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Genetic Polymorphism of Whey Protein Genes *β-LG* and *α-LA* in Three Egyptian Sheep Breeds

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Abstract

The major whey proteins, β-lactoglobulin (*β-LG*) and α-lactalbumin (*α-LA*), are strongly correlated with the nutritional value and the functional properties of milk whey. Ovine whey has a unique protein composition compared to that of bovine whey and it is especially rich in proteins. Genetic polymorphism of *β-LG* using PCR-RFLP and *α-LA* using PCR-SSCP was analyzed in three native Egyptian sheep breeds named Barki, Rahmani and Ossimi.

Digestion of resulting amplified fragments of *β-LG* (452-bp) with *Rsa*I endonuclease differentiated between three different genotypes AA (69.77%), AB (26.74%) and BB (3.49%). The results showed that A allele was present in the Egyptian sheep breeds with a frequency (83.14%) higher than B allele (16.86%). A & B alleles appeared at frequencies 70.4 % & 29.6%, 95.0% & 5.0% and 82.8% & 17.2% in Barki, Rahmani and Ossimi, respectively

SSCP results of the *α-LA* amplified fragments (166-bp) showed that the three native Egyptian sheep breeds possess two different patterns related to the difference in nucleotide sequences with frequencies 88.1% and 11.9%. The frequencies of pattern 1 ranged from 81.48% in Barki to 89.66% in Ossimi and 92.86% in Rahmani breeds whereas the pattern 2 was present at frequencies 18.52%, 7.14% and 10.34% in Barki, Rahmani and Ossimi breeds, respectively

Key words: Sheep, *β-LG*, *α-LA,* RFLP, SSCP.

INTRODUCTION

Sheep dairying is often practiced alongside the cow milk industry due to the unique richness of sheep milk and its special value in human health (**Haenlein, 2006**). It has been suggested that the emphasis in the breeding of sheep should shift from wool to mutton and milk (**Gokhale, 2003**). Ovine whey is especially rich in proteins, the major whey proteins; β-lactoglobulin (*β-LG*) and α-lactalbumin (*α-LA*); are strongly correlated with the nutritional value and the functional properties of whey products (**Moatsou et al., 2005**).

Since the first report of genetic polymorphism of bovine β-lactoglobulin was published by **Aschaffenburg and Drewry (1955**), the genetic polymorphism of milk proteins has been of considerable interest in animal breeding and in the dairy industry, and possible relationships between milk protein polymorphism and production traits, milk composition, and quality have been widely studied (**Elmaci et al., 2006**).

β-Lactoglobulin is the major whey protein of ruminant milk; it is also found in several other species but is lacking in humans and rodents. It consists of 162 amino acids and forms stable dimmers in milk. Apart from its ability to bind and transport small hydrophobic molecules in milk, e.g. retinol and small fatty acids, its biological function is still unclear (**MacLeod et al., 1996**). Investigations in various countries have shown that *β-LG* whey protein is polymorphic in sheep **(Dario et al., 2005**; **Kucinskiene et al., 2005** and **Barillet et al., 2005**). Three genetic polymorphisms of *β-LG* (A, B, and C) have been reported in sheep (**Arora et al., 2010**).

The alpha-lactalbumin encoding gene is essential for the biosynthesis of lactose in the mammary gland and is a potential quantitative trait locus in dairy animals (**Jain et al., 2009**). Alpha-lactalbumin, a calcium metalloprotein, is one of the major serum-proteins in ruminant milk (**Jenness, 1982**) and induces lactose synthesis in the mammary gland by interacting with the enzyme UDP-galactosyl-transferase, giving rise to the heterodimer enzyme lactose synthase (**Kuhn, 1983** and **Hayssen and Blackburn, 1985**). Two variants of *α-LA*; A and B; have been detected in different sheep breeds and the variant B of α-*LA* seems to be rare and confined to very specific breeds (**Amigo et al., 2000**).

This study aimed to identify the genetic polymorphism of *β-LG* and *α-LA* genes in three native Egyptian sheep breeds; PCR-RFLP and PCR-SSCP were used to detect the genetic polymorphism within *β-LG* and *α-LA* genes, respectively.

MATERIALS AND METHODS

Animals

Whole blood samples were collected from sheep animals belonging to three main sheep breeds reared in Egypt. The blood samples were collected from different farms belonging to Animals Production Institute. The three breeds used in this study are, Rahmani (from Animal Breeding Research Station in Sero, Domiata), Barki (from Animal Breeding Research Station in Borg El-Arab, Alex) and Ossimi (Animal Breeding Research Station in Seds, Bani Swif). The samples were collected from both males and females at different ages.

DNA extraction

Genomic DNA was extracted from the whole blood according to the method described by **Miller *et al.* (1988)** with minor modifications. Briefly, 10ml of blood taken on EDTA were mixed with 25ml of cold 2X Sucrose-Triton and 15ml double distilled water. The tubes were placed on ice for 10 min and mixed by inversion several times. After centrifugation, at 5000rpm for 15min at 4ºC, the pellet was re-suspended by 3ml of nucleic lysis buffer. The content was mixed with 108µl of 20% SDS and 150µl of Proteinase K. The tubes were placed in a water bath at 37ºC overnight.

After the incubation, the tube contents were transferred to a 15-ml polypropylene tube and 2 ml of saturated NaCl was added and shaken vigorously for 15s. After centrifuging at 3500 rpm for 15 min at 4ºC, the supernatant was transferred to a clean 15-ml polypropylene tube and mixed with absolute ethanol. The tubes were agitated gently to mix the liquids and a fluffy white ball of DNA was formed. The precipitated DNA was picked up using the heat sealed pasture pipette, then washed twice in 70% ethanol and exposed to air to dry completely.

The DNA was dissolved in 200 µl TE buffer in 1.5-ml Microfuge tube and kept overnight in an incubator at 37ºC. DNA concentration was determined and diluted to the working concentration of 50 ng/µl, which is suitable for polymerase chain reaction using **NanoDrop1000 Thermo Scientific** spectrophotometer.

Polymerase chain reaction (PCR)

The DNA fragments of the studied genes were amplified through polymerase chain reaction technique developed by **Mullis *et al.* (1986).** A PCR cocktail consists of 1.0 mM upper and lower primers (specific for tested genes (**Table 1**), 0.2 mM dNTPs and 1.25 units of Taq polymerase. The cocktail was aliquot into PCR tubes with 100 ng of sheep DNA. The reaction was cycled for 35 cycles according to the specific protocol suitable for each primer (**Table 1**). The amplification will be verified by electrophoresis on 2% agarose gel (w/v) in 1x TBE buffer using **GeneRuler** 100-bp ladder as a molecular weight marker for confirmation of the length of the PCR products. The gel will be stained with ethidium bromide (1 µg/µl) and visualized on UV trans-illuminator.

Restriction fragment length polymorphism (RFLP)

RFLP technique was used to identify the genetic polymorphism of *β-LG* gene in eighty-six animals belonging to the three tested breeds. The restriction mixture was prepared by adding 10 units of the restriction enzyme *Rsa*I to 2.5 µl of restriction buffer and the volume was completed to 5 µl by sterile water. This restriction mixture was mixed with PCR product (20 µl) and incubated overnight at 37°C. The digested PCR product were electrophoresed on a polyacrylamide gel containing ethidium bromide and visualized on UV trans-illuminator.

Single strand conformation polymorphism (SSCP)

SSCP technique was used to identify the genetic polymorphism of *α-LA* gene in eighty-four animals belonging to the three tested breeds. PCR products will be resolved by SSCP analysis according to the method of **Orita et al. (1989)**. PCR product was diluted in denaturing solution, denatured at 95°C for 5 min, chilled on ice and resolved on polyacrylamide (14%). The electrophoresis will be carried in a vertical unit in 1x TBE buffer at 180 V for 16 h and the gel was stained with silver staining (**Bassam et al. (1991)**. The gel was visualized on light box and photo by digital camera.

Sequence analysis

The PCR products represent two different patterns of *α-LA* detected in this study were purified and sequenced by Macrogen Incorporation (Seoul, Korea) to identify the SNPs between these two patterns. Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite.

RESULTS AND DISCUSSION

The major whey proteins, β-lactoglobulin (*β-LG*) and α-lactalbumin (*α-LA*), are strongly correlated with the nutritional value and the functional properties (i.e. gelling, film-forming, foaming and emulsifying) of whey and whey products (**Kinsella and Whitehead, 1989** and **De Wit, 1989**). Ovine whey has a unique whey protein composition compared to that of bovine whey and it is especially rich in proteins (**Moatsou et al., 2005**).

Reported relationship between genetic variants of β-lactoglobulin and milk yield, milk composition and cheese-making ability in cattle (**Cubric-Curik et al., 2002**) have raised interest for the polymorphism identification of *β-LG* in dairy sheep populations. Although in recent years the effects of *β-LG* polymorphism on sheep milk production and manufacturing

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

Gene	Sequences 5′ -------- 3′	PCR conditions	PCR product size	References
β-LG	AAA AGC CCT GGG TGG GCA GC TTG GGT TCA GTG TGA GTC TGG	95°C for 50s. 65°C for 40s. 72°C for 40s.	452-bp	**Kučinskiene et al. (2005)**
α-LA	CTC TTC CTG GAT GTA AGG CTT AGC CTG GGTGGC ATG GAA TA	95°C for 30s. 62°C for 30s. 72°Cfor 30s.	166-bp	**Bastos et al. (2001)**

properties of the cheese have been extensively studied, the results are conflicting, indicating either superiority of a given of *β-LG* variant or absence of relationship (**Amigo et al., 2000**). However, there is an ongoing interest, especially in the Mediterranean countries where sheep are mainly kept for the production of cheese, concerning the genetic polymorphism identification of *β-LG* in native sheep breeds.

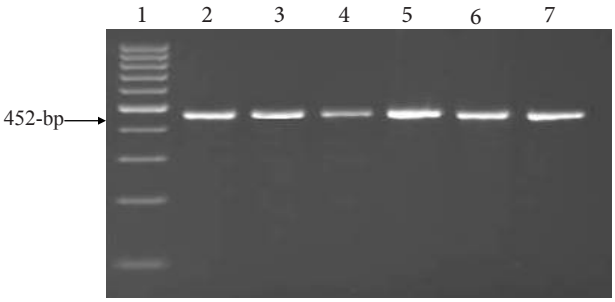


Fig. 1. Ethidium bromide-stained gel of PCR products representing amplification of *β-LG* gene in Egyptian sheep breeds.
Lane 1. 100-bp ladder marker.
Lanes 2-7: 452-bp PCR products amplified from Egyptian sheep DNA

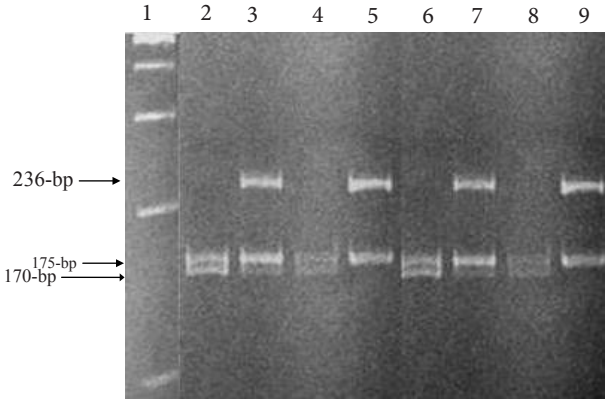


Fig. 2. The electrophoretic pattern obtained after digestion of PCR amplified sheep *β-LG* gene with *Rsa*I restriction enzyme.

Lane 1: 100-bp ladder marker.
Lanes 2, 4, 6 and 8: AA genotypes showed two restricted fragments at 175- and 170-bp.
Lanes 3 and 7: AB genotypes showed three restricted fragments at 236-, 175- and 170-bp.
Lanes 5 and 9: BB genotypes showed two restricted fragments at 236- and 175-bp.
* The small restricted fragments at 66- and 41-bp do not appear in the figure.

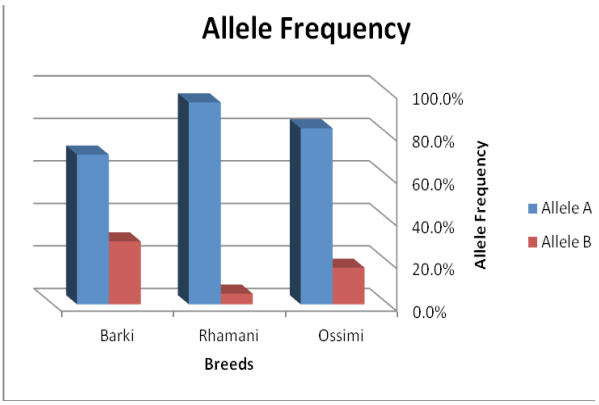
Investigations in various countries have shown that of *β-LG* whey protein is polymorphic in sheep (**Barillet et al. 2005**; **Dario et al. 2005**; **Elyasi et al. 2005**; **Kučinskiene et al. 2005** and **Cengiz et al. 2006**). Three genetic polymorphisms of *β-LG* (A, B, and C) have been reported in sheep. Allelic variants A and B, present in all breeds, differ by a Tyr/His substitution, where *β-LG* A has Tyr and *β-LG* B has His (**Kolde and Braunitzer 1983**). The rare variant *β-LG* C is a subtype of ovine *β-LG* A with a single exchange of Arg-Glu (**Erhardt 1989**).

In the present study, we used the PCR-RFLP technique to identify the genetic polymorphism of *β-LG* gene in three native Egyptian sheep breeds; namely Barki, Rahmani and Ossimi. The primers used in this study flanked a 452-bp fragment (**Fig. 1**) of *β-LG* gene including exon 2 and partially sequences of introns 1 and 2.

Digestion of the resulting amplified fragments with *Rsa*I endonuclease differentiated between three different genotypes: AA with four digested fragments at 175-, 170- , 66- and 41-bp, BB with three digested fragments at 236-, 175- and 41-bp and AB with five digested fragments at 236-, 175-, 170-, 66- and 41-bp (**Fig. 2**).

The results showed that A allele was present in the Egyptian sheep breeds with a frequency (83.14%) higher than B allele (16.86%). A & B alleles appeared at frequencies were 70.4% & 29.6%, 95.0% & 5.0% and 82.8% & 17.2% in Barki, Rahmani and Ossimi, respectively (**Table 2** and **Graph 1**).

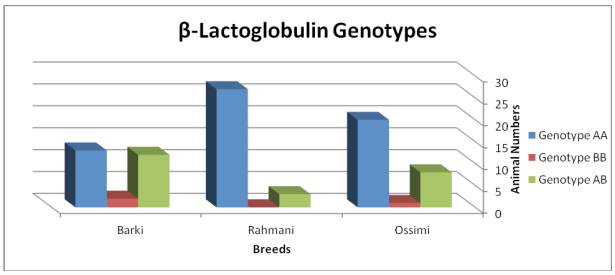
The 3 detected genotypes were present in the eighty-six Egyptian sheep animals at different frequencies with a majority of AA genotype (60 animals, 69.77%) over AB genotype (23 animals, 26.74%) whereas BB genotype appeared at low frequency (3 animals, 3.49%) in the three tested sheep breeds (**Table 2** and **Graph 2**).



Graph 1. Allele frequencies of *β-LG* gene in tested sheep breeds

Table 2. Allele and genotypes frequencies of *β-LG* in tested sheep breeds

Breed	No. of animals	Genotype frequencies						Allele frequencies	
		AA		AB		BB		A	B
		No.	%	No.	%	No.	%	%	%
Barki	27	13	48.15	12	44.44	2	7.41	70.37	29.63
Rahmani	30	27	90.00	3	10.00	0	0.00	95.00	5.00
Ossimi	29	20	68.96	8	27.59	1	3.45	82.76	17.24
Total	86	60	69.77	23	26.74	3	3.49	83.14%	16.86%



Graph 2. No. of animals with different *β-LG* genotypes in tested sheep breeds

Arora et al. (2010) determined the genetic polymorphism of the *β-LG* in Indian sheep breeds. *Rsa*I digestion of a 120-bp PCR fragment of *β-LG* exon 2 revealed two genetic variants, A (0.37) and B (0.63) with three genotypes AA (0.175), AB (0.389), and BB (0.436). The allelic variants revealed that the B allele was more frequent in the Indian breeds than in breeds reported from countries of Southwest Asia, Eastern and Central Europe, and the Mediterranean. This report supports our result where the allele A (83.14%) is more frequent than B (16.86%) allele in Egyptian sheep breeds.

The genetic polymorphism of the β-lactoglobulin gene was investigated in three native Turkish sheep breeds (**Elmaci et al., 2006**). Two genetic variants (A and B) and three genotypes (AA, AB, and BB) of β-lactoglobulin have been identified. The frequencies of *β-LG* A ranged from 0.7632 to 0.9756 and the *β-LG* B frequencies ranged from 0.0244 to 0.2368 in the three Turkish sheep breeds. These frequency ranges follow the hypothesis said that the A allele is more frequent than B allele in European countries.

In Lithuanian Blackface sheep **(Kučinskiene et al., 2005)**, two genetic variants A and B with allele frequencies of A=0.52 and B=0.48 were identified. The most frequent genotype in Lithuanian Blackface breed (66.7%) was AB, whereas homozygous genotypes AA and BB were observed at frequencies of 19.0% and 14.3 %, respectively. In Lithuanian Native Coarsewooled sheep, two genetic variants A (0.69) and B (0.31) were identified and the BB genotype was not frequent (7.8%) in this breed. This result matches with our result where BB genotype appears at the lower frequency (3.49%) with predominant of allele A over allele B in native Egyptian breeds.

Milk samples from 248 Pag ewes, belonging to 14 different flocks and located through the Pag Island (Croatia), were analyzed by isoelectrofocusing and PCR-RFLP (**Cubric-Curik et al., 2002**). According to the allele frequency (A=0.48, B=0.52) and occurrence of genetic variants, the Pag breed is similar to other Mediterranean dairy sheep breeds. The superiority of the AB-lactoglobulin genotype for milk production could be one of possible reasons for the observed excess of heterozygotes and allele frequencies at intermediate level.

To analyze the genotype distribution of *β-Lg* gene in some sheep breeds reared in Taif region of Saudi Arabia and its influence on milk composition, sixty 60 animals belonging to four sheep breeds named were utilized (**El-Shazly et al., 2012**). The results revealed that Noami and Sawakni breeds belong to *β-Lg*-A genotype while Harry and Nagdi belong to genotype *β-Lg*-B. Analysis of milk composition indicated the existence of a significant relationship between *β-Lg*-A genotype and total milk protein content while no clear association between *β-Lg* genotypes and other milk content was proved.

Our sheep breeds possess the predominant of *β-Lg* A allele with the high frequency (83.14%) and according to the results of **Cubric-Curik et al. (2002)** and **El-Shazly et al. (2012),** Egyptian sheep animals characterized by the superiority of milk production with high total milk protein

Alpha-lactalbumin, a calcium metalloprotein, is one of the major serum-proteins in ruminant milk and induces lactose synthesis in the mammary gland by interacting with the enzyme UDP-galactosyl-transferase, giving rise to the heterodimer enzyme lactose synthase. The alpha-lactalbumin encoding gene is essential for the biosynthesis of lactose in the mammary gland and it is a potential quantitative trait locus in dairy animals. It is therefore an interesting candidate gene for marker-assisted selection (MAS) (**Jain et al., 2009**).

The *α-LA* gene has been localized on chromosome 3 in sheep (**Imam-Ghali et al., 1991**). Two variants of *α-LA*, A and B have been detected (**Schmidt and Ebner, 1972**). Variant B of *α-LA* seems to be rare and confined to very specific breeds. This variant has been reported in some Italian breeds, German and Spanish breeds (**Amigo et al., 2000**).

In this study, PCR-SSCP technique was used to determine the different patterns of *α-LA* gene in the native Egyptian sheep breeds. The primers used in this study flanked a 166-bp fragment (**Fig. 3**) from exon 1 of *α-LA* gene.

The SSCP results of the *α-LA* amplified fragments showed that the three native Egyptian sheep breeds possess two different patterns related to the difference in nucleotide sequences (**Fig. 4**).

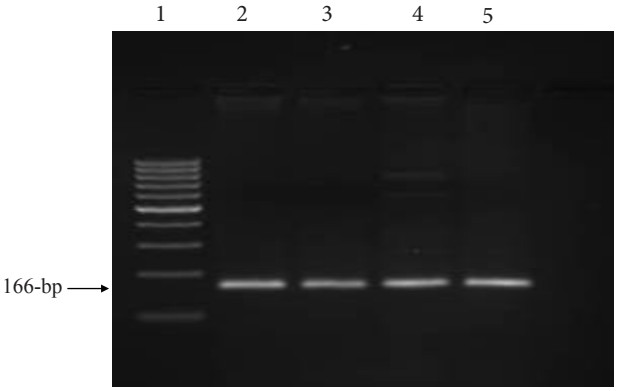


Fig. 3. Ethidium bromide-stained gel of PCR products representing amplification of *α-LA* gene in Egyptian sheep breeds.
Lane 1. 100-bp ladder marker
Lanes 2-5: 166-bp PCR products amplified from Egyptian sheep DNA

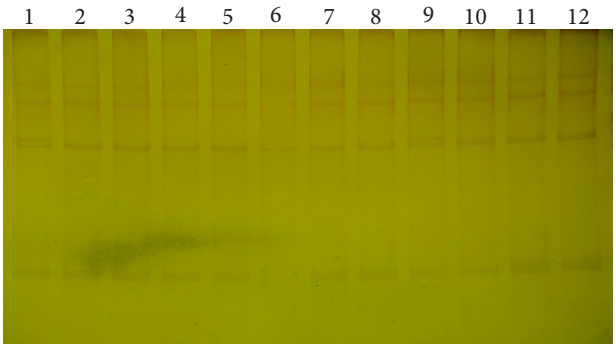
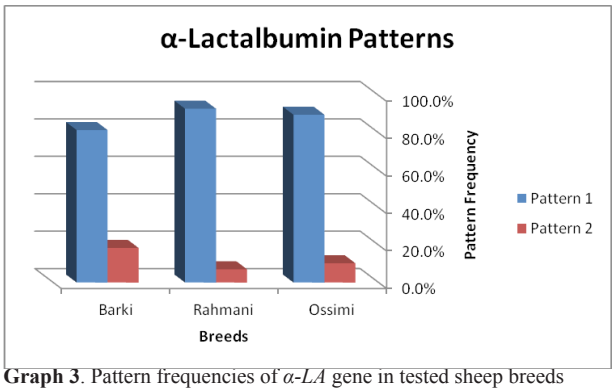


Fig. 4. SSCP patterns of Egyptian sheep *α-LA* gene on 14% silver stained- polyacrylamide gel
Lanes 2-8 and 10-12: Pattern 1
Lanes 1 and 9: Pattern 2

Table 3. Patterns frequencies of *α-LA* in tested sheep breeds

Breed	No. of animals	Pattern frequencies			
		Pattern 1		Pattern 2	
		No.	%	No.	%
Barki	27	22	81.48	5	18.52
Rahmani	28	26	92.86	2	7.14
Ossimi	29	26	89.66	3	10.34
Total	84	74	88.10	10	11. 90



Graph 3. Pattern frequencies of *α-LA* gene in tested sheep breeds

The two different detected patterns were present in the eighty-four Egyptian sheep animals at different frequencies with a majority of pattern 1 (74 animals, 88.1%) over pattern 2 (10 animals, 11.9%). The frequencies of pattern 1 ranged from 81.48% in Barki to 89.66% in Ossimi and 92.86% in Rahmani breeds whereas the pattern 2 was present in frequencies 18.52%, 7.14% and 10.34% in Barki, Rahmani and Ossimi breeds, respectively (**Table 3** and **Graph 3**).

Two-way sequence analysis of *α-LA* amplified PCR product of Egyptian sheep DNA was conducted from animals representing the two different patterns. The sequence analysis between the two patterns indicated two nucleotide substitutions (GC →CT) at nt 41 and 42 (**Fig 5**).

SSCP is a simple and reliable technique, based on the assumption that changes in the nucleotide sequence of a PCR product affect its single strand conformation. Molecules differing by as little as a single base substitution should have different conformers under non-denaturing conditions and migrate differently.

Therefore, those differences can be detected as a shift in the electrophoretic mobility (**Hayashi, 1991**). SSCP analysis proved to be an effective technique for the detection of *α-LA* polymorphisms in different farm animals (**Bastos et al., 2001**; **Ramesha et al., 2002**; **2008** and **Jain et al., 2009**).

Kumar et al. (2006) detected the SSCP in exon 1 of *α-LA* gene of Indian Jamunapari goats. The SSCP analysis resulted in four different patterns designated from P1 to P4 with frequencies 65%, 23%, 7% and 5%, respectively. The *α-LA* polymorphism recorded in their study was higher than that found by **Barracosa (1996)**, who analyzed the same exon using the same methodology and primers but reported only two patterns in Portuguese Serrana goats.

Jain et al. (2009) assessed the genetic variability in 4 exons of *α-LA* gene in Indian Jamunapari goat breed and detected the changes in the nucleotide sequence of PCR products affected by single base substitution. The PCR-SSCP of *α-LA* exons exhibited 9 different conformations, identified in 4 fragments represent 4 exons. The pattern frequencies in *α-LA* gene exon 1, which analyzed in our study, were 0.98 and 0.02 for the two detected patterns in this exon.

Bastos et al. (2001) used SSCP technique to identify the polymorphism within exon 1 of *α-LA* gene in Portuguese indigenous sheep breed "Churra da Terra Quente" and detected three different patterns. The frequencies were 57.5% for pattern 1, 22.5% for pattern 2 and 20% for pattern 3. On the other hand, **Barracosa (1996)**, using the same primers, did not found any SSCP polymorphism within exon 1 of *α-LA* gene in the Portuguese ovine breed "Serra da Estrela".

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Pattern I  CTCTTCCTGGATGTAAGGCTTGATGCCAGGGCCCCTGAGGGCTTTTCCACAAATAAAAGG 60
Pattern II ************************************CT****************** 60

Pattern I  AGGTGAGCAGTGTGGTGACCCCATTTCAGGATCTTGGGGGGTAACCAAAATGATGTCCTT 120
Pattern II ************************************************************ 120

Pattern I  TGTCTCTCTGCTCCTGGTAGGCATCCTATTCCATGCCACCCAGGCT 166
Pattern II ********************************************** 166
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Fig. 5. The sequence of the two patterns of *α-LA* gene in Egyptian sheep

quantitative trait loci for milk production and genetic polymorphism of milk proteins in dairy sheep. Genet. Sel. Evol. 37(Suppl. 1):109- 123

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