Research Article

Polymorphism detection of promoter region of IFN-γ and IL-2 genes and its association with productive traits in Mazandaran native breeder fowls

Hamed Kazemi[1], Mojtaba Najafi[2*], Elaheh Ghasemian[3], Ghodrat Rahimi-Mianji[4], Zarbakht Ansari Pirsaraei[5]

1. Hamed Kazemi, PhD student in Animal Breeding and Genetics, Department of Agricultural and Food Sciences, University of Bologna, Bologna, Italy. *hamed.kazemi@studio.unibo.it, hamed_kazemi23@yahoo.com*
2. Mojtaba Najafi, PhD in Animal Breeding and Genetics, SANRU, Sari, Iran, *mojtaba_najafy@yahoo.com*
3. Elaheh Ghasemian, MSc graduated student in Animal Breeding and Genetics, SANRU, Sari, Iran, *elaheh.ghasemian@yahoo.com*
4. Ghodrat Rahimi-Mianji, Professor in Molecular Genetics, SANRU, Sari, Iran, *rahimimianhi@yahoo.com*
5. Zarbakht Ansari Pirsaraei, Associate Professor in Animal Physiology, SANRU, Sari, Iran, *zarbakht_ansari@yahoo.com*

[*] *Corresponding author: Email address: mojtaba_najafi@yahoo.com* (M. Najafi).

Abstract

In order to identify the polymorphism in Interferon gamma (IFN-γ) and Interleukin-2 (IL-2) genes, blood samples were collected from 380 breeder hens of Mazandaran native fowls breeding station. DNA extraction was performed through modified salting-out method and fragments of 670 and 659 bp from promoter region of IFN-γ and IL-2 genes were amplified by using specific primers, respectively. Following genotyping in IFN-γ gene using *Tsp509*I restriction enzyme, two alleles of A and G with the frequency of 0.55 and 0.45 and three genotypes of AA, AG and GG were observed with the frequency of 0.32, 0.46 and 0.22, respectively. For IL-2 gene, two alleles of A and G were also detected using *Mnl*I restriction enzyme with the frequency of 0.58 and 0.42 and three genotypes of AA, AG and GG with the frequency of 0.33, 0.50 and 0.17, respectively. Statistical analysis revealed significant associations between IL-2 gene SNP and productive traits including the average egg weight at 345-375 days of age, egg number at 345-375 days of age and body weight at 8 weeks of age traits ($P<0.05$). Furthermore, in the mean comparison analysis, there were also significant differences between different genotypes of IL-2 gene in average egg weight at 28 and 30 weeks of age, in which AG genotypes showed higher performance. Additionally, For IFN-γ gene, a significant difference was found between genotypes in average egg weight at 28 weeks of age trait. Therefore, it can be concluded that the above-mentioned polymorphisms could be considered as the pivotal genetic makers to improve Mazandaran native fowls breeding programs in order to achieve the optimum performance in productive traits more efficiently.

Keywords: Polymorphism, IFN-γ, IL-2, Mazandaran native fowls, PCR-RFLP.

Highlights

- SNPs in amplified fragments of IFN-γ and IL-2 genes were detected by RFLP method.
- IFN-γ and IL-2 marker sites showed polymorphic pattern in Mazandaran native fowls.
- In both gene loci, the frequency of AG genotypes was significantly higher.
- Significant effects of IL-2 were detected on egg number, egg weight and body weight.

- AG genotypes had better performance as the pivotal marker for improving productivity.

Introduction

Improving animal health is considered to be the basic goal of the animal breeding industry (Soller and Andersson, 1998). The poultry industry, as one of the most important parts of the agriculture sector for providing the invaluable supply of proteins, fats, minerals and vitamins as well as its worthwhile economic outcomes, has expanded dramatically during the last decade (Vetrivel and Chandrakumarmangalam, 2013). However, it is threatened by progressively prevalent diseases such as Newcastle, Infectious Bronchitis, Avian Influenza, Marek's and Mycoplasmas which can cause catastrophic losses (Roussan *et al.*, 2008). To improve poultry health, reduce disease-associated losses and keep poultry health management at a reasonable cost, efforts need to continue to advance understanding of the genetic bases for resistance and immunity to avian diseases. In this context, it is critically important to focus efforts on identification and characterization of genes and their genetic variants affecting innate and adaptive immunity (van der Poel and Parmentier, 2003). Genetic improvement of the immune responses can also enhance vaccine efficacy and disease resistance in chickens (Lamont *et al.*, 2002). Indeed, reproductive system has key role in assuring the continuity of the species, while the immune system is essential for continued health and survival through internal protection. The chicken has been the primary species which have shed light on reproductive–immune interactions. In the chicken, increase in Ca^{2+} mobilization and its effect on regulation of granulosa cell function (Soboloff *et al.*, 1995), presence of lymphocytes and cells specific to B-cell (Zettergren and Cutlan, 1992) and T-cell (Withanage *et al.*, 2003) lineages in the gonads of birds, stimulation of T-cells in the hen ovary by estrogen (Barua and Yoshimura, 1999), increasing progesterone receptor (PR) expression during sexual maturation (Pasanen *et al.*, 1998), modulation of growth and differentiation of granulosa cells by cytokines (Hales, 2000), participating the immune cells in the ovulatory process (Hellberg *et al.*, 1991) and the formation and demise of the corpus luteum (Benyo and Haibel) have been noted. Cytokines have generally been identified and characterized as the main components of the peripheral immune system (DHAMA *et al*. 2015). Although far from fully comprehended, the role of cytokines as the important immune-modulators in the peripheral immune system is evident (Dhama *et al.*, 2015). Interleukins are regarded as the cytokines that regulate the relationship between lymphocytes and other leukocytes. IL-2 is known as a cytokine that plays an active role in enhancing innate and acquisitive immunity and acquired safety (Kogut *et al.*, 2003), which is produced by type-I T cells and induces natural killer (NK) cells and lymphocyte-

activated killer (LAK) cells, demonstrating strong B-cell growth factor activity and can stimulate monocytic lineage cells (Pintarič *et al.*, 2008) (Nagarajan *et al.*, 2011). It also stimulates progesterone production in granulose cells (Sharma and Gandhi, 2011), the proliferation and differentiation of T cells and the activation of monocytes and macrophages, thus playing a fundamental role in regulating the immune response in animals (Zhao *et al.*, 2011). Additionally, it affects type B lymphocytes and enhances their growth and synthesizes a limited amount of immunoglobulins and also has an inductive role in the production of IFN-γ and IL-15 (Zhou *et al.*, 2001), as well as a direct effect on the activity of heterophils in poultry (Kogut *et al.*, 2002). Chicken IL-2 gene is included 4 exons and 3 introns and located on chromosome 4 from nucleotide *54073166* to *54076514* (*NC_006091.4)* (Kaiser and Mariani, 1999) (Ye *et al.*, 2006). So far, significant associations were identified between SNPs of IL-2 gene with the resistance against gastrointestinal infection by nematodes (Donadoni *et al.*, 2011), the differentiation and homeostasis of so-called natural Tregs which are developed in the thymus of animals (Burchill *et al.*, 2007), and mastitis disease in cattle (Alluwaimi *et al.*, 2003). Researchers reported the presence of transcription factor binding sites in the second intron of the IL-2 gene of dairy cows and stated that the SNPs in these sites may affect the binding of transcription factors resulting in different production patterns in genotypes (Lühken *et al.*, 2005). It has been discovered that in chickens, host immune responses to *Eimeria acervulina* and *Eimeria tenella* infection is due to the high regulation of IL-2 secretion (Choi and Lillehoj, 2000) (Miyamoto *et al.*, 2002), and IL-2 production after re-infection with *Eimeria tenella* could be considered as an important factor causing genetic differences between SC chickens' resistance (an inbred chicken strain which is resistance to coccidiosis) or TK chickens' (an inbred chicken strain which is susceptible to coccidiosis) vulnerability to coccidiosis (Li *et al.*, 2002). Chicken IL-2 has also been used as an adjuvant factor to improve vaccine responses to infectious bursal disease virus (Park *et al.*, 2009), avian influenza virus (Hu *et al.*, 2006), and *Eimeria tenella* (Xu *et al.*, 2008), indicating its functional importance in enhancing immune responses to vaccines (Zhang *et al.*, 2011).

IFN-γ is one of the most important cytokines that controls the primary immune response and cellular immunity (Savan *et al.*, 2009), and is recognized as a macrophage-activating cytokine (Reemers *et al.*, 2012) (Vervelde *et al.*, 2013), representing a substantial link between innate and adaptive immune responses. This cytokine enhances the activity of NK cells, increases the expression of class I and II of major histocompatibility complex (MHC) that modulate immune responses (Zhou *et al.*, 2001), and plays a key role as an adjuvant factor in accelerating the immune response induced by vaccine antigens (Shah *et al.*, 2010). Chicken IFN-γ gene is

located on chromosome 1 from nucleotide *35053221* to *35057368* (*NC_006088.4)* and composed of 4 exons and 3 introns. Polymorphisms of the promoter region of this gene have been identified in White Leghorn strains that were associated with resistance to *E. coli* in lines 6_1 and 7_2 (Kaiser *et al.*, 1998). Like mammalian IFN-γ, production of chicken IFN-γ has been used to regulate cell-mediated immunity (CMI) from T cells upon recall antigen stimulation (Lambrecht *et al.*, 2004), which plays a vital role in influenza A virus annihilation in chickens (Singh *et al.*, 2014). Thus, evaluation of the immune responses can be exploited as an indirect selection for improving genetic resistance and promoting vaccine efficacy in chickens (Kaiser *et al.*, 2008). It was found that heterozygote genotypes of IFN-γ gene in Leghorn and Fayoumi chicken breeds demonstrated the largest primary antibody response to SRBC (Sheep Red Blood Cells) test (Zhou *et al.*, 2003). Considering the importance of the disease resistance issue in poultry industry and the role of IL-2 and IFN-γ genes in the immune system, the present study was performed to identify allelic polymorphisms in the promoter region of these genes and their effects on some productive and reproductive traits in breeder hens of Mazandaran native fowls breeding station.

Material and Methods

Experimental population

The Mazandaran native fowl breeding station was inaugurated in 1988 with the purpose of preserving the population of endangered native fowls. The research center has two major activities comprising reproduction and genetic improvement of this native population. In the present study, 380 native fowls were randomly selected that had been grown in the same condition. The productive and reproductive traits included body weight (BW) at day one, 8 and 12 weeks of age and puberty, age of sexual maturity (ASM), egg number (EN) at 120-270 and 345-375 days of age, laying intensity (LI), egg weight (EW) at puberty, average egg weight (AEW) at 28, 30 and 32 weeks of age and 345-375 days of age, percentage of fertility (PF) and percentage of hatchability (PH) were recorded and measured accurately by researchers.

Blood sampling and DNA extraction

A total of 380 blood samples were collected from the brachial wing vein of selected native breeder fowls and transferred into vacutainer tubes containing disodium ethylene diamine tetra acetic acid (EDTA) and then shipped to our laboratory in an insulated cooler with cold-packs and stored at -20°C until DNA extraction process. DNA was extracted through modified salting-out method and then quantity and quality of the extracted DNA were evaluated by both spectrophotometer and agarose gel electrophoresis.

Amplification of gene fragments and PCR reaction conditions

In order to amplify the targeted loci, PCR reactions were prepared with the volume of 25 µl as follows: 2.5 µl of PCR buffer, 1 µl of each primer, 1.5 µl of genomic DNA, 0.5 µl of dNTP, 0.65 µl of MgCl2, 0.2 µl (500U) of *Taq* Polymerase (Cat.n: TA8109C; sinaclon) and 17.65 µl of distilled water. Initial denaturation and final extension were performed at 95˚C for 240 seconds and 72˚C for 300 seconds, respectively.

Table 1. Primer sequences and other specific information for each genetic locus

Genotyping

In order to perform PCR-RFLP, 5 µl of the PCR product of IL-2 gene was digested with 0.3 µl of restriction enzymes at 37˚C for 12 hours, respectively. Digested products were segregated by agarose gel electrophoresis (2.5%). Then, the gel was stained with Ethidium bromide and the fragments were observed using UV transilluminator and Gel-doc machine. For IFN-γ gene, after digestion of PCR products by *Tsp509*I, digested products were segregated using acrylamide gel 13% for 3 h and 250 V, in order to observe the digested fragments and detecting the alleles.

Statistical analysis

Chi-square test (X^2) was used to verify the equilibrium of the genotypic frequencies with the Hardy-Weinberg Equilibrium (HWE). Gene frequencies were also calculated using Popgene software. Statistical analyzes were performed using SAS 9.1 software and the effects of IL-2 and IFN-γ SNPs on the studied traits were evaluated with GLM procedure.

Results

Amplification of the gene fragments

After DNA extraction, the fragments of promoter region of the IL-2 (659 bp) and the IFN-γ (670 bp) genes were amplified using specific primers. All amplified fragments had strong and clear single band, with no extra band or dimer.

Allelic and genotypic frequencies

In this research, RFLP technique was used to determine the genotypes of specific regions of IL-2 and IFN-γ genes (Table 2). The polymorphism of IL-2 gene was detected by digestion of PCR products using *Mnl*I restriction enzyme (Zhou *et al.*, 2001), and two alleles of A and G and three genotypes of AA (19, 115, 165 and 251 bp), GG (19, 112, 115, 139 and 165 bp) and AG (heterozygote of AG and GG) were observed (Figure 1). The polymorphism of IFN-γ gene was also obtained by digestion of PCR products using *Tsp509*I restriction enzyme (Zhou *et al.*, 2001), and then, two alleles of A and G and three genotypes of AA (28, 53, 55, 56, 64, 88, 99,

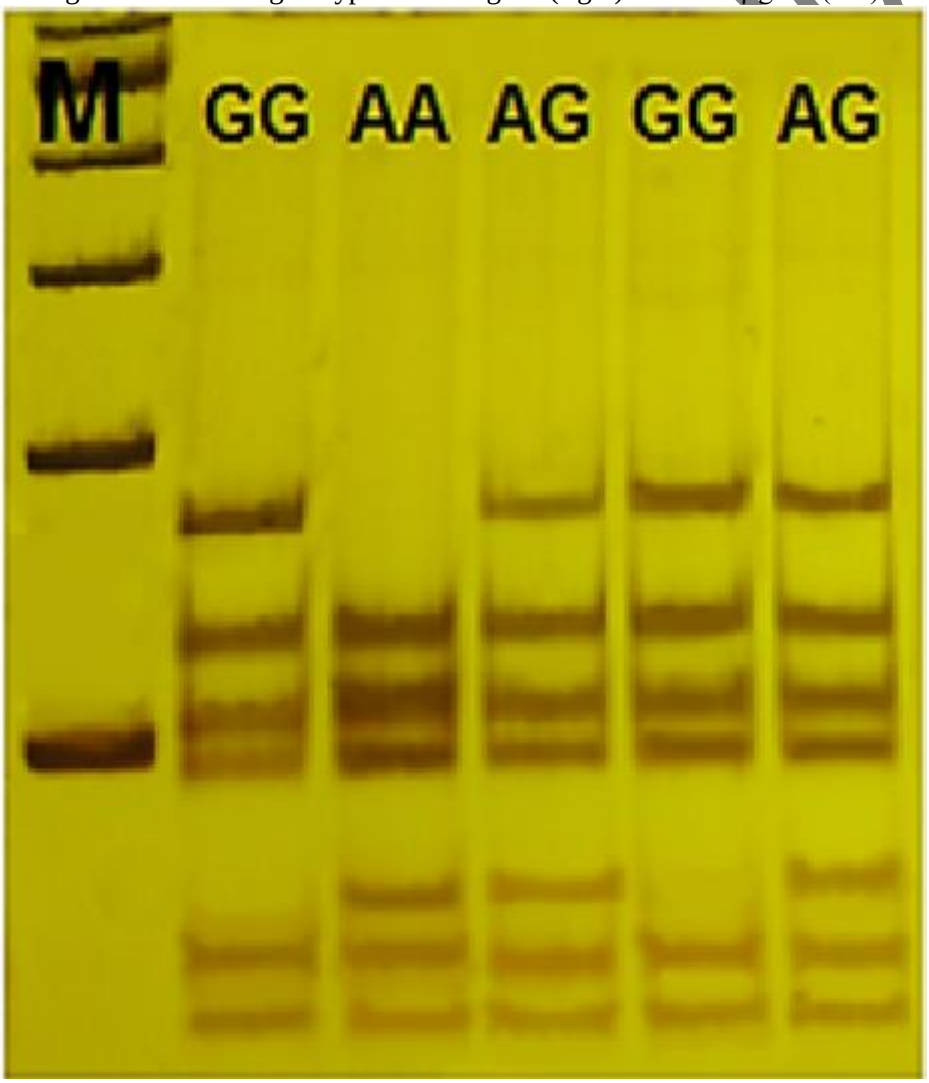
104 and 123 bp), GG (28, 53, 55, 56, 88, 99, 123 and 168 bp) and AG (heterozygote of AG and GG) were detected (Figure 1). Chi-square test was used to determine whether the subjects met the Hardy-Weinberg equilibrium, for in IL-2 and IFN-γ loci, the χ^2 value was 0.86 and 0.15 respectively, both $p > 0.05$ (Table 3). Also, Observed and effective number of alleles of IL-2 and IFN-γ loci are shown in Table 4.

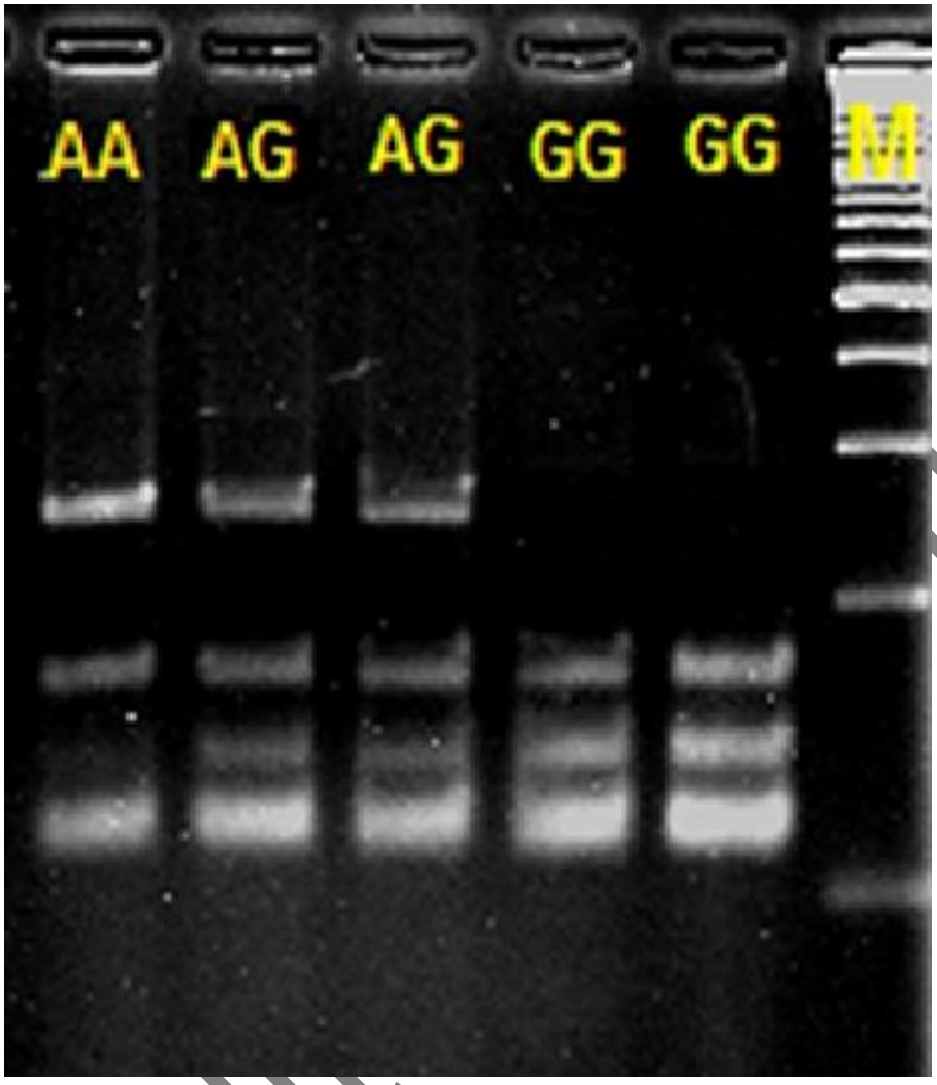
Table 2. SNP variants position found in IL-2 and IFN-γ loci

Table 3. Allelic and genotypic frequencies of IL-2 and IFN-γ loci and X^2 test for HWE

Table 4. Observed and effective number of alleles of IL-2 and IFN-γ loci

Figure 1. Obtained genotypes of IL-2 gene (right) and IFN-γ gene (left).





Association analysis

The association analysis between genotypes of two genes with the studied traits in Mazandaran native fowls population showed significant correlations between IL-2 gene polymorphism and some of the studied traits. In IL-2 locus, significant associations were found between SNP and AEW at 375-345 days of age ($P<0.05$), EN at 345-375 days of age and BW at 8 weeks of age traits (almost achieved significance, $P\leq0.058$) (Table 5). Furthermore, mean comparisons analysis indicated significant differences between different genotypes of IL-2 gene in AEW at 28 and 30 weeks of age and also genotypes of IFN-γ gene only in AEW at 28 weeks of age traits ($P<0.05$). It was also determined that the AG genotypes, despite their lower BW at 8 weeks of age, had better performances in EN and AEW traits than other two genotypes. Therefore, these genotypes could be used as a useful marker for the next generations in order to improve performance in egg production traits.

Table 5. Least Mean Squares test of observed genotypes in Mazandaran native breeder hens

Discussion

Genetic selection in poultry industry was hitherto based on producing broilers with high grow rate and less feed conversion ratio (FCR). However, positive selection towards these traits can adversely impact on immune competence and lead to increase the susceptibility of birds to various disease (Cheema *et al.*, 2003; Janss and Bolder, 2000). Nowadays, in the poultry industry there is genetic selection for egg layers as well as broilers. Selection poultry with an early innate immune response efficiently is an important task in poultry industry because innate immune response directs the acquired response (Parish and O'neill, 1997). Moreover, in broiler selection there is multi environment selection to allow balanced breeding of production and health traits. Therefore, balanced genetic selection for both production and immune competence in contradictory environments is an efficient strategy to improve performance in productive traits. Numerous reports have already revealed a remarkable association between strong pro-inflammatory cytokine and chemokine profile and increased resistance against diverse pathogens as well as various diseases (Coussens *et al.*, 2004; Heinrich *et al.*, 2001; Sebastiani *et al.*, 2002; Swaggerty *et al.*, 2004; Withanage *et al.*, 2004). Swaggerty et al (2008) indicated the mRNA levels of pro-inflammatory cytokines and chemokines observed in the sires are influenced on the innate immune. Cytokines are identified as important players in different tissues and mostly have variety of functions (Goldsby *et al.*, 2003). Moreover, former studies have indicated that cytokines act as intermediators for the two-way relationship between the neuro-endocrine and immune systems (Felten *et al.*, 1997). In mammals, it has proven that cytokines has key role in regression of different tissues such as the corpus luteum (Neuvians *et al.*, 2004; Nishimura *et al.*, 2004), follicles (Knight and Glister, 2003), endometrium (Rahman *et al.*, 2004) and mammary gland (Clarkson *et al.*, 2003). Additionally, cytokines including TGF-β, IFN-y and TNF-α, IL-1α, IL-1β, IL-6, IL-8, IL-10 are constitutively expressed in oviduct and ovary in mammalian (Dimitriadis *et al.*, 2005; Kayisli *et al.*, 2002). Also, increased cytokine mRNA expression was associated with follicular atresia and oviduct regression in chicken (Sundaresan *et al.*, 2007). Although a number of former reports indicate that cytokines can have influence on autocrine and paracrine pathways in the ovary and oviduct, the exact mechanism of cytokine function and normal cytokine profile in the reproductive system of chicken are not known (Sundaresan *et al.*, 2007). Detection of the remarkable effect of the polymorphisms on interest traits in chickens illustrates its potential value for use in marker-assisted selection to improve their performance of birds such as immune function as well as reproduction and production efficiency. Considering the importance of these major genes and the role of cytokines in the immune system, reproduction system and even production, the present study was performed to

identify different allelic forms in the flanking regions of IL-2 and IFN-γ genes and their associations with some productive and reproductive traits in breeder hens of Mazandaran native fowls breeding station. In the present study, in promoter region of IL-2 and IFN-γ genes, two alleles of A and G and three genotypes of AA, AG and GG were identified. The results obtained from the association analysis of significant effects of each gene on different traits and the mean comparisons analysis between genotypes with studied traits are comparable with recent studies in this field.

IL-2

Regarding our findings, two alleles of A and G and three genotypes of AA, AG and GG were detected with different frequencies in promoter region of IL-2 gene in Mazandaran native hens population, demonstrating polymorphic pattern of this gene site in this native population. Also, significant correlations were discovered between IL-2 gene polymorphism at promoter region with EN ($P<0.015$) and AEW at 345-375 days of age traits (almost achieved significance, $P\leq0.058$), while the AG and GG genotypes had higher performance in both traits compared with AA genotypes, which could indicate the positive and additive effect of the G allele in this population. In addition, mean comparisons analysis identified significant differences between genotypes for AEW at 28 and 30 weeks of age ($P\leq0.05$), except that the overall effects of SNP on these traits were found not to be significant. In recent studies, significant correlations were found between SNPs of IL-2 gene with body weight at 7 days of age ($P<0.01$), body weight at 40 days of age and food conversion ratio ($P<0.05$). In addition, investigation of the polymorphism in intron 2 of IL-2 gene in chickens has revealed 15 new haplotypes in 66 breeds of native chickens and commercial lines (Zhang *et al.*, 2011). In a study, genetic polymorphism of IL-2 gene with egg production traits in a native turkey population was detected using PCR-SSCP method. Two alleles of A (52.65) and B (47.35) and three genotypes of AA, AB and BB were observed with the frequency of 13.83, 77.66 and 8.51, respectively. The allelic and genotypic frequencies had a similar pattern as the obtained results from the polymorphism of IL-2 gene promoter in Mazandaran native fowls population. These researchers found significant differences between genotypes in terms of egg number and weight of egg mass production, while the AA genotypes showed better performances than other genotypes ($P\leq0.01$). However, no significant association was revealed between this polymorphism and the average egg weight trait (Erfaniasl *et al.*, 2015).

IFN-γ

Herein we found two alleles of A and G and three genotypes of AA, AG and GG with different frequencies in promoter region of IFN-γ gene in Mazandaran native hens population and significant difference was observed between the genotypes of IFN-γ gene in AEW at 28 weeks of age trait and the effect of IL-2 gene on BW at 8 weeks of age trait was shown to be significant (P≤0.05). The findings of recent researches have indicated significant relationships between polymorphism in promoter region of IFN-γ gene even among other species such as total milk yield, lactation length and daily milk yield in Indian crossbreed cattle (P<0.05) (Prakash *et al.*, 2010), and rectal temperature as well as resistance to heat stress in Nigerian goat breeds (P<0.05) (Yakubu *et al.*, 2016). But, in chickens, significant associations of the polymorphism in promoter region of IFN-γ gene were observed with food conversion ratio (P<0.01) in broiler chicken lines (Ye *et al.*, 2006), egg number and weight of egg mass production in Iranian indigenous turkey breed (P≤0.01) (Erfaniasl *et al.*, 2015), *Salmonella enteritidis* burden in the cecum and spleen of Malaysian indigenous chickens (P<0.05) (Tohidi *et al.*, 2012). Also, in previous researches, the polymorphism analysis of the IFN-γ gene has shown to be significantly associated with the initial antibody response to SRBC, BA (Brucella abortus) and body weight at 12 weeks of age in two layer lines (P<0.05) (Ahmed, 2010), the log transformed number of Ascaridia galli in the brown layer line (P<0.05) (Lühken *et al.*, 2011), IFN-γ protein expression after both primary and secondary immunizations in chickens (P<0.05) (Zhou *et al.*, 2002), and the secondary humoral immune response to the HPAI vaccine in a red jungle fowl population (P<0.01) (Ji *et al.*, 2015). By studying the SNP and expression of IFN-γ gene and its role against *Haemonchus Contortus* in two Indian indigenous sheep breeds, in addition to one SNP detected at exon 3 of IFN-γ gene in all resistant sheep groups, researchers also reported that the level of mRNA in susceptible groups was significantly higher (P≤0.05) in comparison with resistant groups (Patra *et al.*, 2016). In a previous survey, researchers analyzed the polymorphism of the promoter regions of IFN-γ and IL-2 candidate genes with the primary and secondary antibody response to BA and SRBC antigens in Leghorn (G-B1) and Fayoumi (M15.2 and M5) chicken lines (ZHOU *et al.* 2001). The fragments obtained through enzyme digestion, alleles and genotypes discovered for these two genes were identical with the alleles and genotypes observed in Mazandaran native fowls population, namely two alleles of A and G and three genotypes of AA, AG and GG were demonstrated. The results of this researcher's study showed that there was a significant relationship between IFN-γ gene promoter region polymorphism with primary and secondary antibody responses to both BA and SRBC antigens (Zhou *et al.*, 2001). In another study, besides the confirmation of the results mentioned above

relating to the immune response to antibodies, there were also significant differences between genotypes in M5.1 line for body weight at 6 and 12 weeks of age traits, and in M15.2 line for body weight at 2 and 6 weeks of age traits, respectively ($P<0.05$) (Ahmed, 2010).

Conclusion

The association between single nucleotide polymorphisms in IL-2 and IFN-γ genes with some productive and reproductive traits in Mazandaran native fowls population was investigated. Significant effects were detected from the IL-2 gene polymorphisms at promoter region on BW at 8 weeks of age, EN at 345-375 days of age (almost achieved significance, $P\leq0.058$), and AEW at 345-375 days of age ($P<0.01$). Also, in the mean comparisons analysis, in addition to above-mentioned traits, there were significant differences between the genotypes of IL-2 gene in AEW at 28 and 30 weeks of age traits, and for IFN-γ gene only in AEW at 28 weeks of age trait. At the IL-2 gene locus, besides their higher frequency, the AG genotypes had better productive performance than AA and GG genotypes, representing the fact that the AG genotypes could be considered as the competent parents for the next generation due to their higher indexes in EN and AEW traits. In fact, this higher performance of AG genotypes could be related to the association between their lower BW at 8 weeks of age and higher records in EN and AEW traits. Therefore, it can be concluded that the immune system genes were positively affective on some economically important productive traits in this native population. Consequently, the polymorphisms and associations found at these gene loci can be exploited as the pivotal genetic makers to improve Mazandaran native fowls breeding programs in order to achieve increased livability by increased resistance against pathogens as well as the optimum performance in reproductive and productive traits more efficiently.

References

Ahmed A. 2010 Associations of polymorphisms in four immune-related genes with antibody kinetics and body weight in chickens. *Asian-australas. J. Anim. Sci*. **23**, 1089-1095.

Alluwaimi A., Leutenegger C., Farver T., Rossitto P., Smith W. and Cullor J. 2003 The cytokine markers in Staphylococcus aureus mastitis of bovine mammary gland. *Zoonoses Public Health*. **50**, 105-111.

Barua A., Yoshimura Y. 1999 Effects of aging and sex steroids on the localization of T cell subsets in the ovary of chicken, Gallus domesticus. *Gen Comp Endocr*. **114**: 28-35.

Benyo D., Haibel G., Laufman. HP. and Pate. JL. 1991 Exoression of maior histocompatibility complex antigens on th; bovine corpus luteum during the estroos cyile, luteolysis and early pregnancy. *Biol. Repmd.* **45**: 229-234.

Burchill M.A., Yang J., Vang K.B. and Farrar M.A. 2007 Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. *Immunol. Lett*. **114**, 1-8.

Cheema M., Qureshi M., Havenstein G. 2003 A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* **82**: 1519-1529.

Choi K.D. and Lillehoj H.S. 2000 Role of chicken IL-2 on γδ T-cells and Eimeriaacervulina-induced changes in intestinal IL-2 mRNA expression and γδ T-cells. *Vet. Immunol. Immunopathol*. **73**, 309-321.

Clarkson RW., Wayland MT., Lee J., Freeman T. and Watson CJ. 2003 Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res*. **6**: R92.
Coussens PM., Verman N., Coussens MA., Elftman MD. and McNulty AM. 2004 Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with Mycobacterium avium subsp. paratuberculosis: evidence for an inherent proinflammatory gene expression pattern. *Infect. Immun.* **72**: 1409-1422.
Dhama K., Saminathan M., Jacob S.S., Singh M., Karthik K., Tiwari R. et al. 2015 Effect of immunomodulation and immunomodulatory agents on health with some bioactive principles, modes of action and potent biomedical applications. *Int. J. Pharmacol*. **11**, 253-290.
Dimitriadis E., White C., Jones R., Salamonsen L. 2005 Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum. Reprod. Update*. **11**: 613-630.
Donadoni F., Tizioto P., Meirelles S., Malago Junior W., Giglioti R., Ibelli A. et al. 2011 Identification of a SNP in the gene IL2 and its association with resistance against gastrointestinal infection by nematodes in goat. Journal of Animal Science, v. 89, e-suppl.; Journal of Dairy Science, v. 94, e-suppl. 1, p. 280, 2011. Abstract T45. *Abstracts of ADSA-ASAS Joint Annual Meeting*, Jul., 2011, New Orleans.
Erfaniasl Z., Hashemi A., Zarringhabaie G.E. and Farhadian M. 2015 Association of the Interleukin-2 gene polymorphism with egg performance in a native turkey population. *Genetika*. **47**, 417-424.
Felten S., Madden K., Bellinger D., Kruszewska B., Moynihan J., Felten D. 1997 The role of the sympathetic nervous system in the modulation of immune responses. *Adv. Pharmacol*. **42**: 583-587.
Goldsby R., Kindt T., Osborne B. 2003 Kuby. *J. Immunology*. 5th ed. New York: WH Freeman and Company.
Hales DB. 2000 Cytokines and testicular function. *Cytokines Hum. Reprod.* 17-42.
Heinrich J-M., Bernheiden M., Minigo G., Yang KK., Schütt C., Männel DN., Jack RS. 2001 The essential role of lipopolysaccharide-binding protein in protection of mice against a peritoneal Salmonella infection involves the rapid induction of an inflammatory response. *J. Immunol.* **167**: 1624-1628.
Hellberg P., Thomsen P., Olof Janson P., Brännström M. 1991 Leukocyte supplementation increases the luteinizing hormone-induced ovulation rate in the in vitro-perfused rat ovary. *Biol. Reprod.* **44**: 791-797.
Hu H., Wang H., Yuan B., Zhou S., Li X., Huang Y. et al. 2006 Immunoenhancement of eukaryotic expression plasmids with chicken IL-2 or IL-15 genes on H5 subtype avian influenza vaccine. *Vet. Sci. China*. **10**, 805-810.
Janss L., Bolder N. 2000 Heritabilities of and genetic relationships between salmonella resistance traits in broilers. *J. Anim. Sci.* **78**: 2287-2291.
Ji B., Sun T., Ma Z., Lu Q., Hu W., Jian Z. et al. 2015 Possible Association of IFN-g Gene -316A/G SNP with Humoral Immune Response to Killed H5N1 HPAI Vaccine in a Red Jungle fowl Population. *J. Interferon Cytokine Res*. **11**: 844-849.
Kaiser P., Howell J., Fife M., Sadeyen J., Salmon N., Rothwell L., Young J., Van Diemen P., Stevens M., Poh T. 2008 Integrated immunogenomics in the chicken: deciphering the immune response to identify disease resistance genes. In: Animal Genomics for Animal Health: Karger Publishers. p 57-66.
Kaiser P., Mariani P. 1999 Promoter sequence, exon: intron structure, and synteny of genetic location show that a chicken cytokine with T-cell proliferative activity is IL2 and not IL15. *Immunogenetics* **49**: 26-35.
Kaiser P., Wain HM., Rothwell L. 1998 Structure of the chicken interferon-γ gene, and comparison to mammalian homologues. *Gene* **207**: 25-32.
Kaiser P., Howell J., Fife M., Sadeyen J., Salmon N., Rothwell L. et al. 2008 Integrated immunogenomics in the chicken: deciphering the immune response to identify disease resistance genes. *Animal Genomics for Animal Health*. **132**. 57-66.
Kayisli UA., Mahutte NG., Arici A. 2002 Uterine chemokines in reproductive physiology and pathology. *Am. J. Reprod. Immunol.* **47**: 213-221.
Knight PG. and Glister C. 2003 Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Anim. Reprod. Sci.* **78**: 165-183.
Kogut M., Rothwell L. and Kaiser P. 2002 Differential effects of age on chicken heterophil functional activation by recombinant chicken interleukin-2. *Dev. Comp. Immunol*. **26**, 817-830.
Kogut M.H., Rothwell L. and Kaiser P. 2003 Priming by recombinant chicken interleukin-2 induces selective expression of IL-8 and IL-18 mRNA in chicken heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized Salmonella enterica serovar enteritidis. *Mol. Immunol*. **40**, 603-610.
Lambrecht B., Gonze M., Meulemans G. and van denberg T.P. 2004 Assessment of the cell-mediated immune response in chickens by detection of chicken interferon-γ in response to mitogen and recall Newcastle disease viral antigen stimulation. *Avian Pathol*. **33**, 343-350.
Lamont S., Kaiser M. and Liu W. 2002 Candidate genes for resistance to Salmonella enteritidis colonization in chickens as detected in a novel genetic cross. *Vet. Immunol. Immunopathol*. **87**, 423-428.
Li G., Lillehoj E.P. and Lillehoj H.S. 2002 Interleukin-2 production in SC and TK chickens infected with Eimeria tenella. *Avian Dis*. **46**, 2-9.

Lühken G., Gauly M., Kaufmann F. and Erhardt G. 2011 Association study in naturally infected helminth layers shows evidence for influence of interferon-gamma gene variants on Ascaridia galli worm burden. *Vet. Res*. **42**, 84.
Lühken G., Stamm I., Menge C. and Erhardt G. 2005 Functional analysis of a single nucleotide polymorphism in a potential binding site for GATA transcription factors in the ovine interleukin 2 gene. *Vet. Immunol. Immunopathol*. **107**, 51-56.
Miyamoto T., Min W. and Lillehoj H.S. 2002 Kinetics of interleukin-2 production in chickens infected with Eimeria tenella. *Comp. Immunol. Microbiol. Infect. Dis*. **25**, 149-158.
Nagarajan U.M., Sikes J., Prantner D., Andrews C.W., Frazer L., Goodwin A. et al. 2011 MyD88 deficiency leads to decreased NK cell gamma interferon production and T cell recruitment during Chlamydia muridarum genital tract infection, but a predominant Th1 response and enhanced monocytic inflammation are associated with infection resolution. *Infect. Immun*. **79**, 486-498.
Neuvians T., Schams D., Berisha B., Pfaffl M. 2004 Involvement of pro-inflammatory cytokines, mediators of inflammation, and basic fibroblast growth factor in prostaglandin F2α-induced luteolysis in bovine corpus luteum. *Biol. Reprod.* **70**: 473-480.
Nishimura R., Bowolaksono A., Acosta TJ., Murakami S., Piotrowska K., Skarzynski DJ., Okuda K. 2004 Possible role of interleukin-1 in the regulation of bovine corpus luteum throughout the luteal phase. *Biol. Reprod.* **71**: 1688-1693.
Parish CR., O'neill ER. 1997. Dependence of the adaptive immune response on innate immunity: Some questions answered but new paradoxes emerge. *Immunol. Cell Biol.* **75**:543-552.
Park J.H., Sung H.W., Yoon B.I. and Kwon H.M. 2009 Protection of chicken against very virulent IBDV provided by in ovo priming with DNA vaccine and boosting with killed vaccine and the adjuvant effects of plasmid-encoded chicken interleukin-2 and interferon-γ. *J. Vet. Sci*. **10**, 131-139.
Pasanen S., Ylikomi T., Palojoki E., Syvälä H., Pelto-Huikko M., Tuohimaa P. 1998 Progesterone receptor in chicken bursa of Fabricius and thymus: evidence for expression in B-lymphocytes. *Mol. Cell. Endocrinol.* **141**: 119-128.
Patra G., Jas R., Ghosh J., Borthakur S.K. and Paul A. 2016. Single nucleotide polymorphism and expression studies of the interferon gamma gene and its role against Haemonchus contortus in Garole and Sahabadi sheep. *Asian Pac. J. Trop. Dis*. **6**, 106-112.
Pintarič M., Gerner W. and Saalmüller A. 2008 Synergistic effects of IL-2, IL-12 and IL-18 on cytolytic activity, perforin expression and IFN-γ production of porcine natural killer cells. *Vet. Immunol. Immunopathol*. **121**, 68-82.
Prakash V., Bhattacharya T.K. and Pandey O. 2010. Genetic polymorphism study of promoter region of interleukin-2 gene and its association with certain milk associated traits in Indian crossbred cattle. *J. Mol. Genet*. **2**, 15-19.
Rahman A., Snibson KJ., Lee CS., Meeusen EN. 2004 Effects of implantation and early pregnancy on the expression of cytokines and vascular surface molecules in the sheep endometrium. *J. Reprod. Immunol.* **64**: 45-58.
Reemers S.S., Van Haarlem D.A., Sijts A.J., Vervelde L. and Jansen C.A. 2012 Identification of novel avian influenza virus derived CD8+ T-cell epitopes. *PLoS One*. **7**, e31953.
Roussan D., Haddad R., Khawaldeh G. 2008 Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. *Poul. sci*. **87**: 444-448.
Savan R., Ravichandran S., Collins J.R., Sakai M. and Young H.A. 2009 Structural conservation of interferon gamma among vertebrates. *Cytokine Growth Factor Rev*. **20**, 115-124.
Sebastiani G., Blais V., Sancho V., Vogel SN., Stevenson MM., Gros P., Lapointe J-M., Rivest S., Malo D. 2002 Host immune response to Salmonella enterica serovar Typhimurium infection in mice derived from wild strains. *Infect. Immun.* **70**: 1997-2009.
Shah M.A.A., Song X., Xu L., Yan R., Song H., Ruirui Z. et al. 2010 The DNA-induced protective immunity with chicken interferon gamma against poultry coccidiosis. *Parasitol. Res*. **107**, 747-750.
Sharma R.K. and Gandhi E. 2011 Effect of interleukin-2 on steroidogenesis of goat ovary. *J. Immunol. Immunopathol*. **13**, 20-24.
Singh S., Pei J. and Collisson E. 2014 Viral specific CD8 + T lymphocyte immunity provides protection against heterotypic stains of avian influenza viruses and avian coronaviruses. *Int. Trends. Immun*. **2**, 36-46.
Soboloff J., Desilets M., Tsang K. 1995 Influence of tumor necrosis factor alpha on intracellular Ca^{2+} in hen granulosa cells in vitro during follicular development. *Biol. Reprod.* **53**: 546-552.
Soller M. and Andersson L. 1998 Genomic approaches to the improvement of disease resistance in farm animals. *Rev. Sci. Tech*. **17**, 329-345.
Sundaresan N., Anish D., Sastry K., Saxena V., Mohan J., Ahmed K. 2007 Cytokines in reproductive remodeling of molting White Leghorn hens. *J. Reprod. Immunol.* **73**: 39-50.

Swaggerty CL., Kogut MH., Ferro PJ., Rothwell L., Pevzner IY., Kaiser P. 2004 Differential cytokine mRNA expression in heterophils isolated from Salmonella-resistant and-susceptible chickens. *Immunology* **113**: 139-148.
Tohidi R., Idris I.B., Panandam J.M. and Bejo M.H. 2012 The effects of polymorphisms in IL-2, IFN-γ, TGF-β2, IgL, TLR-4, MD-2, and iNOS genes on resistance to Salmonella Enteritidis in indigenous chickens. *Avian Pathol*. **41**, 605-612.
Van der Poel J., Parmentier H. 2003 Genetic bases for resistance and immunity to Avian Disease. S.l.: S.n. (Annual Report, contributing project to NE-1016) - 4 p.
Vervelde L., Matthijs M., Van Haarlem D., de Wit J. and Jansen C. 2013 Rapid NK-cell activation in chicken after infection with infectious bronchitis virus M41. *Vet. Immunol. Immunopathol*. **151**, 337-341.
Vetrivel S. and Chandrakumarmangalam S. 2013 The role of poultry industry in Indian Economy. *Rev. Bras. Cienc. Avic*. **15**, 287-293.
Withanage G., Kaiser P., Wigley P., Powers C., Mastroeni P., Brooks H., Barrow P., Smith A., Maskell D., McConnell I. 2004 Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with Salmonella enterica serovar typhimurium. *Infect. Immun.* **72**: 2152-2159.
Withanage G., Sasai K., Fukata T., Miyamoto T., Lillehoj H., Baba E. 2003 Increased lymphocyte subpopulations and macrophages in the ovaries and oviducts of laying hens infected with Salmonella enterica serovar Enteritidis. *Avian Pathol.* **32**: 583-590.
Xu Q., Song X., Xu L., Yan R., Shah M.A.A. and Li X. 2008 Vaccination of chickens with a chimeric DNA vaccine encoding Eimeria tenella TA4 and chicken IL-2 induces protective immunity against coccidiosis. *Vet. Parasitol*. **156**, 319-323.
Yakubu A., Salako A., Donato M., Takeet M., Peters S., Wheto M. et al. 2016 Interleukin-2 (IL-2) gene polymorphism and association with heat tolerance in Nigerian goats. *Small Rumin. Res.* **141**, 127-134.
Ye X., Avendano S., Dekkers J. and Lamont S. 2006 Association of twelve immune-related genes with performance of three broiler lines in two different hygiene environments. *Poult. Sci*. **85**, 1555-1569.
Zettergren LD., Cutlan RT. 1992 Immunoglobulin-containing cells in chick embryo urogenital tissues: a new site for early B lineage cells in endothermic vertebrates. *J. Exp. Zool., Part A* **262**: 458-461.
Zhang D., Zhao H., Luo Y. and Han J. 2011 Characterization of haplotype diversity defined by discontinuous insertions/deletions within the intron 2 of iInterleukin 2 in different domestic chicken populations. *J. Biol. Sci*. **11**, 261-267.
Zhao K., He W., Gao W., Lu H., Han T., Li J. et al. 2011 Orf virus DNA vaccines expressing ORFV 011 and ORFV 059 chimeric protein enhances immunogenicity. *J. Virol*. **8**, 562.
Zhou H., Buitenhuis A., Weigend S. and Lamont S. 2001 Candidate gene promoter polymorphisms and antibody response kinetics in chickens: interferon-γ, interleukin-2, and immunoglobulin light chain. *Poult. Sci*. **80**, 1679-1689.
Zhou H., Li H. and Lamont S. 2003 Genetic markers associated with antibody response kinetics in adult chickens. *Poult. Sci*. **82**, 699-708.
Zhou H., Lillehoj H.S. and Lamont S.J. 2002 Associations of interferon-γ genotype and protein level with antibody response kinetics in chickens. *Avian Dis*. **46**, 869-876.

Table 1. Primer sequences and other specific information for each genetic locus

Gene	Primer sequence	Enzyme	Annealing	Accession	Reference
IL-2	F: 5'- TGCTTTTAACCGTCTTTG -3' R: 5'- GATGCTCCATAAGCTGTAGT -3'	*Mnl*I	53°C	*AJ22451*	Zhou et al., 2001
IFN-γ	F: 5'- GTAAGGAACTTCAGCCATTG -3' R: 5'- GACGAATGAACTTCATCTGCC -3'	*Tsp509*I	64°C	*Y079221*	

Table 2. SNP variants position found in IL-2 and IFN-γ loci

Gene	Restriction enzyme	SNP	Position	Accession
IL-2	*Mnl*I	A / G	-425	AJ224516
IFN-γ	*Tsp509*I	A / G	-277	Y07922
		C / T	-317	
		A / G	-318	

Table 3. Allelic and genotypic frequencies of IL-2 and IFN-γ loci and X^2 test for HWE

Gene	Allelic frequency		Number of genotypes			Genotypic frequency			X^2 test
IL-2	**A**	**G**	**AA**	**AG**	**GG**	**AA**	**AG**	**GG**	0.861
	0.58	0.42	127	187	66	0.33	0.50	0.17	
IFN-γ	**A**	**G**	**AA**	**AG**	**GG**	**AA**	**AG**	**GG**	0.158
	0.55	0.45	120	175	85	0.32	0.46	0.22	

Table 4. Observed and effective number of alleles of IL-2 and IFN-γ loci

Gene	Sample size	na	ne
IL-2	760	2	1.949
IFN-γ	760	2	1.983

na: observed number of alleles
ne: effective number of alleles

Table 5. Least Mean Squares test of observed genotypes in Mazandaran native breeder hens

Gene	Trait	Genotypes and Means			p-Value
		AA	**AG**	**GG**	
IL-2	**AEW[1]**	57.36±0.44 [b]	58.83±0.37 [a]	58.81±0.59 [a]	0.0155[*]
	AEW[2]	51.26±0.45 [b]	52.25±0.36 [a]	51.55±0.61 [ab]	0.1477 [ns]
	AEW[3]	51.22±0.43 [b]	52.27±0.35 [a]	52.09±0.58 [ab]	0.1519 [ns]
	EN[H]	12±0.64 [b]	14±0.51 [a]	13±0.86 [ab]	0.0560[•]
	BW[8]	832.27±10.22 [a]	801.84±8.23 [b]	813.45±13.77 [ab]	0.0584[•]
IFN-γ	**AEW[2]**	51.42±0.47 [ab]	51.03±0.39 [b]	52.50±0.53 [a]	0.0737 [ns]

ns: non-significant, *P<0.05, •P≤0.05(almost achieved significance), AEW[1]: AEW at 345-375 days of age, AEW[2]: AEW at 28 weeks of age,
AEW[3]: AEW at 30 weeks of age, EN[H]: EN at 345-375 days of age, BW[8]: BW at 8 weeks of age