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Molecular Markers Associated with the Development of New Phenotypes of Japanese Quail in Egypt

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ABSTRACT

In this report, genomic variation within four isolated phenotypes of Japanese quail (*Coturnix japonica*) in Egypt were investigated using two different molecular marker systems which target different genomic regions: RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat). Different dendrograms were constructed based on RAPD and ISSR results, individually and collectively, revealing that similarity and clustering is highly dependent on the marker system used.

Keywords: *Coturnix japonica*, ISSR, marker-assisted selection, RAPD

INTRODUCTION

Japanese quail, as a poultry bird, is appreciated for its meat and eggs and is a valuable laboratory species in Egypt and all over the world because of its small body size, rapid generation interval and high prolificacy (Roussot *et al*. 2003). Prior to 1939, Egypt exported as many as 3 million captured live quail (Woodard *et al*. 1973). Japanese quail is found in Japan, Korea, Eastern China, Mongolia and Sakhalin as migrating birds (Mizutani 2003). Since its immigration to Egypt new breeds with new phenotypes have appeared. To improve quail production and its attributes as a poultry bird, different pheno- and genotypes are often produced and evaluated under different conditions (Salter *et al*. 1999; Amirinia *et al*. 2007; Vali 2007; Lee *et al*. 2008). Moreover, differences in behavior (e.g., endocrine fear responses) were studied in Japanese quail using motivational systems (Richard *et al.* 2008).

Japanese quail belongs to the family Phasianidae, order Galliformes (same as the chicken, *Gallus gallus*), genus *Coturnix*, species *japonica* (Nishibori *et al.* 2002), The scientific designation for Japanese quail is *Coturnix coturnix japonica* (Wakasugi 1984), now renamed *Coturnix japonica* (Crawford 1990), different from the common quail (*Coturnix coturnix*).

At the genomic level, both Japanese quail and chicken have a similar genome length and karyotype. Japanese quail karyotype is composed of 2n = 78 chromosomes, some of which are morphologically distinguishable macrochromosomes (1-8) and the ZW sex chromosomes, while the rest are indistinguishable microchromosomes (Ryttman and Tegelstrom 1981). Recent studies on Japanese quail microchromosomes showed many polymorphic heterochromatic segments which can be used for more in-depth genetic studies (Krasikova *et al*. 2009).

Microsatellite primers from *G. gallus* are not suitable to build a genetic linkage marker map in Japanese quail (Inoue-Murayama *et al*. 2003). The first genetic linkage map of Japanese quail was revealed using an AFLP marker system (Roussot *et al*. 2003), however, the first microsatellite linkage map of Japanese quail was developed one year later (Kayang *et al*. 2004).

Genetic analyses were performed in Japanese quail with plumage color with plumage three color mutations (Gunnarsson *et al*. 2007), which are sex-linked recessive roux color (ro, Ro+) (Cheng and Kimura 2003), autosomal extended brown (E, e+) (Somes 1980), white bib (imperfect albino) autosomal (wp, WP) (Roberts *et al*. 1978) and cinnamon (Minvielle *et al*. 2000). Plumage colour mutations and melanins in the feathers of Japanese quail are determined by single mutations in one of seven genes (Minvielle *et al*. 2009). A recent study describes the causal mutation microphthalmia-associated transcription factor (MITF) gene responsible for the "silver" plumage color in Japanese quail which is associated with effects on growth and body composition (Minvielle *et al*. 2010).

Roux quails, which have characteristics similar to those reported for the albino mutation, and brown quail have similar plumage color, but roux quail is paler (Minvielle *et al*. 1999). Roux color in Japanese quail is associated with a single-nucleotide substitution in the *TYRP1* gene (Nadeau *et al*. 2007). Three plumage color loci have been reported on the Z chromosome. The first locus (AL) has two kinds of mutations: imperfect albinism (Lauber 1964; Sittmann *et al.* 1966; Somes 1988; Gunnarsson *et al*. 2007) or sex-linked white (Homma *et al*. 1968; Wakasugi and Kondo 1973; Gunnarsson *et al*. 2007), and red-eyed brown (Wakasugi and Kondo 1973; Nadeau *et al*. 2006) or cinnamon (Homma and Jinno 1969; Truax and Johnson 1979; Somes 1988; Minvielle *et al*. 2009). The second locus has only one mutation, brown (Homma 1968; Wakasugi and Kondo 1973; Somes 1988; Seabrook-Davison *et al*. 2009), which is caused by a recessive allele. Roux, another sex-linked plumage color mutation, was independently discovered in France and is reportedly controlled by a recessive gene (Somes 1988; Minvielle *et al*. 2003). Japanese quail has also emerged as an important model species for deepening an understanding of circadian clock genes and photoperiodism in poultry (Yasuo *et al*. 2003; Yoshimura group 2010) and for development of transgenic avian species (Poynter *et al*. 2009). Knowledge about genetic diversity and phylogenetic relationships among quail phenotypes could be an invaluable aid in its improvement strategies.

A number of methods are currently available for analyses of genetic diversity in the breeding of avian strains and populations. These methods have relied on performance,

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pedigree, morphological, biochemical and more recently DNA-based molecular data. With DNA-based markers, it is theoretically possible to exploit the entire diversity in a DNA sequence that exists in any cross (Dodgson *et al*. 1997) or within different population (Chang *et al*. 2005). Molecular typing methods provide an opportunity for a powerful and reproducible approach of estimating relatedness within and among genotypes based on DNA sequence variation. Different types of locus-specific markers are available, i.e., minisatellite, microsatellite (Gupta *et al*. 1994; Chang *et al.* 2005), AFLP markers (Chi *et al.* 2005), and random amplified polymorphic DNA (RAPD) markers (Sharma *et al*. 2000).

Microsatellites, also known as simple sequence repeats (SSRs), are tandem repeats of 1-6 bases in length that occur abundantly and at random in most eukaryotic genomes (Stallings *et al.* 1991) and show a codominance mode of inheritance. Microsatellites, which can be typed using the polymerase chain reaction (PCR), have made them the markers of choice in genome mapping and linkage analyses (Kim *et al.* 2007) as well as analyses of genetic diversity and evolution of quail genomes (Amirinia *et al.* 2007).

On the other hand, RAPD-PCR provides a powerful tool for identification of populations and detection of genetic variability within and among populations (Măruțescu *et al.* 2009). The procedure is relatively simple, fast, inexpensive and without requirement for target DNA sequence information (Zhang *et al.* 2002). However, the potential of the original RAPD assay to generate polymorphic DNA markers with a given set of primers was further increased by combining two (Klein-Lankhorst *et al*. 1991) and three sets of primers in a single PCR (Mansour *et al*. 2008).

Knowledge about genetic diversity and phylogenetic relationships among breeding quail could be an invaluable aid in improvement strategies of such poultry birds. In this investigation, we studied genomic variation within four isolated phenotypes of Japanese quail in Egypt using two different molecular marker systems, RAPD and ISSR (inter-simple sequence repeat) to reveal similarity and evolution of Japanese quail in its new habitat in Egypt.

MATERIALS AND METHODS

Quail genotypes

A total of four quail genotypes were used in the present study: Plumage color quail wild type, roux color quail (ro, Ro^+) (mutant type), brown color quail (E, e^+) (mutant type) and white bib autosomal (wp, WP) quail (mutant type). All bird samples were phenotypically normal and healthy. The studied quails were obtained from the Faculty of Agriculture, El-Azhar University.

Blood samples

Eggs were collected and incubated in a single lot for 2 weeks, but eggs from different families were separated in the incubator to prevent intermingling. Chicks were wing-tagged at hatching and placed for 10 d in two large group cages where the temperature was maintained at 35-37°C. They were then transferred to other group cages at 30°C and finally at 25°C, where they remained until breeding, at 28 d of age. After 35 days, chicks were randomly sampled within each type and placed in individual cages at 22°C, where they remained under a 14 h/d artificial lighting regimen until the end of the experiment, at the age of 120 d. Ten healthy individuals were recruited from each genotype, and blood samples were collected from the wing vein of individual birds into plastic tubes containing 0.1% EDTA solution (pH 8.0) as anticoagulant reagent and stored at -20°C until DNA extraction. Feed and water were provided throughout the experiment. Chicks received a mash starter diet (2901 kcal of ME/kg, 11.5% moisture, 7% ash, 27% total protein, 8% fat, and 4% crude fiber) *ad libitum*. Adults received a commercial diet (Sharkia Vetrenary supplies, Zagazig, Egypt) (2709 kcal of ME/kg, 11.5% moisture, 12% ash, 20% total protein, 4% fat, and 4% crude fiber).

Statistical analysis

All obtained data in the experiment was subjected to statistical analysis of variance according to Snedecor and Cochran (1967) and the comparison of means between cultivar data was performed using the LSD test at $P = 0.05$.

Genomic DNA extraction

DNA samples were extracted from blood samples, three from each selected individual, using the Promega Genomic Wizard kit following the manufacturer's instructions. Extraction was followed by treatment with RNase-A (Sigma, St. Louis, MO; R-4875) for 30 min at 37°C according to Mansour *et al*. (2008). The quality and quantity of extracted DNA was measured (2 µl) by a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Delaware, USA). DNA samples were adjusted to 50 ηg/µl with ddH_2O before PCR amplification.

RAPD-PCR reactions

Amplification reactions were performed, three times per sample, according to Williams *et al*. (1990) in 25 µl volumes. Briefly the reaction mixture containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 µM of each dATP, dCTP, dGTP and TTP (Pharmacia), 0.2 µl primer, 25 ng of genomic DNA, and 0.5 U of *Taq* DNA *Polymerase* (Promega). Amplification was performed in a Perkin Elmer 2400 Thermal Cycler programmed for 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 34°C, 2 min at 72°C, using the fastest available transitions between each temperature (ramp time), followed by one cycle of 72°C for 20 min and 4°C thereafter. The annealing temperature varied according to the melting temperature (™) for the primer (**Table 1**). The core program increased from 40 to 45 cycles, if amplification was weak, to get a slight increase in the amount of PCR products (Mansour *et al*. 2008).

Table 1 Sequence of the RAPD and ISSR primers applied

RAPD	Sequences (5′- 3′)
P1	GTA GAC CCG
P2	GGA CCC TTAC
P3	GTC GCC GTC A
P4	GGT CCC TGA C
P5	TGG ACC GGT G
P6	AGG GGT CTT G
P7	TTC CCC CGC T
P8	TTC CCC CCA G
P9	ACT TCG CCA C
P10	CAA TCG CCG T
P11	AGG GAA CGA G
P12	TGC GCC CTT C
P13	TTC GCA CGG G
P14	GTG AGG CGT C
P15	CAA ACG TCG G
P16	CTG CTG GGA C
P17	GTG ACG TAG G
P18	CCA CAG CAG T
P19	TGA GCG GAC A
P20	GTG AGG CGT C
ISSR	
814	$(CT)_8TG$ (#814)
844A	$(CT)_8AC$ (#844A)
844B	$(CT)_8G$ (#844B)
17898A	$(CA)_6AC$(#17898A)
17898B	$(CA)_6GT$ (#17898B)
17899A	$(CA)_6AG$ (#17899A)
17899B	$(CA)_6GG$ (#17899B)
HB8	$(GA)_6GG$ (#HB8)
HB9	$(GT)_6GG$ (#HB9)
HB10	$(GA)_6CC$ (#HB8)
HB11	$(GT)_6CC$ (#HB11)
HB12	$(CAC)_3GC$(#HB12)
HB13	$(GAG)_3GC$ (#HB13)
HB14	$(CTC)_3GC$ (#HB14)
HB15	$(GTG)_3GC$ (#HB14)

Table 2 Comparison of DNA marker systems in quail phenotypes.

Mean of band frequency	Polymorphism (%)	Average № of bands/primer	Gel polymorphism			№ of Primers	Marker system
			Polymorphic (with unique)	Unique bands	Polymorphic (without unique)		
0.361	85.31	20 ± 2	25 ± 2	17 ± 2	8 ± 2	20	Single-primer RAPD
0.333	94.10	10 ± 2	15 ± 2	13 ± 2	3 ± 2	15	ISSR
0.694		30	40	30	11	35	Total ≈

ISSR-PCR reactions

A set of 15 anchored microsatellite primers was procured from Metabion (Germany) (**Table 1**). PCR amplification was performed, three times per sample, according to Dangi *et al*. (2004). Briefly, 20 ng of DNA was added to 10 mM Tris-HCI pH 7.5, 50 mM KCI, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 2% formamide, 0.1 mM dNTPs, 0.3 µM primer and 0.8 U of *Taq* DNA *Polymerase* (Promega, USA) in a 25 µl reaction using a Perkin Elmer 2400 thermocycler. All the chemicals required for the reaction mixture were obtained from Sigma-Aldrich. After initial denaturation at 94°C for 5 min, each cycle consisted of 30 sec denaturation at 94°C, 45 sec of annealing at 50°C, 2 min extension at 72°C along with 5 min extension at 72°C at the end of 40 cycles. The annealing temperature varied according to the ™ of each primer. Moreover, the core program increased from 40 to 45 cycles, if amplification was weak, to get a slight increase in the amount of PCR products.

Gel electrophoresis

10 µl of amplified fragments were separated by agarose (1.6%) gel electrophoresis, stained with ethidium bromide (0.5 ηg/µl) at 80 V in 1X TBE buffer and photographed on a UV transilluminator (Pharmacia) by a Canon S5 digital camera with a UV filter adaptor. A negative control which contained all the necessary PCR components except template DNA was included in the PCR runs.

Fragment analysis

Fragment analysis was conducted according to Mansour *et al.* (2008). Briefly, amplification products were scored independently as 1 and 0 for the presence and absence of bands, respectively, and the obtained binary data were used for the analyses. Only sharp PCR fragments were scored (not "ghost"). Fragments at low intensities were only scored as present when they were reproducible in repeated experiments using GelAnalyzer 3 (Egygene) software. Unequivocally reproducible bands were scored and entered into a binary character matrix (1 for presence and 0 for absence). The genetic similarity among accessions was determined by Nei's genetic distance (Nei 1987) modified to accommodate dominant (e.g., RAPD-like) markers. A dendrogram was constructed based on the matrix of distance using the Unweighted Pair Group Method with Arithmetic averages (UPGMA). All the calculations were performed by using the NTSYS-pc 2.02 software package (Numerical Taxonomy System, Exeter Software) (Rohlf 2000). The statistical stability of the clusters was estimated by a bootstrap analysis with 1000 replications using Winboot software (Yap and Nelson 1996).

RESULTS AND DISCUSSION

Genetic diversity based on RAPD markers

In quail, many molecular markers techniques have been used extensively such as SSR to measure allele frequencies of eight microsatellite loci (Amirinia *et al.* 2007), microchromosomes, AFLP markers to detect fearfulness-related QTLs (Beaumont *et al*. 2005) and RAPD markers to detect polymorphism in various quail lines to measure variability within and between populations (Sharma *et al.* 2000).

In this investigation, single, random oligodeoxyribonucleotide primers were used to generate PCR-amplified fragments, RAPD markers, from the bulked genomic DNA of each genotype. The 20 primers that were initially screened (listed in **Table 1**) exhibited a high level of polymorphism among the bulked DNA samples and were therefore chosen for this study. These primers detected scorable polymorphisms in banding patterns among the four quail genotypes under investigation (**Fig. 1A**).

All polymorphic RAPD products were confirmed by repeating the PCR reaction three times. Each of the 20 primers used for analysis of individual quail genotypes amplified a different number of bands. The average number of amplified fragments from all primers for all genotype is summarized in **Table 2**.

Phylogenetic relationship among quail genotypes based on amplified RAPD fragments (bands)

The potential application of RAPD analysis to assess the relationships among the four quail genotypes was assessed through cluster analysis using a large number of scorable polymorphic markers from 20 different random primers. The dendrogram of genetic distances among quail genotypes was based on band polymorphisms generated by RAPD-PCR after using all primers. This dendrogram clustered the quail genotypes into three clusters, described in **Table 2**. Cluster III, the largest, contained two genotypes, while clusters I and II had a single genotype each. Based on RAPD analysis, the plumage and white phenotypes were clustered in the same group while roux and brown were in separate clusters (**Fig. 1B**). That result is similar to that of Roberts *et al*. (1978) who indicated that the white bib color (imperfect albino) is an autosomal color mutation (wp, WP) derived from the plumage color. However, both brown and roux were grouped individually in the separate group.

Genetic diversity based on ISSR markers

After the generation of the first microsatellite linkage map of Japanese quail was reported (Kayang *et al.* 2004), microsatellite-based markers were used extensively in quail genetic analysis such as estimating inbreeding by pedigree (Kim *et al.* 2007) and analyses of genetic diversity and evolution of quail genomes (Amirinia *et al.* 2007).

In this study, genetic diversity (allele frequency, mean polymorphic information content, etc.) of the preserved population, separated in the incubator to prevent intermingling, of 4 genotypes of quail was analyzed by 15 microsatellite markers with high polymorphism (**Fig. 2A**). The clustering dendrogram was eventually obtained based on Nei's genetic distances of these markers. The average number of alleles of each locus was 10 ± 2 and the range of allele frequencies was 0.333; the four quail genotypes shared 13 ± 2 alleles (average number of each locus). The average polymorphism (without unique) of 15 microsatellite loci was 3 ± 2 (**Table 2**). A dendrogram of genetic distances among quail genotypes based on band polymorphisms was generated by ISSR-PCR after using all primers (**Fig. 2B**). This dendrogram clustered the quail genotypes into two clusters. Cluster II was the largest, having three genotypes, while cluster I had a single genotype (**Fig. 2B**).

Genetic diversity of quail genotypes as determined by combined analysis of ISSR and RAPD band differences

Depending on the marker used, the genetic similarity analyses varied dramatically. Both RAPD and ISSR are based on different strategies for exploring genetic diversity (Man-

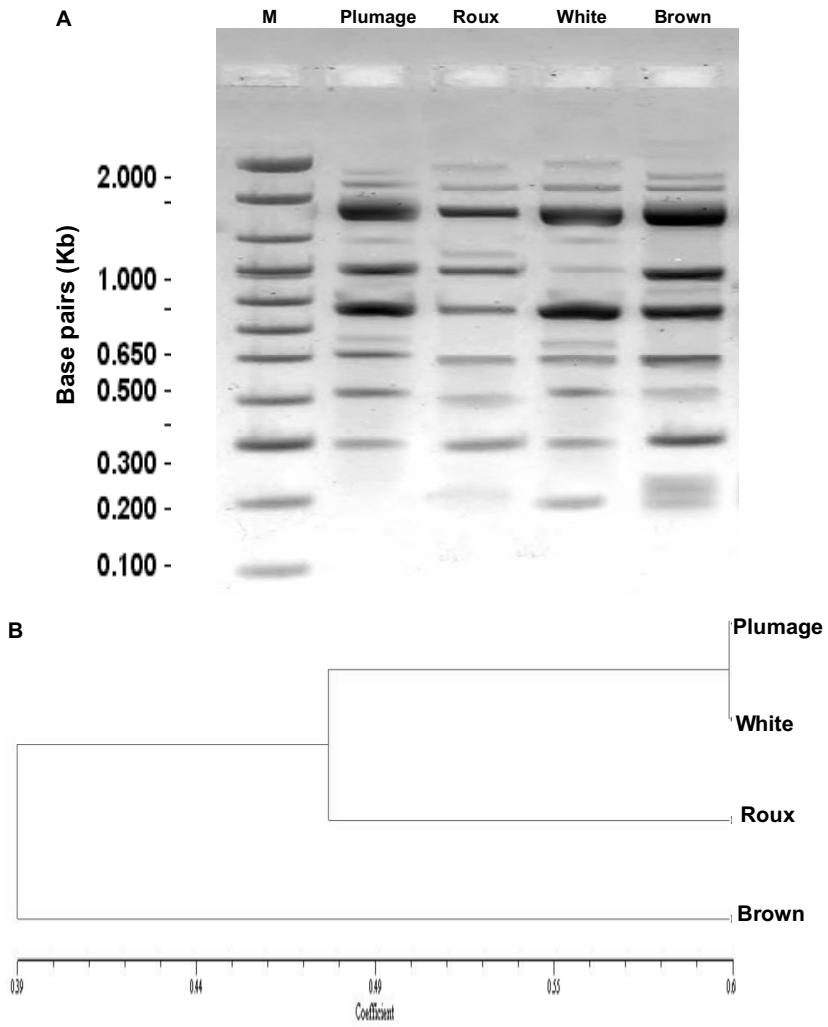

Fig. 1 Genetic diversity based on RAPD markers. (**A**) Band polymorphism generated by RAPD-PCR. (**B**) Dendrogram clustered the quail genotypes into three clusters.

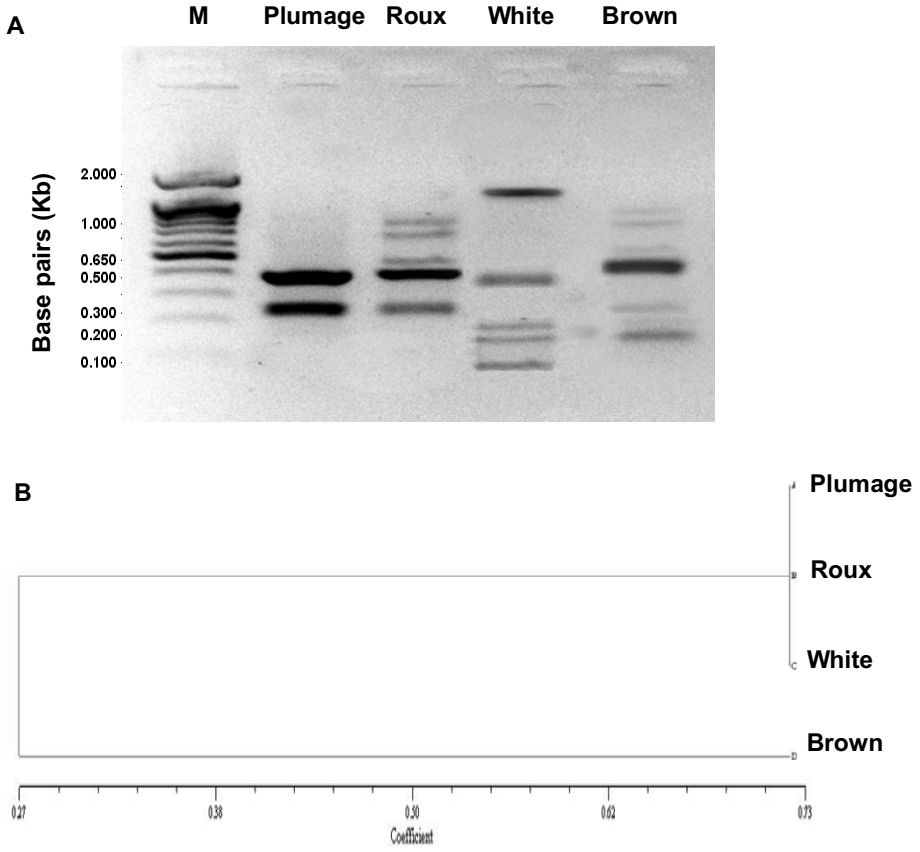

Fig. 2 Quail genotypes diversity analyzed with microsatellite markers. (**A**) Band polymorphisms generated by ISSR markers. (**B**) Dendrogram clustered quail genotypes into two clusters: Cluster II was the largest, having three genotypes, while cluster I had a single genotype.

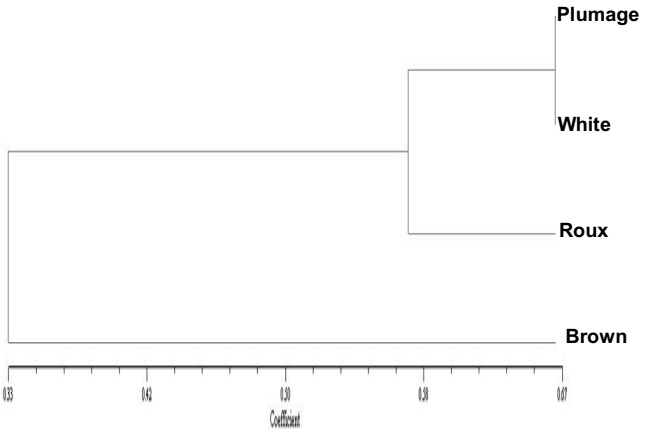

Fig. 3 Collective polymorphisms analysis between quail phenotypes using ISSR and RAPD molecular markers.

sour *et al*. 2008). While RAPD primers randomly target complementary and homologous genomic regions in the genome (Williams *et al*. 1990), ISSR primers amplify the highly repetitive inter-simple sequence repeats of the microsatellite regions (Ziêtkiewicz *et al*. 1994). The combination of both techniques will enhance the screening of diversity between and within genomes. The combination of ISSR and RAPD markers has been used for evaluating genetic variability in cashew (Archak *et al*. 2003); chickpea (Rao *et al*. 2006), strawberry (Kuras *et al*. 2004) and sorghum (Medraoui *et al*. 2007).

A dendrogram was produced from genetic distances between quail genotypes (**Fig. 3**). The linkage dendrogram provides a visual idea about the clustering and variability present in the population. The grouping pattern and distribution of quail genotypes into different clusters is given in **Table 2**. Cluster II was the largest, having three genotypes, while cluster I had a single genotype.

CONCLUDING REMARKS

Depending on consumers' needs and desires, quail breeders usually prefer to breed one color more than others. Thus, it is important to understand color development and evolution of quail inside its new habitat. However, the plumage color phenotype was the original color from which the other colors had segregated, and results from RAPD and ISSR markers indicated that roux (ro, Ro+) and white (wp, WP) mutant quail phenotypes are genetically closer to plumage than the brown (E, e+) mutant phonotype. This result reveals that some of the quail segregates with different colors are more genetically closer to the original plumage color from which they originally evolved. The genetic basis and inheritance of those different colors is yet to be studied extensively. However, more research is still needed to explain the genetic basis behind the development of one color to another. In this case, brown color seemed to be a very distant phenotype from the original.

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