Zagazig J. Agric. Res., Vol. 39 No. (3) 2012 397-407

Zagazig Journal of Agricultural Research
www.zu.edu.eg/agr/journals

THE USE OF MICROSATELLITE MOLECULAR MARKERS FOR DETECTING FecB GENE IN SEVEN SHEEP BREEDS REARED IN EGYPT

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ABSTRACT

Booroola gene (FecB) was the first major gene for prolificacy identified in sheep. In this study Blood samples were collected via jugular vein from seven sheep breeds, three pure Egyptian breeds (Farafra, Rahmany and Saeedy), two foreign breeds (Finnsheep and Romanov) and two crossbreds; CB1 (1/2 Rahmany X 1/2 Romanov) and CB2 (1/2 Rahmany X 1/2 Finnsheep). Genomic DNA was isolated and amplified with two microsatellite primers (BM1329 and OarAE101), which are closely linked to FecB locus. Egyptian sheep were shown to have no FecB mutation in all analyzed samples. On the other hand, both of the foreign breeds and their crosses with the Egyptian breeds showed the presence of the FecB linked allele in most samples, which explain the fact that a half Finnsheep or Romanov sheep is more prolific than an Egyptian pure breed. This result is extrapolated that the target gene is transmitted from the foreign breeds to Egyptian breeds by meaning of crossbreeding without complex breeding programs. The present study indicated that the two microsatellite markers are suitable markers for the introgression of the FecB locus into different local sheep breeds. This study introduce a useful tool for the genetic determination of FecB carriers and help in improving fecundity of the Egyptian sheep breeds by crossing with foreign breeds without influencing of the acclimatization traits of these breeds to the environmental conditions in Egypt.

Keywords: Egyptian sheep, Booroola FecB gene, microsatellite markers.

INRODUCTION

Reproductive traits, which are under the control of multiple genes, are economically important traits in livestock. To improve the fecundity, i.e. litter size, is of special meaning in the selection of animals with high reproductivity, especially for animals raised by people as economic source. Litter size is a character with very low heritability of about 0.1 (Sun *et al.,* 2010). Therefore, it is hard to use traditional breeding methods to improve litter size. The utilization of marker-assisted selection (MAS) may accelerate the breeding process for this character (Chu *et al.,* 2002a, Wang *et al.,* 2003, Lei *et al.,* 2003 and Ji et *al.,* 2003).

Souza *et al.* (2001) proved that The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. This mutation in the subdomain 3 of the kinase domain that could result in an alteration in the expression and/or phosphorylation of SMADs, resulting in the phenotype characteristic of the Booroola animals, which is the 'precocious' development of a large number of small antral follicles resulting in an increased ovulation rate.

The Booroola (FecB) is a dominant gene located on sixth autosomal chromosome and is responsible for increasing the ovulation rate and litter size in sheep (Davis, 2005 and Gootwine, 2005). Most identified genetic mutations in sheep associated with reproduction aspects and many of these have been mapped to specific chromosomal regions (Montgomery *et al.,* 1994, Cockett *et al.,* 2001, Rohrer 2004 and McNatty *et al.,* 2005). Several causative mutations for fecundity traits such as Booroola have been identified (Mulsant *et al.,* 2001, Souza *et al.,* 2001 and Wilson *et al.,* 2001).

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Through analysis of sheep genome resulted in indication of 5 microsatellite loci OarAE101, BM1329, OarHH55, BM143 and BMS2508 (Lord *et al.*, 1996; Vaiman *et al.*, 1996; Ihara *et al.*, 2004 and Weimann *et al.*, 2001). They stated that these microsatellites are linked to the fecundity gene FecB in the sheep, which are closely associated with the high reproduction trait in sheep. These microsatellites could be useful as molecular markers in markers-assisted selection (MAS) in sheep.

In a study to fingerprint FecB gene in five Egyptian sheep using forced PCR-RFLP, Digestion of FecB gene, 190 base pair fragment with AvaII restriction enzyme resulted in non carrier 190 bp fragment (wild type) in all the animals belonging to the five studied Egyptian breeds revealing absence of this restriction site in those five breeds (EL-Hanafy and El-Saadani 2009). Also same results were obtained by Abulyazid *et al.* (2011) Using another Egyptian local breeds and crossbreds.

The aim of this study was to assess the utilization of microsatellites BM1329 and OarAEl0l as specific markers for the introgression of the FecB locus into different sheep breeds and to determine polymorphism pattern of Egyptian` sheep breeds for FecB gene as compared with foreign sheep breeds. This may result in identifying the genotype of this gene in foreign breeds, which might help in improving genetic performance of Egyptian sheep breeds with respect to this important fecundity gene.

MATERIALS AND METHODS

Seven sheep breeds living in Egypt were used in this study. Three of them were pure Egyptian breeds (Rahmany, Saeedy and Farafra), two pure foreign breeds (Romanov, Finnish) and two crossbreds produced by crossing Egyptian (Rahmany) with European (Romanov and Finnsheep) breeds (CB1, CB2). Their ages ranged from two to three years. They were phynotypically normal, healthy and sexually fertile ewes.

Blood Samples Collection

Blood samples, of seven breeds were collected from ten replicate animals for each breed via jugular vein using vacutainer tubes contain ethylene diamine tetra acetic acid (EDTA) as anticoagulant under aseptic conditions.

Blood samples of Rahmany, were collected from the farm of Sheep and Goat Research Center, EL- Serw, Domiat, Egypt. Romanov and CB1 obtained from Animal Production Research Station, Sakha, Kafr el Shaikh, while of Finnish and CB2 were collected from the farm of Buffalo and Sheep Research Center, Mahalet Musa, Kafr El- sheikh, Egypt. From Saeedy and Farafra breeds samples were collected from Animal Production Research Station, Mallawy, and El-Menia, Egypt. All samples were collected during 2010 - 2011.

DNA Extraction

DNA was isolated with the phenol-chloroform extraction method as described by Sambrook *et al.* (1989) as follows:

To 300 µl blood, 300 µl volume of lysis buffer, 30µl SDS 20%, 5 µl Rnase and 10 µl proteinase K were added in 1.5 ml eppendorf tubes. Tubes were incubated overnight at 50 °C or 2 h at 65 °C in shaker water bath. The resulted lysate was purified by adding an equal volume (25 phenol: 24 cloroform: 1 isoamyl alcohol) and centrifuged at 10,000 rpm for 10 min at 4°C. The upper phase (supernatant) was transferred in a new tube, adding (25 phenol: 24 cloroform: 1 isoamyl alcohol) and centrifuged at 10,000 rpm for 10 min at 4°C.

The final supernatant mixed with two volume of ice-cold absolute ethanol to the remainder of DNA extraction from the previous step, and then kept at -20°C in a deep freezer. Centrifugation was achieved and ethanol was gently discarded from the DNA pellets. The pellets were washed using appropriate volume of 70% ethanol, and centrifuged. The 70% ethanol was subsequently discarded from the DNA pellets which were dried at the room temperature for about 30 min. The DNA pellets were re-suspended in 50 µl of distilled water or EDTA buffer pH 8 (TE) and kept at room temperature for two hours, and then stored at 4°C.

PCR Analysis Using Microsatellite Markers

The extracted genomic DNA of 9 animals of each of the 7 breeds were analyzed for 2

microsatellite markers (BM1329 and OarAE101) which are recommended for the introgression of the FecB locus and to identify FecB gene carriers (Chu MX *et al.*, 2001, Zhang *et al.*, 2010 and Saberivand *et al.*, 2010).

The PCR for amplifying microsatellites BM1329 and OarAE101 was performed in total volume of 25 µl in an eppendorf tube containing the following:

dNTPs (2.5 mM)	2.50 µl
$MgCl_2$ (2.5 mM)	1.50 µl
Primer (2 mM)	2.50 µl
(10 x) buffer	2.50 µl
Taq DNA polymerase (250 U)	0.35 µl
Template DNA (25 ng)	2.00 µl
H2O (d.w)	13.65 µl

The reaction mixture was over laid with a drop of sterile mineral oil.

PCR conditions

Amplification reactions were carried out using a TC-3000 thermo cycler.

The conditions were programmed as follows:

Thermal cycling began with an initial cycle of 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec., annealing at the temperature optimized for each primer pair for 1 min (Table 1) and 72°C (1 min), and ultimately with a final extension at 72°C (5 min) to ensure that the amplified DNA are double-stranded and stored at 4°C.

PCR products were electrophorresesed in a 1.5% agarose gel that stained with ethidium bromide and visualized by UV transillumination then photographed.

Allelic frequencies, different genotype frequencies, homogeneity and heterogeneity percentage were estimated and analyzed.

RESULTS AND DISCUSSION

Analysis of electrophoretic data from genomic DNA of seven studied sheep breeds amplified with the two microsatellite primers (OarAE101 and BM1329) showed the occurrence of different microsatellite alleles with different frequencies.

Microsatellite OarAElOl

It is clear from Figures 1-5 and Table 2 that four polymorphic DNA band alleles were identified with microsatellite OarAE1O1 with molecular sizes of 97bp, 113bp, 123pb and 125bp with frequencies 0.242 0.017, 0.558 and 0.183, respectively.

The studied breeds showed high occurrence of the allele 97bp in most samples. The allele 113 occurred only in Finnsheep breed, while the allele 123 was found in all animals of the three Egyptian sheep breeds, Romanov and CB1, while the allele 125 is present only in CB2 and Finnsheep breed.

Microsatellite BM1329

Four alleles were identified with microsatellite BM1329 with molecular sizes of 136 bp, 144 bp, 162 bp and 188 bp (Figures 6 - 10 and Table 2) with frequencies of 0.008, 0.059, 0.342 and 0.592, respectively.

The studied breeds showed occurrence of the allele 162bp in most samples. The alleles 136bp and 144bp occurred only in Romanov breed while the allele 188 was found in all animals of the three Egyptian sheep breeds, Finnsheep, CB1 and CB2.

Table 1. Primer sequences and annealing temperature

Marker	Primer sequences	Annealing temperature (°C)
BM1329	5'-TTGTTTAGGCAAGTCCAAAGTC-3' 3'-AACACCGCAGCTTCATCC-5'	60
OarAE101	5'-TTCTTATAGATGCACTCAAGCTAGG-3' 3'-TAAGAAATATATTTGAAAAAACTGTATCTCCC-5'	59

Table 2. Allele frequencies of polymorphic BMI329 and OarAE101 microsatellites

Primer	Allele (bp)	Allele frequeucy
OarAE101	97	0.242
	113	0.017
	123	0.558
	125	0.183
BM1329	136	0.008
	144	0.058
	162	0.342
	188	0.592

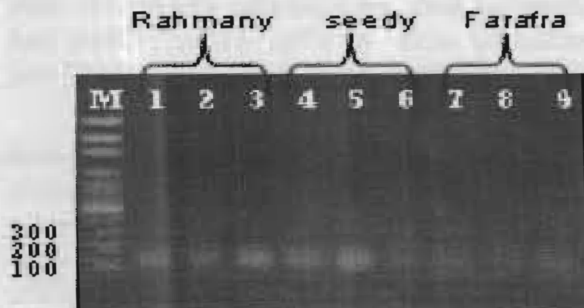

Fig. 1. SSR fingerprints of individual samples from three Egyptian sheep breeds detected with OarAE101 microsatellite

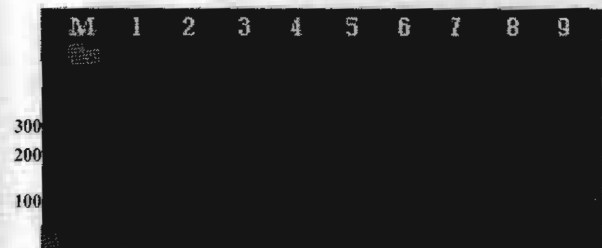

Fig. 2. SSR fingerprints from individual samples for Romanov breed detected with OarAE101microsatellite

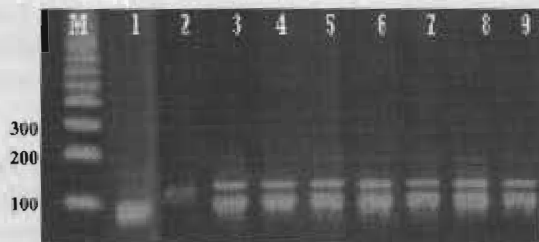

Fig. 3. SSR fingerprints from individual samples for finnsheep breed detected with OarAE101microsatellite

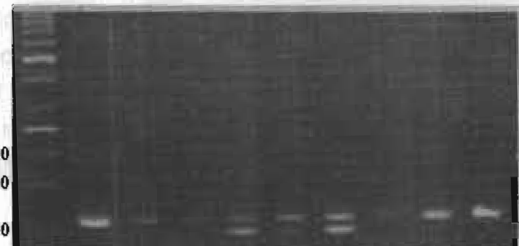

Fig. 4. SSR fingerprints from individual samples for CB2 breed detected with OarAE101 microsatellite

Fig. 5. SSR fingerprints from individual samples for CB1 breed detected with OarAE101 microsatellite

The allele 97bp at OarAE101 and the allele 162 at BM1329 are linked to the FecB allele and are found to have positive effects on the litter size in sheep (Weimann *et al.,* 2001).

Analysis of Microsatellite Markers in Egyptian Sheep Breeds

It is apparent from the analysis of the polymerase chain reaction products using microsatellite OarAE1O1, one common band (allele) in all Egyptian sheep breeds with molecular sizes of 123bp was found (Fig. 1).

There is one genotype detected consistently (123/123) as homozygous genotype with frequency of 1.0 and 100% homogeneity (Table 3).

Microsatellite BM1329 identified one common allele with molecular sizes of (188 bp) in all studied animals belonging to the Egyptian sheep breeds (Fig. 1 and Table 3). There is only one genotype was detected (188/188) as homozygous genotype with frequency of 1.0 and 100% homogeneity (Table 3).

The allele 97 showed higher frequency more than the allele 123.

The obtained results of PCR showed the same band pattern (123 bp at the OarAE101 locus and 188 at the BM1329 locus in all samples belonging to the Egyptian breeds improving the absence of FecB allele. The absence of FecB gene in the Egyptian studied animals agreed with the results of Davis *et al.* (2005), EL-Hanafy and El-Saadani (2009) and Abulyazid *et al.* (2011).

The absence of FecB gene in the Egyptian breeds could be explained on the basis of low litter size in these breeds. In this respect Galal *et al.* (1996) reported that Egyptian sheep breeds are of medium size, low growth rate, breed all year round and have small litter size ranging from 1.03 to 1.40. In addition, Almahdy *et al.* (2000) characterized the Egyptian sheep breeds by extended breeding seasons, high fertility, and low prolificacy, they added that currently in Egypt efforts are being made to intensify production systems, primarily through changing reproductive management and crossing native breeds with introduced breeds.

Analysis of Microsatellite Markers in Foreign Sheep Breeds

Romanov sheep breed

Microsatellite OarAElOl identified two alleles with molecular sizes (97 bp and 167 bp). Two genotypes (97/97) and (97/123) were observed in Romanov sheep breed, with frequency of 0.44 and 0.56 for each genotype respectively, (Figure 2 and Table 3) with heterozygous genotypic distribution constituting 56%

With microsatellite BM1329, two genotypes (136/162) and (144/162) were observed in Romanov sheep breed. With frequency of 0.11 and 0.89 for each genotype respectively, the distribution of these genotypes shows that the heterozygous genotype is found in all animals of Romanov breed.

Finnsheep breed

Microsatellite OarAElOl identified three alleles with molecular sizes (97 bp, 113bp and 125 bp). Three genotypes were observed (97/97), (113/113), (97/125) and with different frequencies of 0.111, 0.111 and 0.778, respectively (Table 3).

The predominant genotypes in this breed are the heterozygous genotypes which are constitute 78% of the random sample under investigation.

Microsatellite BM1329 showed different amplified fragment alleles (Fig. 3) with molecular sizes of 162 bp and 188 bp .

Genotypes of (162/162) and (188/188) were observed in this crossbreed, the two genotypes frequencies of (0.22 and 0.78). The two genotypes in this breed are 100% homogenous (Table 3).

It is clear from Figures (2, 3, 7 and 8) that the polymerase chain reaction using microsatellite markers BM1329 and OarAElOl that linked to FecB allele resulted in carrier of 97bp and 162bp bands, which linked to the FecB allele that have positive effects on the litter size in sheep (Weimann *et al.,* 2001) revealing the occurrence of FecB mutation in Romanov and Finnsheep. Romanov and Finnsheep is known as two of the most prolific breeds such as, Booroola Merino, Barbados Blackbelly and British Milk Sheep.

Table 3. Genotypes, frequency of Genotype, homozygosity and Heterozygosity percentage obtained with the BMI329 and OarAE101 microsatellites

breed	genotype	Genotype frequency	homozygosity	Heterozygosity
		OarAE101		
Rahmany	123/123	1	100%	
Saeedy	123/123	1	100%	
Farafra	123/123	1	100%	
Romanov	97/97	0.556	56%	44%
	123/97	0.444		
Finusheep	97/97	0.111	22%	%78
	113/113	0.111		
	97/125	0.778		
CB1	123/123	0.5	50%	50%
	97/123	0.5		
CB2	125/125	0.667	67%	33%
	97/125	0.333		
		BM1329		
Rahmany	144/144	1	100%	
Saeedy	144/144	1	100%	
Farafra	144/144	1	100%	
Romanov	136/126	0.11		100%
	144/162	0.89		
Finnsheep	162/162	0.22	100%	
	188/188	0.78		
CB1	162/188	0.57	43%	57%
	188/188	0.43		
CB2	162/162	0.44	44%	56%
	162/188	0.56		

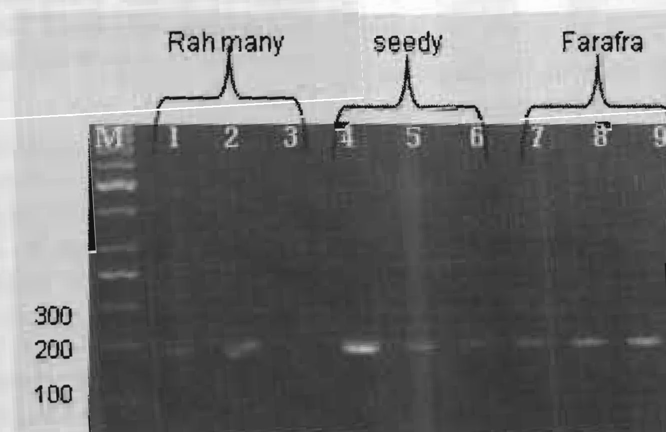

Fig. 6. SSR fingerprints of individual samples from three Egyptian sheep breeds detected with BM1329 microsatellite

Finnsheep is an extremely prolific breed, exhibiting several components of fertility such as early sexual maturity, high ovulation rate, out-of-season lambing, and large litter size (Maijala and Österberg, 1977; Maijala, 1984, 1988 and 1997).

Analysis of Microsatellite Markers in Cross Breeds

CB1 (1/2 Rahmany X 1/2 Romanov)

This study showed different amplified fragments (alleles) with molecular sizes of 97 bp and 123 bp with higher occurrence of the allele 123, than the other allele (97) (Figure 5). Genotypes of (97/123) and (123/123) were observed in this crossbreed, the two genotypes had equal frequencies (0.5)

The two genotypes represent the two states of homogeneity and heterogeneity which have equal contribution of 50% for each genotype (Table 3).

As it is clear from Figure (10), microsatellite BM1329 identified two alleles with molecular sizes (162 bp, and 188 bp). The allele 188 was the more repeatable allele than the allele of 162 bp. In this breed two genotypes were observed (188/188), (162/188) with different frequencies of 0.57 and 0.43, respectively (Table 3). The homozygous genotype showed contribution of 43%, while the heterozygous genotype contributes with 57 %.

CB2 (1/2 Rahmany X 1/2 Finnsheep)

This crossbreed alleles had molecular sizes of 97 bp and 125 bp (Figure 4) with the highest observable occurrence of the allele (97), on the other hand, the allele of 125 bp molecular size showed less occurrence.

The genotypes observed within this crossbreed were (125/125) and (97/125) with relative high frequency of the former of about 0.67, while the other genotype (97/125) had lower frequency of (0.33). The comparison of genotypes of this sample of animals showed the increase of homozygosity vs. heterozygosity, 67% % and 33% (Table 3).

With BM1329 microsatellite, these crossbreed alleles had molecular sizes of 162 bp and 188 bp with the highest occurrence for the allele (162), on the other hand, the allele of 188 bp molecular size showed less occurrence.

The homozygous genotype (162/162) represents contribution of 44% while the heterozygous genotype (162/188) contributes with 56 %. The genotypes observed within this crossbreed were (162/162), (162/188) with different frequencies of 0.44 and 0.56, respectively.

It is clear from the results that the detection of FecB specific linked alleles 97 bp and 162 bp with microsatellite markers OarAElOl and BM1329 in some individuals in the crossbreeds of Finnsheep and Romanov with the breed (Rahmany). This result might explain the extremely increased litter size rate in these crossbreeds. The results are in agreement with those of Almahdy *et al.* (2000), who stated that Incorporation of Finnsheep genes into native Egyptian breeds will improve both biological and economic efficiency under annual or accelerated lambing systems. Half Finn – half Rahmany sheep are expected to be most efficient under annual lambing systems, whereas composites of ¼ Finn - ¾ Rahmany will be easier to maintain because they can be managed like a single breed.

The effect of the FecB mutation is additive for ovulation rate (the number of ova shed at each ovulatory cycle) and partially dominant for litter size. On average, one copy of the FecB mutation increases litter size by one extra lamb (Montgomery *et al.*, 1993)

In this respect, flocks with litter sizes above two tend to be those with ewes from the more prolific breeds such as the North European Short Tail group (Finnish Landrace and Romanov) or those carrying the FecB gene of Booroola origin. Mortality rates for triplets tend to approach 30% (even with intense management) and with four or more lambs the mortality tends to be 50% and above (Willingham and Shelton, 1990).

In conclusion, this study has highlighted the importance of the two microsatellites BM1329 and OarAElOl as an efficient and robust genotyping system for FecB carriers.

This study revealed the absence of FecB allele of any studied Egyptian breeds. On the other hand,

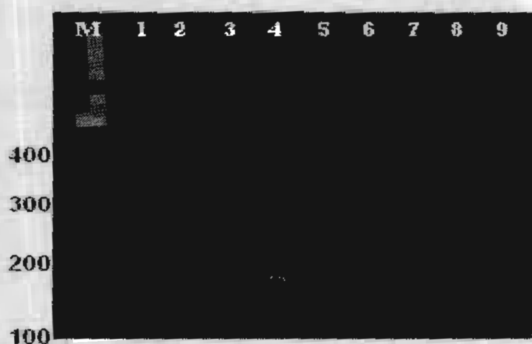

Fig. 7. SSR fingerprints of individual samples from Romanov breed detected with BM1329 microsatellite

Fig. 8. SSR fingerprints of individual samples from finnsheep breed detected with BM1329 microsatellite

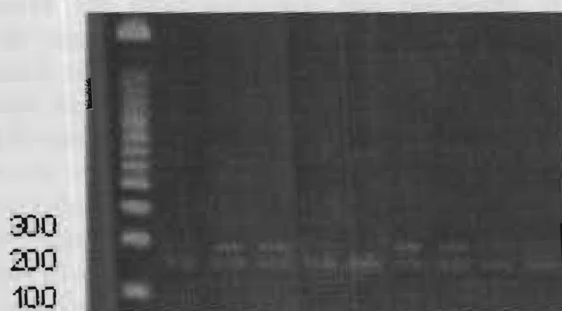

Fig. 9. SSR fingerprints of individual samples from CB2 breed detected with BM1329 microsatellite

Fig. 10. SSR fingerprints of individual samples from CB1 breed detected with BM1329 microsatellite

the investigation identified FecB allele in the foreign sheep breeds living in Egypt. The most important result in this research is the transmission of that important gene into the Egyptian breeds by means of simple breeding system like crossbreeding programs with foreign breeds carrying the favorite genotypes of this gene, which will lead to increase of expression of this gene in the local Egyptian breeds without affecting their acclimatization traits to the environmental conditions in Egypt.

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استخدام واسمات الميكروساتلايت الجزيئية للكشف عن جين التوأمة فى سبع سلالات من الأغنام فى مصر

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جين التوأمة (جين الخصوبة) هو أول جين أساسى لتعدد الخلفة تم التعرف علية فى الاغنام. فى هذه الدراسة تم تجميع عينات الدم من الوريد العنقى للحيوانات فى وجود مادة مانعة للتجلط. استخدمت فى هذه الدراسة سبع سلالات هى: رحمانى، فرافرة، صعيدى كسلالات مصرية والرومانوف والفنلاندى كسلالات اجنبية واثنين من الهجن ٢/١ رحمانى X٢/١ رومانوف (هجين ١) و ٢/١ رحمانى X٢/١ فنلاندى (هجين ٢). تم عزل الحامض النووى الجينومى وتم عمل تفاعل البلمرة المتسلسل باستخدام اثنين من بادئات الواسمات الجزيئية هما BM1329 و OarAE101 لهما ارتباط قوى بالموقع الوراثى الخاص بجين الخصوبة. وقد اوضحت النتائج غياب هذا الجين من السلالات المصرية فى حين ان السلالات الاجنبية والهجن المصرية مع الاجنبية اظهرت وجوداً واضحا للجين فى مادتها الوراثية. وهذا يفسر حقيقة ان الهجن المصرية مع السلالات الاجنبية ترتفع فيها نسبة تعدد الخلفة عن السلالات المصرية النقية. اكدت هذه الدراسة على انتقال هذا الجين من السلالات الاجنبية الى السلالات المصرية من خلال التهجين دون اللجوء الى برامج التربية المعقدة . أمكن لهذه الدراسة أن تقدم وسيلة سهلة وفعالة وغير مكلفة للتعرف على الأفراد الحاملة لجين الخصوبة فى القطيع بالاضافة الى المساهمة فى رفع الكفاءة التناسلية للسلالات المصرية دون التاثير على صفات الاقلمة البيئية مع الظروف البيئية المصرية.