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Research Article

PHYLOGENETIC AND NUCLEOTIDE ANALYSIS OF THE CYTOCHROME C OXIDASE GENE (COXIII) SEQUENCE IN KHORASAN NATIVE CHICKENS

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ABSTRACT
Use of Iranian native chickens has increased due to their resistance to diseases and it is very important to discover new genetic resources for the conservation of genetic and economic resources. One of the most important sources available for the study of genetic variation is the study of mitochondrial genome and hence, the gene CoxIII, 783 bp in length, was considered in order to evaluate phylogenetic analyses. For this purpose, specific primers were designed for amplification of a target with a length of 1070 bp and PCR was performed. In order to identify the sequence of the genes, 10 samples were submitted for bi-directional sequencing. Sequence analysis of the gene CoxIII showed that it has 98% similarity with the red jungle fowl and 89% similarity with the species *Ithaginis cruentus, Tragopancaboti, Bambusicola thoracica, and Phasianus colchicus.* Khorasan native chickens show the lowest genetic distance from and the highest percentage of similarity with the native chickens Xuefeng black-boned, White Plymouth Rock and Jiangbian and they display the highest genetic distance from *Gallus gallus domesticus*. Genetic information obtained from this study can serve as a genetic resource for improvement of future strategies used for Khorasan native chickens.

Keywords: *Mitochondrial Genome, Polymerase Chain Reaction, Cytochrome C Oxidase*

INTRODUCTION
Classification of domestic poultry includes Galliformes, Phasianidae, and Gallus. Domestication will lead to fundamental changes in poultry behavior, physiology and products. However, there are still similarities between the ancestors and the domestic chickens. Currently, most researchers have recognized the red junglefowl as the ancestor of each region's native chickens. One of the most important attempts to understand the origin of the native chickens' family tree was the mitochondrial DNA sequence analysis. Researchers described the theory of monophyletic origin of native chickens which are mainly obtained from the Southeast Asian red junglefowl. Their results were confirmed through microsatellite analysis by Hill *et al.*, (2003) and single-nucleotide polymorphism (SNP) in a large population of chickens. In addition, SNP analysis of the lysozyme gene for the genus *Gallus* showed that there is a close relationship between different native chickens and the red junglefowl. On the other hand, Nishibori *et al.*, (2005) developed the theoretical phylogenetic study of total mitochondrial DNA and two clear genome fragments for the genus *Gallus*. They observed a link of the two genera gray jungle fowl with red jungle fowl. According to the theory of polyphyletic origin, Eriksson and colleagues (2008) tracked the SNP's of the gene beta-carotene dioxygenase 2 that causes the Yellow Skin in many chickens. They believed that even though the red jungle fowl is the origin and ancestor of this gene, this gene is derived from a hybrid breed of the gray jungle fowl with the red jungle fowl. Nevertheless, there are several theories on the geography of domestic fowl. Many believe that native chickens originated from the Indus Valley in 2000 BC but archaeological evidence suggests that domesticated Chickens existed in mainland China around 6000 BC. The main strategy for poultry production involves creation of a line of several breeds in large homogeneous populations. In contrast, native chickens are often maintained in small populations with high genetic and morphological diversity. Village fowls are also hybridized for mating with industrial chickens and this has led to the dilution of maternal genetic contribution. Such an approach not only does not cause genetic progress but also threatens rural genetic resources with genetic erosion. Although the

loss of genetic resources does not matter in industrial breeding strategies, these precious resources may have certain unique characteristics that could meet future needs of poultry production. In addition, maternal genetic resources in rural areas could be retained as the main source of changes in population. Hence, the aim of the current study was to investigate the genetic and phylogenetic analysis of the gene CoxIII related to the aerobic metabolism oxidase enzyme in order to the genomic identification of Khorasan native chickens. Information about the sequence of this region of the mitochondrial genome of Khorasan native chickens along with comparison to other breeds and display of differences can track genetic changes in the future.

MATERIALS AND METHODS

Sample Collection

10 blood samples from RazaviKhorasan native poultry were collected in collaboration with RazaviKhorasan Province Jihad-e-Agriculture Breeding Centerand lack of consanguinity between samples was ensured. Blood samples were stored until extraction in tubes containing EDTA in a freezer at -20 ° C.

Target Amplification and Sequencing

DNA was extracted using # K0721 (Thermo, US) kit according to the kit instructions. The quantity of DNA was measured using NanoDrop® spectrometry (ND2000, Thermo, US) and its quality was evaluated on a 1% agarose gel. Primers for amplifying a fragment containing sequence of regions upstream and downstream of CoxIII in mtDNA were designed using the software Primer premier 5 and total mitochondrial genome of domestic chicken (accession number X52392). Target sequence was amplified using the forward primer 5'CTACCAATAATGCCATCAATCTC '3 and the reverse primer 5'TGGGCTAGTCAAAAGTTTATAGT'3. The thermal PCR program included initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 59°C for 25 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 10 min in 35 cycles. The PCR product was electrophoresed on 1% agarose gel and evaluated by ethidium bromide staining and UV radiation. 30 µl of PCR product was purified and sent with 10 µl of both forward and reverse primers used (10 pmol) to MacroGen company (South Korea) for bi-directional sequencing. These sample were sequenced using the ABI 3130 machine according to Sanger automated approach.

Bioinformatics Analyses

The program Bioedit (Hall *et al.*, 1999) was used for assessing and editing the sequence data. The obtained sequences homology level was measured using the accurate BLAST tool and blast method in NCBI database. In order to study the phylogenetic relation between the target breeds, the phylogeny tree was drawn using the alignment sequences UPGMA approach by MEGA 5 software.

RESULTS AND DISCUSSION

Results

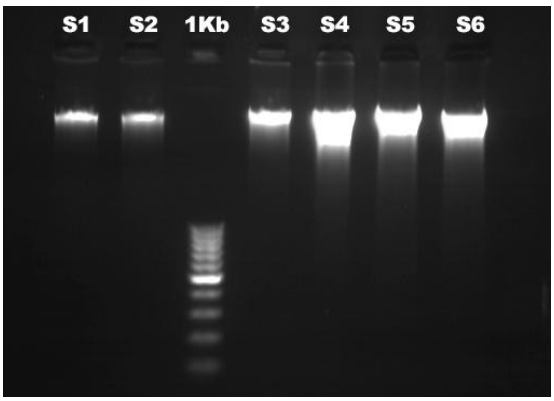

Figure 1: Electrophoresis of DNA extraction on %1 agaros gel. S1-S6 Khorasan native chicken blood DNA, Ladder is 1kb

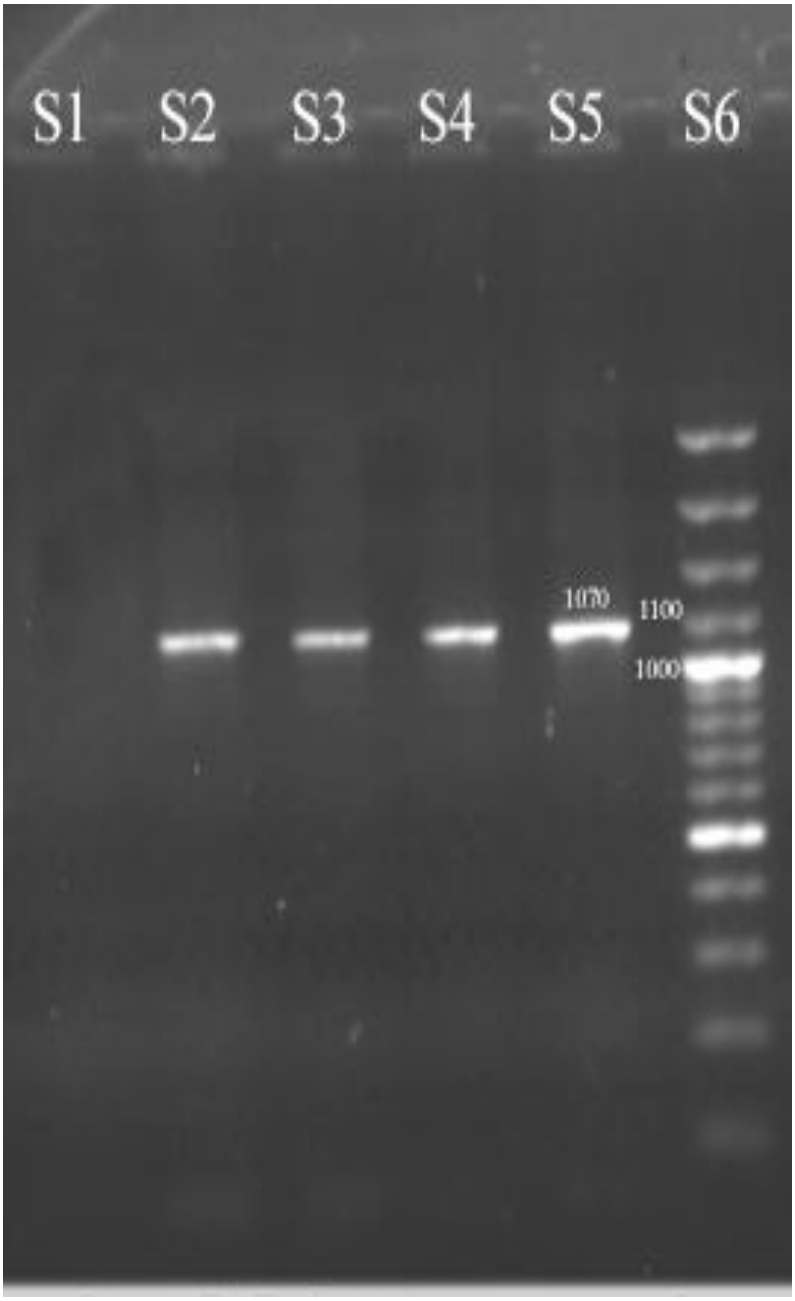

Figure 2: Electrophoresis of Cox III PCR product on %2 agarosgele.S1: negative control, S2-S5: Cox III PCR Product, S6: M100 plus ladder

DNA isolation from all samples was successful. Spectrophotometry and electrophoresis results suggested that the extracted DNA was of suitable quality (Figure 1). Gene bioinformatics analysis, primers annealing to the target sequence was performed using the software CLCWorkBench5.5. Amplified products electrophoresis on 1% agarose showed that the designed primer had acted well and amplified CoxIII specific fragments of 1070 bp length (Figure 2).

Mitochondrial genomic regions of CoxIII were bi-directionally sequenced for 10 samples (Figure 3). Nucleotide composition of CoxIII region sequence was calculated which included 28.8% adenine, 22.68% cytosine, 15.54% guanine, and 33.5% timing and it showed frequencies of 49.04% G+C content and 50.96% A+T (Figure 4).

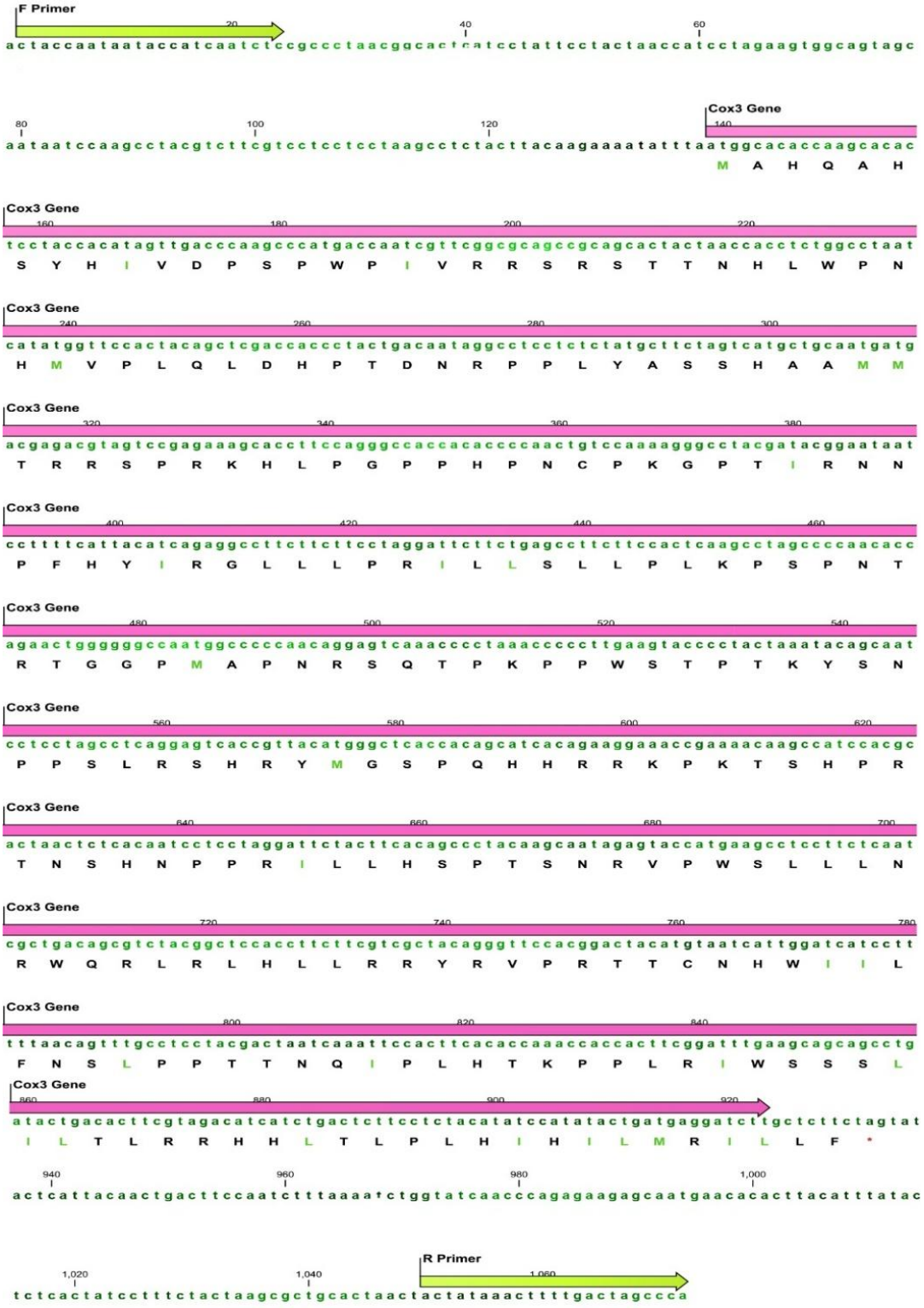

Figure 3: Characterization of nucleotide and protein Cox III gene. Forward and Reveres Primers were showed by green arrow

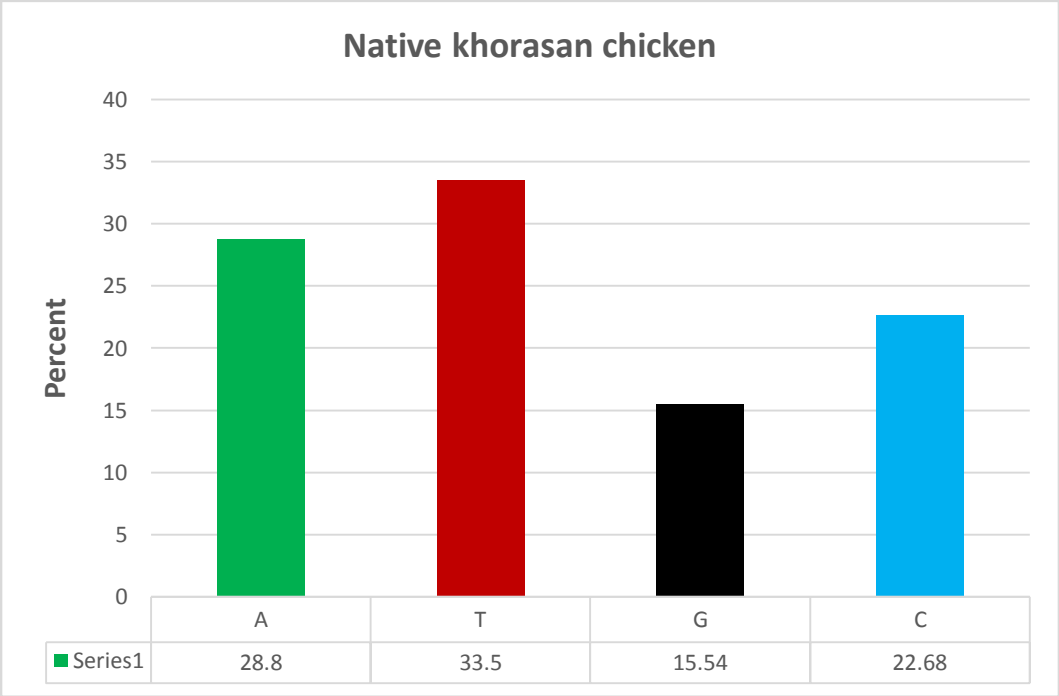

Figure 4: Frequency of nucleotides sequence of CoxIII consensus region for Khorasan native chicken

Comparison between 10 sequences was performed using the Neighbor-joining method in the software MEGA5. A comparison between the obtained sequences by the mentioned method showed only a nucleotide mutation at position 783 (T to C). This mutation was also observed in Laotian native poultry. The reason for this insignificant divergence could be the small sample size or population homogeneity due to consecutive selection in several generations for commercial goals. Relative frequency of nucleotides in consensus sequences in the gene region CoxIII is very close to nucleotides percentage in the regions of mtDNA of domestic poultry recorded in the NCBI database and also the study of the distance matrices specified that the degree of homology overlap between the studied sequences and the existing ones in very high.

		1	2	3	4	5	6	7	8	9	10	11	12	13
NativekhorasanChicken2	1		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.17	0.07	0.10	0.13	0.12
Xuefengblack-bonedchicken	2	99.36		0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.07	0.09	0.12	0.11
Chigulumitochondrion	3	99.36	100.00		0.00	0.00	0.00	0.00	0.00	0.16	0.07	0.09	0.12	0.11
GallusgallusisolateJ41breedJiangbianmitochondrion	4	99.36	100.00	100.00		0.00	0.00	0.00	0.00	0.16	0.07	0.09	0.12	0.11
Xuefengblack-bonedchicken-2	5	99.36	100.00	100.00	100.00		0.00	0.00	0.00	0.16	0.07	0.09	0.12	0.11
WhitePlymouthRockJapan	6	99.36	100.00	100.00	100.00	100.00		0.00	0.00	0.16	0.07	0.09	0.12	0.11
nativechickeninLaos	7	99.11	99.75	99.75	99.75	99.75	99.75		0.01	0.16	0.07	0.09	0.12	0.12
Redjunglefowlmitochondrion	8	99.11	99.74	99.74	99.74	99.74	99.74	99.49		0.17	0.07	0.09	0.12	0.11
G.domesticus	9	84.73	85.37	85.37	85.37	85.37	85.37	85.50	85.11		0.25	0.25	0.30	0.29
Phasianuscolchicus	10	93.24	93.62	93.62	93.62	93.62	93.62	93.38	93.37	79.01		0.10	0.11	0.13
Bambusicolathoracica	11	90.94	91.58	91.58	91.58	91.58	91.58	91.35	91.58	78.50	90.43		0.11	0.11
Tragopancaboti	12	88.39	88.78	88.78	88.78	88.78	88.78	88.55	88.78	75.45	89.54	89.54		0.14
Ithaginiscruentus	13	88.90	89.54	89.54	89.54	89.54	89.54	89.31	89.54	76.08	88.27	89.92	87.63	

Figure 5: Genetic distance and percent identity matrix of Cox III genes than source study

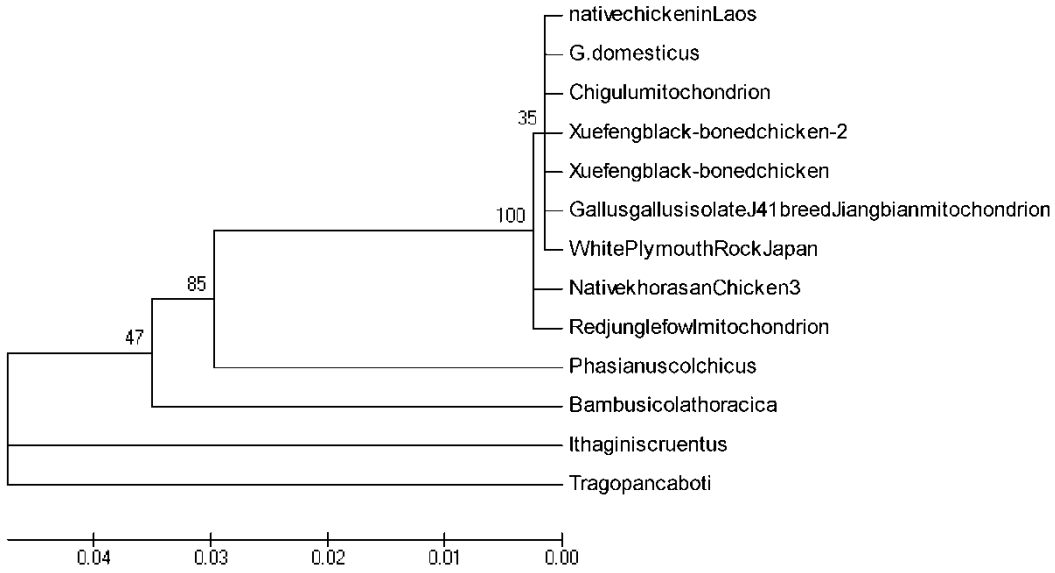

Figure 6: Phylogenetic tree of coding sequences CoxIII native chickens and resources study

Sequence analysis of CoxIII gene showed that it has 99% similarity with the red junglefowl and 89% similarity with the species *Ithaginiscruentus, Tragopancaboti, Bambusicolathoracica, and Phasianuscolchicus.* Khorasan native chickens show the lowest genetic distance from and the highest percentage of similarity with the native chickens Xuefeng black-boned, White Plymouth Rock and Jiangbian and they display the highest genetic distance from *Gallus gallusdomesticus* (Figure 5). Results from the phylogenetic tree analysis confirm the results from the distance matrices. The important point the phylogenetic tree is that khorasan native chickens are closely related to all native chickens red junglefowl (Figure 6). Although the gene CoxIII in the species *Ithaginiscruentus, Tragopancaboti, Bambusicolathoracica, and* [1]*Phasianuscolchicus* is very similar to domestic poultry, the depicted Phylogenetic tree has classified them as separate groups. Oka *et al.,* (2007) reported that several species of Japanese native chickens show high similarity with the native chickens of Korea, China and Indonesia. In another study, Dana *et al.,* (2010) identified the ultimate origin of the Dutch Bantam (chicken) in the Indian native chicken. Silva *et al.,* (2009) reported a Genetic relationship between domesticated fowl of Cambodia, China, Laos and Thailand. Dorji *et al.,* (2012) argued that Bhutanese native chickens should be categorized as the maternal genetic resource of South East Asia. The results of this project show that CoxIII gene phylogenetic study can particularly help one understand genetic relationships and distances. Although the study of one gene will not provide us with detailed information on its ancestors, results showed that investigating the total mitochondrial DNA for Khorasan native chickens can introduce a common ancestor more precisely.

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