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ARTICLE

Genetic polymorphism of two genes associated with carcass trait in Egyptian buffaloes

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Abstract Leptin and μ-calpain have been considered as two candidate genes for carcass performance and meat quality traits in the farm animals. The micromolar calcium-activated neutral protease (*CAPN1*) gene encodes μ-calpain that degrades myofibril proteins under the postmortem conditions which appears to be the primary enzyme in the postmortem tenderization process. Leptin is the hormone product of the obese (*LEP*) gene. The role of leptin as a lipostatic signal regulating whole-body energy metabolism makes it one of the best physiological markers of body weight, food intake, reproduction and immune system functions.

Genomic DNA extracted from 100 healthy buffaloes was amplified using primers that were designed from the cattle *CAPN1* and *LEP* gene sequences. The amplified fragments of *CAPN1* obtained from all tested buffalo DNA at 670-bp were digested with *Fok*I endonuclease. The result showed that all tested buffaloes are genotyped as CC for *CAPN1*. For *LEP* gene, the amplified fragments obtained from all tested buffalo DNA at 400-bp were digested with *Sau*3AI endonuclease. All buffalo animals investigated in the present study are genotyped as AA for *LEP* gene.

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1. Introduction

There is a considerable interest in the application of molecular genetic technologies in the form of specific DNA markers that are associated with various QTL to promote more efficient and relatively easy selection and breeding of farm animals with an advantage for the inheritable traits of growth rate, body weight, carcass merit, feed intake as well as milk yield and composition [48]. Leptin and μ-calpain have been considered as two candidate genes for carcass performance and meat quality traits in the farm animals.

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Calpain is a ubiquitous cytoplasmic cysteine protease and its activity is dependent on the concentration of calcium [47]. In cattle there are two genes of calpain that have been identified: *CAPN1* (μ-calpain) and *CAPN2* (m-calpain) [50]. Bovine *CAPN1* has been mapped to the telomeric end of BTA29 [46]. Recently, a QTL for meat tenderness was found to be segregating in this region of BTA29 [6,35]. The evaluation of *CAPN1* as a candidate gene for meat tenderness was supported by Meat Animal Research Center, MARC [1].

The *CAPN1* gene encodes a μ-calpain that degrades myofibril proteins under postmortem conditions and appears to be the primary enzyme in the postmortem tenderization process [26–28,3]. Regulation of μ-calpain activity has been correlated with variation in meat tenderness in cattle [20].

In *CAPN1* gene, more than 100 single nucleotide polymorphisms (SNPs) have been identified in cattle [46,41,24]. Among them, four polymorphisms, two non-synonymous SNPs (G316A and V530I) and two intronic SNPs (C4685T and C4751T) have been found to have significant effects on meat tenderness [41,42,52,36,44,51].

Leptin is the hormone product of the obese (leptin) gene that has been mapped to bovine chromosome (BTA) 4 [49,43]. It is a 16-kDa protein that is synthesized by white adipose tissue [55,22]. The role of leptin as a lipostatic signal in the regulation of whole-body energy metabolism makes it one of the best physiological markers of the body weight, food intake, energy expenditure [21,53,2], reproduction [11,18] and certain immune system functions [33].

Leptin has been considered as a candidate gene for performance, carcass and meat quality traits in beef cattle [17,4,29].

Genetic polymorphisms in the coding regions of the leptin gene in cattle have been associated with serum leptin concentration [31], feed intake [30,39], milk yield [32,5] and body fat [4,37].

With the aim of assessing the presence of sequence polymorphisms in the buffalo leptin gene, Orrù et al. [40] sequenced the entire coding region and part of the introns on a panel of Italian river buffaloes. They identified a new set of single nucleotide polymorphism (SNP) that is useful for the association studies between the sequence polymorphisms and the traits like the milk yield, feed intake, fat content and carcass and meat quality. To the best of our knowledge, this is the first published data on the genetic polymorphism of *CAPN1* gene in river buffalo.

In the present study, the PCR–RFLP technique was used to detect the genetic polymorphism within intron 14 of *CAPN*1 and intron 2 of *LEP* genes. These two introns exhibited many polymorphisms in cattle. Due to gene mapping conservation and nucleotide sequence homology between cattle and buffalo, we focused on the detection of genetic polymorphism within these two regions in Egyptian buffaloes for the first time.

2. Materials and methods

2.1. Animals

Water buffalo belongs to genus *Bubalus*, species *bubalis*. Water buffalo includes both river buffalo (*Bubalus bubalis bubalis*, $2n = 50$) and swamp buffalo (*Bubalus bubalis carabanesis*, $2n = 48$), the Egyptian buffaloes are of the river type. Egyptian river buffaloes have been classified according to minor phenotypic differences and their geographical locations, such as Beheiri, Menoufi and Balady which are found mainly in Northern-Egypt and Saiedy found in Southern-Egypt [12]. These differences are not well defined to be relied on as taxonomic classification. Few studies [15,14] have investigated the genetic variations in Egyptian buffaloes and concluded that all Egyptian river buffaloes belong to one population (*B. bubalis bubalis*).

2.2. Genomic DNA extraction

Genomic DNA was extracted from the whole blood of 100 unrelated healthy female buffaloes according to the method described by John et al. [23] with minor modifications. Briefly, 10 ml of blood taken on EDTA were mixed with 25 ml of cold sucrose-triton and double distilled water. The tubes were placed on ice for 10 min and mixed by inversion several times. After centrifugation, the pellet was re-suspended by nucleic lysis buffer using plastic pipette. The content was mixed with 108 μl of 20% SDS and 150 μl of proteinase. The tubes were placed in a water bath at 37 °C overnight.

After 24 h of incubation, the tube contents were transferred to a 15-ml polypropylene tube and 1 ml of saturated NaCl was added and shaken vigorously for 15 s. After centrifuging at 3500 rpm for 15 min at 4 °C, the supernatant was transferred to a clean 15-ml polypropylene tube and mixed with absolute ethanol. The tubes were agitated gently to mix the liquids and a fluffy white ball of DNA was formed. The precipitated DNA was picked up using the heat sealed pasture pipette, then washed twice in 70% ethanol and exposed to air to dry completely.

The DNA was dissolved in 200 μl TE buffer in 1.5-ml Microfuge tube and kept overnight in an incubator at 37 °C. DNA concentration was determined and diluted to the working concentration of 50 ng/μl, which is suitable for polymerase chain reaction.

2.3. Polymerase chain reaction (PCR)

The primers used in this study were designed from cattle *CAPN1* and *LEP* gene sequences. The 670-bp DNA fragment (exons 14–18) of *CAPN1* gene [24] and the 400-bp DNA fragment (intron 2) of *LEP* gene [30] were amplified using buffalo DNA.

CAPNI:
Primer forward: TTC AGG CCA ATC TCC CCG ACG
Primer reverse: GAT GTT GAA CTC CAC CAG GCC CAG

LEP:
Primer forward: TGG AGT GGC TTG TTA TTT TCT TCT
Primer reverse: GTC CCC GCT TCT GGC TAC CTA ACT

A PCR cocktail consists of 1.0 μM forward and reverse primers and 0.2 mM dNTPs, 10 mM Tris (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$ and 1.25 U of Taq polymerase. The PCR cocktail (~23 μl) was added to 100 ng of buffalo DNA.

The reaction was run in MJ research PTC-100 Thermocycler for 1 min at 94 °C (denaturation), 2 min at 62 °C for *CAPN1* or 55 °C for *LEP* (annealing) and 2 min at 72 °C (extension) for 30 cycles. The PCR reaction products were electrophoresed on 1.5% agarose/1× TBE gel stained with ethidium bromide to test the amplification success.

2.4. RFLP and agarose gel electrophoresis

Twenty microliters of PCR products were digested with 10 units of restriction enzymes *Fok*I (for *CAPN1*) or *Sau*3AI (for *LEP)* in a final reaction volume 25 µl. The reaction mixture was incubated at 37 °C in water bath for 5 h. After restriction digestion, the restricted fragments were analyzed by electrophoresis on 2.5% agarose/1× TBE gel stained with ethidium bromide. The 100-bp ladder was used as a molecular size marker. The bands were visualized under UV light and the gels were photographed using Mp4 plus Polaroid Camera.

3. Results and discussion

Several QTL for meat performance and carcass trait production in farm animals have been identified. A number of potential candidate genes have been identified and selected for analyses based on a known relationship with physiological or biochemical processes and production traits. It is seldom reported that one particular polymorphism in a candidate gene would influence several traits of economic importance in livestock at the same time. Leptin and µ-calpain have been considered as two candidate genes for carcass performance and meat quality traits in farm animals.

Calpain is a ubiquitous cytoplasmic cysteine protease and its activity is dependent on the concentration of calcium [47]. Two isoforms of calpain have been identified; µ-calpain (CAPN1) that requires calcium at micromolar concentrations for activity and m-calpain (CAPN2), for which calcium at millimolar concentrations is needed [50]. Both calpains have been shown to degrade most of the myofibrillar protein excluding actin and myosin [3]. Regulation of µ-calpain activity has been correlated with variation in meat tenderness [20]. Bovine *CAPN1* and *CAPN2* genes have been mapped to chromosome 29 [46].

In this study by using PCR and RFLP techniques, we aimed to detect the genetic polymorphism within intron 14 of buffalo *CAPN1*. The amplified fragments obtained from all the tested buffalo DNA (100 animals) were at 670-bp (Fig. 1).

These PCR amplified fragments were digested with *Fok*I endonuclease. Depending on the alteration of the presence of C/T at position 4685 (within intron 14), it is easy to differentiate between 3 different genotypes: TT with undigested one fragment at 670-bp, CC with two digested fragments at 530-bp and 140-bp and TC with three fragments at 670-bp, 530-bp and 140-bp. All buffalo animals investigated in this study are genotyped as CC where all the tested buffalo DNA amplified fragments were digested with *Fok*I endonuclease and gave two digested fragments at 530- and 140-bp. The restriction site is resulted due to the presence of C base at position 4685 in all animals and the absence of T base in this position within intron 14 (Fig. 2).

Thirty-eight SNPs in bovine *CAPN1* were identified in two sires [41]. They identified six SNPs in introns being two substitutions T/C (intron 7), one substitution C/T in intron 8 and three mutations: T/C, C/T and G/A (intron 18). Two SNPs in exon 6 were synonymous substitutions: G/A and T/C.

The two SNPs were reported by Page et al. [41,42] in bovine *CAPN1* gene representing exon 9 (A/G) and exon 14 (G/C) predicted amino acid changes: substitution in exon 9 (Gly^{316}/Ala^{316}) and exon 14 (Val^{530}/Ile^{530}). Analysis of genotypes and shear force values in the two sires revealed a difference between *CAPN1* alleles in which the allele encoding isoleucine at position 530 and glycine at position 316 associated with decreased meat tenderness (increased shear force values) relative to the allele encoding valine at position 530 and alanine at position 316 ($p < 0.05$).

The genetic analysis of these both SNPs at 316 and 530 positions was done in two bovine populations by Page et al. [42] and indicated an association of genotype and phenotype for both markers. Animals homozygous for the C allele at marker 316 had lower shear force ($p = 0.02$) than animals of CG or GG genotype, whereas the animals with homozygous GG genotype at marker 530 had lower shear force ($p = 0.04$) than animals of AG or AA genotype.

Juszczuk-Kubiak et al. [24] reported the appearance of three SSCP patterns – within intron 14 of bovine *CAPN1* – in 141 tested animals belonging to seven cattle breeds. These three SSCP patterns were confirmed by RFLP and gave three different genotypes, CC (35%), CT (35%) and TT (30%) due to the transition of C $\rightarrow$ T at position 4685 in tested animals which showed T allele. The allele frequency was 0.62 and 0.38 for C and T allele, respectively. The authors reported that

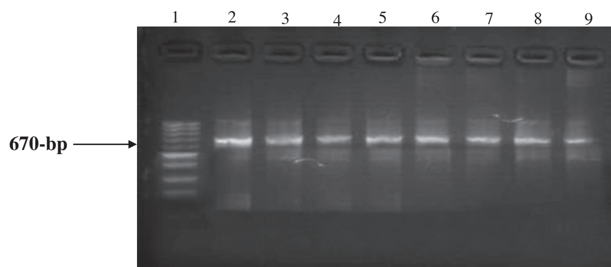

Figure 1 Ethidium bromide-stained gel of PCR products representing amplification of *CAPN1* gene in Egyptian buffaloes. Lane 1: 100-bp ladder marker. Lanes 2–9: 670-bp PCR products amplified from Egyptian buffalo DNA.

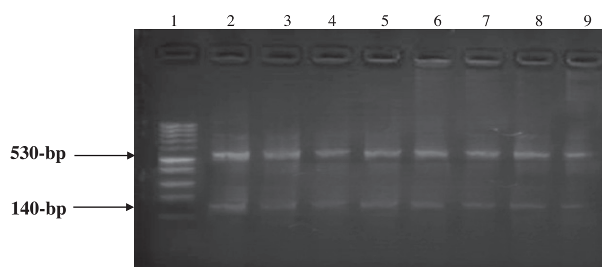

Figure 2 The electrophoretic pattern obtained after digestion of PCR amplified buffalo *CAPN1* with *Fok*I restriction enzyme. Lane 1: 100-bp ladder marker. Lanes 2–9: Homozygous CC genotypes showed two restricted fragments at 530- and 140-bp.

the TT genotype was significantly associated with a higher lean share in valuable cuts; the advantage over the CC genotype was 4.5% ($p \leqslant 0.01$).

Casas et al. [7] assessed the association of four SNPs in the bovine *CAPN1* gene with meat trait in Brahman population. The CAPN530 marker was uninformative in this population, the G allele seems to be fixed or at high frequency. The marker CAPN316 was associated ($p < 0.05$) with sensory panel tenderness score. The three markers CAPN316, CAPN4753 and CAPN5331 were associated with hump height ($p < 0.02$). Another new SNP marker in this population was reported by White et al. [52]. This marker, CAPN4751, was associated with shear force on postmortem d 7 ($p < 0.01$), 14 ($p = 0.015$) and 21 ($p < 0.001$) in this population demonstrating that genetic variation important for tenderness segregates in cattle at or near *CAPN1*.

By direct DNA sequencing of 24 unrelated Korean cattle, Cheong et al. [9] identified 39 polymorphisms in Korean cattle *CAPN1* gene within exons and their flanking regions. Among them, 12 common polymorphic sites were selected for genotyping in the beef cattle ($n = 421$). Associations of *CAPN1* polymorphisms with cold carcass weight (CW) and marbling score (MS) were analyzed. One polymorphism (479C → T) showed significant associations with MS. The T allele revealed an additive effect on MS, i.e., the lowest MS was found in T/T (MS = 0.94), intermediate in C/T (MS = 1.56) and the highest in C/C (MS = 2.34) ($p = 0.02$).

Leptin is the hormone product of the obese (*LEP*) gene [55] which is located on bovine chromosome 4 (BTA 4) [49]. It is a 16-kDa protein that synthesized by white adipose tissue [30]. The role of leptin as a lipostatic signal regulating whole-body energy metabolism makes it one of the best physiological markers of body weight, food intake, energy expenditure [21,53,2,16,13,54,19], reproduction [11,18] and certain immune system functions [33].

Polymorphisms have been reported in the coding regions of the *LEP* gene that show considerable associations with feed intake [30,29,39], carcass merit [4,37,25], serum leptin concentration [31] and milk quantity and quality [5,32,34,8] in cattle.

In the present study by using PCR, the genomic buffalo DNA was amplified using oligonucleotide primers that were designed from the cattle *LEP* gene sequence. The amplified fragments obtained from all tested buffalo DNA (100 animals) were at 400-bp (Fig. 3).

The PCR amplified fragments were digested with *Sau*3AI endonuclease to detect the genetic polymorphism within intron 2 of buffalo *LEP* gene. With this technique, it is easy to differentiate between 3 different genotypes: AA with one undigested fragment at 400-bp, BB with two digested fragments at 300- and 100-bp and AB with three fragments at 400-, 300- and 100-bp. All buffalo animals investigated in the present study are genotyped as AA where all the tested buffalo DNA amplified fragments were digested with *Sau*3AI endonuclease and gave one undigested fragment at 400-bp (Fig. 4).

The presence of A allele in high frequency is also reported in cattle by Liefers et al. [30] and Madeja et al. [34]. By using the same primer and *Sau*3AI enzyme which were used in the present study, Liefers et al. [30] genotyped 613 Holstein Friesian heifers and found that the genotyping frequencies were 0.813, 0.185 and 0.002 for AA, AB and BB, respectively. Also, Madeja et al. [34] genotyped 117 Polish Black- and White-bulls and reported the frequencies of alleles as 0.86, 0.11 and 0.03 for A, B and C alleles, respectively.

Liefers et al. [30] studied the restriction fragment length polymorphism located in intron 2 of the leptin gene in cattle. The authors showed significant differences between genotypes AA and AB (BB genotype was excluded from the analysis because it appeared only in one animal) for milk yield, protein yield, feed intake and lactose yield. The difference of milk yield increased from 1.23 to 1.32 kg/d. Significance levels for mean live weight and live weight at week 15 were between 0.05 and 0.10. The difference for live weight at week 15 between the two genotypes was 10.8 kg ($p = 0.077$) and for mean live weight over the first 15 weeks of lactation, the difference was 9.1 kg ($p = 0.097$). The genotype AB tended to show the higher body weight in comparison with genotype AA.

Allelic variation (cytosine C to thymine T transition that results in an Arg25Cys) in the *LEP* gene has been associated with higher leptin mRNA levels in adipose tissue and increased fat deposition in mature beef cattle [25]. Buchanan et al. [5] reported that this genetic variant is also present in dairy breeds. Body fat reserves play an important role in sustaining high milk production in early lactation when the energy intake is limited. Animals homozygous for the T allele produced more milk (1.5 kg/d vs. CC animals) and had higher somatic cell count linear scores without significantly affecting milk fat or protein percent over the entire lactation. The increase in milk yield is most prominent in the first 100 days of lactation (2.44 kg/d) and declining to 1.74 kg/d between 101 and 200 days in lactation. These results indicated that the leptin TT genotype is associated with increased milk and protein yield without changing yield of the fat of the milk.

Nkrumah et al. [38] reported an association between SNP in the 5 untranslated promoter region of the bovine leptin gene

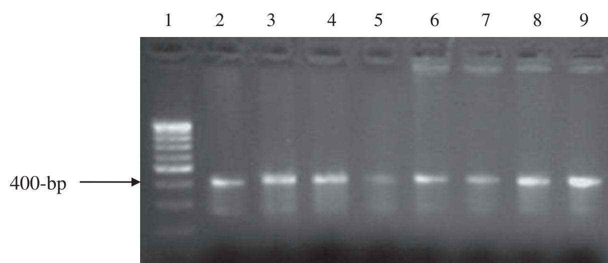

Figure 3 Ethidium bromide-stained gel of PCR products representing amplification of *LEP* gene in Egyptian buffaloes. Lane 1: 100-bp ladder marker. Lanes 2–9: 400-bp PCR products amplified from Egyptian buffalo DNA.

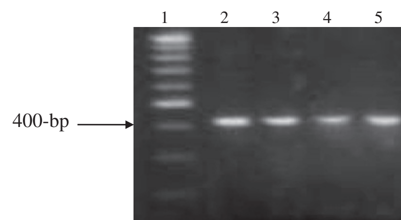

Figure 4 The electrophoretic pattern obtained after digestion of PCR amplified buffalo *LEP* products with *Sau*3AI restriction. Lane 1: 100-bp ladder marker. Lanes 2–5: Homozygous AA genotype showed one undigested fragment at 400-bp.

with serum leptin concentration, growth, body weight, feed intake, feeding behavior and carcass merit in cattle. The authors detected that animals with the TT genotype of a less frequent cytosine/thymine (C/T) substitution detected at position 528 in the bovine leptin promoter showed 48% and 39% increases in serum leptin concentration ($p < 0.001$), 39% and 31% increases in backfat thickness ($p < 0.001$) and 13% and 9% increases in marbling score ($p = 0.01$) compared with CC or CT genotypes, respectively. Animals with the TT genotype also showed significantly higher feed intake ($p < 0.001$), growth rate and metabolic body weight ($p < 0.05$) and live weight at slaughter ($p < 0.01$).

The same authors reported that the animals with the GG genotype of a more frequent cytosine/guanine (C/G) substitution at position 1759 in the bovine leptin promoter also showed higher feed intake ($p = 0.001$), growth rate ($p < 0.1$) and body weight ($p < 0.01$). The thymine allele at position 528 and the guanine allele at position 1759 were separately associated with higher feeding duration ($p < 0.05$).

Five SNPs (UASMS1, UASMS2, UASMS3, E2JW and E2FB) were genotyped by Schenkel et al. [45] in bovine. Only four SNPs were analyzed because UASMS1 and UASMS3 were completely linked. The two leptin exon-2 SNPs were associated with grade fat, lean yield and grade fat (E2JW, $p < 0.01$ and E2FB, $p < 0.05$) and they interacted in their effect on lean meat tenderness ($p < 0.01$).

The association of E2FB with fat yield, grade fat and lean yield was previously reported by Nkrumah et al. [37], where E2FB was genotyped in 144 commercial cattle from five genetic lines with different foundation breeds. These authors concluded that animals carrying the T allele vs. the C allele produce carcasses with poorer grades and lower lean meat yields, but do not differ in carcass marbling. Buchanan et al. [4] also reported a significant E2FB genotype effect on grade fat and average fat where the T allele associated with higher fat but with no significant association with carcass marbling score. Crews et al. [10] did not find any association of E2FB with carcass traits of 433 Charolais and Charolais-cross steers, which included backfat thickness and carcass marbling.

On conclusion of the first study was done to detect the genetic polymorphism of carcass trait genes in Egyptian buffaloes, the monomorphism of *CAPN1* and *LEP* genes declared that all Egyptian buffaloes belong to one population without genetic difference in these two carcass trait genes. The absence of molecular genetic variation in Egyptian buffaloes was previously reported by El-Kholy et al. [15] and El-beltagy et al. [14] who studied the genetic diversity of North- and South-Egyptian buffalo populations using microsatellites and reported that all Egyptian buffaloes belong to one population.

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