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RESEARCH ARTICLE

Genetic characterization of Red Junglefowl (*Gallus gallus gallus*) and commercial chicken lines (*Gallus gallus domesticus*) using molecular markers

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Abstract

Molecular markers such as restriction fragment length polymorphism (RFLP) and DNA fingerprinting (DFP) have so far proved to be useful in establishing genetic relationship among the livestock populations including poultry. The present study was conducted to evaluate the genetic characterization and relationships of Red Junglefowl (*Gallus gallus gallus*) and commercial chicken lines (*Gallus gallus domesticus*) using PCR-RFLP Technique. Samples belonging to one subspecies of Red Junglefowl and three commercial lines, White broiler (White domesticated fowl; Red broiler (Red domesticated fowl), Sasso broiler (Sasso domesticated fowl), were studied. The electrophoresis analysis of PCR amplification of studied chickens shows DNA bands at 412 bp., 465 bp., 546 bp., 650 bp. and 666 bp. as dominant PCR products for primers P1, P2, P3, P4 and P5 respectively. The results of this study revealed that there is a wide intraspecific COI (Cytochrom Oxidas I), SEMA3E (Semaphorin-3E) and NR2E1 (TLX) (Nuclear receptor subfamily 2 group E member 1) variability among these chickens using restriction enzymes *Nla*III where this enzyme produced different restriction fragments in white, Red and Sasso broilers and did not show any fragments in Red Junglefowl except two primers P4 and P5. It was confirmed through the present study that the RFLP is a valuable tool to evaluate genetic variation of the studied chickens.

INTRODUCTION

Chicken (*Gallus gallus domesticus*) is a domesticated fowl, a subspecies of the Red Junglefowl and is raised all over the world for its delicious meats and eggs. The Red Junglefowl (*Gallus gallus gallus*) is a tropical member of the Phasianidae family. Native breeds are considered as a national asset and a key factor in creating sustainable agriculture in developing countries. Therefore, precise assessment of such native genetic resources is of great importance and could be utilized for the purpose of their conservation, management, reproduction and exploitation (Shahbazi *et al*., 2007).

Amongst the poultry species, chicken is the most extensively exploited species to meet the demands of egg and meat production. However, in order to meet the present diverse demand for poultry products, there is a need to explore the other poultry germplasms, which in fact, has received rather less attention such as indigenous chicken in Egypt. The rapid development in modern molecular genetics has given rise to many new nucleic acid fingerprinting techniques. Molecular markers such as restriction fragment length polymorphism (RFLP) and DNA fingerprinting

(DFP) have so far proved to be useful in establishing genetic relationship among the livestock populations including poultry (Hillel *et al*., 1992).

The previous diversity studies of the local chickens reported were based mostly on morphological characterizations, including adult body weight, egg weight, reproduction performance and immune responses to various diseases (Gueye, 1998; Msoffe *et al*., 2001; 2004).With millions of species and their life-stage transformations, the animal kingdom provides a challenging target for taxonomy. Recent work has suggested that a DNA-based identification system, founded on the mitochondrial gene, cytochrome *c* oxidase subunit 1 (COI), can aid the resolution of this diversity (Hebert *et al*., 2003). DNA barcoding, the molecular characterization of species using a standardized DNA region, is a potentially effective tool for identifying species across the eukaryotes of the world (Frezal and Leblois 2008).

For animals, a 648-bp. fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) has been chosen as the standard barcoding marker due to its high interspecific variation, low intraspecific variation, and relatively universal primers for taxonomic groups at the level of orders and even classes (Hebert *et al*., 2003). The *SEMA3E* gene encodes a secreted class 3 semaphorin that triggers repulsion of endothelial cells in specific vascular beds and modulates axonal growth and synaptic connectivity for the correct wiring of the CNS (Cariboni *et al*., 2015). TLX as a transcription factor that is expressed in early embryonic development and adult stem cell niches. TLX is an important target in neural development by regulating cell cycle progression in NSCs (Neural stem cells) by recruiting histone deacetylases (Song *et al*., 2015). Moreover, data on genetic variation can provide crucial input to plan effective strategies for conservation and rehabilitation of natural populations. The present study examined the relationship and genetic characterization of four chickens using RFLP-PCR. The result aimed at providing essential information for use, collection, conservation and research of the entail genetic resources.

MATERIALS AND METHODS

Chicken strains and sample sizes

Twenty-eight samples belonging to one subspecies of Red Junglefowl (n = 7), and three commercial lines, White broiler (White domesticated fowl), n = 7; Red broiler (Red domesticated fowl), n = 7; Sasso broiler (Sasso domesticated fowl), n = 7 were studied.

DNA extraction

Tissue samples (10 mg) were taken from each chicken and DNA was isolated as described in Aljanabi and Martinez (1997) and modification by Hassab El-nabi and Elhassanin (2008).

RFLP-PCR Primers

Five primers were used in this study, the code and sequences of these primers are shown in table (1).

PCR-RFLP

PCR was carried out in 25 µL reaction volume. Amplification process was started by adding approximately 100 ng isolated DNA accompanied by 10 pmole of each forward and reverse primer to Ready-to-go PCR beads tube. The mixture was then added by H_2O tube and mixed by gentle flicking followed by vortex and short spin centrifugation. The PCR tubes were then put into thermalcycler machine and programmed for predenaturation at 95 °C for 5 min, 30 cycles of chain reaction using the following condition: denaturation at 95 °C for 1 min, annealing at the corresponding specific temperature (Table 1) for 1 min and extension at 72 °C for 1 min, followed by post extension step at 72 °C for 5 min. The PCR product was analyzed with 2% agarose gel electrophoresis and estimated its concentration by means of UV spectrophotometer to predict volume of PCR product needed for RFLP.

RFLP by digestion with *Nla*III

For *Nla*III digestion, approximately 0.5 ng PCR fragment was used. The digestion was started by mixing PCR fragment with 2 µL *Nla*III buffer 10x, 20 U of *Nla*III enzyme and added with nuclease free water to final volume 20 µL. The mixture was incubated at 37 °C for 24 h., then electrophoresed in 2% agarose gels and stained with ethidium bromide for visualization.

Data analysis

Only the reproducible and intense bands were scored to maintain the consistency across the samples of different populations. Bands observed in each lane were compared with all the other lanes of the same gel and reproducible bands were scored as present (1) or absent (0). Data was analyzed using the MVSP software package version 3.1 (Kovach, 1999). A dendogram was constructed by unweighted pair-group method of arithmetic average (UPGMA).

RESULTS

PCR-RFLP

The electrophoresis analysis of PCR amplification of chickens can be seen in Fig. 1a each sample show DNA fragments with size of approximately 412 base pairs (bp.) as dominant PCR product for primer P1. It means that PCR succeed to amplify part of COI gene in studied chickens. Although the size of the fragments in each sample is same, they have different nucleotide sequenced. Meanwhile the electrophoresis of digestion product of PCR fragment with digestion enzyme can be seen in Fig. 2a. The results revealed that digestion enzyme produced two fragments, one of 80 bp. and another of 332 bp. in white broiler. Instead in Red broiler two fragments was obtained, one of 50 bp., 362 bp. In addition, performed digestion with *Nla*III of Sasso broiler obtained the same restriction band patterns of one of 66 bp., 346 bp. on the other hand, a different restriction profile with one fragment in Red Junglefowl of 412 bp., this means that no digestion of restriction enzyme occur in these chickens.

In the current study, a single amplification product of approximately 465 bp. as showed in Fig. 1b for primer P2 in each chicken was obtained. Digestion of this fragment with *Nla*III produced highly polymorphic intraspecific and interespecific restriction profiles, when analyzed individuals from four chickens. White broiler was characterized by two fragments, one of 50 bp. and another of 415 bp. Instead in both of Red and Sasso chickens two fragments for each one were obtained, one of 110 bp. and 355 bp. while, no digestion of enzyme occurs in Red Junglefowl and only one fragment of 465 bp. characterizes restriction profile (Fig. 2b).

For primer P3, a single amplification product of approximately 546 bp. in each chicken was obtained (Fig. 1c). Digestion of this fragment with restriction enzyme produced two fragments in White broiler, one of 211 bp. and another of 335 bp. In Red and Sasso broilers two fragments were obtained, one of 200 bp. and 346 bp. in each broiler. No digestion of enzyme occurs in Red Junglefowl and only one fragment of 546 bp. characterizes restriction profile (Fig. 2c).

A single amplification product of approximately 650 bp. for primer P4 in each chicken was indicated in Fig. 1d. In White broiler no digestion of enzyme occurs, only one fragment of 650 bp produced, while in Red broilers three fragments were obtained, one of 70 bp., 110 bp. and 470 bp. in each broiler. Also three fragments were obtained in Sasso broiler one of 110 bp., 260 bp. and 280 bp. For this primer, digestion of enzyme in Red Junglefowl produced three fragments, one of 50 bp., 150 bp. and 450 bp. (Fig. 2d).

In the studied chickens, PCR product of approximately 666 bp. was obtained for primer P5 (Fig. 1e). Digestion of this fragment with NlaIII produced three fragments in White broiler of 80 bp., 120 bp. and 466 bp. In Red broiler three fragments were obtained, one of 70 bp. and 100 bp. and 496 bp. Instead, three fragments characterize the restriction profile of Sasso broiler one of 50 bp. and 100 bp. and 516 bp. In Red Junglefowl two fragments were obtained, one of 150 bp. and 516 bp. (Fig. 2e).

Genetic relationship and phylogenetic tress

An unweighted pair-grouping method analysis (UPGMA) tree based on Nei's unbiased genetic distance matrices revealed that Red broiler was most closely related to sasso broiler. White broiler fall also in the same clade of the two previous chickens, and closely related to red than sasso broilers. On the other hand, red junglefowls were in a separate group with a node connected to the three studied broilers. In addition, in the dendrogram there are two separate genetic groups, one group formed by red junglefowls and the other group formed by Red, Sasso, and White broilers (Fig. 3).

Table 1: primers and primer sequences used for amplification and sequencing in this study.

No.	Primer code	Gene name	Nucleotide sequence (5` to 3`)	Annealing temp. °C
1	P1	*SEMA3E*	Forward-ATACTCCAGCTGAGTGGGGA	60
			Reverse-CAGAAGTATGAGGGAGATCAG	
2	P2	COI	Forward- CGAATGAATAACATAAGCTT	59
			Reverse- CC TCC TGCAG GGTCGAAGAA	
3	P3	TLX	Forward-ACACTAGGAACATAATGGGCT	57
			Reverse-TCACTGTGGCGTTTCAGATT	
4	P4	COI	Forward-GCACAGGATGGACAGTTTAC	55
			Reverse-ATAGCATAGGGGGGTCTCAT	
5	P5	COI	Forward-TTCTCCAACCACAAAGACATTGGCAC	58
			Reverse-ACGTGGGAGATAATTCCAAATCCTG	

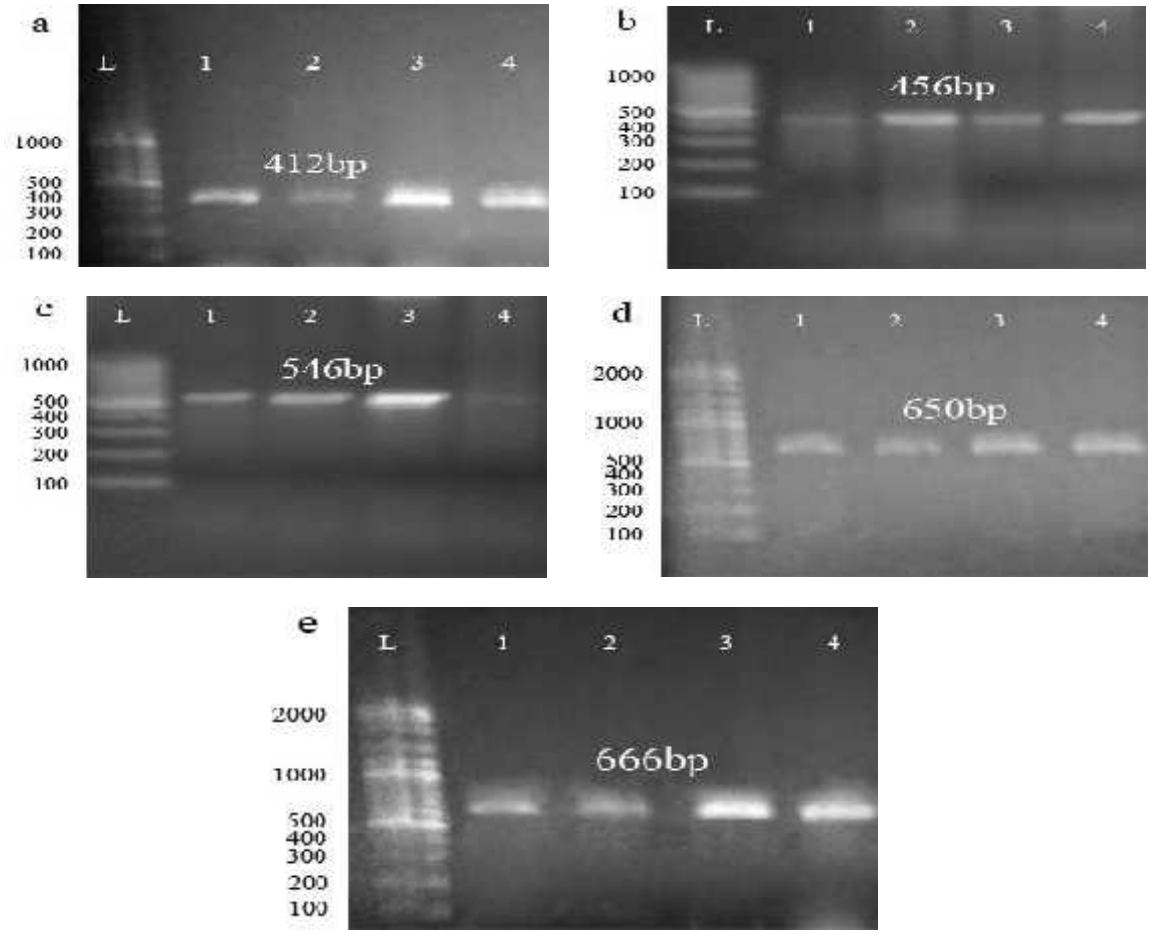
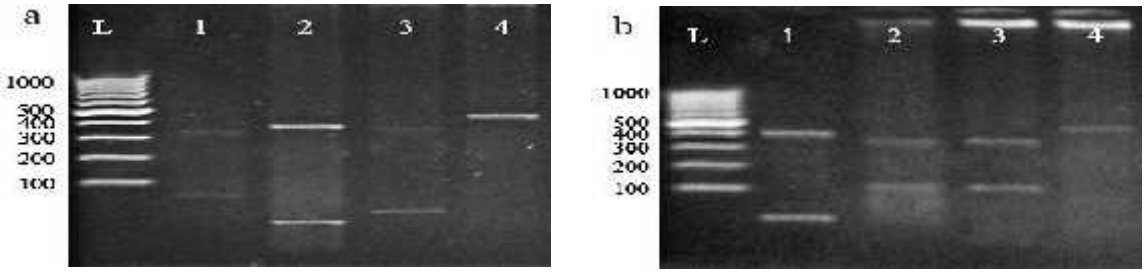


Fig. 1. Polymerase chain reaction based restriction fragment length polymorphism profiles of mitochondrial cytochrome c-oxidase subunit I genes (a: primer P1, b: primer P2, c: primer P3, d: primer P4 and e: primer P5) of chickens. Lane L: DNA marker, lane 1: White broiler, lane 2: Red broiler, lane 3: Sasso broiler and lane 4: Red Junglefowl.



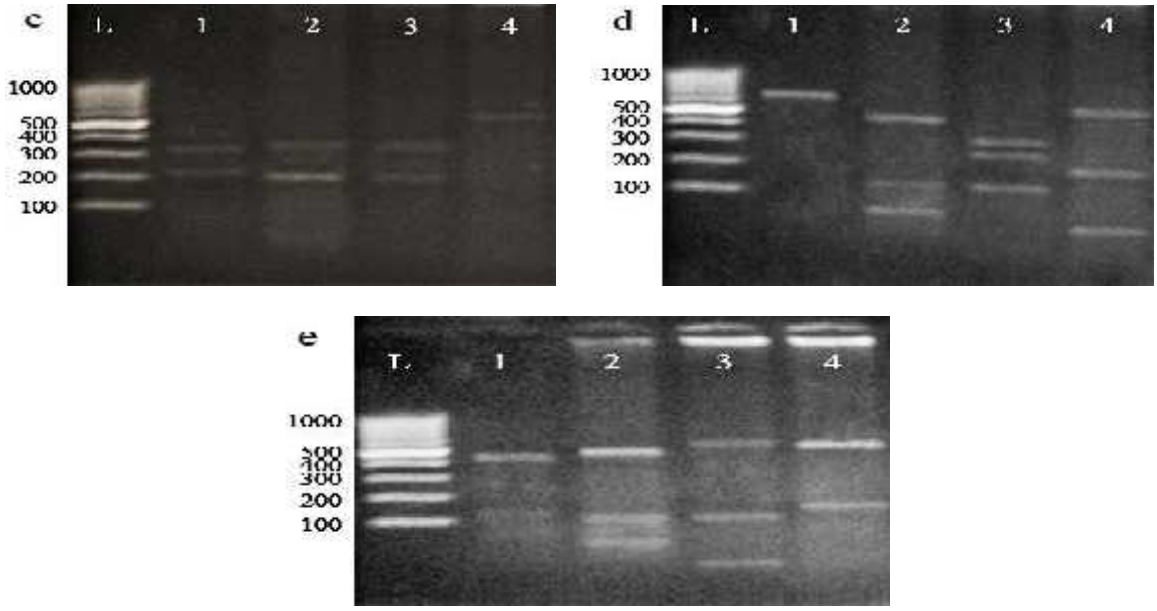


Fig. 2. Polymerase chain reaction based restriction fragment length polymorphism profiles of mitochondrial cytochrome c-oxidase subunit I genes (a: primer P1, b: primer P2, c: primer P3, d: primer P4 and e: primer P5) of chickens digested with restriction endonuclease *NlaIII*. Lane L: DNA marker, lane 1: White broiler, lane 2: Red broiler, lane 3: Sasso broiler and lane 4: Red Junglefowl.

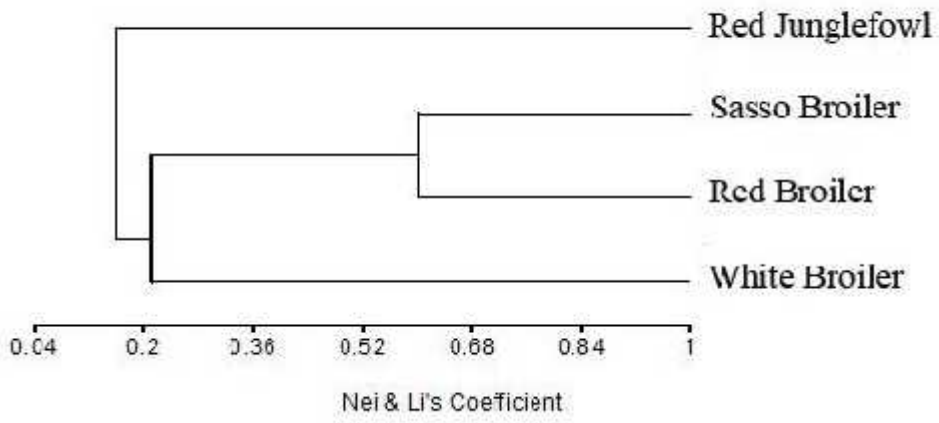


Fig. 3. Dendrogram constructed using UPGMA cluster analysis from MVSP software of four studied chickens.

DISCUSSION

Divergence levels between closely related species were highly variable, ranging from approximately 0-16%; however, some of these values may be inflated for under sampled genera and families. Recent studies have detached rate variation in the mitochondrial genome from factors such as population size, body size, and other life-history traits (Bazin et al., 2006; Nabholz et al., 2008; Nabholz et al., 2009). While some authors contend that rate variation in birds is highly irregular (Nabholz et al., 2009), a recent thorough review demonstrated relatively minor variation andupheld the occurrence of clock-like evolution (Weir and Schluter, 2008).

Consequently, we attribute the limited divergence between some sister species to recent speciation events. Studies documenting recent and rapid diversifications often address subspecific variants rather than full species (Milá *et al*., 2007a and Milá *et al*., 2007b). Still, low sequence divergence does not necessarily indicate that species should be synonymised (Joseph and Omland, 2009). Low sequence divergence is particularly common in super

species complexes, including those divided between continents, but the species within them remain valid units for both ecological studies and conservation.

A method based on the amplification of part of mt DNA-COI region was successfully used, followed by its digestion with restriction enzymes. In as much as the advantages of mithocondrial DNA applicability on systematic and phylogenetic studies for several organisms, including snails (Casiraghi *et al*. 2001, Clark *et al* .2001, DeJong *et al*. 2001), are considerable.

The analysis of generated fragments (RFLP), aimed at obtaining bands to enable genetic characterization of the red junglefowl, Red, White and Sasso chichens was carried out in this study. Also a digestion using one restriction enzyme was performed. *Nla*III digestion produced highly polymorphic intraspecific and interespecific restriction profiles, this enables the differentiation of these chickens and there is wide intraspecific genes (COI, SEMA3E and NR2E1) variability among studied chickens where this enzyme produced restriction fragments in some chickens and did not show any fragments in the red junglefowl except two primers P4 and P5. This differentiation may be providing essential information for use, collection, conservation and research of the entail genetic resources.

Genetic relationship and phylogenetic trees

The unweighted pair-grouping method analysis (UPGMA) tree constructed from PCR RFLP data showed that the red junglefowl, White, Red, Sasso and Red chickens belonged to different populations. (Tunim *et al*., 2010). The findings of this results indicated that red junglefowls should be classified as being genetically close to these three studied broilers; they also showed that Red broiler and Sasso broiler were related to White broiler and fall genetically far from red junglefowls. The phylogenetic tree showed that Sasso chickens and Red chickens were sufficiently genetically similar to suggest that Sasso chickens may be suitable for meat production. The Red Junglefowl may be particularly valuable as a source of genetic variability because it is close to the root of the phylogenetic tree.

In conclusion, evaluating genetic characterization might be useful for conservation of chickens as a genetic resource and natural monument. It was confirmed through the present study that the RFLP is a valuable tool to evaluate genetic relationships of chickens.

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