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Full Length Article

Phylogenetic Trees Reconstruction for Jordan Indigenous Chickens and Commercial Broiler from DNA Sequencing

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Abstract

Different phylogenetic and evolutionary analyses were performed for Jordan indigenous chickens and its genetic relatedness to available commercial broiler chickens based on DNA sequencing. The sequence of 0.5 kb from sixteen individuals of the indigenous chicken, Ross, Lohaman and Hubbard Broiler was performed. Maximum Composite Likelihood (MCL) Method was considered as the best substitution pattern test of homogeneity between sequences to compute evolutionary distances between studied populations. The shortest evolutionary distance was found between Hubbard and Ross chickens revealing their evolutionary closeness. On the other hand, Lohmman showed closer evolutionary distance to Hubbard than to Ross chicken. The longest evolutionary distance found between indigenous chicken and Ross, whereas the shortest evolutionary distance was with Lohmman broiler chicken. As a consequence, different phylogenetic trees were reconstructed providing evidences for a close phylogenetic alliance among commercial broiler and indigenous chickens. The genetic relatedness between the three commercial strains clustered them into one group away from the indigenous that was in separated cluster. However, there were different patterns of clustering for the three commercial populations forming different phylogenetic types. These results might be attributable to different models used for estimating evolutionary distances. Overall, the resulted evolutionary sequencing and phylogeny trees of studied broiler populations may help getting decisions on choosing best match for crossbreeding with the indigenous, taking into consideration climate change alarming in tropical areas.

Keywords: Phylogeny; DNA sequences; Indigenous chicken; Commercial chicken

Introduction

The first genome sequence of an ancient chicken, the red jungle fowl (*Gallus gallus*) was published in 2004 (Hillier *et al*., 2004). Red jungle fowl was the nearest ancestor to the current domestic chicken, as suggested by Darwin (1896) http://www.nature.com/nature/journal/v432/n7018/full/nature03154.html - B14. This fact was later confirmed by mitochondrial DNA analysis (Fumihito *et al.*, 1994). Chickens were domesticated in Asia as early as 8000 years BC (Crawford, 1995; Fitzpatrick and Ahmed, 2000), whereas their genetic improvement was dated to the start of the twentieth century (Punnett, 1923) when hundreds of well-characterized commercial strains and inbred lines have been developed (Pisenti *et al.*, 1999). Furthermore, Asian chickens were genetically contributed to those strains and lines in the world (Moiseyeva *et al.*, 2003). In general, chicken domesticates in and around the ancient Arabian Peninsula, a tropical and Asian region which occupies a key geographic junction, with Africa, Europe and Asia. In particular, there was suggestion that the chicken found in Mediterranean region were sometimes ago the chickens brought into Europe and they were the most closely related to the RJF (Moiseyeva *et al.*, 1996). Therefore, it is assumed that Mediterranean chickens might be in close evolutionary relation to current commercial populations of the world as reported by Crawford (1995).

Chickens are the first non-mammalian have had their genome sequenced, providing a new perspective on its genome evolution studies. The chickens' genome has about the same number of genes (20,000-23,000) as the human genome (20,000-25,000 genes). However, those genes are contained in only 1 billion DNA base pairs, a nearly third of human DNA's 2.8 billion base pairs (Hillier *et al*., 2004). Therefore, as an example, many conserved non-coding sequences are far from genes and cannot be assigned to defined functional classes but for evolutionary studies. The evolutionary studies of many chicken breeds were reported covering evolutionary distance and phylogeny utilizing DNA sequencing approach (Nidup *et al.*, 2002; Mwacharo *et al.*, 2011; Babar *et al.*, 2012). The most large sequences technology has been determined using of bacteriophage T7 promoter (Dunn and Studier, 1983). The technology, that implements the chain termination method, is universally applicable to any piece of DNA and it has become the method of choice in most sequencing projects. The

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determination of genetic evolution based on DNA provides accurate information for genetic distance analysis that allows a ranking of populations according to level of phylogenetic distinction (May, 1990). For chickens, establishment of genetic distance among populations and commercial strains will be important for identifying unique genetic recourses not represented in industrial strains as well as for future crossbreeding and introgression plans (Notter, 1999). The DNA sequencing technology is widely used in studying the evolutionary distances and phylogeny reconstruction of chicken populations (Muir *et al.*, 2008; Sawai *et al.*, 2010; Mwacharo *et al.*, 2011). There is no scientific study, so far, describing the evolutionary distances and phylogenetic of indigenous chicken with other available commercial strains in Jordan using DNA sequencing. The indigenous chickens breed is composed of different ecotypes of a total population to be one million (Abdelqader *et al*., 2008). While, exotic commercial breeds population was 24 million of both layers and broilers (FAOSTAT, 2007). The Broiler commercial breeds (White Leghorn) are mainly broilers Lohmann®, Hubbard®, Ross® and Cobb®). The available evolutionary information about Hubbard and Ross populations is stating that their common origin of United States, whereas Lohmann originated from Germany. The latter is the closer to indigenous chickens of Mediterranean countries (Moiseyeva *et al.*, 1996; Abdelqader *et al.*, 2007). In addition, Al-Atiyat (2009) reported that Jordan indigenous chicken was genetically closer to Lohmann commercial broiler among other studied commercial strains based on Morphological characteristics.

The main aim of this study was to reconstruct evolution phylogenies for indigenous chicken population with the available commercial chickens using DNA sequencing data.

Materials and Methods

Chicken Populations and Samples Collection

Indigenous chickens and three available broiler chickens populations, Ross, Hubbard and Lohmman, were sampled from Jordan for a total of four individuals per strain. The three commercial broiler strains were chosen in this study because they are the most common strains successfully reared in poultry industry in Jordan. The sixteen chicken individuals were randomly sampled at the rearing rural and commercial farms. Sampling was performed by taking a tissue punch of approximately 0.1 cm of the wattle area using an animal punch applicator; samples were transferred into Eppendorf tube, stored immediately in ice-box then were stored in deep freezer at – 20^{o}C until the DNA extraction was performed.

DNA Extraction and Quantification

DNA extraction was performed using a commercially available kit/protocol of E.Z.N.A® Micro Elute Genomic DNA extraction Kit (OMEGA Bio-Teck; www.omegabiotek.com). Concentrations of DNA were estimated by Nano-DNA spectrophotometer (AlphaSpec®, www.alphainnotech.com) in which the quality of DNA was evaluated using the ratio of A260/A280. The DNA concentration of each sample was made of 10 ng/µL.

DNA Sequencing

The sequences technology using bacteriophage T7 promoter was applied following Dunn and Studier (1983). The technology implemented the universally chain termination method applied on a piece of 600 bp of DNA. The universal primers, T7promoter (5′-TAATACGACTCACTATAGGG-3′), was incorporated into a PCR reaction following the protocol suggested by Dunn and Studier (1983). The resulted PCR products were fragmented using high throughput Applied Biosystems 3730XL sequencer. The DNA sequencing service was provided by Macrogen® Commercial Company (www.macrogen.com).

Analysis of DNA Sequences

Resulting DNA sequences were edited and assembled into contiguous sequences (470 to 560 bp) by use of the MEGA software program, version 5 (Tamura *et al.*, 2011). The resulting DNA sequence information was analyzed for following basic evolutionary analyses (Kumar and Gadagkar, 2001), evolutionary distances and phylogenetic reconstructions (Nei and Kumar, 2000; Tamura *et al.*, 2011).

Results and Discussion

Basic Evolutionary Statistics

Evolutionary statistics related to find best model for evolutionary distances and phylogeny that can be computed by MEGA software were performed and presented. First, best DNA model for estimating evolutionary distances was assumed and considered as nucleotide substitutions Model (Table 1). The model computed evolutionary distance between a pair of sequences as the number of nucleotide substitutions occurring between them in a comparison way of nucleotide-by-nucleotide base pair (Nei and Kumar, 2000). The models with the lowest Bayesian Information Criterion (BIC) scores were considered for best substitution pattern (Table 1). The results showed that Jukes-Cantor (JC) distance method was the best model followed by Kimmura-2 parameter method. For each model, Akaike Information Criterion Corrected value (AICc) and estimated values of transition/transversion bias (R) are shown for each model, as well. The analysis involved four nucleotide sequences and codon positions included were 1st, 2nd, 3rd and Noncoding. In Jukes-Cantor distance model, the rate of nucleotide substitution was the same for all pairs of the four nucleotides A, T, C and G (Jukes and Cantor, 1969).

Therefore, the substitution pattern tests of homogeneity between sequences were performed in order to choose best test to compute evolutionary distances between studied chicken populations. The performed tests were Pattern Disparity Index, Maximum Likelihood Estimate (MLE) of Substitution Matrix and Maximum Composite Likelihood (MCL) Method (Kumar and Gadagkar, 2001). Table 2 shows disparity index values with their probability (*P*-values) of rejecting null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases for each sequence pair of studied chicken populations. The estimates of the disparity index per site were significant for all pairwise comparisons except that of Lohmman with Hubbard, stating that both were more homogenous together. The best explanation that significant pairwise comparisons might be that high heterogeneous substitution patterns between each pair. The second test, MLE of Substitution Matrix, was also estimated in which the probability of substitution from one base to another was estimated (Table 3). Rates of different transitional substitutions and those of transversionsal substitutions were also estimated (Table 3). The relative values of instantaneous rates were considered as sum of the values made equal to 100 and the nucleotide frequencies were A=20.06%, T/U=32.66%, C = 21.32% and G = 24.96%. The last test, MCL method, which is defined as a sum of related log-likelihoods (Tamura *et al.*, 2004) was also computed. Since all pairwise distances in a distance matrix have correlations due to the phylogenetic relationships among the sequences, the sum of their log-likelihoods was a composite likelihood (Table 4). Similar to previous method, the probability of substitution from one base to another and the sum of the values was also made equal to 100%. The nucleotide frequencies were 22.43% (A), 29.00% (T), 20.36% (C) and 28.13% (G). Therefore, it seems that both methods reported similar results of transitional substitution rate for T and C nucleotide and different rates for A and G nucleotides. On the other hand, the transversional substitutions rates were higher in MLE for all nucleotides except nucleotide A. Generally, the sums of nucleotides substitution pattern and those of substitution rates among sites in both methods were makeable different. In summing up, the Maximum Composite Likelihood (MCL) model was found to be more reliable for performing evolutionary analyses in this study. This result is in agreement with Tamura *et al.* (2004) who reported that pairwise distances and the related substitution parameters are accurately estimated by maximizing the composite likelihood than other methods. Moreover, Tamura *et al.* (2004) found that, unlike the cases of ordinary independent estimation of each pairwise distance, a complicated model had virtually no disadvantage in the Composite Likelihood Method for phylogenetic analyses. Therefore, estimation evolutionary distances and phylogeny reconstruction in following section were conducted using the MCL model as implemented in Tamura *et al.* (2011).

Table 1: Maximum Likelihood fits of 24 different nucleotide substitution models

Model*	BIC	AICc	R
JC	1740.689	1718.638	0.50
K2	1746.715	1720.273	0.64
HKY	1749.492	1709.920	0.77
T92	1751.588	1720.763	0.64
TN93	1755.908	1711.972	0.78
GTR	1772.247	1715.261	0.72

*GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor. BIC: Bayesian Information Criterion, AICc: Akaike Information Criterion, and *R:* estimated values of transition/transversion bias

Table 2: Test of the homogeneity of substitution patterns between sequences

Population 1	Population 2	Disparity index	*P*-values*
Ross Broiler Chicken	Lohmann Broiler Chicken	1,922	0,004
Ross Broiler Chicken	Hubbard Broiler Chicken	1,553	0,034
Lohmann Broiler Chicken	Hubbard Broiler Chicken	0,000	1,000
Ross Broiler Chicken	Indigenous Chicken	7,913	0,000
Lohmann Broiler Chicken	Indigenous Chicken	1,359	0,030
Hubbard Broiler Chicken	Indigenous Chicken	1,592	0,016

**P*-values < 0.05 are considered significant

Table 3: Maximum Likelihood Estimate of Substitution Matrix

Nucleotide	A	T	C	G
A	-	*9.00*	*6.15*	10.67
T	*5.74*	-	10.02	*7.15*
C	*5.74*	14.66	-	*7.15*
G	8.57	*9.00*	*6.15*	-
Total	20.06	32,66	22,32	24,96

Table 4: Maximum Composite Likelihood Estimate of Nucleotide Substitution

Nucleotide	A	T	C	G
A	-	*7.15*	*5.02*	16.67
T	*4.59*	-	10.32	*5.73*
C	*4.59*	14.7	-	*5.73*
G	13.33	*7.15*	*5.02*	-
Total	22.43	29.00	20.36	28.13

- Each entry shows the probability of substitution from one base (row) to another base (column)
- Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*

Table 5: Estimates of evolutionary divergence between sequences

Population	Ross Broiler Chicken	Lohmann Broiler Chicken	Hubbard Broiler Chicken
Lohmann Broiler Chicken	1.828		
Hubbard Broiler Chicken	1.390	1.707	
Indigenous Chicken	2.417	2.078	2.234

Estimating Evolutionary Distances

Most of the widely used methods for distance estimation

between a pair of sequences usually were measured by the number of nucleotide substitutions occurring between them. The number of base substitutions per site from between sequences is shown in Table 5. Analyses were conducted using the MCL model (Tamura *et al.*, 2004). The number of base substitutions per site between sequences revealed reliable differences between the studied strains (Table 5). The results showed the lowest number of base substitutions per site and the shortest genetic distance, (1.390) was found between Hubbard and Ross chickens. This revealed that both were much more closely related to each other than with the rest. On the other hand, Lohmann broiler showed closer evolutionary distance to Hubbard broiler (1.707) than to Ross broiler. A quite reliable differences found between indigenous chicken with Ross (2.417); being the longest, with Hubbard (1.707) and with Lohmann, being the shortest and the evolutionary closer chicken. It might be assumed that substitution rate of bases per site between indigenous chicken and Lohmann was low for the studied sequences DNA. Furthermore, it might be explained, as there was possibility of closer ancestry as well as low selection pressure in studied sequence of Lohammn in comparison with other two commercials populations. This comes in agreement with the geographical information about these two populations and their common origin of United States, whereas Lohmann originated from Germany, the closer to Mediterranean countries and Jordan (Moiseyeva *et al.*, 1996; Abdelqader *et al.*, 2007). In addition, Al-Atiyat (2009) reported that Jordan indigenous chicken was genetically closer to Lohmann commercial broiler among other studied commercial strains based on Morphological characteristics. Overall, the results of evolutionary distances were fundamental for the study of molecular evolution and were useful for phylogenetic reconstructions and the estimation of divergence times as earlier reported by Nei and Kumar (2000).

Phylogenetic Reconstruction

Phylogenetic relationships of the chicken populations under investigation were presented in a treelike form with a root utilizing three methods for constructing phylogenetic trees from molecular sequencing data (Nei and Kumar, 2000) (Figs. 1-3). They were Maximum Likelihood (ML), Neigbor-Joining (NJ) and UPGMA methods. These methods are explained in Li (1997), Page and Holmes (1998) and Nei and Kumar (2000). The phylogenetic trees revealed a considerable degree of differentiation of populations supporting the results of evolutionary distances presented in Table 5. Fig. 1 shows the ML tree construction of two clusters. The indigenous chicken was grouped in one separated cluster from the commercial broilers that were in the second cluster. The commercial broilers were clustered together with one root in which Hubbard had shorter topology from the same root with Lohmann. This ML phylogenetic tree is corresponding with the results of evolutionary distances (Table 5). The results of ML tree also indicated most probable nucleotide ancestor for pair of populations as indicated by nucleotide A which was common in first cluster of commercial broilers (Fig. 1). While G nucleotide shared by indigenous chicken with the commercial chickens. In general, these results help in reconstruction of the evolutionary history of genes and species of current populations based of these molecular evolution evidences. The reliable phylogenies produced might shed light on the sequence of evolutionary events that generated into present genes of studied populations. Furthermore, the results help us to understand the mechanisms of evolution regard the chicken population history in studied area.

Fig. 2 shows the Neighbor-Joining tree construction of two roots and three clusters. The indigenous chicken considered in one cluster separated from the commercial broilers that were in two clusters. The first cluster included Ross and Lohmann together stating that more evolutionary relation to each other than to Hubbard that clustered in the second group. In general, the populations were assorted into same cluster have similar features. Such situation was likely due to sharing common sequences with less substitution rates from previous ancestors. On the other hand, Fig. 3 shows the UPGMA tree among the studied chicken strains with three clusters and two roots. The tree was clustering the populations as same as those clusters of Neighbor-Joining tree regardless topology length. The indigenous chicken considered in one cluster separated from the commercial broilers that were in two clusters. The first commercial broilers cluster included Hubbard and Lohmann together stating that more evolutionary relation to each other than to Ross that clustered in the second group. These results support results of ML tree (Fig. 1) and evolutionary distances (Table 5). In general, they were however in contrast with Neighbor-Joining tree in which Ross and Lohmann grouped in one cluster. This contrary result was most likely attributable to different models used for estimation evolutionary distances. The Jukes-Cantor (JC) distance was used as a best model for estimation distances for ML tree, whereas MCL estimate of Nucleotide Substitution was model of choice for The Neighbor-Joining and UPGMA tree.

Finally, it would be concluded that, based on results of the three phylogenetic methods, high evolutionary differentiation between indigenous chicken and the studied commercial broiler chickens. Similar results were reported earlier in which genetic relatedness within indigenous chickens was high when compared to different world exotic chickens (Rosenberg *et al.*, 2001; Msoffe *et al.*, 2005; Al-Atiyat, 2010). Furthermore, an example of studies reported a phylogenetic tree among various chicken populations, Romanov and Weigend (2001) reconstructed three major phylogenetic tree groupings, the most interesting group was the second group that comprised commercial layer lines and chicken breeds that were subject to intense selection in the

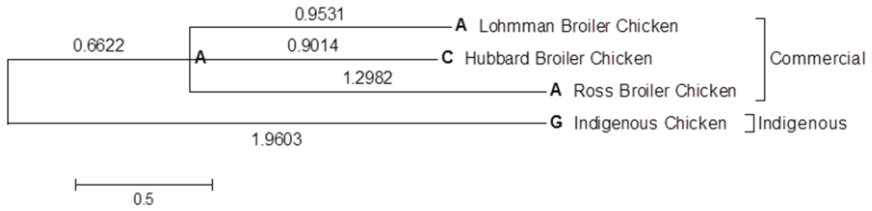

Fig. 1: ML tree with most probable ancestor of nucleotides (A, C, G) among the studied chicken populations

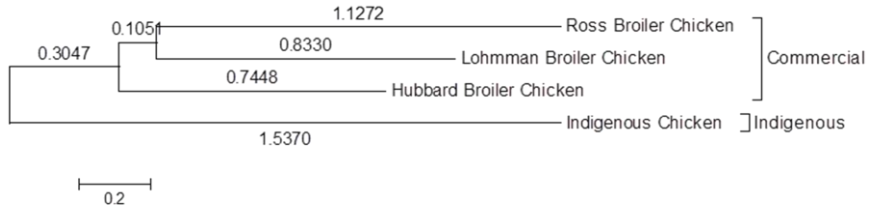

Fig. 2: The Neighbor-Joining tree among the studied chicken populations

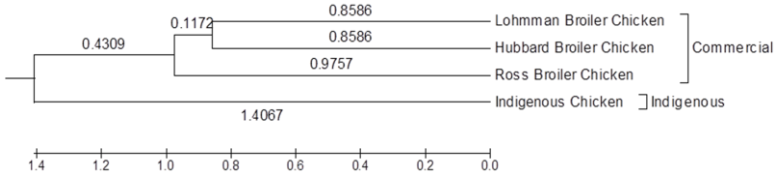

Fig. 3: UPGMA tree among the studied chicken populations

past or had common ancestral breeds with commercial strains. Their results are in agreement with the results found in this study in which indigenous chicken was subjected to intense selection in sometimes ago as well as gene flow from indigenous chicken into commercial ones in past times. On the other hand, regarding Egyptian chickens, Eltanany *et al.* (2011) reported similar results of three main clusters of Egyptian chicken populations encompassing selected Fayoumi lines and Doki-4 in first cluster, native Dandarawi as second cluster, and Sinai, and other six commercial breeds in third cluster. Finally, the importance of these phylogeny results, that drawn from determination of the mean genetic distances of this population to other studied populations of broilers in the species, may help for decision to preserve the indigenous population as a unique genetic resource for commercial industry, as it was earlier suggested by Wimmers *et al.* (2000). In addition, the results might be useful for making decision on best crossbreeding and introgression program for indigenous chickens with evolutionary close broiler population in favor of meat production.

MCL method was considered as the best substitution pattern test of homogeneity between sequences to compute evolutionary distances between studied chicken populations. As a result, the evolutionary distances showed the shortest distance found between Hubbard and Ross broiler chickens revealing their more closely relation other than the others. On the other hand, Lohmann showed closer evolutionary distance to Hubbard than to Ross chickens. A quite reliable differences found between indigenous chicken with Ross; being the longest, and with Lohmann, being the shortest and the evolutionary closer chicken. As a consequence, different phylogenetic trees were reconstructed from 0.5-kb sequences and then provided reliable evidences for a close phylogenetic alliance among commercial broiler chickens and with indigenous chickens. The genetic relatedness between the three commercial strains clustered together and the indigenous that is originally out group was revealed as another separated cluster. However, there was no same phylogenetic branching of grouping for the three commercial chicken populations in one cluster or two clusters considering different models applied. This different pattern was most likely attributable to different models used for estimation distances.

Conclusion

The overall results support conclusions reached previously from DNA markers for such chicken populations in Mediterranean countries of the study. It also shed light on the sequence of evolutionary events that generated the present day chickens. Summing up, resulted phylogeny trees, on one hand, may help for getting decision to preserve the indigenous population as a unique genetic resource for commercial industry. On the other hand, the genetic sequence level of commercial broiler may attribute for which there is a limited information of indigenous chicken performance at the time of selection in a range of

environments, taking into tropical areas and climate change phenomenon. In addition, they might be useful for choosing best crossbreeding and introgression program of indigenous chickens with broiler population in favor of meat production under sub-tropical condition of Jordan.

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