Short Commiunication

Association of prolactin and prolactin receptor gene polymorphisms with economic traits in breeder hens of indigenous chickens of Mazandaran province

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Abstract
Polymorphisms in 5'-flanking region of prolactin (PRL), exon 2 and exon 5 of prolactin receptor (PRLR) genes and its association with growth and egg traits were examined in breeder hens of Mazandaran native fowls breeding station. A single nucleotide polymorphism at site C-2402T and a 24 bp nucleotide sequence insertion at situation -382 in 5'-flanking regions of PRL gene were identified. PCR amplification together with Restriction Fragment Length Polymorphism (RFLP) and direct agarose gel electrophoresis were used to identify different genotypes at C-2402T and a 24 bp indel (insertion-deletion) at the site of -358 of PRL gene, respectively. Nucleotide substitution of C to T and a 24 nucleotides insertion (I) or deletion (D) in promoter region of PRL gene resulted in three genotypes with the frequency of CC (0.10), CT (0.84), TT (0.06) and II (0.39), ID (0.40), DD (0.21), respectively. There were no heterozygous females and only two genotypes A/A (0.54), B/B (0.46) and AA (0.72), BB (0.28) were identified in exon 2 and exon 5 of PRLR gene using PCR-Single Strand Conformation Polymorphism (PCR-SSCP) and PCR-RFLP analyses, respectively. A novel mutation consists of a *Bam*HI restriction site found in the exon 5 of PRLR gene. The results showed significant association between SNP in exon 2 with body weight at hatch, age at sexual maturity, and between SNP in exon 5 and egg number. Individuals with AA genotype produced higher eggs than BB genotype ($P<0.05$). These results showed that the PRLR locus can be considered as a major gene that may influence the production traits in chicken.
Keywords**:** Breeder hens; native fowls; prolactin; prolactin receptor; polymorphism

The expression of prolactin (PRL) depends on the 5′-flanking region sequence. Studies with mammals and birds have shown that Pit-1, CCAAT-enhancer binding protein-α (Day *et al*., 2003; Enwright *et al*., 2003), estrogen receptors (Maurer and Notides, 1987), and other proteins are essential in regulating the expression of PRL via specific promoter binding sites. Polymorphisms in the promoter region, especially those that result in changes of promoter binding sites, most likely influence mRNA expression, thus influencing hen incubation behavior and egg production (Cui *et al*., 2006). The 5′-flanking region (promoter region) of the PRL gene has been considered as an excellent experimental model for studying both tissue-specific and hormonally regulated activation of gene transcription (Elsholtz *et al*., 1991; Seyfred and Gorski, 1990). PRL is thought to be involved in modulating a great variety of physiological processes that are involved with development, metabolism, the immune and neural systems and reproduction. The variable effects of PRL may be due to receptor variations because alternative splicing gives rise to different

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isoforms of mammalian prolactin receptor (PRLR) (Pitts *et al*., 2000). In avian species, a single form of PRLR containing a cytoplasmic domain comparable to that of the longest isoform of mammalian PRLR has been identified by cDNA cloning (Yamamoto *et al*., 2003). The pituitary hormone of PRL is a ligand of PRLR. Chicken PRLR gene was mapped onto the chicken chromosome Zp23-22 (Cheng *et al*., 1995) and disperses over 34 kb on chromosome Z and consists of 15 exons and 14 introns (Leung and Wang, 2005). The membrane-bound PRLR is closely related to the growth hormone receptor and is a member of the cytokine receptor family (Bole-Feysot *et al*., 1988). Consistent with its diverse effects, the PRLR is expressed ubiquitously in chickens and turkeys (Leclerc *et al*., 2007 a,b) and levels of receptor in target tissues are up and down-regulated in response to changes in the concentration of circulating PRL. At around the time of hatch, the secretion of PRL significantly increases in chickens and turkeys in concert with increases in PRLR. Presumably, the increase in circulating PRL is associated with adaptation of the embryo to ex ovo life although in what capacity is not clear (Hiyama *et al*., 2009). As described above, prolactin and its receptor are involved in the growth and development, control of water and electrolyte balance, reproduction, endocrine signaling and metabolism. Due to different biological activities attributed to PRL and PRLR, they can be used as the major candidate genes in molecular animal breeding programs. The aim of the present study was to determine the polymorphism of PRL and PRLR genes and to evaluate their association with some important economic traits in breeder hens of Mazandaran native fowls breeding station.

Native fowls breeding station of Mazandaran located in Northern Iran was established in 1988 with the objective of conserving the endangered population of native fowls in rural areas. In 1986 about 5000 cocks and hens were purchased from rural communities across the Mazandaran province and were transferred to a quarantine farm. In 1987 and after practicing quarantine procedures about 2500 birds of two sexes were kept to produce hatching eggs and chicks produced from these eggs were transferred to the station in 1988. Since then birds have been individually tagged and trap nest has been used for pedigree recording. A multiple trait animal model are being used for genetic evaluation of the birds for body weight at 8 weeks, age of the hens at first egg, number of eggs laid during first 12 weeks after flocks maturity (when 5% of the flock are in egg production) and average egg weight. Economic indexes are calculated for these traits and birds of two sexes are selected based on their aggregate genotypes for these traits. The station has two main activities, namely extension and genetic improvement. The extension part is continuously producing and distributing 8 weeks old chicks among rural communities with the aim of increasing the population f native fowls in Northern provinces of Iran. Genetic improvement is done by selecting the best 100 cocks and 800 hens as parents of the next generations. Parents of each generation are selected from among about 6000 pedigreed and performance recorded birds produced each generation.

A total of 156 blood samples were collected randomly in EDTA treated tubes as an anticoagulant. The collected blood samples were transferred to the labora-

Table 1. PCR forward (F) and reverse (R) primers for the promoter region of prolactin and exon 2 and exon 5 prolactin receptor genes in breeder hens of native fowls.

Locus	Primer sequences	Annealing	Amplicon size (bp)
PRL5	F: 5'-CTAAAGGACCTGGAAGAAGGG-3' R: 5'-AACTTGTCGTAGGTGGGTCTG-3'	62°C, 30s	439
PRL24	F: 5'-GGCTCTCCATGGGTATTAGGA-3' R:5'-GCTGGTGAACCAATCTCGGTT-3'	54°C, 30s	154 and 130
PRLR2	F: 5'-TTTTGCTCCTTGTGTTTTAGGA-3' R:5'-TGGTTTCCTACCGAAAGGATT-3'	59°C, 60s	162
PRLR5	F: 5'-TTGTCTGCTTTGATTCATTTCC-3' R:5'-TGCATTTCATTCTTCCCTTTTT-3'	59°C, 60s	250

tory using cooling chain and stored at -20°C for further analysis. Genomic DNA was isolated by standard salting-out procedure described by Miller *et al*. (1988). The quality and quantity of the extracted DNA was checked by spectrophotometer and agarose gel electrophoreses. DNA samples were adjusted to a concentration of 100 ng/µl and exactly 1 µl of the DNA samples were used as template for polymerase chain reaction. Four pairs of primers provided by Cui *et al*. (2006) and Jiang *et al*. (2005) were used to amplify the 5′-flanking region of the PRL and PRLR loci, respectively. The primer sequences for each marker loci are presented in Table 1. Primer pair PRL5 was used to amplify the 439 bp fragment of the chicken PRL gene containing AGCT sequence for *Alu*I (Fermentas, Germany) restriction enzyme. Primer pair PRL24 was used to amplify the fragment (130 or 154 bp) containing the 24 bp insertion or deletion (indel) at the site of -358 in promoter region of prolactin gene. The primer pairs PRLR2 and PRLR5 were used to amplify a fragment of 160 and 250 bp from exon 2 and exon 5 of prolactin receptor gene, respectively. The fragment amplified by PRLR5 containing GGATC sequence for *Bam*HI endonuclease (Fermentas, Germany). The PCR was performed in a final volume of 20 µl containing 100 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase and 1× reaction buffer with the following profile: initial denaturation of 5 min at 94°C; 35 cycles of 94°C for 30s, 62°C (primer pair PRL5), 54°C (primer pair PRL24), and 59°C for 60 s (primer pairs PRLR2 and PRLR5), with a final extension of 5 min at 72°C. Genotypes were recorded according to band patterns.

The PCR products resulted from PRL5 or PRLR5 were treated with restriction enzymes *Alu*I and *Bam*HI, respectively. For the PCR-RFLP assays, 10 µl of PCR products from PRL and PRLR genes were digested with 5 units of each *Alu*I and *Bam*HI restriction endonuclease at 37°C overnight. A direct agarose gel electrophoresis was used for detection of a 24 nucleotide sequences as a result of insertion or deletion for marker site of PRL24 in promoter region of prolactin gene. The 50 bp DNA ladder (Fermentas SM0251) was used in each gel as molecular size standard. The agarose gels were stained with ethidium bromide and the fragments were visualized using UV trans illuminator. The single strand conformation polymorphism (SSCP) analysis was used for detection of polymorphism in exon 2 of prolactin receptor gene (PRLR2). The PCR products for this marker site were separated by electrophoresis using polyacrylamide gel and visualized by silver staining. The alleles were scored manually from the silver staining gel. Genotypes of individual birds at the different polymorphic loci were recorded by direct counting of the bands. The gene frequencies were calculated by counting method as:

$p = 2(AA) + (AB) / 2N$ And $q = 2(BB) + (AB) / 2N$

where p = the gene frequency of allele A, q = the gene frequency of allele B and N = the total number of birds tested. A chi-squared test for goodness-of-fit was performed to verify if genotype frequencies agreed with Hardy-Weinberg equilibrium (HWE) expectations. Associations of single nucleotide polymorphisms with BWH, BW8, BW12, ASM, EN, body weight at sexual maturity (BWSM) and mean egg weight at sexual maturity (EWSM) were analyzed using the GLM procedure of SAS software (SAS Institute, Inc., Cary,

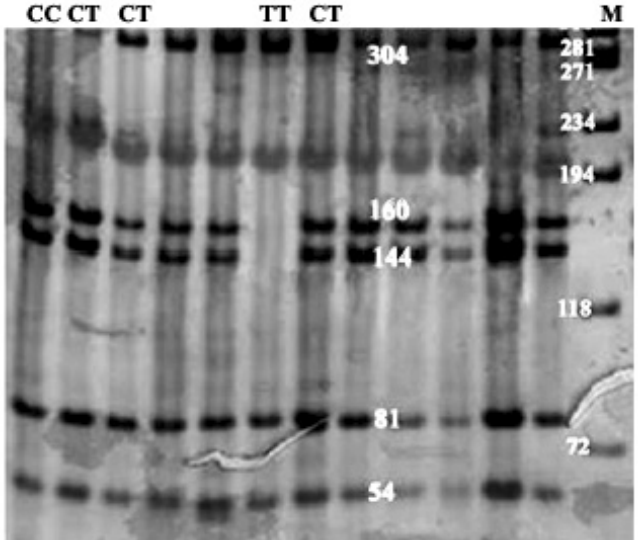

Figure 1. Genotypes of PRL5 marker site revealed on poly acrylamide gel electrophoresis. M: Molecular weight marker of SM0251 (Fermentas).

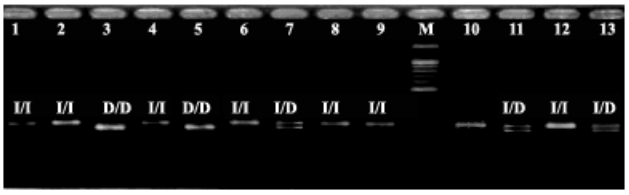

Figure 2. Genotypes of the 24 bp indel at the site -358 of the 5′-flanking region in chicken PRL gene by PCR with agarose gel electrophoresis analysis. II=insertion-insertion; DD=deletion-deletion; ID=insertion-deletion; Lane 1, 2, 4, 6, 8, 9, 12: genotype I/I; Lane 3, 5: genotype D/D; Lane 7, 11: genotype I/D; M: Molecular weight marker of SM0251 (Fermentas).

NC). Type III sum of squares was used in each F-test. Values were considered significant at $P < 0.05$ and presented as least squares means ± standard errors.

The following model was used:

$Y_{ijkl} = \mu + G_i + H_j + S_k + e_{ijkl}$

where Y_{ij} is the average performance of ith genotype in j^{th} hatch, μ is mean of the population, G_i is fixed effect of i^{th} genotype (i=1, 2, 3), H_j is fixed effect of j^{th} hatch (j=1, 2, 3, 4), S_k is fixed effect of k^{th} sex (M, F) and e_{ijkl} is random residual error.

The electrophoretic profiles of RFLP analysis of the fragment obtained from primer pair PRL5 for 5′-flanking region of prolactin gene showed three genotypes as a result of single nucleotide polymorphism for C-2402T (*Alu*I) at this site. Individuals with 4 bands (160, 144, 81, 54 bp), 3 bands (304, 81, 54 bp) and 5 bands (304, 160, 144, 81, 54 bp) were designated as CC, TT and CT genotypes, respectively (Fig. 1). Allele and genotype frequencies observed in the analyzed samples for this marker site are reported in Table 2.

The electrophoretic genotyping of the 24 bp indel for primer pair PRL24 at the site of -358 in promoter region of PRL gene is shown in Figure 2. Three genotypes of II (presence of segment of 24 bp), DD (absence of segment of 24 bp), and ID (presence/absence of segment of 24 bp) were observed in the marker site of PRL24. The frequency of I allele (0.59) was higher than D allele (0.41), and the frequency of observed three genotypes was estimated for homozygous II (0.39), heterozygous ID (0.40) and homozygous DD (0.21) in this population (Table 2). The probability of random mating in the population was estimated by χ^2 test to examine HWE at both marker sites of PRL promoter region. The chi-squared test showed that both marker sites at prolactin promoter region deviated from Hardy-Weinberg equilibrium (P<0.05).

In the present study two fragment sizes of 162 and 250 bp were amplified in exon 2 and exon 5 of PRLR gene using PRLR2 and PRLR5 marker loci, respectively. The resulted fragment by PRLR5 marker (250 bp) was digested with *Bam*HI restriction enzyme and produced two fragments with 195 and 55 bp. The A allele was cleaved into two fragments 195 and 55 bp, while the B allele remained uncut at 250 bp because of the absence of a *Bam*HI restriction site (Fig. 3). The identified A variant is a novel mutation that consist a *Bam*HI target site in fifth exon of PRLR gene. The frequency of A allele was higher (0.72) than B allele (0.28) at PRLR5 marker site (Table 3). The PCR product of PRLR2 marker locus (160 bp) was genotyped by SSCP analyses (Fig. 4). The allele and genotype frequencies observed in the analyzed samples for these marker sites are reported in Table 3. The frequency of A (0.54) allele was greater than B (0.46) allele at PRLR2 marker site (Table 3). Because the PRLR is located on the Z chromosome, there were no heterozygous females, and 2 genotypes were identified at both PRLR2 and PRLR5 marker sites. The test for HWE at each marker site was conducted separately and showed that both marker sites at prolactin receptor gene deviated from Hardy-Weinberg equilibrium.

The effect of polymorphism of PRL gene on economic traits was estimated. The SNP in exon 2 was significantly associated with Body Weight at Hatch (BWH) and Age at Sexual Maturity (ASM) (P<0.05). Individuals with BB genotype had lower ASM and higher BWH than those with AA genotype (Table 4). Whereas the, genotypes of exon 2 were not significant-

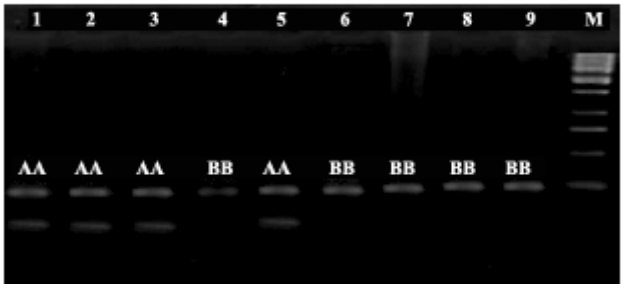

Figure 3. Genotypes of PCR fragment amplified by primer pair PRLR5 for exon 5 of chicken PRLR5 gene using *Bam*HI digestion. Lane 1, 2, 3, 5: genotype AA; Lane 4, 6, 7, 8, 9: genotype BB; M: Molecular weight marker of SM0251 (Fermentas).

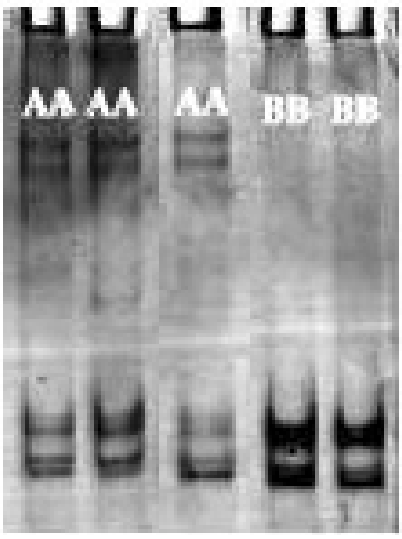

Figure 4. SSCP profile of chicken PRLR2 marker site in Mazandaran native chickens.

Table 2. Alleles and genotypes frequencies of the 2 polymorphic sites for C-2402T (*Alu*I) and -358 (24 bp indel) on 5′ flanking region of chicken PRL gene (N=156).

Marker sites	Allele		Genotype			Allele		Genotype		
	C	T	CC	CT	TT	I	D	II	ID	DD
PRL5	0.52	0.48	0.1	0.84	0.06	-	-	-	-	-
PRL24	-	-	-	-	-	0.59	0.41	0.39	0.40	0.2

Table 3. Alleles and genotypes frequencies for 2 polymorphic loci, PRLR2 (exon 2) and PRLR5 (exon 5) of chicken PRLR gene (N=156).

Marker sites	Allele		Genotype		
	A	B	A/A	A/B	B/B
PRLR2	0.54	0.46	0.54	0	0.46
PRLR5	0.72	0.28	0.72	-	0.28

ly associated with BWH, BW8, BW12, BWSM and EWSM. But, the SNP in exon 5 of PRLR gene showed significant association with EN (P<0.05) as AA genotype produced five eggs higher than BB genotype during recorded laying time (Table 4). In the present study, significant effect of hatch were found on BWH, BW8 and BW12 and sex on BW8 and BW12 (Table 5). No effect of genotype at any other SNP site of PRLR gene on studied traits approached a statistically significant level according to total type III probability of significance.

In birds the role of PRL are not yet understood precisely, but it is believed that the major function of PRL is manifested during incubation and feeding of nestlings (Hui-Fang *et al*., 2009). The allelic frequencies obtained for 5′ flanking region of prolactin gene by primer pairs of PRL5 in the present study differ from those reported by Cui *et al*. (2006). They have been shown that the frequency of C allele at nucleotide -2402 of 5′-flanking region of prolactin gene varied from 0.02 (Taihe Silkies F0 generation), 0.05 (Yangshan), 0.23 (Taihe Silkies F1 generation), 0.35 (White Rock), 0.42 (Nongdahe) in indigenous chickens to 1.0 in commercial White Leghorn chickens. In our experiment, the frequency of C (0.52) and T (0.48) alleles was close to each other but the frequency of heterozygous CT genotype (0.84) was the highest in comparing with homozygous CC (0.08) and TT (0.06) genotypes. It has been reported that CCAAT-enhancer binding protein-α (Day *et al*., 2003; 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'-flanking promoter region of PRL may lead to changes in transcriptional factor binding sites and alter the expression of PRL. The frequency of I allele studied in six Chinese chicken populations changed from 0.02 (Taihe Silkies F0 generation), 0.05 (Yangshan), 0.17 (Nongdahe), 0.20 (Taihe Silkies F1 generation), 0.22 (White Rock) to 1.0 in White Leghorn chickens (Cui *et al*., 2006). In our experiment, the frequency of I allele (0.59) was higher than D allele (0.41) and the frequency of observed three genotypes was estimated 0.39, 0.40 and 0.21 for homozygous II, heterozygous ID and homozygous DD birds, respectively. In a study on other Iranian native fowl population from Yazd province, Emamgholi-Begli *et al*. (2010) reported the frequency of 0.761 and 0.239 for I and D alleles and

Table 4. Least square means ± standard errors of economic traits according to exon 2 and exon 5 genotypes of PRLR gene.

Traits*	PRLR2		P- value	PRLR5		P- value
	AA	BB		AA	BB	
BWH**	38.00±0.43^a	39.13±0.48^b	0.05	38.25±0.67	39.14±0.62	0.38
BW8	518.90±12.98	496.94±14.21	0.25	484.47±23.64	502.23±22.02	0.74
BW12	941.49±20.99	935.43±22.9	0.84	917.45±36.66	953.47±34.15	0.38
BWSM	1680.76±30.22	1661.97±31.36	0.66	1643.82±43.59	1567.84±40.60	0.68
ASM	164.25±2.45^a	156.66±2.54^b	0.03	153.42±3.56	154.91±3.31	0.77
EWSM	40.95±1.06	39.38±1.11	0.30	38.89±1.59	38.87±1.48	0.15
EN	32.54±2.77	31.35±2.58	0.63	34.99±2.46^a	28.90±3.06^b	0.04

*body weight at hatch (BWH), 8(BW8) and 12 (BW12) weeks of age, body weight at sexual maturity (BWSM), age at sexual maturity (ASM), mean egg weight at sex maturity (EWSM) and egg number (EN). **Means with different letter in each row are significantly different (P<0.05).

Table 5. Least square means ± standard errors of economic traits according to different levels of hatch and sex.

Trait	Hatch				Sex**	
	1	2	3	4	M	F
BWH	37.89±0.58^{b}	40.11±0.62^{a}	38.65±0.58^{b}	37.60±0.64^{b}	38.85±0.63	38.27±0.31
BW8	561.54±14.68^{b}	516.69±15.81^{b}	612.46±14.79^{a}	555.31±16.12^{b}	651.28±15.95^{a}	471.72±8.032^{b}
BW12	1111.06±24.30^{a}	1006.16±26.17^{b}	1041.63±24.48^{b}	930.811±26.68^{c}	1163.08±26.41^{a}	881.75±13.29^{b}

Body weight at hatch (BWH), 8 (BW8) and 12 (BW12) weeks of age. *Means with different letter in each row are significantly different (P<0.05). M: male, F: female.

0.566, 0.389 and 0.044 for II, ID and DD genotypes at the 24-bp indel site of prolactin promoter, respectively. In their study, the II and ID genotypes were significantly associated with increased egg number. In the present study, only polymorphism at PRLR5 marker site had significant effect on EN since chickens with AA genotype produced higher eggs than BB chickens. The studied marker of PRLR gene (PRLR2) showed significant effect on ASM and BWH as BB genotype had lower ASM and higher BWH than those with AA genotype (Table 4). It was revealed that the individuals with high BWH had earlier sexual maturity. There were no significant associations between polymorphisms of 24 bp indel site and growth traits in native fowl of Yazd province (Emamgholi-Begli *et al*., 2010). Insertion of this sequence in the promoter may inhibit a transcriptional factor binding site for PRL gene and, hence, decrease the expression of PRL, which contributes to non broodiness in II hens (Jiang *et al*., 2005). The higher frequency of C and also I alleles at 5′-flanking region of prolactin gene in Mazandaran native fowls comparing with Chinese indigenous chicken may be because of breed differences and also selection strategies used in former population. However, only the significant association of the 24 bp indel with egg production was observed when an association studies were carried out at both polymorphic site in 5′-flanking region of PRL gene using the F2 population of the Nongdahe × Taihe Silkies cross (Cui *et al*., 2006). It has been shown that a possible ecotropic viral integration site-1 encoded factor binding site was presented in the 5' flanking region of the chicken PRL gene because of the 24-bp insertion (Cui *et al*., 2005). This viral integration site has 2 zinc-finger domains and represses transforming growth factor-β (TGF-β) signaling and antagonizes the growth-inhibitory effects of TGF-β. Moreover, ecotropic viral integration site-1 has been reported to be involved in transcription of many genes as a repressor (Izutsu *et al*., 2001; 2002). It was assumed that this binding site represses the expression of PRL gene in White Leghorn chickens and further prevents broodiness (Jiang *et al*., 2005), which can improve egg production to some extent (Cui *et al*., 2006). In the present study, the effect of hatch on BW was significant and individuals of hatch 4 had lowest BW values (Table 5). The sex had no significant effect on BW chicks at hatch but this effect was significant at 8 and 12 weeks of age since males had higher BW than females. In a study conducted by Jiang *et al*. (2005), thirteen sets of primer pairs were used to detect polymorphisms in exon 1 to exon 8 of PRLR gene in Chinese local chicken breed. Two out of thirteen used primer pairs showed the incidence of SNP in exon 3 (one SNP) and exon 6 (two SNP) in PRLR gene. An association analysis showed that the genetic polymorphisms at exon 3 and exon 6 of PRLR gene were not related to broodiness in Chinese local chicken breed. Mutation in exon 3 of PRLR did not lead to amino acid changes in PRLR gene due to the mutation in 5′-untranslated region of PRLR cDNA. This may be the reason that the mutation was not associated with broodiness. At the exon 6 of PRLR locus, the SNP of G14820A did not contribute to broodiness because it was synonymous mutation. The T14771C in exon 6 of PRLR, a missense mutation, leads to Leu340Ser in PRLR gene. This amino acid variation occurred in the cytoplasmic tail of the PRLR and may not influence the functional structure and, therefore, did not influence broodiness (Jiang *et al*., 2005). The method of sexing based on differences in the rate of feather growth provides a convenient and inexpensive approach in poultry industry. The actual cause of delayed feathering is still unknown. It was suggested that due to the inhibitory role of PRLR in follicle activation, it is the most likely candidate gene involved in the delay of feather growth (Martin *et al*., 2008). The SSCP and RFLP-PCR analyses in our experiment revealed that a polymorphism occurring in the exon 2 and exon 5 of PRLR gene in breeder hens of Mazandaran native fowls population. The novel SNP variants created a *BamHI* enzyme site in exon 5 of PRLR gene.

In the present study, we showed that polymorphism occurring in the 5′-flanking regions of PRL gene in breeder hens of native fowls population. There was a SNP at C-2402T revealed by endonuclease *Alu*I digestion and a 24 bp nucleotide sequence insertion at situation -382 in the 5'-flanking region of PRL gene. The sequence variation in the 5'-flanking region of PRL gene may lead to changes in transcriptional factor binding sites and alter the expression of PRL hormone. In our study, genetic variants were observed in both exon 2 and exon 5 of PRLR gene. Marker-traits association analysis showed significant association between SNP in exon 2 on BW at hatch and age at sexual maturity and also between SNP in exon 5 with egg number. But there was no significant effect of genotype at any other SNP site of PRLR gene on studied traits (P<0.05). Therefore, the exon 2 and exon 5 of chicken PRLR gene could be considered as potential molecular marker for selection of indigenous chicken.

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