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Genetic diversity of Hajar1 and Hajar2 local Saudi chicken lines using mitochondrial DNA D-loop markers

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Abstract

This study was conducted to assess genetic diversity of Hajar1 and Hajar2 local Saudi chicken lines using mitochondrial DNA (mtDNA) D-loop partial sequences. One hundred blood samples were obtained equally from Hajar1 and Hajar2 Saudi chicken lines as 50 samples from each line. The D-loop region was partially amplified from genomic DNA with a conserved primer set, and the fragments were sequenced. Eight published reference mtDNA sequence data from the GenBank were used for comparisons, and multiple alignments were performed. The most common haplotype was assigned as a basic sequence for comparing within each line. Entropy plot and conserved region analysis were performed. Genetic distances and neighbour-joining (NJ) phylogenetic trees were estimated. The results indicated haplotype variations within and between local Saudi chicken lines, which could explain the phenotypic variation reported earlier. A close genetic relationship was shown between the Saudi local chicken lines. Unique conserved regions and nucleotide substitutions were observed between the two lines. Both lines have a close relationship with the reference Asian local chicken population, especially local Chinese and Indian chicken breeds. The current results are considered the first report of mtDNA sequence diversity for Hajar1 and Hajar2 lines. Further detailed molecular genetic studies of both lines are indispensable to genetic conservation and development.

Keywords: chicken population, diversity, D-loop, Hajar1, Hajar2, mtDNA
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Introduction

There have been significant losses of experimental chicken lines, which are conserved mostly at research institutes. This is because of the difficulty in funding for necessary conservation of these lines (Pym, 2013). These losses in those genetic resources are contemporaneous with the limited genetic base of commercial chicken lines. This may have serious consequences, including a severe decrease in chicken genetic diversity worldwide in the near future (Pym, 2013). Although native and indigenous chicken breeds have further significance for sustainable development (Van Marle-Köster *et al*., 2008), they have not been used commercially. In this context, two local Saudi chicken lines, Hajar1 and Hajar2, were recently characterized for their phenotypic characters (Ahmed & Alabbad, 2014). The genetic pool of the current lines dates back to the early 1990s when they were collected from various areas of Saudi Arabia and represented most of the chicken genetic pool of the country (Al-Yousef 2007). Recently, an ongoing conservation process that included four thousand birds of both lines reported some phenotypic and quantitative genetic parameters (Alabbad, 2014). There were significant variations in certain productive and physiological parameters between the two lines, including bodyweight and some blood biochemical factors (Ahmed *et al*., 2014). The assumption is that exposure to specific environments, feedstuff and pathogens would eventually lead to genetic differences caused by the requirement to adapt (Groeneveld *et al*., 2010; Lenstra *et al*., 2012). Measuring the level of genetic diversity, defined as the variation in the genetic composition of individuals across breeds, is central to the design of sustainable development programmes (Kosba *et al*., 2009). There is a need for research in genetic diversity in chicken populations worldwide (Granevitze *et al*., 2009). Genetic diversity and variation can be characterized with various molecular techniques, including microsatellite markers (Berima *et al*., 2013), single nucleotide polymorphisms (SNPs) (Granevitze *et al*.,

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2014), and mtDNA (Park *et al*., 2011). mtDNA has been used intensively because it has proved suitable for phylogenetic comparisons within and among closely related species (Avise & Zink, 1988). The mtDNA polymorphism has largely been applied for understanding genetic relationships in chickens (Lee *et al*., 2007; Ramadan *et al*., 2011; Bondoc, 2013) and other avian species (Patel *et al*., 2010; Kerr, 2011).

mtDNA D–loop is a non-coding region. It has played a role in the replication and transcription of mtDNA. The D–loop segment shows a higher level of variation compared with protein-coding sequences, owing to reduced functional constraints and relaxed selection pressure (Arif & Khan, 2009). Hamuri *et al*. (2004) emphasized that the D-loop region of chicken mtDNA is more polymorphic than the genomic and other mitochondrial regions. Since the D-loop region evolves much faster than other regions of the mtDNA genome, it is the most valuable and sensitive region that is suited to genetic variation studies within species (Niu *et al*., 2002). The efficiency of mtDNA D-loop in genetic variation assessment has been reported with White Leghorn, Plymouth Rock, and Rhode Island Red chickens in Japan (Harumi *et al*., 2004), Korean chickens (Lee *et al*., 2007), Japanese native chickens (Oka *et al*., 2007), and Egyptian local chickens (Ramadan *et al*., 2011). The aim of this research is to investigate the potential genetic diversity in the newly characterized Saudi chicken lines Hajar1 and Hajar2 and other exotic Indian, Chinese, Japanese, Mediterranean and commercial chicken populations, using a mtDNA marker-based technique, and to construct a phylogenetic tree with them.

Materials & Methods

Blood samples were collected from the Hajar 1 and Hajar 2 Saudi chicken lines (50 samples from each) at King Faisal University Research Centre. For each bird, 1 mL of blood was obtained in 3 mL sterile vacutainer tubes using ethylenediaminetetraacetic acid (EDTA) as the anticoagulant agent. DNA was extracted from the blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, Calif, USA, Cat no. 51104) according to the supplier's instructions, with slight modifications for diluting samples to 25% with normal saline before extraction. All samples were quantified with NanoDrop 2000 (Thermo Scientific, USA), and 45 and 47 samples of Hajar1 and Hajar2 lines, respectively, were selected for further processing according to DNA concentration adequacies. The DNA samples were then stored at -20 ºC. The analysis included previously published mtDNA sequence data for Red Jungle Fowl, Chinese local chickens, commercial broilers, commercial white egg layers, Indian local chickens, Japanese local chickens, Rhode Island Red and White Leghorn chicken populations (GenBank accession numbers AF512151, AY644973, AM746036, AM746033, GU448400, AB098697, AB268517 and AB268521, respectively).

The D-loop regions of all samples were amplified from genomic DNA using polymerase chain reaction (PCR). A conserved primer set was used F (5'-AGGACTACGGCTTGAAAAGC–3') and R (5'-ATGTGCCTGACCGAGGAACCAG -3') to amplify the first 539-base of the D-loop region, as described by Niu *et al*. (2002). Polymerase chain reactions (PCR) were performed using thermal cycler reaction of 20 µL volume which included 20 ng genomic DNA, 250 nM Deoxynucleotides (dNTPs), 2 µL primer, 2 mM $MgCl_2$, and 1U Taq polymerase. The reaction mixture was preheated at 94 ºC for 10 min, followed by 35 cycles (94 ºC for 45 s, 63 ºC for 45 s, and 72 ºC for 1 min). Then a final extension was applied at 72 ºC for 10 min. PCR products were separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide in the presence of DNA ladder to ensure the amplicon was produced before the sequencing step. The image was obtained under UV light. The PCR product sequencing was conducted with a 3730XL automated DNA sequencer (Macrogen Inc., Seoul, South Korea)

The sequences that were obtained in this study, in addition to the reference sequences, were aligned with BioEdit sequence alignment editor software version 7.0.5.2 (Hall, 1999). Multiple alignments were performed with Clustal W multiple alignment in BioEdit version 7.0.5.2 (Hall, 1999). The most common haplotype was assigned as a basic sequence for comparing within each line, while Red Jungle Fowl mtDNA sequence accession number AF512151 was assigned as the basic sequence for comparing all lines. All positions containing gaps and missing data were eliminated. The variable sites in each local line were shown in the entropy plot. Conserved region analysis was performed using BioEdit program version 7.0.5.2 (Hall, 1999). The entropy value is a measure of nucleotide variation in a given position of aligned sequences. Identical sequences in each line were regarded as a single haplotype, then the genetic distances and neighbour-joining (NJ) phylogenetic tree were estimated with MEGA software version 6 (Tamura *et al*., 2013). Analyses were conducted with the maximum composite likelihood model (Tamura *et al*., 2004).

Results

In the analysis of the partial sequences of d-loop mtDNA of the selected samples (45 and 47 from Hajar 1 and Hajar 2, respectively) 24 nucleotide positions recorded changes (Table 1). These changes were grouped into 15 haplotypes, based on the sequence differences. Haplotype group 1 comprised 26.6% of the total

Table 1 Sequence variation of mitochondrial D-loop among Hajar1 local Saudi chicken line

Haplotype	Nucleotide position*																							
	21	42	44	56	145	174	187	214	215	227	261	266	315	322	325	371	376	380	395	397	399	400	407	501
Type1 (12)**	C	G	T	A	T	C	G	C	C	C	T	G	G	T	A	G	A	G	C	T	C	A	G	T
Type2 (5)	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
Type3 (4)	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Type4 (3)	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Type5 (3)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Type6 (3)	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
Type7 (3)	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
Type8 (3)	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Type9 (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	C	.	.
Type10 (2)	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Type11 (1)	.	.	.	.	.	.	A	T	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.
Type12 (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
Type13 (1)	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.
Type14 (1)	.	.	.	.	.	.	.	.	A	.	.	A	A	.	G	.	.	A	.	.	.	.	.	.
Type15 (1)	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	C	.	A	.	.	.	.	C	.

*Numbers indicate nucleotide position in the first 510 bp of mtDNA D-loop region; dots represent the identical nucleotides compared with type1 sequence
** Number of birds observed in each type is indicated in parentheses

haplotypes (12 individuals out of 47), followed by haplotype group 2, and then group 3. The rest of the haplotype groups each represented between one and three individuals from the total number of experimental birds. Analysis of partial sequences of d-loop mtDNA from 43 individuals in Hajar 2 (Table 2) revealed changes in 17 nucleotide positions. The changes were grouped into 12 haplotypes. The first haplotype comprised 23.2% of the total haplotypes, while the second, third and fourth haplotypes together comprised 37.2% of the total. The rest of the haplotypes represented between one and three birds each. Sequence variations in the mtDNA d-loop were assessed for Hajar 1, Hajar 2, and eight reference mtDNA D-loop sequences, using the Red Jungle Fowl mtDNA D-loop sequence as a reference sequence (Table 3). Following this assessment (Table 3), Hajar 1 line sequences were grouped into three haplotypes, comprising 38, 3, and 4 individuals, respectively. Hajar 2 line sequences were grouped into three haplotypes, comprising 40, 2, and 1 individuals, respectively. Each haplotype in both lines differed from the others at a single nucleotide. The first haplotypes in Hajar 1 and Hajar 2 were identical (Table 3), and comprised 87.6% of the total haplotypes for the chickens involved in this study. Comparisons were then performed of the variability within the partial sequence of mtDNA D-loop for Hajar 1 and Hajar 2. Entropy (Hx) values along the 510 bp were calculated and plotted using the entropy BioEdit plot tool (Figure 1). The results indicated different entropy values for the two local lines in terms of maximum entropy value and positions of entropy (Figure 1). An average entropy value of 0.2 was observed in both lines.

Table 2 Sequence variation of mitochondrial D-loop in Hajar2 local Saudi chicken line

Haplotype	Nucleotide position*																
	29	**40**	**42**	**56**	**214**	**225**	**266**	**295**	**315**	**323**	**327**	**334**	**380**	**384**	**427**	**429**	**446**
Type1 (10)**	C	G	G	A	C	T	G	C	G	A	G	A	G	G	T	G	C
Type2 (7)	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Type3 (5)	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.
Type4 (4)	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.
Type5 (3)	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Type6 (3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.
Type7 (3)	.	.	T	.	.	C	A	.	A	.	C	G	.	A	.	C	.
Type8 (3)	.	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.	.
Type9 (2)	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	T
Type10 (1)	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Type11(1)	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
Type12 (1)	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

* Numbers indicate nucleotide position in the first 510 bp of mtDNA D-loop region; dots represent the identical nucleotides compared to type1 sequence

** Number of birds observed in each type is indicated in parentheses

In the present study, the genetic distance between Hajar 1 and Hajar 2 was recorded as 0.0053, based on the mtDNA D-loop sequence (Table 4). mtDNA D-loop partial sequence information from GenBank for Red Jungle Fowl, Chinese local chickens, commercial broilers, commercial white egg layers, Indian local chickens, Japanese local chickens, Rhode Island Red and White Leghorn chickens provided the researchers with extra information about the genetic distances between local and exotic chicken populations (Table 4). The genetic distance between Hajar 1 and Hajar 2 and the Chinese local chicken population was 0.0073 and 0.0071, respectively.

Table 3 Sequence variation of mitochondrial D-loop among local Saudi chicken and reference sequences of other exotic chicken populations

Chicken line	Nucleotide position*																					
	148	188	191	193	198	202	206	211	223	224	227	237	242	262	287	291	296	323	344	348	380	427
Red Jungle Fowl (AF512151)**	C	G	C	G	T	C	T	T	G	T	T	T	C	A	T	C	C	A	C	T	G	C
Chinese local (AY644973)	T	A	.	.	C	.	C	.	.	.	C	.	T	.	.	T	.	.	.	.	.	T
Commercial broiler (AM746036)	T	A	.	A	.	.	C	.	.	.	T	.	.	.	.	.	T	.	.	.	.	C
White egg layer (AM746033)	C	A	T	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C
Indian local (GU448400)	T	A	.	.	.	T	C	.	.	.	C	C	T	G	C	T	.	G	.	.	.	C
Japanese local (AB098697)	.	A	.	.	.	.	C	C	A	.	C	C	.	G	.	T	.	G	T	C	A	C
Rhode Island Red (AB268517)	C	A	.	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C
White Leghorn (AB268521)	C	A	T	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C
Hajar1 (38)***	T	A	.	.	C	.	C	.	.	C	C	C	T	.	.	T	.	.	.	.	.	T
Hajar1 (3)	T	A	.	.	C	.	C	.	.	C	.	C	T	.	.	T	.	.	.	.	.	T
Hajar1(4)	T	A	.	.	C	.	C	.	.	C	C	C	T	.	.	T	.	.	.	.	A	T
Hajar2 (40)	T	A	.	.	C	.	C	.	.	C	C	C	T	.	.	T	.	.	.	.	.	T
Hajar2 (2)	T	A	.	.	C	.	C	.	.	C	C	C	T	.	.	T	.	G	.	.	.	T
Hajar2 (1)	T	A	.	.	C	.	C	.	.	C	C	C	T	.	.	T	.	.	.	.	A	T

* Numbers indicate nucleotide position in the first 510 bp of mtDNA D-loop region; dots represent the identical nucleotides compared with Red Jungle fowl sequence.
** Genbank accession number
*** Observed number of birds in each line that represented the sequence is indicated in parentheses

Both values were the lowest values recorded for local Saudi chicken lines compared with any other reference population. The mtDNA D-loop partial sequences of local and reference populations were used to construct an NJ phylogenetic tree (Figure 2). The phylogenetic tree demonstrated the proximity between Hajar 1 and Hajar 2 when they are located in the same cluster. The reference chicken populations were located in a different cluster. Chinese local chickens showed the closest relationship with the local chicken lines compared with the other reference populations.

Table 4 Genetic distances among local Saudi chicken lines and reference exotic chicken populations based on mtDNA D-loop region partial sequence

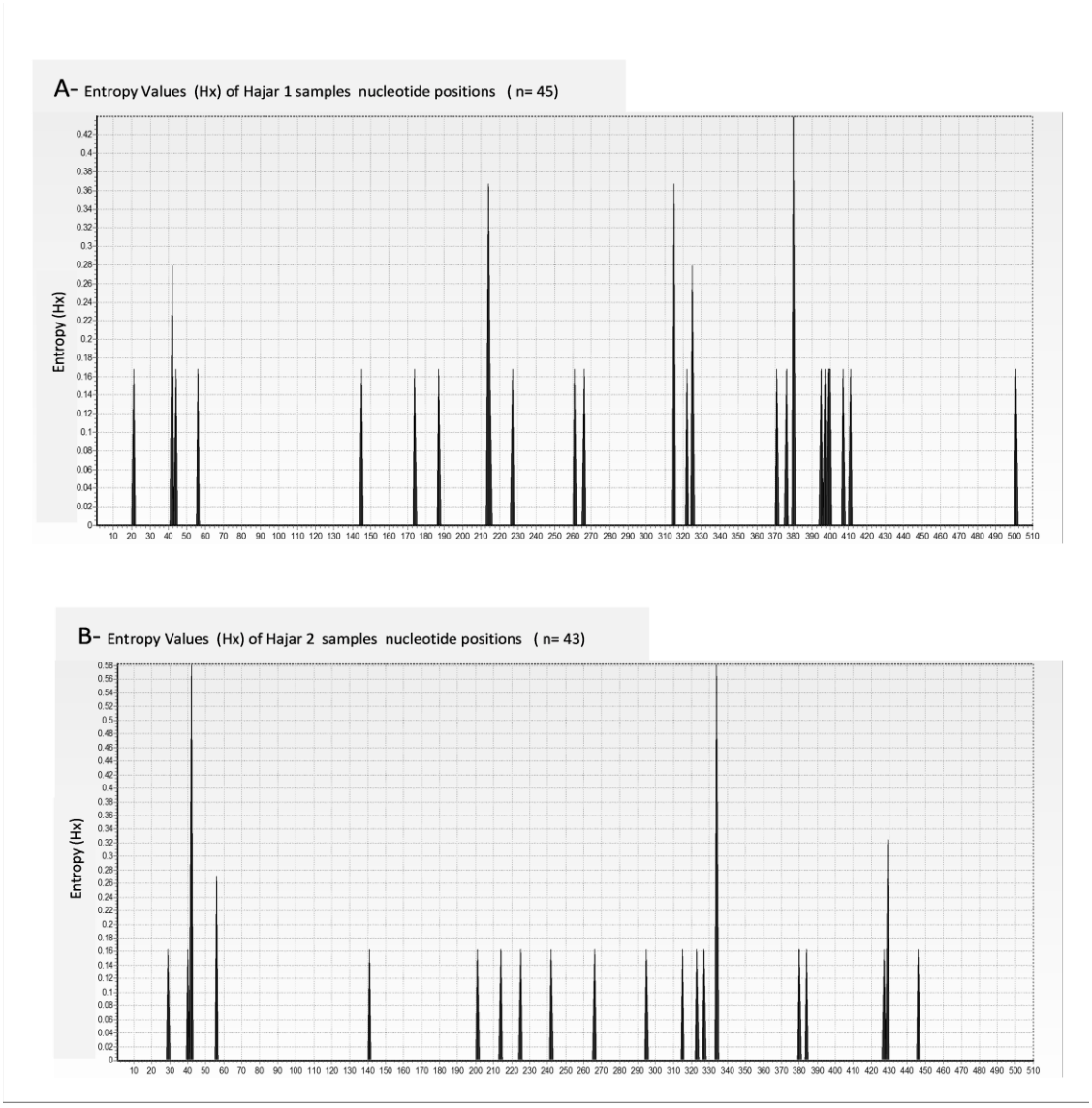
	Hajar2	**Hajar1**	**White Leghorn**	**White egg layer**	**Rhode Island Red**	**Red Jungle**	**Japanese local**	**Indian local**	**Chinese local**
Hajar1	0.0053								
White Leghorn	0.0227	0.0230							
White egg layer	0.0227	0.0230	0.0000						
Rhode Island Red	0.0204	0.0207	0.0022	0.0022					
Red Jungle	0.0250	0.0251	0.0066	0.0066	0.0044				
Japanese local	0.0246	0.0245	0.0291	0.0291	0.0268	0.0314			
Indian local	0.0158	0.0162	0.0246	0.0246	0.0223	0.0268	0.0178		
Chinese local	0.0071	0.0073	0.0155	0.0155	0.0133	0.0178	0.0268	0.0178	
Commercial broiler	0.0227	0.0228	0.0133	0.0133	0.0111	0.0111	0.0291	0.0246	0.0155

Discussion

The results indicated 24 single nucleotide substitution positions in Hajar 1, comprising 8 transition single nucleotide polymorphisms (SNPs) and 16 transversion SNPs substitutions. Hajar 2, meanwhile, showed 17 nucleotide substitution positions, comprising 12 transition and 5 transversion SNP substitutions. Data observed for the Hajar 1 line indicated a clear bias toward transversion. The ratio of transition to transversion should be approximately 0.5 if mutations are random, and 2.36 in the non-coding region for chickens (Vignal *et al*., 2002). The observed bias in local chicken lines requires further detailed study. One possible reason for the transversion bias may be the expected variation along the genome, as reported by Hodgkinson & Eyre-Walker (2010). As regards the low variation within each line, the entropy values could show the differences in variables, with the region between the two lines clearly conserved. Five conserved regions were recorded for Hajar 1 sequences at nucleotide positions 1:41, 43:213, 215:314, 326:379, and 381:510, with average entropy values (Hx) of 0.0041, 0.0049, 0.0076, 0.0062, and 0.009, respectively. Hajar 2 sequences, however, recorded four conserved regions at nucleotide positions 1:41, 57:333, 335:428, and 430:510 with average entropy values (Hx) of 0.008, 0.0059, 0.0052, and 0.002, respectively. Average polymorphism percentages of 4.7 and 3.3 in the mtDNA D-loop partial sequences were recorded for Hajar 1 and Hajar 2, respectively.

Low polymorphism percentages in the current study have also been reported in mtDNA for Chinese local chickens (Fu *et al*., 2000) and Korean local chickens (Lee *et al*., 2007). This low polymorphic percentage could be interpreted in a number of ways. The long-term history of the local chickens in the present study is not well known, so the mtDNA may have gone through an evolutionary bottleneck owing to the domestication process, as described by Niu *et al*. (2002). While an evolutionary bottleneck may have occurred, low mtDNA variation could from recurrent selective sweep as the result of strong natural selection (Hebert *et al*., 2003; Bazin *et al*., 2006). This selective sweep hypothesis is reasonable because of the harsh natural environment to which local chickens are subject in this area of the world. The geographical location of the chicken lines may elevate inbreeding frequency and isolate them from their ancestral population, which could contribute to this low genetic variation, as discussed by Peters *et al*. (2012) and Lyimo *et al*. (2014a). Recent reports about the European chicken population over the past 150 years suggest that these birds are directly descended from Asian chicken breeds or have been crossed with them (Lyimo *et al*., 2014b). In addition, there is a close relationship between various Asian chicken populations and Red Jungle Fowl (Cheng *et al*., 1996; Berthouly *et al*., 2009; Cuc *et al*., 2010). The present study used Red Jungle Fowl as a

reference sequence for comparison, as well as three other reference sequences of Asian origin, in addition to the rest of the mtDNA reference sequences. Hajar 1 and Hajar 2 showed high similarities with Chinese local chicken populations. This may be the result of geographical proximity and trading of animals that has taken place for centuries. The ten transition SNPs between Red Jungle Fowl and local chickens could point to a recent isolation of the current lines. The genetic distance results (the genetic distance between both lines of Saudi local chickens and Chinese local chickens was the lowest observed) may support the authors' theory of geographical proximity and the historic trading of Saudi local chickens and Chinese local chickens. The NJ tree may signify an overlap between the two Saudi chicken lines, indicating their close genetic relationship (Ahmed & Rezk, 2015). This overlap is thought to be because of their similar origin.



A: Variation of Hajar1 samples sequence shown by entropy plot
B: Variation of Hajar2 samples sequence shown by entropy plot
Entropy values (Hx) are a measure of variation at each nucleotide position in a set of aligned sequences

Figure 1 Variation of mitochondrial D-loop sequence of Hajar1 and Hajar2 samples

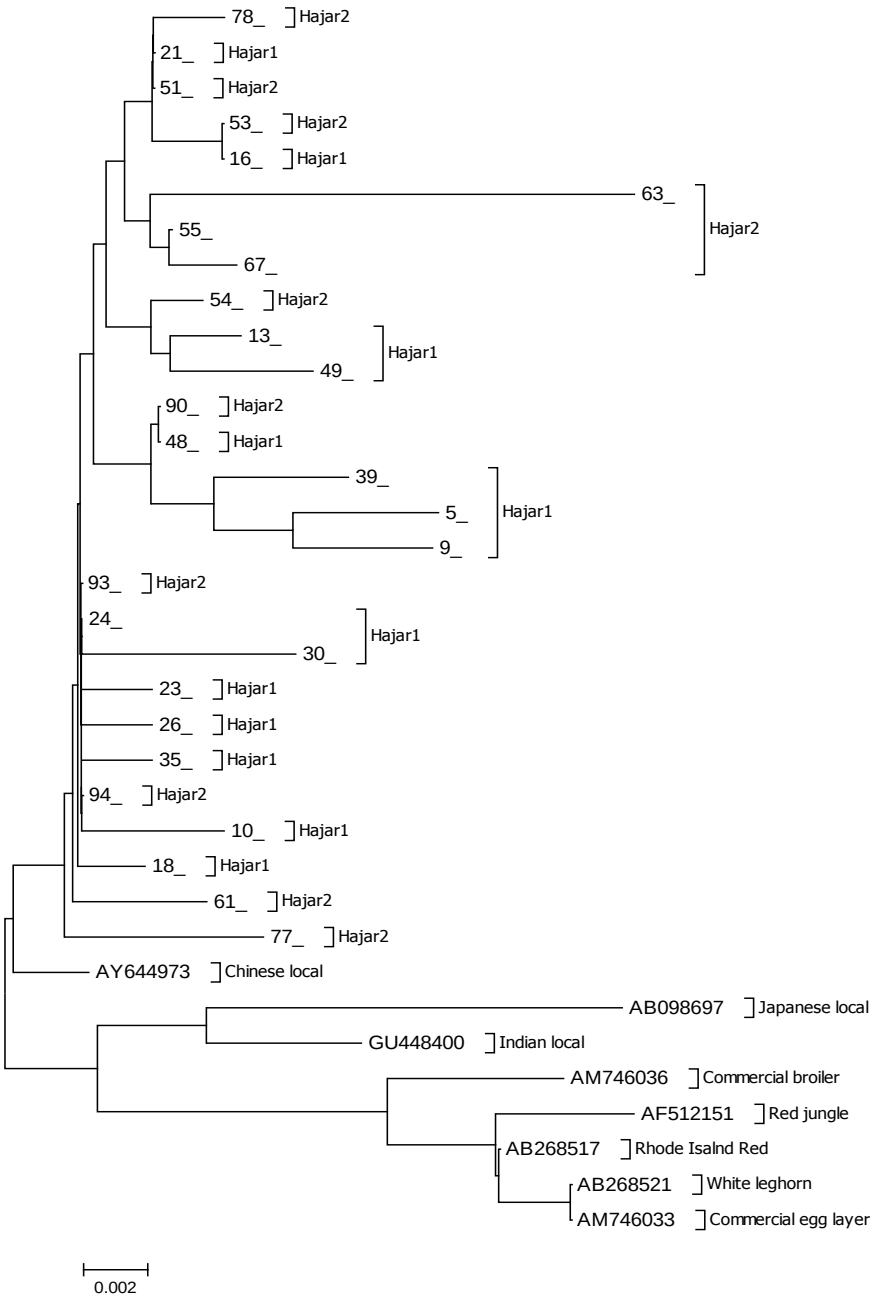


Figure 2 Neighbour-joining tree obtained from Hajar1, Hajar2 Saudi chicken lines and eight reference chicken populations

* To construct the neighbour-joining (NJ) tree each haplotype of local chicken lines was represented by a single sequence Accession numbers are shown next to reference populations

Conclusion

The current study emphasizes the importance of the mtDNA D-loop as a powerful tool for genetic analysis. There were haplotype variations within and between local Saudi chicken lines, which supported the reported phenotypic variation. Despite the unique conserved regions of each line, results indicated the close genetic relationship between the two lines based on partial mtDNA sequence variation. These two lines are

closely related to the referenced Asian local chicken population, especially Chinese and Indian chickens. To sustain and improve the genetic variation of the local Saudi lines, an appropriate breeding programme is required. Equally, further detailed molecular genetic studies are needed using alternate approaches to investigate different genetic locations.

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Authors' Contributions

KAA and AMS were in charge of lab work design and implementation at the Pests and Plant Diseases Unit Molecular facility. ASA was in charge of project design, statistical analysis, results interpretation and writing the manuscript.

Conflict of Interest Declaration

We wish to confirm that there is no known conflict of interest.

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