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A Comparable Genetic Diversity between Chicken Ecotypes of Different Zones using DNA Barcoding

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Abstract
Objectives: The purpose of the current study was to verify the reliability of COI bar-codes in the assessment of genetic diversity of two ecotypes from different ecozones.
Methods: The DNA sequences of cytochrome oxidase I (COI) barcodes of 50 hens belonging to two ecotypes of Ismalia Egypt (ISM) and Taif Saudi Arabia (TA) were isolated and analyzed.
Results: This study results showed that no noticeable great differences among all barcode's sequences of both ecotypes. The average length of both ecotypes was 589 bp. ISM ecotypes have a relatively wider length range. The overall mean of GC% content was 48 ± 0.01. Both ecotypes have the same number of sites 548 bp. ISM ecotype has 523 monomorphic sites whereas TA ecotype has slightly fewer monomorphic sites 517. The ISM ecotype has 7 singleton sites and 18 Parsimony informative sites. TA ecotype has little more polymorphic, that is 12 singleton sites and 19 Parsimony informative sites. The number of mutations (η) was larger in ISM (46) compared to 38 mutations for TA ecotype. Both ecotypes had the same number of Haplotypes (25), and haplotypes diversity (1) as well as the variance of haplotype diversity.
Conclusion: These results indicated a comparable level of genetic diversity of both ecotypes, which in turn may refer to a similarity of evolutionary forces that affect both ecotypes. Based on the present results, COI gene can be used in barcoding. The COI provides an objective the foundation for identification of ecotypes and therefore could be used for a rapid establishment of a variety of identifications.
Key words: DNA barcoding, haplotype diversity, chicken ecotypes

Introduction

In many economically developing nations chickens' ecotypes (*Gallus Gallus domesticus*) are an important asset for rural smallholders. This importance is owing to the fact of limited production inputs, scavenging competency (i.e. birds get the foremost part of their daily ration by scavenging) as well as acclimatization to tough and exhausting environmental circumstances.[1,2] For example, in most of Africa rural poultry (e.g. ecotypes) alone supplies 70% of poultry goods and 20% of animal protein intake.[3] Ecotypes are outputs of years of natural selection under stringent conditions, consequently, ecotypes turned out to be immune to many diseases especially bacterial, and protozoic as well as endoparasites and ectoparasites. These ecotypes survive better than the commercial hybrid strains under such harsh production conditions. Still, ecotypes are characterized by its low egg productivity and light mature body size.[4,5] On the other hand, the world-wide poultry industry is strategically based on commercial chicken breeds. These commercial breeds are a product of a small number of chicken genotypes. Such strategy has a drawback effect of casting away ecotypes. Therefore, ecotypes are negatively selected regardless their good quality of egg and meat, disease resistance as well as adaptation to local environment. As a consequence, these ecotypes are under threat of extinction. Setting up of frame for conserving these genetic resources is of massive need.[1,6–9] Many studies addressed the genetic makeup of ecotypes. Msoffe et al., 2001;[10] Tadelle et al., 2003;[11] Muchadeyi et al., 2007[12] and Rudresh et al., 2015[13] and have been used as an example in biodiversity studies.[14]

At Taif governorate (≈ 1.7 km above sea level) indigenous chickens are adapted to the coarse environment of high altitude. This coarse environment is known for extraordinary natural conditions, such as low air oxygen percentage, partial reduction of oxygen pressure, as fluctuated daily temperature.[15,16] These indigenous chickens are an outcome of many years of acclimatization which in turn are representing a valuable genetic resource.

Mitochondrial DNA mtDNA (aka DNA barcoding) has been widely used to differentiate among and within species.[17,18] DNA barcoding is a short string of uniform genomic region and each type has a specific barcode. DNA barcoding is based on the principle that determining a specific sequence for a particular gene that distinguishes between individuals of a species because of the genetic variation between species exceeds the genetic variation within a species.[19] The genes most commonly used for species identification are cytochrome b (Cyt b).[20] It has also been reported that the mtDNA cytochrome c oxidase I (COI) gene could be used as barcoding for most animals and fishes.[20] The expected growth in COI data recently has led to the use of a dedicated barcoding section to propagate the COI sequence, paving the way for the COI gene to become a major tool for taxonomic identification. According to recent reports, approximately 600 base pairs (bp) part of the 1 subunit of mitochondrial cytochrome oxidase (COI) could be a good choice for the coding gene because it may be involved in most animal species (Roe & Sperling, 2007;[21] Bondoc & Santiago, 2012[22]) used COI to differentiate 31 domestic chicken breeds and strains (*Gallus gallus domesticus*) and 25 red jungle fowls (*Gallus*

gallus philipensis Hatchisuka) in the Philippines. Results of this study indicated that use of DNA barcodes can effectively distinguish chicken breeds and strains, but not differentiate nor identify commercial hybrid chicken.

Osaman et al. (2016)[23] devised the complete sequence of mitochondrial DNA D-loop to clarify the origin of Egyptian native chicken and Asian chicken. Results of this study revealed that both Egyptian native chicken and West and Central Asian chicken are sharing the same common ancestor as they cluster together in the same clade. These results imply that both Saudi and Egyptian native breeds are genetically closer to each other. Inspired by the outcomes of Osaman et al. (2016),[23] the purpose of the current study was to verify the reliability of COI bar codes in identifying native Saudi chickens. In this study, differences in the selected COI gene and population genetic structure of four local Saudi chicken breeds were investigated. The genetic diversity of these chicken breeds has also been studied using the COI gene as a DNA barcode.

Materials and Methods

Collection of Blood Samples & DNA Isolation

Fifty blood samples of native chicken from two locations (Ismailia, Egypt and Taif, Saudi Arabia) were collected (twenty-five of each). Then, the genomic DNA was isolated from each blood sample using Qiagen DNase kit (Germany) as described by the producer's directions Khan et al., 2019.[24] DNA samples were stored at –20°C for use after concentration test with UV spectrophotometer.

COI Gene of mtDNA Amplification

A total of 415 bp of COI gene of mtDNA was amplified using two universal primer sets (BirdF1 and BirdR1 according to Amer et al. (2013).[25] The sequence of the forward primer was 5′-TTCTCCAACCACAAAGACATTGGCAC-3′ while that of the reverse primer was 5′-ACGTGGGAGATAATTC-CAAATCCTG-3′. The reaction was performed in 50 µl of total volume consisting of 12.5 µl GoTaq buffer master mix from Promega (USA), 25 ng of template DNA, 0.5 µl of each amplification primers and up to a final desired volume with deionized distilled water. The PCR thermocycler protocol was achieved as reported previously.[26]

Sequencing, Purification, and Data Analysis

Cycle sequencing of all samples was carried out in a total reaction volume of 20 µl using BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction-100 mix (Thermo Fisher Scientific, Applied Biosystems), BigDye®.Terminator v1.1 and v3.1 × 5 Sequencing Buffer (Thermo Fisher Scientific, Applied Biosystems), forward/reverse primers, and 50–70 ng/µl purified PCR product (Gel extracted DNA) on 2720 thermal cycler (Applied Biosystems). Cycle sequencing conditions consisted of 95 for 5 min, followed by 32 cycles of 95°C for 20s, at 55°C for 15s, and at 60°C for 4 min. All sequenced reactions were purified using Zymo Research DNA Sequencing Clean-up TM Kit (The Epigenetics Company, USA) and sequenced by capillary electrophoresis on an automated DNA sequencer (ABI PRISM 3500 Genetic Analyzer). All the raw sequences were curated and assembled using bioinformatics tools, namely, Sequencing Analysis 5.2 (Thermo Fisher Scientific, Applied Biosystem, India) and Clone Manager Suite 9 (Sci Ed Software, Westminster, Colorado, USA). All the consensus sequences were then aligned and trimmed using bioinformatics software, namely, CLUSTALW and Bio Edit Sequence Alignment Editor for the haplotyping analysis. The haplotyping was done using bioinformatics software DnaSP v6.12.03,[27] considering G. gallus as reference.

20 µl using BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction-100 mix (Thermo Fisher Scientific, Applied Biosystems), BigDye®. Terminator v1.1 and v3.1 × 5 Sequencing Buffer (Thermo Fisher Scientific, Applied Biosystems), forward/reverse primers, and 50–70 ng/µl purified PCR product.

Results and Discussion

The DNA sequences of cytochrome c oxidase I (COI) barcodes of 50 hens belonging to two ecotypes of Ismalia Egypt (ISM) and Taif Saudi Arabia (TA) were analyzed. Figure 1 shows the base frequencies of all sequenced barcodes. No great differences were noticed among all barcodes of all ecotypes.

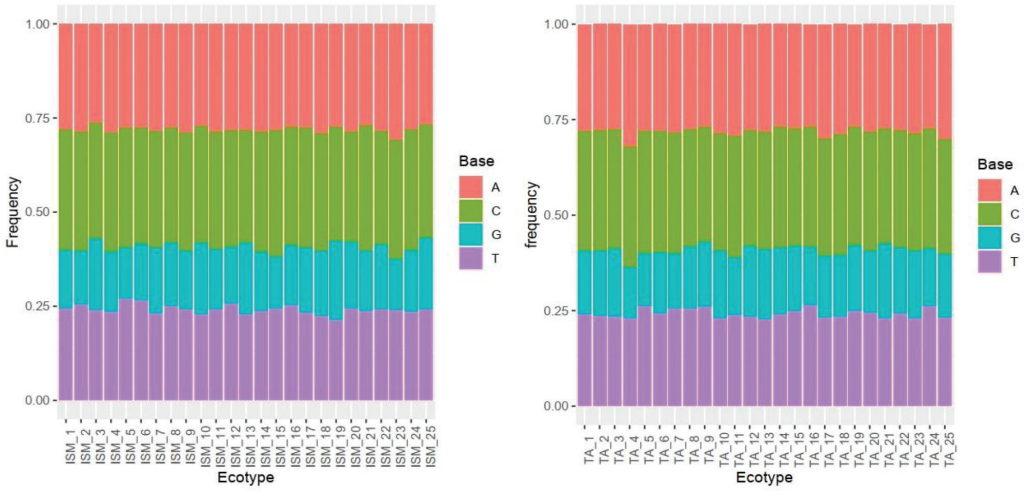

Fig. 1 **Base frequencies of Ismailia (ISA) and Taif (TA) ecotypes.**

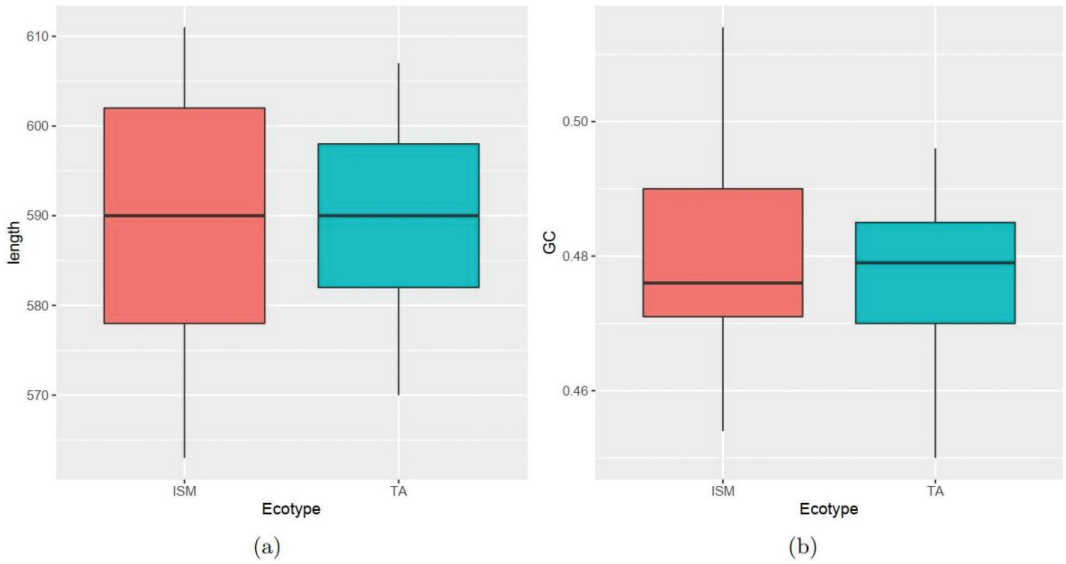

Fig. 2 **Boxplot of sequence length and percentage of GC content in Ismailia (ISA) and Taif (TA) ecotypes.**

COI sequence lengths and percentages of GC content for both ecotypes are presented in Figure 2. Results showed that the average length, after deletion of the primers' sequences, for both ecotypes was 589 bp. Although ISM ecotypes have a relatively wider length range (563–611 bp) compared to TA ecotype (570–607 bp). The average sequence length was shorter than what was reported by Xun-he et al. (2016)[28] on Chinese indigenous chickens, wild jungles and mallard (695) as well as, Peng et al. (2019)[29] on Chinese local and imported chicken breeds (650 bp). Cui et al. (2017)[30] analyzed bar1 and bar2 of COI barcodes sequence for 4 different Chinese native chicken breeds (16 individuals/breed), sequence length averaged 590 bp and 624 bp. Dave et al. (2021)[31] found that the sequence length of cytochrome c oxidase subunit I length for two native Indian chicken breeds averaged 608 and 756 bp. However, it is also important to note that due to the fact that the molecular evolution rate fluctuate among various segments of the genome and across taxa, there no species-specific standard sequence length.[32]

The overall mean of GC percentage was 48 ± 0.01. The range of GC% for ISM (45–51) was slightly wider in comparison with TA (45–50).

To our surprise, both ecotypes have the same number of sites 548 bp (Table 1). Nevertheless, ISM ecotype has 523 monomorphic sites where TA ecotype has slightly fewer monomorphic sites 517. The ISM ecotype has 7 singleton sites and 18 Parsimony informative sites. The TA ecotype has little more polymorphic, that is 12 singleton sites and 19 Parsimony informative sites. These results might indicate TA ecotype had a slightly higher genetic diversity. Nevertheless, our findings are much higher than what reported by Huang et al. (2016b)[28] on 203 individuals of 10 indigenous Chinese chicken breads who reported only 110 sites of which 90 were singleton variable sites and the remaining 20 were parsimony informative sites. Although the number of sties reported by Huang et al. (2016b)[28] were very much lower than what were reported in the present study, which might be ascribed to the larger number of breeds that used by Huang et al. (2016b),[28] but the number of parsimony informative sites were relatively close. Though, Cui et al. (2017)[30] reported only 4 polymorphic sites on four Chinese native breeds. Dave et al. (2021)[31] found that the total number of sites of two native Indian chicken populations was 596 and 647 of which merely 3 and 4 polymorphic sites were detected. Yu-Shi et al. (2011)[33] on 26 individuals of two newly discovered chicken breeds found only 10 variation sites, of which only 6 sites were single polymorphism sites while 4 were simple information sites.

Table 1. **Number of sites, monomorphic and polymorphic sites for ISM and TA ecotypes**

Number of sites	Monomorphic sites	Polymorphic sites Singleton sites Parsimony informative
ISM 548	523	7 18
TA 548	517	12 19

Parameters of DNA polymorphism for ISM and TA ecotypes are shown in Table 2. The number of mutations (η) was larger in ISM ecotype (46) compared to 38 mutations for TA ecotype. However, Huang et al. (2016b)[28] found that (η) ranged from 11 to 22 mutations sites in 10 Chinese indigenous chickens' breeds. This smaller number of mutations sites might be attributed to smaller number of sample size in that study, as sample size ranged from 18 to 23.

Haplotype diversity (Hd) and nucleotide diversity (π) are both of principle importance for assessment of genetic diversity of either population or breed.[31] Haplotype diversity implies the distinctiveness of a certain haplotype in a particular population. Therefore, the higher Hd's mean and π the richer genetic diversity in population. Haplotype diversity refers to the presence of specific haplotype in a particular population (Yu- Shi et al., 2011;[33] Cui et al., 2017[30]). Both ecotypes had the same number of Haplotypes (H = 25), and haplotypes diversity (Hd = 1) as well as variance of haplotype diversity (Table 2). These results indicated to a comparable level of genetic diversity of both ecotypes, which in turn may refer to a similarity of evolutionary forces that affecting both ecotypes. Cui et al. (2017)[30] found that number of haplotypes only

Table 2. **Parameters of DNA polymorphism in ISM and TA ecotypes**

No. of mutations *n*	No. of haplotypes (H)	Haplotype (gene) diversity, (Hd)	SD haplotype diversity
ISM 46	25	1	0.01
TA 38	25	1	0.01

ranged from 2 to 8 in two Chinese native chicken breeds, while Yu-Shi et al. (2011)[33] found seven kinds of haplotypes on newly discovered Chinese chicken breeds. Huang et al. (2016b)[28] defined 84 different haplotypes on from 203 individuals of 10 indigenous chickens in China. Our estimates of Hd were higher than what reported by Huang et al. (2016b)[28] (0.83), Cui et al. (2017)[30] (0.84) where Yu-Shi et al. (2011)[33] found Hd ranged from 0.3 to 0.9. On native Indian breeds, Dave et al. (2021)[31] estimated Hd of 0.34 and 0.93, and Yu-Shi et al. (2011)[33].

Table 3 shows nucleotide diversity (π), the average number of nucleotides differences (k), the total variance of nucleotide differences (free mutations) for ISM and TA ecotypes. Again, no differences were noticed between these two ecotypes for these three parameters. Once more, the equality of these parameters is an emphasis of what we stated earlier of similarity of evolutionary forces that affect both ecotypes regardless of the ecozones. The estimates of the present study were higher than what Yu-Shi et al. (2011)[33] estimated on newly discovered Chinese chicken breeds (0.004). Our estimates for (π) were moderate to what was reported by Dave et al. (2021)[31] (0.228 & 0.0023), as well as Cui et al. (2017)[30] on Chinese native chicken breeds, where Chin π ranged from 0.00102 to 0.00305.

Table 4 shows the Start and End of conserved regions of both ISM and TA ecotypes as well as the conservation, homozygosity, and *P*-value of each conserved region. Conservation is measured as the proportion of conserved sites in the alignment region, homozygosity is defined as (1-Heterozigosity), where *P*-value was estimated under the hypergeometric distribution.[34] The ISM ecotype has 2 conservative regions compared to only one for TA ecotypes. No differences were found in conservation value for all the conservation regions.

Table 3. **Nucleotide diversity, average number of nucleotide differences (k), total variance of nucleotide differences (free mutations) for ISM and TA ecotypes**

	Nucleotide diversity *n*	Average no. of nucleotide differences (k)	Total variance of k (free recombination), V(k)
ISM	0.02	8.4	3.0
TA	0.02	8.3	3.0

Table 4. **Conserved regions, conservation, homozygosity and *P*-values of ISM and TA ecotypes**

Start-End	Conservation	Homozygosity	*P*-value
	ISM		
1–215	1.0	1.0	<0.001
217–488	1.0	1.0	<0.001
	TA		
1–495	1.0	1.0	<0.001

Equal estimates of conservation and homozygosity values were attained for all conservation regions in both ecotypes (1.0). All *P*-value were smaller than 0.05 ranged from <0.001 to 0.04.

Conclusion

This is the first diversity study to use COI markers of two ecotypes in Egypt and Saudi Arabia. Based on the present results, COI gene can be used in barcoding. The COI provides an objective foundation for the identification of ecotypes and therefore could be used for a rapid establishment of a variety of identifications. The results of the present study showed that both ecotypes had a comparable level of genetic diversity. Therefore, it could be concluded that the similarity of evolutionary forces affects both ecotypes.

Conflict of Interest

None.

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