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Studying the Genetic Variation Among Low and High Milk Producer Egyptian Buffalo *(Bubalus bubalis)* using Microsatellite and Scot Markers

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ABSTRACT

The future bio-economy of dairy industry relies on the identification of an affordable approach for increasing milk production and its constituents. Egyptian buffalo *(Bubalus bubalis)* contributes by about 50% of total milk production in Egypt, therefore it is considered as an essential dairy animal. This study aimed to differentiate between high and low milk producer buffaloes (*Bubalus bubalis*) using microsatellite and SCoT markers. The results of the six microsatellite primers showed bands of 350, 377, 496, 247, 262 and 280 bp, where three of which were digested by restriction enzyme. SCoT results of bulked samples showed unique bands that were sequenced and aligned to *Bubalus bubalis* (taxid: 89462) sequences. Alignment results showed similarity to the following encoding genes, Class V myosin, Ubiquitin-conjugating enzyme E2 D4 and acyl-phosphatase 2 (ACYP2), which play a crucial role in organelle trafficking and many pathways, that may affect milk production traits. These results showed that SCoT marker was better than microsatellite in clarifying the difference between high and low milk producing traits in Egyptian buffalo (*Bubalus bubalis).*

Keywords: *Bubalus bubalis,* SCoT, microsatellite markers

INTRODUCTION

The world population is increasing at a rapid rate and is expected to reach 8–9 billion by the end of 2030. Consequently advancements in scientific and technological fields linked to animal production and related biotechnologies are urgently needed (Deb *et al*., 2016). In Egypt buffaloes fed on poor quality nutritional resources, like crop-residues and industrial by-products containing high fibrous materials, due to their ability to utilize poor value feed resources. This is the main reason why water buffalo (*Bubalus bubalis*) is considered as an efficient converter of poor quality resources into high quality milk and meat, in addition to power, fuel, and by-products such as hides, hoof, and bones, as well as manure to be used as fertilizer (Qureshi *et al*., 2002; Sarwar *et al*., 2009).

Buffaloes are also more resistant to many diseases than domestic livestock. This feature helps buffalo to survive in hot humid regions (Marai *et al*, 2010). However, improper feeding regimen and low food availability and quality certainly have a negative impact on reproductive and productive performances, by increasing mortality rates, longer calving interval, and reduction of growth rates (Tiwari *et al*., 2007; Pasha and Khan, 2010). Buffalo population is distributed worldwide, even though the majority (around 97%) present in Asia, and 3 717 million are in Africa, mostly in Egypt (2.24 %); 3.3 million. Buffaloes are the second largest source of milk worldwide, in Egypt it contributes by about 50% of total milk production – 1041533 heads- with Lactation duration of 210-280 days, and Milk yield of 2034030 tones (FAOSTAT 2017). Buffalo milk is more preferred by the consumer for its white color and rich nutrition as it contains higher content of fat (6.5-7.0 %), lactose, casein, whey proteins, and minerals than cow milk, which is responsible for its high energy and nutritive value. Therefore, it is more desired in several dairy industries as cheese, yogurt and ice cream (Hamad *et al*., 2014; Khedkar *et al*., 2016). Egypt suffers from a massive gap in milk and meat production, detected through annual imports. Recent studies give more attention for genetic improvement of domestic buffalo breeds to increase their role in the agricultural production system (Borghese, 2005; FAOSTAT 2017).

Molecular markers are essential in identifying any phenotypic difference that is genetically controlled. In addition, molecular markers are also used to discriminate groups of individuals (Reinosa and Abad, 2012). One of the main critical molecular markers are microsatellite markers, which are repetitive regions of DNA where short nucleotide sequences (ranging in length from 1– 6 or more bp) are repeated 5–50 times, and the number of repeats differs between individuals of the same species. They have high mutation rates than other areas of DNA, which results in high genetic diversity (Brinkmann *et al.* 1998; Richard *et al*., 2008; Gulcher 2012). PCR-RFLP is the amplification of genes with specific primers, and then the amplified PCR products are subjected to digestion using restriction enzymes (Reinosa *et al.*, 2012). K-casein gene: K -casein is a milk protein that determines the size and function of milk micelles, also caseins are raw materials for cheese making industry (Otaviano *et al.* 2005; Shende *et al.*, 2009). Leptin gene: Leptin is a hormone occupied in the regulation of nourishment intake, fat metabolism, and reproduction. A primer amplifying the exon 3 of the leptin gene is used to detect any SNPs that may be related to milk production (Vallinoto *et al.*, 2004). β- lactoglobulin gene: β-lactoglobulin is a milk protein that is related to milk production. It has a main influence on the composition of milk and on the processing property of milk (Meignanalakshmi *et al.*, 2009). Start codon targeted polymorphism (SCoT) gene expression analysis is vital in studying genetic difference; which is mainly based on short conserved region flanking the ATG start codon in plant genes. It was known that SCOT method uses single primers of 18-mer in single primer polymerase chain (Wu *et al*., 2013). These primers are start codon targeted, which would help in studying the expressed genes regardless RNA complications. SCoT primers were used after verifying their sequence similarity to the published buffalo sequences found in the NCBI website. This study aimed to relate some sequences of buffalo milk genes with the milk production traits and to discriminate the difference between high and low milk producing animals.

MATERIALS AND METHODS

Animal blood samples and DNA extraction:

Twenty Egyptian Buffaloes blood samples were kindly provided by Animal Production Research Institute (APRI). Those animals were divided into two groups according to their breeding value. The first group (12 animals) was high producers and the second group (8 animals) was low milk producers. The blood samples were collected using falcon tubes containing 200 µl EDTA from the jugular vein and stored at -20°C until processed. DNA was extracted from frozen blood according to thermo scintific Kit protocol. DNA samples were quantified using NANO drop. DNA samples were then checked on 1% agarose gel.

Microsatellite markers:

Six microsatellite primers were selected from previous studies to detect different milk genes (Table 1).

Table 1.Primers names, sequences, milk genes, expected fragment size, the restriction enzyme and reference.

Primer name	Primer sequence 5'→3'	Gene	Expected fragment size	Restriction enzyme used	Reference
JK5-F JK3-R	ATCATTTATGGCCATTCCACCAAAG GCCCATTTCGCCTTCTCTGTAACAGA	k-casein	350-bp	*Hinf*I	Stipp *et al., 2013*
K1-F K2-R	CCCACCGAGTCCTGCCAC GTTGAAGGACTTAAAGGAGA	k-casein	377bp	None	Damiani *et al*., 2000
Exon3-F Exon3-R	CCCTCTCTCCCACTGAGCTC TAAAGGATGCCCACATAGGC	Leptin	496 bp	None	Orrù *et al*., 2007
Lac-10-F Lac-10-R	TGTGCTGGACACCGACTACAAAAA GCTCCCGGTATATGACCACCCTCT	β-lactoglobulin	247bp	*Hae* III	Karimi *et al,* 2009
I-10-F II-10-R	GTCCTTGTGCTGGACACCGACTACA CAGGACACCGGCTCCTGGTATATGA	β-lactoglobulin	262 bp	*Hae* III	Meignanalakshmi *et al.*, 2009 Stipp *et al., 2013*
ex1-10-F ex2-10-R	TGTGCTGGACACCGACTACAAAAAG GCTCCCGGTATATGACCACCCTCT	β-lactoglobulin	280 bp	*Hae* III	Ilie *et al.*, 2010

Polymerase chain reaction-restriction fragment length polymorphism PCR-RFLP

PCR amplification was performed in a final volume of 25 µl containing 50 ng DNA, (10 pM/ µl) 1 µl of each primer and (5u/ µl) 0.15 µl GoTaq (Promega) 5x green buffer (Promega)5 µl and (2mM) 2.5 µl dNTPs and 1 ml MgCl2. The amplification reaction was performed using PCR profile with an initial cycle at 95º C for 5 min, followed by 35 cycles of 95º C for 30 sec., 60º C for 30 sec. and 72º C for 30 sec., and a final extension at 72º C for 7 min. Thereafter, PCR products were digested with restriction enzymes in a 20 µl of restriction mixture (10U R.E / reaction) and the digestion reaction were incubated at 37º C for 20 minutes and then terminated by increasing the temperature to 80º c for 15 minutes.

SCoT markers:

Fifteen SCoT primers were used in this study. All primers aligned with *Bubalus bubalis* (taxid: 89462) sequences using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database. Thus SCoT primers were used to identify expressed genes for the first attempt in Egyptian buffalo *Bubalus bubalis* (Table 2).

Table 2. SCoT primer sequences used in all the 20 samples.

Primer number	Primer sequence 5'→3'
1	CAACAATGGCTACCACCA
2	CAACAATGGCTACCACCC
11	AAGCAATGGCTACCACCA
13	ACGACATGGCGACCATCG
16	ACCATGGCTACCACCGAC
33	CCATGGCTACCACCGCAG
35	CATGGCTACCACCGGCCC

Bulked samples

SCoT primers used to amplify bulked genomic DNA representing high and low milk production traits, generated by mixing equal concentration from six of the DNA highest and lowest milk producer according to the breeding value. (Al-Soudy *et al*., 2018).

Table 3. SCoT primers sequences used in bulked samples.

Primer Number	Primer sequence 5'→3'
1	CAACAATGGCTACCACCA
2	CAACAATGGCTACCACCC
3	CAACAATGGCTACCACCG
4	CAACAATGGCTACCACCT
11	AAGCAATGGCTACCACCA
12	ACGACATGGCGACCAACG
13	ACGACATGGCGACCATCG
14	ACGACATGGCGACCACGC
16	ACCATGGCTACCACCGAC
20	ACCATGGCTACCACCGCG
22	AACCATGGCTACCACCAC
28	CCATGGCTACCACCGCCA
33	CCATGGCTACCACCGCAG
35	CATGGCTACCACCGGCCC
36	GCAACAATGGCTACCACC

Bands purification, sequencing and data analysis:

Only three of the polymorphic bands were excised and purified using Invitrogen purification kit, for sequencing. Sequencing was performed using Big Tri Dye sequencing kit (ABI Applied Biosystem). The sequences of the three bands were aligned with Bubalus bubalis (taxid:89462) sequence using The Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) data base, using the database (nucleotide collection (nr/nt), and then aligned using the database [whole-genome shotgun contigs (wgs)].

RESULTS AND DISCUSSION

Results

Animal selection

Milk production quantity wasn't the only factor for animal selection in this study; it was subjected to different criteria as number of milking seasons and the average

number of milk production in each season. Moreover sire genes contributes in milk production traits of their daughters as well as dames genes. Accordingly animal selection was based on breeding value that gives evidence on milk production traits, as samples from 1 to 12 are of high BV and samples from 13 to 20 are of low B.V (Table 4).

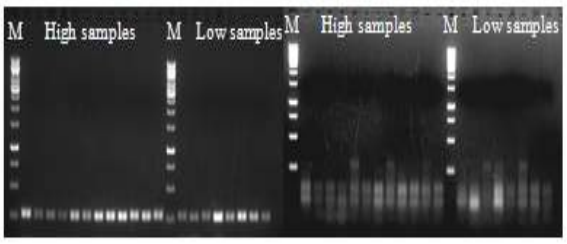
Table 4. Animal number, B.V. (breeding value), sire number, dame number, dame weight at calving, milk production at sampling season and number of milk production season for each buffalo.

Animal No.	B.V[a]	Sire	Dame	Weight of dame at calving (Kg)	Milk production at sampling season (Kg)[b]	Milking Seasons number
1	*106.8	12043	14853	550	2117	6
2	*103	13066	14746	550	1766.5	5
3	*81	13066	2845	500	2105	2
4	*60	12933	2774	400	1865	6
5	*58	1361	2966	400	1691	2
6	*56	13066	2863	550	1544	3
7	*51	12933	2914	550	1847	6
8	*47	18038	15253	580	1941.5	6
9	*40	14727	16529	570	1801	12
10	*27	14727	14746	550	2096	10
11	*18	12148	2007	450	2302	10
12	*8	12043	2629	580	1704	10
13	0	17224	2272	550	2016.5	11
14	-73	13442	2206	550	1544	1
15	-49	1361	1740	450	1559	2
16	-44	13061	2903	400	1934	6
17	-40	1361	2902	500	2119	4
18	-22	12043	16085	580	1716	5
19	-22	15280	2262	550	1532	10
20	-2	12043	2074	550	1655	10

[a] **B.V. breeding value (it is used to clarify high and low milk producing individuals).**
[b] **Milk production at sampling season (season when blood sample was taken).**
*** High producers.**
- Low producers.

Microsatellite markers and PCR-RFLP:

Most of the used microsatellite primers provide monomorphic results. Only three primers showed polymorphism between individual samples within β-LG gene. Namely, primer I-10 amplifying the β-LG gene from exon IV to intron IV that resulted in a 262 bp fragment (Fig. 1A), Primer ex-10 amplifying a 280 bp fragment and Primer Lac-10 amplifying 247 bp. These fragments were then subjected to digestion by *Hae* III enzyme. Digestion resulted in different fragments which differ in number between different individuals (high and low producers), with no significant differences between high and low yielders (Fig. 1 B).



(A) (B)

Fig .1. (A) Primer I-10 amplifying β-LG gene from exon IV to intron IV, (B) Result of digestion of the band by *Hae III* enzyme. (M) Marker 1kb Ladder, High samples (1-12), Low samples (13-20).

SCoT primers

Most of the SCoT primers used resulted in monomorphic profiles (Fig. 2).

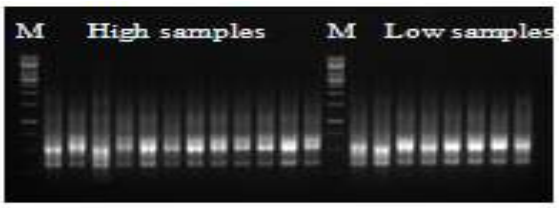


Fig . 2. SCoT marker result. (M) Marker 1kb Ladder, High samples (1-12), Low samples (13-20).

Bulked samples

Fifteen different SCoT primers were used to detect the difference in the bulked samples representing high and low milk production traits (Table 3).Only three of the polymorphic bands were excised and purified.

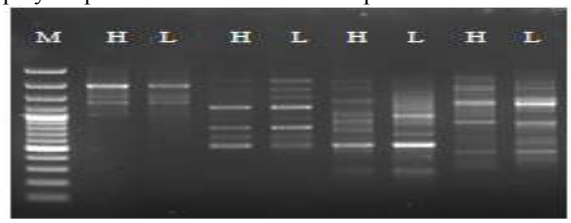


Fig . 3. Results of bulked samples amplification using primers (p3, p4, p12, and p14) respectively from left side, (H) represent high yielders (L) represents low yielders, (M) 1kb Marker.

Sequencing and analysis of amplified bands:

The three sequenced bands contained 543, 528, and 534 nucleotides, respectively. First sequence had 543 nucleotides and showed similarity to the gene expressing Class V myosin with accession number: XM_025295606,

when using (nr/nt) database, This sequence when aligned using the (wgs) database it showed greater similarity to the *Bubalus bubalis* genome. It resulted in similarity to Mediterranean breed chromosome 6 accession number PZYV01000021, 11, 9 and 2, and some contigs and scaffolds.

Second sequence had 528 nucleotides and showed similarity to the gene expressing Ubiquitin-conjugating enzyme E2 D4 with accession number: XM_006074776, when using (nr/nt) database, This sequence when aligned using the (wgs) database it showed greater similarity to the *Bubalus bubalis* genome. It resulted in similarity to Mediterranean breed chromosome 6 accession number PZYV01000021, X, 4, 23 and 2, and some contigs and scaffolds.

Third sequence had 534 nucleotides and showed similarity to the gene expressing acyl-phosphatase 2 (ACYP2) with accession number: XM_025261021 using (nr/nt) database. This sequence when aligned using the (wgs) database it showed greater similarity to the *Bubalus bubalis* genome. It resulted in similarity to Mediterranean breed chromosome 4 accession number PZYV01000019, 3, 19, 13 and 10, and some contigs and scaffolds.

Discussion

In the present study six microsatellites were used for screening different milk genes. Primer K1 amplifying 377 bp of K- casein gene, and primer exon 3 amplifying 496 bp of exon3 leptine gene, resulted in monomorphic profile detecting no SNPs that may be related to milk production traits. β- lactoglobulin gene amplification resulted in a 262, 280, and 247 bp bands using three different primers. In addition, digestion reaction using *Hae* III enzyme resulted in different bands which differ in number. However, it is impossible to assess the influence of these genes on milk production traits, since all animals in this study showed non-significant difference between high and low milk producing animals.

It may be the first attempt to use SCoT primers to identify expressed genes in Egyptian buffalo *Bubalus bubalis*. The results were able to amplify buffalo DNA samples, providing a monomorphic profile. Nevertheless, we weren't able to differentiate between high and low milk producers' animal.

SCoT primers that were used on two bulked samples representing the high and low milk yielders; provides unique bands, therefore the results were able to differentiate between high and low yielders. Only three of the purified bands were sequenced.

First sequence had 543 nucleotides and showed similarity to the gene expressing Class V myosin, which are group of molecular motors. Class V myosins have a preserved structure that can be divided into a head, neck, and tail. One of the class V myosins is Myo5 which is divided to a, b and c, Myo5 is expressed intensively in many tissues as brain, epithelial and glandular tissues including pancreas, prostate, mammary, stomach, colon and lung (Olga *et al.*, 2002). Although class V myosins plays critical roles in organelle trafficking and have been the subject of intensive research, relatively little is known about the localization, dynamics, or functions of Myo5c. Mammals express three different class Vmyosins, myosin-Va (Myo5a), myosin-Vb (Myo5b), and myosin-Vc (Rodriguez and Cheney, 2002).

In a previous study, the first immunolocalization of endogenous Myo5c in a cell line and the first live-cell imaging of GFP-Myo5c was reported by Jacobs *et al.*, (2009). This study revealed that Myo5c localizes the secretory granules; consequently that dominant negative Myo5c dramatically perturbs the distribution of secretory granule markers. Accordingly, this results was able to define the fundamental cell biology of Myo5c and prove that Myo5c functions as a molecular motor in the trafficking of exocrine secretory granules. Additionally, our investigations provide a strong evidence that Myo5c associates with secretory granules and is required for normal secretory granule trafficking.

Previous studies indicated that Myo5c localizes on mature exocrine secretory granules in rabbit lacrimal gland acinar cells (Marchelletta *et al.*, 2008). Interestingly, the expression of Myo5c tail partially inhibits carbachol which consequently stimulates secretion, indicating that Myo5c is required for normal secretion in actual exocrine secretory cells. In accordance to a previous study, it was reported that Myo5c is a class V myosin that functions in the trafficking of exocrine secretory granules (Chen *et al.*, 2006).

Throughout aligning the resulted sequence against *Bubalus bubalis* genome using (wgs) database, results showed greater similarity. Hence the provided sequence showed similarity to chromosome 6, where the relation between some chromosome 6 expressed genes or sequences and milk production was determined, as well as the same for chromosomes 11, 9 and 2.

Results showed that similar sequences are related to milk production especially the sequence that was isolated from the bulked low breeding value animals, which may indicate that the presence of such a sequence is truly related to low milk producing animals. Nevertheless, more developments are required to verify this study.

The second sequence had 528 nucleotides and showed similarity to the gene expressing Ubiquitin-conjugating enzyme E2 D4, which functions as an acceptance of ubiquitin from the E1 complex and catalyzes it to covalently attach to other proteins, one of its other functions is membrane trafficking (David *et al*, 2010). One of its functions is an impact on membrane trafficking. The manner in which ubiquitin is added determines the outcome for the protein. These modifications can include: monoubiquitination, the addition of a single ubiquitin to one lysine residue in the protein, Multi-monoubiquitination, the addition of single ubiquitin molecules to multiple lysine residues in the target protein and polyubiquitination, the addition of multiple ubiquitin residues to a single lysine residue in the protein in the form of a chain which can take a number of different conformations.

These modifications alter cellular process in different ways. Such as, monoubiquitination can impact on membrane trafficking; however, some forms of polyubquitination can result in a protein being targeted for degradation via the proteasome. The ubiquitin pathway has been implicated in a number of genetic disorders and diseases including cancer and neurological diseases which

result from the build-up of protein aggregates such as; Alzheimer's disease, Parkinson's disease and Huntingdon's disease (horizon discovery.com). This sequence was isolated from the bulked low breeding value animals, and is associated with membrane trafficking which is essential in mammary glands.

This sequence when aligned against *Bubalus bubalis* genome using (wgs) database, it showed greater similarity, where this sequence resulted in similarity to chromosome 6. This result showed the relation between some chromosome 6 expressed genes or sequences and milk production, this is also for chromosomes X, 4, 23 and 2. To relate the chromosome genes with low milk production validation experiments are required, in which the intended band was isolated from low bulked sample.

The third sequence had 534 nucleotides and showed similarity to the gene expressing acyl-phosphatase 2 (ACYP2). ACYP2 (Acylphosphatase 2) is a protein coding gene, and is a muscle type isozyme that can hydrolyze the phosphoenzyme intermediate of different membrane pumps, particularly the Ca^{2+}/Mg^{2+}-ATPase from sarcoplasmic reticulum of skeletal muscle (NCBI).

Two isoenzymes have been isolated, called muscle acylphosphatase and erythrocyte acylphosphatase on the basis of their tissue localization. This gene encodes the muscle-type isoform (MT). An increase of the MT isoform is associated with muscle differentiation. Acylphosphatase 2 is expressed in many different tissues including male and female tissues (breast) (NCBI). This sequence when aligned against *Bubalus bubalis* genome using (wgs) database, it showed greater similarity. This sequence resulted in similarity to chromosome 4, where such result show the relation between some chromosome 4 expressed genes or sequences and milk production, this is also for chromosomes 3, 19, 13 and 10. This sequence was isolated from the bulked high breeding animals.

CONCLUSION

Microsatellite markers provide amplified parts of k-casein gene, Leptin gene, and β- lactoglobulin gene. A 262, 280, and 247 bp bands resulted from β- lactoglobulin gene amplification which was then digested by *Hae III* enzyme. It is impossible to assess the influence of these genes on milk production traits, since all animals studied resulted in monomorphic profile.

SCoT primers were used after showing great similarity with buffalo sequence when aligned to it. They successfully amplified buffalo DNA samples, resulting in a monomorphic profile when used individually on the 20 samples.

SCoT primers were then used on two bulked samples representing the high and low milk yielders, this resulted in unique bands differentiating between high and low yielders, three of which were excited, purified and sequenced. The alignment of these sequences with *Bubalus bubalis* (taxid:89462) sequence (BLAST) in (NCBI) using (nr/nt) database showed similarity to three important genes expressing Class V myosins, Ubiquitin-conjugating enzyme E2 D4, and acyl-phosphatase 2 (ACYP2) proteins, which have a crucial role in many pathways and could be related to milk production traits. When aligning these sequences using (wgs) database, it showed much higher similarities but with no specific function with some buffalo chromosomes, which may have an important effect on milk production traits. Additional studies and investigations are recommended for the improvement of Egyptian buffalo milk genes.

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دراسة التباين الوراثي بين الجاموس المصرى Bubalus bubalis ذي الإنتاجية العالية و المنخفضة من الألبان باستخدام معلماتSCoT و microsatellite

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يعتمد الاقتصاد الحيوي المستقبلي لصناعة الألبان على تحديد طريقة قابلة للتحقيق لزيادة إنتاج الحليب ومكوناته. يساهم الجاموس المصري بحوالي 50٪ من إجمالي إنتاج الحليب في مصر ، لذلك يعتبر حيوانا أساسيا فى إنتاج الألبان. تهدف هذه الدراسة لاستخدام المعلمات الجزيئية microsatellite و SCoT فى التفرقة بين سلالات الجاموس ذات الانتاجية العالية و المنخفضة للألبان. و قد اوضحت نتائج استخدام ست معلمات جزيئية من Microsatellite وضوح مقاطع من المادة الوراثية DNA ذات أطوال 280, 262, 246, 496, 377, 350 زوج قاعدى. و من هذه المقاطع تم تعريض ثلاثة فقط لإنزيمات القطع المتخصصة . كما أوضحت معلمات SCoT ظهور مقاطع مميزة للعينات المجمعة و قد تم تنقية هذه المقاطع و قراءة التتابع النيوكليوتيدى بها و تحديد مدى التشابه بينها باستخدام BLAST . و قد أوضح ذلك وجود تشابه بين هذه المقاطع من DNA و *Bubalus bubalis* (taxid: 89462) فى جينات gacyl-phosphatase (ACYP2) 2 و V myosin, Ubiquitin-conjugating E2 D4 والتي تلعب دورا هاما فى العديد من المسارات الحيوية داخل الحيوان و انتقال المركبات المختلفة بين أعضاء الحيوان . و من هذه النتائج يتضح أن استخدام معلمات SCoT كان أفضل من microsatellite فى الحصول على مقاطع مميزة يمكن استخدامها للتفرقة بين الحيوانات ذات الإنتاجية العالية و المنخفضة للألبان فى الجاموس المصرى.