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Identification of Novel Mutations in *IL-2* Gene in Khorasan Native Fowls

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Abstract

The intron-exon structure of Khorasan native fowl interleukin-2 (*IL*-2) was investigated. For this purpose, twenty chickens were selected from the Native Fowl Breeding Station of Khorasan province, and genomic DNA was extracted using a modified conventional DNA extraction protocol. An 875 bp fragment of *IL*-2 was successfully amplified, including a small part of the promoter, exon 1, intron 1, and exon 2. To report a novel mutation in *IL*-2, PCR-sequencing techniques were applied to the gene obtained from Khorasan native fowls. Then, the sequences were compared to evaluate gene mutations using CLC Main Workbench V. 5.5. The I-TASSER server was used to predict the 3-dimensional structure and function of protein molecules from amino acid sequences. The results of the comparison showed mutations in the DNA fragments of the sample birds, which caused changes in the amino acid content of IL-2. Substitutions at points 733, 738, 744, and 794 bp in the sequence were for C/A, A/T, C/A, and C/T, respectively. The translation of nucleotide to protein using BLASTx in the NCBI revealed that nucleotide substitutions caused three amino acid substitutions including A/E, R/W, and P/T.

Introduction

The consequence of genetic selection for faster growth traits in broiler has negatively affected health status regarding bacterial and viral diseases. Therefore, it may be expected commercial chicken with high productivity but poor immune system characteristics (Zekarias *et al*., 2002).

Diseases are a serious challenge for the economic poultry industry in the Asian subcontinent (Mengesha, 2012). Research on immune system function in poultry has a long story with complexity within specific immune-related organs, a different type of white cells for protection of birds against pathogen entrance.

The general function of the immune system in chicken birds reflected the degree of natural resistance or susceptibility and response to common regional disease. There are two different research scenarios for investigation of genetic background in poultry; candidate gene approach and identification of favorable single nucleotide polymorphisms controlling health status and susceptibility to diseases (Muir and Aggrey, 2003).

Cytokines are essential for the activation, differentiation, and control of the host immune system. They may also play a role in the protection conferred by vaccines. Cytokines are proteins or peptides that play a vital role in stimulating the immune system. Different cytokines participate in several immune responses, including proinflammatory activity and regulating the function of T helper lymphocytes (Wigley and Kaiser, 2003). Interleukin-2 is a cytokine that plays a role in stimulating the T cells, B cells, and natural killer cells (Gaffen and Liu, 2004).

The structural and functional study of cytokines helps to better understand their mechanisms of response to infections. For the first time, a cDNA library from chicken spleen-activated cells was produced to identify the *IL*-2 gene in chickens. A protein created from a single gene had 24 to 25% amino acid identity and 44 to 46% similarity to both bovine IL-2 and IL-15, respectively (Sundick and Gill-Dixon, 1997). However, genetic distance analysis showed that ch*IL-2* is more closely related to

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IL-2 of mammals (Kaiser and Mariani, 1999). Furthermore, it was indicated that the gene encoding a chicken cytokine with T cell proliferative activity is *IL-2* and not *IL-15*. Ch*IL-2* is located on chromosome 4 and involves four exons and three introns, but exon 2 and introns 2 and 3 in the chicken are shorter than those in mammals (Kaiser and Mariani, 1999). Based on the similarity of ch*IL-2* to IL-2 and IL-15 of mammals as well as its potential for use as a vaccine adjuvant, a mutational analysis of ch*IL-2* was performed and a model was developed for the interaction between chIL-2 and its receptor. Asp17 was shown to have a critical role in IL-2 function. Additionally, Asp17 is an N-terminal contact residue between chIL-2 and its receptor (Kolodsick *et al*., 2001). Genetic association studies revealed that polymorphisms in the promoter region of chIL-2 were associated with Salmonella burden in caecum and spleen (Kramer *et al*., 2003; Tohidi *et al*., 2012). Intramuscular injection of recombinant plasmids containing chIL-2 and Spike gene (S) derived from infectious bronchitis virus (IBV) indicated more clinical protection in chicken compared to intramuscular administration of plasmids containing only S gene (Zhang *et al*., 2009). A study on single nucleotide polymorphisms (SNPs) in *IL-2* of different domesticated chickens identified 13 SNPs in the coding sequences, eight of which changed eight amino acids. Also, one in/del mutation was found to lead to one amino acid deletion, and eight SNPs and one in/del were identified in intron 2 that were novel (Zhang *et al*., 2011). Nucleotide polymorphism in such genes results in a varied response of individuals to a variety of pathogens/antigens. DNA polymorphism also has an association with the nature of antibody response (Zhang *et al*., 2011; Scepanovic *et al*., 2018).

In recent years, advances in technology, such as Sanger sequencing, microarray technique, and whole-genome sequencing, have provided the most comprehensive collection of genetic variation among species, breeds, and individuals (Ng and Kirkness, 2010). Whole-genome comparative studies have indicated genome in avian species has been stabilized during the history of evolution similar to events that have been occurred in the mammalian genome (Griffin *et al*., 2008). Around 4 kb insert within the SLCO1B3 gene was identified in the chicken genome by the use of whole-genome sequencing (Oh *et al*., 2016). However, it is necessary to characterize the genomic DNA sequences of ch*IL-2* in different chicken populations of various backgrounds (Zhang *et al*., 2011).

Candidate gene approach is a strong tool to identify single nucleotide polymorphism and variation through candidate genes has key roles in health traits (e.g., immune response) in livestock. As commercial chicken strains directed genetically to homozygosity in appearance and DNA information therefore, limited genetic variation was expected to identify within the genome. Considering the importance of IL-2 in immune responses and generating DNA vaccines as well as a DNA marker in one side, and the critical role of animal genetic resources conservation for future purposes such as adaptation to climatic changes in another side, the exonic and intronic regions of *IL-2* were investigated in Khorasan native chicken to find genomic variations and possibly amino acid changes.

Materials and Methods

Sampling

In the present study, blood samples from 20 roosters (grown at the Native Fowl Breeding Station of Khorasan province, Iran) were first collected from the peripheral vein of the wing using EDTA tubes and transferred immediately to the laboratory in the ice flask. Then, blood samples were stored at a -20 °C until DNA extraction. DNA was extracted by a modified salting-out procedure (Miller *et al*., 1988). The DNA quality was analyzed using spectrophotometry and electrophoresis on 0.8% agarose gel.

Primer design

A pair of primers were synthesized for *IL-2* proliferation based on a sequence of registered chicken reference genomes (GenBank: AJ224516) in the NCBI database using Primer Premier 5 software as it replicated a specific genetic region of 875 bp. The full details are given in Table (1).

Table 1. Specification and sequence of primers used to proliferate the *IL-2* fragment

Primer	Nucleotide Sequence	Fragment Length (bp)	Annealing Temp.
Forward	5' GCAGGAGGACAAACATACACCAGTA 3'	875	64 °C
Reverse	3' GGAAACCATCCTACCCTCACACTG 5'		

Polymerase chain reaction (PCR)

PCR was performed in a 50-mL master mix containing 300 ng of chicken genomic DNA, 1 μM of each primer, 200 mM of each dNTP, 1.5 U Taq DNA polymerase (Promega, Madison, Wisconsin, USA), 1X reaction buffer, and 2 mM $MgCl_2$. The PCR program included primary denaturation at 95 °C for

10 minutes followed by 33 cycles of denaturation at 95 °C for 30 seconds, annealing at 61 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes. The PCR products were electrophoresed on a 1.5% agarose gel with ethidium bromide at 80 V for 1 h.

Sequencing and mutation screening

The PCR products were sent to Bioneer Co., South Korea, for sequencing. Sequence read quality was evaluated using BioEdit software and the sequences were confirmed by the BLAST tool in the NCBI. Then, the sequences were compared to evaluate gene mutations using CLC Main Workbench, V. 5.5. The I-TASSER server was used to predict the 3-dimensional structure and function of protein molecules from amino acid sequences (Roy *et al*., 2010).

Results

An 875 bp fragment of *IL*-2, including a small part of the promoter, exon 1, intron 1, and exon 2, was successfully amplified. The sequencing results of 15 PCR samples had the appropriate quality for the next analysis. The DNA sequenced fragments of Khorasan native chickens were compared with those obtained from NCBI (AJ224516). The results of the comparison showed mutations in the DNA fragments of Khorasan native fowls. The mutations in exon 1 caused changes in the amino acid content (Table 2).

Table 2. Nucleotide substitutions in the sequenced fragments of *IL-2* in Khorasan native fowls

Genomic Region	Position	Substitution	*Frequency %
Intron 1	820	A/G	36
Intron 1	838 and 840	T/G	100
Exon 1	733	A/C	50
Exon 1	738	T/A	50
Exon 1	744	A/C	50
Exon 1	794	T/C	50

*Frequency of nucleotides substitutions in the data

Sequence analysis of the exon 1 region showed that there were replacement substitutions in six of 15 sequenced samples compared to the reference sequence. The substitutions in the exon 1 region for the six samples were similar. Substitutions at points 733, 738, 744, and 794 bp in our sequence were for C/A, A/T, C/A, and C/T, respectively (Figure 1). The translation of nucleotide to protein using BLASTx in the NCBI revealed that nucleotide substitutions caused three amino acid substitutions including A/E, R/W, and P/T (Table 3).

Exon 2 sequences in this study were completely similar to the reference sequence (Figure 2). Also, three nucleotide substitutions for intron 1 were found at positions 820, 838, and 840 bp in our sequence for G/A, G/T, and G/T, respectively (Figure 3).

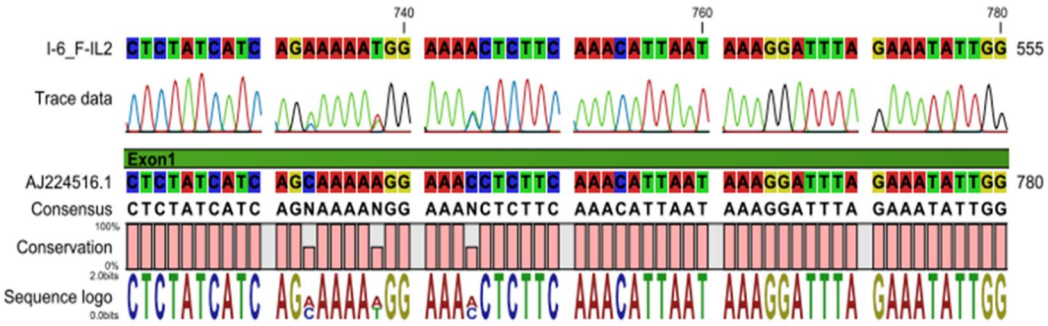


Figure 1. The exon 1 of *IL-2* in Khorasan native chicken compared to the reference sequence (E value = 3e-79)

Table 3. Comparison of amino acid sequence derived from exon 1 and partial exon 2 of Khorasan native chicken and reference sequence for IL-2

Source of Sequence	Amino Acid Sequence
Sample	MMCKVLIFGCISVAMLMTTAYGASLSSEKWKTLQTLIKDLEILENIKNKIHLELYTPTET
AJ224516.1	MMCKVLIFGCISVAMLMTTAYGASLSSAKRKPLQTLIKDLEILENIKNKIHLELYTPTET

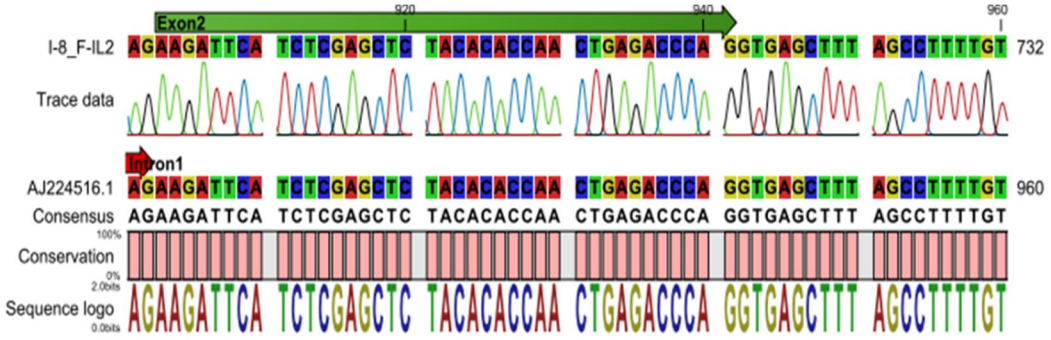


Figure 2. The exon 2 of *IL-2* in Khorasan native chicken compared to the reference sequence

The results of determining the structure and function of IL-2 using I-TASSER are shown in Figures 4-6. The predicted secondary structure of the mutated proteins revealed α-helix instead of coil compared to the reference sequence (Figure 4). The comparison of tertiary structures showed a slight difference in the position of the β-sheet between the reference sequence and the sequence derived from Khorasan native fowls (Figure 5). These differences may cause variations in binding sites between the two sequences (Figure 6).

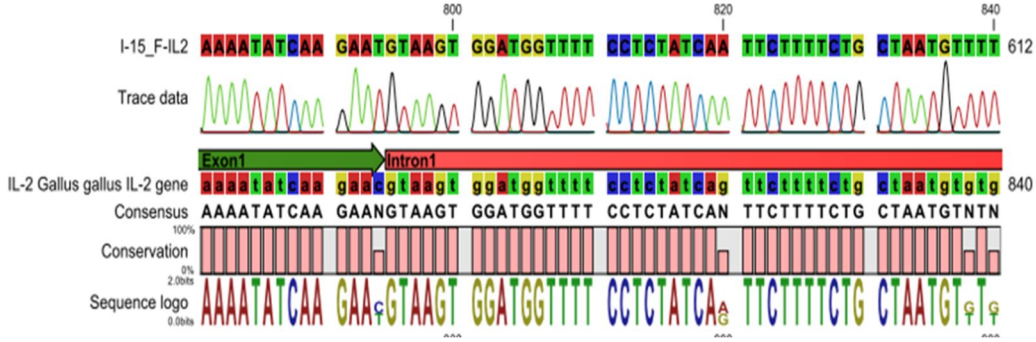


Figure 3. The intron 1 of *IL-2* in Khorasan native chicken compared to the reference sequence

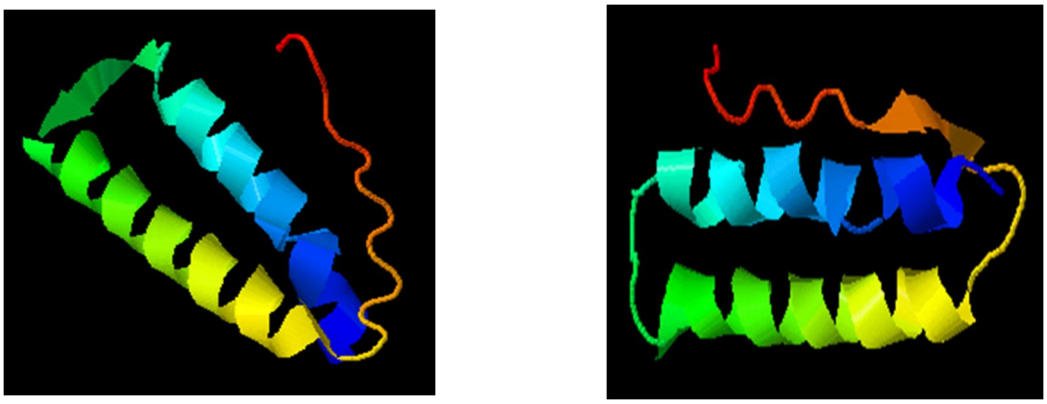
(a)

```
                    20                  40                  60
                    |                   |                   |
Sequence    MMCKVLIFGCISVAMLMTTAYGASLSSEKWKTLQTLIKDLEILENIKNKIHLELYTPTET
Prediction  CCCSSHHHHHHHHHHHHHHHCCCCCHHHHHHHHHHHHHHHHHHHHCCSSSSSSCCCCC
Conf.Score  95120358899999998872697885672058999999999986312524888589999
            H:Helix; S:Strand; C:Coil
```

(b)

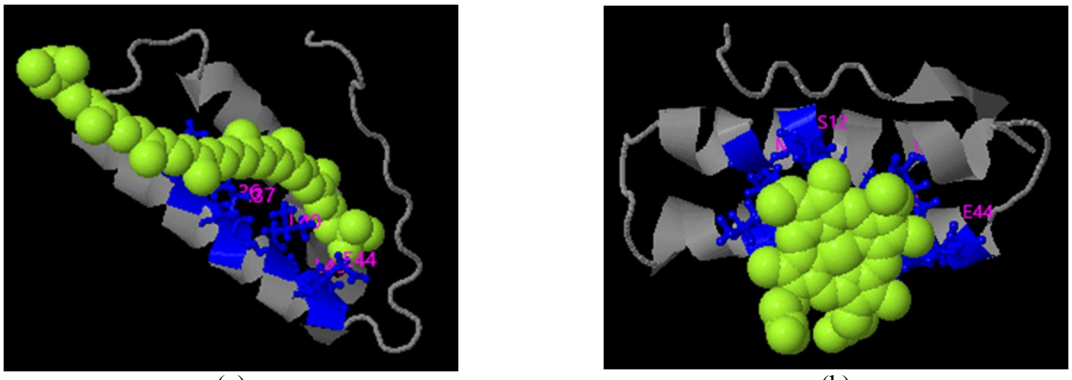
```
                    20                  40                  60
                    |                   |                   |
Sequence    MMCKVLIFGCISVAMLMTTAYGASLSSAKRKPLQTLIKDLEILENIKNKIHLELYTPTET
Prediction  CCCSSHHHHHHHHHHHHHHHCCCCCHHHCCCHHHHHHHHHHHHHHHHCCSSSSSSCCCCC
Conf.Score  95120358899999999872697893544618899999999986313624898589999
            H:Helix; S:Strand; C:Coil
```

Figure 4. The predicted secondary structure of the IL-2 (**a**, Khorasan native fowl; **b**, reference sequence)



(a) (b)

Figure 5. The tertiary structure of the IL-2 (**a**, Khorasan native fowl; **b**, reference sequence)



(a) (b)

Figure 6. The ligand-binding sites of the IL-2 (**a**, Khorasan native fowl; **b**, reference sequence)

Discussion

The promoter region of ch-*IL*-2 is about 650 bp (Kaiser and Mariani, 1999), and in the present study, 70 bp of this genomic region was sequenced and compared to the reference sequence in the NCBI. There were no mutations in this region. However, polymorphisms were reported in 659 bp fragment of the chicken promoter region of *IL*-2 (Zhou *et al*., 2001).

Since the intron has a regulatory role in the gene function (Chorev and Carmel, 2012), the observed substitutions in this study may alter the activity of *IL*-2. Previously, 13 SNPs were reported for coding sequences of *IL*-2 in red jungle fowl and Leghorn, eight of which yielded eight amino acid replacements. Besides, eight novel SNPs were found in chicken populations from China, Cambodia, Egypt, Fiji, Lao, and Uganda (Zhang *et al*., 2011).

Intensive selection in domesticated chickens has been applied for a long period and led to highly productive commercial strains (FAO, 2007). Summary of previous literature on the genetic background of the immune system in avian and mammalian species has been discussed intensively and heritability index for innate and adaptive immune response traits was highlighted between 0.06 to 0.053 in different types of chickens (Muir and Aggrey, 2003; Wijga *et al*., 2009), 0.19 to 0.41 in bovine (Thompson-Crispi *et al*., 2012), and 0.20 to 0.90 in pigs, accordingly (Flori *et al*., 2011).

Domestication and intensive selection have reduced the genetic diversity in animals relative to their wild ancestor (Zhang *et al*., 2013). However, native and backyard chickens are kept away from intensive selection. Therefore, these breeds may show higher genetic variation between and within populations (Li *et al*., 2019). While the Khorasan native fowls are not originally pure, and they have been collected from different villages by the Native Fowl Breeding Station of Khorasan province, thus, the observed substitutions may transfer from the other breeds.

Mutations in a polypeptide sequence may change or disrupt the biological activity of the protein. There are 19 amino acids to be involved in the binding of IL-2 to the IL-2Rα chain that are not conserved in chickens (Zelus *et al*., 2000). Chemical and structural properties of amino acids determine the function of a polypeptide. A single amino acid substitution of Trp666 to Ala in the membrane-proximal interbox1/2 region of *IL*-6 signal transducer gp130 abrogates the activation of signal transducer and activator of

transcription factors and prevents the association of Janus kinase JAK1 (Haan *et al*., 2000). The two cysteines (C78-C125) form a disulfide bond that plays a key role in IL-2 folding in all mammals (Zelus *et al*., 2000). The intramolecular disulfide bridge stabilizes the IL-2 protein in a biologically active conformation (Wang *et al*., 1984). Moreover, mutations at three cysteine residues in the active site of human IL-2 revealed its decreasing stability as the sulfide bridge was disrupted (Liang *et al*., 1986). However, mutations at the position of an amino acid other than the receptor binding site, including valine111 to arginine, lysine117 to glutamine, and threonine133 to asparagine, caused higher stability (Dakshinamurthi *et al*., 2009). Alanine is a non-polar, hydrophobic amino acid that is very small and capable of being wedged into tight loops or chains. It is rarely involved in protein function (Betts and Russell, 2003). Conversely, glutamic acid is a hydrophilic amino acid and is very stable in water. It is quite frequently involved in protein active or binding sites. The substitution of glutamic acid instead of alanine may change the structure and function of the protein. Mutations at points T117A, E116A, E118K, and E118R in loop 9 for IL-1β revealed reduction of the biological activity (Chen *et al*., 2016). The substitution of alanine for glutamic acid decreased the biological activity of IL-1β compared to wild type chickens. Also, it would not be able to form the wild type hydrogen bond and hydrophobic interaction (Chen *et al*., 2016). Also, tryptophan is a non-polar hydrophobic amino acid that can bind to non-protein atoms. The substitution of arginine, a positively charged polar amino acid, to tryptophan can devastate the protein function. It was shown that specific types of amino acid substitutions, for instance, R38W, could affect the functionality of IL-2 without principally affecting the cytokine characteristics (Hu *et al*., 2003). Furthermore, proline is generally assumed as a specific amino acid that destroys the formation of α-helices (Zurawski *et al*., 1993).

The substitution of proline by threonine may change the function of the protein as the hydrophilic group for threonine amino acid can form hydrogen bonds with a variety of polar substrates (Betts and Russell, 2003).

Conclusion

Candidate gene approach may be a substantial tool for identification of DNA polymorphism and detection of nucleotide variations in the genes responsible for key traits of health importance (e.g., immune response) in farm animals. As commercial chicken strains directed genetically to homozygosity in appearance and DNA information therefore, limited genetic variation was expected to identify within the genome. The results of the comparison showed mutations in the DNA fragments of Khorasan native fowls, of which exon 1 mutations caused changes in the amino acid content. Therefore, based on the results, alanine, arginine, and proline have been changed to glutamic acid, tryptophan, and threonine, respectively, and the function of IL-2 may change in the mutated group. To determine the possibility of variation in the IL-2 activity, an in vivo and in vitro study on protein function is suggested. Also, it is necessary to evaluate the other local breeds for these substitutions.

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