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# Characterization and Estimation of Genetic Diversity in Two Syrian Chicken Phenotypes Using Molecular Markers

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**Abstract:** In order to establish pure Syrian local breeds, two chicken phenotypes were collected from the coastal area in Syria. 17 individuals (3 males and 14 females) from each phenotype were analyzed using molecular markers. 11 Operon primers and 10 SSR Primer pairs were used to characterize the chicken genotypes and to estimate their genetic diversity. The level of genetic diversity detected in the black phenotype using the two kinds of markers (0.24533) was higher than that detected in the grey phenotype (0.20453). Dendrogram based on the RAPD and SSR data showed a clear separation between individuals belonging to each phenotype. Based on the obtained results, genotypes with small genetic distance from each phenotype can be selected as parents for the establishment of pure breeds.

**Key words:** *Gallus gallus*, Syria, molecular marker, SSR, RAPD, genetic diversity

## INTRODUCTION

Chicken are domesticated fowl belonging to the subspecies *Gallus gallus domesticus* and is raised all over the world for its delicious meats and eggs.

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).

Native chickens are known to be good foragers and efficient mothers and minimal care is required for their growth. They are, therefore, most suited for raising under village conditions. These birds do, however, need special attention with respect to their conservation and improvement (Kaya and Yildiz, 2008; Fulton, 2008).

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).

Many new markers have been developed over the past two decades and used to detect and estimate the genetic diversity within and between chicken populations. Out of them the molecular markers based on DNA molecules. These last markers allow the detection of real variations affecting the genetic content at the DNA level (Rahimi *et al*., 2005).

DNA based typing methods provide a rapid and reliable method for differentiating individuals in a genetically diverse populations (Bidwell, 1994; Parham and Ohta, 1996). Many types of molecular markers have been developed. Among the most popular and fast markers were the Random Amplified Polymorphic DNA (RAPD), described first by Welsh and McClelland (1990) and Williams *et al*. (1990). It is a quick and effective method that can be applied to generate genotype specific banding patterns. It was used for the analysis of genetic diversity in Bangladeshi chicken which had a significant impact on the breeding and conservation of native chicken genetic resources in Bangladesh (Mollah *et al*., 2009), in the genetic analysis of six Hungarian indigenous chicken breeds (Bodzsar *et al*., 2009), to estimate the genetic relatedness between Nicobari fowls (Brown, Black and White) and an exotic bird (White Leghorn) (Ahlawat *et al*., 2004), in the analysis of variations within and between Iranian native chicken populations and in the estimation of genetic distances among five native chicken populations (Dehghanzadeh *et al*., 2009), in detecting genetic similarity between local chicken genotypes selected for eggs and meat productions in Egypt (Ali *et al*., 2003) and between chicken lines (Okumus and Kaya, 2005) and to discriminate between both normal and abnormal male of broiler chicken and normal and mutant birds (Salem *et al*., 2005).

The second widely used DNA marker is based on microsatellite DNA (Simple Sequence Repeats, SSR) which is characterized by reliable and reproducible results (VanMarle-Koster and Nel, 2000; Wimmers *et al*., 2000). SSRs are highly polymorphic and abundant molecular markers that are easily typed using PCR (Polymerase Chain Reaction) technique and scored on electrophoresis gels (Rincon *et al*., 2000). They have been used to estimate the genetic diversity among Turkish native chickens, Denizli and Gerze (Kaya and

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Yildiz, 2008), between six Hungarian indigenous chicken breeds (Bodzsar *et al*., 2009), within and between nine Vietnamese local and two Chinese chicken breeds (Cuc *et al*., 2010), for assessment of biodiversity of 2000 chickens randomly selected from 65 different populations of various chicken types and various geographical regions (Hillel *et al*., 2007), for genetic characterization of Fayoumi breed (Roushdy *et al*., 2008), for protecting purebred Tibetan chicken population (Yang *et al*., 2009) and for the assessment of genetic variability in Guangxi three- yellow chickens (Jian-Min *et al*., 2010).

There are no pure local breeds of chicken in Syria where individuals of many and different genotypes exist together and are distributed in different areas of the country. Therefore, a project to develop pure local breeds was launched. The first step in the program was the selection of the parents to be used for the establishment of pure breeds and their characterization. Therefore, the targets of this paper were to characterize the individuals to be used as parents and to estimate the genetic diversity and the relationships between them using the molecular markers.

## MATERIALS AND METHODS

**Sample collection:** Two colors of chicken were choose for the establishment of pure breeds, taking in consideration the morphological similarity between individuals of the same color. 34 individuals, composed of 17 grey (G) and 17 black (B) birds representing two dominant phenotypes in the coastal area, were collected. 14 females (F) and 3 males (M) from each phenotype were analyzed.

**DNA isolation:** DNA was isolated from blood samples according to Boodram protocol (2004) with some modifications. 1 ml of blood was collected from chicken venous and placed into 5 ml tubes containing 0.5 ml of 2 mM EDTA, used as anti-coagulant agent. Blood samples were centrifuged for 2000 t/min at 25°C. After centrifugation, plasma was discarded, buffy-coat was suspended into 8M urea added to fill the 5 mL tube, well mixed and kept at room temperature until DAN extraction (It could be kept in these conditions for a week). 200 µL of buffy-coat (in 8M urea) were transferred to a new 2 mL tube. DNA was extracted by adding 100 µl of Urea (2M), well mixed, then 150 µl of cold 3 M potassium acetate/2M acetic acid were added, mixed and Placed on ice for 10 min. The new mixture was then centrifuged at 10.000 t/min. for 5 min. The supernatant was transferred to a new eppendorf tube. 50 µl of 6 M NaCl were added and vortexed for 5 sec, then extracted with chloroform-isoamylic alcohol (24:1). Aqueous phases containing DNAs were separated by centrifugation for 10 min, at 10.000 t/min at 22°C, then transferred to a 2 mL clean eppendorf tube. Nucleic acids were precipitated by adding 2/3 of their volumes of cold isopropanol, then twice washed with 70% ethanol.

After a brief air drying, DNA pellets were re-suspended in 300 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and kept at -20°C until use.

The analysis was conducted in the laboratory of Molecular Genetic in the Faculty of Agriculture, Tishreen University, Lattakia, Syria.

**PCR amplification and electrophoresis**

**RAPD analysis:** Eighteen decamer primers obtained from Operon Technologies Inc. were tested on 8 DNA samples (four samples from each of the two phenotypes, grey and black). Out of 18, 11 Operon primers (OPB-01, OPB-04, OPG-16, OPH-07, OPH-08, OPJ-14, OPM-05, OPM-13, OPQ-14, OPQ-20, OPR-20) were selected for Random Amplified Polymorphic DNA (RAPD) analysis. The selection of primers was based on the level of polymorphism detected between the tested samples, the specificity and the reproducibility of amplified products.

PCR reaction contained 15 ng of DNA template, 100 µM of dNTPs, 15 pg of Operon primer, 1X of PCR buffer with 1.5 mM of $MgCl_2$ and one Unit of DNA *Taq* polymerase, in a final volume of 15 µl, was performed.

PCR program conditions consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles, each one consisted of 94°C for 1 min, 38°C for 1 min and 72°C for 2 min and finished with a final extension cycle at 72°C for 5 min.

PCR products were separated on 1.4% agarose gel and stained with 0.5 µg/ml of ethidium bromide and photographed in the presence of UV light.

**SSR analysis:** 10 SSR primer pairs were tested on 8 DNA samples. Out of them, six primer pairs have detected polymorphic alleles.

The PCR amplifications were conducted in a total volume of 10 µL solution containing 35 ng of DNA, 1 X of PCR buffer, 200 µM of dNTPs, 3 µM of each primer and 0.5 Unit of DNA *Taq* polymerase. Samples were subjected to a PCR program consisted of an initial denaturation step at 95°C for 5 min, followed by 45 cycles, each cycle consisted of 95°C for 30 Sec, followed by an annealing step at 65°C for 30 Sec. with -0.7°C/cycle for 15 cycles, then at 54°C for 30 cycles, then at 72°C for 45 sec and a final step at 72°C for 7 min.

PCR products were separated on 6% acrylamide gel and stained with silver nitrate (Bassam and Caetano-Anolles, 1993).

**Data analysis:** The amplified products were scored as 1 and 0 for presence and absence of bands (for RAPD) or alleles (for SSR) respectively. The data matrix was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 3.2 (Rohlf, 1993).

Dendrograms were generated by the Un-weighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath and Sokal, 1973). Analysis of gene diversity (H) was calculated as following:

$$H = (1-\Sigma p_{ij}^2)$$

Where (pij) is the frequency of the j th allele generated with the primer I (Weir, 1990). The Genetic Diversity (GD) was calculated according to the following formula of Nei (1987):

$$GD = n(1-\Sigma p^2)/(n-1)$$

Where (n) is the number of samples and (p) is the frequency of one allele.

## RESULTS AND DISCUSSION

**Polymorphism and genetic diversity within and between the two phenotypes:** The amplification of 34 samples (17 samples/phenotype) with 11 Operon primers produced a total of 125 different fragments (bands), out of them 93 bands were polymorphic. The number of fragments amplified per Primer varied from 5 (OPJ-14) to 17 (OPH-08), with an average of 11.4 fragments per primer. The highest number of polymorphic bands was detected by the primer OPH-08 (17 polymorphic bands), while the lowest number was detected with OPM-13 (2 polymorphic bands).

The level of polymorphism detected varied between the two phenotypes (grey and black). The grey phenotype showed higher number of amplified fragments (117 fragments) than the black phenotype (114 fragments). 64.9% of the total amplified fragments (74 fragments) in the black phenotype were polymorphic, while only 57.2% of the total amplified fragments (67 fragments) were polymorphic in the grey phenotype.

A data matrix (0, 1) based on RAPD results was established. This matrix data was used to estimate the genetic distance and to establish a dendrogram showing the genetic relationships between individuals and phenotypes (Fig. 1). All individuals belonging to one phenotype regrouped together and well separated from those belonging to the other phenotype (Fig. 1).

The genetic diversity were estimated with the different Operon primers (Table 1). The highest value (0.3075) was detected with OPR-20, while the lowest value (0.0816) was revealed with OPM-13 over the two

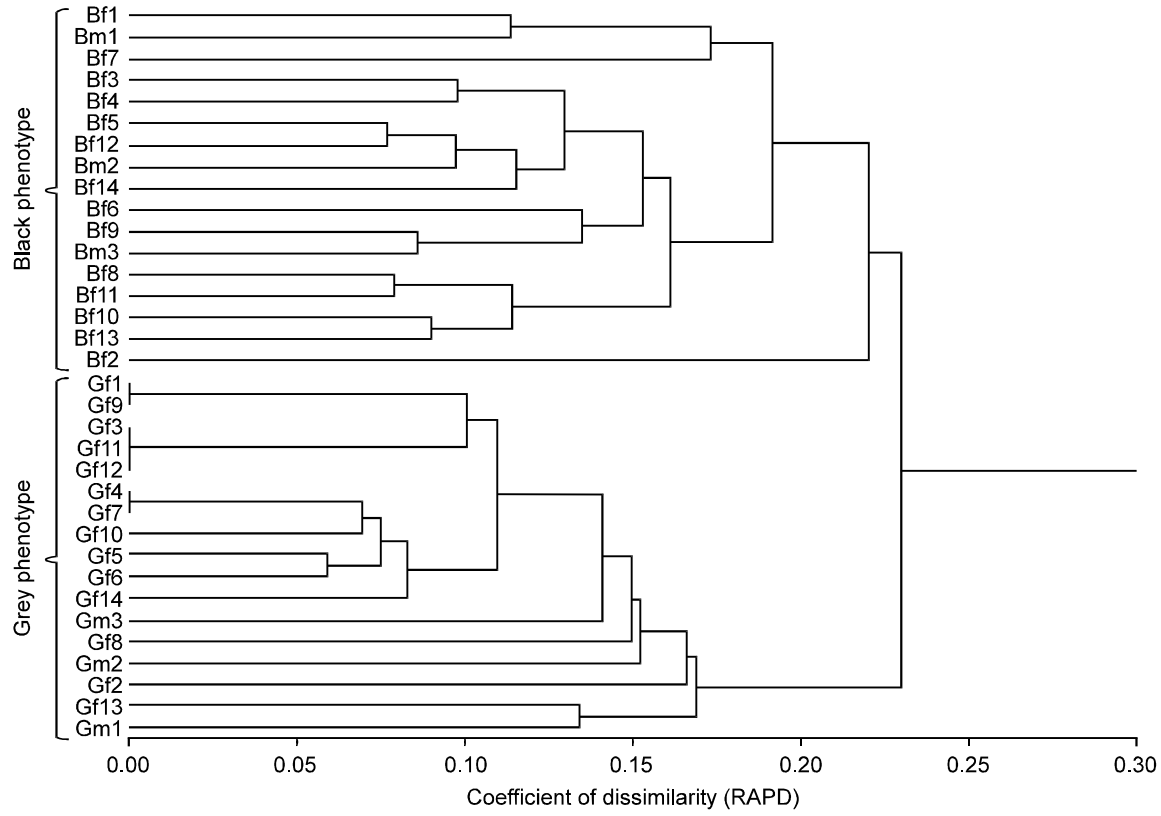


Fig. 1: Dendrogram of genetic distance between two phenotypes of chicken based on RAPD data. Bm1, 2 and 3: Males Bf1, 2,..etc: Females (of black phenotype). Gm1, 2 and 3: Males, Gf1, 2,.....etc: Females (of grey phenotype)

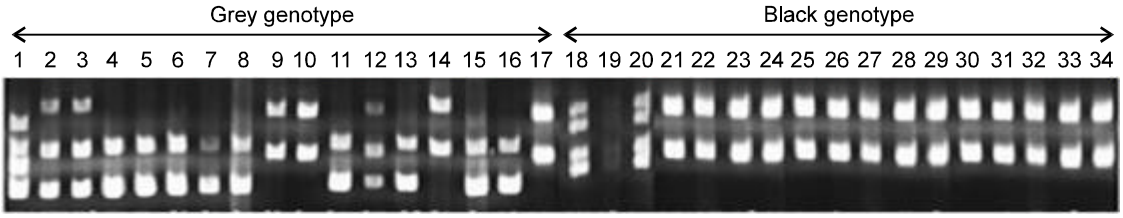


Fig. 2: Analysis of 34 DNA samples with the SSR primer MberA4-10 on 6% acrylamid gel

Table 1: Genetic diversity detected with 11 Operon primers in the two chicken phenotypes

| Operon Primers | Genetic diversity in Grey genotype | Black genotype | All individuals |
|---|---|---|---|
| OPB-01 | 0.23391 | 0.19377 | 0.28287 |
| OPB-04 | 0.16147 | 0.16955 | 0.17531 |
| OPG-16 | 0.16289 | 0.16806 | 0.23727 |
| OPH-07 | 0.13702 | 0.20069 | 0.19896 |
| OPH-08 | 0.18882 | 0.27780 | 0.23234 |
| OPJ-14 | 0.23529 | 0.22145 | 0.24740 |
| OPM-05 | 0.13715 | 0.20228 | 0.17906 |
| OPM-13 | 0.03229 | 0.09688 | 0.08169 |
| OPQ-14 | 0.19476 | 0.16493 | 0.25098 |
| OPQ-20 | 0.16357 | 0.19930 | 0.23199 |
| OPR-20 | 0.25836 | 0.26874 | 0.30752 |
| Average | 0.173233 | 0.196682 | 0.2204946 |

phenotypes. The loci OPH-08 gave the high value for genetic diversity (0.277805) in the black group, while the loci OPR-20 detected the highest value (0.2583) in the grey group.

The average of genetic diversity estimated overall the 34 individuals and with all the Operon primers was 0.22049. The level of genetic diversity detected was higher in the black phenotype individuals (0.19668) than the grey phenotype individuals (0.17323) and that was illustrated in the dendrogram where the highest level of genetic distance was detected between individuals of the black group (Fig. 1).

**For SSR analysis:** Out of 10 SSR primers tested, six primer pairs were able to reveal polymorphic alleles on the correspondent loci (ADL306, LEI0171, MCW55, MberA4-10, WS35, LEI234) (Fig. 2), while the other primers pairs (SCW0248, MberC8-1, MberD8, MberC11-10) showed monomorphic alleles. The alleles detected by SSR primers were scored for the 34 individuals and used for further analysis.

The total number of alleles produced in the two phenotypes on the 6 loci was 26 (the four monomorphic loci were excluded from the analysis) ranging from 2 (on Loci LEI0171 and WS35) to 9 alleles per locus (on Locus MberA4-10), with an average of 4.3 alleles/locus. The highest number of alleles was found in the grey phenotype where 22 different alleles were detected out of 24 alleles revealed on the six loci, while only 17 different alleles were detected in the black phenotype out of 22 revealed ones.

Specific alleles were detected in the individuals belonging to the black phenotype on the two loci recognized by the primer pairs MberA4-10 and MCW55. These primer pairs were able to differentiate between the males and females in the Black phenotype. On The first locus, the primer pair MberA4-10 revealed 2 specific alleles (100 and 150 bp) present in the males and absent in the females, while On the second locus (MCW55) a specific allele (250 bp) has differentiated the males of black genotype. These three alleles were characteristic of black males and were absent in the males and females of grey phenotype. These results are similar to others studies using SSR markers where they were able to identify specific alleles to differentiate between Jungle and domestic populations (Romanov and Weigend, 2001) and between White Leghorn, Rhode Island Red, Red Cornish and an Indian breed of chickens (Sharma *et al*., 2001), between local and other different duck breeds (El-Gendy *et al*., 2005).

Alleles detected on gels after staining were scored as (1) for the presence of allele and (0) for its absence. These data were used to establish a matrix for the calculation of genetic distance between and within the two phenotypes. The values of genetic distance were used to establish the dendrogram showing the genetic relationships between and within phenotypes (Fig. 3).

The level of genetic diversity estimated using the SSR primers varied between loci and between the two analyzed phenotypes. The locus LEI0171 showed the highest level of genetic diversity either in the grey (0.4982) or the black (0.4567) phenotypes (Table 2). The level of genetic diversity was higher in the black group (0.33452) than that in the grey group (0.26191). Although the number of polymorphic alleles was higher in the grey phenotype but their frequency was lower than that in the black phenotype, which affect and decrease the level of genetic diversity in the grey phenotype comparing to the black phenotype.

**SSR and RAPD analysis:** Data obtained with 11 operon primers and 6 polymorphic SSR primer pairs were accumulated and used to estimate and establish a dendogram of genetic distance (Fig. 4) and to evaluate the genetic diversity within and between the two phenotypes. The dendrogram of genetic distance showed a similar pattern to dendrograms based on each marker alone. It showed a good separation

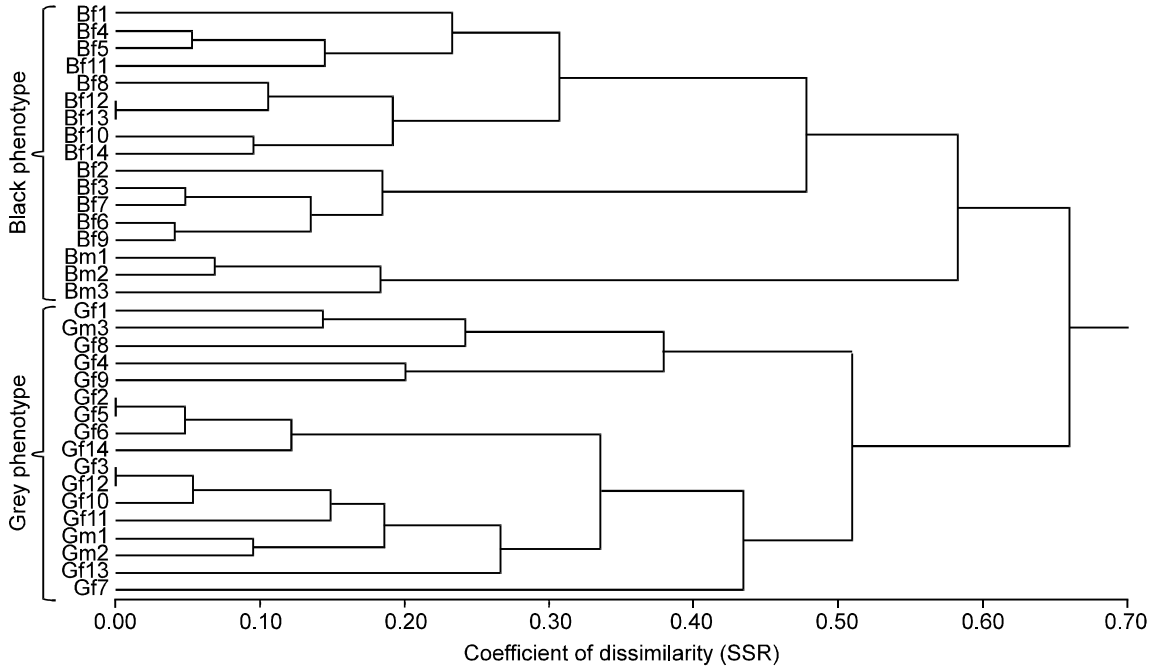


Fig. 3: Dendrogram of genetic distance based on SSR data in the two chicken phenotypes

Table 2: Values of genetic diversity in the grey and black phenotypes on the SSR loci

| Loci | Primer sequences 5' _____ 3' | Genetic diversity Grey | Black | Genetic diversity in 34 individuals |
|---|---|---|---|---|
| ADL306 | 5'-TCAGTTTGACTTTCCTTCAT-3' | | | |
| | 5'-GTTACTGTATCTTGGCTCAT-3' | 0.1268 | 0.3690 | 0.2753 |
| LEI0171 | 5'-CTCAGGGCACCATTTTCACTG-3' | | | |
| | 5'-GAGTGTAGACAGTAGTGTATC-3' | 0.4982 | 0.4567 | 0.4930 |
| MCW55 | 5'-GTTTGCATTGTCTACAGCTCCTTG-3' | | | |
| | 5'-TTTGTAGTTACCTGGTACTGA-3' | 0.1107 | 0.38408 | 0.28287 |
| MberA4-10 | 5'- GTCCTTGCCAGAGGCTTC-3' | | | |
| | 5'- TGGGTCAGACGGGCTTTG-3' | 0.26124 | 0.17439 | 0.34352 |
| WS35 | 5'- CCATGAACCAGGTAAGTACAG-3' | | | |
| | 5'- GATCCAGAGTGAAATTTACAC-3' | 0.207612 | 0.24913 | 0.242215 |
| LEI234 | 5'- ATGCATCAGATTGGTATTCAA-3' | | | |
| | 5'- CGTGGCTGTGAACAAATATG-3' | 0.366782 | 0.37370 | 0.372405 |
| Average | | 0.261918 | 0.33452 | 0.334911 |

between the two analyzed phenotypes which demonstrated the presence of specific alleles able to differentiate between the two phenotypes. The genetic variability between individuals of the black phenotype was higher than that exist within the grey phenotype.

Different levels of genetic diversity were shown between and within phenotypes. The individuals of black group showed higher genetic diversity level than those belonging to the grey phenotype.

The results obtained with the different markers proved the presence of genetic variability between the individuals analyzed within and between the two phenotypes. Based on the presented data, genetically close individuals can be selected to be used as parents in a breeding program to start the establishment of pure black and grey breeds.

Based on the genetic distance (Fig. 4) the selection of grey females (GF3, GF12 and GF11) to be crossed with the grey male (GM3) would be the best selection to have the faster pure grey breed. With the small value of genetic distance of the parents we can expect to have a generation with higher level of genetic similarity comparing to the parents, then many successive selections will be applied for many generations until obtaining a pure grey breed.

For the black phenotype, The female individuals (BF6 and BF9) with the male individuals (BM2 and BM3) will be selected for crossing as they have the most suitable genetic distance among the black individuals.

For the black phenotype, the distance genetic between individuals was higher either among females or between females and males, therefore, it could be

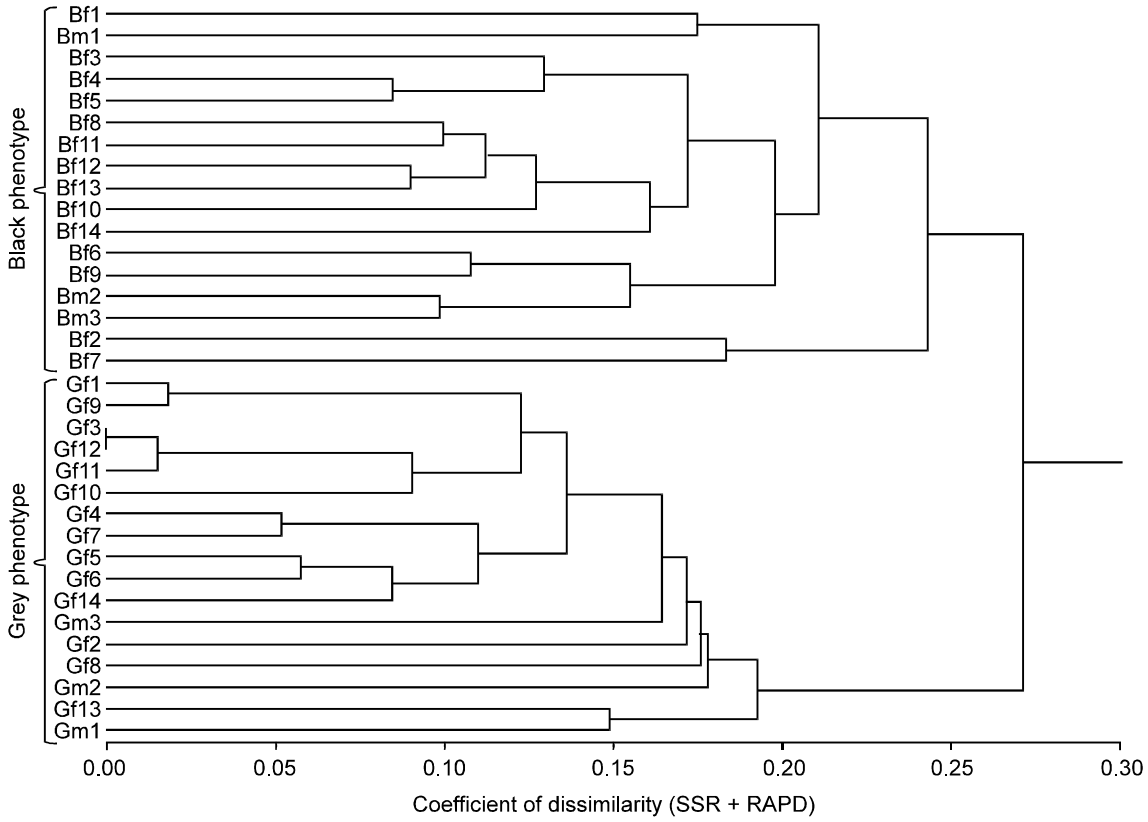


Fig. 4: Dendrogram of genetic distance based on SSR and RAPD data in the two chicken phenotypes

expected that more time will be needed to have a pure breed comparing to the time needed for the grey breed. The successive selections to obtain pure grey and black breeds will be accompanied with the molecular analysis using different types of markers in order to achieve faster our target to possess pure Syrian breeds.

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