MOLECULAR CHARACTERIZATION OF CYTOCHROME P450 AROMATASE (*CYP19*) GENE IN EGYPTIAN RIVER BUFFALOES

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Cytochrome P450 aromatase - as product of *CYP19* gene - catalyzes the last step in steroidogenesis which convert the androgens (16-α-hydroxytestosterone, testosterone and androstenedione) to estrogens (17*β*, 16*α*-estriol, 17-β-estradiol and estrone). This reaction includes 3 steps which employ 1 NADPH and 1 O_2 for each step. The first 2 steps include C19 (methyl group) hydroxylation to 19-hydroxy and 19-oxo compounds followed with the last step that remove C19 as formic acid and the steroid A ring aromatization (Di Nardo and Gilardi, 2013).

Cytochrome P450 aromatase is a member in cytochrome P450 superfamily which has a main role in estrogens biosynthesis. Cytochrome P450 is a hemi containing enzyme which was found in many vertebrates and expresses mainly in the gonads of both sexes in addition to other tissues like brain and placenta (Blakemore and Naftolin, 2016). The cattle *CYP19* gene (*NCBI* Gene ID: 281740) has 11 exons. *CYP19* gene was mapped in river buffalo chromosome 11 in the locus 11q26 (Iannuzzi *et al.*, 2001).

CYP19 gene polymorphism (A1044G) which located in the distal promoter P1.1 (that regulate CYP19 transcription in placenta as the main source for P450 aromatase expression) by PCR-RFLP method using the restriction enzyme *PvuII* (RFLP-*PvuII*) was studied. 472 Polish Holstein-Friesian cows were screened and found that the calving-to-conception interval in the first and third lactation in addition to the calving interval in the third lactation were shorter in AG genotype than AA genotype (Szatkowska *et al.*, 2011).

The effect of CYP19 SNPs on reproduction using RFLP-*PvuII* in 909 Polish Holstein-Friesian cows was investigated. The authors found that the cows with GG genotype had earlier age at first calving, shorter first calving interval and longer second calving interval compared to AA and AG genotypes (Wierzbicki *et al.*, 2014).

Although, the buffalo (*Bubalus bubalis*) can adapt to harsh environments and poor quality forage, the buffalo has been traditionally regarded as a poor breeder due to its low fertility in the majority of conditions under which they are raised. The reproductive problems could be manifested mainly as late maturity, long postpartum anoestrous intervals, poor expression of oestrus, poor conception rates and long calving intervals (Perera, 2011). Depending on the high similarity degree between the buffaloes and cattle genomes (Tantia *et al.*, 2011), the transferability of cow fertility marker in CYP19 promoter to Egyptian buffalo was investigated as a possible tool to select the females with the high reproductive performance. Therefore, a PCR-RFLP assay and DNA sequence analysis were performed to examine the genotype and polymorphism in the distal promoter P1.1 of *CYP19* gene in 81 females of the Egyptian river buffalo.

MATERIALS AND METHODS

This investigation was carried out in the Cell Biology Department, National Research Centre, Giza, Egypt and Genetics Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

1. Blood collection and DNA extraction

A total of 81 blood samples were collected from healthy and unrelated Egyptian river buffalo females. Thirty blood samples were collected from animals of the farm of Cattle Information System/Egypt (CISE) and 51 blood samples were collected from animals of the Agricultural Experiments Station (AES), Faculty of Agriculture, Cairo University, Giza, Egypt. A ten ml of blood samples were collected in sterile 15 ml tubes containing a 0.5 ml of 0.5 M EDTA solution (PH 8.0). Genomic DNA was extracted from the whole blood samples according to the method described by Miller *et al.* (1988) with minor modifications. The DNA concentration was determined using Nano Drop1000 thermo scientific spectrophotometer and then diluted to the working final concentration of a 50 ng/µl.

2. Animals genotyping and CYP19 amplicon sequencing

A DNA fragment which is a part of CYP19-distal promoter P1.1 was amplified using CYP19-F (5` CAAGGGCCTCATATGGTTCA 3`) and CYP19-R (5` CCAGATCAGAACCACCTTTGT 3`) primer pair which were designed using Primer3 online software (http://primer3.ut.ee/; Yazdani *et al.*, 2010) based on the sequence of GenBank record NW_005785802.1. Polymerase chain reaction (PCR) were performed in a 25 µl of reaction volume, which included a 50 ng of genomic DNA, a 50 ng of each primer, a 200 µM of each dNTP, a 2.5 µl of 10X PCR buffer and a 0·5 U of Taq DNA polymerase (Promega, Madison, WI, USA). Amplification was carried out in a thermocycler which programmed as follows: an initial start separation cycle at 94°C for 2 min, 35 cycles including a

denaturation step at 94°C for 30 sec, an annealing step at 60°C for 30 sec, a polymerization step at 72°C for 45 sec and a final extension cycle at 72°C for 10 minute. The PCR products were screened by electrophoresis on a 2% agarose gel in a 0.5X of TBE buffer which stained with ethidium bromide and visualized with an UV transilluminator.

PCR product was digested by *PvuII* restriction enzyme (Thermo Scientific, Dreieich, Germany) at 37°C for 30 min according to the procedure provided by the manufacturer. Digested products were separated by electrophoresis on a 2% agarose gel in a 0.5X of TBE buffer which stained with ethidium bromide and visualized with an UV transilluminator.

3. *Purification and sequencing of PCR products*

The PCR products of selected samples were purified using GeneJET Gel Elution Kit (Thermo Scientific, Dreieich, Germany). The purified PCR products were sequenced using an automated sequencing service (Macrogen, Korea).

4. *Data analysis*

Alignment of the sequences were performed with GenBank database BLAST tool (Yazdani and Ahmed, 2010) to identify the homology between CYP19 target promoter and the orthologues sequences on the GeneBank database. AliBaba2 software (Grabe, 2002) was used to predict the potential transcription factor binding sites within the regulatory sequences based on TRANSFAC database.

RESULTS AND DISCUSSION

1. *PCR-RFLP pattern of CYP19 gene*

Amplification of a fragment from the CYP19 promoter in the Egyptian river buffalo blood samples produced a 419 bp PCR product (Fig. 1-A) which digested with *PvuII* restriction enzyme, and the pattern of PCR-RFLP was analyzed by electrophoresis (Fig. 1-B). The PCR-RFLP pattern showed that A allele was not detected as homo- or heterozygote genotype and all the animals represented the GG genotype in the form of 187 and 232 bp fragments. Sequencing result of the PCR products provided that the DNA sequence had a guanidine nucleotide located in the SNP target position which creates *PvuII* restriction site (Fig. 1-C). In the opposite to these results in which A allele was not detected, the A allele frequency in the Polish Holstein-Friesian cows was ranged from 92.27 to 93.2% (Szatkowska *et al.*, 2011; Wierzbicki *et al.*, 2014).

2. *Sequencing of amplified region from CYP19 gene and in silico sequence analysis*

The nucleotide sequence of amplified region of the distal P1.1 promoter of the buffalo *CYP19* gene showed the size of 419 bp (GenBank accession numbers MF490278 and MF490279; Fig. 2-A). The 3` end of this fragment was located at 21213 bp upstream the translation initia-

tion site of *CYP19* gene (NCBI Reference Sequence: NW_005785802.1). G to A transition SNP (G197A) was detected by sequencing (Fig. 2-A and B) and the Egyptian river buffalo had both of the G and A alleles while the other submitted similar sequences in the GenBank database (NW_005785802.1, KT596715.1 and KT596714.1) had the A allele only.

The target regulatory region was scanned to investigate the possible transcription factor binding sites (TFBSs) and 17 known TFBSs span along the sequence was predicted (Table 1). The discovered G197A SNP was localized out of the 17 known TFBSs in the wild type sequence and did not created any new TFBS in the muted sequence.

3. Comparison of the amplified region in CYP19 gene among some species

Blasting results of the amplified region from CYP19 promoter in the Egyptian river buffalo against the GenBank records belong to different organisms showed that there were no significant similarity values among the amplified segment of CYP19 promoter in buffalo, human and mouse sequences. In the contrary, cattle represented the highest identity score with buffalo target sequence (97%) compared to sheep and goat which represented 95% and 93% sequences identity, respectively (Fig. 3).

SUMMARY

Cytochrome P450 aromatase (*CYP19*) gene catalyzes the last step in the steroidogenesis which convert the androgens to estrogens. Therefore, the objective of this study was to investigate the polymorphism in *CYP19* gene and its potential effects in female buffaloes fertility. The DNA was extracted from the blood samples of 81 Egyptian river buffalo females and a 419 bp fragment contained a part of CYP19- distal promoter P1.1 was amplified by PCR which subsequently treated with *PvuII* restriction enzyme. The PCR-RFLP pattern showed that all the animals had a fixed GG genotype and A allele was not detected. Sequencing of the amplified fragment (GenBank accession No. MF490278 and MF490279) followed by sequence alignment with the GenBank database revealed that the homology among the Egyptian river buffalo target sequence and its orthologues sequences in cattle, sheep and goat was 97, 95 and 93%, respectively. G to A transition SNP (G197A) was detected among individuals representing the Egyptian river buffalo by sequencing. G allele was detected only in the Egyptian buffalo and not in the other buffalo records in the GenBank. Seventeen transcription factor binding sites (TFBSs) span along the sequence were predicted.

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Table (1): The predicted transcription factor binding sites within amplified region of the Egyptian river buffalo CYP19 promoter.

Factor	Start	Stop	Factor	Start	Stop
1-Oct	16	25	COUP	325	334
CPE_binding_pro	219	228	Kr	330	339
TEC1	226	235	CREB	333	342
C/EBPalpha	240	249	NF-kappaB	351	360
Sp1	254	263	NF-kappaB1	352	361
11-Oct	268	277	NF-kappaB	375	384
PU.1	294	303	C/EBPalpha	376	386
GATA-1	295	304	GATA-1	405	414
Sp1	312	324			

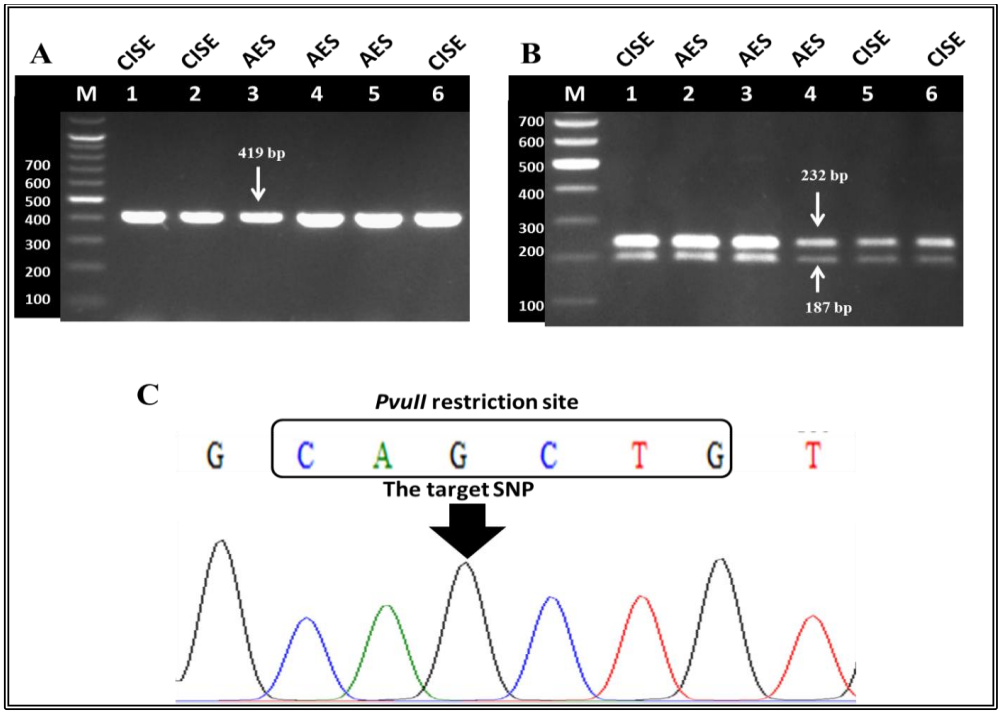


Fig. (1): The PCR product, PCR-RFLP pattern and sequencing result of the amplified CYP19-promotor fragment. A) The PCR product of CYP19 fragment. B) The PCR-RFLP pattern of the *CYP19* gene amplicon. C) A part of CYP19 fragment sequencing chromatogram. CISE: Cattle Information System/Egypt. AES: Agricultural Experiments Station. M: 100 bp molecular marker.

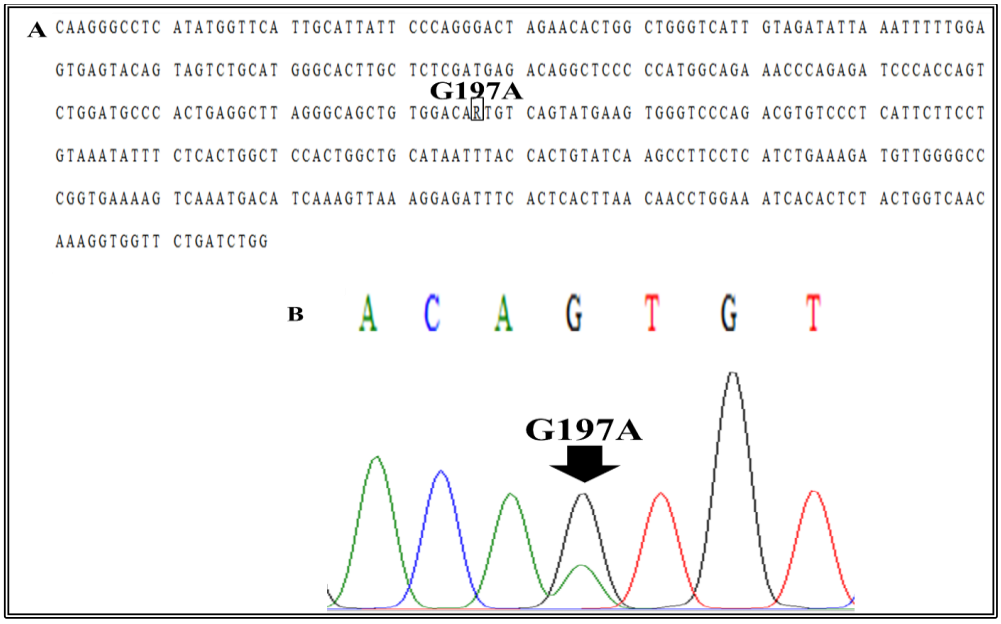
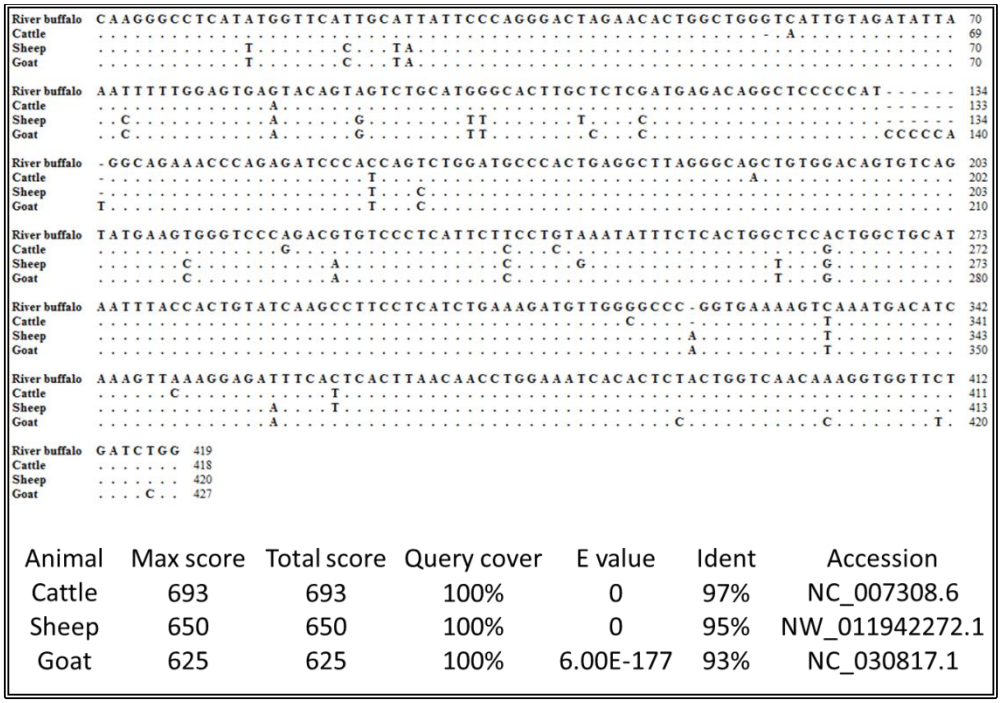


Fig. (2): The nucleotide sequence and the detected SNP in the Egyptian river buffalo CYP19 promoter amplified fragment.



Animal	Max score	Total score	Query cover	E value	Ident	Accession
Cattle	693	693	100%	0	97%	NC_007308.6
Sheep	650	650	100%	0	95%	NW_011942272.1
Goat	625	625	100%	6.00E-177	93%	NC_030817.1

Fig. (3): The nucleotides sequence homology among the amplified region of CYP19 promoter in Egyptian river buffalo and some species.