

Genetic variations of β - and K-casein genes in Egyptian sheep breeds

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ABSTRACT

Objective: Casein genetic polymorphisms are important and well known due to their effects on quantitative traits and technological properties of milk manufacturing. The casein fraction of ruminant milk proteins consists of four caseins, namely α s1-, α s2-, β - and K-casein. At the DNA level, polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) allow for the simultaneous typing of several alleles at casein loci, as well as the detection of unknown polymorphisms. The genetic polymorphism of two ovine milk protein casein genes, β - and K-caseins, was studied in sheep animals belonging to three main breeds reared in Egypt (Rahmani, Barki and Ossimi), as a tool for genetic improvement of milk trait characteristics.

Methodology and results: SSCP of β -casein exon 7 revealed two different patterns in eighty-five tested animals. The sequence analysis of the PCR product (299-bp) of these two different patterns showed two single nucleotide substitutions; A→C and C→T without any amino acid exchange. The frequencies of these two different patterns were 96.67% and 3.33% in Rahmani; 65.52% and 34.48% in Ossimi and 88.46% and 11.54% in Barki, respectively. The nucleotide sequence of β -casein in Egyptian sheep was submitted in database NCBI/ GenBank with the accession number **JX080379**. The polymorphism of K-casein gene was also detected in eighty-six animals using PCR-SSCP technique. PCR amplified a fragment with 406-bp in exon 4 of this gene. SSCP results showed that all tested sheep animals are monomorphic. The alignment between our sequences with published sequence revealed two nucleotide substitutions; C→T and T→C. The nucleotide sequence of K-casein in our tested animals was submitted in database NCBI/ GenBank with the accession number **JX050176**.

Conclusion and application of findings: This study aimed to identify the genetic polymorphism of β and K-casein genes, which are strongly related to economically important milk quantitative traits in some Egyptian sheep breeds, as a tool or genetic markers for improvement of these breeds.

Key words: Sheep, β -casein, K-casein, PCR-SSCP, SNP.

Accession numbers: The nucleotide sequence data were submitted to nucleotide sequences database NCBI/ Bankit/ GenBank with the accession numbers: JX080379- JX050176

INTRODUCTION

More than 95% of the proteins contained in ruminants' milk (mainly goats, sheep and cattle) are synthesized from 6 structural genes encoding proteins: α -lactalbumin and β -lactoglobulin, the two main whey proteins in ruminants, and the four caseins α s1, α s2, β and K which are encoded by four tightly linked and clustered genes (Ferretti *et al.*, 1990 and Threadgill and Womack, 1990). Casein genes form a cluster in a 250-kb genomic DNA fragment, where α s1- is very close to β - followed by α s2- and K-casein (Marletta *et al.*, 2005). The genetic polymorphism of milk proteins has been of considerable interest in animal breeding and in the dairy industry. Studies on sheep milk protein polymorphism and its effects on milk yields of sheep are principally carried out in the Mediterranean countries, in which great importance is attached to milk performance (Mroczkowski *et al.*, 2004). Some evidences indicated that ovine genetic polymorphisms affect the physicochemical properties of milk (Rampilli *et al.*, 1992 and Pirisi *et al.*, 1999). Several studies have been carried out on cattle and goats (Dayal *et al.*, 2006; Jain *et al.*, 2009; Ma *et al.*, 2010). In this respect, a more in-depth knowledge of the genetic polymorphism of ovine milk proteins and their impact on ovine milk technological properties is essential for the improvement of the quality of ewe's milk cheese (Amigo *et al.*, 2000). β -casein is the most abundant casein with an average content of at least 50% in sheep (Dove, 2000). It was believed that β -casein had only a non-genetic polymorphism due to varying degrees of phosphorylation until Chianese (1997)

MATERIALS AND METHODS

Animals and genomic DNA extraction: Whole blood samples were collected from sheep animals belonging to three main sheep breeds reared in Egypt (Rahmani, Barki and Ossimi). The blood samples were collected from different farms belonging to Animals Production Institute. Genomic DNA was extracted from the whole blood according to the method described by Miller *et al.* (1988) with minor modifications.

differentiated between three genetic variants of β -casein designated A, B, and, C. The only sequence difference found between A and C was the amino acid substitution of Glu at position 2 in variant A for Gln in variant C but no sequence data for the B variant.

Kappa casein is highly heterogeneous, soluble in the presence of calcium and differs considerably in structure from the calcium sensitive caseins (Fox and McSweeney, 2003). It is essential for micelle formation and stabilization, and influences the manufacturing properties of milk. Cheese making is based on the cleavage of the k-Casein Phe¹⁰⁵-Met¹⁰⁶ peptide bond by enzymes or heat (Yahyaoui *et al.*, 2001).

Although K-casein is widely polymorphic in cattle with six variants characterized by Kaminski (1996) and two variants localized in the N-terminal region of the protein in goat (Di Luccia *et al.* 1990 and Law & Tziboula, 1993), it is considered to be monomorphic in sheep (Yahyaoui *et al.*, 2001). The present study is focusing on the genetic polymorphism of two major fractions of casein protein genes, which are strongly related to economically important milk quantitative traits in some Egyptian sheep breeds, as a tool for genetic improvement of these breeds. On the other hand, promoting selection programs that are depending on the use of genetic markers will help improving breeding strategies. This study aimed to identify the genetic polymorphism of β - and K-casein genes in three main Egyptian sheep breeds- Rahmani, Barki and Ossimi- using PCR-SSCP technique.

Polymerase Chain Reaction (PCR): The DNA fragments of the studied genes were amplified through PCR technique developed by Mullis *et al.* (1986). A PCR cocktail consisted of 1.0 M upper and lower primers specific for the tested genes (Table 1), 0.2 mM dNTPs and 1.25 units of *Taq* polymerase. The cocktail was aliquoted into PCR tubes with 100 ng of sheep DNA. The reaction was cycled according to the specific protocol suitable for each primer (Table 1). PCR was

performed using MJ research PTC-100 thermocycler, the amplification was verified by electrophoresis on 2% agarose gel (w/v) in 1x TBE buffer using 100-bp ladder. The gel was stained with ethidium bromide (1 µg/µl) and visualized on UV transilluminator.

SSCP analysis: PCR products were resolved by SSCP analysis according to the method of **Orita *et al.* (1989)**. Each PCR product was diluted in denaturing solution, denatured at 95°C for 5-8 min, chilled on ice and resolved on polyacrylamide gel. The SSCP conditions for the tested genes are presented in **Table 2**. Electrophoresis was carried out in a vertical unit in 1x

TBE buffer and the gel was stained with silver staining, where the method of **Bassam *et al.* (1991)** was used with some modifications.

Sequence analysis: The PCR products representing different patterns of the tested β- and K-casein genes were purified and sequenced by MacroGen Incorporation (Seoul, Korea) to identify the SNPs between different patterns. Sequence analysis and alignments were carried out using CLUSTAL-W (**Gasteiger *et al.*, 2003**).

Table 1: The sequences and information of primers used in this study

Tested gene	Primer sequences 5' ----- 3'	PCR conditions	PCR product size	Reference
β-casein	CGT GCT GTC CCT TTC TC GTT TTC CAG CTT ATT CTA TTT AT	30 cycles 94°C for 45s 58°C for 45 s 72°C for 3min	299 bp	Ceriotti <i>et al.</i> (2004)
K-casein	GGT ATC CTA GTT ATG GAC TCA AT GTT GAA GTA ACT TGG GCT GTG T	35 cycles 94°C for 1min 59°C for 45s 72°C for 3min	406 bp	

Table 2: SSCP conditions for the tested genes

Tested gene	Gel composition	Denaturation conditions	Reference
β-casein	Acrylamide: Bisacrylamide gels: 29:1 Polyacrylamide: 10 % Glycerol: 0.5% Current: 280V, 20mA Time of run: 15h Temp: 4°C	12µl of PCR product, 16µl of denaturing solution 95°C for 7min.	Ceriotti <i>et al.</i> (2004)
K-casein	Acrylamide: Bisacrylamide gels: 29:1 Polyacrylamide: 9.25% Glycerol: 1% Current: 340 V, 15mA Time of run: 25h Temp: 4°C	12µl of PCR product, 16µl of denaturing solution 95°C for 8min.	

RESULTS and DISCUSSION

Casein genetic polymorphisms are important and well known due to their effects on quantitative traits and technological properties of milk (**Ceriotti *et al.*, 2004**). Globally, research on the polymorphism of ewes' milk is not yet as extensive as this in cows or goats (**Hristova, 2011**). Caseins, in particular, have been proposed as

polymorphic markers for the selection in order to improve the yield and the quality of cheese (**Bonifácio *et al.*, 2001**). In this respect, **Othman *et al.* (2012)** studied the genetic polymorphisms of whey protein genes, α- latalbumin and β- lactoglobulin, in three sheep breeds reared in Egypt; in addition to another

study in the same field analyzing the genetic polymorphism of α s1- and α s2-casein genes in Egyptian sheep using PCR-SSCP and PCR-RFLP, respectively (Othman *et al.*, unpublished data). With the goal of milk trait improvement for Egyptian sheep, the present study was performed to investigate the genetic polymorphism of β - and K-casein genes. The study was carried out on eighty five animals belonging

to three major native sheep breeds reared in Egypt; Barki, Rahmani and Ossimi. Sequence analysis of different patterns and alleles was done to identify the single nucleotide polymorphism (SNP) for each gene.

β -casein gene: PCR-SSCP technique was used to detect the polymorphism of β -casein gene in three Egyptian sheep breeds. PCR amplified a fragment of 299-bp in size (Fig. 1).

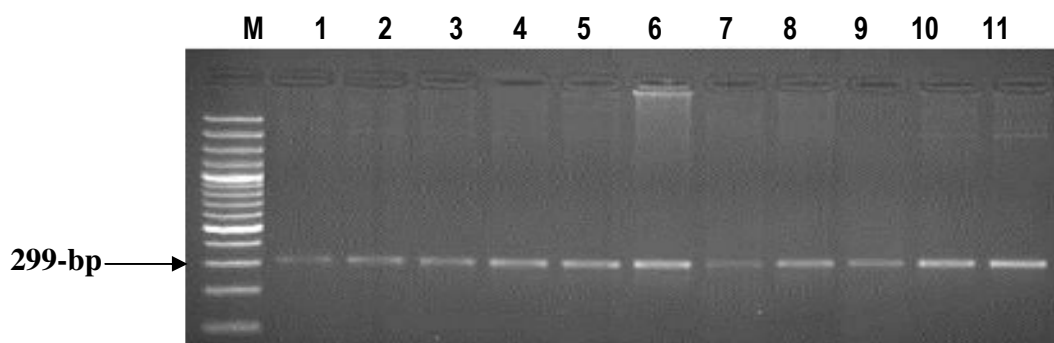


Fig. 1: Agarose gel stained with ethidium bromide showing the PCR product of β -casein gene
M: 100-bp ladder
Lanes 1-11: 299-bp PCR product of β -casein gene.

SSCP results recorded two different patterns in eighty-five tested Egyptian sheep animals (Fig. 2).



Fig. 2: Two SSCP patterns of β -casein gene for Egyptian sheep on 10% silver stained-polyacrylamide gel
Lanes 1 and 2: pattern I
Lanes 3-11: pattern II

Pattern I was recorded in this study with high frequency in all tested breeds; its frequency was around five times greater than pattern II. Within breeds, pattern I had higher frequencies (88.46%, 96.67% and 65.53%) than pattern II (11.54%, 3.33% and 34.48%) in Barki, Rahmani and Ossimi breeds, respectively. Rahmani breed possessed the highest frequency of pattern I

whereas Ossimi breed had the highest frequency of pattern II (Table 3). The sequence analysis of these two patterns showed the presence of two nucleotide substitutions: the first (A→C) at position 104 and the second (C→T) at position 193 (Fig. 3). The sequence was submitted to GenBank with the accession number **JX080379**.

Table (3): The pattern frequencies of the β -casein gene in three tested sheep breeds

Breeds	No. of Animals	Pattern frequencies			
		Pattern I		Pattern II	
		No. of animals	Frequency	No. of animals	Frequency
Barki	26	23	88.46%	3	11.54%
Rahmani	30	29	96.67%	1	3.33%
Ossimi	29	19	65.52%	10	34.48%
Total	85	71	83.53%	14	16.47%

Pattern I CGTGCTGTCCCTTTCTCAGCCCAAAGTTCTGCCTGTTCCCCAGAAAGCAGTGCCCCAGAG 60
Pattern II ***** 60

Pattern I AGATATGCCCATCCAGGCCTTTCTGCTGTACCAGGAGCCTGTAAATTGGTCCTGTCCGGGG 120
Pattern II *****C***** 120

Pattern I ACCCTTCCTATTCTTGTAAGTCTAAATTTACTAACCTGTGCTGTTAACTTCTGATTGT 180
Pattern II ***** 180

Pattern I TTGTATGATATTGGAGTAATTAAGAGCCCTACAAAAAATCAATAATGAATGGTTCCAAA 240
Pattern II *****T***** 240

Pattern I ATAAGCATAGCTGAGATTAATGATTCTCAGCATTAGTTATAAATAGAATAAGCTGGAAA 299
Pattern II ***** 299

Fig (3): ClustalW2 alignment of the sequences of two different patterns of β -casein gene in Egyptian sheep

The full sequence of ovine β -casein gene was published by **Provot et al. (1995)** with the accession number (GenBank: X79703.1). They reported that the gene is composed of nine exons that are short with the exception of exon 7 which is included in the coding region, and exon 9 (492- and 323-bp respectively). The present study was applied on exon and intron 7 of β -casein gene. The study recorded two patterns in sheep animals from different Egyptian native breeds, pattern I had higher frequency in all breeds. **Bastos et al. (2001)** and **Ceriotti et al. (2004)** also obtained two distinct patterns in β -casein exon 7, where **Ceriotti et al. (2004)** demonstrated that the two different patterns were corresponding to SNP (A→G) at position 12029 of the referring sequence (**Provot et al., 1995**), resulting in the deduced amino acid exchange Met¹⁸³→Val¹⁸³.

The sequence analysis of the present results showed different nucleotide substitution positions, (A→C) at position 12069 of the referring sequence, which deduced amino acid exchange (Iso¹⁹⁶→Leu¹⁹⁶) and (C→T) at position 12155 in intron 7. **Ceriotti et al. (2004)** and **Sztankóová et al. (2011)** recorded the A variant with greater frequency than G variant in the sheep breeds in their studies. The A variant was found in the two patterns of the present study.

K-casein gene: The polymorphism of κ -casein gene was detected in this study using PCR-SSCP technique. PCR amplified a fragment of 406-bp in size (**Fig. 4**). SSCP results for K-casein gene showed that all tested sheep animals (86 animals) are monomorphic and possess the same SSCP pattern (**Fig. 5**).

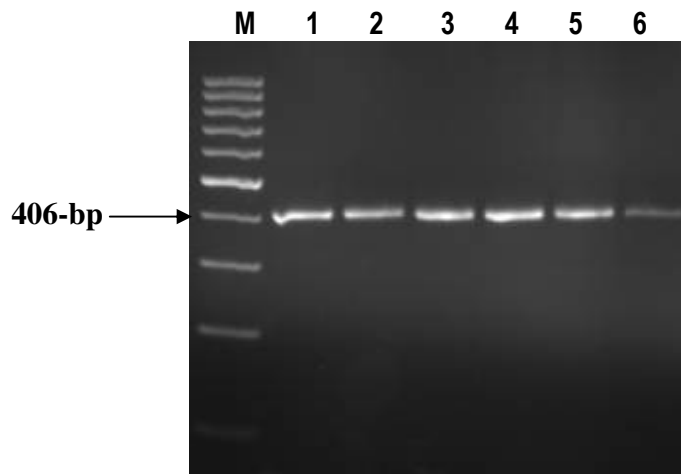


Fig. 4: Agarose gel stained with ethidium bromide showing the PCR product of K-casein gene. M: 100-bp ladder
Lanes 1-6: 406-bp PCR product of K-casein gene.

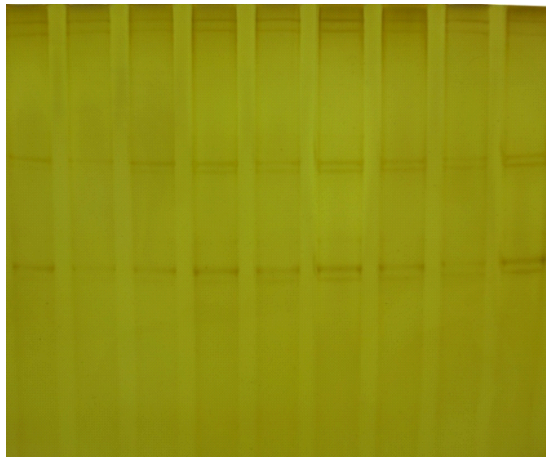


Fig. 5: SSCP pattern of K-casein gene for Egyptian sheep on 9.25% silver stained-polyacrylamide gel.

The sequence analysis of representative samples from the tested animals revealed that all tested animals had the same sequence without any nucleotide substitution. The nucleotide sequence was submitted to GenBank with the accession number **JX050176**. Alignment of the tested sheep sequence with K-casein sequence

published in database (accession number AY444505.1) showed the presence of two nucleotide substitutions, the first (C→T) at position 18 and the second (T→C) at position 164 (**Fig. 6**).

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AY444505.1  CTAATTAATAATCAATTTCTGCCATACCCATATTATGCGAAGCCAGTTGCAGTTAGGTCA 60
Our seq.    *****T***** 60

AY444505.1  CCTGCCCAAACCTCTTCAATGGCAAGTTTTGCCAAATGCTGTGCCTGCCAAGTCTGCCAA 120
Our seq.    ***** 120

AY444505.1  GACCAGCCAACCTGCCATGGCAGTCACCCACACCCACATTATTTATTATGGCCATTCCA 180
Our seq.    *****C***** 180

AY444505.1  CCAAAGAAAGATCAGGATAAAACAGAAATCCCTGCCATCAATACCATTGCTAGTGCTGAG 240
Our seq.    ***** 240

AY444505.1  CCTACAGTACACAGTACACCTACCACCGAAGCAGTAGTGAACGCTGTAGATAATCCAGAA 300
Our seq.    ***** 300

AY444505.1  GCTTCCTCAGAATCGATTGCGAGTGCACCTGAGACCAACACAGCCCAAGTT 351
Our seq.    ***** 351

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Fig. 6: The alignment of the published K-casein sequence (GenBank accession no. AY444505.1) with Egyptian sheep K-casein sequence.

Although K-casein is widely polymorphic in cattle with six variants characterized by **Kaminski (1996)** and two variants localized in the N-terminal region of the protein in goat (**Di Luccia *et al.* 1990** and **Law & Tziboula, 1993**), it is considered to be monomorphic in sheep (**Yahyaoui *et al.*, 2001**). The present study examined the genetic polymorphism in exon 4 of K-casein. The results showed that there was no difference recorded between the three breeds tested using SSCP technique. Also, **Bastos *et al.* (2001)** detected monomorphism in the same exon in Portuguese indigenous sheep breed. **Ceriotti *et al.* (2004)** recorded two different patterns of K-casein in three Italian sheep breeds; these differences are attributed to single nucleotide substitution C→T, resulting in the deduced amino acid exchange Ser¹⁰⁴ → Leu¹⁰⁴. The frequency

of pattern T was very low, although its frequency was higher in the study reported by **Feligini *et al.* (2005)** which was applied on Pag sheep using Real-time PCR but it is still lower than C pattern frequency. On contrast, the sequence analysis of the present results showed the dominance of allele C with the complete absence of allele T. The same result was recorded by **Sztankoova *et al.* (2007)** and **Sztankóová *et al.* (2011)** in Czech Sumava and Valachian breeds where the κ-casein locus was found to be monomorphic in these populations.

In conclusion, this study is considered to be a step forward for further studies that may contribute to give more information about the genetic polymorphism of Egyptian sheep milk proteins and the improvement of this economically important trait.

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