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Research Report

Genetic analysis of *ND4* and *ND4L* regions of mitochondrial genome in Khorasan native chickens

Morteza Hashemi Attar and Mohammadreza Nassiri*

Department of Animal Science, Ferdowsi University of Mashhad, Mashhad, Iran.

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Abstract

Preserving genetic diversity of Iranian native chickens is significant in order to perform breeding programs and improved production. Genetic diversity among close populations can be determined by investigating their phylogenetic relations. Different approaches have been applied to determine the phylogenic relations from which genomic sequencing has been considered the most functional approach. In this project, we aimed to evaluate the phylogeny and genetic nucleotide sequences of *ND4* and *ND4L* regions in mitochondrial genome of Khorasan native chickens. Blood samples were collected from 6 random populations and their genomic DNAs were extracted. Results showed that there is no haplotype difference between the studied samples. Lowest genetic distance was observed between Khorasan native chicken and other Asian chickens i.e. Jiangbian, Lvenwv and Red jungle fowl for the *ND4* and *ND4L* genes indicating their close relationship.

***Keywords*:** Khorasan native chicken, *ND4L*, *ND4*, Mitochondrial DNA, Phylogeny tree

Introduction

There are more than 100 native fowl genetic masses in Iran adjusted to environmental conditions. They have obtained relative resistance to local diseases and are considered as important national wealth. These native chickens are genetic reserve and their protection for next generations needs an extensive study (Tavakolian, 1999). There are two hypotheses about the origin of domestic chickens, first, these are originated in gallus gallus or these were derived from several gallus subclass (Crwford1990). Mitochondrial genome sequencing has been considered the most functional method to determine the phylogeny relationship among their different populations and species (Bruforrd et al 2003). A lot of comparing tests have been conducted for different regions sequencing, genetic diversity and species developmental origin using mitochondrial genome. *ND4* (NADH dehydrogenase subunit 4) and *ND4L* (NADH-ubiquinone oxidoreductase chain 4L) are 459 and 98 amino acids containing proteins encoded by mitochondrial region's encoding genes to make NADH_COQ, the respiratory chain protein which is responsible to transfer electrons from NDAH to respiratory chain (Zhang et al 2000). The purpose of this project was phylogeny analyzes of *ND4* and *ND4L* regions of mitochondrial DNA of Khorasan native chickens.

Materials and Methods

Sampling:

Blood samples were collected from 6 Khorasan native chickens and unrelated chickens to ensuring their relationships. Blood samples were stored in EDTA containing tubes at -20^{0}C. DNA was extracted using commercial kit (Thermo, USA). Quantitative and qualitative assessment was done according to spectrometry method using nanodrop–ND 2000 spectrophotometer (Thermo, USA) and running on 1% agarose gel. *ND4* and *ND4L* regions were amplified using their specific primers as shown in table 1.

Polymerase chain reactions (PCR) were carried out to amplify *ND4* and *ND4L* using T-personal model Biometra thermo cycler according to the standard method. The components of PCR mix (25-µl)were as, 100 ng DNA, 0.2 unit *Taq* polymerase enzyme, 2 µl dNTP (10 mM), 1.5 µl MgCl2 and 1 pmol gene specific primers (50 mM). In order to confirm the amplification, samples were electrophoresed on 1% agarose gel. PCR program for*ND4L* fragment was adjusted as,94 ^{0}C for 30s (denaturation), 54^{0}C for 35s (annealing), 72^{0}C for

Corresponding authors E-mail:
* *E-mail: nassiryr@um.ac.ir*

30s (amplification) till 35 cycles, a primary step at 94°C for 10 min and a final amplifying stage at 72°C for 10 min, PCR program for *ND4* gene was adjusted as 94 °C for 30s (denaturation),56°C for 35s (annealing), 72 °C for 30s (proliferation) till 35 cycles, a primary step at 94°Cfor 10 min and a final amplifying stage at 72°C for 10 min. PCR products were electrophoresed on1% agarose gel which was strained by ethidium bromide (EtBr). ١٠٠ µl of PCR product was purified and sent for sequencing (Macro Gen company, South Korea) with 50 µl of each used primers. These samples were sequenced using the ABI3130 machine according to Sanger automated approach. The obtained sequences homology level was measured using accurate BLAST tool 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 5.1 software**.**

Table 1. Specific primers for *ND4L* and *ND4* region using Primer premier-5.

Primers	Sequence
Forward (*ND4L*)	5´- TTCACATTCAGCAGCCTAGGACT-3`
Reverse (*ND4L*)	5`- GCTTTAGGCAGTCATAGGTGTAGTC-3`
Forward (*ND4*)	5`- ACCTACCTGCCTCCTGAACAA-3`
Reverse (*ND4*)	5`-TCTGGTTTGAGGATGAGTGTTAGTA-3`

Results

The quality of extracted DNA was confirmed by spectrophotometery. Electrophoresis of amplified fragments on 1% agarose resulted in the bands of *ND4* and *ND4L* fragments as 913bp and 802 bp respectively, showed the correct size of *ND4* and *ND4L* fragments as shown in figure 1. Sequencing of *ND4* and *ND4L* regions was performed for 6 samples. After sequencing, their alignment was compared using Glusta multiplie alignment tool of Bio Edit 7.2.2 software. The 891and 707 fragments were used in all the samples as the consensus sequence for *ND4L*, *ND4* regions respectively. Comparative analysis of the obtained sequences by these tools revealed that there is no difference between the studied sequences (p=0).Probably it was resulted from few samples or homogenization of the mass because of permanent selection according to commercial goals for several generations.*ND4L* sequence nucleotide compound was calculated as A 30%, C 36%, G 10% and T 24%, indicating the 46% and 54% frequency for G+C and A+T respectively (figure 3).

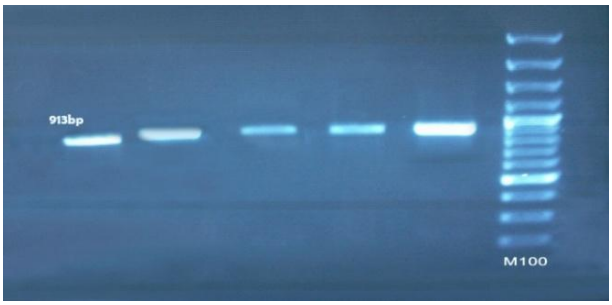

Figure 1A. Electrophoresis of 913 bp PCR Products on 1 % agarose gel

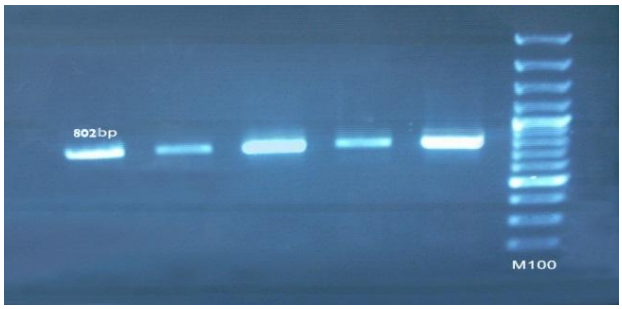

Figure 1B. Electrophoresis of 802 bp PCR Products on 1 % agarose gel

A+T and G+C frequencies were calculated for *ND4* as 23%, 12%, 36%, 29, and the 48, 52% frequency in relation to G+C and A+T, respectively (figure 4).

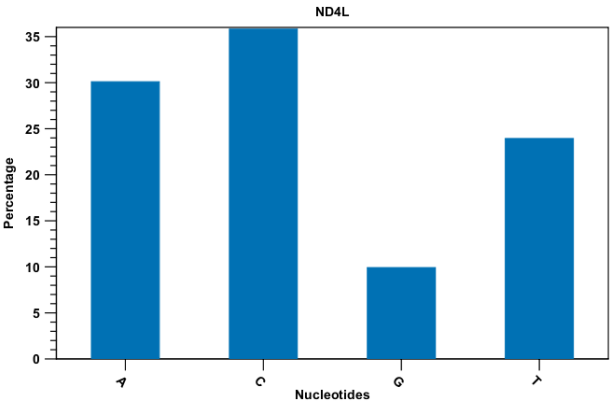

Figure 3. Frequency percentage for constituent nucleotides at *ND4* Lconsensus sequences in Khorasan native chickens.

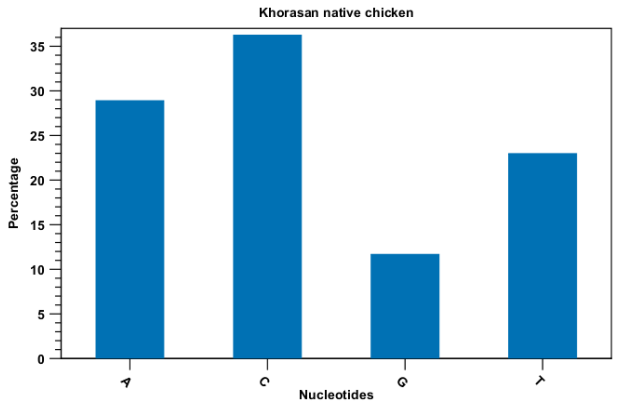

Figure 4. Frequency percentage for constituent nucleotides at *ND4* consensus in Khorasan native chickens.

The constructed phylogenetic tree revealed that *ND4L* region's sequence of Khorasan native chickens comparing with Asian chicken breeds was close to Jiangbian, Lvenue, Red and jungle fowl. Furthermore, results showed that all of them are belonging to the same group except of Nixi breeds (figure 5).

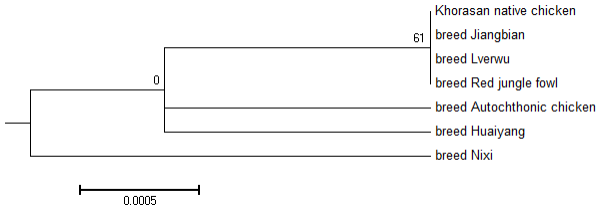

Figure 5. Phylogenetic tree of *ND4L* consensus sequence of Khorasan native chicken and other breeds

Furthermore, the results of *ND4* gene phylogenetic tree showed that mitochondrial genome of Khorasan native chickens are close to Autochtonic, jiangbian, Lvenwva and red jungle fowl, and belonged to the same group but differences were observed with Tulufan and Huaiyang gushi breeds(figure 6).

Studying the *ND4L* region's genetic matrices of mitochondrial genome of Khorasan native chickens indicated that there was lowest genetic distance between Khorasan native chickens and Jiangbian, Lvenwv, Red jungle fowl and Autochthonic chickens. Genetic distance between Khorasan native chickens with Nixi and Huaiyang chickens was also observed (Table 2).

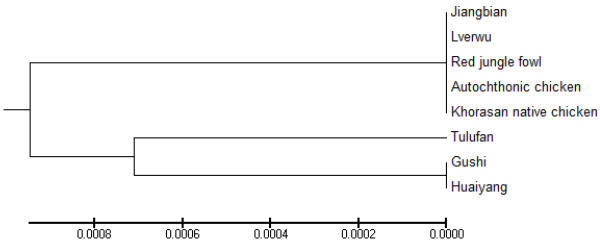

Figure 6. Phylogenetic tree of *ND4* consensus sequence of Khorasan native chicken and other breeds

Table 2. Nucleotide similarities and differences matrix of *ND4L* gene in Khorasan native chickens in relation to other breeds

		1	2	3	4	5	6	7
Khorasan native chicken	1		0	0	0	2	3	2
breed Jiangbian	2	100.00		0	0	2	3	2
breed Lv'erwu	3	100.00	100.00		0	2	3	2
breed Red jungle fowl	4	100.00	100.00	100.00		2	3	2
breed Autochthonic chicken	5	99.78	99.78	99.78	99.78		3	2
breed Nixi	6	99.66	99.66	99.66	99.66	99.66		3
breed Huaiyang	7	99.78	99.78	99.78	99.78	99.78	99.66	

Matrix of *ND4* region's genetic distances of mitochondrial genome indicated that there is a little genetic distance between Khorasan native chickens and Authochthonic, Jiangbian, Lvenwv and Red jungle fowl chickens. A huge distance was observed between them with Tulufan and Huaiyang Gushi (Table 3).

Table 3. Nucleotide similarities and differences matrix of NDL gene in Khorasan native chickens in relation to other breeds

		1	2	3	4	5	6	7	8
Khorasan native chicken	1		1	1	2	2	1	1	3
Autochthonic chicken	2	99.86		0	1	1	0	0	2
Red jungle fowl	3	99.86	100.00		1	1	0	0	2
Gushi	4	99.72	99.86	99.86		0	1	1	1
Huaiyang	5	99.72	99.86	99.86	100.00		1	1	1
Jiangbian	6	99.86	100.00	100.00	99.86	99.86		0	2
Lv`erwu	7	99.86	100.00	100.00	99.86	99.86	100.00		2
Tulufan	8	99.58	99.72	99.72	99.86	99.86	99.72	99.72	

Discussion:

As we find out the genetic conservation of Khorasan native chickens, this genetic reserver may be helpful to enhance production capacity of native chickens and to increase their ability against variable environmental conditions and relative resistance to local diseases. Import of foreign modified breed may lead in the reduction of native chickens production which are the national asset themselves. It should be noted that using the

mentioned sequences and their registration in the world gene bank would be regarded as an indicator to determine native chicken breeds in the Khorasan regions of Iran.

Results showed nucleotide relative frequency in consensus sequences in *ND4* and *ND4L* regions of Khorasan native chickens is very close to nucleotides percentage with the registered domestic chicken mtDNA in NCBI database. Comparison of these obtained sequences with the registered sequences indicated that there are high overlapping and homology among these sequences. These findings showed that the sequenced regions in this study were same as in other studies.

The phylogeny tree of different Asian chicken breeds and genetic analysis of *ND4* and *ND4L*, lead us to the conclusion that these genes are very important to study the breeds and the genetic distances between the Khorasan native chicken. Also results are comparable with the findings of Silvia et al., 2008; Oluwabukola et al., 2005; Pirany et al., 2005 and Siming, 2011. Zardoya et al., 1996 suggested *ND4* as an ideal gene to analyze the mitochondrial proteins. They also considered the *ND4L* gene as a weak gene in phylogeny analysis. Our results indicated the genetic diversity among these populations.

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