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Identification of RAPD molecular markers linked to phenotypic characteristics in Rabbits breeds

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Abstract

The study was planned to compare six pure rabbit breeds (Gabali, Chinchilla, V-line – Bouscat, New Zealand White and California) using random amplified polymorphic (RAPD) molecular markers and phenotypic characteristics. A total of 115 amplified bands were scored out of them 98 were polymorphic. The percentage of polymorphism (%P) ranged from 42.86% to 100.00%. The cluster analysis revealed similarity coefficient values ranged from 0.31 (Gabali and California) to 0.66 (Bouscat and New Zealand). A cluster analysis realized using percentage of similarity method for phenotypic data, revealed similarity coefficient values ranged from 77.74 (California and Gabali) to 96.37 (Bouscat and V Line). Results of single marker analysis showed seven, five and four RAPD markers related to body weight, body tall and mortality rate (MR) traits, respectively. The marker OPA-13_{200bp} was significantly associated with litter's weight at weaning (LWW) (r=0.010, *p*=0.041). The associated markers each explained a maximum regression of 69.00 (LWW) to 93.00% (MR) of the total available variation for individual associated traits. The phenotypic data showed that New Zealand rabbits had the highest body weight and the body tall compared with the other pure breeds. The highest pre-weaning mortality rate was for Bouscat rabbits compared to other pure breeds. In the same direction, the highest LWW was for California rabbits.

Introduction

Rabbits are small mammals in the family *Leporidae* of the order *Lagomorpha*, found in several locations of the world. Their genome is estimated to be three billion base pairs long, almost equal to the size of the human genome. Rabbit meat is rich in high quality proteins, certain vitamins and minerals compared with the meat of other species (especially pork and beef), and it has less fat. People in Egypt, as a developing country with high human populations suffer from animal protein insufficiency due to the wide spread of Avian Flu and the enormous increase in the prices of poultry feeds, the annual poultry products deduced pronouncedly, which consequently minimized the daily average of animal protein consumption (Shafiq *et al.*, 2009). Therefore, it is worth to mention that raising rabbits could cover economically a considerable part of the Egyptian requirements from animal protein (El-hammady *et al.*,

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2010).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). One of such techniques is the use of random amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990). The advantages of RAPD is its Simplicity, applicability and low cost which gave this technique wide range of applications in many areas of genetics and molecular biology (Khalil *et al*., 2008 and Al-Saef *et al*., 2012), RAPD assay is applied by using 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). Also, RAPD technique provides a useful approach for evaluating genetic differentiation (Kresovich *et al*., 1992; Welsh and McClelland, 1990) particularly in phylogenetic study (Halward *et al*., 1992) and for identifying the markers linked to traits of interest without the necessity for mapping the entire genome (Bardakci *et al*., 2001).The objectives of this work were: 1) to evaluate genetic similarities determined by RAPD technique for the identification of the genetic relationship among the rabbit breeds and 2) to find out the association between phenotypic characteristics and RAPD molecular markers.

Materials and Methods

This study was carried out at the Rabbit research farm, Department of Poultry Production and Biotechnological laboratory, Department of Genetics, Faculty of Agriculture, Sohag University, Egypt, during the period from November 2012 to January 2015.

Animal materials:

A total number of 54 (9 per breed) rabbits from six pure breeds (Gabali, Chinchilla, V-line, Bouscat, New Zealand White and California, Table 1) were used in this study.

Rabbitry and housing:

Rabbits were raised in a semi-closed rabbitry of 54 m^2 (6 m width and 9 m length) with wire netted windows for natural ventilation and hoods to get rid of ammonia. The windows were oriented with an elevation of 150 cm from the floor. During cold, windy days and at night, windows were closed for protection from severe atmosphere and electric heaters were used to keep the air warm in winter. In

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summer cooler is used to maintain the proper temperature (25-30ºc) in the rabbitry by using fan to stir the air.

Phenotypic characteristics:

The rabbits were weighed and body tall was measured at predefined anatomical points using a measuring tape (cm). For the measurement procedures, the rabbits were put on a table and the same person measured the animals during the experiment **(Chineke *et al.*, 2006)**.

The descriptions of the measurements are as follows:

Body Tall (cm): was measured from atlas to the first coccygeal vertebra.

Mortality rate (MR): Pre-weaning litter mortality rate in pure litters (PLM) was calculated by the equation:

$$PLM = \frac{\text{Litters number at birth} - \text{Litters number at weaning}}{\text{Litters number at birth x 100}}$$

Whereas: Litter number at birth was measured by direct counting of kits immediately after kindling. It included number of still birth, while litter number at weaning was the number of fryers in each litter at 28 day according to Oke and Iheanocho (2011).

Litter weight at weaning (LWW): Litter weight at weaning was measured by weighing all the fryers (weaners) in a litter individually and summing up their weight (Oke and Iheanocho, 2011).

Statistical analysis:

Data of means of studied traits were statistically analyzed using SAS (1997), Duncan Multiple range Test was used to compare the differences between means (Duncan and Duncan, 1955).

Blood collection and DNA extraction

Blood samples (500µl) were collected from 3 animals (bulk) each breed (from the central ear vein) in 2 ml eppendorf tubes containing 500 µl extraction buffer (Tris-HCl, 100 mM; 1.576 grams NaCl, 1.4 M; 8.18 grams EDTA, 20 mM; 0.744 grams 2% CTAB; 2 grams and PVP, 1.0 grams). Genomic DNA (Figure 1) was extracted directly from whole blood using cetyltrimethyl ammonium bromide (CTAB) protocol as described by Poresbski *et al.*, (1997). The quality of the genomic DNA was checked by electrophoresis in 1% agarose gel containing ethidium bromide (0.5 mg ml-1) in ½ x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA). A total of twenty-six varied 10-mer random primers (Metabion International AG, Germany) were scanned across the parental genotypes.

PCR procedures

Amplification was carried out in a DNA Thermal Cycler (Primus 25, Germany) according to the method described by Williams *et al.*, (1990). The RAPD assay was performed in a 20 µl volume containing 12.5 µl of Go Taq®Green

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Master Mix (Promega, Madison, USA), 2.5 µl of primer 5 pmol, 3 µl of nuclease-free water and 2 µl of 150 ng of genomic DNA templates. PCR amplification was programmed for conditions with an initial denaturation cycle at 95°C for five minutes. The following 35 cycles were composed of: denaturation step at 95°C for 1 min, annealing step at 35°C for 1 min 30 s and elongation step at 72°C for 2 min. The final cycle of polymerization was performed at 72°C for 7 min. The amplification products were electrophoresed in a 1.0% agarose gel stained with 0.1 µl ethidium bromide. The amplified fragments were visualized and photographed using UVP Bio Doc-It imaging system (USA).

Data of RAPD markers analysis

The DNA banding patterns generated by RAPDs were analyzed by Gene Profiler software (Scanalytics, version 4.03). The presence (1) or absence (0) of each band was recorded for each breed for all the tested primers. Analysis of variance (ANOVA) was conducted using the 1–0 data according to Moore and McCabe (2003). The association analysis was conducted using simple linear regression. Data on final mean of individual character were regressed on whole 1–0 binary marker data for each individual marker using MS Excel program. The coefficient of determination (R^2) was calculated as $R^2 = 1 - (SSE / SST)$, where SSE and SST are the sum of squares of error and the total sum of squares, respectively.

In order to detect patterns of genetic relationship among breeds, similarity analysis of RAPD data and final means of all studied characters were constructed on the Jaccard's coefficient (Jaccard, 1908). A cophenetic matrix was derived from each matrix to test goodness of fit of the clusters by comparing the two matrices using the Mantel test (Mantel 1967). Dendrograms were generated with the unweighted pair group method with arithmetic mean (UPGMA) algorithm using the computational package (Multi variable statistical package) MVSP version 3.1. Finally, the correlation between each distance pair was calculated using NTSYS-pc version 2.2 (Rolhf 2000).

Results and Discussion

Phenotypic characteristics:

1- Final body weight (gm):

Data presented in Table (2) showed the effects of pure breeds, hybrid and sex on body weight and body measurements, it noted that New Zealand rabbits had the highest body weight (2821.7 gm) compared with the other pure breeds, where the lowest body weight (2212gm) was noted in V Line rabbits. Differences between pure breeds were highly significant ($p<0.01$). These results are similar to results of Abdel-Hamid (2014) who showed that breed effects on all body dimensions were significant ($p<0.05$).

2- Final body tall (cm)

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New Zealand rabbits had the highest body tall compared with other pure breeds when it was (52 cm), but V Line rabbits had the lowest body tall (47.6 cm). Differences among pure breeds were not significant ($P>0.05$). These results are in agreement with those of Adeyemo *et al.* (2014) who reported that in Chinchilla, Dutch and New Zealand white, all other parameters measured for linear body measurement were not significantly ($p>0.05$).

3- Mortality rate at weaning (%):

As shown in Table 2, it could be observed that the highest pre-weaning mortality rate was for Bouscat rabbits compared to other pure breeds, it was (32.89±11.9), while the lowest pre-weaning mortality rate observed for California rabbits (4.17±4.1). These results are disagreed with Topczewska *et al.* (2013) who observed a high percentage of deaths in the Californian breed (26.45%). Differences between pure breeds were highly significant ($p<0.01$). This result is similar to that of Nwakpu *et al.* (2015) who observed a significant ($P>0.05$) differences in the pre-weaning mortality of three purebreds of rabbits (Chinchilla, New Zealand White and Dutch breeds). However, this result is disagreed with Ramesh Chandra *et al.* (2015) who observed non-significant ($P<0.05$) effect of breed on pre-weaning mortality.

4- Litter weight at weaning (gm):

Data presented in Table 2, showed that in pure breeds the highest litter weight at weaning was in California rabbits (335.48 gm ±26.2) while the lowest litter weight at weaning in New Zealand rabbits (241.84 gm ±15.9). Differences among pure breeds were highly significant ($p<0.01$). These results were disagreed with the findings of Oke and Iheanocho (2011) in their studies on New Zealand White and Chinchilla rabbits. They showed that breed had no significant effect ($p>0.05$) on most of reproductive traits measured including litter weight at weaning.

Level of polymorphism based on RAPDs

The six rabbit breeds were differentiated using 26 RAPD primers, out of them, 16 primers (Table 3) were generated different degrees of polymorphism (%P). A band was considered as polymorphic if it the band differentiates at least any 2 of the 6 genotypes (Figure 1). In this study, the number of amplification products per primer varied from 5 (OPAM-01, OPA-13 and OPA-08) to 10 (OPH-01 and OPA-18), with an average of 7.91 per primer. The number of polymorphic bands ranged from 3 (OPP-05 and OPA-08) to 9 (OPH-01 and OPA-10) with an average of approximately 6.13 bands per primer (Table 3). Rangoju *et al.,* (2007) assessed genetic variability and phylogenetic relationship among three rabbit breeds using six RAPD primers (OPA-1 OPA-8 OPA-10 OPA-18 OPB-3 OPB-5). They found

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that the number of bands was between 6.4 and 13.2 per primer.

Ninety-eight out of 115 amplified bands were scored polymorphic. The percentage of polymorphism (%P) ranged from 42.86% (OPP-05) to 100.00% (OPAV-13, OPAM-01, OPG-09, OPW-13, OPAR-05, OPA-13, OPAT-08 and OPA-10) with an average of 85.14% (Table 3). In this study, the %P presents a kind of genetic diversity which was higher than that (35.44%) obtained by Galal *et al.,* (2013) among 4 rabbit genotypes, named: Animal Production Research Institute "APRI", New Zealand White "NZW", Balady Black "BB" and Gabali "GAB". They showed that the highest level of polymorphism (100%) was observed in primer OP-B10, but the lowest level of polymorphism was 20% in primer OP-B14. In the same direction, El-Bayomi *et al.,* (2013) showed that 39 bands (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%). Likely, RAPD-PCR fingerprints have been successfully used in defining genetic diversity among different species of horse, buffalo, beef, venison, rabbit, and kangaroo (Yang *et al.,* 2013).

In this work, the number of primers (16 out of 26) which was able to detect the polymorphism among rabbit genotypes was bigger than that mentioned by Al-Saef *et al.,* (2012). They showed that from a total of 40 primers used, only five primers were able to identify five polymorphic fragments at molecular weight of 1500, 1100, 1200, 700 and 900 bp, respectively. The band size obtained in this study ranged from 80 to 1000 bp generated by primers OPAT-08 and OPA-08, respectively (Table 3). This band size is less than that obtained by Osman *et al*., (2010), who showed that eight RAPD primers produced a total of 71 bands with a molecular size ranging from 150 to 2000 bp. They mentioned that the similarity percents ranged from 64.8% (between Rix and New Zealand White) to 92.4% (between Line M and Line V). Also, El-Sabrout and Aggag (2015) reported that RAPD profiles at molecular weight ranged from 600 to 1800 bp with a total of 86 of polymorphic band patterns and nine monomorphic band patterns.

Cluster analysis based on RAPD markers

The genetic similarity values among the six rabbit genotypes were calculated according to the analytical results of electrophoretic band patterns of RAPD markers and were used for UPGMA cluster analysis according to Jaccard`s Coefficient (Jaccard 1908). A cluster analysis realized using similarity coefficient for parental genotypes, revealed similarity coefficient values ranged

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from 0.31 (between Gabali and California) to 0.66 (between Bouscat and New Zealand White) with an average of 0.49 (Table 4).

The UPGMA cluster analysis based on RAPD markers separated the parental genotypes into five different clusters (Figure 2). The first cluster contains the genotype Bouscat which branched at 0.66 level of similarity with the genotype New Zealand. The genotype V line branched at 0.58 of similarity level with the first cluster. The genotypes Chinchilla, Gabali and California were belonged to the third, fourth and fifth clusters, respectively. These results indicate that RAPD primers revealed a kind of genetic diversity among these genotypes, which suggested that RAPD markers can be used as a tool to understand the genetic variability and phylogenetic relationships among rabbit genotypes. Knowledge of the genetic distances among different genotypes is very useful for genetic improvement (Ceron and Angel, 2001). The results of Galal *et al.,* (2013) indicated that BB genotype was closely related with GAB breed, while the APRI genotype was the most different. This may be due to the fact that BB and GAB are Egyptian genotypes.

Cluster analysis based on phenotypic data

A cluster analysis realized using percentage of similarity method for phenotypic data, revealed similarity coefficient values ranged from 77.74 (between California and Gabali) to 96.37% (between Bouscat and V Line) with an average of 88.90% level of similarity (Table 4). In this regard, the dendrogram gathered the genotypes into two clusters, which separated at 84.25% similarity coefficient. The first cluster contains genotype "Gabli". The second cluster sub-divided into two sub-groups, genotypes Bouscat and V Line grouped together belong the first sub-group. The second sub-group was with genotype California which branched at 91.89% of similarity with genotypes New Zealand and Chinchilla (Figure 2).

Single Marker analysis (SMA)

The present study involved a set of 6 rabbit breeds, which constitute important and diverse genotypes, exhibiting moderate to high genetic variability for characteristics analyzed during this work. Using simple linear regression method, a total of 98 polymorphic RAPD molecular markers were identified, 17 of which showed significant and highly significant association with 4 characters (Table 5). The single marker analysis results showed, seven RAPD markers (OPAW-10$_{350bp}$, OPAV-13$_{270b}$p, OPAM-01$_{110bp}$, OPH-01$_{170bp}$, OPW-13$_{500bp}$, OPC-05$_{240bp}$ and OPA-18$_{375b}$p) were identified for body weight character. Also, five RAPD markers (OPAW-10$_{350bp}$, OPAV-13$_{270bp}$, OPAM-01$_{110bp}$, OPH-01$_{170bp}$ and OPH-01$_{120bp}$) were highly significant associated with the body tall character. The RAPD markers;

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OPAW-10_{350bp} (Figure 1), OPAV-13_{270bp}, OPAM-01_{110bp} and OPH-01_{170bp} were regarded as candidate markers, linked to the mortality rate (MR) character. Finally, the marker OPA-13_{200bp} was significantly associated with litter's weight at weaning (LWW) (0.010*, p=0.041) character (Table 5). The associated markers each explained a regression ranged from 69.00 (LWW) to 93.00% (MR) of the total available variation for individual associated traits.

Markers identified during the present study need to be subjected to validation and/or functional analysis of respective traits, which is beyond the scope of the present work. However, we believe that at least one of the markers identified would be validated and used for marker-assisted selection. Similar findings were obtained by Keliang *et al.*, (2008) who reported that the RAPD markers; OPA1, OPA7 and OPA14 are correlated with the performance of birth weight traits of the Rex rabbit; OPA1, OPA7,OPA14 and OPA15 are correlated with birth litter size character, and OPA14 and OPA15 are correlated with the performances of litter size and living litter size character. Likely, Khalil *et al.*, (2008), showed that out of 40 RAPD primers, three (OPA-19, OPF-09 and OPF-12) showed significant linkage with body weight, litter weight and gain traits, milk yield. Also, Al-Saef *et al.*, (2012) identified five primers (OPA12, OPA19, OPA20, OPF09, and OPF12) which could be used as markers in differentiating between animals of Saudi-2 line since these markers showed significant linkages with respiration rates, body temperatures and daily gain rate traits.

Table 1: List and data of the six rabbit breeds used in the study.

Breeds	**Origin**	Breeds	Origin
Gabali	Egypt	Bouscat	France
Chinchilla	United States of America	New Zealand White	United States of America
V-line	Spain	California	United States of America

Table 2: Means of rabbit characters linked to RAPD molecular markers.

Breeds	**Body weight (gm)**	**Body tall (cm)**	**Mortality rate at weaning (%)**	**Litter's weight at weaning (gm)**
Pure Breeds				
Gabali	2533.8^{bac}	50.75^{a}	14.6^{c}±6.2	288.75^{bc}±28.6
Chinchilla	2621.8^{ba}	50.8^{a}	13.22^{c}±6.9	248.13^{c}±15.1
Bouscat	2415.8^{bdc}	50.667^{a}	32.89^{a}±11.9	316.0^{a}±30.4
V line	2212^{dc}	47.6^{a}	11.46^{c}±7.9	276.19^{bc}±20.6
New Zealand	2821.7^{a}	52^{a}	23.60^{b}±6.9	241.84^{c}±15.9
California	2469.2^{bdc}	51.0^{a}	4.17^{d}±4.1	335.48^{a}±26.2
Probability				
significance	**	*NS*	**	**

Means in the same column have different letters are significantly different.*= p≤0.05, **=p≤0.01 and NS = p>0.05.

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Table 3: Primers used in RAPD analysis, their sequences, total number of fragments detected by each primer and percentage of polymorphism (%P).

Primer Name	Primer Sequence 5'→3'	Amplified bands		%P	Fragment size (bp)	
		BN	PB		Larger	Smaller
OPAW-10	GTTGTTTGCC	6	5	83.33	800	200
OPAV-13	CTGACTTCCC	8	8	100.00	430	100
OPAM-01	TCACGTACGG	5	5	100.00	500	110
OPH-01	GGTCGGAGA A	10	9	90.00	790	100
OPG-09	CTGACGTCAC	6	6	100.00	890	260
OPW-13	CACAGCGACA	8	8	100.00	900	200
OPAR-05	CATACCTGCC	7	7	100.00	650	190
OPC-05	GATGACCGCC	7	5	71.43	920	140
OPP-05	CCCCGGTAAC	7	3	42.86	400	100
OPA-13	CAGCACCCAC	5	5	100.00	400	100
OPF-20	GGTCTAGAGG	8	7	87.50	890	110
OPAT-08	TCCTCGTGGG	7	7	100.00	700	80
OPA-01	CAGGCCCTTC	7	4	57.14	775	225
OPA-08	GTGACGTAGG	5	3	60.00	1000	250
OPA-10	GTGATCGCAG	9	9	100.00	725	175
OPA-18	AGGTGACCGT	10	7	70.00	675	150
Total	-	**115**	**98**	-	-	-
Mean	-	**7.19**	**6.13**	**85.14**	-	-

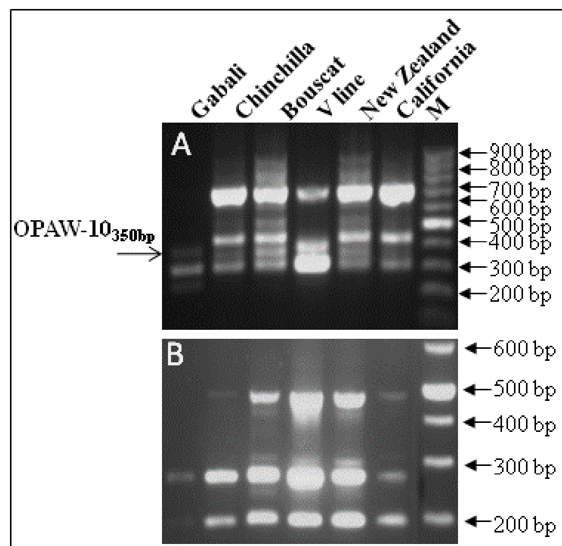

Figure 1 : RAPD Profile obtained with primers (A) OPAW-10 and (B) OPA-08.

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Table 4: Similarity matrix for 6 parental rabbit genotypes obtained from 115 RAPD fragments (bottom diagonal) and phenotypic data (above diagonal).

Genotypes	Gabali	Chinchilla	Bouscat	V Line	New Zealand	California
Gabali	-	83.91	88.97	87.23	83.39	77.74
Chinchilla	0.47	-	92.65	89.73	95.11	93.26
Bouscat	0.38	0.58	-	96.37	91.39	88.02
V Line	0.38	0.50	0.55	-	88.6	86.68
New Zealand	0.41	0.51	0.66	0.62	-	90.52
California	0.31	0.36	0.41	0.41	0.44	-

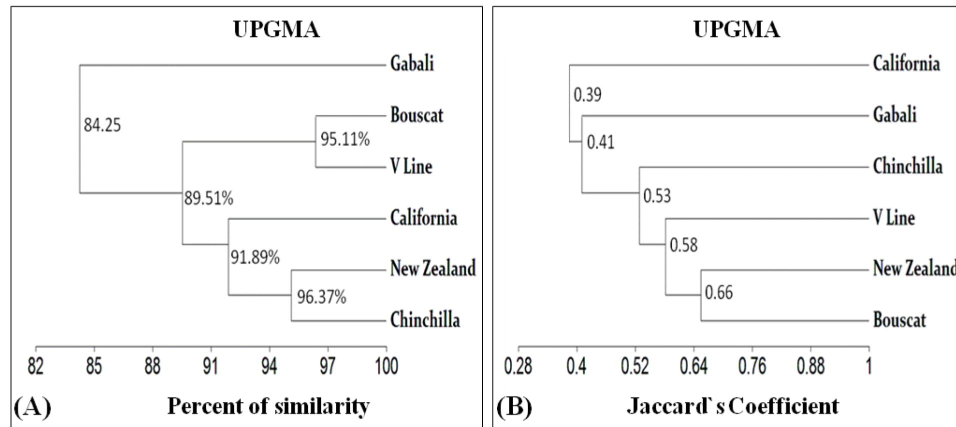

Figure 2: dendrogram of six rabbit genotypes obtained using (A) phenotypic data and (B) RAPD data.

Table 5: Details analyses of variances (ANOVA) involving simple linear regression for traits using 98 RAPD polymorphic bands.

Marker	trait	SV	df	SS	MS	R^2(%)	*P-value*
OPAW-10_{350bp} OPAV-13_{270bp} OPAM-01_{110bp} OPH-01_{170bp} OPW-13_{500bp}	body weight	Genotypes Error Total	1 4 5	224817.188 77182.813 302000	224817.188* 19295.703	74.44	**0.027**
OPC-05_{240bp} OPA-18_{375bp}		Genotypes Error Total	1 4 5	246037.5 55962.5 302000	246037.5** 13990.625	81.47	**0.014**
OPAW-10_{350bp} OPAV-13_{270bp} OPAM-01_{110bp} OPH-01_{170bp}	body tall	Genotypes Error Total	1 4 5	7.521 1.188 8.708	7.521** 0.297	86.36	**0.007**
OPH-01_{120bp}		Genotypes Error Total	1 4 5	7.042 1.667 8.708	7.042* 0.417	80.86	**0.015**
OPAW-10_{350bp} OPAV-13_{270bp} OPAM-01_{110bp} OPH-01_{170bp}	mortality rate at weaning	Genotypes Error Total	1 4 5	0.165 0.012 0.177	0.165** 0.003	93.00	**0.002**
OPA-13_{200bp}	litter's weight at weaning	Genotypes Error Total	1 4 5	0.010 0.004 0.014	0.010* 0.001	69.00	**0.041**

SV, source of variance df, degrees of freedom
SS, sum square MS, mean square
R^2 % , coefficient of determination

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