

Sequencing and Bioinformatics Analysis of Kappa-Casein Exon 4 Gene in Iranian *Bactrianus* and *Dromedaries* Camels

Research Article

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ABSTRACT

Kappa-casein, as a major protein component in mammalian milk, plays an essential role in formation and stabilization milk micelles and preventing them from aggregating and therefore, helping to keep calcium phosphate in solution and transfer of calcium and phosphors from animal milk to consumers. Therefore, the objective of the current study was to investigate genetic and phylogenetic analysis of exon 4 of k-casein (CSN3) gene in Iranian camels using 10 blood samples from Dromedary camels and 5 blood samples from Bactrian camels. After DNA extraction, partial DNA fragment (713 bp) of the k-casein gene was amplified and sequenced using specific primers by PCR reaction. Obtained sequences were edited and subsequently were compared with other k-casein gene sequences which were submitted in NCBI data base. The results showed that there were no significant variations among analyzed sequences. Furthermore, the Neighbor-Joining phylogenetic analysis showed that according to k-casein gene sequence, Iranian Dromedary and Bactrian camels have the lowest genetic distance with *Lama* species.

KEY WORDS bioinformatics analysis, *Camelus bactrianus*, *Camelus dromedaries*, κ-casein gene.

INTRODUCTION

Camels belong to the family of Camelidae, suborder of Tylopoda, order of artiodactyla and class of mammals (Schwartz, 1992). The family Camelidae has two old world species, double-humped camel (*Camelus bactrianus*) and single-humped camel (*Camelus dromedaries*) and four new world (tribe Lamini) species, guanaco (*Lama guanicoe*), llama (*Lama glama*), alpaca (*Lama pacos*) and vicuna (*Lama vicugna* or *Vicugna vicugna*) at present time (Wilson, 2005; Wilson, 1984). The single-humped camel inhabits Afro-Arabia, Ethiopia and west Central Asia while the double-humped inhabits eastern Central Asia and China (Cui *et al.* 2007). Camel has been historically and economically an important species worldwide especially in the Africa and Asia. Camel has unique characteristics enable it to

adapt its desert environment (Schwartz, 1992; Nagarajan *et al.* 2012; Pauciuolo *et al.* 2012). The total worldwide camel population at present estimated to be about 23 million in the world. Somalia and Sudan together hold approximately 50% of the whole camel population. In the last 40 years, the number of camels has increased by almost 45% (Pauciuolo *et al.* 2012). In 2010 nearly 5.25 million camels were producing 2.12 million tons of milk which is an important indicator for its role as protein source for humans (Giambra *et al.* 2013). Iranian single-humped and two-humped camels exist in the number of 150000 and 100 heads respectively. Two-humped camel's population in Iran is very limited and only found in north western of Iran (Ansari- Renani *et al.* 2010). Polymorphism of the blood groups, enzymes and milk proteins could be used as biological tools to improve the genetic merit in animal breeding studies. Researchers

have shown that there are relationship between diversity in milk protein and genetic variations (Ikonen *et al.* 2008; Schlee *et al.* 1992). In this regards, there are some studies which evaluated the associations between milk protein variability and milk performance traits in ruminants (Giambra *et al.* 2013). Casein is a glycosylated protein belonging to a family of phosphoproteins (α s1, β , α s2, κ) that represents the major protein component in mammalian milk (approximately 80%) (Giambra *et al.* 2013; Ikonen *et al.* 2008; Medrano *et al.* 1990). K-casein is a mammalian milk protein involved in a number of important physiological processes (Lutfuaalah *et al.* 2011). Besides, κ -casein stabilizes milk micelles, preventing them from aggregating and therefore, helping to keep calcium phosphate in solution and transfer of calcium and phosphors from animal milk to consumers (Ikonen *et al.* 2008). The length of camel κ -casein gene is 13000 bp and contains of 5 exons and 4 introns. Exon 4 is the longest (approximately 494 bp) and the most important exon in the mature protein (Schlee *et al.* 1992; Pauciullo *et al.* 2012). Allelic polymorphism studies in κ -casein gene usually have been focused on farm animals particularly on bovine. In this regards, screening for mutations in exon IV of the bovine CSN3 gene determine two allelic variants, A and B. These variants were distinguished by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis in the indigenous Sahiwal and Tharparkar cattle breeds (Rachagani *et al.* 2008). Pauciullo *et al.* (2012) characterized for the first time nucleotide sequence of the whole κ -casein-encoding gene plus 1045 nucleotides at the 5' flanking region in *Camelus dromedaries* (Pauciullo *et al.* 2012). They identified 17 polymorphic sites in camels for promoter of casein loci. The objective of this study was to investigate genetic and phylogenetic analysis of exon4 of κ -casein gene in Iranian camels.

MATERIALS AND METHODS

In order to investigate genetic and phylogenetic analysis of exon 4 of κ -casein (CSN3) gene in Iranian camels, blood samples from the jugular vein of 10 unrelated Dromedaries (Tapeh salam, Mashhad, Iran) and Bacterianus camels (Ardebil, Iran) at slaughterhouse were taken in tubes containing ethylenediaminetetraacetic acid (EDTA). The blood samples were numbered and labeled with full information and then was stored in -80 °C until DNA extraction. Genomic DNA was extracted from 100 μ L blood samples with Bioneer spin-column kits according to the manufacturer's instruction (Bioneer DNA Extraction Kit, Cat .NO .K-3032, USA). The quality of the extracted DNA was analyzed by electrophoresis on 0.8% agarose gel and the purity of the obtained DNA was verified by NanoDrop ND-2000 spec-

trophotometer (THERMO, America), then the DNA were stored at -20 °C until PCR analysis.

To amplify the fragment of CSN3 gene (Part of the intron 3, whole of the exon 4 and Part of the intron 4), the specific primers:

CSN3-F: 5'GCTGAAAATCAAGAAGTGAAAGG'3 and

CSN3-R: 5'TTTG TGTTTTGCTCATT TACCTG'3

were designed by using Primer Premier 5 (www.PremierBiosoft.com) according to the available nucleotide sequence on the NCBI GenBank database (Accession no: HE863813) and synthesized commercially (MACRO GEN). The PCR reaction was carried out using the Personal Cycloer™ thermocycler (Biometra, Germany) in a final volume of 25 μ L, containing 100 ng DNA template, 1 unit of Taq DNA polymerase, 0.5 μ L dNTPs (10 mM), 1 μ L MgCl₂ (50 mM), 1 μ L of mix primer (5 pmol), 2.5 μ L of 10X PCR buffer and 18 μ L deionized water. Details of the PCR program was as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 45 sec. A final extension period of 72 °C for 10 min then was performed.

After the PCR reaction, the PCR products were analyzed in 1.5% agarose gel electrophoresis in TBE buffer with ethidium bromide staining. The purified PCR products were extracted from the gel by Ron's Agarose Gel Miniprep Kit (BioRon, Germany) according to the manufacturer's instruction and were subjected to sequencing and the primers for PCR reaction were used for sequencing (Bioneer, Korea). The nucleotide sequences obtained were edited using the PHRED software (<http://www.phrap.org/phredphrapconsed.html>). After editing, basic local alignment search tool (BLAST) were used in order to find the homology of sequences. Further analysis of the sequences were carried out, by using the other software's such as Chromas Lite (<http://www.techneylum.com.au>), Bio Edit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) and the obtained sequences, were aligned with other exon 4 CSN3 gene of camel and other species, using CLC Main workbench 5.5 software (<http://www.clcbio.com>). The sequences were conducted using the maximum composite likelihood method by MEGA software (www.megasoftware.net, version v.5.2). Phylogenetic tree was constructed using the Neighbor-Joining method by the same software.

RESULTS AND DISCUSSION

DNA was successfully extracted from all samples. The quality and quantity of extracted DNA were confirmed. The PCR products were assessed by electrophoresis on 1.5% agarose gel and visualized under UV light.

A 713 bp specific fragment of k-casein (CSN3) gene which successfully amplified is shown in Figure 1.

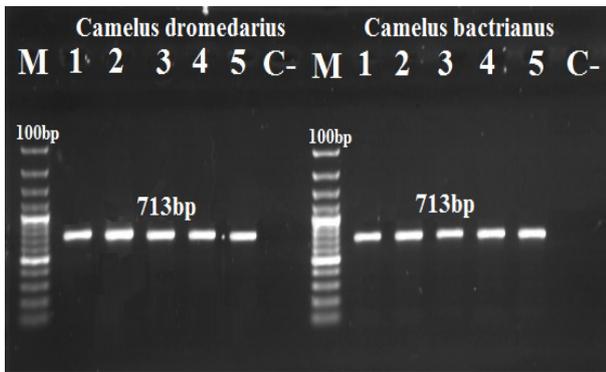


Figure 1 PCR products of fragment of CSN3 gene on agarose 1.5%. M: M100 ladder, 1 to 5 column: amplified CSN3 fragment in Iranian Dromedarius and Bactrianus camel, C: PCR reaction without DNA as template

The sequencing of the PCR products with specific primers was performed and the sequences were analyzed using Chromas Lite and Bio Edit software program (Figure 2).

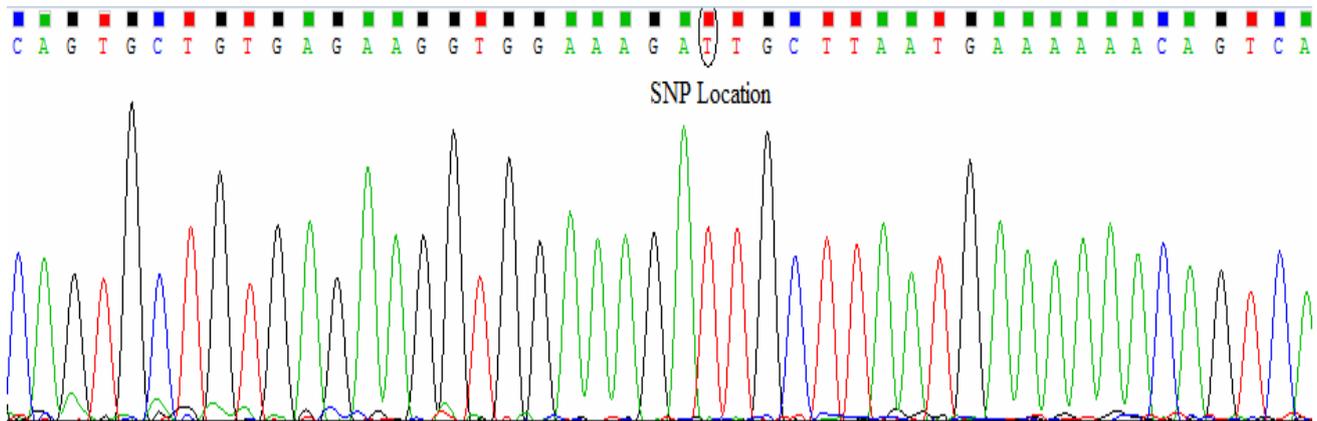


Figure 2 The chromatography of the obtained sequence

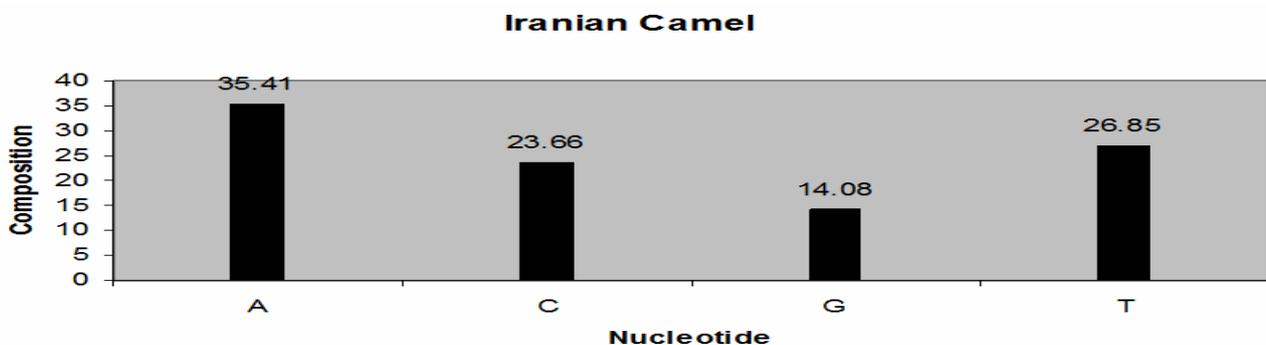


Figure 3 Estimated percentage of nucleotides in CSN3 gene of Iranian camels

Some studies completely sequenced the CSN3 exon 4 from sheep, Pauciuillo *et al.* (2012) sequenced the whole k-casein gene from camel, sequenced the CSN3 exon 4 from horse.

The content of nucleotide sequences (Figure 3) showed that the estimated frequencies of C + G and A + T were in the range of 37.74 and 62.26 percents, respectively. These results were comparable with other submitted sequences in NCBI for exon 4 of CSN3 gene (494 bp). High level of homology was obtained in the Camelidae family (Table1). Pauciuillo *et al.* (2012) report percents of the T/A contact between bovine and camel also they report. The camel CSN3 gene is also characterized by high A/T content compared to G/C (69.6% vs. 30.4%). Some studies report this feature seems to be conserved among the species.

The nucleotides analysis of Bactrianus and Dromedaries camels, using disparity index analysis of mega version v.5.2 software program, did not show any differences between these two sequences (P=0). Consequently, no haplotypes were identified from these sequences.

Comparing the exon 4 of k-casein (CSN3) gene with other reported sequence in this region in camel (Accession no: HE863813) showed 98% homology.

The sequence analysis of Dromedaries camels (10 samples) indicated one single nucleotide polymorphism (SNP) in intron 3 region of sample 7, (Figure 4-a) and one change of amino acid in exon 4 of sample 3 (Figure 4-b). For Bactrian camels (5 samples), the nucleotide analysis showed one SNP in exon 4 (Figure 5).

These SNPs in intron 3 and exon 4 of Dromedaries camels were reported for the first time. The details of these SNPs are listed in Table 2. Some studies report two SNP in exon 4 in kappa casein in hours. Pauciullo *et al.* (2012) report three SNP in Exon4 in kappa casein in camel.

According to the Table 3, Iranian camels showed the lowest genetic distance (0.031) with lama (*Lama guanicoe*) that is in line with the results of phylogenetic tree illustrated in Figure 6.

Table 1 Estimated A/T and G/C nucleotide content of the k-casein gene in different species

Species	Accession no	T/A	G/C
Bos grunniens	AF030327	56.97	43.03
Bubalus bubalis	FJ770200	57.11	42.02
Camelus bactrianus	AF165632	61.29	37.33
Camelus dromedarius	Y10082	61.35	38.65
Giraffe	GCU53886	60.8	39.2
Ovis aries	AY670679	55.01	44.99
Lama guanicoe	LGU53890	64	36
Iranian camel	KF998219	62.26	37.74

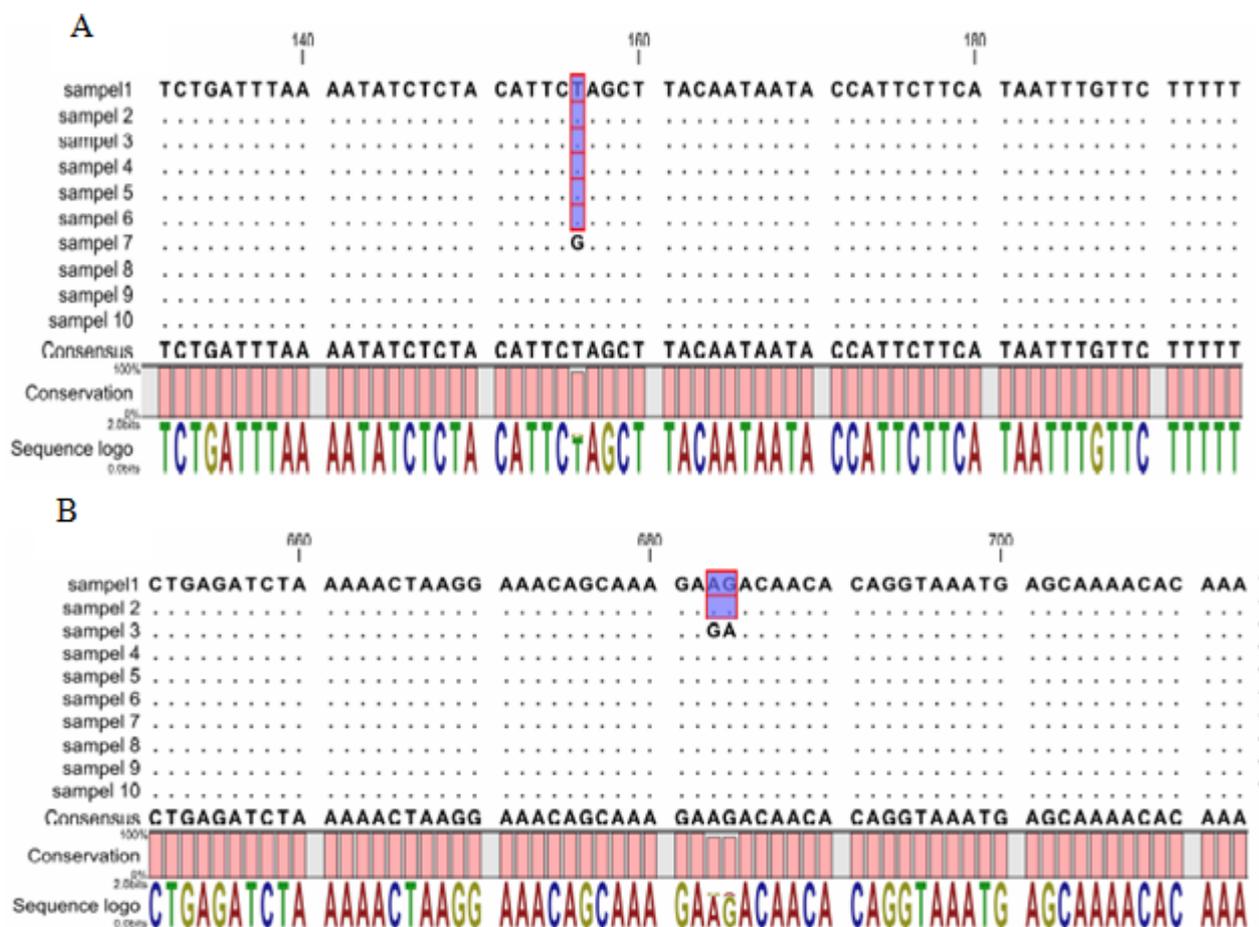


Figure 4 Alignment of nucleotide sequences of *Camelus dromedaries* A: intron 3, B: exon 4

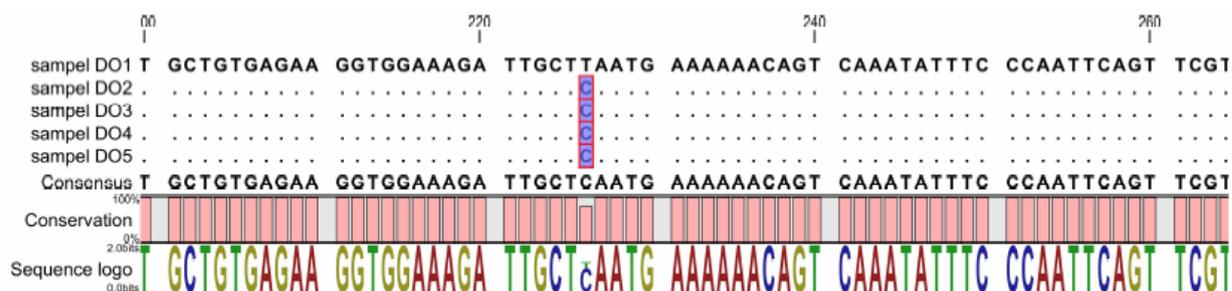


Figure 5 Alignment of nucleotide sequences of *Camelus bactrianus*

Table 2 The details of the SNPs

Location	Animal	Position	I
Intron 3	<i>Camelus dromedaries</i>	156	G
Exon 4	<i>Camelus dromedaries</i>	684/683	G/A
	<i>Camelus bactrianus</i>	226	T

Table 3 Genetics distance between sequences of the Iranian Bacterianus and Dromedaries camels and other species

	Accession no	1	2	3	4	5	6	7	8	9	10	11	12	13	14
<i>C. dromedarius</i>	KF998219	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. bactrianus</i>	KF998220	0.000	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. dromedarius</i>	Y10082	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. bactrianus</i>	AF165632	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
<i>L. guanicoe</i>	LGU53890	0.031	0.031	0.031	0.031	-	-	-	-	-	-	-	-	-	-
<i>E. zebra</i>	EU448385	0.220	0.220	0.220	0.220	0.236	-	-	-	-	-	-	-	-	-
<i>E. caballus</i>	AF165600	0.225	0.225	0.225	0.225	0.242	0.015	-	-	-	-	-	-	-	-
<i>C. hircus</i>	AF486523	0.289	0.289	0.289	0.289	0.296	0.291	0.302	-	-	-	-	-	-	-
<i>O. aries</i>	AY670679	0.301	0.301	0.301	0.301	0.303	0.308	0.302	0.021	-	-	-	-	-	-
<i>B. bubalis</i>	FJ770200	0.309	0.309	0.309	0.309	0.306	0.287	0.298	0.057	0.067	-	-	-	-	-
<i>B. indicus</i>	EU295526	0.312	0.312	0.312	0.312	0.320	0.306	0.312	0.085	0.088	0.047	-	-	-	-
<i>E. asinus</i>	EU448385	0.315	0.315	0.315	0.315	0.312	0.318	0.329	0.097	0.107	0.078	0.103	-	-	-
<i>C. elaghus</i>	CEU37504	0.315	0.315	0.315	0.315	0.312	0.318	0.329	0.097	0.107	0.078	0.103	0.000	-	-
<i>C. duvauceli</i>	CDU37507	0.316	0.316	0.316	0.316	0.312	0.309	0.321	0.086	0.096	0.068	0.093	0.021	0.021	-
<i>B. taurus</i>	AJ841946	0.317	0.317	0.317	0.317	0.325	0.310	0.317	0.086	0.092	0.050	0.003	0.107	0.0107	0.096

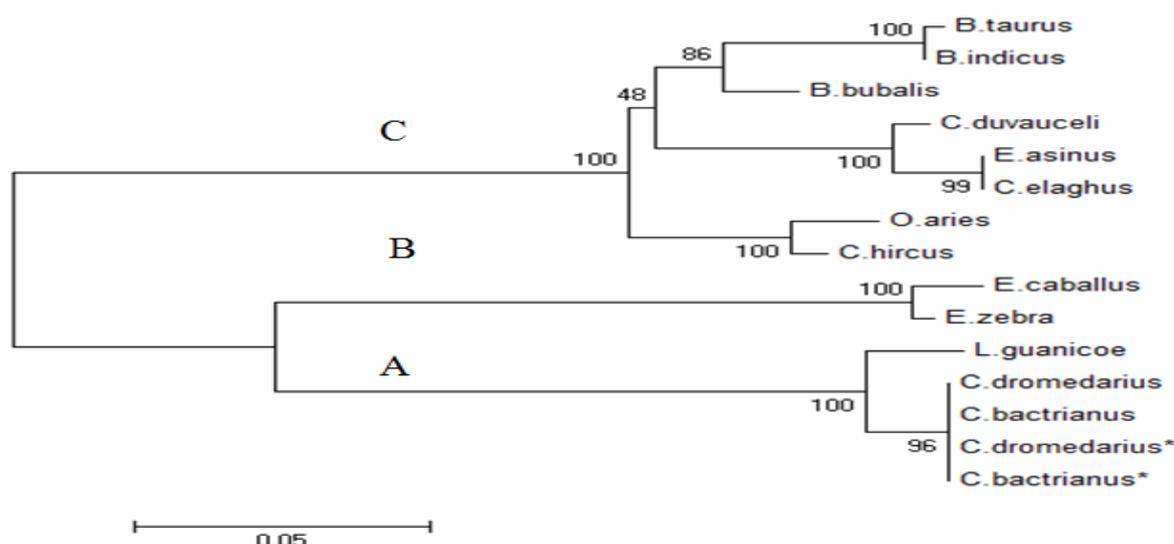


Figure 6 Phylogenetic tree of promoter region of CSN3 in Iranian Bactrianus and Dromedaries camels*

CONCLUSION

Exon 4 of k-casein sequence analysis shown that Iranian Dromedarius and Bactrianus camels had high level homology in sequence and nucleotide content. SNP screening in exon 4 of CSN3 gene in Iranian Dromedarius and Bactrianus, indicated that there were 3 and 1 single mutations in sample population in this study, respectively. In the light of above facts, increasing the number of camel population in future study could identify more SNP in exon 4 of CSN3 gene which may be useful in milk camel breeding strategies.

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