DETECTING THE MOLECULAR ASSOCIATIONS OF PROLACTIN GENE GENOTYPES WITH MILK TRAITS IN FRIESIAN AND BALADI COWS USING RFLP TECHNIQUE

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SUMMARY

Four years of experimental work were conducted applying PCR-RFLP to detect the SNP polymorphic associations of the prolactin candidate gene (PRL) with milk traits in cattle. A total of 494 lactations for 180 milking cows of Friesian in Elkarda herd (FK) and Sakha herd (FS) and Baladi cows in Elserv herd (BS) were used. The PCR products were digested using Nael and Smal restricted enzymes to investigate the molecular associations of the PRL gene genotypes with milk traits. The highest expected numbers of alleles (NE) in CC, CD and DD genotypes of the PRL gene were recorded in the DD genotype in both Friesian and Baladi herds, while the lowest expected numbers were recorded in CC genotype in all herds. The highest NE in GG, GT and TT genotype was in the TT genotype and the lowest NE was in the GG genotype in all herds. PRL gene was not in Hardy-Weinberg Equilibrium in all herds. The allelic frequency for the D allele was much higher than the frequency of the C allele, while the frequency of the T allele was higher than the frequency of the G allele.

The observed and expected values of heterozygosity for the PRL gene were mostly moderate in the three herds studied. The values of polymorphic information content (PIC) were mostly moderate in all herds. The values of fixation index (reduction in heterozygosity due to inbreeding, FIS) were high in the FK herd (0.82), FS herd (0.62) and BS herd (0.94). The molecular associations between PRL gene genotypes and milk traits indicate that the CD genotype of the PRL gene has strong molecular associations with yields of milk, fat and protein relative to CC and DD genotypes. GG genotype was significantly associated with all milk traits relative to GT and TT genotypes.

Keywords: Friesian and Baladi cows, PRL gene, PCR-RFLP, polymorphic associations, milk yield and its compositions

INTRODUCTION

In the last two decades, some genes are used as potential candidate genes associated with milk traits in Marker Assisted Selection (MAS) and genomic selection (GS) in dairy cattle. With the advancing of molecular techniques in terms of availability and costs of genotyping, GS has become more affordable (Raschia et al., 2018). According to genome-wide association studies (GWAS), milk traits are affected by several candidate genes and these genes could be used in GS. Among the various candidate genes, prolactin gene (PRL) seems to be promising, because it has diverse biological functions such as water and electrolyte balance, growth and development, immunity traits and reproductive functions and it plays crucial roles in mammary gland development, milk secretion and initiation and maintenance of lactation (Khatami et al., 2005; Zhang et al., 2008; César et al., 2012; Ozdemir, 2020; Özcan-Gökçek and İşık, 2023; Samuel et al., 2023).

In practice, most of the Quantitative Trait Loci (QTLs) studies have been supported the possibility to increase the efficiency of GS compared to traditional selection (Hu et al., 2009; Yudin and Voevoda, 2015; Patel and Chauhan, 2017; Samuel et al., 2023). Due to the importance of this PRL candidate gene, most of the studies suggested that the PRL variants could be useful in selection programs for improving milk yield and its composition in dairy cattle (e.g. Bukhari et al., 2013; Deepika and Salar, 2014; Fontanesi et al., 2014; Sonmez and Ozdemir, 2017; Thuy et al., 2018; Dudule et al., 2020; Ozdemir, 2020, and Shah et al., 2021). Recently, Ilie et al. (2023) stated that the bovine PRL gene is essential for the initiation and maintenance of lactation and exerts multiple effects on mammary alveoli to promote the synthesis and secretion of major components of milk.

The Bovine PRL gene has been mapped to chromosome 23 at 43 cM close to QTL, about 10 kb in size, including 5 exons coding for 199 amino acids and 4 introns (Mahajan et al., 2012; Patel and Chauhan, 2017, and Samuel et al., 2023). Using polymerase chain reaction and the Restriction Fragments Length Polymorphism technique (PCR-RFLP), these polymorphic structures of the PRL gene have been studied by many researchers, who confirmed significant associations between PRL
polymorphic variants and milk production traits in dairy cattle (He et al., 2006; Hu et al., 2009; Mehmannaz et al., 2009; Alfonso et al., 2012; Cesar et al., 2012; Thuy et al., 2018, and Ilie et al., 2023).

Based on recent worldwide molecular studies, strong associations among the PRL gene genotypes and milk traits were detected, emphasizing the potentiality of the PRL gene as a candidate gene to be used in selection for improving milk traits in dairy cattle (Alipanah et al., 2007; Lu et al., 2010; Alfonso et al., 2012; Cesar et al., 2012; Das et al., 2012; Mahajan et al., 2012; Bukhari et al., 2013; Patel and Chauhan, 2017; Thuy et al., 2018, and Samuel et al., 2023). But, the molecular studies practiced in developing countries concerning the associations of the PRL gene with milk traits in dairy cattle are limited and scarce. In Egypt, no systematic efforts have been undertaken to assess the status of PRL polymorphism in dairy cattle breeds. Therefore, the main aims of the present study were: (1) To characterize the PRL candidate gene of Friesian and Baladi cows, on SNPs molecular basis, in terms of Hardy Weinberg equilibrium, genotypic and allelic frequencies, observed and expected heterozygosity, polymorphic information content and fixation index, and (2) To investigate the molecular associations of the PRL gene genotypes with yields of milk, fat and protein in Friesian and Baladi cattle using Nael and Smal restriction enzymes and applying PCR-RFLP. Accordingly, this is the first attempt to report the associations between the PRL gene and milk production in dairy cattle in Egypt.

**MATERIAL AND METHODS**

**Animals used and genomic DNA extraction:**

A study was conducted on 494 lactations of 142 Friesian and 38 Baladi cows in herds of Elkarda Friesian (FK), Sakha Friesian (FS) and Elserw Baladi (BS) to characterize prolactin gene on molecular basis and to detect the polymorphic associations between SNP genotypes of this gene and milk traits applying PCR-RFLP method (Kumari et al., 2008; Dudule et al., 2020, and Samuel et al., 2023). Cows in the first four groups were used, depending on available records. However, the polymorphism in exon 3 and 4 of the prolactin gene in dairy cattle was clarified and protocolled by several investigators (e.g. Zhang et al., 2008; Hu et al., 2009; Mehmannaz et al., 2009; Das et al., 2012; Karuthadurai et al., 2019; Bayil and Bozkurt, 2019; Dudule et al., 2020; Shah et al., 2021, and Samuel et al., 2023).

Data on yields of milk, fat and protein of unrelated cows in Friesian herds were recorded for 10 months in FK and FS herds, while the data of unrelated cows in BS herd was available only for six months of lactation. These yields were collected over a period of four years from 2013 through 2016. Cows were milked twice a day at 7 am and 4 pm throughout the lactation period. Fat and protein yields were quantified by the automated method of infrared absorption spectrophotometry (Milk-o-Scan; Foss Electric, Hillerød, Denmark) at the Dairy Services Unit, Animal Production Research Institute, Sakha, Kafr Elsheikh governorate, Egypt.

Blood samples were collected from the jugular vein of 180 cows in test tubes containing an anticoagulant of ethylenediamine tetraacetic acid tripotassium salt (K3EDTA). Genomic DNA extraction was performed and the RFLP method was applied to detect PCR products using Nael and Smal restriction enzymes (Kumari et al., 2008; Sacravarty et al., 2008; Dudule et al., 2020, and Samuel et al., 2023).

**PCR amplification:**

On chromosome 23, the PCR amplification was performed in a volume of 25 μl using 1.5 mM MgCl2, 200 μM dNTPs (deoxyribonucleotide triphosphate), 5 pmol of each primer, 50-100 ng of genomic DNA and 0.5 Units of Taq DNA polymerase (Matrix Scientific Trade CO., Egypt). Two fragments of 232 and 168 bps in length were used in the amplification process, based on the available sequences of the bovine PRL gene exon 10 (Gene Bank accession number: FJ901285, SNP location 486 and 828 bps). In the case of the 232-bp fragment containing a polymorphic Nael restriction site, the Forward Primer was 5'- CCTATTTTCTTGCCAAATGGA-3’ and the Reverse Primer was 5'-TCTGACTCCCTGCTGTGTTTGGT-3’, while in the case of the 168-bp fragment containing a polymorphic Smal restriction site, the Forward Primer was 5' AGATGGAGTGCTGCTGCTGTGCT-3' and the Reverse Primer was 5'- GCCTTCTTGGGTCCCTTCTC3'. Amplification reactions were carried out by a program starting with 5 minutes of denaturation at 94°C followed by 35 cycles of 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for one minute, with a final extension of 10 minutes at 72°C.

**PCR Products Digestion:**

PCR products were separated by using an electrophoresis unit on 3.5 % agarose gel at a constant 75 V, stained with ethidium bromide and excised for digestion by specific enzymes. Since the DNA fragment of PRL gene is large and too long, therefore two restricted enzymes were used to ascertain the molecular analyses. Digestion using Nael restriction enzyme was performed in 10 μl mixture containing 8.8 μl PCR products + 0.2 μlNael + 1 μl Buffer. The reaction system was performed under 37°C for three hours and the resulting fragments were separated on 3% agarose gel and detected by electrophoreses unit and U.V. unit to detect the number of bands for each genotype. Digestion by Smal restriction enzyme was performed in a 10 μl mixture containing 4.9 μl PCR products + 0.1 μlSmal + 5 μl Buffer. The reaction system was performed at 55°C overnight and the product of fragments was separated on 3 % agarose gel and detected by electrophoreses unit and U.V. unit to
detect the number of bands for each genotype. All the molecular investigations were carried out in the Laboratories of Molecular Genetics of Research Labs Park, Faculty of Agriculture, Benha University, Egypt.

Characterizing PRL gene in Friesian and Baladi herds:

The genetic molecular weights were detected for each genotype using POPGENE software, version 1.32 (Yeh et al., 1999; https://genepop.curtin.edu.au/). Hardy-Weinberg Equilibrium (HWE; https://genepop.curtin.edu.au/genepop_op1.html), the observed (NO) and expected (NE) numbers of alleles (https://genepop.curtin.edu.au/genepop_op4.html), and genotypic and allelic frequencies (https://genepop.curtin.edu.au/genepop_op5.html) of the PRL gene were estimated within each herd. The observed (HO) and expected (HE) heterozygosities were estimated using GENALEX software, version 6.5 (Peakall and Smouse, 2006; https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1471-8286.2005.01155.x). The polymorphic information content (PIC) was calculated using CERVUS software, version 3 (Kalinowski et al., 2007). The reduction in heterozygosity due to inbreeding within each herd (i.e. fixation index, FIS) was calculated using POPGENE program, version 1.32 (Yeh et al., 1999; https://genepop.curtin.edu.au/genepop_op5.html).

The following equations were used in the estimation of the previous parameters:

\[
H_o = \text{No. heterozygosity} / n
\]

\[
H_e = 1 - \sum_{i=1}^{n} P_i^2
\]

\[
PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n} \sum_{j=1}^{n} 2P_i^2P_j^2
\]

\[
F_{IS} = (H_e - H_o) / H_e
\]

Where \( P_i \) = the frequency of the \( i \)th allele, \( P_j \) = the frequency of the \( j \)th allele, \( n \) = the number of alleles (Peakall and Smouse, 2012), FIS= fixation index or the reduction in heterozygosity due to inbreeding.

Estimation of molecular associations between PRL gene genotypes and milk traits:

Test day (TD) records for milk, fat and protein yields were collected and data available was mostly for 10 months of lactation in the Friesian herds, while the data available in the Baladi herd were collected mostly for six months of lactation. For data analysis, TD records were used to predict 305 days of milk yield in Friesian herds using Fleishman method as mentioned by Kong et al. (2018). The polymorphic associations between PRL gene genotypes and yields of milk, fat and protein were analyzed by applying the "Mixed procedure" of SAS, package 9.0 (Kamp et al., 2002) and using the following mixed model (in matrix notations):

\[
y = Xb + Za + Zp p + e
\]

Where: \( y \) = the vector of milk trait observations; \( X \) = the incidence matrix relating the fixed effects to \( y \); \( b \) = the vector of the fixed effects of year-season of calving, parity and genotypes of PRL gene restricted by \( Nael \) and \( Smal \) restriction enzymes (three genotypes for each enzyme, genotypes CC, CD and DD for \( Nael \) enzyme and genotypes GG, GT and TT for \( Smal \) enzyme); \( Za \) = the incidence matrix relating the cow additive effects to \( y \); \( a \) = the vector of the random additive effects associated with the incidence matrix \( Za \); \( Zp \) = the incidence matrix relating the permanent environmental effects to \( y \); \( p \) = the vector of random permanent environmental effects associated with the incidence matrix \( Zp \); \( e \) = the vector of random residual effects.

RESULTS AND DISCUSSION

Molecular weights:

The amplified DNA fragment of 230 bp was digested using the \( Nael \) restriction enzyme and three genotypes of CC, CD and DD of the PRL gene were detected. The banding patterns of the PRL gene yielded in PCR product were one band in the CC genotype (230 bp), three bands in the CD genotype (230, 150 and 80 bps) and two bands in the DD genotype (150 and 80 bps). Udina et al. (2001) using Black Pied, Russian Ayrshire and Gorbato Red cattle reported that variation of the microsatellite of the PRL gene was monomorphic in Gorbato Red cattle (164 bp) and two alleles were detected in Black Pied and Russian Ayrshire cattle (164 and 162 bps). Mahajan et al. (2012) with Frieswal cattle amplified a 156 bp of the PRL gene in the exon-3 segment by PCR-RFLP method using \( RsaI \) restriction enzyme. Sonmez and Ozdemir (2017) using the PCR-RFLP method detected three fragments of the PRL gene in native EAR cattle raised in Turkey (GG genotype with 210 bp, AA genotype with 120 bp and AG genotype with 90 bp).

The DNA fragment of 180 bp was digested using the \( Smal \) restriction enzyme and three genotypes of GG, GT and TT of the PRL gene were detected. The banding patterns of the PRL gene yielded in PCR product were one band in GG genotype (180 bp), three bands in GT genotype (180, 123 and 57 bps) and two bands in TT genotype (123 and 57 bps). These genotypes and bands were comparable with those obtained by Deepika and Salar (2014) who observed three genotypes of GG, GT and TT in indigenous Indian grey cattle. Similar RFLP patterns using \( RsaI \) restriction enzyme were reported by Sacravarty et al. (2008) in Kankrej cattle. Mehmammadavaz et al. (2009) detected a mutation in 294-bp fragment of PRL gene located in exon 4 in Iranian Holstein cattle using PCR-RFLP technique and \( RsaI \) restriction enzyme. Karuthadurai et al. (2019) in Sahiwal cattle found that the fragments of genotype GA had 156, 84 and 72 bps, the genotype GG had 84 and 72 bps and the genotype AA had 156 bp.
The observed and expected number of alleles and Hardy-Weinberg equilibrium in each herd:

The observed (NO) and expected (NE) numbers of alleles and Hardy-Weinberg equilibrium (HWE) in the three genotypes of the PRL gene are presented in Table 1 for each separate herd. In case of using the Nael enzyme, the highest NE was recorded in DD genotype in all herds (59.4, 37.0 and 12.9 for FK, FS and BS herds, respectively). In Smal polymorphism genotypes, the highest NE was recorded in TT genotype for all herds (56.1, 25.1 and 17.1 for FK, FS and BS herds, respectively), while the lowest NE was recorded in CC genotype (9.8, 11.3 and 11.9 for FK, FS and BS herds, respectively). In Smal polymorphism genotypes, the highest NE was recorded in TT genotype for all herds (56.1, 25.1 and 17.1 for FK, FS and BS herds, respectively), while the lowest NE was recorded in GG genotype (2.1, 8.2 and 4.1 for FK, FS and BS herds, respectively).

Table 1. The observed (NO) and expected (NE) number of alleles and Chi-square values ($\chi^2$) for Hardy-Weinberg equilibrium (HWE) for the PRL gene genotypes in the three cattle herds studied

<table>
<thead>
<tr>
<th>Herd</th>
<th>No of cows</th>
<th>Observed number of PRL alleles (NO)</th>
<th>Expected number of PRL alleles (NE)</th>
<th>$\chi^2$ value for HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>CD</td>
<td>DD</td>
</tr>
<tr>
<td>FK</td>
<td>80</td>
<td>2</td>
<td>11</td>
<td>67</td>
</tr>
<tr>
<td>FS</td>
<td>62</td>
<td>2</td>
<td>16</td>
<td>44</td>
</tr>
<tr>
<td>BS</td>
<td>38</td>
<td>3</td>
<td>11</td>
<td>24</td>
</tr>
</tbody>
</table>

Polymorphic genotypes in case of using Nael restriction enzyme:

<table>
<thead>
<tr>
<th>Herd</th>
<th>No of cows</th>
<th>Observed number of PRL alleles (NO)</th>
<th>Expected number of PRL alleles (NE)</th>
<th>$\chi^2$ value for HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>GT</td>
<td>TT</td>
</tr>
<tr>
<td>FK</td>
<td>80</td>
<td>11</td>
<td>4</td>
<td>65</td>
</tr>
<tr>
<td>FS</td>
<td>62</td>
<td>17</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td>BS</td>
<td>38</td>
<td>12</td>
<td>1</td>
<td>25</td>
</tr>
</tbody>
</table>

**FK= Friesian in Elkarada herd, FS= Friesian in Sakha herd, and BS= Baladi in Elserw herd. *= P<0.05, **=P<0.01

In both Nael and Smal enzymes, Chi square values ($\chi^2$) of the PRL gene genotypes were significant in all herds studied (P<0.05 or P<0.01; Table 1). These significant values of $\chi^2$ indicate that all the herds were not in HWE for PRL gene. The result of Mahajan et al. (2012) in Frieswal cattle showed that the population was not in HWE for PRL gene. Dong et al. (2013) found that the haplotype frequencies of the PRL gene in Chinese Holstein cows were not in HWE (P<0.01). Akyuz and Cinar (2014) using East Anatolian Red, Zavot, Brown Swiss and Simmental breeds reported that the Zavot breed was the only breed in HWE. Patel and Chauhan (2017) found that the Chi-square values for Gir and Kankrej cattle in India were 13.46 and 12.58, indicating that the populations were not in HWE for PRL gene. Sonmez and Ozdemir (2017) applying PCR-RFLP in native East Anatolian Red cattle found that the population was in HWE for PRL gene. Bayil and Bozkurt (2019) reported that the Holstein cattle population was in HWE for PRL gene since the Chi-square value was not significant ($\chi^2 = 3.74$).

Genotypic and allelic frequencies in each herd:

In case of Nael restriction enzyme, the highest genotypic frequency in both Friesian and Baladi herds was recorded in DD genotype of the PRL gene (0.837, 0.709 and 0.631 in FK, FS and BS herds, respectively; Table 2), while the lowest frequency was in CC genotype (0.025, 0.032 and 0.079 in FK, FS and BS herds, respectively). The allelic frequency for D allele was much higher than for C allele (0.906, 0.838 and 0.776 vs 0.093, 0.161 and 0.223 in FK, FS and BS herds, respectively; Table 2). The allelic variation in the sequences of the PRL gene in dairy cattle would be of interest because the PRL gene has a possible direct or indirect effect on milk production (Hu et al., 2009; Boleckova et al., 2012; Thy et al., 2018). Udina et al. (2001) found that the frequencies of B allele of PRL gene detected by PCR-RFLP method were 0.141 and 0.869 in Russian Ayrshire and Gorbatov Red cattle, respectively. Mehmannavaz et al. (2009) found that the frequency of G allele (0.931) was much higher than A allele (0.069). Boleckova et al. (2012) reported that the frequencies in Holstein cattle were 0.12 for A allele and 0.88 for G allele. Thy et al. (2018) stated that the allelic frequencies were 0.886 for L allele and 0.114 for V allele in Holstein Friesian dairy cows. Results of Mahajan et al. (2012) using PCR-RFLP method and Rsal restriction enzyme in Frieswal cattle revealed that the allelic frequencies were 0.630 for A allele and 0.370 for B allele and the genotypic frequencies were 0.315 for AA genotype, 0.629 for AB genotype and 0.056 for BB genotype.
In case of using **NaeI** enzyme:

<table>
<thead>
<tr>
<th>Herd</th>
<th>Number of cows</th>
<th>Genotypic frequency</th>
<th>Gene frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>FK</td>
<td>80</td>
<td>CC 0.025, CD 0.137, DD 0.837</td>
<td>C 0.093, D 0.906</td>
</tr>
<tr>
<td>FS</td>
<td>62</td>
<td>CC 0.032, CD 0.258, DD 0.709</td>
<td>C 0.161, D 0.838</td>
</tr>
<tr>
<td>BS</td>
<td>38</td>
<td>CC 0.079, CD 0.289, DD 0.631</td>
<td>C 0.223, D 0.776</td>
</tr>
</tbody>
</table>

In case of using **SmaI** enzyme:

<table>
<thead>
<tr>
<th>Herd</th>
<th>Number of cows</th>
<th>Genotypic frequency</th>
<th>Gene frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>FK</td>
<td>80</td>
<td>GG 0.137, GT 0.050, TT 0.812</td>
<td>G 0.162, T 0.837</td>
</tr>
<tr>
<td>FS</td>
<td>62</td>
<td>GG 0.274, GT 0.177, TT 0.548</td>
<td>G 0.362, T 0.637</td>
</tr>
<tr>
<td>BS</td>
<td>38</td>
<td>GG 0.315, GT 0.026, TT 0.657</td>
<td>G 0.328, T 0.671</td>
</tr>
</tbody>
</table>

FK = Friesian in Elkarada herd, FS = Friesian in Sakha herd, and BS = Baladi in Elserw herd.

In case of **SmaI** restriction enzyme, TT genotype of the PRL gene was the highest frequency (0.812, 0.548 and 0.657 in FK, FS and BS herds, respectively) relative to GG genotype (0.137, 0.274 and 0.315) and GT genotype (0.050, 0.177 and 0.026) as presented in Table 2. The frequency of T allele was higher than G allele in all herds studied (0.837, 0.637 and 0.671 for T allele vs 0.162, 0.362 and 0.328 for G allele in FK, FS and BS herds, respectively). Accordingly, these allelic frequencies gave a noticeably higher incidence of allele G in genotypes of PRL gene as cited by Mehmannavaz et al. (2009) in Holstein (0.93) and by Singh et al. (2015) in Holstein crossbred (0.75). Khatami et al. (2005) in Russia using RFLP analysis and Rsal enzyme found clear differences in allelic and genotypic frequencies of PRL gene in Russian and German Black and White and Yaroslavl cattle. Thuy et al. (2018) found that only two genotypes were detected with genotypic frequencies of 0.780 and 0.220 for LL and LV genotypes, respectively.

The variation of PRL allelic frequencies obtained in both Friesian herds (FK and FS herds, Table 2) may be due to the differences in herd size and selection programs practiced in these herds, indicating also that PRL gene is considered as candidate gene potentially associated with milk performance traits to be used in marker-assisted selection program in dairy cattle in Egypt. However, the genotypic and allelic frequencies of GG, GT and TT genotypes of the PRL polymorphism reported here are similar to those findings reported in various regions of the world for different breeds (Mehmannavaz et al., 2009; Lu et al., 2010; Alfonso et al., 2012; Boleckova et al., 2012; Das et al., 2012, and Sonmez and Ozdemir, 2017). Kumari et al. (2008) in India reported frequencies of AA, BB and AB genotypes to be 0.22, 0.13 and 0.65 in Jersey cattle with allelic frequencies of 0.55 for A allele and 0.45 for B allele. Karuthadurai et al. (2019) reported also in India that the frequencies of AA, GG and GA genotypes of Sahiwal cattle were 0.30, 0.45 and 0.25, respectively with allelic frequencies of 0.425 for A allele and 0.575 for G allele. In Croatia, Maurie et al. (2017) stated that most of the animals of Simmental and crossbred Holstein cattle were genotyped as GG homozygous genotype for PRL gene.

**Heterozygosis in PRL gene genotypes:**

In both **NaeI** and **SmaI** restricted enzymes, the observed values of heterozygosity (HO) for PRL gene (Table 3) were moderate and nearly similar in magnitude and trend in FK herd (0.47 and 0.36), FS herd (0.26 and 0.21) and BS herd (0.28 and 0.19). Similarly, Akyuz et al. (2012) reported moderate values of HO for PRL gene to be 0.28 in Black and White cattle and 0.43 in Jersey cattle. Sonmez and Ozdemir (2017) in native East Anatolian Red cattle found that the observed heterozygosity for PRL polymorphism was also moderate (0.34).

The expected values of heterozygosity (HE) were moderate and similar in the three herds studied (0.23 and 0.39; Table 3). Patel and Chauhan (2017) reported that the expected frequencies of heterozygosity of PRL gene obtained by PCR-RFLP technique in Gir and Kankrej cattle were high (0.83 and 0.70).

The values of fixation index (FIS) were high and nearly similar in magnitude and trend for both **NaeI** and **SmaI** restricted enzymes in FK herd (0.70 and 0.82), FS herd (0.75 and 0.62) and BS herd (0.70 and 0.94) (Table 3). Sonmez and Ozdemir (2017) applying PCR-RFLP in native East Anatolian Red cattle found that the value of FIS was of low rate (0.072).

The values of polymorphic information content (PIC) in both **NaeI** and **SmaI** restricted enzymes were moderate and nearly similar in magnitude in FK herd (0.22 and 0.23), FS herd (0.20 and 0.36) and BS herd (0.28 and 0.34) (Table 3). These moderate values of PIC measure the ability of a molecular marker to detect polymorphisms, and therefore have enormous importance in selecting markers for genetic studies in dairy cattle. These values can also serve as screening for genetic studies of the Egyptian herds with the objectives to investigate evolutionary and ecological
processes. Udina et al. (2001) in Russian Ayrshire, Gorbatov Red and Black Pied cattle found that the estimate of PIC applying PCR–RFLP in exon 3 of PRL gene was moderate (0.21). Dong et al. (2013) reported that the intron 3 and 4 of the PRL gene in Chinese Holstein cows indicated that the PIC values of the haplotype block (0.194) should be considered as slightly polymorphic information content.

Table 3. The observed (HO) and expected (HE) heterozygositys, fixation index (FIS) and the polymorphic information content (PIC) for genotypes of PRL gene in Friesian and Baladi cattle herds

<table>
<thead>
<tr>
<th>Herd</th>
<th>No. of cows</th>
<th>H_o± SE</th>
<th>H_L± SE</th>
<th>F_IS</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In case of using NaeI restriction enzyme:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FK</td>
<td>80</td>
<td>0.47±0.03</td>
<td>0.23±0.03</td>
<td>0.70</td>
<td>0.22</td>
</tr>
<tr>
<td>FS</td>
<td>62</td>
<td>0.26±0.04</td>
<td>0.23±0.04</td>
<td>0.75</td>
<td>0.20</td>
</tr>
<tr>
<td>BS</td>
<td>38</td>
<td>0.28±0.02</td>
<td>0.23±0.02</td>
<td>0.70</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>In case of using Smal restriction enzyme:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FK</td>
<td>80</td>
<td>0.36±0.03</td>
<td>0.39±0.03</td>
<td>0.82</td>
<td>0.23</td>
</tr>
<tr>
<td>FS</td>
<td>62</td>
<td>0.21±0.04</td>
<td>0.39±0.04</td>
<td>0.62</td>
<td>0.36</td>
</tr>
<tr>
<td>BS</td>
<td>38</td>
<td>0.19±0.02</td>
<td>0.39±0.02</td>
<td>0.94</td>
<td>0.34</td>
</tr>
</tbody>
</table>

FK= Friesian in Elkarada herd, FS= Friesian in Sakha herd, and BS= Baladi in Elserw herd. SE= Standard error.

**Polymorphic associations between PRL gene genotypes and milk traits within each herd:**

The molecular analyses in each separate herd revealed that three genotypes of the PRL gene of CC, CD and DD for Nael enzyme and GG, GT and TT for Smal enzyme were detected. However, there were abundant reports evidencing that the PRL gene is associated with milk production and/or composition traits in dairy cattle (e.g., Fontanesi et al., 2014; Singh et al., 2015; Mauriæ et al., 2017; Raschia et al., 2017; Shah et al., 2021; Ilie et al., 2023). Therefore, the results obtained in this study confirmed the molecular associations reported previously in the other populations of dairy cattle.

In the Friesian of Elkarada herd, the least square means for yields of milk, fat and protein were 3805, 52.8 and 32.2 kg in CC genotype, 3960, 54.8 and 39.6 kg in CD genotype and 3113, 50.5 and 35.8 kg in DD genotype in case of using Nael enzyme, while the means were 2996, 59.8 and 41.5 kg in GG genotype, 2865, 47.5 and 27.6 kg in GT genotype and 2920, 53.6 and 40.1 kg in TT genotype in case of using Smal enzyme, respectively (Table 4).

Table 4. The polymorphic associations of PRL gene genotypes with yields of milk, fat and protein in Friesian of Elkarada herd expressed as least square means (LSM) of 10 months lactation

<table>
<thead>
<tr>
<th>Trait</th>
<th>LSM</th>
<th>SE</th>
<th>LSM</th>
<th>SE</th>
<th>LSM</th>
<th>SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In case of using NaeI restricted enzyme:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC Genotype (N=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>3805b</td>
<td>82</td>
<td>3960b</td>
<td>85</td>
<td>3113b</td>
<td>119</td>
<td>0.003</td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>52.8a</td>
<td>3.4</td>
<td>54.8a</td>
<td>3.5</td>
<td>50.5a</td>
<td>2.4</td>
<td>0.015</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>32.2a</td>
<td>2.8</td>
<td>39.6a</td>
<td>2.3</td>
<td>35.8a</td>
<td>1.6</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>In case of using Smal restricted enzyme:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG Genotype (N=52)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>2996a</td>
<td>147</td>
<td>2865b</td>
<td>170</td>
<td>2920b</td>
<td>90</td>
<td>0.014</td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>59.8a</td>
<td>4.2</td>
<td>47.5b</td>
<td>4.8</td>
<td>53.6ab</td>
<td>2.5</td>
<td>0.016</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>41.5a</td>
<td>2.8</td>
<td>27.6b</td>
<td>3.2</td>
<td>40.1a</td>
<td>1.7</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a,b Means within each classification, not followed by the same letters differed significantly (P < 0.01), SE= Standard error.

The differences in yields of milk, fat and protein among CC, CD and DD genotypes of the PRL gene in Friesian of Elkarada herd were significantly in favour of CD genotype (p<0.01; Table 4). Also, the differences among the three genotypes of GG, GT and TT were significantly in favour of GG genotype relative to GT and TT genotypes. However, high means for CD and GG genotypes of the PRL gene indicate that there are strong associations of these genotypes with milk traits. Therefore, CD and GG
genotypes could be used in selection to improve milk traits of Friesian cows in Egypt (for milk, fat and protein yields). Singh et al. (2015) found that GG genotype had significantly greater milk yield in Indian Frieswal cattle (P<0.05) than AG genotype in crossbred Holstein cattle and this AG genotype had significantly higher milk yield compared to AG genotype in Simmental cattle (P<0.05). Maurie et al. (2017) stated that AG genotype of the crossbred Holstein cattle had significantly higher milk yield than GG genotype (P<0.05). Shah et al. (2021) reported that the differences in yield traits between RR and Rr genotypes in Jersey cows were significantly in favour of Rr genotype (p<0.05) relative to RR genotype.

Table 5. The polymorphic associations of the PRL gene genotypes with yields of milk, fat and protein in Friesian of Sakha herd expressed as least square means (LSM) of 10 months lactation

<table>
<thead>
<tr>
<th>Trait</th>
<th>NaeI restricted enzyme</th>
<th>SmaI restricted enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC Genotype (N= 14)</td>
<td>CD Genotype (N= 25)</td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>2920±a</td>
<td>3093±a</td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>63.2±a</td>
<td>66.5±a</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>50.4±a</td>
<td>52.3±a</td>
</tr>
<tr>
<td></td>
<td>GG Genotype (N= 40)</td>
<td>GT Genotype (N= 10)</td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>2852±b</td>
<td>2670±a</td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>70.9±b</td>
<td>53.1±a</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>44.7±b</td>
<td>43.5±b</td>
</tr>
</tbody>
</table>

Table 6. The polymorphic associations of the PRL gene genotypes with yields of milk, fat and protein in Baladi cattle herd expressed as least square means (LSM) of 10 months lactation

<table>
<thead>
<tr>
<th>Trait</th>
<th>NaeI restricted enzyme</th>
<th>SmaI restricted enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC Genotype (N= 14)</td>
<td>CD Genotype (N= 25)</td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>2920±a</td>
<td>3093±a</td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>63.2±a</td>
<td>66.5±a</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>50.4±a</td>
<td>52.3±a</td>
</tr>
<tr>
<td></td>
<td>GG Genotype (N= 40)</td>
<td>GT Genotype (N= 10)</td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>2852±b</td>
<td>2670±a</td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>70.9±b</td>
<td>53.1±a</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>44.7±b</td>
<td>43.5±b</td>
</tr>
</tbody>
</table>

The differences among the three genotypes of CC, CD and DD in yields of milk, fat and protein were significantly in favour of CD genotype of the PRL gene (p<0.01; Table 5). On the other hand, the differences among the three genotypes of GG, GT and TT in all milk traits were significantly in favour of GG genotype comparable to GT and TT genotypes. High least square means for CD and GG genotypes of the PRL gene indicate that these genotypes were considerably associated with milk traits in Friesian of Sakha herd. Therefore, CD and GG genotypes could be used in selection to improve milk traits of the Friesian cows in Egypt. He et al. (2006) using SNP in Chinese Holstein dairy cattle reported that BB genotype of the PRL gene was significantly associated with milk yield, fat yield, protein yield and protein percentage (p<0.05 or p<0.01), i.e. cows with BB genotype had significantly higher milk yield, fat yield and protein yield than that of AA genotype. Recently, Ilie et al. (2023) reported that AA genotype of the PRL gene was significantly associated with fat and protein percentages in the milk of the Romanian Brown cattle (p<0.05), i.e. AA genotype was associated with higher fat and protein percentages in milk compared to GG genotype.

In the Baladi cattle of Elserw herd, the molecular analysis of the PRL gene revealed that there are significant associations for CD and GG genotypes of the PRL gene with milk productivity parameters (Table 6), i.e. PRL gene is promising to be used as effective marker for milk productivity of Baladi cattle in Egypt. The least square means of yields of milk, fat and protein were 802, 25.0 and 22.0 kg in CC genotype, 815, 27.3 and 24.8 kg in CD genotype and 662, 22.7 and 20.3 kg in DD genotype in case of using NaeI enzyme, while the averages of yields of milk, fat and protein were 724, 25.7 and 22.9 kg in GG genotype, 294, 11.1 and 9.6 kg in GT genotype and 584, 19.2 and 17.2 kg in TT genotype in case of using SmaI enzyme, respectively (Table 6). The differences in yields of milk, fat and protein among CC, CD and DD genotypes of PRL gene in the local cattle of Elserw herd were significantly in favour of CD genotype (p<0.01). Also, the differences among the three genotypes of GG, GT and TT in the milk traits were significantly in favour of GG genotype (p<0.01). Therefore, CD and GG genotypes could be used in selection to improve the milk traits of Baladi cattle in Egypt.
Table 6. The polymorphic associations of the PRL gene genotypes with yields of milk, fat and protein in Baladi of Elserw herd expressed as least square means (LSM) of six months lactation

<table>
<thead>
<tr>
<th>Trait</th>
<th>LSM</th>
<th>SE</th>
<th>LSM</th>
<th>SE</th>
<th>LSM</th>
<th>SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>In case of using NaeI restricted enzyme:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC genotype (N=5)</td>
<td>802&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68</td>
<td>815&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71</td>
<td>662&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40</td>
<td>0.013</td>
</tr>
<tr>
<td>CD genotype (N=17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD genotype (N=48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>25.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2</td>
<td>27.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4</td>
<td>22.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4</td>
<td>0.015</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>22.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8</td>
<td>24.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1</td>
<td>20.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2</td>
<td>0.015</td>
</tr>
<tr>
<td>In case of using Smal restricted enzyme:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG genotype (N=17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GT genotype (N=2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT genotype (N=51)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>724&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70</td>
<td>294&lt;sup&gt;b&lt;/sup&gt;</td>
<td>206</td>
<td>584&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41</td>
<td>0.002</td>
</tr>
<tr>
<td>Fat yield, kg</td>
<td>25.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4</td>
<td>11.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.9</td>
<td>19.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.4</td>
<td>0.017</td>
</tr>
<tr>
<td>Protein yield, kg</td>
<td>22.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9</td>
<td>9.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.6</td>
<td>17.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2</td>
<td>0.012</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Means within each classification, not followed by the same letters differed significantly (P<0.01), SE= Standard error.

The molecular associations between exon 3 and exon 4 of the PRL gene with milk production traits in cattle were significantly evidenced in the last two decades by several investigators (e.g., Brym et al., 2005; Alipanah et al., 2007; Boleckova et al., 2012; Mahajan et al., 2012; Bukhari et al., 2013, and Ilie et al., 2023). Hu et al. (2009) in Chinese Holstein cows identified three genotypes within exon 4 of PRL gene (AA, AB and BB) using the SSCP technique of polymorphism where the cows with BB genotype showed higher milk yield in comparison to cows with AA and AB genotypes (P<0.05). Mahajan et al. (2012) in Frieswal cattle stated that the animals with AB genotype had higher milk yield relative to AA and BB genotypes, concluding that the PRL gene could be used as a considerable marker for genetic selection of milk traits in dairy cattle. Recently, Ilie et al. (2023) showed that PRL gene favored significantly higher fat and protein percentages in the milk of the Romanian Brown cattle compared to the Romanian Spotted breed.

CONCLUSION

Considering NaeI and Smal as restriction enzymes, CD and GG genotypes of the PRL gene were strongly associated on the molecular basis with milk traits and therefore both genotypes could be the main target in selection to improve yields of milk, fat and protein in Friesian and Baladi dairy cattle in Egypt, i.e., PRL gene could be used as a powerful tool in gene selection.

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The discovery of the genetic associations with milk yield in cattle is a crucial step in genetic improvement programs. This paper provides a detailed analysis of the relationship between the milk yield traits and the prolactin gene polymorphism in cattle populations of Ethiopia. The study used a cross-sectional design and analyzed the association between milk yield traits and the prolactin gene polymorphism using statistical methods. The results showed that the prolactin gene polymorphism had a significant effect on milk yield in cattle populations of Ethiopia. The study suggests that the prolactin gene polymorphism could be used as a genetic marker for milk yield improvement in cattle populations of Ethiopia.