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Prediction of desirable genotype patterns in Baladi beef cattle and water buffalo by identification of new leptin gene SNPs

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ABSTRACT

The leptin gene is considered to be an excellent candidate gene for predicting desirable economic traits in both beef and dairy cattle. Leptin gene polymorphism has been analyzed in different livestock species and the polymorphic pattern has been demonstrated to be associated with energy balance, milk production, live body weight and reproductive performance. The present study was designed to analyze genetic polymorphisms in the coding region of leptin gene in native beef cattle and water buffalo in comparison to Holstein cattle. A total number of 180 animals (60 animals of each breed) were used for blood sampling and DNA extraction. Target sites in leptin gene (first 94 bp fragment of exon 2 and 330 bp fragment including first part of exon 3) were amplified by polymerase chain reaction (PCR) using two specific primers pairs. Genotyping for R25C single nucleotide polymorphism (SNP) in exon 2 was carried out using the Kpn21-RFLP method. Variations in the exon 3 coding sequence were investigated by PCR-SSCP analysis. Two alleles (C and T) were observed in exon 2 giving rise to three R25C variants (CC, CT and TT). The highest frequency in all populations was the homozygote genotype (CC) where it recorded 91.6%, 98.3% and 100% for Baladi cattle, Holstein cattle and buffalo, respectively. Four alleles (A, B, C and D) and six genotypes (AA, AB, BB, CC, DD and CD) were identified in all studied breeds upon exon 3 analysis. Genotype AA was found to be the most dominant in all studied breeds followed by genotype CC in Holstein and Baladi cattle but followed by AB genotype in buffalo. All observed and expected genotypes were found to be statistically significant ($P \leq 0.05$) when subjected to chi-square analysis. Two point mutations have been identified in the first part of exon 3 coding sequence (a3033 > t and c3051 > t) of Baladi cattle samples. Polymorphisms that were detected in this study indicated that these breeds have high genetic variability in the leptin gene. These results implicate the prospective use of leptin gene polymorphisms for association studies with different productive and reproductive performances and marker assisted selection (MAS).

1. Introduction

Genotyping and marker-assisted selection (MAS) have been extensively used to improve meat quality in beef cattle. The potential of these technologies has been well demonstrated (Ibañez-Escriche and Gonzalez-Recio, 2011; Rezende et al., 2012). Leptin is one of the most useful biomolecules to serve as a genetic marker for selecting high quality individuals for better productivity and adaptability (Agarwal et al., 2009; Anton et al., 2012). Leptin plays important roles in controlling body weight, feed intake, energy expenditure, immune function and reproduction (Komisarek, 2010; Patricia et al., 2010). Moreover, leptin acts as a body barometer providing a critical link between energy homeostasis, appetite and reproductive function (Nassiry et al., 2007). Due to its coordinating effect on the whole body metabolism, leptin may be classified as a metabolism modifier (Farooqi and O'Rahilly, 2009).

The leptin gene is highly conserved across species. The bovine leptin gene (LEP) consists of three exons separated by two introns (Pomp et al., 1997) and has been mapped to chromosome 4 and 8 in cattle and buffalo, respectively (Konfortov et al., 1999; Vallinoto et al., 2004). The coding region of leptin gene is contained in exons 2 and 3 which encode a protein of 167 amino acids (Konfortov et al., 1999; Liefers and Te Pas, 2002). Several studies on the bovine leptin gene polymorphisms have been reported in cattle (Pomp et al., 1997; Yang et al., 2007; Guo et al., 2008) and in buffalo (Javanmard et al., 2005; Orrù et al., 2007). The leptin gene was found to be highly polymorphic (Dandapat et al., 2010). Twenty single nucleotide polymorphisms (SNPs) have been identified by sequence analysis of the bovine leptin gene giving a frequency of 1 SNP per 89 bp (Konfortov et al., 1999).

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To date, many studies in different cattle breeds have shown a close relationship between polymorphisms in the bovine LEP gene locus and traits of economic interest like carcass quality (Pinto et al., 2011; Woronuk et al., 2012), lean meat yield (Schenkel et al., 2005), milk production (Buchanan et al., 2003; Anton et al., 2012) and growth (Lusk, 2007). A single nucleotide polymorphism (C/T substitution) at position 305 in exon 2 in bovine leptin, resulting in coding of arginine instead of cysteine amino acid (R25C) displayed a high association with growth, carcass quality and milk production traits that were first reported by Buchanan et al. (2002) and have been shown to have an impact on growth and carcass quality (Woronuk et al., 2012). However, there are few studies on exon 3 polymorphism of leptin gene, particular in buffalo breeds.

Studying polymorphisms in the leptin gene as a marker gene for enhancing the productivity and quality of livestock and as a potential candidate for marker assisted selection (MAS) have been being employed as part of a more efficient selection in a number of countries (Agarwal et al., 2009). Native breeds of cattle often have a slower growth rate and a lower yield of beef production, compared to foreign beef cattle breeds, but have a relatively favorable meat quality. On the other hand, native buffalo are known for high fat percentage in milk, better quality of meat, and a higher adaptability to tropical climates. However, the genetic potential of this livestock species is yet to be fully exploited as there are only few reports on characterization of candidate genes governing meat and milk production traits in buffalo (Tanpure et al., 2012).

The present study was undertaken with the objective of detecting leptin gene polymorphisms in native cattle and buffalo as the main food producing animals in our countries. With the aim of providing a set of genetic markers potentially useful for MAS in native cattle and buffalo, we carried out this study to characterize potential SNPs in the LEP gene and to evaluate their allelic and genotypic frequencies. This LEP gene based genotyping may be implicated in selection of high producing animals of these species.

2. Material and methods

2.1. Animals and DNA extraction method

Three different breeds [Baladi beef cattle ($n=60$), Holstein cattle ($n=60$), and Egyptian buffalo ($n=60$)] that reared in farms of Research Institute of Animal Production were used for this study. Blood samples were obtained from the jugular vein using EDTA as anticoagulant and in accordance for the code of ethics of EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). Genomic DNA was extracted using salting out extraction protocol (Miller et al., 1988). DNA quantity and integrity were estimated by spectrophotometery and 1% agarose gel electrophoresis.

2.2. Analysis of leptin gene polymorphisms

Primer designing and PCR Amplification for leptin gene: Two pairs of oligonucleotide primers were used to amplify two leptin fragments based on the published nucleotide sequence information of exon 2 and exon 3 of the bovine leptin gene (GenBank accession no. U50365). The primer sequences, the size of the amplified fragments and the analytical method used are shown in Table 1. PCR was carried out in 25 μl volumes using PCR master mix containing 0.04 u/μl *Taq* DNA Polymerase in PCR reaction buffer, 3 mM $MgCl_2$ and 0.04 mM of each dNTP, 10 pmol of each primer and about 100 ng of extracted DNA as template. The thermal profile consisted of 5 min at 95 °C, followed by 35 cycles of 40 s at 94 °C, 40 s at, 64 °C for exon 2 and 62 °C for exon 3, and 40 s at 72 °C, with a final extension of 10 min at 72 °C. PCR products were electrophoresed on a 2% agarose gel.

RFLP for leptin exon 2: The 94 bp PCR product of leptin exon 2 containing the *Kpn21* SNP were subjected to genotyping using RFLP method according to Buchanan et al. (2002). The PCR products were digested with FastDigest Kpn2I (Fermentas) restriction enzyme. Each digestion contained 8 μl PCR products, 2 μl Buffer 10 ×, 5 U *Kpn2I* enzyme and up to 20 μl deionized water and incubated at 55 °C for 3 h. Restriction fragments from the above digestion reactions were electrophoresed on 15% PAGE in 1 × TBE (120 V for 2 h) and stained with ethidium bromide prior to visualization under UV light.

Single strand confirmation polymorphism (SSCP) for leptin exon 3: the amplified 330 bp-coding fragment for exon 3 of bovine leptin was screened by SSCP according to Yang et al. (2007). Briefly, 8 μl aliquot of each PCR amplicon was mixed with 8 μl of denaturing loading dye [0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromophenol blue in 95% formamide]. the mixture was denatured at 95 °C for 10 min and was snap chilled on ice for 15 min to prevent re-annealing of the single-stranded product (Zhou et al., 2009) and then loaded on 12.5% polyacrylamide gel with crosslinking ratio of 29:1 acrylamide: Bisacrylamide. electrophoresis was performed using Vertical slab unit (BioRAD) at room temperature and constant 140 V for 4 h in 1 × TBE buffer (Tris 100 mM, boric acid 9 mM, EDTA 1 mM). Polyacrylamide gels were then stained with ethidium bromide.

2.3. DNA sequencing

PCR products representative for each SSCP variant of exon 3 of leptin gene were sequenced. Briefly, the PCR products were purified by Gel Elution kit (Sigma, USA). The sequencing reaction was done by using Big Dye TM Terminator cycle sequencing kit (Germany) according to manufacturer instructions. After purification of sequence reactions they subjected to DNA sequencing using ABI PRISM 3730 XL analyzer (Applied Biosystem). The sequencing data were analyzed using free BioEdit program for single sequence alignment using pairwise alignment to the published leptin gene.

2.4. Statistical analysis

The allelic and genotypic frequencies were determined by counting. The chi-square test (χ^2) was done for each population using GENEPOP (Version 1.31) to check whether the populations were in Hardy–Weinberg equilibrium (HWE). A value of $P \leq 0.05$ indicated deviation from HWE.

Table 1
Primer sequence, annealing temperature and PCR product size used for both RFLP and SSCP analysis of leptin gene in buffalo, Baladi cattle and Holstein cattle.

Amplified region	Primer type	Primer sequence	Annealing temp.	Fragment size	Analysis method	Ref.
Exon2	Forward	ATGCGCTGTGGACCCCTGTATC	64 °C	94 bp	RFLP	Buchanan et al. (2002)
	Reverse	TGGTGTCATCCTGGACCTTGCG				
Exon3	Forward	GGGAAGGGCAGAAAGATAGG	62 °C	330 bp	SSCP	Yang et al. (2007)
	Reverse	AGGCAGACTGGTGAGGATC				

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3. Results

Bovine *leptin* gene polymorphisms were characterized in native beef breeds of cattle and water buffalo in comparison to high producing Holstein cattle. Two fragments spanning the leptin coding region (parts of exon 2 and exon 3) were amplified by PCR using two specific pairs of primers (Table 1 and Fig. 4) on the three sample populations.

3.1. *Kpn2I-RFLP Polymorphism in leptin exon 2*

A PCR fragment of 94 bp spanning between nucleotides 1008 and 1201 within exon 2 was amplified by PCR (Fig. 1A) and genotyped for the R25C SNP by *Kpn2I* -RFLP. On digestion by *Kpn2I* restriction enzyme, three genotypes were determined; CC (75 and 19 bp), CT (94, 75 and 19 bp) and TT (94 bp, uncut product) (Fig. 1B). The C and T allele frequencies were calculated for all studied breeds (Table 2). The C allele frequency was significantly dominant over T allele frequency for all breeds studied. We observed frequencies of 97.5, 99.1 and 100% in Baladi cattle, Holstein cattle and buffalo, respectively. The homozygous genotype (CC) also displayed the highest frequency in all populations where it displayed frequencies of 91.6, 98.3 and 100% for Baladi cattle, Holstein cattle and native buffalo, respectively (Table 2). The heterozygote (CT) genotype frequency expected for Baladi breed was significantly lower than that observed ($P \leq 0.05$) while it was not significantly different for the other two breeds of Holstein cattle and buffalo.

3.2. *PCR-SSCP analysis of LEP gene*

DNA samples from all breeds yielded a specific 330 bp single band PCR product of leptin exon 3 without any nonspecific band (Fig. 2). Therefore, the PCR products were directly subjected for SSCP analysis. Six unique banding patterns representing four different allelic variants (A–D) of bovine leptin exon 3 could be resolved by the SSCP technique and the proposed patterns for genotyping are represented in Fig. 3A and calculated in Table 3. The denatured polyacrylamide gel electrophoresis pattern enabled the visualization of a total of six SSCP patterns in the examined breeds (Fig. 3B–D). Based on patterns of exon 3-SSCP analysis, five genotypes were found in both Holstein and Baladi cattle as shown in Fig. 3C and D, respectively. The CD genotype was not observed in Holstein cows while the AB genotype was not observed in Baladi cattle and only three genotypes were observed in buffalo samples (Fig. 3B). As demonstrated in Table 3, similar dominant allele and genotype frequencies were observed in all breed groups, while the frequency of mutant homozygotes (AA) was very high. Allele A was found to be the most dominant in all studied breeds followed by allele C in Holstein and Baladi cattle and allele B in buffalo. Consequently, genotype AA was found to be the most dominant in all studied breeds followed by genotype CC in Holstein and Baladi cattle and followed by AB genotype in buffalo. All observed and expected genotypes were found to be statistically significant ($P \leq 0.05$) when subjected to chi-square analysis. Comparison of the sequence of the PCR product of leptin exon 3 (330 bp) with the published leptin gene sequence (GenBank accession no. U50365) revealed that two point mutations have been identified in the first part of exon 3 coding sequence (a3033 > t and c3051 > t) along the 330 bp amplified fragment of native cattle DNA (Fig. 4).

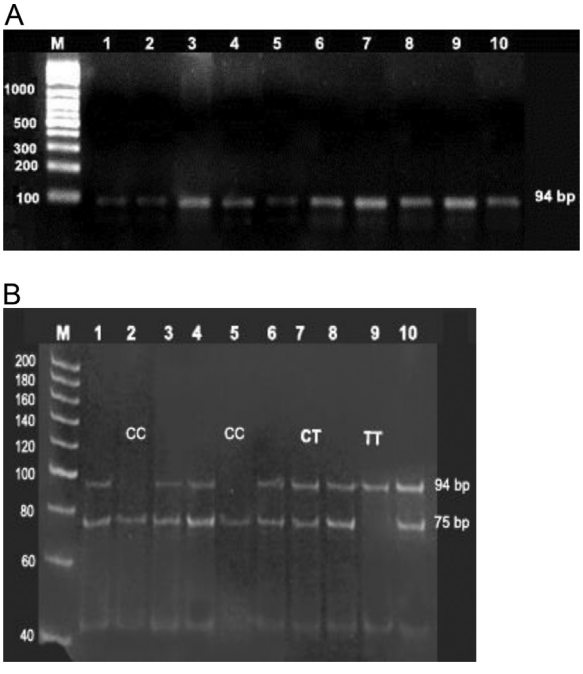

Fig. 1. PCR products of leptin gene exon 2 and genotyping patterns. (A) Agarose gel electrophoresis of amplified region (94 bp fragment). Lane M: 50 bp ladder, lanes 1–10: representative PCR products of buffalo, Baladi cattle and Holstein cattle. (B) A 15% polyacrylamide gel displaying a *Kpn2 I* restriction digestion of an amplified region. PCR-RFLP was used for the detection of a C/T substitution at position 1180 (C1180T SNP) within exon 2 region of the leptin gene in all breeds under study. Lane M, 20 bp ladder, lanes 2 and 5 are genotype CC, lanes 1, 3, 4, 6, 7, 8, 10 are genotype CT, and lane 9 is genotype TT.

Table 2
Genotype and allele frequencies of buffalo, Baladi cattle and Holstein cattle according to polymorphism in Leptin exon 2.

Breed type		Genotype frequency			χ^2 value	Allele frequency	
		CC	CT	TT		C	T
Buffalo, $N=60$	Obs.	100	0	0	0.00^{NS}	100	0
	Exp.	100	0	0			
Baladi cattle, $N=60$	Obs.	91.60	6.70	1.70	29.57*	97.50	2.50
	Exp.	95	4.91	0.09			
Holstein cattle, $N=60$	Obs.	98.30	1.70	0	0.009^{NS}	99.10	0.90
	Exp.	98.10	1.90	0			

N: Number of animals.
Obs.: Observed frequency.
Exp.: Expected frequency under Hardy–Weinberg equilibrium (HWE).
χ^2: chi-square value.
NS: non-significant ($P \leq 0.05$); suggesting that the population is in HWE.
* Statistically significant; the deviation between observed genotypic frequencies and those expected under HWE was statistically significant ($P \leq 0.05$) suggesting that this population sample was not in HWE.

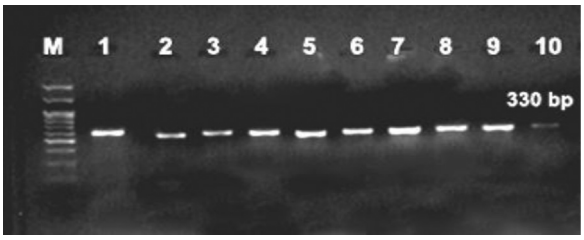

Fig. 2. Agarose gel electrophoresis of PCR products of leptin gene exon 3 (330 bp fragment). Lane M: 50 bp ladder, lanes 1–10: representative PCR products for various samples of buffalo, Baladi cattle and Holstein cattle.

4. Discussion

Leptin is considered to be an important regulator of energy metabolism in adipocytes and reproduction, and may be linked to meat quality determinants such as marbling (Saleem et al., 2015).

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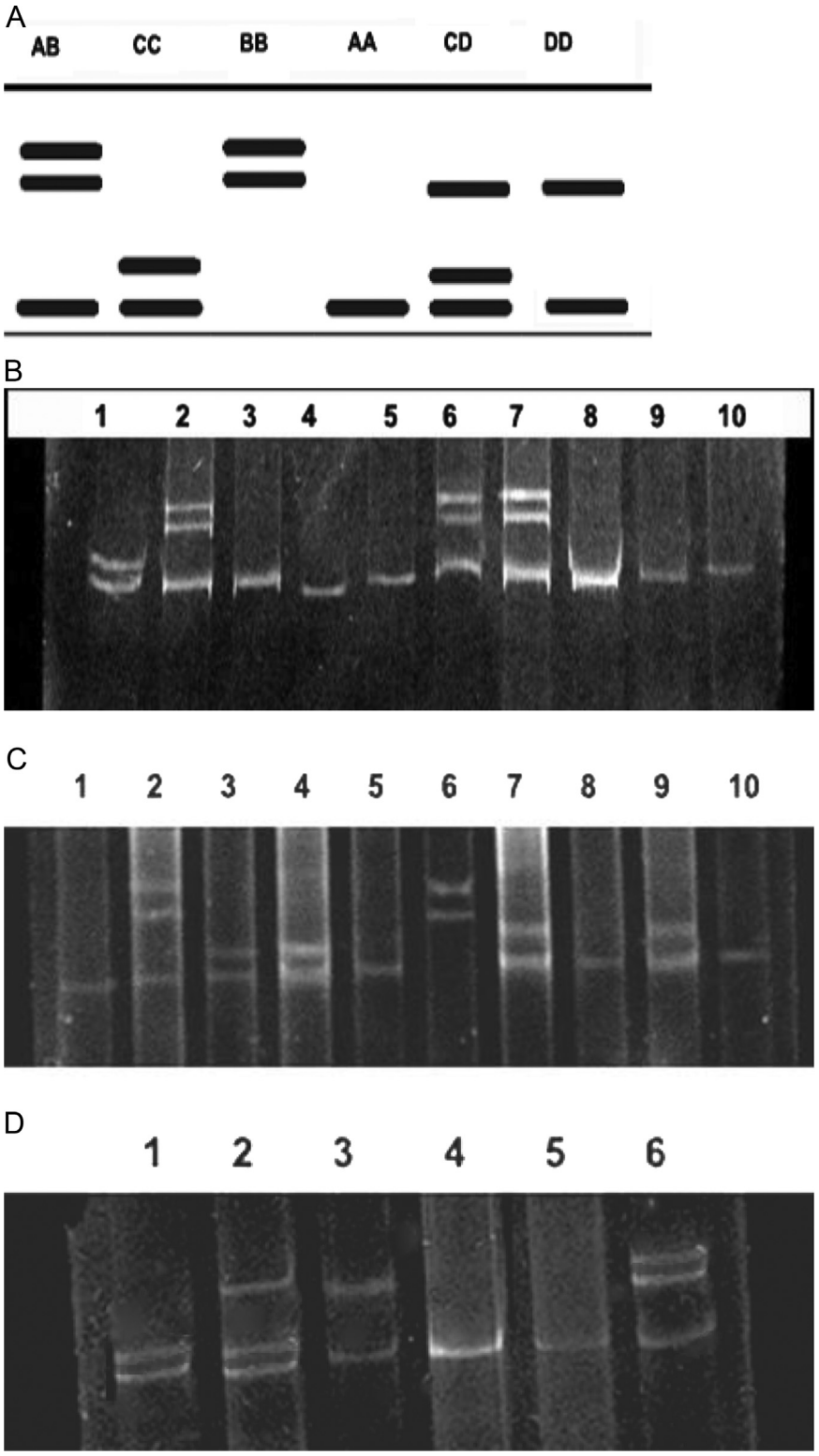

Fig. 3. SSCP-genotyping of buffalo, Baladi cattle and Holstein cattle based on leptin gene polymorphisms. (A) Schematic representation of six unique banding patterns involving four different allelic sequences (A–D) of Bovine leptin exon 3 could be resolved by SSCP technique using 12.5% polyacrylamide-formamide gel. (B) Polyacrylamide gel showing three different genotypes of exon 3 of the leptin gene in water buffalo. Lane 1: CC genotype; lanes 2, 6, 7: AB genotype and lanes 3–5 and 8–10: AA genotype. (C) Polyacrylamide gel showing four different genotypes of exon 3 of the leptin gene in Holstein cattle. Lanes 1, 5, 8, 10: AA genotype; lane 2: AB genotype; lanes 3, 4, 7, 9: CC genotype and lane 6: BB genotype. DD genotype is not shown in this figure. (D) Polyacrylamide gel showing five different genotype patterns of leptin gene exon 3 in Baladi cattle. Lane 1: CC genotype; lane 2: CD genotype; lane 3: DD genotype; lanes 4, 5: AA genotype and lane 6: AB genotype.

The fact that leptin is found in adipose tissue makes it a logical choice to serve as an excellent genetic marker for desirable carcass traits in beef cattle. One of the main goals of animal breeders is to select superior animals for breeding. Screening favorable alleles for selection at the DNA level provides an ideal tool for MAS. To the best of our knowledge, leptin gene polymorphisms in native beef breeds and buffalo have not yet been studied. Based on polymorphism in leptin exon 2 for all studied breeds, the C allele

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Table 3
Allelic and genotypic frequencies of buffalo, Baladi cattle and Holstein cattle as revealed by PCR-SSCP of leptin exon 3 and HWE analysis.

Animal breed		Genotypic frequencies						χ^2 value	Allelic frequency			
		AA	BB	AB	CC	DD	CD		A	B	C	D
Buffalo, $N=0$	Obs.	56.50	0	37	6.50	0	0	19.96*	0.75	0.18	0.07	0
	Exp.	56.25	3.32	27	7	0	0					
Baladi cattle, $N=60$	Obs.	48.30	0	3.30	33.40	10	5	46.68*	0.50	0.02	0.38	0.10
	Exp.	25	0.04	2	14.44	7.60	1					
Holstein cattle, $N=60$	Obs.	61.70	5	5	25	3.30	0	58.28*	0.64	0.08	0.26	0.02
	Exp.	41	0.67	10.23	6.67	1.04	0.40					

Obs.: Observed frequency.
Exp.: Expected frequency under Hardy-Weinberg equilibrium (HWE).
χ^2: chi-square value.

* Statistically significant; the deviation between observed genotypic frequencies and those expected under HWE was statistically significant ($P \leq 0.05$); suggesting that this population sample was not in HWE.

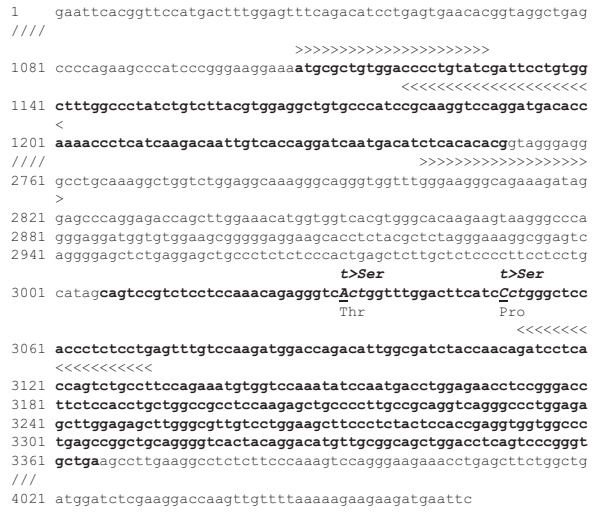

Fig. 4. Locations of primers used for amplification of exons 2 and 3, point polymorphisms and amino acids changes of Baladi cattle leptin gene (compared to sequence of GenBank: U50365.1). Bold nucleotides are representing mRNA sequences for exons 2 and 3. Locations of primers used in this study are indicated as > > > > > for forward primers and < < < < < for reverse primers. Capital letter nucleotides are two polymorphic points in Baladi cattle sequenced leptin exon 3 that changed to those in the upper line. Threonine (Thr) and Proline (Pro) are the amino acids of original leptin sequence which changed to Serine (Ser) upon polymorphism.

frequency was significantly dominant over the T allele frequency similar to that observed in Angus and Hereford breeds (Buchanan et al., 2002). The homozygous genotype (CC) had the highest frequency in all populations: 91.7%, 98.3% and 100% for Baladi, Holstein and buffalo, respectively. However, in Holstein–Friesian dairy cattle, the frequency of CC and CT genotypes was nearly equal (C/C 0.40, C/T 0.47) with lower TT genotype (0.13) (Giblin et al., 2010). In the present study, the heterozygote (CT) genotype frequency expected for Baladi cattle was significantly lower than those observed ($P < 0.05$). The lack of planned mating programs and closed mating systems using a few sires are probably the main explanation for the low level of heterozygosity observed, especially in native breeds of cattle and buffaloes. No information is currently available for native breeds to be compared with our results, so we studied Holstein cattle as a main stream breed for comparison. In addition to breed type, several factors are also integrated in studying the production of meat quality. Undoubtedly, increasing the number of animals investigated, considering the role of other major genes and the physiological status of animals simultaneously will perfectly explain more facts in this regard. It must be pointed out that, frequency of any allele can be altered simply by mating strategies in different herds based on economic and population demands. Unfortunately, phenotypic records were not available in our study to indicate how the allele and genotype frequencies associate with phenotypes. Therefore, genotyping the superior cow did not clarify the situation unless productivity correlation is well studied.

Previous reports revealed some conflicting results for the association of R25C with desirable traits. The T allele at R25C has been reported to be associated with increased milk yield, milk protein yield and decreased milk fat concentration (Buchanan et al., 2003). The CT heterozygous Holstein cows had greater yields of milk fat and milk protein compared to CC cows (Chebel and Santos, 2011). In other data sets no associations were found between R25C and milk production traits (Banos et al., 2008). The discrepancy between data of these studies could be due to a multitude of factors: (1) The frequency of the different alleles in the sample populations. (2) The experimental design (e.g., using estimated breeding value or actual phenotypes as the dependent variable) or the statistical models and data used (e.g., period during which the phenotype was measured. (3) Genotype by environment interaction or (4) the genetic background of the animals. The T allele of R25C has been reported to be associated with significantly heavier calves at weaning, fatter carcasses, slower growth rates, and higher leptin mRNA levels in adipose tissue compared to the C allele, albeit in beef cattle (Lusk, 2007; Di Stasio et al., 2007; DeVuyst et al., 2008).

By SSCP analysis of exon 3, a total of six genotype patterns were observed in the examined breeds. Similar gene and genotype frequencies were observed where AA genotype is the dominant one in Baladi cattle and buffalo similar to that of Holstein cattle. The HWE test is commonly used as genotyping quality control. However, the deviation between observed genotypic frequencies and those expected under HWE was found to be statistically significant in all studied breeds ($P \leq 0.05$), suggesting that these population samples were not in HWE. Deviation from HWE can be due to sampling error or alternatively to selective pressure acting on the locus. None of the polymorphism found in the Italian Buffalos showed a significant departure from HWE (Orrù et al., 2007).

Phenotypic information is required to correlate these various genotypes with production rates. Anton et al. (2012) found that leptin CC animals produced significantly higher 305 day milk protein percent values ($P < 0.05$) than other genotypes in Hungarian Simmental breed. They also reported that TT cows at the TG locus showed the highest milk fat percentage values, although differences between genotypes proved to be significant ($P < 0.05$) only in the Jersey breed. We have identified a set of new SNPs in the leptin gene of buffalo and Baladi cattle that may be useful for further association studies. Two polymorphic nucleotides have

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been characterized in exon 3 at position a3033 > t and c3051 > t of the cattle leptin cds. Upon translation, these two nucleotides polymorphism result in changing amino acids 58 and 64 of leptin from threonine and proline, respectively, into serine. Association of leptin gene polymorphisms with meat quality and carcass traits have been demonstrated by de Oliveira et al. (2013) and Tian et al. (2013) in Nellore cattle and Chinese steers. How the results reported in this study affect leptin function and whether it is correlated with food production and reproduction efficiency requires further study. The leptin sequence variations investigated in this study raise the question the potential importance of these leptin variants, or indeed leptin, in selection for improving productivity in Baladi cattle and buffalo. Further studies will be required to determine the relationship between LEP gene polymorphisms and the performance traits, and the association of leptin concentration and genotypes in these breeds.

5. Conclusions

The goal of this study was to elucidate genetic polymorphisms of the leptin gene in native beef breeds. The results obtained using PCR-RFLP and SSCP genotyping revealed that several leptin gene polymorphisms were observed in all breeds studied. This study also identifies interesting aspects for future selection programs, especially marker assisted selection. The analysis of leptin gene polymorphism indicated a degree of variability of the beef cattle populations herein investigated, suggesting that the artificial selection applied to the breeds has not reduced their diversity in these systems.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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