Genetic Diversity and Phylogenetic Relationship among Some Rabbit Breeds Using Random Amplified Polymorphic DNA Markers

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Abstract: Random Amplified Polymorphic DNA (RAPD) marker was employed to assess the genetic variation and phylogenetic relationship among three rabbit breeds viz. *New Zealand White*, *Californian* and *Flander* reared in Egypt. Initially, a total of 20 random primers of arbitrary sequence were used but 14 of them generated reproducible, scoreable and polymorphic bands. Out of 120 bands scored using these primers, 39 (33%) were recognized as polymorphic and 81 (67%) as monomorphic bands. The highest percentage of polymorphic bands was recognized for primers OPA-10 and OPA-06 (56%) while the lowest percentage of polymorphic bands was recognized for primers OPE-19 (7%) and OPF-12 (14%). The band sharing frequencies (BSF) was found higher between *New Zealand White-Californian* (0.88±0.029), followed by *Californian-Flander* (0.87±0.024) and *New Zealand White-Flander* (0.84±0.034). Overall, there was no significant difference (P>0.05) in BSF values between breeds. The highest genetic distance was found between *New Zealand White-Flander* (5.568) followed by *Californian-Flander* (5.000) and *New Zealand White-Californian* (4.690). One primer (OPE-11) in *New Zealand White*, three primers (OPA-10, OPC-02, OPF-09) in *Californian*, five primers (OPA-01, OPA-06, OPA-10, OPB-14, OPF-09) in *Flander* were found to be specific for these breeds. The study suggests that RAPD can be successfully utilized for detecting genetic variation among the studied rabbit breeds.
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1. Introduction

In the last decade, popular meat breeds (*New Zealand White* and *Californian*) of rabbit were introduced in Egypt, being used in large scale commercial production throughout Egypt. They exhibit outstanding maternal abilities as related to maternal behavior, fecundity, lactation, and preweaning growth and survival (**Khalil, 1993**). Recently, the rabbit has attracted more attention from the biotechnology community. Several features make it an attractive model for transgenic, cloning, and an ideal choice for genomic analysis including; the rapid onset of sexual maturity, a short gestation period, a relatively larger number of offspring per litter, year-round reproductive capacity, and an average life-span of 9 years. Also, the rabbit genome is estimated to be 3 billion base pairs long, almost equal to the size of the human genome. In addition, rabbits have similar lipid metabolism to humans, making them good models of atherosclerosis (**Dove, 2000**).

Characterization at the molecular level is undertaken mainly to explore genetic diversity within and between animal populations, and to determine genetic relationships among such populations. The estimation of genetic variability of a species is an important criterion for its conservation and further genetic improvement (**Rahimi *et al*., 2005**).

Molecular markers derived from polymerase chain reaction (PCR) amplification of genomic DNA are an important part of the toolkit of evolutionary geneticists (**Holsinger *et al*., 2002**). By detecting genetic variation, genetic markers may provide useful information at different levels; population structure, levels of gene flow, phylogenetic relationships, patterns of historical biogeography and the analysis of parentage and relatedness (**Feral, 2002**). PCR-based multi-locus DNA fingerprints represent one of the most informative and cost-effective measures of genetic diversity (**Bagley *et al*., 2001**).

Randomly Amplified Polymorphic DNA (RAPD) technique, described firstly by **Williams *et al*., (1990)**, is a simple, fast and comparatively low cost assay that uses short oligonucleotide primers of arbitrary sequences to amplify anonymous fragments of genomic DNA (**Stepniak *et al*., 2002**), and no prior knowledge of the genome under investigation is necessary to perform the assay (**Bowditch *et al*., 1993**). Due to those features, the RAPD analysis has found many uses in different fields of study in both plants and animals. Polymorphism of RAPD fragments is detected as a band's presence or absence and may result from deletion, insertion or differences in the nucleotide sequences in or between the priming regions (**Clark and Lanigan, 1993**). RAPD markers are the randomly amplified target regions of less

functional part of the genome that do not strongly respond to selection on the phenotype level. Such amplified regions may accumulate more mutations thereby offering a wider potential in assessing the interbreed/population genetic differentiation.

The objective of this study was to assess the genetic diversity and phylogenetic relationship among three rabbit breeds viz. *New Zealand White* (NZW), *Californian* (C) and *Flander* (F) using Random Amplified Polymorphic DNA (RAPD) Markers.

2. Materials and Methods

This research was performed in the laboratory of Genetics and Genetic Engineering, Department of Animal Wealth Development, Faculty of Veterinary Medicine, Zagazig University.

2.1. Animals studied:

A total of thirty rabbits viz. *New Zealand White* (NZW, n=10), *Californian* (C, n=10) and *Flander* (F, n=10) were taken for this study. These breeds were obtained from San-El-Hagar Agricultural Company Farm, Sharkiya Governorate. Rabbits were housed in an open sided house and were fed commercial pelleted ration. All rabbits showed the signs of health and were free from any clinical disorders.

2.2. Blood collection and DNA extraction:

Blood samples were collected from each individual (from the central ear vein) aseptically in sterilized vacutainer tubes containing EDTA as anticoagulant, then stored at −20°C until extraction of DNA. Genomic DNA was extracted from whole blood using Gene JET whole blood genomic DNA purification mini kit (Cat. no. #K0781, Fermentas) following the manufacturer protocol. The quantity and quality of DNA were determined by spectrophotometer and agarose gel electrophoresis, respectively. The intact DNA showing no smearing was selected for further analysis

2.3. RAPD-PCR conditions and Electrophoresis:

Twenty random primers (Operon Technologies Inc, USA) of arbitrary sequence with 60-70% GC content were screened on pooled rabbit DNA Table (1). The amplification reaction was carried out in 25 µl final volume containing 1.5µl primer (10 pmol/µl), 12.5µl 2X Taq mastermix, 5µl template DNA (50 ng/µl) and 6µl dd-H_2O. Amplification of DNA was performed in Tprofessional thermal cycler (Biometra, Germany) with the following amplification conditions: initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 1 min, annealing at 34°C for 1 min, extension at 72°C for 1.5 min for 45 cycles and final extension at 72°C for 7 min. The PCR products were run on 1.5% agarose gel. Gel photographs were captured through gel documentation system (Bio Doc Analyse, Biometra, Germany).

Table (1): Name, Sequences and G+C (%) of primers used for amplification of RAPD loci.

Primers	Sequences (5′-3′)	G+C (%)	Primers	Sequences (5′-3′)	G+C (%)
OPA-01	5'-CAG GCC CTT C-3'	70 %	**OPC-02**	5'-GTG AGG CGT C-3'	70 %
OPA-06	5'-GGT CCC TGA C-3'	70 %	**OPC-08**	5'-TGG ACC GGT G-3'	70 %
OPA-10	5'-GTG ATC GCA G-3'	60 %	**OPE-11**	5'-GAG TCT CAG G-3'	60 %
OPB-05	5'-TGC GCC CTT C-3'	70 %	**OPE-19**	5'-ACG GCG TAT G-3'	60 %
OPB-13	5'-TTC CCC CGC T-3'	70 %	**OPF-09**	5'-CCA AGC TTC C-3'	60 %
OPB-14	5'-TCC GCT CTG G-3'	70 %	**OPF-12**	5'-ACG GTA CCA G-3'	60 %
OPC-01	5'-TTC GAG CCA G-3'	60 %	**OPX-02**	5'-TTC CGC CAC C-3'	70 %

2.4. Data Analysis:

Only distinct and clear bands of RAPD products on agarose gel were scored. 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. The BSF between individuals x and y was calculated as:

$$BSF_{xy} = \frac{2N_{xy}}{N_x + N_y}$$

Where N_{xy} is the number of common fragments observed in individuals x and y. N_x and N_y are the total number of fragments scored in x and y individuals, respectively. The BSF values between breeds will be statistically analyzed by analysis of variance (ANOVA) using SPSS 21 program.

Significant differences between means will be detected using Duncan's multiple range tests (**Kramer, 1957**). Genetic distances are designed to express the genetic differences between two populations as a single number. The genetic distances (D) were calculated between breeds based on Euclidean distance. Dendrogram was constructed using Unweighted Pair Group Method with Arithmetic average (UPGMA) by ***STATISTICA*** **(Stat Soft, Inc., 2007)**.

3. Results and Discussion

In the present study, RAPD technique was used to assess the genetic variability and phylogenetic relationship among three rabbit breeds. Twenty random primers were tested to amplify pooled genomic DNA from these breeds. 14 of them were chosen for further analysis, on the basis of the presence of reproducible and distinct RAPD profiles in one or more rabbit breeds (Figure 1). These primers amplified on average 6 to 14 bands of sizes varying from 174 bp to 2860 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**).

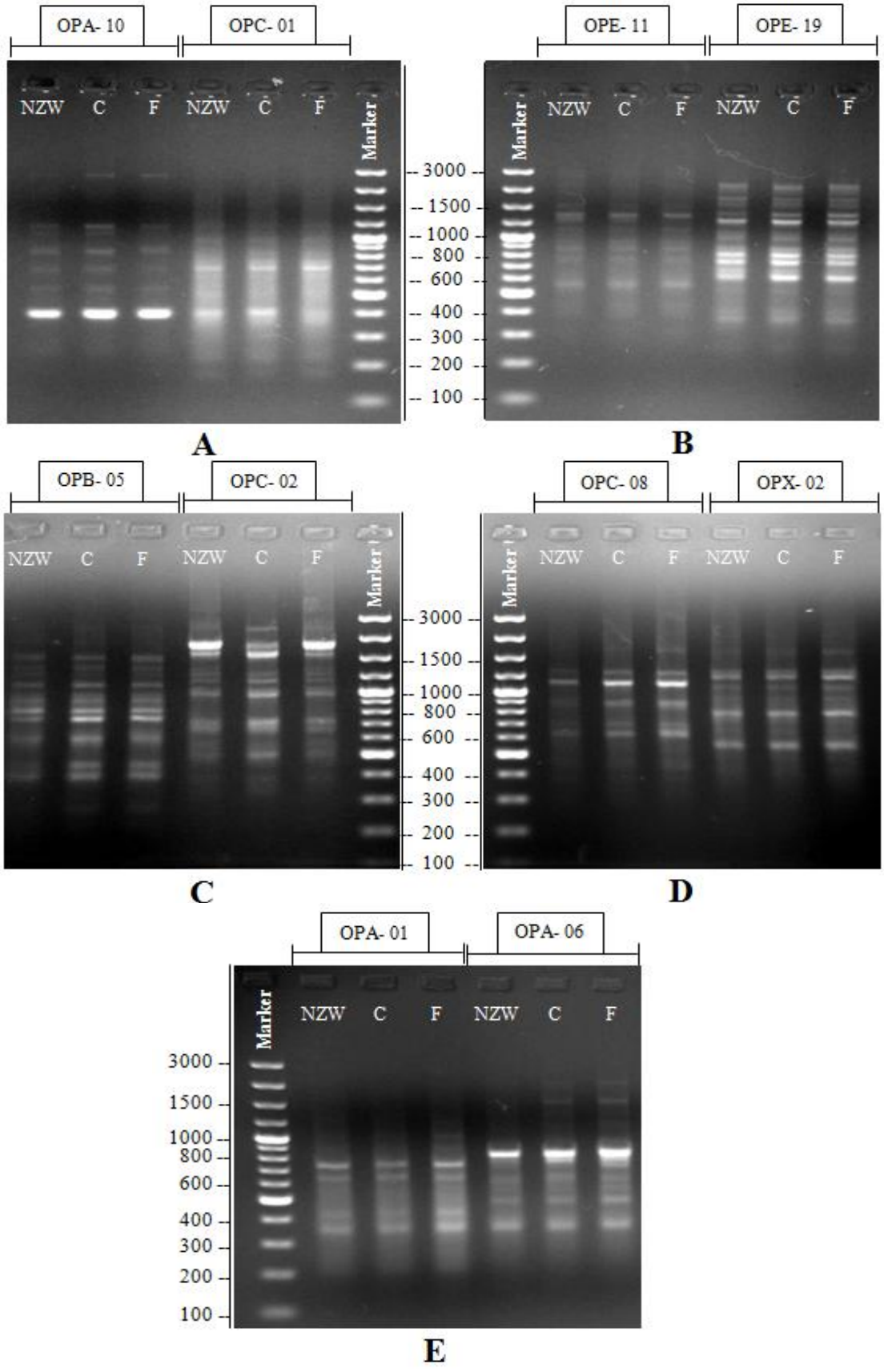
A total of 120 diagnostic bands were scored within RAPD profiles amplified by these 14 primers. The number of detected bands (TDB) per primers, number of polymorphic bands (NPB) and percent of polymorphic bands (PB) are presented in Table (2). Among 120 scorable bands 39 (33%) were recognized as polymorphic and 81 (67%) as monomorphic bands (Table 2). The average number of polymorphic bands per primer varied from 1 to 5. The highest percentage of polymorphic bands was recognized for primers OPA-10 and OPA-06 (56%) while the lowest percentage was recognized for primers OPE-19 (7%) and OPF-12 (14%), (Table 2).

Table (2): Summary of the results of RAPD analysis with 14 random primers: total number of detected bands (TDB), number of polymorphic bands (NPB) and percentage of polymorphic bands (PB %).

Primers	TDB	NPB	PB %	Primers	TDB	NPB	PB %
OPA-01	06	02	33	**OPC-02**	09	03	33
OPA-06	09	05	56	**OPC-08**	06	03	50
OPA-10	09	05	56	**OPE-11**	08	03	38
OPB-05	10	02	20	**OPE-19**	14	01	07
OPB-13	09	03	33	**OPF-09**	08	04	50
OPB-14	09	03	33	**OPF-12**	07	01	14
OPC-01	09	02	22	**OPX-02**	07	02	29
				Total	120	39	33

The number of bands amplified per primer was variable among the three rabbit breeds (Table 3). The maximum numbers of bands were found in *Californian* breed and *New Zealand White* (14) followed by *Flander* (13) using primer OPE-19. Primer OPE-19 gave the maximum numbers of bands (41) while the minimum numbers of bands were obtained using primer OPA-01 (14). **Khalil, *et al*., (2008)** studied association between RAPD markers and some reproductive traits in rabbits. They found five polymorphic fragments at molecular weight of 1500, 1100, 1200, 700 and 900 bp, respectively for five RAPD markers (OPA-12, OPA-19, OPA-20, OPF-09, and OPF-12) linked to these traits.

In a similar study conducted by **El Sayed (2010)**, he used eight RAPD primers (OPA-10, OPB-05, OPC-01, OPC-02, OPC-08, OPE-11, OPE-19 and OPX-02) to assess the genetic diversity among six rabbit breeds viz; *New Zealand White, Black Rex, Hyplus strain, Spanish line V, Moshtohor or Line M, and Sinai*. He found that, all primers yielded informative and identifiable bands revealing differences between breeds.


OPA- 10
OPC- 01
NZW C F NZW C F
Marker
3000
1500
1000
800
600
400
300
200
100
A
OPE- 11
OPE- 19
NZW C F NZW C F
Marker
B
OPB- 05
OPC- 02
NZW C F NZW C F
Marker
C
OPC- 08
OPX- 02
NZW C F NZW C F
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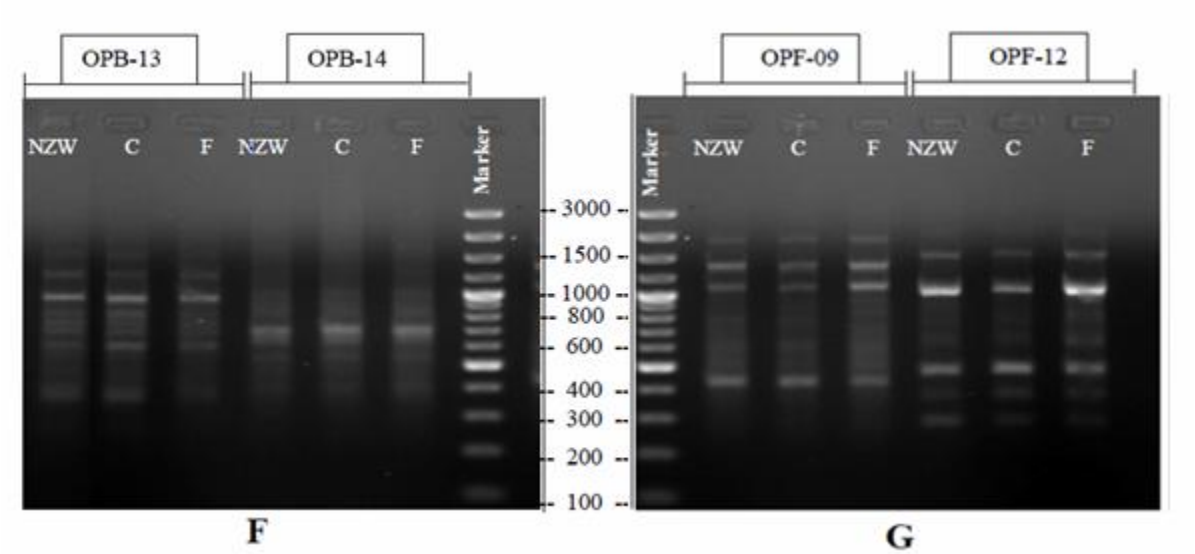

Figure (1): RAPD profile in different rabbit breeds; *NZW: New Zealand White, C: Californian* and *F: Flander* using primers; A: OPA-10 and OPC-01; B: OPE-11 and OPE-19; C: OPB-05 and OPC-02; D: OPC-08 and OPX-02; E: OPA-01 and OPA-06; F: OPB-13 and OPB-14; G: OPF-09 and OPF-12. Molecular marker (100 bp plus ladder).

Table (3): Number of bands per primer in different rabbit breeds

Primers	NZW	C	F	Total	Primers	NZW	C	F	Total
OPA-01	04	04	06	14	**OPC-02**	06	09	06	21
OPA-06	04	08	09	21	**OPC-08**	03	06	06	15
OPA-10	06	08	06	20	**OPE-11**	07	07	06	20
OPB-05	09	09	10	28	**OPE-19**	14	14	13	41
OPB-13	09	09	06	24	**OPF-09**	05	07	06	18
OPB-14	07	08	08	22	**OPF-12**	07	06	07	20
OPC-01	08	09	08	25	**OPX-02**	07	06	06	19
					Total	96	110	103	309

NZW: *New Zealand White* C: *Californian* F: *Flander*

Similar type of the study was conducted by **Mamuris *et al.*, (2002)** they used RAPD primers (OPA-02, OPA-9، OPA-10, OPA-20 and OPF-1) for assessment of genetic variability among brown hare (L.europaeus) population from different geographical regions. In their study, all primers produced polymorphic bands in the range of 5 to 11. **Rangoju *et al.*, (2007)** assessed genetic variability and phylogenetic relationship among three rabbit breeds using six random primers (OPA-1 OPA-8 OPA-10 OPA-18 OPB-3 OPB-5). They found that, the maximum number of bands was (13.2 ± 0.4), while minimum number of bands were (6.4 ± 0.2) in all the breeds, which is very similar to our study.

RAPD markers for breed differentiation:

The RAPD profiles of *New Zealand White*, *Californian* and *Flander* breeds generated by 14 random primers were studied for identifying breed specific markers i.e. the marker unique to a particular breed only. One primer (OPE-11) was identified in *New Zealand White*. Three primers (OPA-10, OPC-02 and OPF-09) were identified in *Californian*. Five primers (OPA-01, OPA-06, OPA-10, OPB-14 and OPF-09) were identified in *Flander*, Table (4). These breed specific primers can be used in identification of the breeds; however, these results need to be validated by using large sample size.

Table (4) Breed specific markers

No	Primers	NZW	C	F
1	**OPA-01**	**********	**********	1034 bp, 898bp
2	**OPA-06**	**********	**********	1191 bp
3	**OPA-10**	**********	489 bp	872 bp
4	**OPB-14**	**********	**********	1688 bp
5	**OPC-02**	**********	2453 bp, 1224 bp, 527 bp	**********
6	**OPE-11**	1248 bp	**********	**********
7	**OPF-09**	**********	625 bp	991 bp

NZW: *New Zealand White* C: *Californian* F: *Flander*

Band Sharing Frequency (BSF) is an indicator of relatedness between breeds (**Nei & Li, 1979**). Interbreed BSF being the highest between *New Zealand White-Californian* (0.88±0.029), followed by *Californian-Flander* (0.87±0.024) and *New Zealand White-Flander* (0.84±0.034) Table (5). Similarity between fingerprint patterns expressed by band sharing values provides a reliable method for evaluating genetic distance among population (**Kuhnlein *et al.*, 1989& Dunnington *et al.*, 1991**).

Table (5) Band sharing frequency between rabbit breeds

Primers	NZW-C	NZW-F	C-F	Primers	NZW-C	NZW-F	C-F
OPA-01	1.00	0.80	0.80	**OPC-02**	0.80	1.00	0.80
OPA-06	0.66	0.61	1.00	**OPC-08**	0.66	0.66	1.00
OPA-10	0.85	0.66	0.71	**OPE-11**	0.85	0.76	0.92
OPB-05	0.88	0.94	0.94	**OPE-19**	1.00	0.96	0.96
OPB-13	1.00	0.80	0.80	**OPF-09**	0.83	0.72	0.76
OPB-14	0.93	0.80	0.87	**OPF-12**	0.92	1.00	0.92
OPC-01	0.94	0.87	0.94	**OPX-02**	0.92	0.92	0.83
				Overall	0.88±0.029	0.84±0.034	0.87±0.024

NZW: *New Zealand White* C: *Californian* F: *Flander*

Rangoju *et al.*, (2007) studied genetic variation among three rabbit breeds viz. *White Giant (WG), Soviet Chinchilla (SC) and Grey Giant (GG)* using 40 RAPD primers. Six of them were found polymorphic and the overall BSF value between breeds was found higher in SC-GG followed by WG-SC and WG-GG. The Nei's genetic distance (D) was found highest between WG-GG followed by WG-SC and SC-GG.

Genetic distances among the studied three rabbit breeds were shown in Table (6). The genetic distances ranged from 4.690 between *New Zealand White -Californian* (more related) to 5.568 between *New Zealand White -Flander* (distantly related).

Table (6): Genetic distances between rabbit breeds using RAPD data.

Breeds	*New Zealand White*	*Californian*	*Flander*
New Zealand White	0.000		
Californian	4.690	0.000	
Flander	5.568	5.000	0.000

The genetic distance was higher as compared to the results shown by **Rangoju *et al.* (2007)**, which indicated less genetic distances (D=0.1605) between *White Giant-Grey Giant* followed by (D=0.1403) between *White Giant-Soviet Chinchilla* and (D=0.1295) between *Soviet Chinchilla-Grey Giant* rabbit breeds. These variations might be due to the different breeds and different geographical and climatic conditions, which cause variability in the gene pool. **El Sayed, (2010),** found that the highest value of the genetic distances (37.0) was between *New Zealand White/Black Rex and New Zealand White/ Spanish Line V* while the lowest value was 10.0 (more related) between *Spanish Line V/ Line M* (*Moshtohor*).

The phylogenetic relationships among the studied three rabbit breeds based on genetic distance were given in Figure (2). There were two separate clusters

formed from the three rabbit breeds. The topologies differ some what with respect to clustering arrangements as *New Zealand White* and *Californian* breeds clustered together in one cluster however, *Flander* breed clustered alone. These variations might be due to the different geographical climatic conditions and different genus/species, which cause variability in the gene pool.

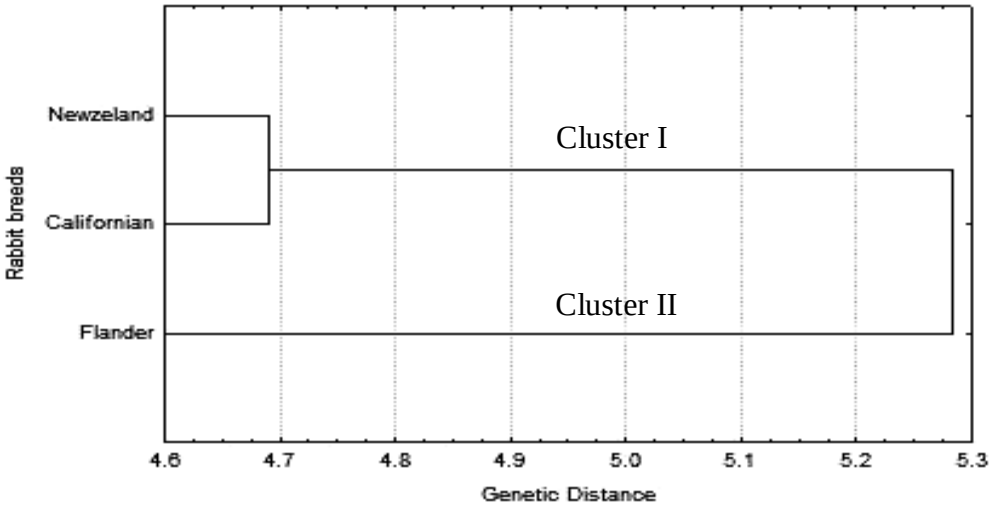

Figure (2): Dendrogram of studied rabbit breeds based on genetic distances among them.

In this concept, **Rangoju *et al.*, (2007)** illustrated the phylogenetic relationship among some rabbit breeds and their dendrogram revealed that *Soviet Chinchilla and Grey Giant* are closer, *while White Giant and Grey Giant* are distant to each other. Moreover, **El Sayed (2010),** showed that *Spanish Line V* and *Line M* (*Moshtohor*) were close to each other while *New Zealand White* and *Hyplus* were more distant breeds.

RAPD analysis has been used to discriminate animal species other than rabbits and to determine the genetic diversity and phylogenetic relationship between animals such as cattle breeds (**Rincón *et al.*, 2000; Ramadan, 2004; Devrim and Kaya, 2006 & Joshi *et al.*, 2007**), buffalo (**Sodhi *et al.*, 2006; & Abdel-Rahman and Hafez, 2007**), horse breeds (**Alves do Egito *et al.*, 2007 & Saleh, 2011**), sheep and goat (**Ali, 2003; Abd Rabou, 2007; Elmaci *et al.*, 2007; Mahfouz, *et al.*, 2008 & Kunene *et al.*, 2009**), chicken (**Zhang *et al.*, 2002; & Ahlawat *et al.*, 2004 & El Araby, 2006**), geese (**Maciuszonek *et al.*, 2005**), quail (**Sharma *et al.*, 2000**), duck breeds (**El-Gendy *et al.*, 2005 & Gholizadeh *et al.*, 2007**), turkey breeds (**Smith *et al.*, 2005**) and fish species (**Callejas and Ochando, 2002; Hassanien, 2004 & Abel Wahab, 2009**).

Conclusion:

The present study suggests that RAPD can be used as a tool to understand the genetic variability and phylogenetic relationship among three rabbit breeds. The wide genetic diversity between *New Zealand White* and *Flander* allows scientists' further research in rabbit breeding programs to obtain hybrid vigor and improve rabbit production. The study also provided unique molecular genetic markers for the studied rabbit breeds which may be useful in differentiating between these breeds at the molecular level.

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