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An evaluation on genetic polymorphism of β-Lg locus using PCR-RFLP in sheep breeds reared in Taif

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β-Lactoglobulin (*β*-Lg) is one of the most important proteins in mammals' milk. It plays a crucial role in milk quality. The polymorphism of *β-LG* gene can be used as a marker system. 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 Noami, Sawakni, Harry and Nagdi, were utilized. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test was performed and genetic polymorphism was detected by the digestion of a 452 bp PCR fragment of exon II of *β-LG* gene with the endonuclease *RSA*I. The results revealed that Noami and Sawakni breeds belong to *β-LG*-A genotype while Harry and Nagdi belong to genotype *β-LG*-B. Sequence analysis of a 340 bp fragment in the promoter region of *β-LG* showed polymorphism AMONG the examined breeds. Analysis of milk composition in the different breeds indicated that the total protein content of milk was the highest in Noami breed followed by Sawakni, Nagdi and Harry. Concerning milk total fat and total solids contents, Harry breed was the highest, while no significant difference was evident among different breeds in lactose or non-fat solid contents. These results indicate the feasibility of PCR-RFLP test for differentiating sheep breeds and 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.

Key words: Sheep, *β*-lactoglobulin, genetic polymorphism, PCR-RFLP, milk composition.

INTRODUCTION

Among all foods, milk is the most complete and balanced in nutritional contents. The presence of similar levels of protein, fat and carbohydrate gives milk the unique indestructibility over any period of time. Therefore, milk does not spoil nor become inedible or harmful; it only changes from sweet to sour, liquid to increasingly solid, but retains very acceptable food qualities in the form of sour milk, yogurt and cheeses (Martin et al., 2002).
Breeding of sheep for dairy purposes has a long tradition throughout the world. Sheep milk accounts for only 2% of the global volume of milk obtained, but in some countries, such as Yemen, this proportion reaches as much as 50 or 90% (Molik et al., 2008).

The physico-chemical characteristics of sheep milk have unique properties as compared to goat and cow milk. Sheep milk contains higher levels of total solids and major nutrients than goat and cow milk. Also, mineral and vitamin contents of sheep milk are mostly higher than that of cow milk (Park et al., 2007). Sheep milk is more nutritious, richer in vitamins A, B and E, calcium, phos-phorus, potassium and magnesium than cow milk (Coni

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et al., 1999). Also, it contains a higher portion of short and medium chain fatty acids, which have recognized health benefits (Jandal, 1996).

Studies on the milk protein system have been in progress for more than 100 years, and constituted a rather difficult matter because of the intrinsic complexity of the subject. The occurrence of genetic polymorphism in milk proteins was described for the first time by Aschaffenburg and Drewry (1955). Since then, many studies have been performed to investigate milk protein polymorphism and great progress has been made with the introduction of analytical techniques that offer a greater degree of resolution. This has led to an extensive investigation of genetic polymorphism of the six main milk proteins (Ng-Kwai-Hang, 1998; Martin et al., 2002).

Among specific genes that may affect economically important traits, is the β-lactoglobulin (*β-Lg*) locus that has been considerably studied (Tsiaras et al., 2005). β-Lg is the major whey protein in the milk of ruminant representing 60 to 65% of total protein in milk whey. It is also found in the milk of the mammals, but absent in milk of rodents, lagomorphs, human and probably camels. The native protein is found as dimer with a molecular weight of 36.4 kDa corresponding to 162 amino acids while in other species it has been found as monodimer (Yahyaoui, 2003). β-Lg protein belongs to the lipocalin protein family, constituted by small secreted proteins which are characterized by their affinity to bind hydrophobic molecules.

The attention surrounding sheep milk and its cheese production characteristics, especially in the Mediterranean countries, has spurred considerable research into the genetic structure of native sheep populations and the possible relationship between the genetic variants of milk protein genes and milk related traits (Schlee et al., 1993; Feligini et al., 1997; Cubric-Curik et al*.,* 2002; Nassiry et al*.,* 2007; Dario et al., 2005). This in turn, has generated a substantial interest in the practical applications of genetic markers in breed development programs and preservation strategies, as well as the awareness of the cultural value connected to the safe-guard of autochthonous dairy sheep breeds biodiversity.

The *β-Lg* encoding gene has been sequenced in sheep (Harris et al*.*, 1988) and assigned to chromosome 3. Three genetic variants have been identified; A, B and C (Kolde and Braunitzer, 1983; Erhardt, 1989; Schlee et al., 1993). Variants A and B were described in order of decreasing mobility (Elmaci et al., 2006) and differ by an amino-acid replacements at position 20 (TyrA→HisB) (Bell and McKenzie, 1967). This difference can be distinguished by PCR-RFLP using *Rsa*I endonuclease as (GT/AC) for the A allele, while allele B has no such restriction site. The A and B forms of *β-Lg* are ubiquitous (Bell and McKenzie, 1967), while the *β-Lg*-C isoform is a subtype of A form with a single amino acid exchange of Arg to Glu at position 14. This form has been reported only in milk from German and Spanish merino breeds Erhardt, 1989; Recio et al., 1995; Anton et al., 1998).

In recent years, many studies on the relationships between the genetic polymorphism of *β-Lg* and the qualitative characteristics of ovine milk have been performed, particularly on Italian breeds like Massese (Rampilli et al., 1997) and Spanish breeds like Lacha, Merino (Recio et al., 1997) and Manchega (López-Gálvez et al., 1993). The results are inconsistent, indicating either the superiority of a given phenotype (López-Gálvez et al., 1993) or the absence of relationships (Recio et al., 1997).

Milk protein binding factor (MPBF) is a factor that has recognition sites in the promoters of many milk protein genes including three sites in the promoter of the sheep β-lactoglobulin (*β-Lg*) gene. Mutagenesis of these sites reduced expression of the *β-Lg* gene in lactating mammary glands (Burdon et al., 1994). Many research focused on searching for differences in transcription factors binding caused by point mutations in the milk protein gene promoters in BLG gene (Lum et al., 1997) and αS1, β- and αS2-casein (Martin et al., 2002). Recently, Prinzenberg et al. (2003) found several polymorphisms within 5' region of αS1-casein gene strongly correlated with protein content of milk.

The objective of the present study was to investigate the genetic polymorphism of *β-Lg* locus using PCR-RFLP in sheep breeds reared in Taif, KSA and its relationship with milk composition. The studied breeds included Harry breed, locally known as a dairy breed, Nagdi breed as a mutton breed and both Noami and Sawakni breeds as a dual purpose breeds.

MATERIALS AND METHODS

Blood samples and genomic DNA extraction

Blood samples were collected from four sheep breeds living in natural habitats belonging to the Taif region, KSA. Breeds included Noami, Sawakni, Harry and Nagdi. Blood samples were COLLECTED from 15 animals in each breed into potassium EDTA evacuated blood collection tubes, transported to the laboratory in ice box and then preserved at -20°C till analysis.

Genomic DNA was extracted from whole blood using EZ-10 spin column genomic DNA Minipreps kit (Bio Basic INC, Canada). DNA concentration and purity were examined using UV spectrophotometer at 260/280 nm.

Polymerase chain reaction (PCR) amplification of β-Lg gene and promoter area

Amplification of the *β-Lg* gene from exon II region was performed using PCR. The PCR was carried out in a 50 µl reaction mixture containing: 100 ng genomic DNA, 0.5 mM of each primer, 1.0 U of *Taq* DNA Polymerase, dNTPs each at 150 mM and 5.0 µL of 10 × PCR buffer containing 1.5 mM $MgCl_2$. Primers used were according to Jurate et al*.* (2005); forward 5́-TTG GGT TCA GTG TGA GTC TGG -3́and reverse 5́-AAA AGC CCT GGG TGG GCA GC-3́with an expected size of 452 bp. DNA samples were amplified for 33 cycles (94^{0},C 66^{0},C 72°C for one minute each) with final elongation of five min at 72°C.

For the amplification of *β-Lg* gene promoter region, the primers were designed using Primer 3 program (http://frodo.wi.mit.edu/primer3/)

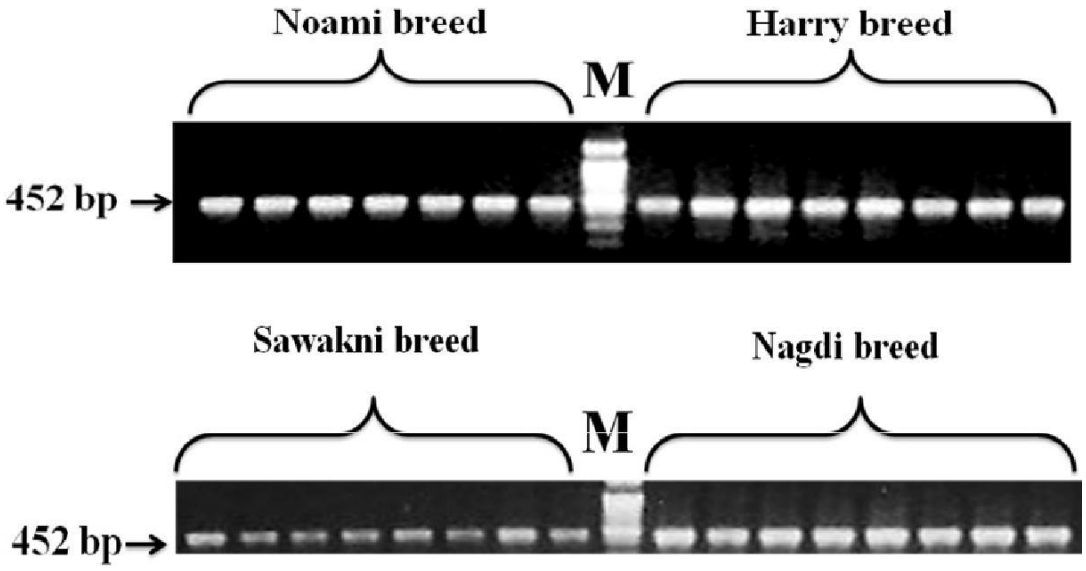

Figure 1. Ethidium bromide stained agarose gel electrophoresis of PCR products from exon II of ovine *β-Lg* gene. M indicates 100 bp DNA size marker and other lanes are PCR products from different individuals from four breeds: Harry, Noami, Sawakni and Nagdi breeds.

and ovine *β-Lg* genomic DNA published in gene bank with an accession no. X12817.1 (Harris et al., 1988). The forward primer sequence was 5´-CAC CTG TGC CCC CGC TTC TG -3´ and the reverse was 5´- TGA CGA TGATGG CCT GGA CGC -3´. DNA amplifications were performed for 33 cycles ($94^{0,}$C $65^{0,}$C and 72°C for one minute each) with a final elongation of 5 min at 72°C and predicted product size of 320 bp.

Agarose gel electrophoresis

PCR product electrophoreses were performed using 15 µl of PCR product on 2% agarose (FMC, Rokland, ME) gel in 0.5X Tris-borate-EDTA buffer at 100 V for 45 min. The gel was stained in 1% ethidium bromide and washed with distilled water. PCR products were visualized a UV transilluminator and photographed using a digital camera.

Restriction enzyme digestion

PCR products were purified using BioFlux BioSpin Gel Extraction kit (Bioer technology Co., Ltd, Japan). A total volume of 10 µl of each purified PCR product was digested overnight at 37°C with 10 U of *Rsa*I endonuclease (Boehringer Mannheim, Germany). Amplicons as well as digested products were analyzed by electrophoresis in 3% agarose gel and then stained with 1% ethidium bromide.

Promoter nucleotide sequence analysis

Following *β-Lg* promoter region amplification, PCR products were purified using BioFlux BioSpin Gel Extraction kit (Bioer technology Co., Ltd, Japan). Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using forward and reverse primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, USA). Nucleotides sequences were analyzed and the phylogenic studies were conducted using DNasis program.

Milk samples collection and composition analysis

Ten milk samples were collected from different sheep breeds from their home tract. The samples were transported, on ice, to the laboratory and stored at -20°C for further analysis. Milk composition (total protein, total fat, lactose, total solids and non fat solid contents) were determined using Milko- scan 130 series system maintained at International Live Stock Management Training Center (ILMTC) which follows Animal Production Research Institute (APRI).

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) and Fischer's protected least-significant difference test, by StatView program for Macintosh computer with $P < 0.05$ regarded as statistically significant. Neighbor-joining analytical method was used to construct the genetic relationship under 1000 bootstrap replications (Sowfford, 2002).

RESULTS

PCR-RFLP

Amplification of *β-Lg* gene from different sheep breeds produced a fragment of 452 bp, which was BASED ON THE USED primers (Figure 1). Digestion of the PCR amplified *β-Lg* gene with *Rsa*I restriction endonuclease resulted in two patterns. The first with four fragments: 175, 170, 66 and 41 bp corresponding to A genotype (*β-Lg*-A) and

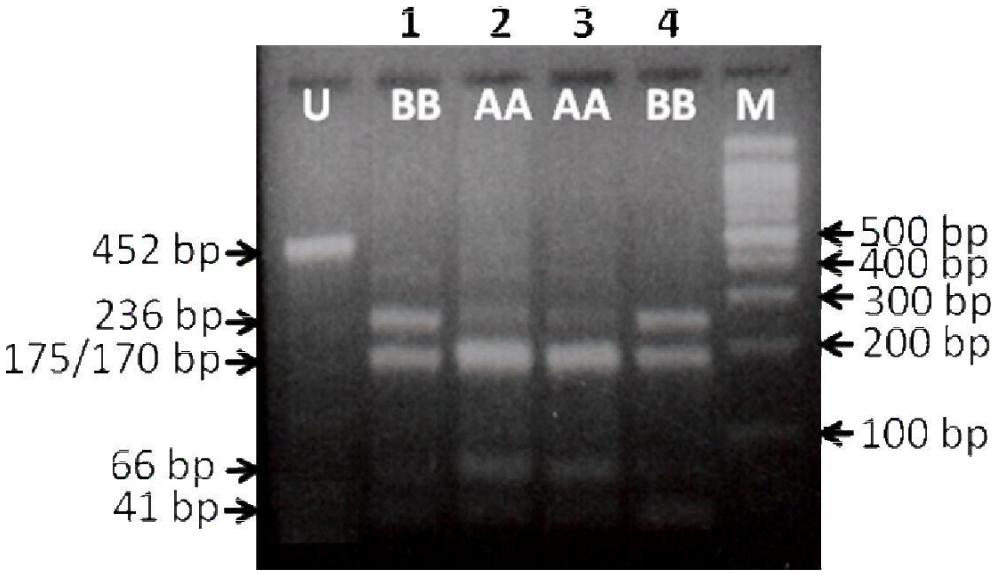

Figure 2. RFLP analysis of 452 bp fragment of exon II of ovine *β-Lg* gene by *Rsa*I enzyme on 2% agarose. U: uncut PCR product; lane 1 from Harry breed shows BB genotype, lane 2 from Noami breed shows AA genotype, lane 3 from Sawakni breed shows AA genotype and lane 4 from Nagdi breed shows BB genotype. M is 100 bp DNA ladder.

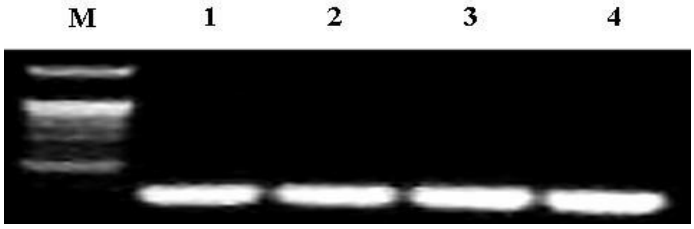

Figure 3. Ethidium bromide stained agarose gel electrophoresis of PCR products from promoter area of ovine *β-Lg* gene. M indicates 100 bp DNA size marker and lanes 1, 2, 3 and 4 are PCR products from different individuals from the four examined breeds: Harry, Noami, Sawakni and Nagdi respectively.

the second with three fragments: 236, 175 and 41 bp, which corresponds to B genotype (*β-Lg*-B). Both Harry and Nagdi breeds were assigned to *β-Lg*-A, while both Sawakni and Noami breeds belonged to *β-Lg*-B type (Figure 2).

β-Lg promoter sequence analysis

PCR amplification of a 340 bp at the promoter region of *β-Lg* gene, between nucleotide number 565 and 905, resulted in the same size (340 bp) in all breeds under investigation (Figure 3). The PCR products were subjected to sequence analysis in comparison to each other and to that published in gene bank (accession no. X12817). The results revealed a substitution of G by C at nucleotide no. 636 in both Noami and Sawakni breeds. Another substitution of G by T was proved at nucleotide no. 665 in the same breeds. Meanwhile, the four breeds showed an insertion of A at position 682 and deletion of G at nucleotide no. 686 as well as GC deletion at nucleotide no. 760 and 761 and a C deletion at nucleotide no. 787 (Figure 4A). The phylogenic relationship of the four breeds using promoter nucleotide sequence as compared to that published in gene bank showed a close relationship between Harry and Sawakni breeds with considerable statistical support of about 88% (Figure 4B).

Milk chemical composition

The chemical composition of milk from the 4 examined sheep breeds are summarized in Figure 5. Although, milk chemical composition did not differ greatly among tested breeds, however, remarkable differences were recorded. The highest total protein content was found in the milk of

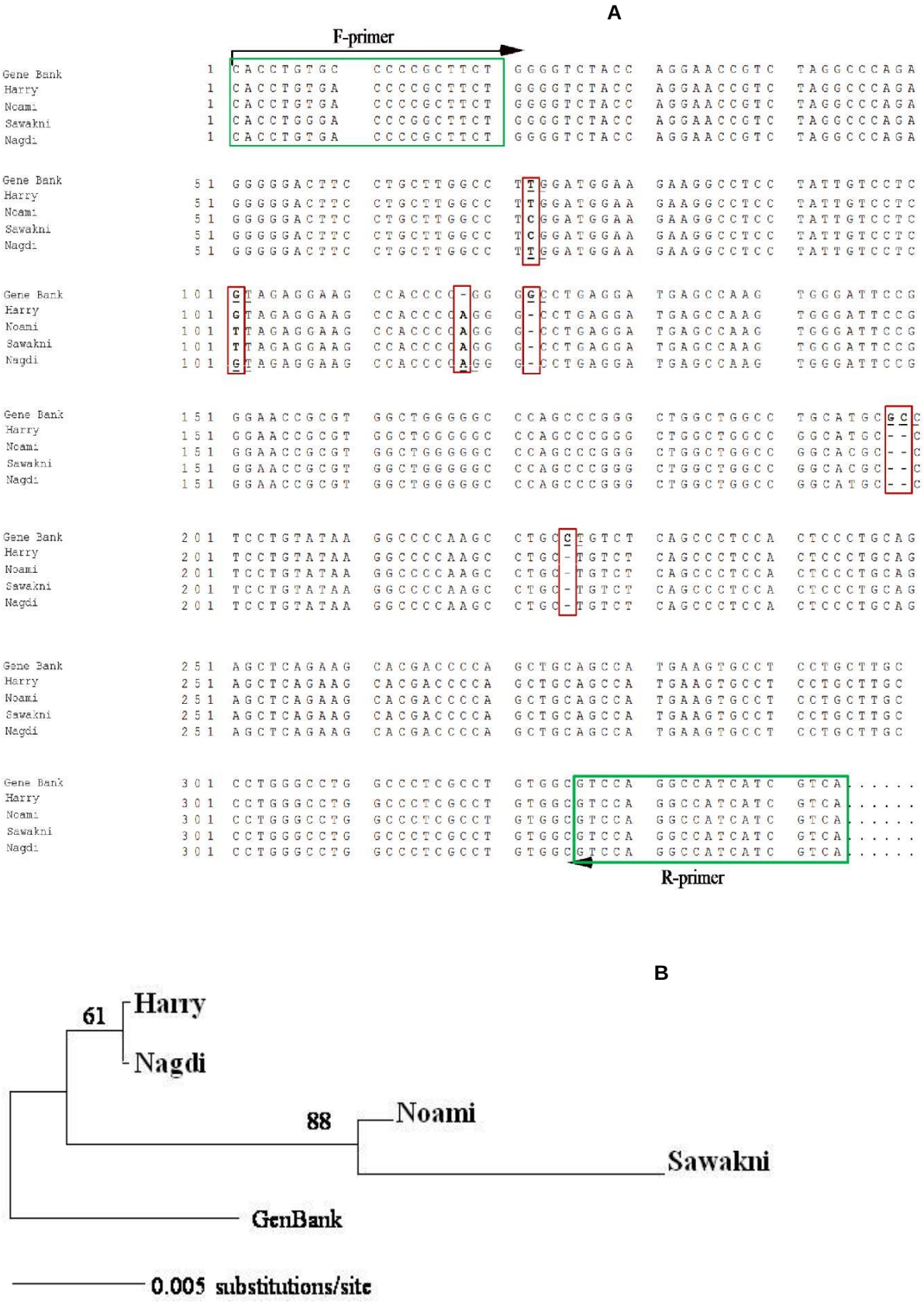

Figure 4. Panel A: Sequence analysis of the four different breeds when compared with that cited in gene. Panel B: Dendogram according to the obtained promoter sequence data from different breeds and Nagdi breeds, respectively.

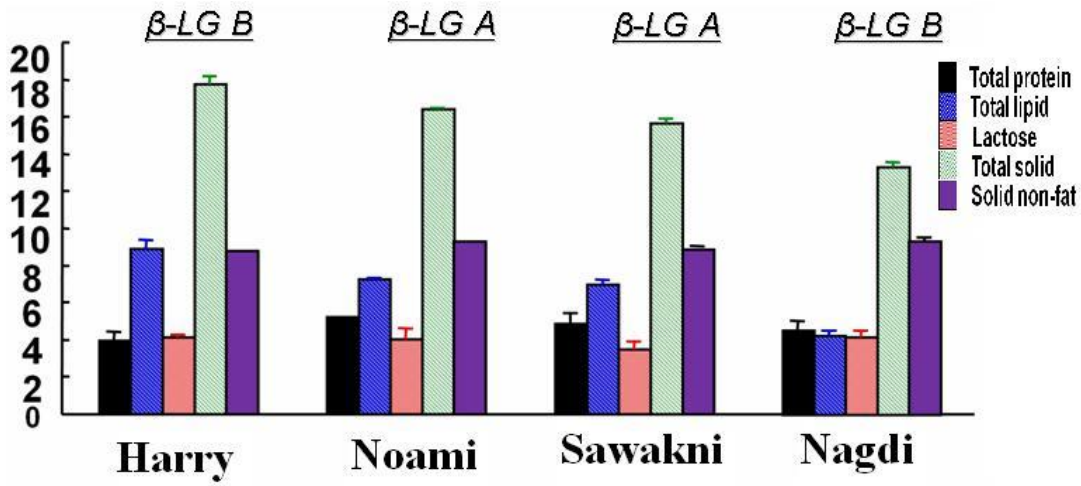

Figure 5. Milk chemical composition (total solids, total protein, fat content, non-fat solid and lactose contents) in different breeds.

Noami and Sawakni when compared with Harry and Nagdi breeds. Values of total fat content indicated that Harry breed had the highest content followed by Noami, Sawakni and finally Nagdi. Meanwhile, total solids contents exhibited similar pattern. On the other hand, regarding lactose content as well as non-fat solid contents, there were no significant differences among the four investigated breeds.

DISCUSSION

Currently, genetic polymorphisms are playing an increasingly important role as genetic markers in many fields of animal breeding. With the development of molecular genetic techniques, it has become possible to establish a new class of gene markers based upon the variability at DNA sequence level (Meignanalakshmi and Nainar, 2009). In dairy cattle, *β-Lg* polymorphism was described in considerable details and in many cases the genetically determined diversity of this protein was shown to be associated with the yield of milk and milk components (Litwinczuk et al., 2002). On the other hand, the issue of polymorphism has not been adequately studied among the breeds of sheep raised for milk production (Kawecka and Radko, 2011). Previous studies have shown that the protein is polymorphic in many breeds of sheep. This is a result of single base pair substitution in *β-Lg* gene that rises to *Rsa*I RFLP (Elyasi et al., 2010). In the present study, RFLP was evidenced in the *β-Lg* locus after digestion with *RsaI* endonuclease. Both Noami and Sawakni breeds appeared to belong to *β-Lg*-A type, while Harry and Nagdi breeds belong to *β-Lg*-B genotype.

Several transcription factors are known to bind to recognition sequence in *β-Lg* promoter (Folch et al., 1994). Milk protein binding factor (MPBF) is a factor that has recognition sites in the promoters of many milk protein genes including three sites in the promoter of the sheep beta-lactoglobulin (*β-Lg*) gene. Mutagenesis of these sites reduced expression of the *β-Lg* gene in lactating mammary glands (Burdon et.al., 1994). Herein, the promoter region was investigated by sequencing of a 340 bp fragment in the promoter area of *β-Lg* gene. The results showed a polymorphism in this area between the four sheep breeds including substitution, deletion and insertion. Further studies are needed to evaluate the impact of these polymorphisms on the expression of *β-Lg* gene.

Information on composition and physico-chemical characteristics of sheep milk is essential for successful development of dairy sheep industry as well as for the marketing of the products. The effect of milk protein polymorphism on milk production traits has been investigated during the past decades and in some cases results are still conflicting (Prinzenberg et al., 2003; Kucerova et al., 2006). For this reason, *β-Lg* locus has been extensively studied as one of the genes that may affect the economically important traits in sheep. Polymorphism has been detected in several breeds, but studies of the effect of *β-Lg* alleles on milk production traits have given inconsistent results. Some studies observed that *β-Lg* polymorphism significantly affects milk yield (Bolla et al., 1989; Faraghì et al., 1996), fat and protein content (Garzon and Martínez, 1992), cheese yield and composition (Di Stasio et al., 1997; Rampilli et al., 1997) and only fat content (Pirisi et al., 1999). However, other studies failed to detect any effect of genetic polymorphism on milk production traits (Barillet et al., 1993; Recio et al., 1997; Piwczynski et al., 2002). In accordance, milk chemical composition did not differ substantially among *β-Lg* genotypes and the present investigation failed to find a remarkable relationship among the determined genotypes and milk constituents, except for total protein that was significantly higher in *β-*

Lg-A type. Other milk constituents including total fat, total solids, non-fat solid content as well as lactose content showed non-significant difference between the two identified *β-Lg* genotypes. These inconsistencies are similar to those reported for dairy cattle, were explained by breed differences, population size, frequency distribution of the genetic variants and a failure to consider relationships among animals (Sabour et al., 1996).

In conclusion, the present study contributes to polymorphism of *β*-Lg locus in sheep breeds. It revealed that PCR-RFLP can be used to differentiate among different sheep breeds. Two genotypes exist in the examined breeds where both Harry and Nagdi breeds belong to *β-Lg*-A, while both Noemi and Sawakni breeds belong to *β-Lg*-B. Although, the milk chemical composition failed to show a clear relationship with *β-LG* genotype, only protein content was high in *β-Lg-A* genotype breeds and more investigation of the effect of *β-Lg* polymorphism and milk composition is still needed.

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