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### Genetic Analysis of Mitochondrial Genomic D-Loop and tRNA Regions in Khorasan's Native Chicken

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#### ABSTRACT

Mitochondrial sequencing of D-loop is considered as most functional approach to determine the phylogeny relation between close populations. The purpose of this project was to evaluation of the phylogeny and genetic nucleotide sequences of D-loop and tRNA regions in mitochondrial genome of khorasan's native Chickens. Blood samples were collected from randomly 6 khorasan's native chickens and after DNA extraction, the D-loop and tRNA regions with 818 bp lengths were amplified using specific primers. Sequencing was done according to Sanger method and based on automate system. Phylogenetic tree and Genetic distances matrix between khorasan's native chickens and other breeds for D-loop and tRNA of mitochondrial genome were drawn using the same sequences of mitochondrial genome in other available breeds in NCBI database. Results showed no haplotype difference between the studied samples sequences. The results of phylogeny test revealed that lowest genetic distance was observed between khorasan's native chicken with other Asian chickens such as Huang Lang, Lv'erwu, White Leghorn and White Plymouth rock for the D-loop and tRNA genes. So we could conclude that there is close relationship between khorasan's native chickens and other Asian chickens.

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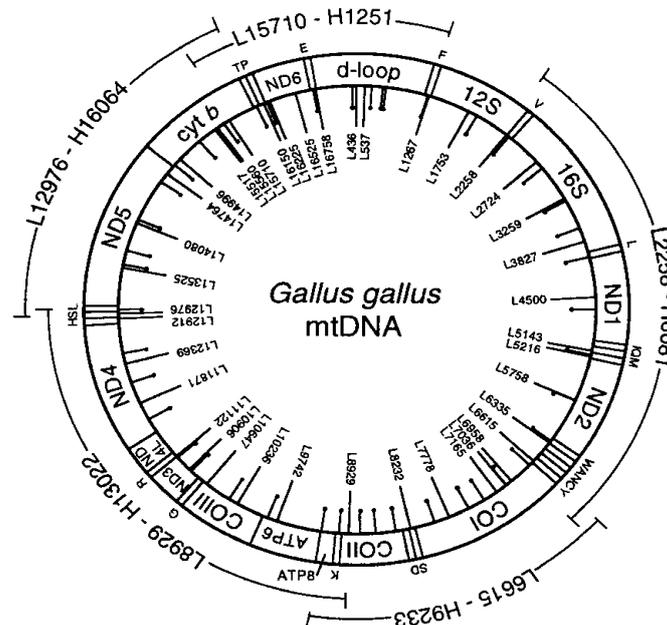
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#### INTRODUCTION

According to the study done by FAO up to 30% of Global mammalian and avian Livestock breeds are faced currently at risk of being lost and could not be replaced [12]. Conservation genetics or the application of genetics to the preservation of species has received increasing attention in recent years [1]. In conservation genetics, knowledge of the relatedness between individuals is particularly important in captive breeding programs that seek to reduce incestuous mating in order to minimize inbreeding and the loss of genetic variation [2]. It is well known that a decline in genetic variation reduces the ability of a population to adapt to environmental changes and therefore decreases its long term survival. The loss of genetic diversity also results in lower individual fitness and poor adaptability [7]. In order to perform breeding programs and improve production of the native chickens the preserving genetic diversity in different areas of Iran is important because of their few population. Several advantages could be mentioned for mitochondrial DNA (mtDNA) such as more than 1000 copies (transcripts) for each cell, small size comparing with genome DNA, maternal heritability, haploidy, no recombination in them, and the ability to amplify using distinctive triggers, and finally protected and non protected regions (D-Loop) sequencing for dependent species developmental studying [3]. Mitochondrion is a cytoplasm organ existing in most body cells. This organ who is able to produce energy for the cells having a distinctive DNA that is independent nuclear DNA, encoding 37 genes in animal species including 13 genes encoding respiratory chain, 22 genes encoding tRNA and 2 genes encoding rRNA. Expressing this gene seems to be necessary in vertebrate because their roles in energy producing, metabolism, homeostasis and cell death [5]. In most species, the mitochondrial DNA control region (D-loop region) is the most variable part of the mitochondrial DNA (mtDNA) molecule, presumably because of the lack of coding constraints and often used for phylogenetic analysis within species [4]. [8] Sequenced a total of 31 Korean Ogol chicken for evaluating genetic structure and phylogenetic analysis and grouped into four haplotypes and the large haplotype was represented in 12 individuals. The purpose of this project was phylogeny analyzing of D-loop and

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tRNA regions of mitochondrial DNA of khorasan's native chickens in order to obtain valuable information about these regions of mitochondrial, also comparing these sequences with other breeds.



**Fig. 1:** Schematic diagram of avian mitochondrial DNA.

## MATERIALS AND METHODS

Six blood samples from unrelated chickens of khorasan's native chickens for ensuring of any relationship were collected and were stored until extraction in tubes containing EDTA in  $-20\text{ }^{\circ}\text{C}$ . DNA was extracted using commercial kit. Quantity of the extracted DNA was measured according to spectrometry method using nano drop –nd 2000 spectrophotometer of USA Thermo Company and the quality was calculated on Agarose gele 1%. Specific primers for amplifying D-loop and tRNA fragments designed by using Primer premier 5 Software as mentioned in below:

Forward (D-loop and tRNA)-5'- TACACCTGCGTTGCGTCCTATC -3'

Reverse (D-loop and tRNA)-5'- CTGGCACAAGATTTACCAACCCT-3'

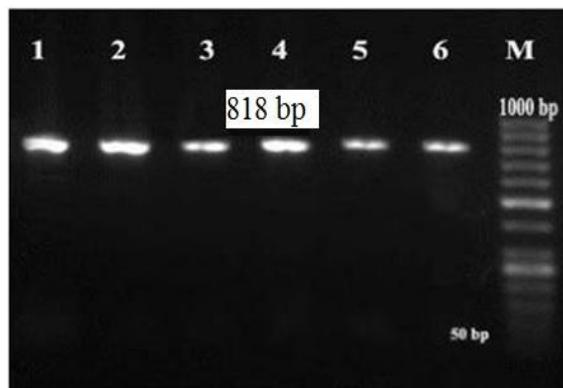
Polymerase chain reaction for amplifying 818 bp of D-loop and tRNA fragments were carried out using T-personal model Biometra thermo cycler according to standard method. The component of polymerase chain reaction in the final volume were included 25- $\mu\text{l}$  PCR mixture containing 100 ng DNA, 0.2 unit taq polymerase enzyme, 2  $\mu\text{l}$  dNTP (10 mM), 1.5  $\mu\text{l}$   $\text{MgCl}_2$ , 1pmol gene specific of the primers (50 mM). Also, in order to confirm the amplification, samples were electrophoresis on 1% agarose gel. Also PCR program for D-loop and tRNA genes was included 94  $^{\circ}\text{C}$  for 30 s , annealing in 54  $^{\circ}\text{C}$  for 35 s, proliferation in 72  $^{\circ}\text{C}$  for 30 s, a primary step in 94  $^{\circ}\text{C}$  for 10 min and a final amplifying stage in 72  $^{\circ}\text{C}$  for 10 min on 35 cycle. The PCR Products were electrophoresis on 1% agarose gel and coloration of gel was performed using etidium bromide. 100 ml of PCR products was purified and sent with 50 ml of each used primers forward and reverse (10 pmol) to MacroGen company(south Korea)for sequencing. These sample are sequenced using the ABI3130 machine according to Sanger automate approach. The obtained sequences homology level was measured using accurate BLAST tool and blastn method in NCBI database. In order to study the phylogenetic relation between target breeds, we draw the phylogeny tree using the alignment sequences UPGMA approach by MEGA 5 software.

## RESULTS AND DISCUSSION

The gel electrophoresis suggests that the methodology used to isolate DNA from various samples was reliable and that the specific 818 bp D-loop and tRNA mitochondrial DNA genes fragment can be amplified using the specific DNA primers (figure 2).

Mitochondrial genomic regions of D-loop and tRNA were sequenced for 6 samples. But one of the samples was excluded due to poor quality and was not used further. After sequencing the samples, comparison between the five sequences using Glusta Multiple alignment and Bio Edit software was conducted [6]. After controlling the quality of sequencing nucleotides, those sequences with poor quality were removed from both ends and common areas between the five sequences were isolated and this result leads to produce five pieces with 804 nucleotides length which contain D-loop and tRNA genes in all samples. Comparison between sequences

showed that there was no differences between studied sequences ( $P=0$ ) and this reason may be due to small sample size and population homogeneity because of consecutive selection for commercial goals. (Figure 3)

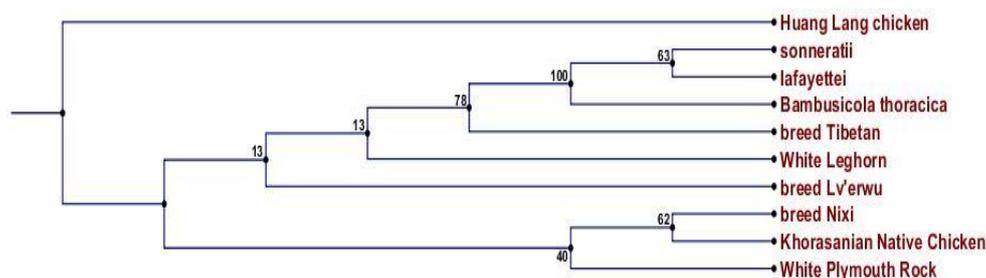


**Fig. 2:** Electrophoresis of 818 bp PCR Products on 1 % Agarose gel

|                            |        |       |         |     |            |            |            |
|----------------------------|--------|-------|---------|-----|------------|------------|------------|
| Khorasanian Native Chicken | CTCAA  | ACTAT | ACAAACG | TTT | ATCGTATAAT | ATATATACAT | TATTGTTTAT |
| Huang Lang chicken         | ctcaa  | actat | acaaacg | ttt | atcgtataat | atatatacat | tattgtttat |
| breed Lv'erwu              | ctcaa  | actat | acaaacg | ttt | atcgtataat | atatatacat | tattgtttat |
| White Leghorn              | CTCAA  | ACTAT | ACAAACG | TTT | ATCGTATAAT | ATATATACAT | TATTGTTTAT |
| White Plymouth Rock        | ctcaa  | actat | acaaacg | ttt | atcgtataat | atatatacat | tattgtttat |
| breed Nixi                 | ctcaa  | actat | acaaacg | ttt | atcgtataat | atatatacat | tattgtttat |
| breed Tibetan              | ctcaa  | actat | acaaacg | ttt | atcgtataat | atatatacat | tattgtttat |
| lafayettei                 | ctcaa  | actat | acaaacg | ttt | atcgtatagt | atatatacat | tattgtttgt |
| sonneratii                 | ctcaag | ctat  | acaaacg | ttt | atcgtatagt | atatatacat | ta-gtttgt  |
| Bambusicola thoracica      | ctgaa  | accat | acaaacg | ttt | atcgcgt-gt | atatatacat | cattgtatgt |

**Fig. 3:** Comparison between some Parts of D-loop and tRNA regions in Khorasan's native chicken with Lv'erwu, Huang Lang, White Leghorn, White piymouth Rock, Nixi, Tibetan, Sonneratii, Lafayettei, Bambusicola thoracica.

There was not any SNP diversity caused by mutation. Phylogenetic tree analysis showed that mitochondrial genomic regions of D-loop and tRNA sequences of khorasan's native chickens were closely related with the Huang Lang (Asian) and had farthest distance with Bambusicola thorcica (Asian) breed. (Figure 4).



**Fig. 4:** Phylogenetic tree for mitochondrial genomic regions of D-loop and tRNA sequences of khorasan's native chickens and other breeds which are taken from GenBank.

Genetic distances Matrix is obtained by pair wise comparison of each sequences between species using Maximum Composite Likelihood model [14]. Results represented the nucleotide substitution rates between studied sequences; therefore genetic distance between two populations is the same as phylogenetic relationship between them. So this index can be used to determine Genetic distances between breeds. By investigation of genetic distances matrix for mitochondrial genomic regions of D-loop and tRNA among khorasan's native chickens and others, we found that the lowest genetic distance existed between khorasan's native breeds with Huang Lang and Lv'erwu breeds from China and White Leghorn and White Plymouth rock with a 99.75 % overlap and two nucleotide replacement and genetic distance between khorasan's native breeds with Bambusicola thoracica has 91.73 % overlap. These results confirm the accuracy of the phylogenetic tree (Table1).

**Table 1:** Similarity Matrix for nucleotide D-loop and tRNA genes in Khorasan's native and other breeds.

|                            | 1  | 2  | 3     | 4      | 5      | 6      | 7     | 8     | 9     | 10    | Percent Identity |       |
|----------------------------|----|----|-------|--------|--------|--------|-------|-------|-------|-------|------------------|-------|
| Khorasanian Native Chicken | 1  |    | 99.75 | 99.75  | 99.75  | 99.75  | 99.63 | 99.63 | 96.65 | 95.54 |                  | 91.73 |
| Huang Lang chicken         | 2  | 2  |       | 100.00 | 100.00 | 100.00 | 99.88 | 99.88 | 96.89 | 95.78 |                  | 91.96 |
| breed L'Verwu              | 3  | 2  | 0     |        | 100.00 | 100.00 | 99.88 | 99.88 | 96.89 | 95.78 |                  | 91.96 |
| White Leghorn              | 4  | 2  | 0     | 0      |        | 100.00 | 99.88 | 99.88 | 96.89 | 95.78 |                  | 91.96 |
| White Plymouth Rock        | 5  | 2  | 0     | 0      | 0      |        | 99.88 | 99.88 | 96.89 | 95.78 |                  | 91.96 |
| breed Nixi                 | 6  | 3  | 1     | 1      | 1      | 1      |       | 99.75 | 96.77 | 95.66 |                  | 91.84 |
| breed Tibetan              | 7  | 3  | 1     | 1      | 1      | 1      | 2     |       | 96.77 | 95.66 |                  | 92.08 |
| lafayettei                 | 8  | 27 | 25    | 25     | 25     | 25     | 26    | 26    |       | 96.76 |                  | 91.69 |
| sonneratii                 | 9  | 36 | 34    | 34     | 34     | 34     | 35    | 35    | 26    |       |                  | 91.45 |
| Bambusicola thoracica      | 10 | 67 | 65    | 65     | 65     | 65     | 66    | 64    | 67    | 69    |                  |       |

Divergence

According to phylogenetic tree and genetic distances matrix survey results for the two genes in our study, it can be founded that using these two markers for representing genetic relationships among Khorasan's native chickens and other breeds is very important and Mitochondrial genomic sequences has been used successfully to determine genetic diversity in Asian chicken breeds [13]. In a study to determine the sequence of HVS-I region in chicken's mitochondrial D-Loop of Marandi's breed, phylogeny analysis revealed that Marandi's breed which are one of the Iranian native breeds, are more closely related with Azerbaijan native breeds, Plymouth Rock and White Leghorn breeds [9].

The results showed moderate to low genetic diversity in population. Low genetic diversity in these birds requires special attention to protect genetic resources [7]. Similarities in Number of polymorphic loci, indicating closely genetic relationship between these breeds and other South East Asia's poultry breeds therefore, it is confirmed that Asian countries including Southeast Asia was the origin of domesticated chickens [10, 11].

Importing improved poultry breeds from foreign countries has led to neglect the capacity of our native poultry. Genetic resources conservation is an expensive activity therefore according to indigenous chicken breeds which are adapted to their endemic environment and their relative resistance to diseases in that region; it is possible to increase their production capacity with breeding strategies. Using mitochondrial genomic regions sequencing for D-loop and tRNA and due to less complicated and time effectiveness of this technique for data analyzing and in comparison with some highly productive commercial breeds, we realize that these breeds have some similarities with highly productive breeds which represents the productive ability of these breeds.

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