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GENETIC DIVERSITY ANALYSIS OF INDIGENOUS LAYERS IN COMPARISON WITH EXOTIC LAYERS

Raed M. Al-Atiyat

Animal Production Department, King Saud University, Kingdom of Saudi Arabia
Email: ralatiyat@ksu.edu.sa

ABSTRACT

Genetic variation of a hundred indigenous layers chicken and its genetic relatedness to twenty exotic layers chickens of each Pakistani chicken and commercial layers were assessed in Jordan using six Microsatellite markers. The Microsatellite markers showed an average allele number of 5.8 per locus, expected heterozygosity of 55.8% and high polymorphism of 54% in Jordan indigenous chicken. On the other hand, the genetic relatedness between the three breeds, that were originally independent, was described by pairwaise genetic distance. The genetic distance was the shortest (0.135) between indigenous chicken and Pakistani chicken, revealing indigenous chicken was more closely related to Pakistani layer chicken than the commercial layers. This result confirmed the historical information about their common origin. Long genetic distance (0.205) was showed between indigenous chicken and commercial layers. This might be a result of gene flow by crossing individuals of both sometimes ago. Finally, the longest genetic distance (0.288) was found between commercial layers and Pakistani chicken. The overall results are valuable for sustainable genetic conservation program to reserve indigenous Jordan layer chicken for its unique genetic sources.

Keywords Indigenous layers, Exotic layers, Genetic diversity, Genetic distances, Microsatellite markers.

INTRODUCTION

It is widely accepted that all populations of domesticated chickens, *Gallus gallus*, in the world are descendant from a single ancestor; the Red Jungle Fowl (RJF), originating in Asia. It has been also claimed that other wild species of Gallus might have contributed to all domesticated chicken (Crawford, 1984). The more accepted finding is that RJF alone is sufficient to account for the maternal ancestry of the domesticated chicken (Fumihito *et al.*, 1994). In general, breeds and lines in different parts of the world were developed mostly from Asian chickens that are mainly Pakistani, Chinese, Malaysian and Egyptians (Moiseyeva *et al.*, 2003; Anjum *et al.*, 2012). In general, chicken domesticates in and around the ancient Arabian Peninsula, a tropical region which occupies a key geographic junction with Africa to the West and the Asian continent to the East, was reported by Boivin and Fuller (2009). In particular, Mediterranean domesticated chicken are the most closely related to the RJF (Moiseyeva *et al.*, 1996). All of these breeds contributed to the modern biodiversity of indigenous chicken breeds in each country of Asia (Crawford, 1990). In Jordan, a Mediterranean subtropical country, there is no scientific study, so far, describing the genetic variation of indigenous chicken breed and its genetic relatedness with other available breeds.

Genetic diversity of indigenous Jordan chickens has accumulated over a long time and as a consequence many crosses and/or strains differences are found (Al-Atiyat, 2010). A complete description of each hybrid or strain would entail ascertaining all genes that contribute to any phenotypic trait (Barker *et al.*, 1993). The different sources of chicken breeds were therefore, assumingly used to develop the indigenous chickens that are currently raised in all rural places of Jordan (Al-Atiyat, 2009). Abdelqader (2007) reported that the most predominant tropical chicken breeds reared in Jordan were Indigenous (67.3%) followed by Pakistani (27.7%) and Brahma (5%). These percentages are representing population of one million of Chickens. On the other hand, the total exotic commercial breeds were estimated to be 24 millions (FAOSTAT, 2004). Therefore, Jordan indigenous chicken breeds, resulting from centuries of domestication and breeding, are now at the risk of being lost as a result of intensive random crossing with exotic breeds and lines (Abdelqader *et al.*, 2007). These breeds may, based on little information available on its adaptive traits to tropical environments (Abdelqader *et al.*, 2007; Alshawabkeh and Tabbaa, 2001), represent good genetic resources for future breeding and research purposes. Before starting any breeding program, phenotypic and genetic characterization of chicken populations is a prerequisite. Therefore, the evaluation of indigenous chickens as genetic resources includes records of phenotypes and breeding history as well as determination of genetic variation and genetic distances (Hammond, 1994). The determination of genetic variation based on DNA markers provides accurate information for genetic distance analysis that allows a ranking of populations according to level of phylogenetic distinction (May, 1990). For poultry, establishment of genetic distance among populations and commercial strains will be important for identifying unique genetic recourses not

represented in industrial strains (Notter, 1999). Many such populations may exist, likely in the hands of either hobbyists in developed countries or farmers and householders in developing countries. As long as indigenous chicken genetic resources have been identified, their conservation can be assessed and monitored. The recent DNA molecular technologies have identified genetic variability at the DNA level in chicken breeds. Microsatellites markers (MS) are widely used, as polymorphic and randomly distributed in the living-being's genome, to study the biodiversity and genetic relationship between and within chicken populations (Kavaca, *et al.* 1999; Romanov and Weigend, 2001; Rosenberg *et al.*, 2001; Hillel *et al.*, 2003). The main aim of this study was to evaluate the genetic biodiversity of indigenous chicken population and their relationship to exotic populations in Jordan using MS markers.

MATERIALS AND METHODS

Chicken populations: Where relevant throughout this study, chicken population, breed, line and strain will be referred to as population. All work for this research using chickens was performed with the permission of and in accordance with the guidelines set by Ethics Committee of Mutah University (Reference number 120/14/785). One hundred indigenous layer chicken (IC) were collected from the rural areas of Northern (Irbid, Ajlun and Jarash Governorates), Eastern (Al-Mafraq and Az-Zarqa Governorates), Western (Al-Balqa, Amman and Madaba Governorates) and Southern parts of Jordan (Al-Karak and At-Tafilah Governorates) (Figure 1). To avoid bias, at least twenty indigenous chickens were randomly collected from at least three regions or villages in each governorate (Figure 1). A middleman was asked to represent, as many as possible, all chickens in each region/village that cover a wide range of phenotypical different individuals. The individuals were coming from at least three holders in each region. The selected and sampled individual was based on the phenotypes using a detailed phenotypic descriptor list.

On the other hand, two exotic chickens were considered in this study; the Pakistani layer chicken (PC) and commercial layer chicken (CC) of White Leghorn breed. Twenty pure-breed chickens each of PC and CC were randomly sampled on their rearing places. The rearing places were reliable owners of exotic chickens, game bird keepers and commercial farms. Sampling those birds was in order to find genetic destination to IC.

Sample collection: Samples were collected in two forms; blood and tissue. The blood sample was collected from the wing vein and transferred into vacutainer tube. This method was used to sample all exotic chickens as it was mostly preferred by their farmers. However CC farmers who had, in most of cases, not permitted his/her birds to be blood sampled, an alternative tissue sampling was applied. Tissue sampling was performed by taking a punch of ~0.2cm of the wattle tissue using an animal punch applicator and then transferred into eppendorf tube. The samples were immediately stored at a recommended temperature for DNA extraction process.

DNA extraction and quantification: A commercial kit, Wizard® Genomic DNA purification kit – Promega®, was used as a simple and convenient technique to isolate high-quality genomic DNA from blood and tissue samples (Promega Technical Manual, 2007). The recommended protocols in the technical manual were used for isolation genomic DNA from 10ml blood volume and 0.5cm bird tissue. After performing DNA extraction process the DNA sample was stored at 4°*C* (Sambrook *et al.*, 1989).

Agarose gel electrophoresis of 1% was used to visualize DNA bands and check the integrity of genomic DNA. Figure 2 is an example of visualizing DNA by using 1% Agrose gel for some DNA samples. All DNA samples were then quantified by spectrophotometer. The measurements were taken at λ260nm and λ280nm. The purity of DNA samples ranged from 1.2 up to 1.9. Samples were then diluted to 10ng/µl for use in subsequent PCR (PTC-200 programmable Thermal Controller, MJ Research Inc.) reactions.

Microsatellite markers genotyping: Poultry MS markers primers selected from poultry genome are shown in Table 1. These markers have been selected for their ability to be useful in studies of biodiversities, taxonomic identities, systematic relationships, population genetic structure, species hybridization, and parentage identification (Brandstrom and Ellegren, 2008). The MS genotyping required facilities that were not available at my laboratory. Therefore purified DNA samples were sent to overseas commercial company, Macrogen Inc.®, in order to provide genotyping services. In general, PCR reactions were performed and their products were resolved by polyacrylamide gel electrophoresis using an ABI 373XL automated sequencer. The output of this genotyping was provided as allele information in form of Excel sheet file. Particularly, PCR reaction of 10 µl volume was conducted under the following conditions: 10ng template DNA, 250 nM of each primer, 200 nM dNTPs, 1U Taq polymerase and 1.5 mM $MgCl_2$. The reaction was performed for each type of primer pair following the programs recommended in protocols of Sambrook *et al.*, (1989).

Genetic diversity and distance: Population genetic parameters of the selected chicken populations were investigated using genotypic data of MS markers and by utilizing main software program, GDA® (Lewis and Zaykin, 2001). Using the calculated allele frequencies for each population, gene diversity (expected heterozygosity;

H_e) was calculated under Hardy-Weinberg conditions. Genetic distance matrix, based on allele frequencies, was estimated to measure genetic relatedness between selected populations.

Estimates of allelic frequency of each locus were calculated to describe the genetic variation within and between populations. The observed heterozygosity is calculated as $\hat{H}_O = \frac{\sum N_{ij}}{N}$, and unbiased estimate of expected heterozygosity, H_e, at each locus was calculated as $\hat{H}_e = \frac{2N}{2N-1}(1-\sum_{i=1}^{n} \hat{p}_i^2)$ (Nei, 1987), where N is the number of individuals in the sample, N_{ij} is the number of observed heterozygosity, n is the number of alleles and pi is the frequency of the i-th allele. These two measures were calculated using GDA program. On the other hand, polymorphism information content (*PIC*) has been used to describe the amount of polymorphism at a single locus using the following implemented formula in GDA program;

$$PIC = 2\sum_{i-1}^{n-1}\sum_{j=i+1}^{n} p_i p_j (1 - p_i p_j)$$

(Botstein *et al.*, 1980), where, pi is the population frequency of the i-th allele, and pj is the frequency of the j-th allele.

Genetic distances between populations were also measured using GDA that utilizes the most widely used measure of genetic distances proposed by Nei (1972). Estimates the genetic distances between two populations were provided on the basis of a ratio of allele frequencies that are different between them. The genetic distances were calculated from the allele differences between two populations, whereas the genetic identity estimates the proportion of alleles that are common in the two populations. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software program, version 5 (Tamura *et al.*, 2011).

RESULTS AND DISCUSSION

Genetic diversity and polymorphism of indigenous chicken: Average numbers of genotyped alleles in the studied MS loci for all populations was 5.8 (Table 2). The result indicates a high number of MS alleles in IC in Jordan. MS loci varied in number of described alleles from 4 (*ADL278*) to 8 (*MCW14*) (Table 2). The results are most similar to those of local Ghanaian chickens that showed number of alleles per locus was 6.6 compared with 6.0 alleles for combined control populations of Red Jungle fowl and two European commercial chickens (Osei-Amponsah *et al.* 2010). In four strains of Pakistani Aseel chicken, similar results were reported with an average of 9.1 alleles per locus and number of alleles varied between 4 to 10 using ten MS markers (Babar *et al.*, 2012). For Indian indigenous chicken, number of alleles varied 3 to 10 for twenty five MS markers (Parmar *et al.,* 2007).

The MS loci are varied in the allele sizes in the populations. They varied from 76 bp *(MCW295)* to 221 bp (*MCW206*) in all populations. On the other hand, there was no one-way relationship between degree of polymorphism and MS length. The proportion of polymorphic loci sometimes increased with the repeat length increased such as *MCW14* (0.675) and *MCW67* (0.73). In other times, the polymorphism decreased with increase of MS length. In contrast, polymorphism was previously seen at MS loci with as few as five repeat units and the proportion of polymorphic increases for sequences with 10 repeat units, to reach a maximum of 75%–80% for sequences with 15 or more repeat units. In general, allele sizes for all studied MS within the range reported in chicken genome-wide analysis by Brandstrom and Ellegren (2008). For example, *MCW295* and *MCW14* alleles' sizes range are 76-92 and 152-192, respectively. These ranges are matching results of Dorji *et al.* (2011), who found the average sizes were of 83-146 and 156-253 for *MCW295* and *MCW14*, respectively. On the other hand, allele size of polymorphic loci ranged from 98-356 bp in Indian chicken and showed very large variation across loci (Parmar *et al.,* 2007).

The average gene diversity, H_e, was 0.558 for the IC, (Table 2). These results showed high H_e in IC and for most of studied loci except *ADL278*. However, H_o, in some of the cases, was slightly higher than H_e, resulted in slightly higher average H_o. Average H_o was 0.563 (Table 2) suggesting a tendency towards heterozygote deficiency. In general, if heterozygosity is higher than expected, an isolate-breaking effect would be expected for mixing different populations. Similarly, many studies reported high heterozygosity of both for different indigenous population (Wimmers *et al.*, 2000; Hillel *et al.*, 2003; Kaya and Yıldız, 2008; Osei-Amponsah *et al.*, 2010; Dorji *et al.*, 2011:). For example, Hillel *et al.*, (2003) stated that the range of H_e using 22 MS markers was from 0.11 to 0.75 for 52 chicken populations. Dorji *et al.*, (2011), compared Thai indigenous chickens with commercial lines using 20 MS, found range of H_o and *He* in four populations of Thai indigenous chickens were 0.55-0.63 and 0.79-0.81, respectively. In addition, mean observed and expected heterozygosity values of 0.3941 and 0.7718 were recorded in Pakistani Aseel chicken (Babar *et al.*, 2012), whereas overall heterozygosity value among three varieties of Indian indigenous chicken was found to be 0.701 (Parmar *et al.,* 2007).

On the other hand, the results also showed that polymorphic information content (*PIC*) values were high for all studied loci except the loci that showing low values of either H_o or H_e. The *PIC* averages were 0.540 in the population. The lowest and the highest values of *PIC*

were 0.352 (*ADL278*) and 0.675 (*MCW14*). These values were within the *PIC* range of indigenous chickens of different worldwide ecological zones (0.14-0.72) reported by Wimmers *et al.* (2000), 0.61 by Kaya and Yıldız (2008), 0.51-0.52 by Zhao *et al.* (2010) and 0.65 - 0.71 by Babar *et al.* (2012).Overall, levels of H_e and H_o and *PIC* at the six loci in PC population were relatively high reflecting more number of alleles per locus. These results were quite expected for MS loci, which have demonstrated high polymorphism in all species studied so far (DeWoody *et al.*, 1995; Hillel *et al.*, 2003; Dorji *et al.*, 2011).

In summary, for the studied MS markers, 54% of the IC population was polymorphic. The mean number of alleles was 5.8 across all populations and average gene diversity was ~56%. The most polymorphic MS marker was *MCW14* with 8 alleles on average across populations. The gene diversity of *MCW14* was 71.1%. The results express wide range variation in the sampled populations and the high level of polymorphism. These finding was in agreement with results of Hillel *et al.* (2003) who found wide range of biodiversity and high level of polymorphism in chicken populations. The genetic diversity of the indigenous chickens found in present study reflects its local adaptation to prevailing tropical climatic and environmental conditions.

Genetic distances of indigenous, Pakistani and commercial chickens: The standard genetic distance measure of Nei (1972) revealed reliable differences between the three populations (Table 3). The results showed the pairwaise genetic identity and distance estimates between the three populations. Genetic identity is unity when the two populations have the same alleles in identical frequencies, while it is zero when they have no common alleles (Nei, 1972), while genetic distances, it is unity when they have no common alleles and zero if the alleles are identical. A sum of both identity and genetic distance values of pairwaise populations must be equal to unity. The shortest genetic distance (0.135) was found between IC and PC. This revealed that IC was more closely related to PC than the other population. In other words, this can be drawn from value of genetic identity (0.856) between the two populations (Table 3). This finding was in agreement with the historical information about common origin of these two populations (Moiseyeva *et al.*, 1996; Moiseyeva *et al.*, 2003; Abdelqader *et al.*, 2007). The result may indicate that many efforts made by farmers in the past to improve production performance of the IC by crossing with PC. Another reason might be that IC and PC have almost been kept together in free-range conditions. In addition, both can tolerate the harsh free-rang condition of subtropics in Jordan better than CC which was restricted for intensive production. Although the PC had shortest genetic distance with IC, it showed longest genetic distance (0.288) with CC. It seems that exchange of genetic material between PC and CC has happened very rarely. The IC had smaller pairwaise distance (0.206) with CC. This might be explained, as there was possibility of gene flow from commercial chicken to indigenous chicken. It was supported by morphological characteristics for crossbred individuals that could be expressed by genes integrated into indigenous chicken genome through crossing with CC individuals practiced by farmers and rural people in order to increase egg production.

The phylogenetic tree in Figure 3 revealed a considerable degree of differentiation of populations supporting the data presented in Table 3. Figure 3 shows the neighbor-joining tree construction of two clusters. The CC was considered in a cluster separated from the IC that was gathered with PC in another cluster. The IC and PC were more related to each other than to the CC. In general the populations were assorted into two clusters with chickens from similar features. Such situation was likely due to sharing common genes from previous ancestors. Therefore, it would be concluded that there was high genetic relatedness between IC and PC than CC. Similar results were reported earlier in which genetic relatedness within indigenous chickens was high when compared to different chicken ecotypes (Rosenberg *et al.*, 2001; Msoffe *et al.*, 2005). The decision to preserve the indigenous population as a genetic resource should be drawn from determination of the mean genetic distances of this population to other populations in the species as it was earlier suggested by Wimmers *et al.*, (2000). It was difficult in this study to include all exotic chicken populations other than those chickens already existed in Jordan. On the other hand, there are many studies reported a phylogenetic tree that reflected general patterns of relatedness and genetic differentiation among various chicken populations. For example, Romanov and Weigend (2001) reconstructed three major phylogentic tree groupings, the most interesting group was the second group that comprised commercial layer lines and chicken breeds that were subjected to intense selection in the past or had common ancestral breeds with commercial strains. Their result is in agreement with the results found in this study in which IC and PC was subjected to intense selection in sometimes ago as well as gene flow from CC to IC in past times. In addition, Similar findings was recently reported by Eltanany *et al.* (2011) who identified 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 synthetic breeds in third cluster. On the other hand, Babar *et al.* (2012) found two clusters of four varieties of Pakistani Aseel chicken of close genetic relationships.

Table 1. Sequences of Microstallites primers used for genotyping.

MS Primers	Sequence (5'-3') (forward)	(Reverse)
MCW295	*ATCACTACAGAACACCCTCTC*	*TATGTATGCACGCAGATATCC*
MCW206	*ACATCTAGAATTGACTGTTCAC*	*CTTGACAGTGATGCATTAAATG*
MCW14	*AAAATATTGGCTCTAGGAACTGTC*	*ACCGGAAATGAAGGTAAGACTAGC*
ADL278	*CCAGCAGTCTACCTTCCTAT*	*TGTCATCCAAGAACAGTGTG*
MCW67	*TTTGAAGGGATGCTGCATGCA*	*CTGATTTGCAGCTTGGCTGAG*
MCW216	*GGACCTCTCAGCAGTGCCATAATA*	*GATGAACTCCTTGGATTTGTCCCA*

Table 2. Number of alleles at each locus, expected heterozygosity (*He*), observed heterozygosity (*Ho*) at each loci, polymorphic information content (*PIC*) and allele sizes for six microsatellite loci in the Indigenous population.

Primers	Chromosome No.	Position (cM)	*No.*	*Ho*	*He*	*PIC*	Allele sizes (bp)
MCW295	4	75	5	0.482	0.521	0.490	76-92
MCW206	2	104	6	0.593	0.591	0.596	197-221
MCW14	6	96	8	0.739	0.711	0.675	152-192
ADL278	8	87	4	0.379	0.390	0.352	107-125
MCW67	10	61	7	0.712	0.652	0.673	165-193
MCW216	13	28	5	0.525	0.492	0.452	126-148
Average			**5.8**	**0.563**	**0.558**	**0.540**	

No.: Average number of alleles per locus. *He* : Unbiased expected heterozygosity . *Ho*: Unbiased observed heterozygosity, *PIC*: Polymorphic information content.

Table 3. Pairwise genetic identities* and distances+ between Indigenous, Pakistani and Commercial chicken populations.

Population	Indigenous	Pakistani	Commercial
Indigenous		0.856	0.789
Pakistani	0.135		0.720
Commercial	0.206	0.288	

** Identity above diagonal and distance below diagonal.*
+ The sum of the distances and identity is slightly higher than 1, this is due to rounding of the numbers and separate calculation of each estimate (mention software used)

Finally, the information about the populations and genetic relatedness estimated by MS analysis may be useful as an initial guide in future investigations of IC biodiversity. As a consequence, the results may prove to be valuable for the future conservation of genetic resources of indigenous chicken breeds in Jordan. Here genetic conservation would be attained through full awareness of a negative impact of factors such as destruction and loss of habitat, overexploitation of resources and lack of conservation programs (Meadows, 2011) and climate change (Qureshi and Ali, 2011).For more general view, the importance of Jordan indigenous population as genetic resources lies in potential presence of unique alleles or allelic combination for production and adaptive traits of economical interest.

Fig. 1 Map of Jordan presenting regions*/villages in different governorates where indigenous chicken individuals sampled.
* Dot (•): represents regions where indigenous chicken individuals sampled in each governorate.

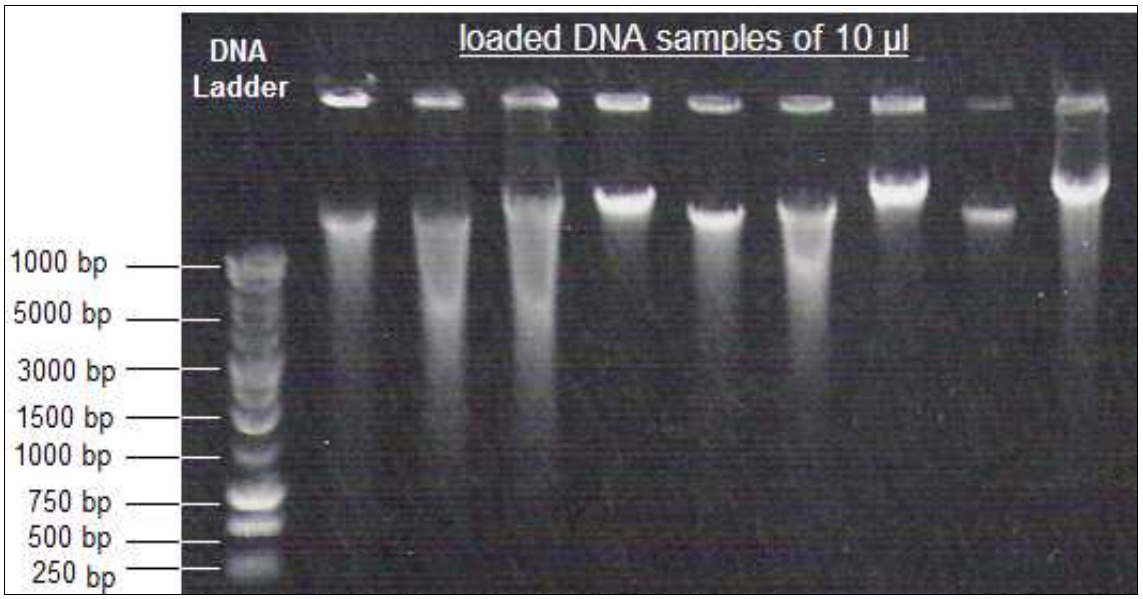

Fig. 2. Agarose gel of 1% loaded with DNA ladder of 1000 base pair (bp) and nine extracted DNA samples of 10µl.

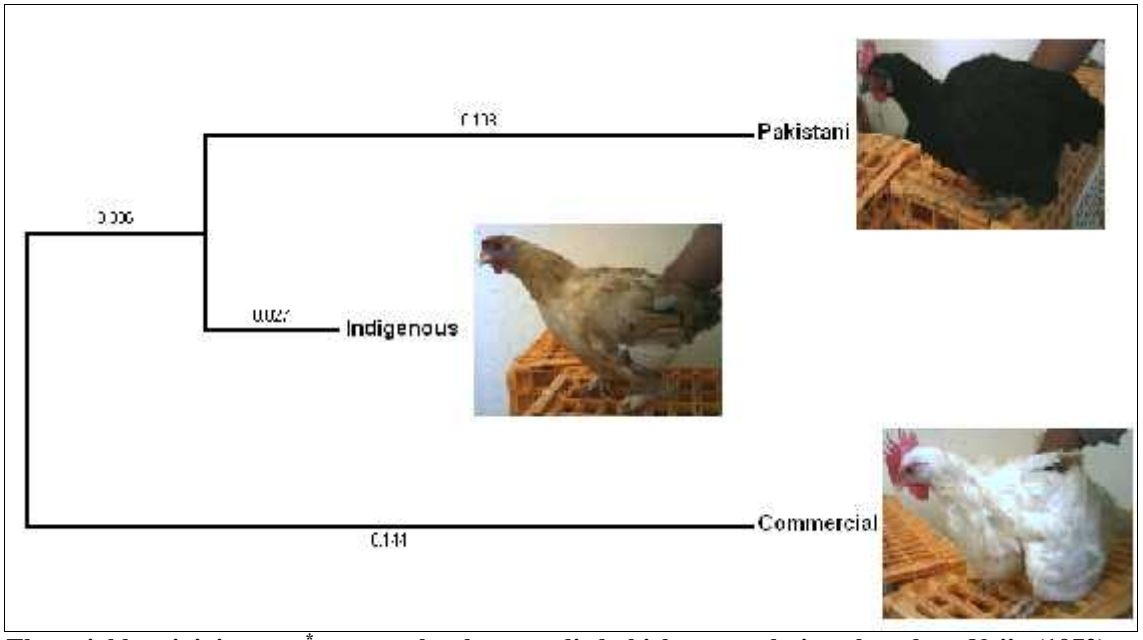

Fig. 3. The neighbor-joining tree* among the three studied chicken populations based on Nei's (1972) unbiased genetic distance method using MEGA software program (Tamura *et al.*, 2011).

* Number showed in tree branches represents distance coefficient.

Conclusion: Biodiversity of indigenous chicken populations was relatively high with high values of gene diversity and *PIC* for the studied markers. Shortest genetic distance was found between IC and PC. This revealed that IC was more closely related to PC than another population. This result is in agreement with the historical information of their common origin and pastime crossing. Even the longest genetic distance was found between CC and PC, the CC showed long genetic distance with IC, This might be a result of gene flow from CC through crossing in past times by farmers. Finally, overall results may be valuable for necessary future genetic conservation program for keeping biodiversity of IC populations and to reserve them for their unique genetic resources. Hence, support global programs to determine genetic distances among indigenous chicken populations and to establish core collections of diversity within species. A greater understanding for long-term genetic improvement and genetic relationships among global chicken populations is

priorities for managing genetic diversity of Jordan's' indigenous chicken.

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