BENHA VETERINARY MEDICAL JOURNAL, VOL. 26, NO. 1:161-170, MARCH 2014

ISOLATION AND SEQUENCING OF *CYP19* GENE (EXON9) IN EGYPTIAN BUFFALOES

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A B S T R A C T

Numerous studies have been investigated to assess the relationship between the fertility related gene, *CYP19*, and reproduction in cow; however only scanty studies were performed on *CYP19* gene in buffalo. The present study was designed to isolate a fragment of *CYP19* gene containing exon 9 and to detect any polymorphism associated with infertility in Egyptian buffalo cows. *CYP19* gene fragment was isolated by using PCR technique and detected by the direct sequencing and single strand conformation polymorphism (SSCP). A fragment (locus) of *CYP19* gene with size of 446bp was successfully amplified. No SNP was detected in this locus as revealed by a monomorphic SSCP pattern observed in normal cyclic and anestrous animals. The results of SSCP were confirmed by nucleotide sequencing. Alignment of nucleotide sequences of this locus showed 100% identity with Indian water buffaloes (EF126034) and 99% identity with *Bos Taurus* (Z69249.1). The present study concluded that the sequence of *CYP19* is highly conserved between cattle and buffalo.

Keywords: CYP19, SSCP, PCR, Sequencing, Anestrum, Buffalo.

(BVMJ-26(1):161-170, 2014)

1. INTRODUCTION

As a result to the gradual increase in the world population and shortage of water as well as decrease of green areas, it becomes necessary for developing countries to maximize the production of their native animals to guarantee a sustainable source for food of an animal origin. Among domestic animals, the water buffaloes (Bubalus bubalis), particularly the river buffalo, which is the main buffalo breed in Egypt. Indeed, selection of high fertile buffaloes on a genotype basis can improve the reproductive efficiency of the breed which will reflect on their productivity and on the national economics. One of the limiting factors for quick genetic improvement in the buffalo population is poor reproduction. Field surveys on reproductive disorders revealed that anestrum was the most common single cause of infertility in buffaloes (Ashturkar et al., 1995; Singh and Sahni, 1995). The common genes of aromatase cytochromes P450 family are CYP19 and CYP21 (Kobylinska, 1994). The role of CYP19 is the conversion of androgens to estrogens, which is necessary to maintain the reproductive pattern in females (Evans et al., 2004; Ghai et al., 2012; Jędrzejczak et al., 2011; Murray et al., 2000). Detection of single nucleotide polymorphisms (SNPs) can be achieved

simply by many techniques including the single strand conformation polymorphism (SSCP) (Orita et al., 1989), RFLP (Cotton, 1993; Youil et al., 1995) and the denaturing gradient gel electrophoresis (Myers et al., 1987; Sheffield et al., 1989). The SSCP has been used for detecting the genetic mutations in humans (Orita et al., 1989), rats (Pravenec et al., 1992), cattle (Kirkpatrick, 1992; Raghavan, 2006), and in various bacteriological (Morohoshi et al., 1991) and viral (Fugita et al., 1992) systems. The SSCP followed by sequencing was implemented in the present study to discover polymorphism in CYP19 gene. Kumar et al. (2009) have analyzed CYP19 gene polymorphism by SSCP in 3 groups of buffaloes of different fertility performance, normal cyclic buffaloes, late matured and late maturing/true anestrous animals (Kumar et al., 2009). Monomorphic SSCP patterns were observed in all three groups for exons 4, 5, 6 and 8. However, 4 allelic variants in coding exons 2, 9 and introns 3, 7 with unaltered protein sequence. Heterozygotic condition (C/ 82G) was observed in CYP19 exon 9 of late matured and true anestrus animals. Though there was a change from GG homozygote (control) to C/G heterozygote of late matured and true anestrus animals, the amino acid encoded by both the codons (CCC and CCG) is proline due to the degeneracy of the genetic code (Watson et al., 2004). Indeed, previous studies have shown that the mutations in CYP19 gene cause aromatase deficiency that results in underdeveloped external genitalia and uteri, arrested follicles, and no corpora lutea (Grumbach and Auchus, 1999; Layman, 2002; Palter et al., 2001). The aim of the present study was to isolate CYP19 gene locus containing exon 9 and to detect any polymorphisms associated with incidence of infertility problems in Egyptian buffalo cows.

2. MATERIAL AND METHODS

2.1. Animal source and grouping

The current study was conducted on a total of 180 buffalo cows and heifers selected from Buffalo Nucleus Herd, Animal Production Research Institute, Ministry of Agriculture. Heifers were naturally served for the first time when they reach 300 to 350 kg of body weight and/or 24 months of age. Buffalo cows should be dried off two months before the expected calving date, and they served not before two months after calving. Animals were assigned in two main groups: normal fertile and infertile due to anestrum. Buffalo cows with 5 successive calving will be grouped as normal fertile, heifers which didn't show oestrus over two years of age and buffaloes which didn't ovulate over sex months after calving was grouped as anoestrus.

2.2. Total DNA extraction

Blood samples were collected in EDTA-containing vacutainer tubes (kept in ice box) from jugular veins of 40 animals in each group. The genomic DNA was extracted from the leucocytes using Gene JET genomic DNA purification kit following the manufacturer protocol (Fermentas, #K0721). The concentration of total extracted DNA was spectrophotometrically determined at 230 and 260 nm using Nanodrop then stored at -20ºC.

2.3. Polymerase chain reaction

The *CYP19* locus was amplified by PCR using primers (Table 1) designed by Primer 3.0 software based on the published sequences of Indian buffalo (GenBank accession number, EF126034). The PCR was carried out in a reaction volume of 50 µL, containing 4.0 µL DNA template (approximately 100 ng), 10 µl Dream Taq Green PCR master mix 5x (Fermentas, #K1071, European Union), 2.0 µL (10 µmol/L) forward primer, 2.0 µL (10µmol/L) reverse primer, and 32 µL nuclease free water. PCR was performed by employing a PCR program as follows: Initial denaturation

step at 94ºC for 2 min, then tubes were subjected to 35 cycles of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min, followed by a final extension step at 72ºC for 10 min. As a negative control, tubes were prepared with water instead of DNA template. Then PCR products were resolved by electrophoresis on 1% agarose gel in 1X TAE, stained with ethidium bromide and visualized with UV light of Gel Documentation System (Biometra Biomedizinische Analytik, GmbH).

2.4. Single Strand Conformation Polymorphism (SSCP).

The procedure adapted in SSCP was described by kumar et al (2009) with some modifications. The PCR products (5 µl) were mixed with 5 µl of SSCP dye (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue) in 0.2 ml PCR tubes, then incubated at 95 °C for 10 min for denaturation and plunged in ice for 5 min to form single strand conformers then electrophoresed in a 12% polyacrylamide gel (ratio of acrylamide to bis-acrylamide was 39:1 [0.5 gram of bis-acrylamide and 19.5 gram of acrylamide was added to 50 ml distilled water and mixed well in water bath at 37ºC till complete solubility, 5 ml 10% TBE (contained 108g Tris, 55g boric acid +40 ml 0.5M EDTA [37.22g EDTA in 150 ml of distilled water] and up to 1000 ml deionized water and mixed well), 12 ml of 20 % acrylamide to bis-acrylamide mix were added to 8 ml deionized water, 20 µl of TEMED (tetra methylene diamine) and finally 200 µl of 10% Ammonium persulfate was added to at a time and mixed well. The optimal polymerization time was about 4 h. The gel was pre electrophoresed at 160 V for 30 min with 0.5x TBE as electrode buffer. The conformers of PCR products were separated in the gel at 14–16 °C using constant voltage of 160 V for 4 h. The DNA fragments in the gel were detected by 500ml of Ethidium bromide 0.5 µg/ml in 1x TBE for 10-30 minutes on a rocking platform for proper fixing of SSCP conformers in the gel. Then the gel was transferred gently in 500ml of sterile distilled water for de-staining. The fragment patterns were visualized on the UV Trans-illuminator and photographed by gel documentation system (UVDI Major Science, USA). After the polymorphism was detected, the PCR products of different electrophoresis patterns were purified and then sent to be sequenced.

2.5. DNA Sequencing

After getting purified PCR products (clones) with expected sizes , the clone was purified using PCR purification kit following the manufacturer protocol **(**Jena Bioscience # pp-201×s**)** to remove primer dimmers, primers, nucleotides, proteins, salt, agarose, ethidium bromide and other impurities. The PCR products were sequenced in automated sequencer (Applied Biosystem, USA) using *CYP19* primers. The Sequences were analyzed using the Chromas Lite 2.1 program(http://technelysium.com.au/?page_id=13) and the identity of the sequenced PCR product was examined using Blast search against Genbank database of Indian buffalo and cattle (*Bos Taurus*) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).The alignments, annotations and assembly of the sequences were performed using Geneious 4.8.4 software http://www.geneious.com/web/geneious/home.

3. RESULTS

The PCR product with the expected size (446bp) was obtained as shown by 1% agarose gel electrophoresis photo (Fig. 1). Then, the resulting PCR products were purified using PCR purification technique. Subsequently, SSCP was carried out in order to detect the genotypes and to detect any SNPs. No polymorphism was detected in *CYP19* locus as revealed by presence of only

Table 1: Forward and reverse primers sequence for *CYP19* locus, annealing temperatures (Ta), size of PCR amplicon (bp) and its localization in the gene.

Gene	Primers		Ta (°C)	Size (bp)	Localization
	Forward (5-3)	Reverse(5-3)			
CYP19	TCTACGGAACAAGCAC AGGA	GGCACGCTCAGTTTTA AGGA	60	446	I8, E9, I9

I = Intron E = Exon

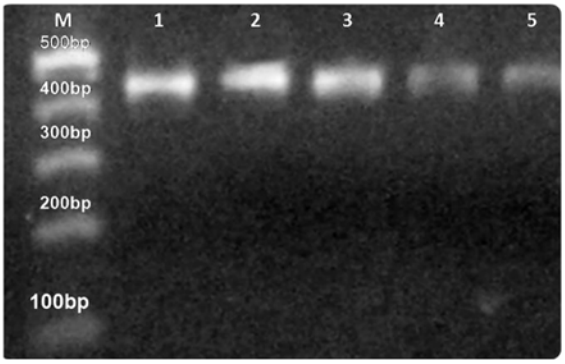

Fig.1. Ethidium bromide stained agarose gel of PCR products representing amplification of *CYP19* locus (lanes1-5) with size of 446 bp in Egyptian buffaloes. M represents 100bp ladder.

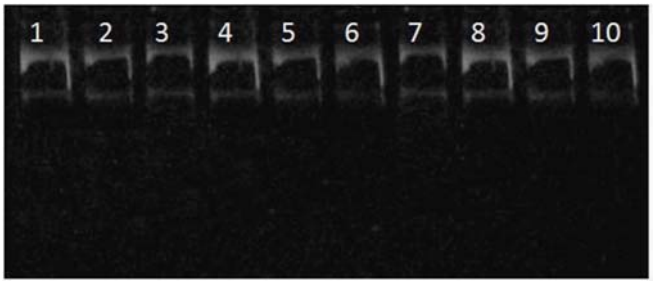

Fig.2. PCR-SSCP patterns of *CYP19* locus in Egyptian buffalo show the genotypes. One SSCP pattern was detected in this locus.

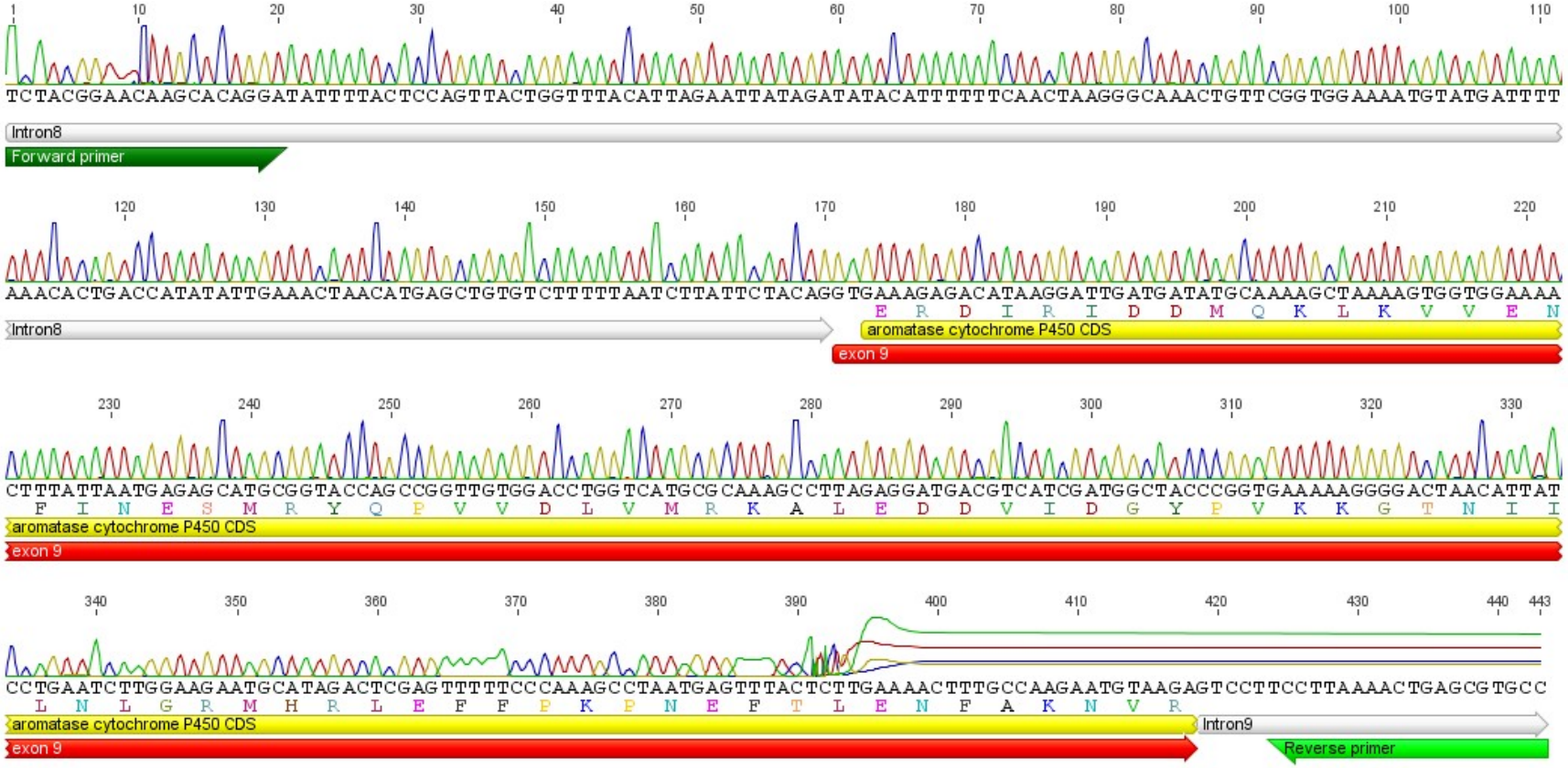

Fig.3. Nucleotide sequences of *CYP19* locus didn't show detected SNP. The corresponding amino acid sequences were shown below nucleotide sequences of exon 9.

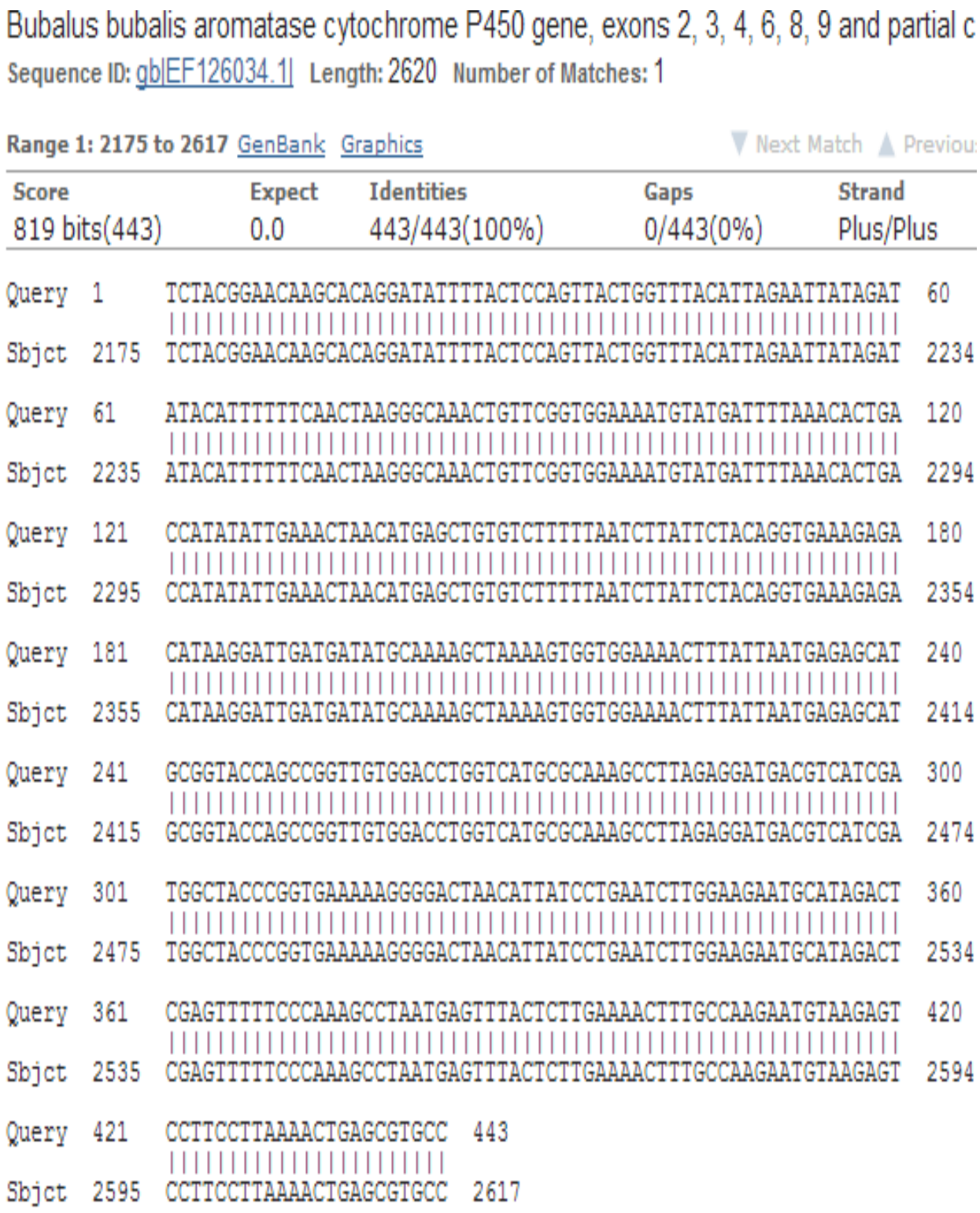

Fig.4. Nucleotide sequences alignment of *CYP19* locus with Indian water buffaloes (EF126034) showed 100% identity.

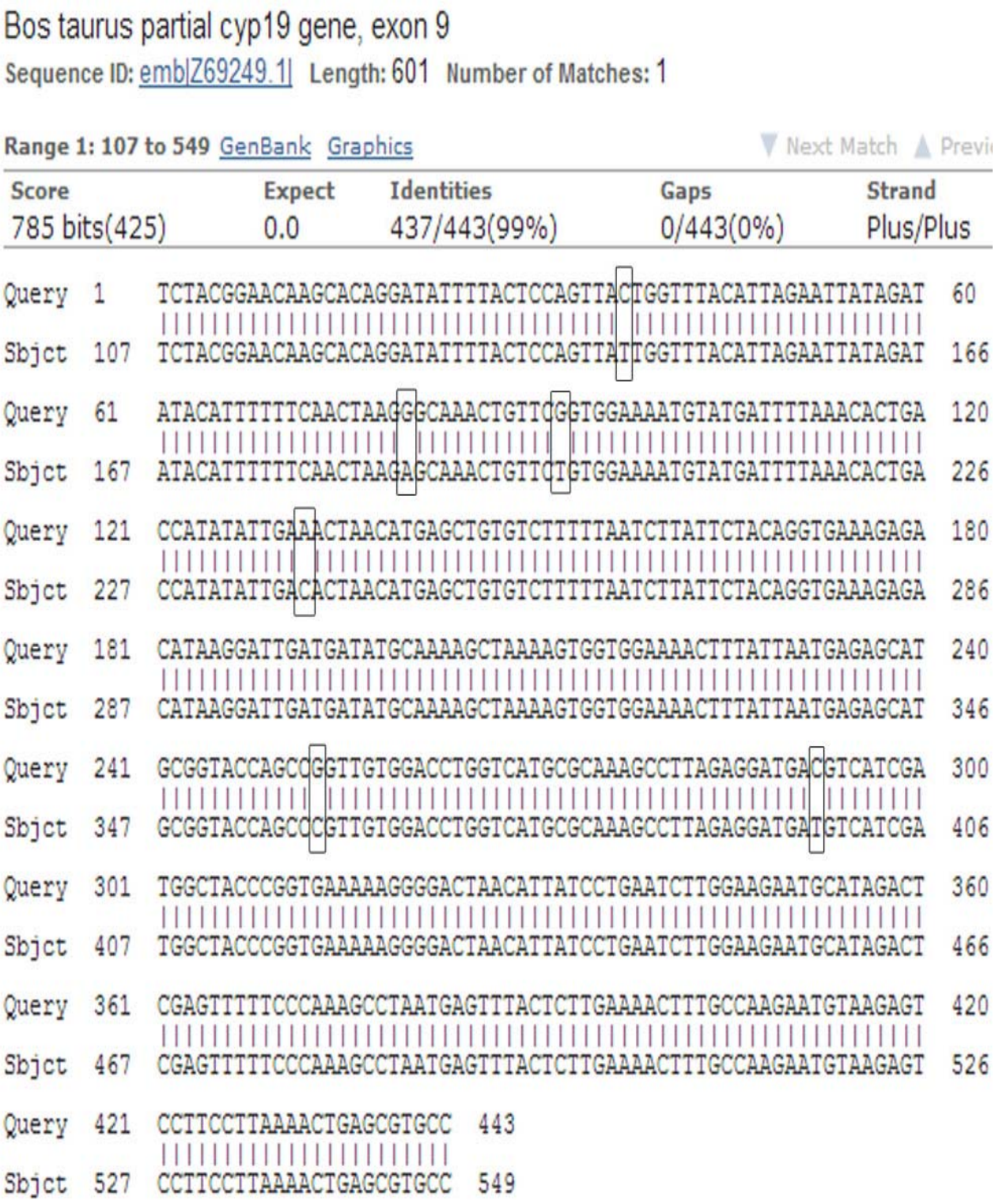

Fig.5. Nucleotide sequences alignment of *CYP19* locus with *Bos Taurus* (Z69249.1) showed 99% identity.

one SSCP banding (monomorphic) pattern (Fig. 2). Sequencing was conducted to verify the results of SSCP. The sequences of *CYP19* locus in Egyptian buffaloes (submitted to GenBank with accession number KF976407) showed no polymorphism (Fig. 3). Nucleotide sequences alignment of *CYP19* locus showed 100% identity with Indian water buffaloes (EF126034) (Fig.4) and 99% identity with *Bos Taurus* (Z69249.1) (Fig.5).

4. DISCUSSION

Identification of genetic markers that can potentially improve reproduction and extension of the animal's productive life seems essential mainly for the breeding practice. Among the potential genetic markers analyzed in the present study is the CYP19 gene which can play a role in predicting anestrum which is the most common single cause of infertility in buffaloes (Ashturkar et al., 1995; Singh and Sahni, 1995), incorporated with the low level of ovarian estrogens (Hafez and Hafez, 2000). The key enzyme in estrogen biosynthesis is cytochrome P450 aromatase, the protein product of CYP19 gene. The present study indicated the presence of an association between CYP19 gene polymorphisms and reproduction of Egyptian buffalo-cows. Kumar et al (2009) found a C82G SNP in coding region of CYP19 exon9 in late matured and true anestrus animals (CG) as compared to GG control animals. Although, this SNP was in the coding region of exon9, the amino acid encoded by both the codons (CCC and CCG) is proline due to the degeneracy of the genetic code (Watson et al., 2004). Thus, the aromatase amino acid sequence will be unaltered, retaining its activity. Therefore, this condition may not be related to anestrum (Kumar et al., 2009). In contrast, we did not find such SNP or any other SNP in exon9 of all examined Egyptian buffaloes. This means that this SNP is not conserved in Bubalus bubalis. Alignment of nucleotide sequences of CYP19 locus in Egyptian buffaloes showed 100% identity with Indian water buffaloes (EF126034) and 99% identity with Bos Taurus (Z69249.1). This result indicates that the sequence of CYP19 is highly conserved between cattle and buffalo. This is a preliminary study that provides the researchers with raw data, which could be used as a basis for further studies to associate other SNPs in CYP19 with fertility in buffalo.

5. REFERENCES

Ashturkar, R.W., Aher, V.D., Bhokre, A.P., 1995. Studies on infertility problems in non-descript buffaloes and cows. Indian Vet. J. , 72: 1050–1052.

Cotton, R.G.H., 1993. Current methods in mutation detection. Mutation Res., 285: 125–144.

Evans, A.C.O., Ireland, J.L.H., Winn, M.E., Longergan, P., Smith, G.W., Coussens, P.M., Ireland, J.J., 2004. Identification of genes involved in apoptosis and dominant follicle development during follicular waves in cattle. Biol. Reprod. , 70: 1475–1484.

Fugita, K., Silver, J., Peden, K., 1992. Changes in both gp120 and gp41 can count for increased growth potential and expanded host range of human immunodeficiency virus type 1. J. of Virol. , 66: 4445–4451.

Ghai, S., Monga, R., Mohanty, T.K., Chauhan, M.S., Singh, D., 2012. Term placenta shows methylation independent down regulation of Cyp19 gene in animals with retained fetal membranes. Res. in Vet. Sci., 92(1): 53-59.

Grumbach, M.M., Auchus, R.J., 1999. Estrogen: consequences and implications of human mutations in synthesis and action. J Clin Endocrinol Metab, 84(12): 4677-94.

Hafez, E.S.E., Hafez, B., 2000. Reproduction in farm animals. seventh ed, Lippincott Williams and Wilkins publications, PP 261–263.

Jędrzejczak, M., Grzesiak, W., Szatkowska, I., Dybus, A., Muszyńska, M., Zaborski, D., 2011. Association between polymorphisms of CYP19, CYP21, and ER1 genes and milk production traits in Black-and-White cattle. Turk. J. Vet. Anim. Sci., 35(1): 41-49.

Kirkpatrick, B.W., 1992. Detection of a three-allele single strand conformation polymorphism (SSCP) in the fourth intron of the bovine growth hormone gene. Animal Genetics, 23(2): 179-180.

Kobylinska, K., 1994. Molecular forms of cytochrome P-450 in rat liver. . Postepy Biochem., 40: 248-253.

Kumar, S.O., Sharma, D., Singh, D., Sharma, M.K., 2009. CYP19 (cytochrome P450 aromatase) gene polymorphism in murrah buffalo heifers of different fertility performance. Res Vet Sci, 86(3): 427-437.

Layman, L.C., 2002. Human gene mutations causing infertility. J.of Medic. Genetics, 39(3): 153-161.

Morohoshi, F., Hayashi, K., Munakata, N., 1991. Molecular analysis of Bacillus subtilis ada mutants deficient in the adaptive response to simple alkylating agents. J Bacteriol, 173(24): 7834-40.

Murray, R.K., Granner, D.K., Mayes, P.A., Rodwell, V.W., 2000. Harper's Biochemistry. Appleton and Lange publications vol.25, 599-603.

Myers, R.M., Maniatis, T., Lerman, L.S., 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol, 155: 501-27.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., Sekiya, T., 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A, 86(8): 2766-70.

Palter, S.F., Tavares, A.B., Hourvitz, A., Veldhuis, J.D., Adashi, E.Y., 2001. Are Estrogens of Import to Primate/Human Ovarian Folliculogenesis? Endocrine Reviews, 22(3): 389-424.

Pravenec, M., Simonet, L., Kren, V., St Lezin, E., Levan, G., Szpirer, J., Szpirer, C., Kurtz, T., 1992. Assignment of rat linkage group V to chromosome 19 by single-strand conformation polymorphism analysis of somatic cell hybrids. Genomics, 12(2): 350-6.

Raghavan, V.S., 2006. Single nucleotide polymorphisms of diacyl glycerol acyl transferase 1 (DGAT 1) gene in indigenous cattle and buffaloes. Ph.D. thesis submitted to National Dairy Research Institute, Karnal, India

Sheffield, V.C., Cox, D.R., Lerman, L.S., Myers, R.M., 1989. Attachment of a 40-basepair G rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc. Nat1 Acad. Sci. USA 86: 232–236.

Singh, B., Sahni, K.L., 1995. Causes of infertility in cattle and buffaloes under field conditions. Indian J of Anim Sci, 65: 1119–1121.

Watson, J.D., Baker, T.A., Bell, S.P., Gann, A., Levine, M., Losick, R., 2004. Molecular Biology of the Gene. fifth ed. , 358 pp.

Youil, R., Kemper, B.W., Cotton, R.G., 1995. Screening for mutations by enzyme mismatch cleavage with T4 endonuclease VII. Proc Natl Acad Sci U S A, 92(1): 87-91.

عزل وتسلسل الإكسون التاسع لجين *CYP19* في الجاموس المصري

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الملخص العربي

تم إجراء هذه الدراسة على عدد مائة وثمانين من الجاموس المصري النقي. وقد تم الحصول على هذه الحيوانات من محطه النطاف بمحلة موسى بكفر الشيخ. وقد تم تحديد الطفرة الموجودة فى الاكسون التاسع جين *CYP19* والتي لم تؤد الى أي تغيير في النيوكليوتيدات فى هذا الموقع من الجين. لقد تمت دراسات عديدة علي الارتباط بين الجين المتعلق بالخصوبة *CYP19* والتناسل في الأبقار ولكن أجريت فقط دراسات ضئيلة علي هذا الجين في الجاموس، لذلك أجريت هذه الدراسة لعزل جزء من جين *CYP19* تحتوي الإكسون التاسع والتعرف علي أي طفرات مرتبطة بعدم الشياع في إناث الجاموس المصري. لتحقيق ذلك تم عزل جزء من جين *CYP19* باستخدام طريقة التفاعل البلمري المتسلسل ثم تم الكشف عن الطفرات بالتسلسل المباشر وطريقة SSCP . تم عزل جزء من جين ال *CYP19* يصل حجمه إلي 446 زوج من القواعد النيتروجينية ولكن لم يتم الكشف عن طفرات في هذا الجزء ولقد تحقق ذلك بظهور طرز أحادي الشكل في الحيوانات الطبيعية والتي لم يتم فيها الشياع. للتأكيد على هذه النتائج تم عمل تسلسل للقواعد النيتروجينية. مقارنة تتابع النيوكليوتيدات في هذا الموقع من الجين أثبت تماثل بنسبة 100 % مع الجاموس الهندي و99% مع الأبقار. هذه النتائج تدل على أن التسلسل لجين *CYP19* عالي التشابه بين الأبقار والجاموس. هذه دراسة مبدئية تمد الباحثين بالبيانات الخام والتي يمكن أن تستخدم كقاعدة لدراسات إضافية لربط الطفرات في نيوكليوتيدة أحادية في جين *CYP19* بالخصوبة في الجاموس.

(مجلة بنها للعلوم الطبية البيطرية: عدد 26(1):161-170, مارس 2014)