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Molecular characterization and genetic variability at κ -casein gene (CSN3) in camels

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ABSTRACT

κ -casein is a glycosylated protein belonging to a family of phosphoproteins (α s1, α s2, β , κ) that represent the major protein component in mammalian milk. κ -casein plays an essential role in the casein micelle stabilization, determining the size and the specific function. In the present paper, we report for the first time the characterization of the nucleotide sequence of the whole κ -casein-encoding gene (*CSN3*) plus 1045 nucleotides at the 5' flanking region in *Camelus dromedarius*. The promoter region and the complete cDNA were also provided for the first time in *Camelus bactrianus*. The gene is spread over 9.3 kb and consists of 5 exons varying in length from 33 bp (exon 3) to 494 bp (exon 4), and 4 introns from 1200 bp (intron 3) to 2928 bp (intron 2). Highly conserved sequences, located in the 5' flanking region, have been found. The regulatory regions of camels seems to be more related to equids than to other compared species. 17 polymorphic sites have been detected, one of these (g.1029T>C) is responsible for the creation of a new putative consensus sequence for the transcription factor HNF-1. In general, these SNPs are the first reported in camels for casein *loci*. Finally, seven interspersed repeated elements were also identified at intronic level.

Keywords: *Camelus dromedarius*, Casein, *CSN3*, Promoter analysis, Interspersed elements, HNF-1.

Abbreviations: *CSN3*, κ -casein-encoding gene; SNP, single nucleotide polymorphism; CN, casein; CMP, caseino-macropeptide; cDNA, DNA complementary to RNA; EDTA, ethylene diamine tetra acetic acid; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; $MgCl_2$, magnesium chloride; dNTP, deoxyribonucleoside triphosphate; TBE, tris boric acid EDTA; C/EBP- α , CCAAT enhancer bind protein-alpha; MGF, Mammary Gland Factor; CRE-BP, cAMP response element binding protein; cAMP, cyclic adenosine 3',5'-

monophosphate; Oct-1, Octamer bind protein; TBP, tata binding protein; GR, glucocorticoid receptor; PRL, prolactin; SP1, specificity protein 1; NF-1, nuclear factor-1; SRF, serum response factor; HNF-1, hepatocyte nuclear factor-1; HNF-3, hepatocyte nuclear factor-3; SINE, short interspersed element; LINE, long interspersed element; RFLP, restriction fragment length polymorphism.

1. INTRODUCTION

The camel stock is at present estimated to be about 23 million in the world. Somalia and Sudan together hold almost 50% of the whole camel population. In the last forty years (1970-2010), the number of animals has increased of almost 45% (www.faostat.fao.org), and such increase is even more evident in Sudan (+92.4%), where camels are mainly bred for milk production and more rarely for dairy purpose. The daily milk production is estimated to vary between 3 and 10 kg during a lactation period of 12-18 months (Farah et al. 2007), depending on breed, stage of lactation, feeding and management conditions, with an average content of 2.9% and 3.1% of protein and fat respectively (Al-haj and Al Kanhal, 2011).

Milk proteins and the corresponding coding genes have been deeply studied in ruminants, whereas such information is still limited in camels. Caseins (α s1, β , α s2 and κ) are coded by single autosomal genes (*CSN1S1*, *CSN2*, *CSN1S2* and *CSN3*, respectively) clustered in a DNA stretch of about 250 kb and mapped on chromosome 6 in cattle, sheep and goat (Rijnkels, 2002). They have been very well characterized both at DNA and protein level. Goats and cows represent the most polymorphic species, for which many alleles associated with different rates of protein synthesis have been identified (Caroli et al. 2006; 2009; Ramunno et al. 2005). In camels, genetic variants are known for α s-1 casein (Kappeler et al. 1998) and recently a new protein variant has been identified for such protein fraction (Salih Shuiep et al. 2012). Among caseins, κ -CN plays an essential role in the casein micelle

1 stabilization (Alexander et al. 1988). It is located predominantly on micellar surface and it is
2 the specific substrate of the chymosin, which is responsible for the hydrolyzation of the κ -CN
3 into the para- κ -CN and the caseino-macropptide (CMP). At least 16 alleles corresponding to
4 13 κ -CN variants have been identified in goat (Caroli et al. 2006), and at least 19 alleles
5 corresponding to 14 κ -CN variants so far in cattle (Caroli et al. 2009).

6 Unlike what has been accomplished in these species, κ -casein gene in camels has not
7 received much attention so far, whereas more information is available from a proteomic point
8 of view. Farah and Farah-Riesen (1985) reported on the first characterization of major
9 components of camel milk casein. Partial sequencing of tryptic digests of κ -casein showed a
10 more stable conformation of the active cleavage site of camel chymosin compared to cow's κ -
11 casein site (Kappeler et al. 1998). A quantitative analysis carried out on camel milk protein
12 showed significantly lower amounts of camel κ -casein compared to the homologous cow's
13 casein (Kappeler et al. 2003), and recently five different isoforms of κ -casein were found in
14 camel milk as result of a strong glycosilation of such protein (Hinz et al. 2012). To date no κ -
15 casein variants have been detected and the investigation at DNA level is limited to Somali
16 camels cDNA sequence (Kappeler et al. 1998) and to a comparison of the 5' flanking regions
17 (Kappeler et al. 2003). Excluding these two studies no additional information is available at
18 DNA level and no genetic variability is reported so far.

19 Keeping these reports in mind, the investigation was undertaken to explore genetic
20 variability at camel κ -CN *locus*. We report on the first polymorphism detected at *CSN3* gene
21 in *Camelus dromedarius*, and we provide the full characterization and an extensive annotation
22 of such gene. A comparison between *Camelus dromedarius* and *Camelus bactrianus* *CSN3*
23 promoter regions and cDNA was also accomplished.

24 25 2. MATERIALS AND METHODS

2.1 Animals

Blood samples were collected from 188 Sudanese she-camels (*Camelus dromedarius*, locally known as *Naga*) reared in five regions of the country (Fig. 1) and belonging to different ecotypes including Shanblai, Kahli, Lahaoi and Arabi camels. Due to the lack of records, local experience was used to determine ecotypes in different locations and also individuals within the same herd. Typical phenotypic characteristics for each ecotype were strictly followed. Blood samples were immediately applied to classic filter paper (FTA[®]Classic Card- Whatman[®]BioScience, Maidstone, UK), allowed to dry at room temperature and stored until DNA isolation.

Additional blood samples were collected from three *Camelus bactrianus* belonging to Wilhelma Zoo (Stuttgart, Germany) and treated according to Spin Blood Mini Kit (Invitex, Germany).

2.2 DNA isolation

The filter paper containing blood samples were soaked (56°C, overnight) in 500 µl sodium-tris-EDTA buffer with 10 µl proteinase K (10 mg/ml) in presence of sodium dodecyl sulfate (SDS). Then DNA was isolated from the emerging lysis according to the procedure described by Sambrook et al. (1989). The isolated DNA was resuspended in 100 µl TE buffer pH 7.6 (10 mM Tris, 1mM EDTA).

DNA concentration and OD_{260/280} ratio of the samples were measured with the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific).

2.3 PCR conditions and sequencing

DNA regions spanning from nucleotide -1045 to +10436 of the camel *CSN3* gene were amplified by means of a iCycler (Bio-Rad). A set of 33 primers for amplification and sequencing were designed by means of DNAsis-Max ver. 3.0 software (Hitachi), using as

preliminary template the complete sequence of camel cDNA available in gene bank (EMBL acc. no. Y10082) and then the new sequences determined in the course of the research. A typical PCR reaction mix (50 µl) comprised: 100 ng of genomic DNA, 1X PCR Buffer (Promega), 2.5 mM MgCl₂, 5 pmol of each primer, dNTPs each at 200 µM, 2.5 U of *Taq* DNA Polymerase (Promega). In Table 1 we report the starting amplicons for the sequencing of the whole gene. PCR was performed under the following conditions: 95°C (4 min), 35 cycles at 95°C (60 s), annealing temperatures depending on amplicon (Table 1) (45 s), 72°C (90 s), final extension at 72°C (10 min).

PCR products were purified and sequenced. The purification was carried out using MSB[®] Spin PCRapace kit (Invitek, Berlin, Germany). Sequence was accomplished by ABI 3100 Genetic Analyzer (Applied Biosystem). Targeted fragments were sequenced in both directions using BigDye chemistry (Applied Biosystems).

2.4 Genotyping by Alu I PCR-RFLP

PCR reaction mixture and thermal conditions for the amplification of the DNA fragment 488 bp long and spanning from -137 bp of 5' flanking region to +351 bp of the camel *CSN3* gene were accomplished by using the following primers: *forward* 5'-CACAAAGATGACTCTGCTATCG-3' and *reverse* 5'-GCCCTCCACATATGTCTG-3', according to standard PCR conditions already reported above. Product specificity was confirmed by ethidium-bromide-stained 1.5% agarose gel electrophoresis.

The entire panel of 188 animals was genotyped for the g.1029T>C SNP using a PCR-RFLP method. Digestion of 17µl of each PCR amplification was accomplished with 10 U of *AluI* endonuclease (AG↓CT) (Fermentas) over-night at 37°C. The digestion products were analysed directly by electrophoresis in 3.5% agarose gel in 1X TBE buffer and stained with ethidium bromide.

2.5 Bioinformatics

The allele frequency and Hardy-Weinberg equilibrium (χ^2 test) were calculated for the investigated population. SNP discovery, homology searches, comparison among sequences, and multiple alignments were accomplished using DNAsis-Max ver. 3.0 software (Hitachi Software, San Bruno, CA), whereas the putative transcription factor binding sites were searched by Transfact 7.0 software. Interspersed elements were found by RepeatMasker Web Server (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>). Estimates of evolutionary divergence between the sequences were conducted using the maximum composite likelihood method by using MEGA version 4.0 software. Phylogenetic tree was constructed using the Neighbor-Joining method by using the same software.

3. RESULTS AND DISCUSSION

The entire sequence of the *Camelus dromedarius* *CSN3* gene plus the analysis of 5' flanking region are described. The promoter region of *CSN3* gene and the complete cDNA is also provided for the first time in *Camelus bactrianus*. The analysis of 5' flanking region of the camel *CSN3* gene provide an important contribution in order to evaluate the role and the importance of factors involved in the regulation of milk protein gene expression and the transcriptional effects of polymorphisms located in such regions. Furthermore the knowledge of the gene structure and the distribution of interspersed elements add new information in a species not investigated so far, as well as the genetic variability detected at camel *CSN3* gene will provide new opportunities to start selection programmes also in such species.

3.1 Analysis of the gene structure

1 The whole gene encoding the camel κ -casein (*CSN3*) plus 1045 nucleotides at the
2 5'flanking region were sequenced (EMBL ID: HE863813). The gene extends over 9391 bp
3 including 823 bp of exonic regions and 8568 bp of intronic regions with a total similarity with
4 the corresponding bovine sequence (EMBL ID: AY380228) of about 58.3%. Approximately
5 the same level of homology was also found by the following comparison to the κ -casein gene
6 in other species: 58.0% vs river buffalo (EMBL ID: AM900443); 71.2% vs donkey (EMBL
7 ID: FR922990) and 67.5% vs human (EMBL ID: U51899).

8 On the whole, the camel *CSN3* gene shares a similar organization with the bovine
9 counterpart, with some differences in intronic size. In fact, the analysis of the camel *CSN3*
10 gene evidenced an exon/intron size ratio higher (1:10.41) than that observed in cattle
11 (1:14.42). The camel *CSN3* gene is also characterized by high A/T content compared to G/C
12 (69.6% vs 30.4%). However, this feature seems to be conserved among the species (Ward et
13 al. 1997). It contains 5 exons, ranging in size from 33bp (exon 3) to 494 bp (exon 4), and 4
14 introns from 1200 bp (intron 3) to 2928 bp (intron 2).

15 The first exon (63 bp) plus 8 bp of the exon 2 are not coding at all. The leader peptide
16 (20 amino acids) is coded by the remaining part of the exon 2 (54 bp) plus the first two triplets
17 of the exon 3. The mature peptide (162 amino acids) is encoded by the rest of exon 3, and
18 almost the entire exon 4. The translation stop codon TAA is realized between the 460th-462th
19 nucleotides of the exon 4, whereas the polyadenylation site is located between the 153rd and
20 158th nucleotide of the exon 5. All splice junctions follow the 5' GT/3'AG splice rule.

21 The translation initiation codon (AUG) and the nucleotide immediately upstream
22 (Kozak consensus sequences: GCCGCCRCCAUGG) are known to play an important role in
23 determining the translation rate and to contribute to the translation process (Kozak, 1991).
24 Camel κ -casein shows 4 nucleotides homologous to Kozak sequence (positions: -6, -5, -3, -1,
25 taking the A nucleotide of the AUG codon as number +1) as also reported in donkey (-6, -5, -

3, -2, EMBL ID: [FR922990](#)), but differently from ruminants, pig, rabbit and rat (Cosenza et al. 2011) which have only 3 homologous nucleotides (positions: -6, -3, -2). The higher homology with Kozak sequence should guarantee a stronger consensus for camels and donkey compared to ruminants and therefore an higher efficiency of mRNA translation (Kozak, 1991). Further investigations need to confirm this statement, but it opens the way to new studies of expression analysis and translation efficiency in these species -so far- never investigated from this point of view.

A comparison of the sequences of the investigated samples shows only 7 nucleotide differences: 1 polymorphic site in the promoter region and 6 at intronic level. No polymorphisms were found in the coding regions (Table 2).

The following comparison with the published sequences of the camel cDNA (Kappeler et al. 1998) and promoter region (Kappeler et al. 2003) shows a total of 10 polymorphic sites (Table 2): 5 insertions/deletions and 5 nucleotide substitutions, one of which results in an amino acid substitution at the second triplet of the exon 4 (TGT^{Cys}→TTT^{Phe}). The latter potential polymorphism was characterized by sequencing 40 randomly chosen animals, and it resulted monomorph in our samples.

One single nucleotide polymorphism (c.185C>T) was also detected at the exon 4 of *C. bactrianus* cDNA sequence (EMBL ID: [HE863815](#)). Although this mutation is synonymous, it represents the first example of polymorphism in *CSN3* coding region for such species.

To date many genetic variants of κ -casein have been identified at protein or DNA level in many species. Camels were not investigated so far, but the absence of polymorphisms in coding regions and the number of mutations detected in the rest of the *CSN3* gene suggests that the level of genetic variations of camel κ -casein is very low in comparison to other species (Carneiro and Ferrand, 2007; Caroli et al. 2006, 2009; Hobor et al. 2008). The homologous bovine gene, for instance, shows 42 polymorphic sites which differentiate only the allelic variants A and B (Robitaille et al. 2005). Therefore, a larger investigation of camel

1 κ -casein should be performed to detect nucleotide variability, and further studies would be
2 necessary in order to evaluate the potential influence on the quality-quantitative properties of
3 camel milk. For instance, in cattle it is known that cows carrying the κ -casein BB genotype
4 produce milk with a significantly higher protein content (Caroli et al. 2009). This led the dairy
5 farmers to preferentially select these cows in order to have an higher cheese yield. Therefore,
6 we can not exclude the presence of quantitative alleles also in camels, which might lead
7 similar advantages also for dairy camel breeders.

8 Furthermore, recently Weimann et al. (2009) showed that in cattle different *CSN3*
9 variants are source of diverse angiotensin I converting enzyme (ACE) inhibitory peptides and
10 demonstrated their potential role for human health. These bio-functional peptides were found
11 also in camel milk (Al-Haj et al. 2011). Therefore, it's likely that the genetic variants of the
12 camel κ -casein might also influence its functional role and make the camel milk even more
13 useful for the human nutrition.

15 ***3.2 Analysis of the CSN3 gene promoter***

16 In absence of polymorphisms in the coding regions, we decided to extend the
17 sequencing to the 5'-flanking region and to analyze the putative regulatory regions of the
18 camel κ -casein gene. About 1050bp of the *CSN3* gene were amplified and sequenced for
19 *Camelus dromedarius* (EMBL ID: [HE863813](#)) and *Camelus bactrianus* (EMBL ID:
20 [HE863814](#)). A high level of homology and similar locations of most putative transcription
21 binding sites were already reported among ovine, caprine, bovine murine and rabbit κ -
22 promoters (Gerencsér et al. 2002), horse, donkey and zebra *CSN3* 5' flanking region (Hobor
23 et al. 2008). Here we performed a comparative analysis which included the aforementioned
24 sequences in addition to river buffalo (EMBL ID: [AM900443](#)), human (EMBL ID: [U51899](#)),
25 pig (ENSEMBL ID: [ENSSSCG000000009267](#)) and the newly sequenced *Camelus*

dromedarius and *Camelus bactrianus* κ -promoters. The level of homology differs between compared sequences. As expected, sequences belonging to species of the same family are closely related each other (Table 3). Camels and equids showed a slightly lower level of divergence (on average 20.8%) compared with ruminants ($\leq 26.6\%$). Phylogenetic data confirmed that camels belong to their own branch (Fig. 2), but their κ -casein promoter sequences appears more related to equids (average homology $>80\%$) than to ruminants, rabbit, mouse, human and pig which showed an average similarity significantly lower at about 60%. For some aspects these data are surprising considering that molecular analysis supported by fibrinogen, from which κ -casein encoding gene is supposed to be originated by a duplication gene event (Jollès et al. 1978), and mitochondrial genome data found *Camelidae* closely related to *Suina* (Geisler et al. 2005). However, our findings are supported by the phenotypic data reported first by Farah and Farah-Riesen (1985) and recently by Hinz et al. (2012). They showed respectively the lack and the low concentration of such milk protein fraction, as well as already observed in horses (Hinz et al. 2012). The close relationship with equids is also confirmed by cDNA comparison from which the level of similarity is $>83\%$, whereas it is lower than 65% in the other cases.

Similar mechanisms of regulation are supposed for these related species, therefore camels κ -promoter sequences were compared with the homologous sequences of donkey promoter -not characterized so far- and bovine, respectively as non-ruminant and ruminant counterpart. 25 putative consensus sequences for transcription factor binding sites were found and reported in Fig. 3. Briefly, the analysis of the camel promoter showed a TATA-box located, with reference to the first nucleotide of the first exon, at nucleotides -32/-25 which is perfectly conserved among the compared sequences. Particularly interesting are also other 6 putative transcription factor binding sites almost perfectly conserved: one CCAAT enhancer bind protein (C/EBP)-alpha in position -1025/-1016, one Mammary Gland Factor/STAT5

(MGF/STAT5) at nucleotides -1018/-1008, one cAMP response element binding protein (CRE-BP) -900/-891, two Octamer bind protein (Oct-1) in position -307/-299 and -277/-268 respectively and one Tata Binding Protein (TBP) at nucleotides -236/-227.

The first two sites were reported to be very well conserved in more than 9 species (Gerencsér et al. 2002; Hobor et al. 2008; Malewski, 1998). Here we confirm them also in camels increasing the probability that these factors are necessary for the regulation of the gene expression. The CRE binding protein (CRE-BP) is a transcriptional co-activator, which potentiates the activity of several groups of transcription. It interacts directly with STAT 5, reported as a crucial functional activator of casein gene expression (Vanselow et al. 2006); but it functions also as a transcriptional co-activator of the glucocorticoid receptor (GR) and prolactin (PRL) (Pfitzner et al. 1998). Oct-1 is not known to be a strong transcriptional activator by itself, but in conjunction with other co-activators. For instance, Zhao et al. (2002) showed that Oct-1 and STAT5 are both involved in the hormonal induction of casein gene expression. They bind DNA cooperatively and they interact in the stabilization of the transcription complex. However, Oct-1 can also interact with the TBP components (Zwilling et al. 1994). Oct-1 and TBP are putative sites very representative of camel κ -casein promoter being characterized by 7 Oct-1 and 3 TBP putative sites. Therefore, it would be reasonable to suggest that Oct-1 may also mediate stimulation of κ -casein expression in mammary gland through this way.

According to the comparative analysis of Gerencsér et al. (2002) on six different species, also in camels we found wide conserved regions (CB1, CB2 and CB4) apparently not characterized by known transcription factor binding sites. In fact, camel CB1 region is only partially covered by glucocorticoid receptor (GR) consensus sequence, whereas no consensus sites have been found for CB2 and CB4. Analogously, the wide region between nucleotides -180/-60 is completely lacking of known sites, whereas in bovine the same region is

1 characterized at least by two SP1 sites, one NF-1 and one Ying Yang-1 factor, and the donkey
2 sequence showed at least one SRF and one C/EBP- α .

3 Two polymorphic sites were detected in camels promoters. The first is a transition
4 g.975A>G in position -112 which characterizes the *C. bactrianus* sequence and not affects
5 any putative regulatory region. The latter, at the nucleotide -17, is a transition g.1029T>C
6 realized in *C. dromedarius* promoter just upstream the exon 1. The presence of thymine is
7 responsible for the creation of a extra putative consensus sequence for the hepatocyte nuclear
8 factor-1 (HNF-1). This transcription factor is reported to regulate a number of genes involved
9 in innate immunity, lipid and glucose transport, metabolism, etc...(Reiner et al. 2009), and to
10 bind the promoters of hepatocyte specific genes including those of the fibrinogen chain
11 (Mendel et al. 1991). However, conflicting are the information regarding the expression of
12 HNF-1 in the mammary gland. Although Dunn et al. (2003) reported that no HNF-1
13 transcripts were detected in the human mammary gland, recently expression microarray
14 analysis of pubertal mouse mammary gland showed the presence of HNF-1 transcripts
15 (McBryan et al. 2007), whereas no additional information is reported in other species.
16 However, another member of hepatocyte nuclear factors (HNF-3) is well known to be a
17 potential regulator of casein genes expression (Schild et al. 1994; Schild et al. 1996), thus
18 further investigation is required to verify also the influence of HNF-1 allelic variant in camel
19 κ -casein gene regulation.

21 **3.3 Interspersed elements**

22 Retroposons are genetic elements that are propagated via a process known as
23 retroposition. Retroposons are gathered in two major groups: Short INterspersed Elements
24 (SINEs; Okada and Ohshima, 1995) and Long INterspersed Elements (LINEs; Eickbush,
25 1994).

1 The camel *CSN3* gene sequence is characterised by 7 DNA fragments (boxes from A to
2 G) showing high similarity (on average 80%) to interspersed elements belonging to both
3 groups (Fig. 4).

4 Three elements (A, B, C) have been found in the intron 1. In particular, two (A and C)
5 located between the nucleotides +1728/+1925 and +3239/+3306 appear to be LINEs, both
6 belonging to the family L1. The C element has a complementary sequence and it seems to be
7 a truncated LINE (68 bp long), flanked upstream by (TA)_n repeat of 78 bp and downstream
8 by an AT rich region of 24 bp. This element appears characteristic of the camel *CSN3*
9 sequence, since it has not been found by the comparison with bovine, buffalo, donkey, and
10 human κ -casein gene.

11 The B element is also complementary and it is situated between the nucleotides
12 +2547/+2670. This is a SINE belonging to the class of mammalian-wide interspersed repeats
13 (MIR-3) and it seems to be absent in bovine and buffalo κ -casein gene sequences, whereas it
14 characterizes the camel, donkey and human *CSN3* gene.

15 Elements D and E are located in the second intron. The former (from +4972 to +5163)
16 shows a similarity of 87.5% with a LINE 1, whereas the latter (+6394/+6622) has a similarity
17 of 81.1% with a complementary Bov-A2 sequence (Lenstra et al. 1993).

18 Two additional complementary SINEs (MIR-b) have been found in the camel *CSN3*
19 gene, one (F) is located between the nucleotides +7548/+7633 of the intron 3, whereas the
20 other (G) is positioned at the level of the intron 4 between the nucleotides +9542/+9627. As
21 for the element B, also the retroposon G is not present in bovine and buffalo *CSN3* sequence,
22 however it characterizes also the human and donkey κ -casein gene.

23 The seven repetitive elements observed at the camel *CSN3* gene represent the 10.4% of
24 the sequence deposited in the EMBL database. This feature raises to 21.4% in the bovine
25 counterpart because of six extra repetitive elements (H, I, L, M, N, O) for a total of 2791bp

1 observed in this species (Fig. 4). The camel *CSN3* is also characterized by several low
2 complexity repeats. All of them belong to AT rich regions and they cover a total of 321bp,
3 representing approximately the 3.4% of the gene.

4 All vertebrate genomes contain repetitive sequences, for instance the 45%, 37% and
5 55% of the human, mouse and opossum genomes are made up of these elements (Lander et al.
6 2001; Gentles et al. 2007). Transposable elements have been reported to have a large
7 influence on evolution of the mammalian genome playing a prominent role in species
8 diversification (Bowen and Jordan, 2002). The lower number of repetitive element in camel is
9 an indication that *Tylopoda* diverged from *Ruminantia* before additional retrotransposition
10 events took place at different times during the divergence of in such suborder (Nijman et al.
11 2002). In such contest, the distinctive retroposon element is typical of camel sequence, and as
12 the extra elements that characterize the bovine sequence, they can be considered powerful
13 phylogenetic markers for evolution and clustering studies.

15 **3.5 Genotyping**

16 The transition g.1029T>C creates a restriction site for the endonuclease *AluI*, thus a
17 PCR-RFLP protocol was set up for the quick genotyping of the samples. The digestion of the
18 PCR product (488 bp) allows the identification of both alleles (Fig. 5).

19 The restriction pattern is characterized by 3 fragments of 203 bp, 158 bp and 127 bp
20 for the TT samples, whereas the band 158 bp long is further restricted into two fragments of
21 120 bp and 38 bp in the presence of cytosine. The restriction pattern of the heterozygous
22 samples results in 5 fragments. The frequency of cytosine in the sample of 188 camels was
23 0.38, with a variation among the breeds between 0.30 and 0.46 (Table 4). This value might
24 open the possibility for a rapid directional selection in favor of the C allele, which is
25 responsible for the creation of an extra putative site for the HNF-1 transcription factor. Hardy-
26 Weinberg disequilibrium was detected for the genotype distribution ($\chi^2=4.97$), but this event

could be the result of several causes. In particular, the restricted number of genotyped individuals and the mating system. In the last case, even though the mating technique in camel is exclusively natural, we can't exclude inbreeding effect due to the use of the same stallion.

4. CONCLUSIONS

1. For the first time, *CSN3* gene was completely sequenced and characterized in *Camelus dromedarius* species. We also provided for the first time the complete cDNA sequence of *Camelus bactrianus*. On the whole, the camel *CSN3* gene shares a similar organization compared to the other species. In particular some differences in intronic size characterize camel sequence. The ratio exon/intron is higher (1:10.41) than that one observed in bovine (1:14.42).
2. Such difference in size is also due to a different number of interspersed elements (7 in camel vs 10 in bovine) which account of 10.4% of the camel sequence vs 21.4% in the bovine counterpart. One LINE truncated appears to be characteristic of the camel *CSN3* sequence, since it has not been found by the comparison with bovine, buffalo, donkey, and human κ -casein gene, and it could be used as genetic marker typical of the specie, as well as for evolution and clustering studies.
3. The promoter sequence was deeply characterized and used as reference to estimate the genetic distance between 13 taxa. The regulatory regions of camels seems to be more related to equids than to ruminants. 25 putative consensus sequences for transcription factors have been identified and 6 of them are very well conserved. Oct-1 and TBP are very representative motifs of camel κ -casein gene promoter, and although further studies are necessary, it would be reasonable to suggest a possible regulation of gene expression through this way.

4. It's also interesting to notice the higher homology of camel translation starting codon with Kozak sequence. This event should guarantee a stronger consensus for camels and therefore an higher efficiency of mRNA translation.
5. A total of 17 SNP have been found, 7 by the comparison of our sequences and 10 by the comparison of the sequences already available in database. The g.1029T>C SNP, located in the promoter region, creates an extra putative site for the transcription factor HNF-1. Further investigations are required to verify the influence of HNF-1 allelic variant in camel κ -casein gene regulation.
6. The polymorphism detected in the promoter region (g.1029T>C) is the first genetic marker for the casein cluster in camels, since no information is available so far. Population data indicate that the frequency of the C allele is 0.38 thus opening the possibility for a rapid directional selection in favor of such allele.

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Amplicon	Region covered	Primers	Sequence	Ta °C	Size
1	5' UTR/Ex 1	Cd CSN3 5'F*	5'-TGAAACTCTGCCATCTTTCTCT-3'	65.5	1213 bp
		Cd CSN3 Ex1R	5'-TTCCCTTGTGCCTGTCAG-3'		
2	Ex1/Intr1/Ex2	Cd CSN3 Ex1F	5'-GGCCAGCTCAACCTACT-3'	61.5	2610 bp
		Cd CSN3 Ex2R	5'-AGTCACAACCTAGGAAGAAGCTC-3'		
3	Ex2/Intr2/Ex3	Cd CSN3 Ex2F	5'-AGCTTCTTCCTAGTTGTGAC-3'	66.0	2993 bp
		Cd CSN3 Ex3R	5'-GTTTTGCACCTCTGCACC-3'		
4	Ex3/Intr3/Ex4	Cd CSN3 Ex3F	5'-GGTGCAGAGGTGCAAAAC-3'	65.0	1726 bp
		Cd CSN3 Ex4R	5'-CTGTGTTGTCTTCTTTG-3'		
5	Ex4/Intr4/Ex5	Cd CSN3 Ex4F	5'-CAAATATTTCCCAATTCAGT-3'	58.5	2535 bp
		Cd CSN3 Ex5R	5'-GCATTCAATTAGCTTTATTA-3'		

Table 1. Primer sequences, annealing temperature and amplicon size used for the characterization of *Camelus dromedarius CSN3* gene. Asterisk refer to primer designed on camel *CSN3* promoter sequence (EMBL acc. No. [AJ409280](#)), whereas the other primers were designed on cDNA sequence (EMBL acc. No. [Y10082](#)).

Position	Nucleotide	A	B	C
promoter	335-336	-	G	*
	640	T	G	*
	702	T	-	*
	721	T	-	*
	1029	Y	T	*
	1039	T	-	*
Exon 1 (5'UTR)	1055-1056	TT	-G	*
Intron 2	3951	Y	*	*
	5019	Y	*	*
	5033	W	*	*
	5041	M	*	*
	5460	Y	*	*
Exon 4	7862	C ^{Cys}	*	T ^{Cys}
	7864	G ^{Cys}	*	T ^{Phe}
	8018	A ^{Ala}	*	C ^{Ala}
Intron 4	8647	Y	*	*

A: Present study; B: Kappeller et al. (2003); C: Kappeller et al. (1998)

Table 2. Polymorphisms detected by the comparison among *Camelus dromedarius CSN3* gene sequence of the present study (**A**), with the *CSN3* promoter sequence (**B**) reported by Kappeller et al. (2003) and the cDNA (**C**) described by Kappeller et al. (1998). Grey cells correspond only to mutations detected in our investigated samples (Y=A/C, W=A/T, M=A/C). Dashes indicate deleted nucleotide, asterisks show unavailable sequence.

	EMBL ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1. <i>C. dromedarius</i>	HE863813													
2. <i>C. bactrianus</i>	HE863814	0.004												
3. <i>E. asinus</i>	EU429803	0.213	0.211											
4. <i>E. zebra</i>	EU429802	0.202	0.201	0.003										
5. <i>E. caballus</i>	AY579426	0.212	0.210	0.010	0.006									
6. <i>B. taurus</i>	AY380228	0.266	0.266	0.335	0.330	0.339								
7. <i>B. bubalis</i>	AM900443	0.221	0.222	0.319	0.313	0.322	0.032							
8. <i>C. hircus</i>	Z33882	0.212	0.213	0.310	0.307	0.312	0.064	0.044						
9. <i>O. aries</i>	L31372	0.216	0.216	0.311	0.311	0.313	0.067	0.053	0.018					
10. <i>O. cuniculus</i>	AJ309572	0.406	0.416	0.416	0.412	0.418	0.541	0.515	0.544	0.548				
11. <i>M. musculus</i>	AJ309571	0.526	0.536	0.499	0.497	0.504	0.618	0.602	0.613	0.596	0.530			
12. <i>H. sapiens</i>	U51899	0.912	0.908	0.726	0.678	0.712	0.793	0.585	0.599	0.532	0.683	0.844		
13. <i>S. scrofa</i>	ENSSSCG000000009267	1.794	1.761	1.757	1.752	1.744	1.832	1.764	1.739	1.777	2.095	2.055	1.330	

Table 3. Estimates of evolutionary divergence between 13 taxa for κ -casein promoter sequence.

Breed	Observed Genotypes				Allelic frequency	
	CC	CT	TT	Total	C	T
Shanbali	9	17	12	38	0.46	0.54
Kahli	7	13	25	45	0.30	0.70
Arabi	4	10	12	26	0.35	0.65
Lahaoi	14	34	31	79	0.39	0.61
Total	34	74	80	188	0.38	0.62

$\chi^2=4.97$ - $p \leq 0,05$ - d.o.f.=1

Table 4. Genotyping data and allele frequency of the g.1029T>C SNP at the *CSN3* promoter gene in Sudanese *Camelus dromedarius* population.



Fig. 1. Locations of sample collection from Sudan: 1. El Shuak (El Gadarif State); 2. west Omdurman (Khartoum State); 3. El Obeid (North Kordofan State); 4. Nyala (South Darfur State); 5. Tamboul (El Butana).

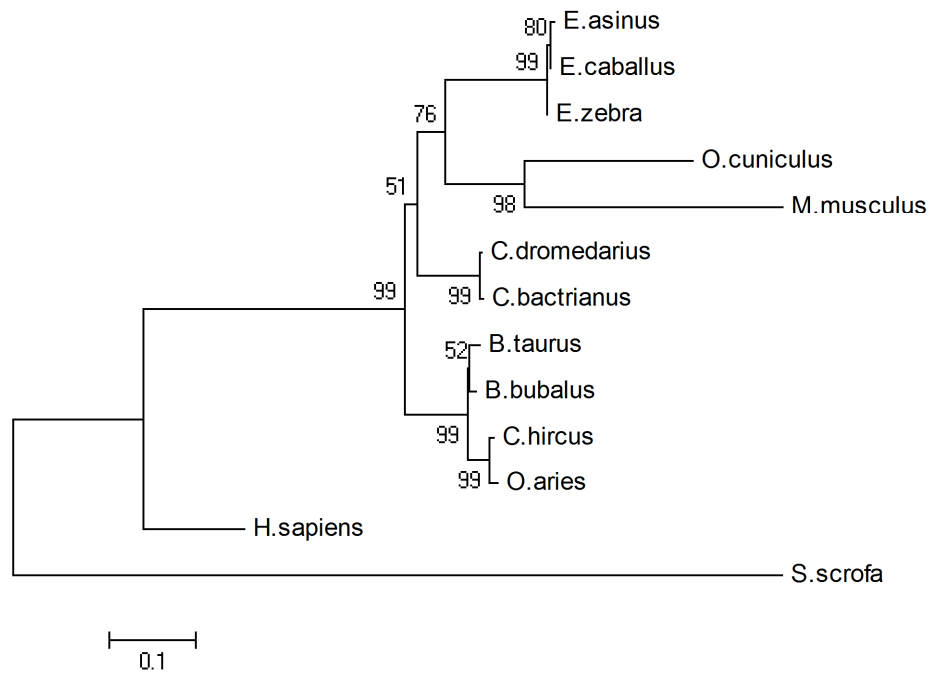


Fig. 2. Evolutionary relationships of 13 taxa for *CSN3* gene promoter. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

ACTCCTAACTTTCTAAGACATGAAATACCTCTAAGAA...GGAAGTACAA...CACAATGCTGTTATTATTTC -974
 ----- -974
 ----- -972
 GT-----G-----CA-----GAATAACT-----AC----- -979
 -1077

 CB1 CB2 CB1
 TTGTTTGTCTCTGGCAGTCCT.CATTGATTCCTGTAAATACCTCAATCTGGCCAAGGACTTCATAGCTAAGGTGAAGTC -895
 ----- -895
 ----- -893
 -----A-----G-T-A-----C-----T-----C-T-A----- -900
 -----A-C-----A-CA-----...TC-C-T---GTG-----..-AA----- -1004

 -BP C/EBP-α Oct-1 MGF/STAT5 Oct-1
 ACAGTTAACAATTTTTTCCCCAGAGAAA TGTATGCAAAAGAAAATATTCTTCTCTGAATCATCTAAGCAAATTTTGGT -815
 ----- -815
 ----- -813
 -----C-C-A-----TC-----T-----G-----C----- -823
 -----G...G-----ACG-----CG-G-----TC-T---T-G--G-A----- -928

 CB4 YY1
 TAGCTATATTTTACCAAATATCTCCCATATTTGGTAGTTTATGATATATACTTTGTAAGTTAGAATGAGCTGTCTTTGA -735
 ----- -735
 ----- -733
 -----G-----C-T-----G-----G-----C-----GC-----TC----- -743
 -----CGAG-----T-----C-C-----G-C-----A-----T-----C-----A-C----- -849

 C/EBP-α
 AACAAAACAATTATT.CTGAATTCAA.TAATTAAATTTGTATTTCCCGAATGTTTCACCTGTATTATTGAAATTTAAAA -657
 ----- -657
 -----G----- -654
 -----G-----G-CC-----G.C-----CAC-A-----GA-----C-----C-A----- -666
 -----G-----G-T-----CA-A-----A-----A-----C----- -771

 ATCTAAATGCAACATT.....GAT.TCATAAAAGGTTAATCAATCAGCA.....AGTATACATTGA -602
 ----- -602
 ----- -599
 -----G-AA---T-C-----G-----G-G---A-----T---A----- -611
 -----G-A-G---A-AAATGCTGAAGAA---G-G-AG---GG---CTC-TA---CTGTTGGTGGC-A-G---A-C---C -691

 A.....TGT.....TTATA.....CCATGAACCTACTGAAGAATA.....AAAT..... -568
 ----- -568
 ----- -565
 -----C-----G-T---G-----TATGT.TGCAGCTC----- -564
 -GAGCCAC---GGAGAACA---GGAGATTCCTT---GA---T-A-AAA---A-A-TCTATGTGCAACTTG-T-CATAAGAA -611
 * **
 YY1-rev HNF-1 Oct-1
GCAAATGGCTTTGCTAC.....TTTAT.....ATTGATTAAAA.....TATTTTCATATT..T -523
 ----- -523
 ----- -520
 -----CCC.T-T-----GC-----G-----C----- -520
 ACTAATCAATCAA---CA-G-G-TA---TGATGAA---CTGAAGA-CAA-CG---ATGGATCCC---C-T---GA- -531

 YY1-rev C/EBP-α
 AGAT.....TTAACTTGATGAAGTTGCTCTTGGGTATTTAAGAAATGGTCTGTTTTCTAATTTCTAAAAATCA -454
 ----- -454
 ----- -451
 G-----G-----AA---C-----G-----G-----G-A-----G---T-----TG--- -450
 TA---ATTTTCATATT---G-T---AACAT---A---AT-----C-A---A-G-----A-----GT---T-CTG -452
 **
 TBP
 ATGATTGTAATTCTGGAAGGAAGATGACCAACCACAGCCCATAATATATATAGAGGGTCACTTGATATCCAGGTTCTTAA -374
 ----- -374

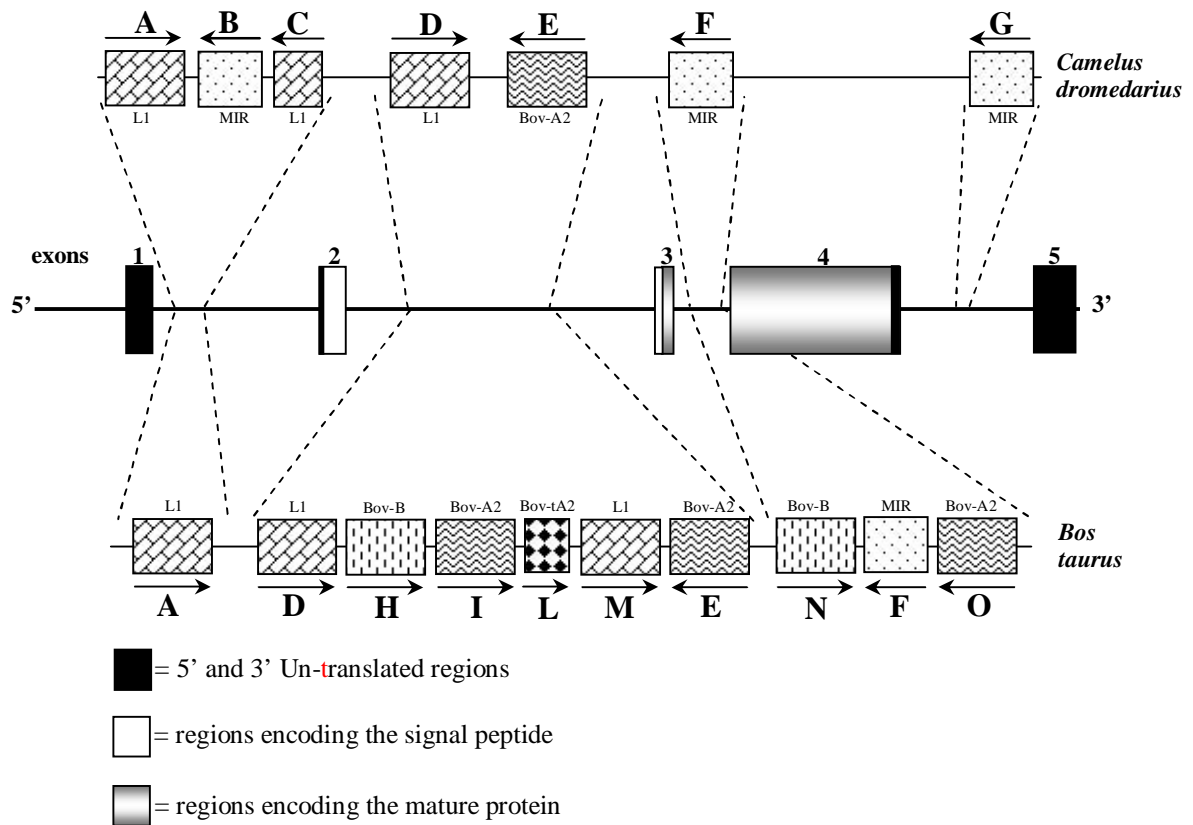


Fig. 4. Schematic representation of the *CSN3* gene and of the interspersed elements observed in camel and cattle. Common interspersed elements are indicated with the same letter.

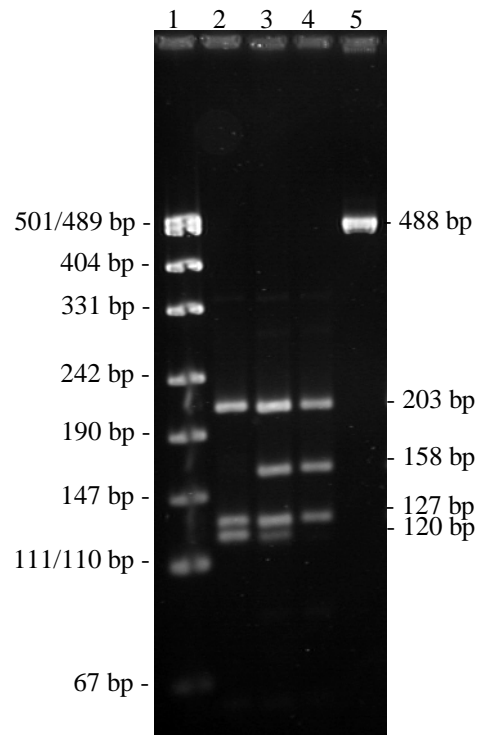


Fig. 5. Genotyping of *C. dromedarius* *CSN3* g.1029T>C SNP by Alu I (AG↓CT) PCR-RFLP. Line 2: CC samples; line 4: TT samples; line 3: CT samples; line 5: undigested sample. In the line 2 and 3 the band 38 bp long is not visible. Line 1 is pUC 19 DNA/MspI (HpaII) Marker, 23 (Fermentas).