Egyptian J. Anim. Prod. (2022) 59(1):9-18

MOLECULAR CHARACTERIZATION OF SOME CANDIDATE GENES IN PURE EGYPTIAN BUFFALOES AND CROSSBRED OF ITALIAN BUFFALOES

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***Submitted:** 11/1/2021; **Accepted:** 1/2/2022; **Published:** 5/2/2022*

SUMMARY

The goal of this work was to describe the sequences of some candidate genes (IGF-I, IGF-I receptor, and Leptin) that are associated with economically important quantitative aspects in dairy buffalo, such as reproductive and productive attributes, as well as milk composition. Ninety-nine dairy buffaloes were used to compare the pure Egyptian buffalo (PE) with the Egyptian–Italian crossbred G1 (25.0%), G2 (50.0%), G3 (62.5%), G4 (75.0%), G5 (87.5%), and G6 (94.0%), respectively. All buffaloes investigated were genotyped BB, which means they were negative for the SnaBI at position 224^225 (TAC^GTA) of the IGF-I regulatory region, and they were genotyped AA-positive for the IGF-I receptor TaqI at position 47^48 (T^CGA). They also tested positive for the leptin gene's Alu1 restriction site yielding three products with genotype GT that was 55-, 118-, and 205-bp in length (AG^CT). Finally, the PE and Egyptian-Italian crossbred demonstrate monomorphism since the two Bubaline populations are closely related and the genes in question are maintained. More research is needed to learn more about Egyptian-Italian buffalo crossbreeds before national crossbreeding initiatives may be expanded.

Keywords: Egyptian-Italian buffalo, insulin-like growth factor, leptin, restriction fragment length polymorphism

INTRODUCTION

Water buffaloes are the second most important species for milk production in the world (Coroian *et al*., 2013). Although buffalo milk production is lower than that of cow breeds (Ibrahim, 2012), buffalo milk has a considerably superior composition (Senosy and Hussein, 2013). Because of its fat, protein, lactose, and mineral content, Buffalo milk is a popular dietary in some areas. (El-Salam and El-Shibiny, 2011). Buffaloes have a high conversion rate, making them more efficient than dairy cows at converting low-quality feed and forage into meat and milk (Ibrahim, 2012). As a result, buffaloes are essential farm livestock animals maintained for various purposes by breeders on small farms in a variety of climates. Buffaloes have recently gained in value, particularly in terms of milk production (Pardal *et al*., 2017).

Researchers are working to develop improved buffalo breeds, but when single traits are selected negative impacts on milk quality and reproductive performance must be avoided (Barros *et al*., 2014). Although Egypt has more buffaloes than Italy (Nasr, 2016b), Egyptian buffaloes produce less milk and have a worse milk efficiency. This distinction can be attributed to the successful programs of selection, breeding, and recording efforts used in Italy (Borghese, 2010). Egyptian dairy farmers have begun to cross pure Egyptian buffaloes (PE) with Italian-breed buffaloes to take benefit of the Italian system, which improves the production traits and reproductive fitness of the Egyptian buffaloes. Imported Italian semen with reliable breeding values for numerous production and type traits is used in this technique (Ibrahim, 2012).

There is currently a scarcity of data on the fulfillment of several buffalo breeds in semi-arid environments (Silva *et al*., 2016 and Boison *et al*., 2017).

The genetic improvement of farm animal productivity is based on quantitative genetics; some traits are controlled by a single gene, but the majority are controlled by several genes and are impacted by environmental factors (Hill, 2016). The genes of leptin and insulin-like growth factor (IGF) could be useful as markers for identifying elite animals, which could lead to improvements in adaptability and production. The *leptin* gene is involved in the regulation of processes such as growth, puberty, reproduction, milk production, and milk constituents in both animals and humans (Ali *et al*., 2018). IGFs which include the *IGF-I* gene and the IGF-I receptor (IGF-IR) are strongly associated with several reproductive and productive characteristics in dairy animals; they are found throughout the body and regulate a variety of pathways that affect body growth (Uniyal *et al.*, 2015). They also influence carcass and meat quality traits (Grochowska *et al.*, 2017). The reproductive parameters of dairy

animals are affected by Polymorphisms in the IGFs (Colli *et al*., 2018). The purpose of this study was to compare *IGF1/SnaBI, IGF-1R/Taq*, and *leptin/Alu1* in Egyptian buffalo and Egyptian-Italian crossbreeds.

MATERIALS AND METHODS

Animal and blood sampling:

This study was conducted using 99 dairy buffalo from the "United Group" farm in the Qaliobeia governorate. Samples from 14 pure Egyptian (PE) and 85 Egyptian–Italian crossbred buffaloes were taken as shown in Table 1. G1 crosses (75% PE and 25% Italian buffalo), G2 crosses (50% PE and 50% Italian buffalo), G3 crosses (25% Egyptian–Italian and 50% Italian buffalo), G4 crosses (75% crosses and 25% PE), G5 crosses (75% crosses and 50% Italian buffalo), and G6 crosses (G5 crosses and 50% Italian buffalo) as shown in figure 1. Five ml blood specimens were collected from all animals through the jugular vein using vacutainer tubes coated with EDTA as an anticoagulant. As far as molecular genetic studies were concerned the blood samples were kept at -20 ℃ in a deep freeze.

Table 1. A number of samples and percentage of hybridization Italian to Egyptian buffalos

Crossbred	% Of hybrid (Italian to Egyptian)	No. of animals
PE	--	14
G1	25.0 %	12
G2	50.0 %	17
G3	62.5 %	12
G4	75.0 %	14
G5	87.5 %	17
G6	94.0 %	13
Total		99

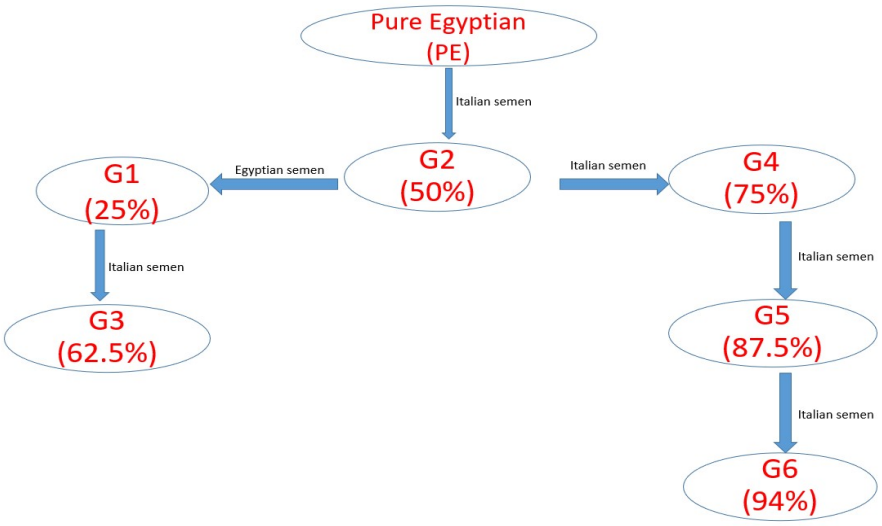

Figure 1. Percentages of crossbreeding between Egyptian and Italian buffaloes.

Molecular study:

Extraction of DNA:

A G-spin™ Total DNA Extraction Mini Kit was used to extract high-quality, whole genomic DNA from previously preserved blood samples according to the manufacturer's instructions. The total DNA concentration and purity were measured using UV-visible absorbance measurements at 260 and 280 nm. The 260/280 optical density (OD) ratios of all the DNA samples were in the range of 1.8 to 2, indicating high purity. The DNA samples were kept at -20 °C until they were used in the PCR test.

Polymerase Chain Reaction for IGF-I, IGF-IR, and leptin genes:

The total volume used for polymerase chain reaction (PCR) (25 µl) consisted of 1.0 µM forward and 1.0 µM reverse primers specific to each gene, 12.5 µl of master mix with loading dye (2x), 3 µl of genomic DNA, and 7.5 µl of distilled water. The primer sequences for each tested gene, as well as PCR conditions and primer sources, are shown in Table 2. The PCR products were electrophoresed on a 2% ethidium bromide-agarose gel to test the amplification success.

Table 2. The sequences and information of primers used in this study

Gene	Primer sequence 5'-3'	PCR condition	Restriction enzyme used	Primer source
IGF-1	F- ATT ACA AAG CTG CCT GCC CC R- ACC TTA CCC GTA TGA AAG GAA TAT ACG T	94ºC 1 min 58ºC 1 min 72ºC 1 min	SnaB1	Othman *et al.,* 2013
IGF-1R	F- CCC AAT GGA TTG ATC CTC ATG T R-GCT GTG TAG TTC CCT GGG TT	94ºC 1 min 56ºC 1 min 72ºC 1 min	TaqI	Othman *et al.,* 2013
Leptin	F- GCA TAG CAG TCC GTC TCC TC R- TTC CCT GGA CTT TGG GAA G	93ºC 1 min 56ºC 30 s 72ºC 1.3 min	Alu1	Sanjoy *et al.,* 2013

Restriction Fragment Length Polymorphism (RFLP):

The PCR products for the tested genes were digested with restriction enzymes specific to the genes (Table 2). The restriction mixture for each sample was prepared by adding 2.5 µl of 10^x restriction buffer to 1 µl of the restriction enzyme and 11.5 µl of sterile water. For IGF-I, this restriction mixture was mixed with 10 µl of PCR product and incubated overnight at 65°C to provide the maximum activity for the restriction enzyme. Subsequently, it was incubated for 20 min at 80°C to inactivate the restriction enzyme.

For IGF-IR, this restriction mixture was mixed with 10 µl of PCR product and incubated overnight at 37°C to provide the maximum activity for the restriction enzyme. Afterward, it was incubated for 20 min at 65°C to inactivate the restriction enzyme. The digested PCR products were electrophoresed on 2% ethidium bromide agarose gels.

For the *leptin* gene, this restriction mixture was mixed with 10 µl of PCR product and incubated for 4 h at 37°C to achieve the maximum activity for the restriction enzyme. Afterward, it was incubated for 20 min at 65°C to inactivate the restriction enzyme. The digested PCR products were electrophoresed on 1.5% ethidium bromide agarose gels to detect the different genotypes of the tested genes.

Genetic identity and Sequence analysis:

The bands of PCR products and fragments after digestion with a restriction enzyme for each tested gene were analyzed using the Gel Doc 2000 data system (Bio-Rad). The PCR products were purified and sequenced at the Reference Laboratory of the Animal Health Institute. Sequence analysis and alignment were performed using ClustalX (version 2.1, http://www.clustal.org).

RESULTS AND DISCUSSION

IGF Gene

IGF-1 Gene

All tested samples showed a 250-bp fragment located in the regulatory region of the buffalo *IGF-I* gene (Fig. 2) as well as had monomorphism for one undigested fragment with the SnaBI endonuclease. Insulin-like growth factor I (IGF-I) is a single-chain polypeptide with 70 amino acids that is encoded by a single gene **(**Van Doorn, 2020). Through binding to a family of specialized membrane-associated glycoprotein receptors, the IGF-1 gene is thought to govern growth, differentiation, and the maintenance of differentiated function in a variety of organs and cell types in mammals (Sarfstein *et al.*, 2019).

Establish the variation in IGF-1 nucleotide sequence between swamp and river buffalo found that there is no genetic difference between swamp and river buffalo, and that river and swamp buffalo (*B. bubalis spp*.) are genetically related to each other (Margawati *et al*., 2019).

Ge *et al*. (2001) detected the IGF-1/SnaB1 polymorphism, which is a T (allele A) to C (allele B) transition in the IGF-1 gene's regulatory region that might impact production features directly or indirectly. In other words, this marker may influence phenotypic traits or be in linkage disequilibrium with a polymorphism that influences these traits.

In four cattle breeds, Curi *et al*. (2005) noticed two genetic variants (A and B) of the IGF1/SnaB1 polymorphism. The presence of two digested fragments at 226- and 23- bp was used to identify genotype AA, while the presence of a single fragment at 249- bp was used to identify genotype BB. Allele B was determined to be fixed in the group of Nellore animals in the investigated samples. In all the groups studied, the frequency of allele B was considerably greater (p=0.05) than that of allele A.

Putra *et al*. (2018) recorded that the IGF1/SnaB1 gene of Pasundan cattle is monomorphic for CC genotype with C allele as the common allele in is monomorphic and cannot be used for molecular selection.

All investigated buffaloes were genotyped as BB, and all tested buffalo DNA amplified fragments at 250-bp located in the regulatory region of buffalo IGF-1 were treated with SnaBI endonuclease, yielding one 250-bp undigested fragment. According to the results of the IGF1/SnaB1 polymorphism. Thus, the PE and Egyptian–Italian crossbreds are genetically closer to the Nellore breed than other cattle breeds such as Canchim and Angus. Also, they have genetic markers that may be directly or

indirectly associated with meat production traits, such as body weight, as previously revealed by Othman *et al*. (2013).

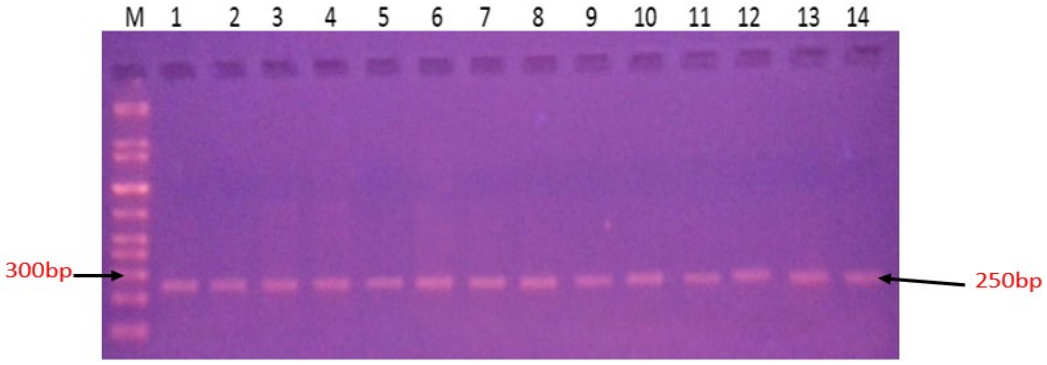

Fig 2. Ethidium bromide-agarose gel of PCR products representing samples from all tested *IGF-I* gene amplifications. Lane M: marker; lanes 1 & 2: PE; lanes 3 & 4: G1; lanes 5 & 6: G2; lanes 7 & 8: G3; lanes 9 & 10: G4; lanes 11 & 12: G5; lanes 13 & 14: G6.

IGR-1R Gene:

The *IGF-IR* gene had a 616-bp fragment located in the regulatory region of the gene (Fig. 3) and monomorphism for two digested fragments at 569- and 47-bp (Fig. 4), which were due to the presence of the restriction site 47^48 (T^CGA) with Taq1 endonuclease; thus, all buffaloes in this study were genotyped as AA for *IGF-IR*.

The IGF-1R gene is likely to be found on the acrocentric buffalo chromosome 20 based on chromosome homology between cattle and river buffalo (Di Berardino *et al*., 1981).

Moody *et al.,* (1996) found a polymorphism in alleles A and B after digesting a 625-bp PCR result using the TaqI restriction enzyme. The low B allele frequency and existence in just *Bos indicus* cattle, they found, may limit the polymorphism's utility.

Szewczuk *et al.* (2011) found that the highest frequency of *IGF-IR* in Holstein–Friesian cows were for the BB and AB genotypes, whereas the lowest was for the AA genotype. In their study, the frequency of alleles was 0.28 and 0.72 for alleles A and B, respectively.

Statistical analysis of the analyzed polymorphism showed that it significantly affected milk yield, milk protein yield, and milk fat yield in favor of the BB genotype.

The prevalence of alleles A and B in IGF-1R polymorphisms revealed by the TaqI digestion was 0.61 and 0.39, respectively. There were no discernible effects of the IGF-1R/TaqI polymorphism on fat and protein output of milk fat content. Compared to other genotype combinations, cows with the IGF-1RBB/IGF-1AB genotype combination produced higher milk, fat, and protein (p=0.05) (Szewczuk *et al*. 2012).

Othman *et al*., (2013) also recorded the same results when evaluating the genetic polymorphism of IGF-I and IGF-IR, in agreement with the observation that the *IGF* gene is a conserved protein family found in most mammalian species and many other vertebrates (Li *et al*., 2021).

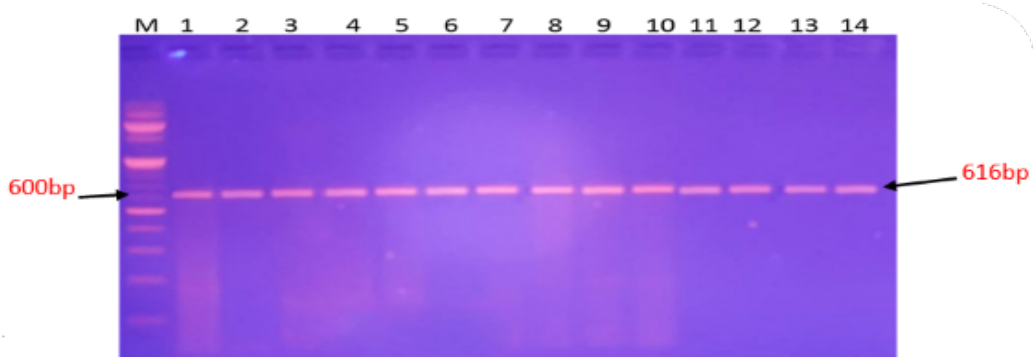

Fig 3. Ethidium bromide-agarose gel of PCR products representing samples from all tested *IGF-IR* gene amplifications. Lane M: marker; lanes 1 & 2: PE; lanes 3 & 4: G1; lanes 5 & 6: G2; lanes 7 & 8: G3; lanes 9 & 10: G4; lanes 11 & 12: G5; lanes 13 & 14: G6.

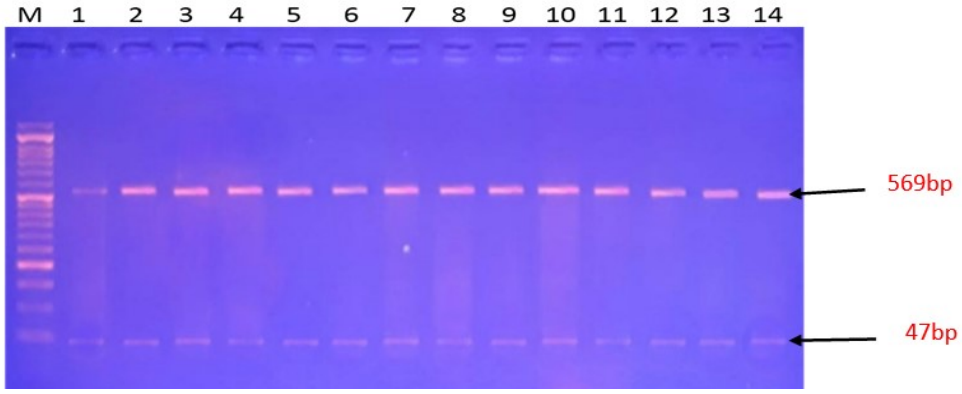

Fig 4. The electrophoretic pattern was obtained after digestion of the PCR-amplified buffalo *IGF-IR* gene with the taq1 restriction enzyme, representing samples from all tested animals. Lane M: marker; lanes 1 & 2: PE; lanes 3 & 4: G1; lanes 5 & 6: G2; lanes 7 & 8: G3; lanes 9 & 10: G4; lanes 11 & 12: G5; lanes 13 & 14: G6.

Leptin gene:

All tested samples had a 405-bp exon III segment for the *leptin* gene (Fig. 5) as well as monomorphism for three products sized 55-, 118-, and 215-bp (AG^CT) with the Alu1 endonuclease (Fig. 6), all buffaloes in this study were genotyped as GT for *leptin*.

The leptin gene is found on chromosome 8 and consists of three exons and two introns that span 18.9 kb, with the first exon not translated into protein (Vallinato *et al*., 2004).

Datta *et al*. (2013) identified monomorphic products for two Alu1 endonuclease-produced fragments of the leptin gene (55- and 350-bp) in Murrah buffaloes, implying high DNA sequence conservation between cattle and buffaloes.

Kaplan (2018) genotyped the bubaline leptin gene T1131G polymorphism in Anatolian buffaloes using DdeI restriction enzyme. Anatolian buffaloes have the TT, GT, and GG genotypes. The T and G allele frequencies are 0.478 and 0.521, respectively, in this study. On the other hand, Aboelenin *et al*. (2017) reported that the G allele was only present in Egyptian buffalo and not in any other buffalo records in GenBank.

Buchanan *et al*. (2002) identified and characterized 416 Holstein cows using the restriction enzyme Kpn21. Animals homozygous for the T allele expressed more milk and had higher somatic cell count linear scores throughout the lactation, without changing milk fat or protein percent.

Orrù *et al*. (2007) sequenced the whole coding region and part of the introns on a panel of Italian River Buffaloes. In both Egyptian and Italian Buffalo, position G3441A was monomorphic. They also found a new set of SNPs (Single Nucleotide Polymorphisms) that could use in association research.

In Egyptian buffaloes, (El-Debaky *et al*., 2020) identified a leptin gene polymorphism and its relationship to reproductive state. The leptin has two variations (AA and BB). Fertile buffalo belonged to genotype AA in 64 %, while infertile buffalo in 36 %. In both fertile and infertile animals, the genotype BB distributed similarly. Sequence examination of normal and polymorphic buffalo revealed many single-nucleotide polymorphisms (SNPs) in the leptin gene; however, these SNPs exhibited no statistical link with the reproductive status (fertile or infertile) of the buffalo studied.

Karima *et al*. (2020) reported that the tested gene, CC, had monomorphic patterns in all the animals. The restriction enzyme Eco91I created the gene's PCR-RFLP pattern. The Leptin gene was amplified and sequenced that obtain a 511-bp fragment.

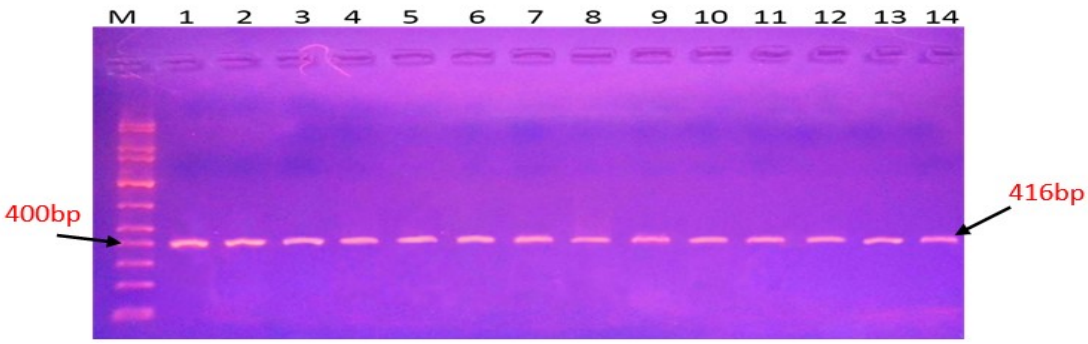

Fig 5. Ethidium bromide-agarose gel for PCR products representing samples from all tested *leptin* gene amplifications. Lane M: marker; lanes 1 & 2: PE; lanes 3 & 4: G1; lanes 5 & 6: G2; lanes 7 & 8: G3; lanes 9 & 10: G4; lanes 11 & 12: G5; lanes 13 & 14: G6.

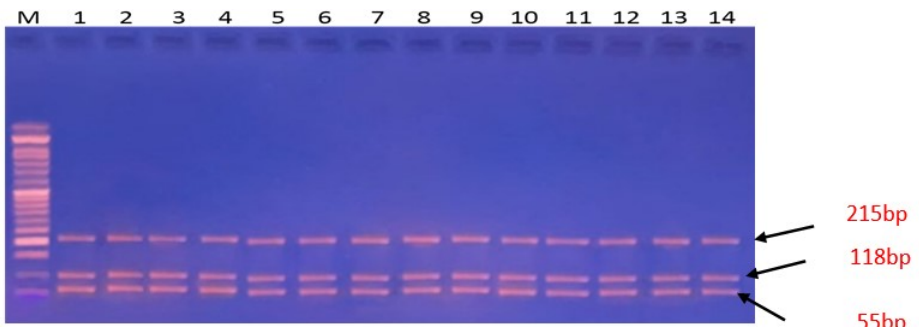

Fig 6. Electrophoretic pattern obtained after digestion of PCR-amplified buffalo *leptin* gene with the Alu1 restriction enzyme, representing samples from all tested animals. Lane M: marker; lanes 1 & 2: PE; lanes 3 & 4: G1; lanes 5 & 6: G2; lanes 7 & 8: G3; lanes 9 & 10: G4; lanes 11 & 12: G5; lanes 13 & 14: G6.

Sequence analysis and alignment

The nucleotides for the *IGF-I*, *IGF-IR*, and *leptin* genes are presented in **Figs. 7, 8,** and **9**, respectively. The sequence obtained of the PE breed compared in alignment with the sequences produced from the Egyptian–Italian crossbreds. There was no change in amino acid sequences among the three examined genes.

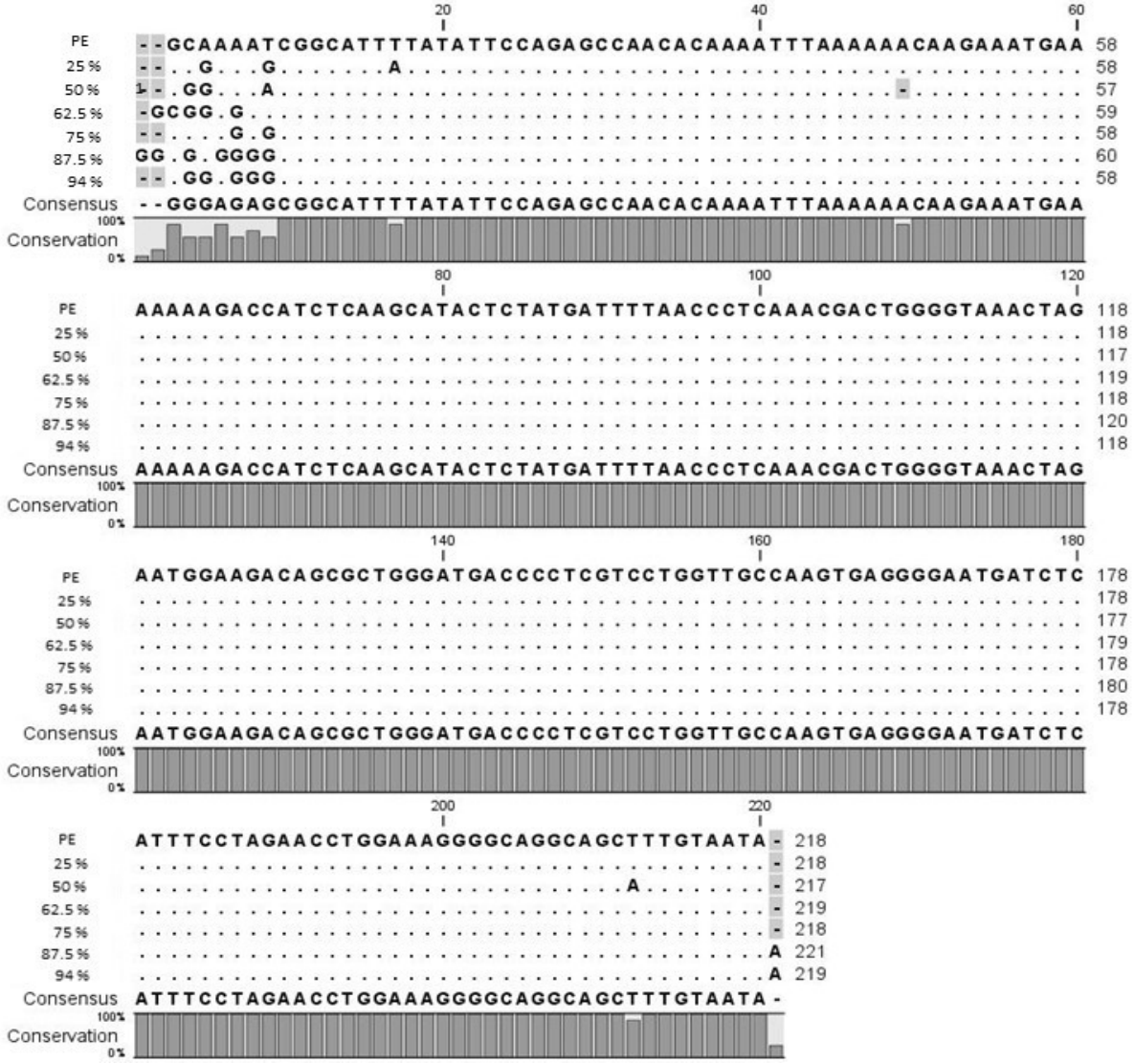

Fig 7. Sequence alignment of the amplified pure Egyptian buffalo *IGF-1* gene fragment with the genes of crossbred buffaloes.

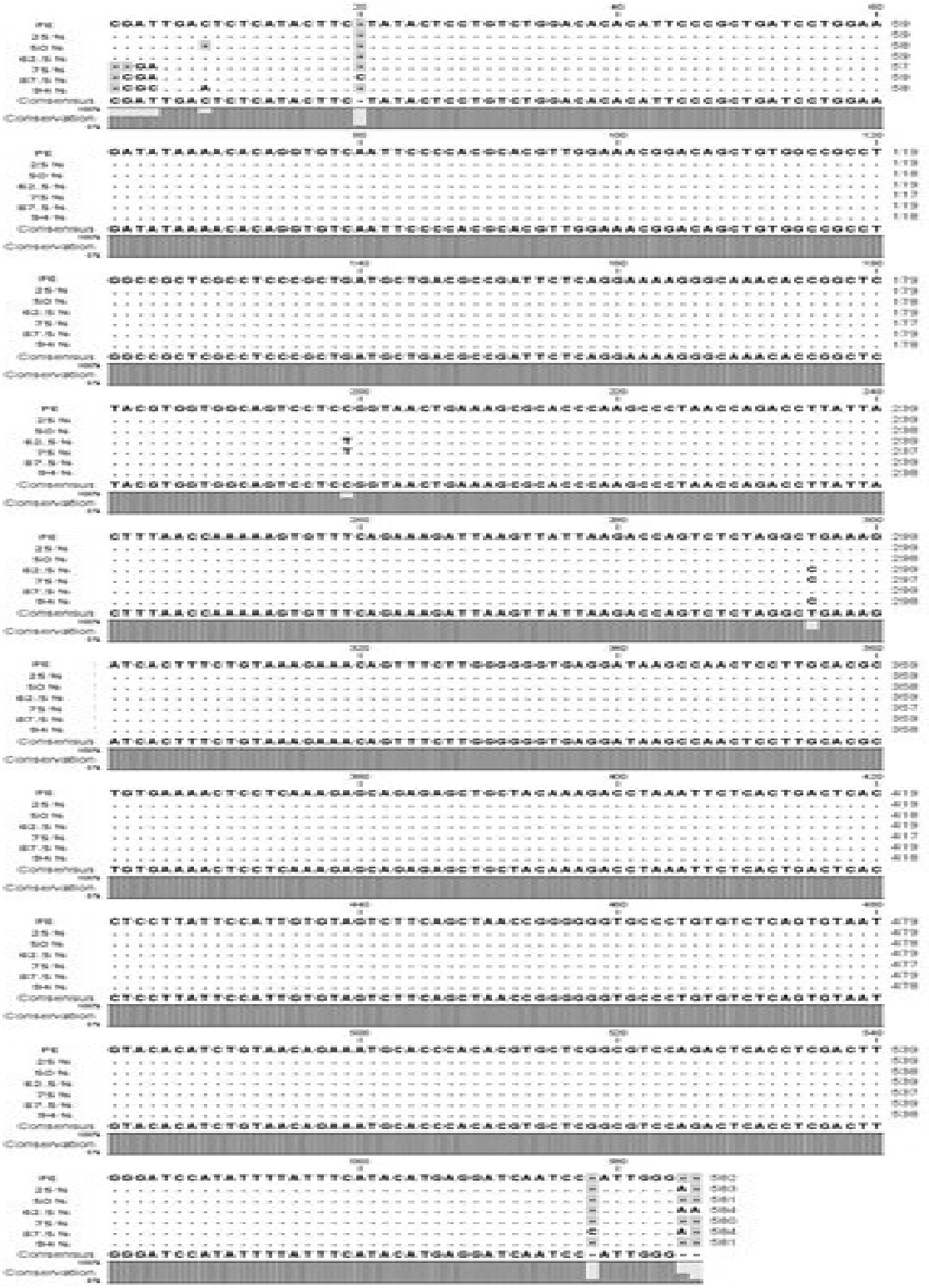

Fig 8. Sequence alignment of the amplified pure Egyptian buffalo *IGF-IR* gene fragment with the genes of crossbred buffaloes.

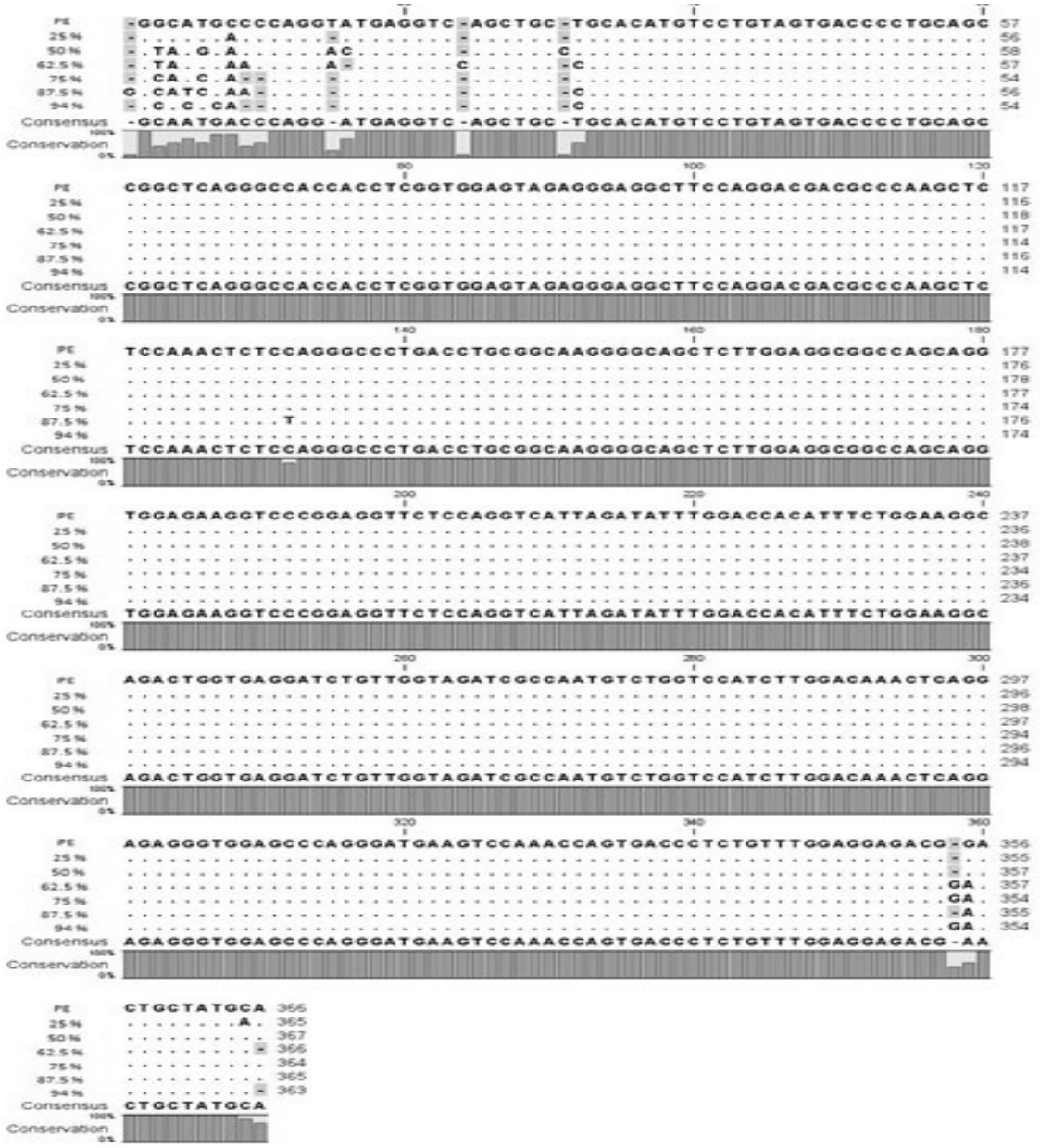

Fig 9. Sequence alignment of the amplified pure Egyptian buffalo *leptin* gene fragment with the genes of crossbred buffaloes.

CONCLUSION

Identifying genetic markers associated with economically important traits in livestock animals is the primary goal of animal genetic research. As a result, the candidate gene approach provided new knowledge for animal genetic research. Many biological functions of IGFs and Leptin genes impacted on characteristics of livestock animals' commercially. Using candidate genes in animal breeding programs can help not only in the selection of young animals but also in the estimate of animal breeding value. It could be concluded that the Egyptian and Egyptian–Italian crossbred buffaloes have monomorphism due to the two Bubaline populations are closely related and the genes in question are preserved.

ACKNOWLEDGMENT

The authors would like to thank the "United Group" farm owner Mr. Francis Abadir and we express our deep gratitude for the help provided by the farm manager veterinarian Dr. Reda Sami in a blood sample and data collection.

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خصـائص بعض الجينات المنتخبة فى الجاموس المصرى والخليط الايطالى على المستوى الجزيئى

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تهدف الدراسة الي توصيف التتابعات و التغيرات الجينية لبعض الجينات (*IGF-I*, *IGF-I receptor,* and *Leptin*)المرتبطة بالصفات الخاصة بالأداء التناسلي والإنتاجي ومكونات اللبن. وقد استخدم عدد 99 رأس من الجاموس الحلاب مشتملين على الجاموس المصري النقي والهجن مع الايطالي (25.0%) G1, (50.0%) G2, (62.5%) G3, (75.0%) G4, (87.5%) G5 و(94.0%) G6 . وقد اوضحت النتائج من العينات التى تم أخذها على المستوى الجزيئي، ان جميع عشائر الجاموس التي تم فحصها في هذه الدراسة من الطراز الجيني BB كانت سلبية لانزيم القطع المحدد SnaBI في الموضع ٢٢٤ ^ ٢٢٥ (TAC ^ GTA) لجين IGF-1 بينما كانت العينات ذات الطراز الجيني AA إيجابية لكل من انزيم القطع المحدد TaqI في الموضع ٤٧ ^ ٤٨ (T ^ CGA) لجين IGF-1R وانزيم القطع المحدد Alu1 لجين Leptin حيث أعطى ثلاثة حزم عند ٥٥ و ١١٨ و ٢٠٥ في الموضع (AG ^ CT) مع طراز جيني GT. ولذا نوصي بالاهتمام بالمزيد من الدراسات علي المستوى الجزيئي وتحديد افضل الجينات الانتاجية وذلك قبل تعميم تهجين الجاموس المصري بالجاموس الإيطالي على المستوى القومي حيث ان استخدام الجينات المرشحة في برامج تربية الحيوان يساعد في اختيار الحيوان في عمر مبكرو أيضًا في تقدير القيمة التربوية له مما يساعد علي زيادة الانتاج.