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# PARTIAL SEQUENCING AND INVESTIGATION OF LEPTIN AND CALPAIN, CANDIDATE GENES FOR MEAT QUALITY IN CAMEL

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## ABSTRACT

Achieving greater improvements in meat quality was very slow before molecular markers became an accessible technology in breeding. Camel meat is a major source of animal protein, therefore investigation for meat quality and related genes have of great importance. LEP (Leptin) and CAPN1 (μ-Calpain) have been considered as two candidate genes for carcass performance and meat quality traits in the farm animals. The micro molar 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 tenderisation process. Leptin is the hormone product of the obese (LEP) gene. The role of leptin as a lipostatic signal in regulating whole-body energy metabolism makes it one of the best physiological markers of body weight and food intake. In current study, genomic DNA was extracted from 25 Iranian native camels and amplified using published primers that were designed based on bovine CAPN1 and ovine LEP gene sequences. The amplified fragments of LEP and CAPN1 obtained from all samples with 471 and 787 bp length, respectively and were sequenced. Alignment results of sequences showed no difference in sequences of the two genes in all of studied animals, and also between one-humped and two-humped camels. All of the sequences identified here shared high similarity with the published LEP and CAPN1 sequences from cattle and other species.

**Key words:** Calpain, camel, Leptin, meat quality, sequencing

Genetic improvement has long been considered an important factor in the competitiveness of meat production. Identification of the genes and polymorphisms underlying quantitative or qualitative traits and their mode of interaction with environment or other genes affecting economic traits might be the keys to successful application of marker-assisted selection in the commercial animal breeding. When associations between the candidate genes and meat quality traits are well established, potential breeding animals may, be genotyped to add information about their genetic potential for producing high quality meat.

A number of polymorphisms in key genes have been reported to be associated meat quality traits. In this work two major candidate genes; LEP (Leptin) and CAPN1 (μ-Calpain); will be studied in an Iranian camel population. Leptin is a peptide hormone primarily produced by white adipose tissue and in lesser extent in the placenta and skeletal muscle. The recently discovered leptin hormone contributes to the regulation of energy balance, feeding behaviour, and reproduction by acting on the central nervous system (Nkrumah *et al,* 2006). A SNP in exon 2 with a transition from a cytosine (C) to thymine (T) changes an amino acid from arginine to cysteine, referred to as marker Ex2FB (Buchanan *et al,* 2002). Results indicate that the T allele is associated with high grade fat and average back fat (Buchanan *et al,* 2002), subcutaneous fat (Schenkel *et al,* 2006) ultrasound backfat gain and carcass grade fat (back fat) (Nkrumah *et al,* 2004). Kononoff *et al* (2005) found an association between highly marbled individuals and animal homozygous for the T allele in this SNP. Moreover, the two SNPs in exon 2 are particularly significant for tenderness (Schenkel *et al,* 2006). Four additional SNPs in the LEP gene have been associated with fat deposition. Three of these are located in the promoter region of LEP and have in several studies been associated to fat characteristics (Schenkel *et al,* 2006) and carcass weight (Kononoff *et al,* 2005). Moreover, there are many studies that investigated ovine LEP gene and its association with productive traits in various breeds of sheep (Zhou *et al,* 2009; Tahmurespoor *et al,* 2009; Shojaei *et al,* 2010).

The Calpain proteolytic enzyme is composed of two subunits with molecular weights of 28 and 80 kDa. At calcium concentration around 1-2 mM, the form m-calpain is activated. When lower

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concentrations about 50-100 µM of the calcium ion are present, the main calpain enzyme (µ-calpain) is active (Goll *et al,* 2003). CAPN1 degrades myofibrillar proteins under postmortem conditions and appears to be the primary enzyme in the postmortem tenderisation process (Beltran *et al,* 1997). Regulation of CAPN1 activity has been correlated with variation in meat tenderness, and previous studies also identified a quantitative trait locus influencing meat tenderness on chromosome 29 where CAPN1 lies (Casas *et al,* 2003). In the CAPN1 gene, more than 100 single nucleotide polymorphisms (SNPs) have been identified in *Bos indicus* or *Bos Taurus* (Page *et al,* 2002; Soria *et al,* 2010). The calpain-calpastatin system (CCS) contains a family of $Ca^{+2}$ that found in most animal tissues and influences many important processes including muscle development and degradation, postmortem meat tenderisation and fertility (Palmer *et al,* 1999). Calpain (CAPN) plays a major role in postmortem tenderisation in beef, lamb and pork by degrading structure of muscles and is essential for the postmortem enzymatic activity. (Geesink *et al,* 2006; Gandolfi *et al,* 2011; Shi *et al,* 2011). A number of studies have shown that the calpain system is also important in normal skeletal muscle growth (Palmer *et al,* 1999; Goll *et al,* 2003). The CAPN1 gene that codes for µ-calpain is located on bovine chromosome 29, but it is unknown in camel. Two SNPs in the bovine CAPN1 gene located in exon 9 and 14 were evaluated by Page *et al* (2002). In 2006, the first knock out of the µ-calpain gene was performed in mice, where the results further support the role of the calpain gene in tenderisation of meat (Geesink *et al,* 2006).

Camels are in the taxonomic order *Artiodactyla,* sub-order *Tylopod,* and family *Camiladae*. There are two genera within this family. The genus *Camelus consists* of *Camelus dromedarius*, dromedary camel (one hump) and *Camelus bactrianus*, Bactrian camel (two humps). Both species have been domesticated; they provide milk and meat. The karyotypes of different camelid species have been studied earlier by many groups, but no agreement on chromosome nomenclature of camelids has been reached. Camel meat has been used as food in various parts of the world for centuries, therefore investigation for meat quality and related genes is important. However, there is no information about LEP and CAPN in the camel, even though these genes could play an important role in the camels' acclimatisation to harsh environments. In this study, we report some information about calpain in camel for the first time. The aim of present work was to identify LEP and CAPN genes in camel using direct sequencing.

## Materials and Methods

### *Animals and Blood sample collection*

This study was approved by the local ethics and welfare committee. A total of 25 camels belonging to two different species *Camelus dromediarus* (n=20) and *Camelus bactrianus* (n=5) were included in this study. Blood samples (approximately 8 ml) were collected by jugular vein from 25 unrelated camels from Yazd and Ardabil provinces of Iran and stored in EDTA-coated tubes and stored at -20°C.

### *Genomic DNA extraction*

Total genomic DNA was extracted from blood samples (0.2 ml of each sample) using Accuprep® Genomic DNA Extraction Kit (Bioneer Corporation, South Korea) according to the manufacturer's protocol. Extracted DNA samples were stored at -20°C until subsequent steps.

### *PCR Primers and Amplification*

Due to the unavailability of any sequence of these genes in camel and also in any of animals in family *Cameliadae*, primers were designed based on sequences of these genes in other species such as cattle and sheep. The 471-bp fragment of the LEP gene was amplified using following primers, 5′-AGGAAGCACCTCTACGCTC-3′ (forward) and 5´-CTTCAAGGCTTCAGCACC-3´ (reverse), as described by Zhou *et al* (2009) in sheep. The PCRs were carried out in thermal profile consisted of 7 min at 94°C, followed by 35 cycles of 30 s at 94°C, 25 s at 60°C and 30 s at 72°C, with a final extension of 5 min at 72°C. Primers for PCR amplification of calpain, were designed based on reported bovine CAPN1 sequences (GenBank AF252504 and AF248054) by Corva *et al* (2007). A 787 bp fragment was amplified with the primers, 5′-AGCGCAGGGACCCAGTGA-3′ (forward) and 5′-TCCCCTGCCAGTTGTCTGAAG-3′ (reverse) using The following thermal profile; 35 cycles of 45 s at 95°C, 45 s at the annealing temperature, 62°C and 50 s at 72°C, with a initial hot start at 95°C for 5 min and a final extension for 7 min at 72°C. PCR reactions were performed in a total volume of 25 µl containing 2.5 µl of standard PCR buffer, 1 mM $MgCl_2$, 0.5 µM of each primers, 1.6 µM dNTP, 0.5 U Taq DNA polymerase and 50-100 ng of template DNA.

### *DNA Sequencing and Sequence Analysis*

The amplified DNA fragments of the LEP and CAPN1 genes were evaluated by electrophoresis on 1.5% agarose gels containing 200 ng/ml of ethidium

bromide. A 100-bp molecular weight size marker was included in each gel to permit the estimation of the size of the fragments. Amplified fragments were purified using GenJetTM Gel Extraction Kit (Fermentase, EU) and the purified products were sequenced by Bioneer corporation (South Korea). In order to perform a phylogenetic analysis, homologous sequences were recovered from GenBank with the Basic Local Alignment Search Tool (BLAST: http: // blast. ncbi.nlm.nih.gov). Sequences longer and shorter than these gene fragments or with ambiguous sites were excluded from analysis. The sequences of two genes were edited, assembled and analysed using the BioEdit Sequence Alignment Editor Software version 5.0.9 (Hall, 1999). These sequences and homologous sequences were aligned using ClC main workbench version 5.6 using default settings and create tree phylogenetic with UPGMA algorithm and perform 100 bootstrap analyses.

**Results and Discussion**

Amplicons comparable to bovine amplicon (771 bp for CAPN1) and ovine amplicon length (471 bp for LEP) were obtained. All of the sequences identified here shared high similarity with the published LEP and CAPN1 sequences from cattle and other species. These sequences were deposited into the GenBank with the accession Nos., KC571189 (*Camelus dromediarus*), KC571190 (*Camelus bactrianus*) for calpain and, KC571191 (*Camelus dromediarus*) for LEP. In this study, we found no consistent differences in the nucleotide sequence of one-humped and two-humped camels in fragments and sequences were very similar.

In a historic perspective, the focus on quantity has been necessary to support the growing population with meat at low costs. However, the meat quality parameters that generally are of importance for consumer acceptance are expensive to measure with available methods. Thus, it would be advantageous if the eating quality of meat of an animal could be assessed based on its genotype. Considerable progress in farm animal breeding has been made in the last few decades, but achieving greater understanding in the improvement of meat quality was very slow before molecular markers became an accessible technology with wide applications in breeding methods. Meat tenderness is an important issue in meat because it has a major impact on consumer satisfaction. However, meat quality is not routinely measured, so a classical selection based on records is not feasible. Under these conditions, study of molecular basis of variations in meat tenderness may provide a solution to improve tenderness by developing marker assisted selection. 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.

LEP and CAPN1 have been considered as two candidate genes for carcass performance and meat quality traits in farm animals. In livestock, variation in the LEP and CAPN genes has been characterised in cattle, sheep and pig, but it has not been reported in camel. Markers in the CAPN1 and LEP genes have been suggested as being associated with meat tenderness (Page *et al,* 2002; Page *et al,* 2004; Schenkel *et al,* 2006; Casas *et al,* 2006; Othman *et al,* 2011; Allais *et al,* 2011). The polymorphism in the exon 3 of the LEP in sheep was reported by Zhou *et al* (2009) using PCR-SSCP technique. In another study, LEP gene polymorphism and its association with skeletal muscle growth and meat quality was investigated using single nucleotide polymorphism (SNP) analysis. Sayed-Ahmed *et al* (2005) reports for the first time information on LEP in camel (*C. dromedarius*). They also present the first investigation of LEP expression and its localisation in mammalian liver tissue. Using in situ hybridisation and immunohistochemistry techniques, they demonstrated the expression of LEP mRNA and LEP protein in alveolar epithelial cells of the one-humped camel mammary gland (Sayed-Ahmed *et al,* 2005). In the study by Sayed-Ahmad *et al* (2005), subsequent partial sequencing of cDNAs for LEP and both isoforms of the LEP receptor revealed the similarity between the actual length of the amplified PCR products,. Because of the short size of published sequences by Sayed-Ahmad *et al* (2005); this sequence was not used for primer designing in our study. One-humped camel cDNAs for LEP and both isoforms of the LEP receptor showed a high percentage of nucleotide and amino acid similarity to partial sequences of the cow (98%), the Egyptian water buffalo (97%), the sheep (96%), and the pig (92%). In present study, all of the sequences identified here shared high similarity with the published LEP sequences from cattle and other species, that percentage of nucleotide similarity to partial sequences of the Arabian camel, cow (*Bos Taurus* and *Bos indicus*) and sheep were: 97.5, 97.3, 84.1 and 94.5 %, respectively (Fig 1). The phylogenetic tree has been shown considering the relationships among camel and some relevant species, using alignment of LEP nucleic acid sequences corresponding to exon 3 and partial flanking to its region (Fig 2).

| | | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| KC571191 - *Camelus dromedarius* (Iranian camel) | 1 | | 97.55 | 97.27 | 94.53 | 84.10 |
| AY247404 - *Camelus dromedarius* (Arabian camel) | 2 | 0.02 | | 98.48 | 95.74 | 85.63 |
| AJ512639- *Bos taurus* | 3 | 0.03 | 0.02 | | 96.67 | 85.15 |
| EF534371- *Ovis aries* | 4 | 0.06 | 0.04 | 0.03 | | 83.28 |
| FJ626852- *Bos indicus* | 5 | 0.18 | 0.16 | 0.17 | 0.19 | |

**Fig 1.** Comparison of LEP nucleic acid sequences corresponding to exon 3 and partial flanking to its region with other species showing percent identities and distances.

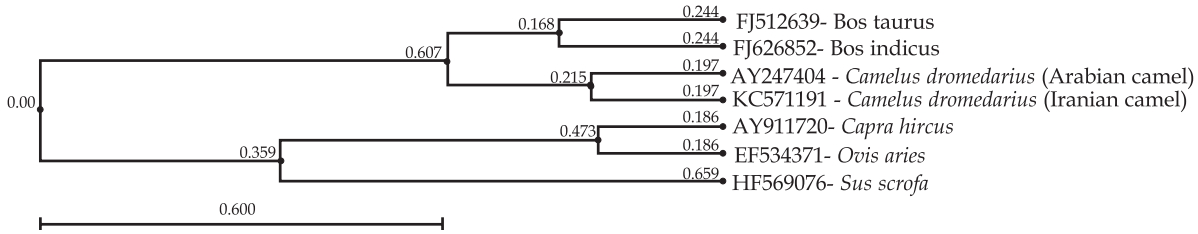


**Fig 2.** Phylogenetic tree showing the relationships among LEP nucleic acid sequences corresponding to exon 3 and partial flanking to its region and some species. Numbers toward the key nodes indicate distances.

| | | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| KC571190 - *Camelus bactrianus* (Iranian camel) | 1 | | 98.07 | 55.24 | 73.93 | 71.67 |
| KC571189 - *Camelus dromedarius* (Iranian camel) | 2 | 0.02 | | 54.79 | 73.15 | 72.34 |
| AF2504S2- *Bos taurus* | 3 | 0.68 | 0.69 | | 73.58 | 54.55 |
| DQ111770- *Bos indicus* | 4 | 0.32 | 0.33 | 0.33 | | 67.76 |
| DQ192642S3- *Sus scrofa* | 5 | 0.36 | 0.35 | 0.70 | 0.42 | |

**Fig 3.** Comparison of CAPN1 nucleic acid sequences corresponding to exon 13-exon 15 with other species showing per cent identities and distances.

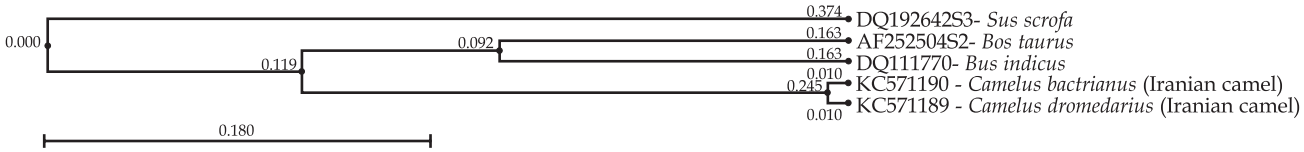


**Fig 4.** Phylogenetic tree showing the relationships among CAPN1 nucleic acid sequences corresponding to exon 13-exon 15. Numbers toward the key nodes indicate distances.

The alignmenting of sequences from CAPN1 showed no sequence differences between one-humped and two-humped camels. Sequences were very similar, except for several nucleotides in some animals (Fig 3), that these nucleotide substitution may be a potential SNPs sites in CAPN1 gene in Iranian camel population, but its verification and validation needs studies with a larger samples size. Creation of an alignment of CAPN1 sequences and homologous sequences of other species showed some percentage of nucleotide similarity i.e. 74% (*Bos indicus*), 55% (*Bos taurus*), and 72% *Sus scrofa* (Fig 3). Relationships among camel and some relevant species was evident in the phylogenetic tree using alignment of obtained CAPN1 nucleic acid sequences shown in Fig 4. The reason for the low similarity in some cases is the lack of homologous sequences with the same size for the desired position corresponding, in those cases. However, in CAPN1 sequences of four camels were at two different positions i.e. 756 and 757 (C>T and T>G). Cheong *et al* (2008) identified 39 polymorphisms in Korean cattle CAPN1 gene within exons and their flanking regions by direct DNA sequencing of 24 unrelated Korean cattle. In their

study, associations of CAPN1 polymorphisms with cold carcass weight (CW) and marbling score (MS) were analysed. One of their reported polymorphisms 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). The present study was the first attempt for the identification of LEP and CAPN genes variation in Iranian camels. Further studies are required to investigate the relationship between polymorphisms of these genes and the performance traits. Moreover association study of genotypes in camels is required to undertake basic and applied research for improvement of camel. Results of this study can be used in genetic improvement of indigenous camels through conventional and molecular means for increasing production.

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## News

### Archaeologists Carbon-Date Camel Bones

Researchers Lidar Sapir-Hen and Erez Ben-Yosef from Tel Aviv University have determined the age of the oldest-known camel bones and found that camels were first introduced to Israel around the 9th century BCE which differs from the facts given in the history laid out in the Bible. The Hebrew Bible, or Old Testament refers to camels as pack animals as early as the story of Abraham. Though there is no archaeological evidence of Abraham's life, many in the religious and scientific communities, including Chabad and the Associates For Biblical Research, cite the 20th century BCE as his time of birth. The researchers scoured ancient copper production sites in the Aravah Valley, where camel bones were only present in sites active in the last third of the 10 century and the 9th century BCE. This suggests that camels were introduced to the region abruptly, perhaps by Egyptians along Mediterranean trade routes.

(Source–"The Huffington Post")

### Camels suspected in deadly MERS coronavirus outbreak in Saudi Arabia

A deadly virus that emerged in Saudi Arabia last year infected people and possibly spread through one-humped camels used in the region for meat, milk, transport and racing. Scientists found strong evidence for this virus being widespread among dromedary camels in the Middle East. The Middle East Respiratory Syndrome Coronavirus (MERS-CoV), which can cause coughing, fever and pneumonia, has been reported in people in the Gulf, France, Germany, Italy, Tunisia and Britain. The World Health Organisation (WHO) says 46 people have died out of a total 94 confirmed cases, the majority in Saudi Arabia. Chantal Reusken of the National Institute for Public Health and the Environment in Bilthoven, the Netherlands, who led the study said that new human cases of MERS-CoV continue to emerge, without any clues about the sources of infection except for people who caught it from other patients, which suggest that dromedary camels may be one reservoir". Experts appreciated these findings as a major step towards solving the mystery of the MERS virus and, ultimately, controlling it. WHO spokesman Tarik Jasarevic says that camels have shown antibodies in the camels, that means that camels have been infected at some point in time and that produced antibodies.

(REUTERS- Friday, August 9, 2013)