sambrook *et al*., 1989). PCR was performed to amplify the fragments of the 5'-proximal region of PRL. The primers (P1) were designed according to the sequence of goose PRL (GenBank accession No.: EF190486) using the online primer design procedure, Primer 3.0 (Primer 3.0, Whitehead Institute for Biomedical Research, www.cbr.nrc.ca/cgi-bin/primer3_www.cgi). The sequences of P1 were: Forward 5'- TGGACATGC ACATCTTTTACG -3', Reverse 5'- ATCCACAAGTGAATGGCTAA -3'. The 20 μL PCR volume included 50 ng of DNA template, 0.20 mM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.20 mM primer and 0.5 U of Taq DNA polymerase (Dingguo Biotechnology Company, Beijing, P. R. China). The PCR protocol was 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were purified using a DNA Fragment Quick purification/Recover Kit (Dingguo Biotechnology Company, Beijing, P. R. China), ligated to the pMD 18-T vector (Dingguo Biotechnology Company, Beijing, P. R. China) and transformed into DH5-α Escherichia coli (Dingguo Biotechnology Company, Beijing, P. R. China) for cloning (Sambrook *et al.*, 1989). Sequencing was performed on an ABI377 sequencer.

PCR products of 20 individuals were individually sequenced to screen the SNP. Because of variation, sites screened in this study only ranged from -402 to -255 bp. To facilitate genotyping, another PCR set of primers (P2) that produced a shorter PCR product (<300 bp) was re-designed to amplify the region covering the variation sites. The P2 was as following: Forward 5'-AAGCCCCCATTATCCCTCTC -3', Reverse 5'-TGGTTTGATGAAGAGAATGCC -3'. The PCR products of P2 for all individuals were genotyped by single-stranded conformational polymorphism protocol: 2 μL of PCR product of each individual were mixed with 5 μL of denaturing buffer (98% formamide, 0.09% xylene cyanole FF, and 0.09% bromophenol blue) and then denatured at 94 °C for 5 min followed by a rapid chill on ice for 10 min. The denatured PCR products were electrophoresed for 14 h at 8 V/cm on 12% acrylamide gels. The DNA bands on the gel were stained with 0.2% $AgNO_3$ for 20 min, followed by 3% Na_2CO_3 for about 5 min (Qu *et al.*, 2005). Genotypes were recorded according to banding patterns. The PCR products of P2 for each homozygote were sequenced to identify the SNP mutation type.

Body weight, FEW and EP were calculated for each genotype. Genotypic effects on BW, FEW, EP and RB were analyzed by one-way ANOVA using the GLM procedure of the SAS Institute (2001) with genotype (G) as the fixed effect, according to the following model:

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

where Y = dependent variable, μ = population mean and e = random error. Significant differences among means of different genotypes or haplotypes were calculated using the Duncan's multiple-range test, and the significance was determined at $P < 0.05$.

The sequence fragment, from -790 to -1, of 5'-proximal region of goose PRL was successfully amplified using the primer P1. By comparing the sequences of 20 individuals, three nucleotide variation sites, A-401G, G-268A and T-266A, were detected. By developing the single-stranded conformational polymorphism protocol, three genotypes were identified at the P2 locus both in Wan-xi White and Rhine

geese and defined as AA, AB, and BB (Figure 1). Sequencing of the different genotypes showed that the homozygote individuals of genotype AA shared the genomic characteristics of -401A, -268G and -266T, and the BB of -401G, -268A and -266A, while the heterozygote AB combined the two.

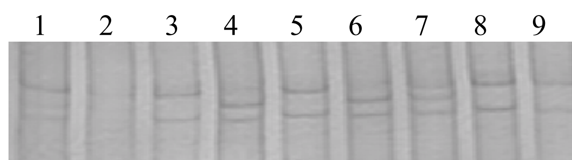

Figure 1 Genotypes at P2 locus of goose *PRL*. Lanes 1 and 7 are genotype AB; lanes 3, 5, 8, and 9 are AA; lanes 4 and 6 are BB.

Table 1 Genotypic and allelic frequencies of P2 locus in *PRL* in Wan-xi White and Rhine geese

Breeds	Items	Genotypes			Alleles	
		AA	AB	BB	A	B
Wan-xi White	No. of animals	123	45	32		
	Frequency	0.62	0.22	0.16	0.73	0.27
Rhine	No. of animals	45	17	18		
	Frequency	0.56	0.21	0.23	0.59	0.41

Genotypic and allelic frequencies of the P2 locus were calculated within each breed (Table 1). In Wan-xi White and Rhine geese, the genotypic frequency for AA (0.62 and 0.56, respectively) was the greatest and, therefore, the allele A was predominant in the populations (0.73 and 0.59, respectively).

As shown in Table 2, no significant difference ($P > 0.05$) was found between genotypes in BW and FEW in the two breeds, and the RB did not differ ($P > 0.05$) between genotypes in Wan-xi White. However, in Wan-xi White geese, the mean EP in the first laying cycle of genotype AA (32.5) was greater ($P < 0.05$) than that of genotype AB (26.07), and in Rhine geese, the mean EP at 45 wk of age of genotype AA (33.73) was greater ($P < 0.05$) than that of genotype BB (26.93).

Table 2 Association of genotypes at the P2 locus in *PRL* with reproduction traits in Wan-xi White and Rhine geese

Breeds	Genotypes	N[1]	Traits ($\mu \pm$ s.e.)[2]			
			BW (g)	FEW (g)	EP[3]	RB
Wan-xi White	AA	123	4214.2 ± 46.6	140.81 ± 1.01	32.5 ^{a} ± 0.88	4.34 ± 0.08
	AB	45	4196.6 ± 65.9	137.97 ± 1.39	26.07 ^{b} ± 1.39	4.00 ± 0.17
	BB	32	4304.8 ± 82.1	138.06 ± 1.76	28.07 ^{ab} ± 1.41	4.89 ± 0.19
Rhine	AA	45	4269.1 ± 63.70	138.07 ± 1.70	33.73 ^{a} ± 0.98	
	AB	17	4160.2 ± 129.6	141.24 ± 2.47	30.43 ^{ab} ± 2.14	
	BB	18	4233.9 ± 120.2	141.91 ± 2.22	26.93 ^{b} ± 2.07	

[1] N - number of individuals.
[2] BW - body weight at first egg; FEW - first egg weight; EP - egg production; RB - repeats of broodiness.
[3] The EP for Wan-xi White was at 60 weeks of age, while for Rhine it was at 45 weeks of age.
[a, b] Means in the same column within a breed with different superscripts differed at $P < 0.05$.

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By screening the 5'-flanking region of goose PRL, three SNPs, A-401G, G-268A and T-266A, were identified both in Wan-xi White, a Chinese breed and in Rhine geese, a European breed, suggesting the abundance of nucleotide variations in the region as exhibited in the chicken. The fact that the three SNPs produced only three genotypes in the experimental populations indicated the linkage heredity of the SNPs. In the study the association of genotypes with egg production in Wan-xi White and Rhine geese shared a similar profile, the geese of genotype AA laid more eggs than genotypes AB or BB, indicating that allele A could be the favourite for egg production or linked with the QTLs of interest. The similar association observed in both breeds with different genetic backgrounds basically ensured the reliability of the result, suggesting that the enrichment of allele A in a population by molecular selection may be helpful for the enhancement of egg production in geese.

The plasma PRL protein expression level is of great importance in regulating avian reproduction ability including incubation and egg production. In avian species, changes in plasma PRL level are associated with the expression of PRL mRNA in the anterior pituitary (Wong *et al.*, 1991). However, the expression of PRL is regulated by the 5′-flanking region sequence by binding with specific transcription factors (Jiang *et al.*, 2005). Studies in mammals and birds have shown that Pit-1/GHF-1 (Frisch *et al.*, 2000), oestrogen receptors (Maurer & Notides, 1987), the CCAAT-enhancer binding protein-α (Enwright *et al.*, 2003) and other proteins are essential in regulating the expression of PRL via specific promoter binding sites. The sequence variation in the 5′-proximal region of PRL may lead to changes of transcription factor binding sites and alter the expression of PRL. In the experiment reported here, there were three single nucleotide variations at -401, -268 and -266 bp sites in the 5′-proximal region of goose PRL. To understand whether and how the SNPs change the transcription factor binding sites, binding modes for pre- and post-mutation were predicted using TFSEARCH ver.1.3 (http://www.cbrc.jp/research/DSB/tfsearch.html). According to the prediction, the binding sites for transcription factor GATA-2 and GATA-1 are lost when the G-268A and T-266A mutation occurs. Although the transcription factors GATA-1 and GATA-2 play key roles in the regulation of gene mRNA expression (Ohneda & Yamamoto, 2002), whether and how the nucleotide variations identified in the present study lead to the alteration of transcription factor binding mode and mRNA expression of PRL in geese are still to be tested in future studies.

In conclusion, three SNPs in the 5'-proximal region of goose PRL were identified. Statistical analysis suggested that the genotype AA characterized by A-401, G-268 and T-266 had positive genetic effects on egg production ($P < 0.05$), and therefore the polymorphisms reported here have the potential for utilization in molecular marker-assisted selection programmes for egg production in geese.

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