USE OF INTER SIMPLE SEQUENCE REPEATS AND PROTEIN MARKERS IN ASSESSING GENETIC DIVERSITY AND RELATIONSHIPS AMONG FOUR RABBIT GENOTYPES

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Abstract: The importance of DNA and protein applications as powerful tools in breeding programmes is revealed. The inter simple sequence repeats (ISSR) technique was used to characterise and determine phylogenetic relationships among 4 genotypes of rabbit, namely Alexandria (Alex), V line (V), New Zealand White (NZW) and California (Cal). Six out of 7 ISSR primers exhibited sufficient variability and were used to characterise the genetic diversity and relationships among studied genotypes. A total of 141 DNA bands were detected. DNA fragments were generated with 87 (61.7%) being polymorphic, indicating considerable genetic variation among the examined genotypes. While protein electrophoresis provides a precise method for assaying variation in serum proteins which play an important part in productive performance. The results demonstrated 2 specific protein markers in Alexandria rabbits; these specific protein markers may be responsible for the superiority of Alexandria line in weight. Phylogenetic analysis based on Nei and Li unbiased genetic distance illustrated that (Alex & V) and (Cal & V) were genetically closely related. Our results showed that ISSR and protein electrophoresis are useful methods to detect different genetic expressions and understand the variability in some productive traits in rabbits.

Key Words: Rabbit, ISSR, protein, genetic diversity, phylogenetic analysis.

INTRODUCTION

Domestic rabbits are one of the most efficient cellulose converter species, ensuring high production of low-cost meat due to their herbivore nature, and they are not in competition with humans regarding diet (Bud *et al.*, 2011). Recently, rabbits have attracted more attention in biotechnology experiments (El-Bayomi *et al.*, 2013). Over the last decade, popular broiler rabbit genotypes (New Zealand White, Californian and V line) have been introduced in Egypt and are currently used in large-scale commercial production (El-Bayomi *et al.*, 2013). The genetic diversity found in domestic strains allows breeders to develop new characteristics in response to changes in environment, diseases or market conditions (Ola *et al.*, 2013). Conservation strategies for rabbit populations should be based on molecular genetics characterisation (Romanov *et al.*, 1996).

Molecular analysis of genetic diversity and relatedness among genotypes is necessary for the recognition of genetic resources that are economically important (Nikkhoo *et al.*, 2011). Molecular genetics knowledge provides modern tools for rabbit breeding with rapid and accurate identification (Fulton, 2008). DNA molecular markers can be successfully used to develop genetic improvement programmes (El-Sabrout *et al.*, 2014). Inter simple sequence repeats (ISSR) analyses have become an important technique for identifying the markers linked to economical traits of interest with no need to map the entire genome. ISSR is a sensitive technique with only a small amount of DNA and low cost, which has been widely utilised in genetics analysis in animals and has shown good results in genetic

diversity population studies (Parthasarathy *et al.*, 2013). Protein electrophoresis is an effective technique for the detection of genetic polymorphism among different breeds. The polymorphism of blood protein markers gives some useful information in animal breeding studies (Ola *et al.*, 2013). Moreover, the protein electrophoresis technique provides comprehensive approaches for studying the biochemical differences among different rabbit genotypes and their productive performance (El-Sabrout *et al.*, 2015).

Therefore, the main objective of the present study was to genetically characterise and determine genetic similarity and diversity in 4 rabbit genotypes, namely Alexandria (El-Raffa, 2005, 2007), V-line (Estany *et al.*, 1989), New Zealand White and California, by studying: (i) DNA fingerprint using ISSR analysis and (ii) protein fingerprint using protein electrophoresis analysis, to provide genetic information for future breeding programmes that will include these breeds.

MATERIALS AND METHODS

Rabbits in this study were housed in cages and given *ad libitum* access to commercial pelleted diet containing 18% crude protein, 13% crude fibre and 2600 kcal/kg. By using high standards of hygiene and good management, the occurrence of dangerous diseases was largely avoided. All rabbits used were phenotypically normal and sexually fertile.

Blood collection

Blood samples were collected from Alexandria (Alex), V-line (V), New Zealand White (NZW) and California (Cal) rabbits (10 rabbits/genotype) reared in Egypt from central artery vein of the ear under vacuum into centrifuge tubes containing EDTA as anticoagulant for molecular genetic analysis, from each individual, and in centrifuge tubes containing heparin sodium as anticoagulant for the molecular protein analysis. Samples were kept at –20°C until use.

Molecular genetic analysis

DNA extraction

DNA was extracted from whole blood following the instruction of Fermentas whole blood genomic DNA extraction kit (Thermo® Corporation, Lithuania). The quantity and quality of the isolated DNA was determined by agarose gel electrophoresis and spectrophotometer (260 nm-280 nm).

ISSR-PCR analysis

To generate ISSR profiles from rabbit DNA, 7 different ISSR markers from Biosearch Technologies (USA) were used in this study (Table 1). The polymerase chain reaction (PCR) analysis included 3 steps: after an initial heating at 95°C for 180 s, 35 cycles of PCR amplification were performed, each consisting of four steps: denaturation at 95°C for 30 s; annealing at 45°C for 30 s; extension at 72°C for 60 s; and final extension at 72°C for 10 min. Amplicons were separated on 1% agarose gel, stained with Ethidium Bromide and visualised under UV Transilluminator.

Molecular protein analysis

Serum was obtained by centrifugation of the blood samples at 4000 rpm for 10 min. Protein profile was analysed by procedure published by Pan *et al.* (1991). Polyacrylamide gel electrophoresis (PAGE) resolving gels (7.5%, 1.5 mm

Table 1: List of seven ISSR primers and their sequences.

N	Primer code	Nucleotide sequence (5'-3')
1	ISSR1	CAC ACA CAC ACA CAC ACA G
2	ISSR2	AGA GAG AGA GAG AGA GAG C
3	ISSR3	GAG AGA GAG AGA GAG AGA C
4	ISSR4	ATG ATG ATG ATG ATG ATG T
5	ISSR5	AGA GAG AGA GAG AGA GGA
6	ISSR6	AGA GAG AGA GAG AGA GGT
7	ISSR7	AGA GAG AGA GAG AGA GCT

thick) were prepared by mixing 3.33 mL of 30% acrylamide (acrylamide+bis acrylamide 30:0.8), 5.0 mL of 1.5 M Tris-HCL (pH 8.8), 50 µl of 10% ammonium persulphate, 4.02 mL of distilled water, and 7.5 µL tetramethylethylenediamine (TEMED). The stacking gel contained 1.3 mL 30% acrylamide, 2.5 mL of 0.5 M Tris-HCL (pH 6.8), 50 µL of 10% ammonium persulphate and 6.1 mL distilled water. After degassing for 10 min, 10 µL TEMED were added, and the gel was poured between glass plates. Gels were run using Mini-PROTEIN II vertical cell (Bio-Rad) at 75 V through stacking gel followed by 125 V to the end of electrophoresis (2 h), 30 mA. Gels were stained in 0.02 M acetate buffer at pH 4.5 containing 0.05% pyrogallol and 0.03% H_2O_2. After staining, gels were immersed in 7% acetic acid for 3 min and then washed with distilled water.

Scoring and analysis of DNA and protein patterns

The resolved DNA and protein bands were documented and processed for data analysis. The clear and reproducible band represented across the DNA and protein samples were scored as (1) and absence of bands was recorded as (0) in the ISSR and protein profile of different rabbit genotypes. The indexes of similarity were calculated across all possible pairwise comparisons among genotypes following the method of Nei and Li, 1979. The similarity matrix results were subjected to unweighted pair group method with arithmetic mean (UPGMA) clustering and PAST software (Hammer *et al.*, 2001) to generate a dendrogram.

RESULTS

Molecular genetic analysis

In the present study, genetic variations were detected in 4 different rabbit genotypes, namely Alexandria, V-line, New Zealand White and California, using ISSR analysis. Seven ISSR primers were examined; 6 of them were employed and amplified (ISSR1, ISSR2, ISSR3, ISSR4, ISSR6 and ISSR7). An example of ISSR pattern obtained with studied genotypes is shown in Figure 1. In the PCR products, ISSR2 and ISSR3 primers amplified the largest number of fragments, which ranged from 250 to 2000 bp. These primers detected 3 and 2 unique bands specific for Alexandria and California respectively. A total of 141 DNA fragments were detected. DNA fragments were generated with 87 polymorphic bands (61.7%).

The UPGMA dendrogram, based on Nei's genetic distance, depicts the relationship among the investigated rabbit genotypes. The genetic similarity values were (0.44), (0.14) and (0.11) between (Alex & V), (Alex & NZW), and (Alex & Cal), respectively (Table 2). Alexandria line was distinctly different from NZW and Cal genotypes. From the phylogenetic analysis, the genotypes Alexandria and V-line appeared to be closely related, and also the genotypes V-line and California (Figure 2).

Molecular protein analysis

Scan of PAGE electrophoretic patterns of proteins of Alex shown in Figure 3. The banding protein patterns showed 15 protein bands are diagnostic to Alex line, while 14 bands are detected for NZW line and 13 bands for
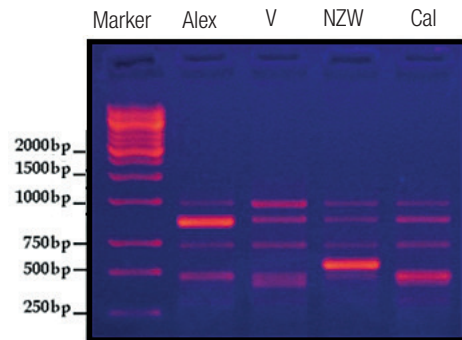

Figure 1: ISSR2 profile of 4 rabbit genotypes: Alexandria (Alex), V-line (V), New Zealand White (NZW) and California (Cal).
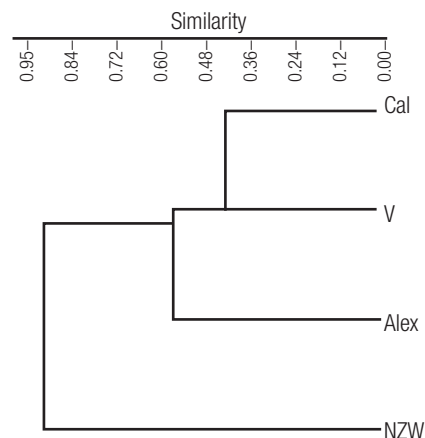

Figure 2: Dendrogram of genetic distance based on ISSR data of four rabbit genotypes: Alexandria (Alex), V-line (V), New Zealand White (NZW) and California (Cal).

Table 2: Genetic similarity estimated among 4 rabbit genotypes: Alexandria (Alex), V-line (V), New Zealand White (NZW) and California (Cal).

	Alex	V	NZW	Cal
Alex	1	0.4395	0.1411	0.1103
V		1	0.1844	0.6901
NZW			1	0.1297
Cal				1

V line as well as Cal line. The results revealed 2 specific bands at 70 and 245 kDa which could be used as specific protein markers to characterise Alex rabbits. In addition, we detected one specific band for NZW at 48 kDa which could be used as specific protein marker to characterise NZW rabbits.

The major serum proteins of rabbit blood can be classified as follows: albumin and globulin proteins. Globulin protein is divided into: α1,2-globulins, transferrins (β1,2-globulins) and immunoglobulins (γ-globulins) (Figure 4).

DISCUSSION

In general, genetic variation allows species to adapt to changes in environmental conditions and assessment of genetic variation among rabbit genotypes could be very useful in rabbit breeding programme improvement. The efficacy of DNA markers in detecting polymorphism among rabbit genotypes and their applicability in population studies and establishing genetic relationships among populations have been reported by El-Sabrout *et al.* (2014).

In this study, we demonstrated that the ISSR primers produced different number of bands with studied genotypes and showing different levels of molecular polymorphisms. These primers were utilised for comparative analysis of genetic diversity and for phylogenic analyses. This was in agreement with Pradeep *et al.* (2002) who found that ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, genome mapping and evolutionary biology. Hakki *et al.* (2007) also reported effective segregation of species with ISSR. The results obtained in this study
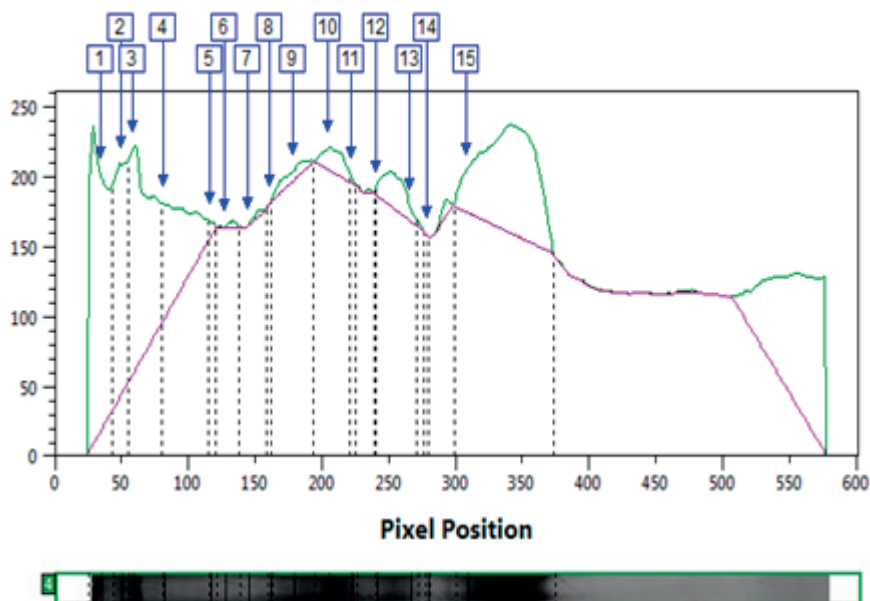

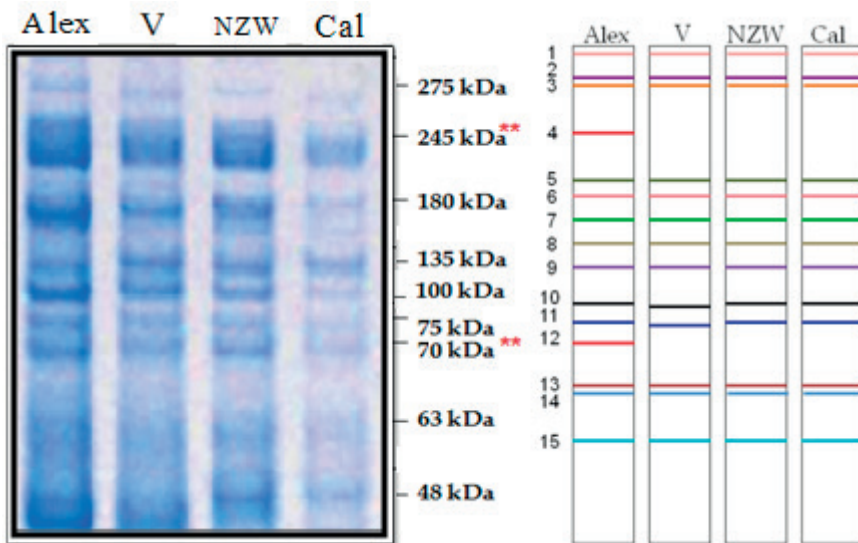Figure 3: Scan of PAGE- electrophoretic patterns of proteins of Alex (by TotalLab Quant® program, 2013).

Figure 4: Photograph and zymogram showing protein fingerprinting of 4 rabbit genotypes: Alexandria (Alex), V-line (V), New Zealand White (NZW) and California (Cal).

confirmed that ISSR is a useful technique, indicating considerable genetic variation among the examined genotypes, which presents a kind of genetic diversity. Furthermore, the possibility of characterisation of every genotype examined offers a promising perspective as a molecular tool for varietal identification and breeding programme applications.

Dendrogram derived from an UPGMA cluster analysis of the ISSR results based on Nei's genetic distance allowed the identification and provided knowledge of the genetic distances among different genotypes, which is very useful for genetic improvement. Simple matching genetic distances among all genotypes were calculated. The values of similarity indices reflect the range of homogeneity and inbreeding within each genotype studied. Alex line had the highest polymorphism and was distinctly different from NZW and Cal genotypes. From previous results, we can expect that the crossbreeding between Alex and Cal will be more successful to obtain many benefits from the 2 genotypes to improve the productive performance of rabbit genotypes and develop new characteristics to become more adapted to environmental changes; this is because they are genetically far apart and Alex is a synthetic line selected for daily weight gain and more adapted to environmental changes. Knowledge of genetic diversity among rabbit breeds and the relationship between their genetic markers and performance play an important role in the conservation of genetic resources of rabbits (Wilkinson *et al.*, 2011).

The polymorphism of protein markers gives some beneficial information in animal breeding studies, such as the relationships among breeds and their expressions. Variation in protein profile reflects changes in the genes that code for them. Native polyacrylamide gel electrophoresis provides an accurate method for assaying variation in serum proteins. The protein banding pattern of each genotype reveals a biochemical genetic fingerprint and each band in the pattern reflects a separate transcriptional level. The 2 specific protein markers which were obtained in Alex line are expression of genes which may be responsible for their superiority in weight from other species in this study or their difference in fur colour. Moreover, the profile of protein classification can be used to estimate, diagnose and observe a variety of diseases and conditions (Pagana, 1998).

CONCLUSION

Molecular markers including ISSR and protein fingerprinting represent reliable tools which may have a great impact in rabbit breeding programmes and genetic improvement of rabbits. This study supplies comprehensive approaches

for studying the genetic differences among different rabbit genotypes. Furthermore, it showed that ISSR-PCR and protein electrophoresis are useful methods to detect different genetic expressions and understand the variability in some productive traits in rabbits.

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