

Assessment of genetic variability among some rabbit breeds using RAPD-DNA technique

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Abstract

Random Amplified Polymorphic DNA (RAPD) markers were employed to assess the genetic diversity and phylogenetic relationships among three Egyptian rabbit breeds (Gabali, Baladi Red and Baladi Black) along with New Zealand White breed as a reference population. A total of 120 animals were used in this study, 30 animals from each breed. Genomic DNA was extracted and PCR amplification was performed and the amplification products were resolved by electrophoresis. A total of 14 RAPD primers of arbitrary sequence were used to amplify pooled genomic DNA from these breeds, and only 10 primers generated reproducible, score able and polymorphic bands. Out of 131 bands scored, 74 bands (56.48%) were recognized as polymorphic and 57 bands (43.52%) as monomorphic. The highest percentage of polymorphic bands was recognized by primers OPB02 (94%) and OPB07 (92%), and the lowest percentage of polymorphic bands was recognized by primers OPA02 (16%) and OPF12 (28%). One marker of OPA19 was identified as specific for New Zealand White, three markers detected by OPB02, OPA19 and OPA02 were specific for Baladi Black, four markers detected by OPA19, OPA20, OPB05 and OPB07 in Gabali, and five markers of OPB05, OPB02, OPA19, OPF12 and OPB14 were specific for Baladi Red. The band sharing frequencies (BSF) were found to be the highest between Baladi Red and Baladi Black (0.80 ± 0.038), followed by Gabali and Baladi Black (0.71 ± 0.079), New Zealand White and Baladi Black (0.70 ± 0.096), New Zealand White and Baladi Red (0.69 ± 0.088) and the least BSF was found between New Zealand White and Gabali (0.64 ± 0.081). Overall, there was no significant difference ($P > 0.05$) in BSF values between breeds. The closest genetic distance was found between Baladi Red and Baladi Black (0.87), while the lowest genetic distance was found between New Zealand White and Baladi Black (0.73).

Key words: Rabbits breeds, genetic diversity, RAPD markers, band sharing frequency, phylogenetic relationships, genetic distance.

Introduction

Recent reports of FAO and CIHEAM have indicated the lack of information and rapid loss in animal genetic resources, including rabbits. Most of the local breeds of rabbits as well as lines synthesized recently in Egypt appear to be in a real danger of extinction due to the low number of animals, while some breeds are currently being disappeared (Khalil and Baselga, 2002). Characterization of native rabbit breeds at the molecular level is currently considered an important criterion for breed conservation and for further genetic improvement (Al-Saef *et al.*, 2012). The FAO and CIHEAM working groups proposed a global programme for characterization of *Animal Genetic Resources* (AnGR), including the molecular genetic characterization, with the recommendations of applying molecular analysis of domestic animal diversity on a global scale via a research programme (FAO, 2011). Therefore, assessment of genetic variability in rabbits will be an important issue to preserve the genetic resources and propose future breeding plans in order to satisfy the demands of changeable markets (Groeneveld *et al.*, 2010). However, genetic diversity among breeds provides us with valuable information, to understand domestication and evolution history, and this will lead us to suggest the conservation and breeding plans.

Molecular analyses performed for economic traits in rabbits are very limited to be used in further genetic improvement programs. RAPD technology has been principally used to study the genetic relationships between varieties/species in the rabbit. For instance, Liping *et al.* (2000 and 2005) analyzed the genetic relationships among three domestic rabbit breeds; Rongqing *et al.* (2000) analyzed evolutionary relationships among five rabbit populations; Rangoju *et al.* (2007) assessed the genetic variability and phylogenetic relationships among three rabbit breeds. RAPD technique is one of the most widely techniques used in the applications of molecular biology to identify the markers linked to traits of interest without the necessity of mapping the entire genome (Bardakci, 2001; Khalil *et al.*, 2008).

The objective of the present study was to assess the genetic diversity and phylogenetic relationships within and among four rabbit breeds (New Zealand White, Gabali, Baladi Red and Baladi Black) using random amplified polymorphic DNA markers (RAPD).

Materials and Methods

Experimental animals:

Three Egyptian rabbit breeds namely Baladi Red (BR), Baladi Black (BB) and Gabali (G) along with

New Zealand White breed as a reference breed were used in the present study. A total of 120 rabbits viz. 30 rabbits from each breed were selected from four accredited farms viz. the rabbitry of the Department of Animal Production, Faculty of Agriculture, Benha University, Egypt; Inshas, Gimmeza and Sakha experimental rabbitries belong to Animal Production Research Institute (APRI), Ministry of Agriculture, Egypt. Baladi Red and Baladi Black rabbits are Egyptian traditional breeds that have not been subjected to followed by a program of genetic improvement after their formation (**Khalil and Baselga, 2002**). These breeds were developed by crossing local Baladi rabbits with Flemish Giant (G) for several generations in rabbitries of Animal Production Research Institute (APRI), Ministry of Agriculture (**Galal and Khalil, 1994; Khalil, 1999; Khalil and Baselga, 2002**). While the Gabali rabbits are raised by the Egyptian Bedouins in Sinai and the north coast of western desert (**Khalil, 1999; Afifi, 2002**). This breed was extensively studied in the last two decades by **Afifi (2002)** and **Iraqi et al. (2007)**. On the other hand, New Zealand White (NZW) is a foreign breed actually originated in the United States and wide spread globally in the world and has been raised in Egypt in alarge scale of commercial production for several decades. New Zealand White exhibits outstanding maternal abilities as related to maternal behavior, fecundity, lactation, and pre weaning growth and survival (**Khaliland Baselga, 2002**).

Blood samples and DNA extraction:

The blood samples were taken randomly from pedigreed animals with the least relationship to decrease the genetic similarity and to have more chance in showing more polymorphism in marker

alleles. The animals were identified depending on their pedigrees and full-sib and half-sibs were avoided. In addition, these relationships were checked by analyzing the polymorphism after genotyping to insure that there is no error in their original farm pedigree.

DNA of each breed were extracted from the whole fresh blood samples, approximately 5 ml blood was collected from the central artery vein of the ear of animal into tubes containing ethylene di amine tetra acetic acid(EDTA) as anticoagulant. The blood samples were kept in ice tank till reaching the laboratory and were then preserved in a freezer at -20°C until extraction of DNA. Genomic DNA was extracted using the Promega Wizard Genomic DNA Purification Kit (Cat No. A 1120) using the manufacture protocol.

PCR amplification and RAPD analysis:

A total of fourteen random primers (Operon Technologies Inc, USA) of arbitrary sequence with 60-70% GC content were screened on pooled rabbit DNA Ten of the primers generate reproducible, score able and polymorphic bands and were used alone to amplify random sequences from genomic DNA(Table 1). These primers amplified on average 2 to 14 bands of sizes varying from 200 bp to 2000 bp. This observed range of products presumably due to limitations in the resolving power of the agarose gels at lower molecular weights as well as inefficiency of the extension reaction under the described PCR conditions at higher molecular weights (**Bowditch et al., 1993**). However, the DNA amplification protocol was performed as described by **Welsh and McClelland (1990)** and **Williams et al (1990)**.

Table 1. List of random amplified polymorphic DNA primers (RAPD) and their nucleotide sequences.

Primer code	Nucleotide sequence (5'→3')	GC (%)
OPA09	5'-GGGTAACGCC-3'	70%
OPB05	5'-TGCGCCCTTC-3'	70%
OPB07	5'-GGTGACGCAG-3'	70%
OPB14	5'-TCCGCTCTGG-3'	70%
OPA19	5'-CAAACGTCGG-3'	60%
OPA20	5'-GTTGCGATCC-3'	60%
OPF09	5'-CCAAGCTTCC-3'	60%
OPF12	5'-ACGGTACCAG-3'	60%
OPB02	5'-TGATCCCTGG-3'	60%
OPA02	5'-TGCCGAGCTG-3'	70%

As shown in Table 2, PCR reactions were carried out in a total volume of 25 µl containing 2 µl of genomic DNA as a template, 30 pmoles of random primer, 2 mM of dNTP's mix (dATP, dCTP, dTTP and dGTP;ABgene, Surrey, UK), 5X PCR buffer, 25 mM MgCl₂, and 1 unitTaq DNA polymerase (MBI Fermentas Inc., Hanover, Madison, Wisconsin, USA). A master mix was prepared in a 1.5 ml eppendorf tube

according to the number of PCR reactions to be performed, with an extra reaction included to compensate the loss part of the solution due to frequent pipetting. An aliquot of 22 µl master mix solution was dispensed in each PCR tube (0.2 ml eppendorf tube); containing 2 µl of the appropriate template DNA PCR reaction components.

Table 2. Components of RAPD-PCR reaction.

PCR Component	Amount of one PCR reaction (1X)
5X PCR buffer	5µl
MgCl ₂ (25mM)	2µl
dNTP's mix (2mM)	2.5 µl
Primer (10 pmoles/µl)	3 µl
Taq (5 U/µl)	0.2µl
DNA (10ng/µl)	2µl
d.dH ₂ O	10.3µl
Total volume	25µl

PCR amplification was performed in a Gene Amp® PCR System 9700 (Applied Bio systems, Foster City, California, USA), programmed to fulfill 35 cycles after an initial denaturation cycle for 4 minutes at 94°C, in this cycle dsDNA converted to ssDNA and Taq DNA polymerase was activated.

Each PCR amplification cycle consisted of three steps, a denaturation step at 94°C for 40 seconds, an annealing step at 36°C for one minute, and an elongation or extension step at 72°C for one minute. After the last cycle, the primer extension segment was extended to 10 minutes at 72°C in the final extending cycle and was followed by soaking at 4°C until reaction removed from PCR machine.

Electrophoresis and visualization of RAPD- PCR products

The amplification products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer. A 10µl of each PCR product was mixed with 2 µl of loading buffer (tracking dye), and loaded into the wells of the gel. The gel was run at 95 volts until tracking dye reached to the end of the gel. After electrophoresis, the amplified RAPD-PCR products (amplicons) were visualized with Gel Documentation System (Gel-Doc 2000 with Diversity Database software Ver. 2.1, Bio-Rad Laboratories, Hercules, California, USA) was used for gel documentation and gel analysis.

RAPD banding patterns on gels scrutinized for variation in presence/absence variation of a band at a specific position on the gel, presume to reflect priming sequence variation or large insertion/deletion variants that preclude successful amplification.

Statistical analysis:

The presence and absence of band was recorded as "1" and "0", respectively. The binary coded characters (1, 0) were used for the genetic analysis. Band sharing frequency (BSF) was used to estimate the genetic similarity for each primer (Lynch, 1990) and a simple expression of similarity measured in terms of sharing bands between breeds. BSF was calculated as an expression of animals from either the same or different breeds using the following formula:

$$BSF = 2N_{ab} / (N_a + N_b)$$

Whereas, N_{ab} is the number of bands common to a and b individuals, N_a is the number of bands present in the animal a, while N_b is the number of bands present in the animal b. The BSF values were statistically analyzed by the ANOVA procedure using SAS program (SAS, 2004). Significant differences between means were detected using Duncan's multiple range test (Duncan, 1955).

The genetic distances (D) were designed to express the genetic differences between two populations as a single number. If there are no differences, the distances could be set to zero, whereas if the populations have no allele in common at any locus the distance may be set to its maximum value. The genetic distances were calculated by POPGENE software (Yeh et al., 1999) using standard genetic distance equation (Nei, 1972). The phylogenetic relationships among rabbit breeds were analyzed by generating the phylogenetic tree for different genetic distances using POPGENE software (Nei 1972; Yeh et al., 1999).

Results and Discussion

Number of bands detected in the breeds:

The number of detectable bands per primer (TDB), number of polymorphic bands (NPB) and percentages of polymorphic bands (PB) are presented in Table 3. A total of 131 bands were scored in the RAPD profiles and were amplified by 10 primers. Among these 131 scoreable bands, 74 bands (56.5%) were recognized as polymorphic and 57 bands (43.5%) as monomorphic. El-Bayomi et al. (2013) detected a total of 120 bands within RAPD profiles amplified by 14 primers, and 39 bands (33%) were recognized as polymorphic and 81 bands (67%) as monomorphic bands.

The average number of polymorphic bands per primer varied from 2 to 17 (Table 3). The highest percentage of polymorphic bands was recognized for primers OPB02 (94%) and OPB07 (92%), and the lowest percentage of polymorphic bands was recognized for primers OPA02 (16%) and OPF12 (28%). Khalil et al. (2008) detected five polymorphic RAPD markers of OPA12, OPA19, OPA20, OPF09, and OPF12 at molecular weights of 1500, 1100, 1200, 700 and 900 bp, respectively. Mamuris et al. (2002) used five RAPD primers (OPA02, OPA09, OPA10, OPA20 and OPF01) to assess the genetic variability

among brown hare populations (*L.europaeus*) from different geographical regions. It was found that all the primers produced polymorphic bands in the range of 5 to 11. **Rangojuet et al. (2007)** used six random primers (OPA01, OPA08, OPA10, OPA18, OPB03 and OPB05) to screen the genomes of three rabbit breeds. All bands were polymorphic and the maximum number of bands was 13.2 ± 0.4 , while the minimum number of bands were 6.4 ± 0.2 . Also, **El-**

Bayomi et al. (2013) used 13 RAPD primers for three rabbit breeds (OPA01, OPA06, OPA10, OPB05, OPB13, OPB14, OPC01, OPC02, OPC08, OPE19, OPE11, OPF09, OPF12 and OPX02) and found that, the highest percentage of polymorphic bands was recognized for primers OPA10 and OPA06 (56%), and the lowest percentage of polymorphic bands was recognized for primers OPE19 (7%) and OPF12 (14%).

Table 3. The total number of detectable bands (TDB), number of polymorphic bands (NPB) and percentages of polymorphic bands (PB %) for the ten random primers used (across the breeds).

Primer	TDB	NPB	PB%
OPA09	13	9	69%
OPB05	9	5	55%
OPB07	13	12	92%
OPB14	14	9	64%
OPA19	15	6	40%
OPA20	8	4	50%
OPF09	15	6	40%
OPF12	14	4	28%
OPB02	18	17	94%
OPA02	12	2	16%
Total= 131 band		Total= 74 band	Average = 56.5 %

Number of bands amplified within each breed

Among the four rabbit breeds, the number of bands amplified per primer was variable (Table 4). Using the primer OPF09, the maximum numbers of bands detected in New Zealand White rabbits were 14 bands, followed by Gabali, Baladi Black and Baladi Red (13 bands each). The maximum number of bands given by primer OPF09 was 53 band, while

the minimum number of bands was 18 bands and were obtained using primer OPB05. Using eight RAPD primers (OPA10, OPB05, OPC01, OPC02, OPC08, OPE11, OPE19 and OPX02), **El Sayed (2010)** found that, all the primers yielded informative and identifiable bands revealing genetic differences between New Zealand White, Black Rex, Hyplus, Spanish line V, Moshtohor line and Sinai rabbits.

Table 4. Number of bands amplified per primer in each rabbit breed.

RAPD primer	New Zealand White	Gabali	Baladi Black	Baladi Red	Total
OPA09	6 ± 0.57^b	12 ± 0.57^a	11 ± 0.57^a	7 ± 0.57^b	36
OPB05	2 ± 0.57^b	3 ± 0.57^b	6 ± 0.57^a	7 ± 0.57^a	18
OPB07	2 ± 0.57^c	9 ± 0.57^a	9 ± 0.57^a	7 ± 0.57^b	27
OPB14	8 ± 0.57^b	9 ± 0.57^b	9 ± 0.57^b	14 ± 0.57^a	40
OPA19	12 ± 0.57^a	11 ± 0.57^a	12 ± 0.57^a	10 ± 0.57^a	45
OPA20	6 ± 0.57^a	7 ± 0.57^a	7 ± 0.57^a	4 ± 0.57^b	24
OPF09	14 ± 0.57^a	13 ± 0.57^a	13 ± 0.57^a	13 ± 0.57^a	53
OPF12	11 ± 0.57^a	11 ± 0.57^a	12 ± 0.57^a	12 ± 0.57^a	46
OPB02	3 ± 0.57^d	6 ± 0.57^c	12 ± 0.57^b	14 ± 0.57^a	35
OPA02	10 ± 0.57^a	9 ± 0.57^a	11 ± 0.57^a	10 ± 0.57^a	40
Total	74	90	102	98	364

Means with the same letters in the row are not significantly different ($p \leq 0.05$).

The RAPD profiles and breed specific markers:

The RAPD profiles of New Zealand White, Gabali, Baladi Black and Baladi Red breeds generated by 10 random primers identified breed-specific markers (Table 5), i.e. the marker unique to a particular breed only. As presented in Table 5, one band of OPA19 at molecular weight of 360 bp was identified as specific for New Zealand White. Three bands of OPB02, OPA19 and OPA02 at molecular weights of 1500, 1500 and 2050 bp were identified as specific for Baladi Black. Four bands of OPA19, OPA20, OPB05 and

OPB07 were identified as specific for Gabali, and five markers of OP05, OPB02, OPA19, OPF12 and OPB14 were identified as specific for Baladi Red. Therefore, these breed-specific markers can be used in identification of the breeds. **El-Bayomi et al. (2013)** used 14 RAPD primers and reported that one marker of OPE-11 was identified to be specific for *New Zealand White*, Three bands of OPA10, OPC02 and OPF09 were identified to be specific for *Californian*, five bands of OPA01, OPA06, OPA10, OPB14 and OPF09 were identified as specific for Flander rabbits.

Table 5. Specific RAPD markers identified in the four rabbit breed studied.

Breed	Molecular marker	Number of bands	Molecular weight (bp)
New Zealand White Baladi Black	OPA19	14	360
	OPB02	16	400
	OPA19	3	1500
	OPB02	3	1500
	OPA02	1	2050
Gabali	OPA19	9	720
	OPA20	7	550
	OPB07	8	620
	OPB07	4	970
	OPB07	2	1250
	OPB05	7	790
	OPB05	6	860
Baladi Red	OPB14	12	440
	OPB14	1	1880
	OPA19	6	920
	OPB02	7	1000
	OPB02	4	1400
	OPB02	2	1750
	OPB02	1	2000
	OPF12	2	1600
	OPB05	1	1880

Band sharing frequency (BSF)

BSF is an indicator of the relationships between breeds (*Nei and Li, 1979*). The sharing frequencies given in Table 6 showed that there were no significant differences ($P > 0.05$) in BSF values among the breeds. Among the primers used, the highest overall BSF was between Baladi Black and Baladi Red rabbits (0.807 ± 0.038), followed by Gabali and Baladi Black rabbits (0.711 ± 0.079) and the lowest BSF was between New Zealand White and Gabali (0.649 ± 0.081). *Rangoju et al. (2007)* used 40 RAPD primers to study the genetic variation among three rabbit breeds viz. White Giant (WG), Soviet Chinchilla (SC) and Grey Giant (GG). The BSF was the highest in SC-GG rabbits, followed by WG-SC and WG-GG rabbits. *El-Bayomi et al. (2013)* found that the highest sharing frequency was between *New Zealand White* and *Californian* rabbits (0.88 ± 0.029), followed by that between *Californian-Flander* rabbits (0.87 ± 0.024) and between *New Zealand White-Flander* rabbits (0.84 ± 0.034).

Genetic distances between the breeds

The genetic distances (D) between the four rabbit breeds are shown in Table 7. The genetic distances ranged from 0.87 between Baladi Black and Baladi Red (more related) to 0.73 between New Zealand White and Baladi Black (distantly related). High value of genetic distance was found between New Zealand White and Gabali rabbits (0.80). *Rangoju et al. (2007)* found that the highest genetic distance was between *White Giant* and *Grey Giant* rabbits ($D = 0.1605$), followed by between *White Giant* and *Soviet Chinchilla* rabbits ($D = 0.1403$) and between *Soviet Chinchilla* and *Grey Giant* rabbits ($D = 0.1295$),

explaining the difference between the breeds to reflect the survival under different geographical and climatic conditions. *El Sayed (2010)* reported that the highest genetic distance was 0.37 between New Zealand White and Black Rex and between New Zealand White and Spanish V line rabbits, while the lowest value was 0.10 between Spanish V line and Moshtohor line rabbits.

Phylogenetic relationships

The phylogenetic relationships among the studied four rabbit breeds based on genetic distance were given in Figure 1. As shown in this dendro gram, two separate clusters were formed in the four rabbit breed used. The local breeds are clustered together in one cluster, while New Zealand White breed clustered alone. In agreement with these findings, *Grimalet al. (2012)* reported that the four Egyptian breeds (Baladi Black, Baladi Red, Giza White and Gabali) were structurally separated from the Spanish New Zealand White line. These variations might be due to that these breeds are raised in different geographical regions and/or raised from different races, which cause variability in the gene pool. In India *Rangoju et al. (2007)* illustrated the phylogenetic relationships among many rabbit breeds and revealed that Soviet Chinchilla and Grey Giant are close to each other, while White Giant and Grey Giant are distant from each other. In Egypt, *El Sayed (2010)* showed that Spanish V line and Moshtohor line were close to each other, while New Zealand White and Hyplus were more distant breeds. *El-Bayomi et al. (2013)* showed also that New Zealand White and Californian breeds were close to each other, while Flander breed was distant to them.

Table 6. Estimates of band sharing frequency (BSF) among the rabbit breed studied [†].

RAPD primer	NZW-G	NZW-BB	NZW-BR	G-BB	G-BR	BR-BB
	BSF±SE	BSF±SE	BSF±SE	BSF±SE	BSF±SE	BSF±SE
OPA09	0.55± 0.005 ^d	0.47± 0.005 ^e	0.92± 0.005 ^b	0.95± 0.005 ^a	0.63± 0.005 ^c	0.55± 0.005 ^d
OPB05	0.40± 0.041 ^b	0.50± 0.041 ^b	0.44± 0.041 ^b	0.22± 0.041 ^c	0.20± 0.041 ^c	0.92± 0.041 ^a
OPB07	0.18± 0.005 ^e	0.36± 0.005 ^d	0.44± 0.005 ^c	0.55± 0.005 ^b	0.37± 0.005 ^d	0.75± 0.005 ^a
OPB14	0.58± 0.005 ^d	0.94± 0.005 ^a	0.72± 0.005 ^c	0.55± 0.005 ^e	0.78± 0.005 ^b	0.78± 0.005 ^b
OPA19	0.86± 0.005 ^b	0.91± 0.005 ^a	0.81± 0.005 ^c	0.86± 0.005 ^b	0.85± 0.005 ^b	0.81± 0.005 ^c
OPA20	0.76± 0.005 ^d	0.92± 0.005 ^a	0.8± 0.005 ^c	0.85± 0.005 ^b	0.72± 0.005 ^e	0.72± 0.005 ^e
OPF09	0.88± 0.005 ^b	0.88± 0.005 ^b	0.88± 0.005 ^b	0.84± 0.005 ^c	0.84± 0.005 ^c	0.92± 0.005 ^a
OPF12	0.90± 0.005 ^b	0.95± 0.005 ^a	0.86± 0.005 ^c	0.95± 0.005 ^a	0.86± 0.005 ^c	0.91± 0.005 ^b
OPB02	0.44± 0.024 ^b	0.13± 0.024 ^c	0.11± 0.024 ^c	0.44± 0.024 ^b	0.40± 0.024 ^b	0.76± 0.024 ^a
OPA02	0.94± 0.23 ^a	0.95± 0.23 ^a	1.00± 0.23 ^a	0.90± 0.23 ^a	0.94± 0.23 ^a	0.95± 0.23 ^a
Overall±	0.649±	0.701±	0.698±	0.711±	0.659±	0.807±
S.E	0.081	0.096	0.088	0.079	0.075	0.038

[†]NZW= New Zealand White, G= Gabali, BB= Baladi Black and Baladi Red (BR).

Means with the same letters in the row are not significantly different (p≤0.05).

Table 7. Genetic distances among the rabbit breeds investigated using the RAPD data

Breed [†]	NZW	G	BR	BB
NZW	1			
G	0.81	1		
BR	0.75	0.86	1	
BB	0.74	0.82	0.87	1

[†]NZW= New Zealand White, G= Gabali, Baladi Red =BR and BB= Baladi Black.

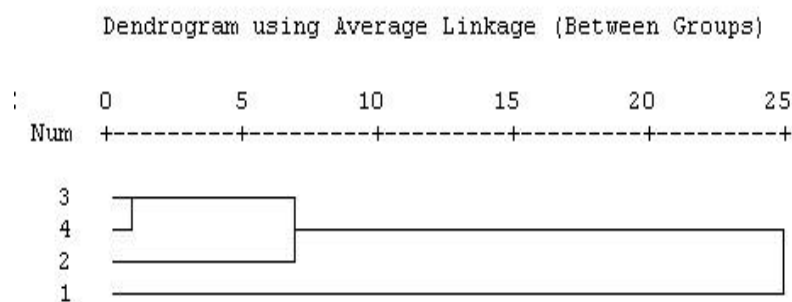


Figure 1. Dendrogram of the studied rabbit breeds based on the genetic distances: (1) NEW Zealand White. (2) Gabali. (3) Baladi Black. (4) Baladi Red.

Conclusions

- 1) RAPD can be successfully utilized as a tool for detecting the genetic variation and phylogenetic relationships among the Egyptian rabbit breed studied (Gabali, Baladi Black and Baladi Red).
- 2) The wide genetic diversity between New Zealand White and local breeds allows the

genetists to carry out further research in rabbit breeding programs to obtain unique molecular genetic markers which may be useful in differentiating between these breeds at molecular level.

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