

CHARACTERIZATION AND SEQUENCING OF MT-COX1 GENE IN KHORASAN NATIVE POULTRY

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ABSTRACT

The aim of this study was to investigate the nucleotide sequence of COX1 gene in mitochondrial genome of Khorasan native chicken and detect the possible mutations in the genome. For this purpose, after sampling and extracting DNA from the whole blood samples, the COX1 gene was amplified using specific primers and cloned in pTG19-T plasmid of the DH5a strain of Escherichia coli bacteria. Finally, the recombinant plasmids were extracted from positive colonies and sequenced. The results of nucleotide sequence indicated maximum of similarity with the same sequence in the Gallus gallus mitochondrial genome. Comparing these two sequences identified the presence of six nucleotide differences. However, only three point mutations led to the altering of dissimilar amino acids. Also, a protein BLAST query showed that the sequence had a similarity of 99% in 100% coverage to cytochrome C oxidase subunit 1. The protein BLAST query suggested the conserved domain cytochrome C, in a family which is part of a complex IV. This complex converts the oxygen into the water by electrons transfer from reduced cytochrome in a reaction associated with mitochondrial electron transport chain. The findings may be useful in identifying phenotypes in poultry.

Keywords: Cloning, Sequencing, MT-COX1, Khorasan native poultry, *Gallus gallus*

INTRODUCTION

Native and local breeds are known due to their adaptation to the climate conditions and resistance against the diseases. It is necessary to protect native animal populations as genetic reserves and national capitals for breeding plans and increasing production. Animal breeding is the science and art of stabilizing genes effective in economic production of animal. Genes are abundant in cell nucleus chromosomes and scarce in mitochondrion. Mitochondrion is a membrane-bound organelle found in most eukaryotic cells (DiMauro, 2004). Each mitochondrion is estimated to contain 2 – 10 mtDNA copies and totally 100 to 10000 copy of DNA in each somatic cell dependent on the number of mitochondria (Wiesner *et al.*, 1992).

The presence of mitochondrial DNA was proved with electron microscope in 1963 (Nass and Nass, 1963). Mitochondrial DNA in poultry is a helical molecule with about 15 to 20 thousand pairs of nucleotides and a non-control region (D-Loop) and the coding region, with 37 genes containing 13 proteins, 22 tRNAs, and 2 rRNAs (Figure 1) (Anderson *et al.*, 1981; Desjardins and Morais, 1990; Paul Desjardins and Réjean Morais, 1991; Wolstenholme, 1992; Chinnery and Schon, 2003). The coding proteins are all subunits of oxidative phosphorylation complexes, which according to DiMauro (2004) are involved in cellular respiration and energy production processes. The COX1 gene is as among mitochondrial genes that encodes a protein called cytochrome C oxidase I.

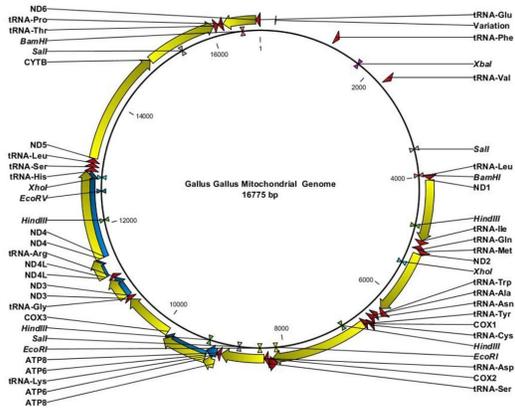


Figure 1: *Gallus gallus* mitochondrial genome (Anderson *et al.*, 1981)

The compound is one of the three subunits of cytochrome oxidase or IV complex, and as the third and the last enzyme in the respiratory chain, converts the oxygen into the water by electrons transfer from reduced cytochrome (Tsukihara *et al.*, 1996).

Substitution speed of nucleotides in some mitochondrial regions of vertebrates is 5 to 10 times as much as nuclear genome and these results from the absence of DNA restorative mechanisms, lack of histone and protecting proteins and destructive effect of proximity to free oxygen radicals produced in oxidative phosphorylation processes (Zachary and McGavin, 2012). Occurrence of mutations in mitochondrial DNA genome can cause changes in its structure and enzymes activity, which are often associated with severe metabolic abnormalities and mortality. Such disorders appear early in the life cycle, and primarily affect tissues such as brain, heart, muscles, retina and the muscles of the eye, which have a high energy consumption. Among mitochondrial genetic defects, the anomalies caused by these genes are known as the most destructive kind (Pecina *et al.*, 2004).

In many species, the mitochondrial genome is inherited only through the mother, and for the same reason, it is considered a suitable tool to find genetic relationships between individuals and groups in a particular species as well as phylogenetic identifying and classifying between different species (Filosto *et al.*, 2003). Also, the variations

in mitochondrial gene COX1 region among different species are as such that can be used as genetic barcode for the identification of biological identity (Hebert *et al.*, 2003), genetic variation detection and to determine the phylogenetic relationships among close populations and species (Xue *et al.*, 2009).

Mitochondrial amino acids genetic codes differ slightly from genomic or standard DNA codes. Thus, AGA and AGG are considered as Stop Codon instead of being arginine amino acid codes; or, AUA is used as methionine code instead of being isoleucine amino acid code. Similarly, UGA instead of being a stop codon is code for tryptophan amino acid. In domesticated chickens and quail, in addition to the start codon of ATG, the GUG code can also be used for this purpose (Desjardins and Morais, 1991). The only exception is the COX1 gene that its start codon is GTG (Yan *et al.*, 2014).

The purpose of this study was to determine the sequence of nucleotides in COX1 gene in Khorasan native chicken and compare it with data from databases gene bank and also to identify the potential mutations at DNA and protein levels in this region of the genome.

MATERIALS AND METHODS

Sampling and Extraction of DNA: Blood sample of native poultry of Khorasan was prepared from blood bank of animal biotechnology laboratory of Agriculture collage of Ferdowsi University of Mashhad, and stored in vacuum pipes containing EDTA anticoagulant material in freezer and at -20°C until extraction. DNA was extracted from whole blood and with Thermo Scientific kit. Due to the importance of the DNA purity, its quality and quantity was determinate with Nanodrop device and light absorption rate was measured in wavelengths of 230, 260 and 28 nanometers. Agarose gel electrophoresis was used as complementary evaluation method. After electrophoresis, not observing smear and lack of protein pollution in gel pit, the suitability of the extraction method was ensured .

Design of Specific Primer: The primer was designed for amplify of the desired part from

mitochondrial genome based on full mitochondrial genome of domestic chickens (reference poultry under access No. x52392.1). Due to length of 2050 nucleotides of the desired segment (location 6280 to 8329 of the MT-genome) and considering ability of sequencing in each reading, this segment was divided into two sections and with considering overlapping area with length of 55 nucleotides, primers were designed using online software (Table 1) (IDT, 2014).

Concentration of Materials in Polymerase Chain Reaction and Reaction Conditions:

Polymerase chain reaction was used for amplification of desired mitochondrial genes area based on standard method. Concentration and components of reaction in 25 microliter of final volume based as specified in Taq Polymerase Kit (Jena Bioscience Company) which included Taq DNA Polymerase 1 unit, dNtp of 0.25 mM, MgCL₂ of 1.5 mM, forward and reverse primer each 10 Pico mole, DNA of 100 Nano gram and distilled water. Polymerase chain reaction was performed in three stages and according to temperature and time schedule as follows. Initialization step was performed at 95°C for 5 minutes and then the second stage including 35 cycles of denaturation step was performed at 95°C for 60 seconds, Annealing stage was performed at 59°C for 60 seconds and Extension/elongation step was done at 72°C for 30 seconds. At the end, a final elongation stage was performed at 72°C for 10 minutes.

Electrophoresis of PCR Product, Its Purification from Gene and Insertion in Plasmid: Product of polymerase chain reaction was electrophoresed on agarose gel of 1%. To purify product of the reaction, the desired band was isolated from gene with sterile scalpel and extracted and purified with Gene All Kit (Sambrook *et al.*, 2003).

To prepare recombinant plasmid and connect the recovered segment to pTZ57R/T vector, they were mixed with each other according to conditions specified in Thermo Scientific Kit and also observe ratio of 1 to 3 between the vector and recovered fragment.

Preparing Susceptible Bacterial Cells, Transfer Recombinant Plasmid to Bacteria and Screening Recombinant Colonies:

The nightly culture of *E. coli*, DH5 α strain was used to prepare susceptible bacterial cells. Thermal shock method was used to transfer recombinant plasmid to bacteria. In transformation stage, the bacterium was incubated at 37 °C and in solid culture medium containing ampicillin for 16 hours. To identify colonies containing plasmid with the agreed genome fragments, resistant colony PCR method was used.

Extracting Plasmid, PCR of Extracted Plasmids and Determining Sequence of the Reproduced Fragments:

Plasmid was extracted from positive colony after nightly culture of liquid containing ampicillin with Gene All kit (Sambrook *et al.*, 2003). After extraction and to confirm the segment in plasmid, the extracted plasmids were diluted with ratio of 1:20 after determining their concentration with Nano drop device and used as pattern in polymerase chain reaction. Concentration of the used materials, temperature condition of the reaction and electrophoresis of the reaction product were based on the previous method.

The extracted plasmid was sent to South Korean Macrogen Company to determine sequence with forward and reverse M13 primers. Then, the obtained nucleotide sequence was edited and studied with MEGA software 5 and CLC Main Workbench version 5 and BioEdit software version 7 and compared to nucleotide sequences of target genes with Basic Local Alignment Search Tool (BLAST) procedure available in NCBI bank (NCBI, 2014).

RESULTS

DNA was extracted successfully from blood sample. Spectrophotometric results showed that the extracted DNA had suitable quality. Electrophoresis of the extracted sample on Agarose gel 1% showed the presence of a straight dedicated band which confirmed high purity and lack of DNA fracture and lack of RNA (Figure 2).

Table 1: Sequence of primers used for PCR amplification

Name of Primer	Access region in MT genome	Sequence of primer	Length of the product
COX1 Part A. F	6228-7367	GCGCTATCAACCACCCTATT	1165
COX1 Part A. R		GGAGGATGAGGATGTAAACTTCG	
COX1 Part B. F	7292-8355	CACCACATTCTTCGACCCA	1082
COX1 Part B. R		GGGATGAGGCGTCTTGAAA	

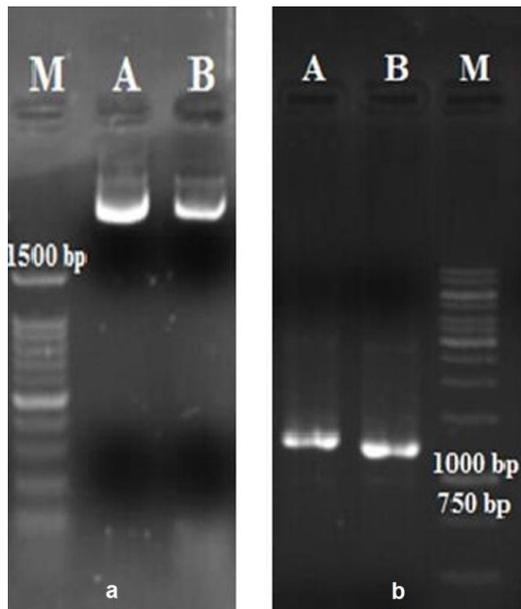


Figure 2: a. Electrophoresis of extracted plasmid, b. Electrophoresis of PCR product (A) 1165 base segment; (B) 1082 base segment; (M) size marker

This issue showed that specific primers could produce the desired products with lengths of 1165 and 1082 and there is no similar sequence for pairing them in other places of genome.

After ensuring specificity of reproduction, the agreed segment was separated and sequenced. The sequence was read from two ends and with M₁₃-F and M₁₃-R primers embedded in vector. Result of sequencing was edited with BioEdit software and finally the existing segment was separated and specified by deleting the additional sequences of vector in two ends of each retrieved sequence and merging overlapping areas.

The presence of sequences of the designed primer at two ends of the obtained sequences proved truth of the performed work. Then, the obtained sequence was compared with perfect sequence of mitochondrial genome relating to *Gallus gallus* (Taxid 9031) in NCBI bank with BLAST tools and nblast procedure.

About 100 sequences were found in this region among which 99 sequences including poultry mitochondrial sequences recorded with the access numbers of X52392.1 (reference for poultry mitochondrial sequences), GU261713.1, KF954727.1, HQ857210.1, KF826490.1, AP003319.1 and AY235571.1 had 99% of overlapping at 100% of their length with the studied sequence, and one sequence with the lowest similarity related to JN793568.1, had 98% of overlapping at 100% of its length (Figure 3).

Phylogenetic analysis of the sequences obtained with the reference sequence and other existing sequences in the database also confirmed the provided reports and high similarity of COX1 gene in Khorasan native poultry with the sequences recorded in the database.

The COX1 gene in the reference poultry genome includes the nucleotides from 6645 to 8192. Comparison of the generated segment sequence with the reference sequence (x52392.1 (CDS 6645...8192)) identified the presence of six nucleotide mutations. With the exception of mutation at position 7530, which is a transversion type, in the remaining positions, the mutations occurred are as transition type; i.e. the substitution of a purine with a purine

Description	Max score	Total score	Query cover	E value	Ident	Accession
Gallus gallus breed Huang Lang chicken mitochondrion, complete genome	2839	2839	100%	0.0	99%	KF954727.1
Gallus gallus mitochondrion, complete genome	2839	2839	100%	0.0	99%	KF826490.1
Gallus gallus isolate vin109 mitochondrion, complete genome	2839	2839	100%	0.0	99%	HQ857210.1
Gallus gallus isolate J41 breed Jiangbian mitochondrion, complete genome	2839	2839	100%	0.0	99%	GU261713.1
Gallus gallus isolate YP20320 breed Lv'erwu mitochondrion, complete genome	2839	2839	100%	0.0	99%	GU261712.1
Gallus gallus murghi isolate qqm1 breed Red jungle fowl mitochondrion, complete genome	2839	2839	100%	0.0	99%	GU261709.1
Gallus gallus isolate YP20548 breed Autochthonic chicken mitochondrion, complete genome	2839	2839	100%	0.0	99%	GU261694.1
Gallus gallus isolate YP20488 breed Autochthonic chicken mitochondrion, complete genome	2839	2839	100%	0.0	99%	GU261686.1
Gallus gallus mitochondrial DNA, complete genome, native chicken in Laos	2839	2839	100%	0.0	99%	AP003319.1
Gallus gallus mitochondrial DNA, complete genome, White Plymouth Rock	2839	2839	100%	0.0	99%	AP003318.1
Gallus gallus mitochondrial DNA, complete genome, White Leghorn	2839	2839	100%	0.0	99%	AP003317.1
Gallus gallus mitochondrial DNA, complete genome	2839	2839	100%	0.0	99%	AP003580.1
Gallus gallus mitochondrion, complete genome	2839	2839	100%	0.0	99%	AY235571.1
Gallus gallus mitochondrion, complete genome	2839	2839	100%	0.0	99%	AY235570.1
Gallus gallus isolate vin46 mitochondrion, complete genome	2828	2828	100%	0.0	99%	HQ857212.1
Gallus gallus isolate vin100 mitochondrion, complete genome	2828	2828	100%	0.0	99%	HQ857209.1
Gallus gallus murghi isolate qqm4 breed Red jungle fowl mitochondrion, complete genome	2828	2828	100%	0.0	99%	GU261708.1
Gallus gallus mitochondrial genome	2826	2826	100%	0.0	99%	X52392.1

Figure 3: Results of nucleotide alignment of the sequenced fragment with *Gallus gallus*

Table 2: Mutations in nucleotide and amino acid sequences of MT-COX1 gene in Khorasan native poultry

Nucleotide Position	Nucleotide sequence		Amino acid	
	Reference genome	Native hen	Reference genome	Native hen
6758	ATT	ATC	Isoleucine	
6800	GAT	GAC	Asparagine	
6899	CCA	CCG	Proline	
7377	ATC	GTC	Isoleucine	Valine
7530	CGA	GGA	Arginine	Glycine
8070	TTC	CTC	Phenylalanine	Leucine

(adenine to guanine or vice versa) or a pyrimidine with a pyrimidine (cytosine to thymine or vice versa).

To translate the nucleotide codes into amino acids, the nucleotide sequence was translated into six amino acid forms using EMBOSS (2014) Transeq tool and vertebrate mitochondrial genetic codes table, among which only form 1 with 515 amino acids lacked the stop codon. This protein form was compared with a similar protein sequence in the reference for poultry. The results showed that the incidence of these mutations in the first three places, due to similar genetic codes and lack of changes in amino acid translation is of silence type, while it has led to different translated amino acids in the next three positions (Table 2).

DISCUSSION

Compared to the protein sequence of the reference poultry, the first substitution is occurred at position of amino acid 245 and conversion of non-polar isoleucine to non-polar valine. Also, the polar arginine amino acid at position 296 has been changed to non-polar glycine, and the non-polar leucine amino acid at position 476 was substituted the non-polar phenylalanine amino acid. Given these cases and 99% similarities of these two sequences, one can say that these two proteins are likely similar regarding configuration and activity.

Protein comparison of the resulting sequence with protein sequences of the mitochondrial COX1 gene of the poultry in the database was conducted using the BLAST tool and blastp procedure on NCBI bank (Figure 4).

Description	Max score	Total score	Query cover	E value	Ident	Accession
cytochrome c oxidase subunit I [Gallus gallus] >sp P18943.1 COX1_CHICK RefName: Full=Cytochrome c oxidase subunit 1; AltName: F	1022	1022	100%	0.0	99%	NP_006917.1
cytochrome oxidase subunit I [Gallus gallus] >qb AAO44987.1 cytochrome c oxidase subunit I [Gallus gallus] >qb AAO44999.1 cytochro	1017	1017	100%	0.0	99%	BAC57577.1
cytochrome c oxidase subunit I [Gallus gallus]	1014	1014	100%	0.0	99%	ADW41554.1
cytochrome c oxidase subunit I [Gallus lafayettei] >ref YP_272074.1 cytochrome c oxidase subunit I [Gallus gallus bankiva] >ref YP_27206	1014	1014	100%	0.0	99%	YP_272048.1
cytochrome c oxidase subunit I (mitochondrion) [Gallus gallus]	1014	1014	100%	0.0	99%	ADB06689.1
cytochrome c oxidase subunit I (mitochondrion) [Gallus gallus]	1013	1013	100%	0.0	99%	AHI87875.1
cytochrome c oxidase subunit I (mitochondrion) [Gallus gallus]	1013	1013	100%	0.0	99%	ADB06871.1
cytochrome c oxidase subunit I [Gallus gallus]	1012	1012	100%	0.0	99%	ABF70970.1
cytochrome c oxidase subunit I (mitochondrion) [Gallus gallus]	1011	1011	100%	0.0	99%	ADB06650.1

Figure 4: Results of protein BLAST alignment of the sequenced fragment with *Gallus gallus*

The results indicated 99% of similarity at 100% of length of cytochrome C oxidase subunit 1 with domestic chickens and other poultry mitochondrial sequences recorded in this database, including access numbers of YP272100.1, ADB06689.1, AHI87875.1, YP272087.1, ADB06871.1, ABF70970.1, BAC57577.1 and ADB06650.1. Furthermore, this comparison revealed that Khorasan native poultry in protein sequences of COX1 gene has 96% similarity with all other poultry.

The nucleotide relative frequency of COX1 gene sequence with the length of 1548 nucleotides in this study compared with the frequency of similar region in the reference poultry mitochondrial genome, and thus showed that these two are very close together. Harumi *et al.* (2004) studying the non-coding region in six broilers chicken observed 11 mutations. In another study, the first 539 base pair segment of this region of mitochondrial DNA in six breeds of indigenous broiler chicken of China were sequenced and compared with the reference poultry (domestic chickens). The results of these comparisons revealed strong differences in this region of mitochondrial genome (Niu *et al.*, 2002). In another study, the 455 base pair segment of the same region of the mitochondrial genome related to 283 chickens of 14 bird populations of Zimbabwe were sequenced that 32 single nucleotide polymorphism (SNP) were identified (Muchadeyi *et al.*, 2008).

Guan *et al.* (2007) studied the entire mitochondrial genome of broilers and Leghorn laying hens and identified a total of 70 SNPs in the coding region and 21 SNPs in the non-coding region. Bao *et al.* (2008a) in a study on

three encoding genes of mitochondrial cytochrome C oxidase (genes of COX1, COX2, COX3) related to five broiler breeds reported 14 SNPs. In a study by Nishburi *et al.* (2003) using White Leghorn chicken and White Plymouth Rock as dominant breeds and with a different breeding synthesis history reported 99.96% of DNA sequence similarity. As can be seen, in most of the conducted studies, the highest genetic variation is reported in the sequence of non-coding region, while in the coding region, according to higher length of base pairs in this region compared to the non-coding region, a much less relative genetic diversity is observed. The above results and the obtained result from the BLAST of conserved domains in the sequenced fragment all imply that the cytochrome C region is a conserved domain, and nucleotide changes occur rarely in.

Mannen *et al.* (2003) in a study on beef cattle reported a significant association between mitochondrial SNPs and carcass characteristics. Lie *et al.* (1998) examining the mitochondrial genome of white Leghorn hens reported a SNP in the gene of NADH dehydrogenase complex subunit 4, which was apparently associated with the resistance to Marek's disease in poultry. Bao *et al.* (2008b) examining three breeds of chickens, reported four SNPs in mitochondrial tRNA genes. They considered such mutations associated with genetic adaptation of Tibet chickens with hypoxia conditions in the biological area.

Given the above, it seems that in breeding animals, including poultry, due to breeding improvement operations applied over the years and natural or artificial processes such as selection of superior animals or deletion of

weaker and inefficient animals, the gene frequency of deleterious mutations with an adverse effect on the fitness and production performance of that animal has dropped, and for the same reasons, unlike human societies, such mutations can be rarely observed in today's farm animals, while mutations leading to increased fitness and productivity are remaining and sustaining in the populations.

Conclusions: The study results revealed six nucleotide mutations in the total of 1548 nucleotides of COX1 gene sequence, which have led to three amino acid changes in a total of 515 amino acid of protein sequence of this gene. Such mutations may be associated with the evolution of Khorasan native poultry to adapt with environmental circumstances of its place of life. This is the first study on sequencing and identification of potential mutations in the mitochondrial COX1 gene of this poultry.

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