Zagazig J. Agric. Res., Vol. 34 No .(4) 2007 685-701

GENETIC DIVERSITY AMONG FOUR SHEEP CROSSBREDS BASED ON DETECTED RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS

Mahmoud[1], A.A., A.S. Mandour[1], H.H. Mansour[2], and Heba S.A. Taha[1]

[1.] Genet. Dept., Fac. Agric., Zagazig Univ., Egypt.
[2.] Theriogenology Dept., Fac. Vet. Med., Zagazig Univ., Egypt

Accepted 8/4/2007

ABSTRACT: **Blood samples were collected via jugular vein from four crossbreds of sheep, CB1, CB2, CB3 and CB4. Random amplified polymorphic DNA analysis was used to fingerprint these crossbreds in order to study the genetic relationships between them and to search for genetic markers in each one. In this respect, many random primers were applied, but only seven primers (A02, A04, A07, A09, A10, A12, and A17) were succeeded in the amplification of genomic DNA from these sheep breeds. Several positive and negative specific bands (markers) which are differentiated between crossbreds were detected by five primers. Primer A02 was the best one, exhibiting markers among the four crossbreds followed by both A04 and A09 primers, which gave markers for three crossbreds, while A12 revealed specific markers for two crossbreds and A17 for only one crossbred. On the other hand, neither A07 nor A10 primers gave any specific band.**

Similarity index and dendrogram presentation showed that the highest differences were scored between CB1 and both CB3 and CB4 followed by those between CB1 and CB2. Results showed high genetic proximity between the CB3 and the CB4. This study might support that RAPD-PCR technique is a useful tool for the genetic characterization of animal breeds to determine their genetic relationships.

Key words: Sheep, crossbreds, RAPD-PCR, genetic diversity, genetic similarity.

INTRODUCTION

Characterization of animal breeds requires knowledge of genetic variation that can be effectively used to measure polymorphism within and between populations (Hetzel and Drinkwater, 1992). 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). Several investigations studied the genetic relationships among sheep breeds (Makaveer and Nakev, 1987, Zanotti *et al*, 1988, Shiquan *et al*, 1990, Hiendleder, 1996, Hiendleder *et al*, 1998, Wafaa Abd-elnaby and Tharwat, 2000, Ali, 2003 and Awad, 2005).

The objective of this study was to perform molecular genetic identification of some sheep crossbreds in order to determine their homogeneity, at the molecular level. The polymerase chain reaction (PCR) technique was used to detect any molecular markers which differentiate between crossbreds and to determine their phylogenetic relationships.

MATERIALS AND METHODES

Four sheep crossbreds, CB1, CB2, CB3 and CB4 were used in this study, the four crossbreds are resulted from the crossing between the Rahmany (Egyptian breed) and other foreign breeds and well adapted under the Egyptian conditions. Their origin and pedigree are presented in Table 1.

Blood Samples Collection

Blood samples, of four sheep crossbreds were collected from two replicate animals for each crossbred. Blood samples of the four crosses were collected from the Farm of the Buffalo and Sheep Research Center, Mahalet Musa, Kafr El- Sheikh, Egypt. All animals sampled were phynotypically normal, healthy and sexually fertile. Blood samples were collected via the jugular vein of each animal using vacutainer tubes contain heparin as anticoagulant. Samples were centrifuged at 5000 rpm for 15 min. at 4°C, and the plasma protein (supernatant) was transferred to clean plastic vials and stored at -20° C until electrophoretic analysis. The pellet was immediately stored at -80°C for

Table 1. The pedigree of the four studied sheep crossbreds.

Crossbreds	Pedigree
CB1	Produced by crossing between Finnish (pure) and Rahmany (pure) breeds (1/2 Finnish – 1/2 Rahmany).
CB2	Produced by crossing between Romanov (pure) and Rahmany (pure) breeds (1/2 Romanve– 1/2 Rahmany)
CB3	The product of backcross of CB2 with pure Romanov breed (3/4 Romanov – 1/4 Rahmany)
CB4	The product of backcross of CB2 with pure Rahmany breed (1/4 Romanov x 3/4 Rahmany)

DNA extraction (Udalova *et al*, 1987).

Genomic DNA extraction

DNA extraction was carried out according to the method of Sharma *et al* (2000). 700 µl of lyses buffer (10 mM Tris – HCl, 100mM NaCl, 1mM EDTA, pH 8.0, 0.5% SDS) and 60 µg of proteinase K 20 mg /ml) were added. To an aliquot of 100 µl blood (after thawing). The mixture was vortexed and incubated at 37°C overnight. DNA was extracted by equal volumes of phenol, phenol-chloroform (1:1) and chloroform – isoamyle alcohol 24:1). DNA was precipitated by adding two volumes of chilled ethanol in the presence of a high concentration of salts (10% 3M sodium acetate). The pellet was washed with 70% ethanol, air dried and subsequently dissolved in (Tris-EDTA) TE buffer (10m M Tris HCl, 1mM EDTA).

RAPD-PCR analysis

PCR was conducted to detect RAPD markers following the procedure of Williams *et al*, 1990. The reaction was carried out in total volume of 25 µl in an eppendorf tube containing 2.5 µl dNTPs (2.5 mM), 1.5 µl MgCl (2.5 mM), 2.5 µl primer (2mM), 2.5 mM buffer (10x), 0.35 µl Taq polymerase (250 U) , 2µ template DNA (25 ng) and 13.65 µl H_2O.

The reaction mixture was overlaid with a drop of sterile mineral oil. Amplification reactions were carried out using a Perkin Elmer Gene Amp. PCR Thermocycler 2400 with a heated lid to reduce the evaporation. The reaction was programed as follows:-

Preliminary, 5 min denaturation at 94°C (one step) for ensuring the complete separation of the template DNA, then 40 cycles of 1 min at 94°C (denaturation), 1 min at 36°C (annealing) and 2 min at 72°C (initial extension for amplification of target DNA) then the final extension (one step) at 72°C for 12 min to ensure that the amplified DNA are double-stranded and stored at 4°C.

A volume of 15µl of the amplified RAPD products were loaded in each well in sub-marine gel agarose electrophoresis apparatus (BIORAD) and DNA molecular weight marker (100 base pair ladder). Agarose gel (1.2% in TBE buffer) was used for electrophoretic separation in 100 ml of TBE buffer for about 40-70 min. Gel was stained with

0.2µg/ml ethidium bromide and then the DNA bands were visualized by UV light. PCR products were photographed by gel documentation system under UV transilluminator.

PCR products were scored across the lanes as variables. The presence of a band of amplified DNA was scored as "+" and absence as "-". The obtained data so was used for calculation of similarity index and dendrogram using the SPSS Computer Software (1995).

All the biochemical and molecular analyses were done at The Genetic Molecular Laboratory, Genet, dept., Agric. Fac., Ain Shams Univ., during 2005-2006.

RESULTS AND DISCUSSION

RAPD-PCR Analysis

Many random primers were examined in this study, but only seven primers were successfully amplified DNA fragments when applied to DNA extracted from crossbreds. These primers and their base sequences were:

Primer	Sequences
A02	5' TGCCGAGCTG 3'
A04	5' AATCGGGCTG 3'
A07	5' GAAACGGGTG 3'
A09	5' GGGTAACGCC 3'
A10	5' GTGATCGCAG 3'
A12	5' TCGGCGATAG 3'
A17	5' GACCGCTTGT 3'

Figure 1 and Tables 1 and 2 illustrated the banding pattern of the seven primers in the four crossbreds.

Sixty bands were detected as PCR products for the seven primers over all the four crossbreds. The number of bands varied from 4 to 16 bands per primer and from 1 to 5 bands per animal. The size of the amplified fragments ranged from 83 bp to 1405 bp.

The amplified bands could be divided into four types: 1- monomorphic bands which were present in all samples (common and undistinguishable bands, 2- polymorphic bands which present in some samples and absent in others, (bands could be used to distinguish among samples), 3- positive specific bands (present only in one sample and absent in the others) and 4- negative specific bands, absent only in one sample

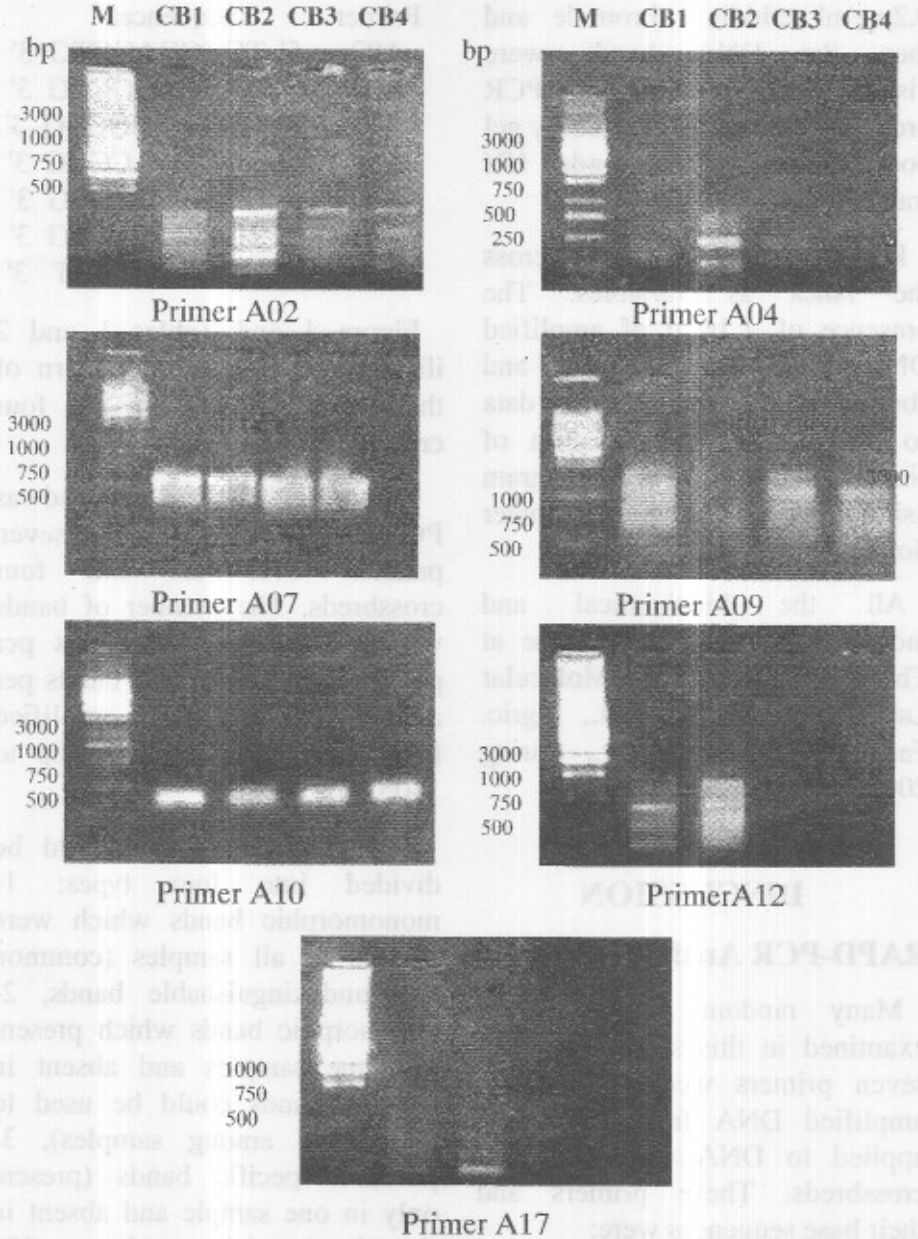

Fig.1: RAPD-PCR fragments produced by the seven primers in the four studied sheep crossbreds.

and present in the others. The later types (positive and negative specific bands) could be used to distinguish one sample from the others and could be considered as a characteristic band.

Primer A02

This primer produced eleven polymorphic bands and did not produce any monomorphic bands. The highest number of bands (five bands) was detected in CB1, while the lowest number (three bands) was found in CB3.

In the CB1, three unique positive bands of molecular weights (MW) 548, 303, and 107 bp and one negative band of 351 bp were detected. In both CB2 and CB3, one unique positive band was detected for each at MW 261, 194 bp, respectively. While, two unique positive bands were resulted in CB4, having sizes of 225 and 134 bp (Fig 1 and Table 2). This primer appeared to be an excellent characteristic primer and could be strongly recommended for distinguishing among crossbreds, since it presented different specific markers for each crossbred under investigation.

Primer A04

Primer A04 reacted only with three crossbreds (CB1, CB2 and CB3) and was not reacted with CB4, (Fig. 1 and Table 3). Seven polymorphic distinguishable bands were obtained by this primer. Their sizes ranged between 661 and 110 bp. The highest number of bands (five bands) was detected in CB2, while only one band was observed in each of CB1 and CB3. All these bands seemed to be as positive specific markers. The five unique positive specific bands in CB2 were detected, having MW of 661, 490, 363, 225 and 110 bp. While one unique positive specific band was detected in each of CB1 and CB3 with MW of 167 and 239 bp, respectively. This primer might be considered as a characteristic primer for these three crossbreds.

Primer A07

As obvious in Figure 1, primer A07 produced two monomorphic bands that presented in all samples having molecular sizes of 277 and 157 bp. Therefore, this primer was unsuitable for distinguishing any of the investigated crossbreds.

Table 2. RAPD-PCR banding pattern produced by A02 primer in the studied four sheep crossbreds.

Band No.	RF	MW(bp)	CB1	CB2	CB3	CB4
1	0.68	794	-	-	+	+
2	0.7	684	+	+	-	-
3	0.73	548	+*	-	-	-
4	0.79	351	-*	+	+	+
5	0.81	303	+*	-	-	-
6	0.83	261	-	+*	-	-
7	0.85	225	-	-	-	+*
8	0.87	194	-	-	+*	-
9	0.89	167	+	+	-	-
10	0.92	134	-	-	-	+*
11	0.95	107	+*	-	-	-

+ band present, - Band absent and +* or -* specific marker

Table 3. RAPD-PCR banding pattern produced by A04, A09, A12 and A17 primers in the four studied sheep crossbreds.

Band No.	RF	MW(bp)	CB1	CB2	CB3	CB4
		Primer A04				
1	0.62	661	-	+*	-	-
2	0.67	490	-	+*	-	-
3	0.72	363	-	+*	-	-
4	0.79	239	-	-	+*	-
5	0.8	225	-	+*	-	-
6	0.85	167	+*	-	-	-
7	0.92	110	-	+*	-	-
		Primer A09				
1	0.52	1277	-	-	+*	-
2	0.59	922	+	-*	+	+
3	0.65	697	+*	-	-	-
4	0.75	438	+	+	+	+
		Primer A12				
1	0.57	1405	+*	-	-	-
2	0.61	1062	-	+*	-	-
3	0.69	607	-	+*	-	-
4	0.70	566	+*	-	-	-
5	0.78	324	+*	-	-	-
6	0.80	281	-	+*	-	-
7	0.87	172	+	+	-	-
8	0.93	113	+*	-	-	-
		Primer A17				
1	0.69	536	+*	-	-	-
2	0.73	393	+*	-	-	-
3	0.81	211	+*	-	-	-
4	0.86	143	+	+	-	-
5	0.93	83	+	+	-	-

+ band present, - band absent and +* or -* specific marker

Primer A09

The PCR products of primer A09 are illustrated in Fig 1 and Table 3. This primer reacted with all crossbreds and produced four bands with molecular sizes ranged between 1277 and 438 bp. A band appeared to be monomorphic band with molecular size of 438 bp which represented in all crossbreds. The other three bands proved to be polymorphic, as present in some crossbreds and absent in others. Some crossbreds had a positive or negative specific bands that used to distinguish among such breeds. For instance, both CB1 and CB3 have unique positive band at MW of 697 and 1277 bp respectively. While CB2 had one unique negative band with MW of 922 bp. On the other hand, this primer was not revealed any specific markers for CB4. So, this primer might be considered as a characteristic primer for each of CB1, CB2 and CB3.

Primer A10

Regarding primer A10, no specific markers or polymorphic bands were detected (Figure 1). Only one strong monomorphic band having MW of 211 bp was found in all crossbreds. So, this primer was not suitable to characterize any of the four investigated crossbreds.

Primer A12

Concerning primer A12, eight bands were detected as PCR products. Having MW ranged between 1405 and 113 bp and all were distinguishable (polymorphic) bands (Fig. 1 and Table 3). Primer A12 was not revealed any bands neither in CB3 nor in CB4, but revealed four positive specific bands in CB1, with MW of 1405, 566, 324 and 113 bp. This primer also revealed three positive specific bands in CB2, their sizes are 1062, 607 and 281. Primer A12 might be considered as a good characteristic primer for both CB1 and CB 2, but not for the other two crossbreds.

Primer A17

Primer A17 reacted with two crossbreds (CB1 and CB2), but not reacted with the other two crossbreds (CB3 and CB4). As shown in Fig .1 and Table 3. This primer produced five polymorphic bands with molecular sizes ranging between 536 and 83 bp. Three positive specific markers were observed in CB1, having MW of

536, 393 and 211 bp. Meanwhile, no specific markers were detected by this primer in the other crossbreds. Primer A17 appeared to be a characteristic primer only for CB1.

RAPD-PCR Genetic Markers

Combining data of the seven primers among the four crossbreds, complete identification could be obtained for the investigated sheep crossbreds. The RAPD-PCR specific markers produced by the different primers over all the studied crossbreds were collected and summarized in Table 4.

It is clear that twenty eight specific markers were obtained by the different primers. Two negative specific markers and twenty six positive specific markers, representing 43.3% of the total bands detected as PCR products for the different primers over all the four crossbreds.

CB1 was characterized by the presence of twelve markers. Primers A02, A04, A09, A12 and A17 revealed 3, 1, 1, 4 and 3 markers respectively, and by the absence of one band (A02: with MW 351 bp). CB2 was characterized by the presence of nine markers, (A02: 1, A04: 5 and A12: 3) and by the absence of one band (A09: with MW 922). CB3 was characterized by the presence of three markers, A02: one for each, A04 and A09.

CB4 was characterized by the presence of two markers produced by the primer A02, having sizes of 225 and 134 bp (Table 4). These might provide useful negative and positive specific markers for each of the investigated sheep crossbreds. The number of specific markers varied according the examined primers. The highest number of specific markers was produced by the primer A02 (7 positive and 1 negative) followed by those produced by both A04 and A12 primers (7 positive). While, the lowest number (3 positive markers) was obtained by the primers A09 and A17. On the other hand, neither primer A07 nor primer A10 revealed any specific markers for any one of the studied crossbreds. Primer A02 seemed to be the best one, producing the highest number of the specific markers, and producing specific markers for each one of the studied crossbreds. Furthermore, it could be recommended as an excellent characteristic primer for those crossbreds.

Table 4. Specific genetic markers for the four studied sheep crossbreds across RAPD-PCR analysis

Primer	MW	CB1	CB2	CB3	CB4
A02	548	+			
	351	-			
	303	+			
	261		+		
	225				+
	194			+	
	134				+
	107	+			
A04	661		+		
	490		+		
	363		+		
	239			+	
	225		+		
	167	+			
	110		+		
A09	1277			+	
	922		-		
	697	+			
A12	1405	+			
	1062		+		
	607		+		
	566	+			
	324	+			
	218		+		
	113	+			
A17	536	+			
	393	+			
	211	+			

\+ Positive specific marker and – Negative specific marker

Our results revealed the possibility of DNA analysis to differentiate between the crossbreds. The majority of random primers examined gave distinctly reproducible patterns among all crossbreds studied. Whatever, the primers varied in the extent of information that generated. Some produced highly polymorphic patterns, whereas others produced less polymorphic products. Some DNA fragments were apparently similar in size among the four crosses (monomorphic). However others were unique to a particular crossbred that could be used as markers for such breed.

These results demonstrate the usefulness of the RAPD-PCR technique for detecting DNA polymorphism in sheep. Our results are in agreement with several previous studies on sheep (Hiendleder, 1996, Appa Roa *et al*, 1996, Hiendleder *et al*, 1998, Wafaa Abdel-naby and Tharwat, 2000, Ali, 2003 and Awad, 2005), on goat (Appa Roa *et al*, 1996, Li *et al*, 2002 and Alia El-seody, 2005).

Similarity Index

Similarity Index and dendrogram presentatuion demonstrating the relationships between the four crosses as detected by RAPD-PCR, were given in (Fig. 2 and Table 5). The results indicated that the widest differences observed between CB1 and both CB3 and CB4, followed by between CB1 and CB2. However, the lowest difference was noticed between CB3 and CB4 reflecting genetic similarity index. While, the lowest similarity was between CB1 and the other three crosses. This might be due to their genetic constitution, 50% of CB1 genome came from Finnish sheep, while the genomes of CB2, CB3 and CB4 possessed 50, 75 and 25 % of Russian sheep (Romanov) genome, respectively.

This work revealed the existence of genetic diversity among the four crossbreds studied. The RAPD profile generated for each crossbred might be effectively used as a supporting marker for taxonomic identification. In taxonomic and molecular systematic species, specific RAPD markers could be an invaluable tool for species variation and establishing the status of organisms and its evolution (Allard *et al*, 1992 and Appa Roa *et al*, 1996).

Table 5. Similarity index between the four studied sheep crossbreds based on RDPD-PCR data.

Crossbreds	CB1	CB2	CB3	CB4
CB1	-	-	-	-
CB2	43.9	-	-	-
CB3	31.3	34.5	-	-
CB4	32.3	35.7	73.7	-

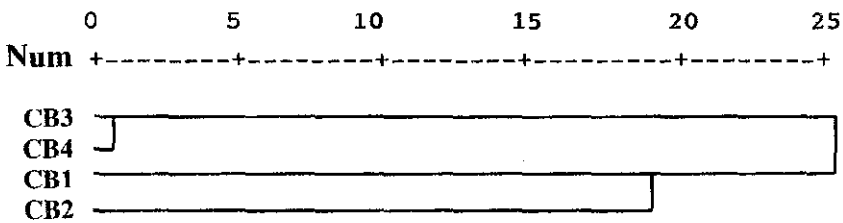

Fig. 2. Dendrogram presentation based on RAPD-PCR data analysis among the studied four crossbreds of sheep.

REFERENCES

Allard M.W., M.M. Miyamoto, I. Jarecki, F. Kraus and M.R. Tennant 1992 . Proc. Natl. Acad. Sci. USA 18: 3972-3976.

Ali, B.A. 2003. Genetics similarity among four breeds of sheep in Egypt detected by random amplified polymorphic DNA markers. African J. Biotech. Vol. 2 (7), pp. 194 – 197.

ALIA, A. El-Seoudy, Nermin M. Abdel Gawad, Asmaa M. Abu Shady, and A.Z.E. Abdelsalam 2005. Biochemical and molecular genetic characterization of Egyptian goat breeds. Egypt. J. Genet. Cytol. 34(1): 63-73.

Appa Rao K.B.C., K.V. Bhat and S.M. Totey 1996. Detection of species specific genetic markers in farm animals through random amplified polymorphic DNA (RAPD). Genetic analysis: Biomolecular Engineering 13:(1) 35 –38.

Awad, N.S. 2005. Molecular genetic studies on some Egyptian sheep breeds. Ph.D. thesis. Ain-Shams University, Cairo, Egypt.

Feral, J.P. 2002. How useful are the genetic markers in attempts to understanding and manage marine biodiversity. J. Exp. Mar. Biol. Ecol. 268: 121-145.

Hetzel, D. J. S. and R. D. Drinkwater, 1992. The use of DNA technologies for the conservation and improvement of animal genetic resources. FAO, Expert Consultation on Management of Global Animal Genetic Resources, Rome, April.

Hiendleder, S. 1996. Molecular characterization of the sheep metoconderial genome. J. Animal. Breed. Genet.113: 293-302.

Hiendleder, S., K. Mainz, Y. Planty, and H. Lewalski 1998. Analysis of mitoconderial DNA indicates that domestic sheep are derived from two different ancestral maternal sources. J. Heredity 89: 113-120.

Li, B., M. Du, X. Guo and Z. Zhou 2002. Genetic analysis of Shanxi Native goats using RAPD markers. Proc. 7th World Con. Genet. Appl. to Livestock Production, August 19-23, Montpellier, France.

Makaveer, T. S. and S. Nakev, 1987. Genetic polymorphism of blood serum in a population of Shumen sheep. Zhivolnov dni-Naunki 24, 10: 83-87.

Sharma, D., K. B. C. Appa Rao and S. M. Totey 2000. Measurement of within and between population genetic variability in quails. Br. Poult. Sci. 41: 29-32

Shiquan, Wang, W. C. Foate and T. D. Bunch 1990. Transferrin and hemoglobin polymorphism in domesticated goats in the USA. Animal Genetics 21: 91-94.

SPSS 1995, Computer Software Users Guide, USA.

Udalova, M. N., A.S. Akhmetshiev and T.A. Pak 1987. Blood protein and enzyme polymorphism in sheep of different breeds. Sel'-skokhozyaistvennaya-Biologiya. (3): 45-47.

Wafaa A.M. Abd El-Naby and E.E. Tharwat 2000. Biochemical and molecular genetic characterization of some sheep breeds in Egypt. Arab J. Biotech. 3(1):9-20.

Williams, G. K., A. R. Kulelik, K. J. Livak, J. A. Rafalski and S. V. Tingey 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucl. Acid Res., 18: 6531-6535.

Zanotti, M., G. C. Candini, P. Lione and G. Rognoni, 1988. Genetic relationship among four sheep breeds of the Italian Alpine. Arc. J. Anim. Breed. Genet.105: 135-142.

التباين الوراثى بين أربعة من هجن الأغنام باستخدام واسمات الرابيد RAPD

أحمد عبدالسلام محمود[1]- عبدالرحمن السيد مندور[1]

حسن حسن منصور[2]- هبة سيد احمد طه[1]

[1] قسم الوراثة – كلية الزراعة – جامعة الزقازيق – الزقازيق – مصر

[2] قسم التوليد والتناسل-كلية الطب البيطرى-جامعة الزقازيق- مصر

تم تجميع عينات الدم من الوريد العنقى لاربعة من هجن الاغنام تحت الدراسة وتم تحليل تلك العينات باستخدام تفاعل الـ RAPD-PCR لتحديد بصمة وراثية للتمييز بين كل من الهجن الاربعة ودراسة العلاقة الوراثية بينهم وايجاد واسمات وراثية لكل منهم. وقد تم استخدام عدد من البادئات العشوائية نجح منها سبعة (A2, A4, A7, A9, A10, A12, A17) فى التكامل مع الـ DNA الجينومى الخاص بتلك الهجن. هذا وقد اعطت خمس بادءات تعدد صور فى طرز حزم واسمات الرابيد بين الهجن، حيث اعطى كل بادئ عدداً مختلفاً من الحزم. واظهر البادئ A2 افضل النتائج حيث اعطى واسمات متخصصة لكل من الهجن الاربعة يليه A4,A9 اللذان اظهرا واسمات متخصصة لثلاثة من الهجن الاربعة بينما البادئ A12 اعطى واسمات متخصصة لاثنين فقط من الهجن، اما البادئ A17 اعطى واسمات متخصصة لهجين واحد فقط، بينما لم يعط اى من البادئين A10 ,A7 اى واسمات متخصصة لاى من الهجن المدروسة. دل التحليل الاحصائى للبيانات ان اعلى تباعد وراثي كان بين الهجين الاول وكل من الهجينين الثالث والرابع يليه التباعد بين الهجين الاول والثانى، كما اظهرت النتائج قرابة وراثية عالية بين الهجين الثالث والرابع.

هذه الدراسة دعمت اهمية تقنية الـ RAPD-PCR كوسيلة فعالة للتعرف على تعدد الصور الوراثية بين الطرز الوراثية وتقدير العلاقات الوراثية بينها وكذلك فى عمل بصمة وراثيه لها.