**Associations between prolactin receptor (PRLR) polymorphisms and milk production traits in dairy buffalo**

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Associations between prolactin receptor (PRLR) polymorphisms and milk production traits in dairy buffalo

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ABSTRACT

Buffalo milk is economically important, and an understanding of the genetic basis of milk production traits may therefore facilitate buffalo breeding. In this study, polymerase chain reaction–single-strand conformation polymorphism was used to analyze polymorphisms in prolactin receptor (PRLR) exons 3, 5, 7, and 10 in Murrah, Nili-Ravi, and crossbred buffaloes and Holstein cows. A site in PRLR exon 3 exhibited variation in all three buffalo breeds and cows, and the three genotypes GG, GT, and TT were detected. In PRLR exon 5, nucleotide differences were detected between buffalo and Holstein dairy cow populations; the genotypes AA, AG, and GG were observed. In PRLR exon 7, polymorphisms were not detected. In PRLR exon 10, at 9637 bp, the TT, TC, and TT genotypes were observed. At the three polymorphic loci, Hardy–Weinberg equilibrium was reached in the crossbreed buffalo population for exons 3 and 5 and in all three buffalo populations for exon 10. In an analysis of the associations of the three polymorphisms with milk production traits, only PRLR exon 10 in Murrah
buffaloes was associated with milk yield, with a significant difference between the TT and CC genotypes.

**Key words:** Buffalo, Prolactin receptor, Polymorphism, Milk production trait

**INTRODUCTION**

Buffalo is well-adapted to subtropical and tropical climates, with many biological traits that render it valuable as livestock. According to the FAO, in 2008, global buffalo herds totaled 1.852 M heads distributed in 129 countries, with 97 % (179,752,900), 2.18 % (4,052,600), 0.62 % (1,153,400), and 0.2 % (370,000) in Asia, Africa, the United States, and Europe, respectively. India has the largest buffalo population, accounting for 56.74 % (105,127,000) of all buffaloes worldwide, followed by Pakistan with 15.65 % (29,000,000) and China with 12.56 % (3,271,900).

Based on appearance and life habits, water buffalo can be subdivided into swamp buffalo and river buffalo. China has the largest swamp buffalo population, representing 80 % of the global population; they are mainly distributed in the vast southern region of the Yangtze River. China has a long history of buffalo domestication, dating back approximately 7,000 years. Long-term natural and artificial selection in swamp buffalo has resulted in excellent biological characteristics, including a sturdy physique; power; resistance to high temperatures, humidity, and crude feed; docility; low disease incidence; and easy feeding. Accordingly, it is becoming an important livestock resource and an indispensable source of labor for the development of agricultural production in southern China.

However, owing to economic and urban development and improvements in agricultural mechanization, rural productivity has become less reliant on the labor value of buffaloes, which have become, for many farmers, a “non-performing asset.” Therefore, it is important to develop buffalo from
labor animals to animals for use in dairy and meat production and to utilize them effectively. Buffalo is an important economic animal in Guangxi, with high milk production value. The protein, amino acid, fat, vitamin, and trace element content in buffalo milk is higher than that in milk from Holstein cows (Zhu et al. 2008). Thus, promoting the buffalo dairy market has broad market potential. Lactation is an important economic trait in dairy cattle; it is controlled by a number of minor genes and is an ideal candidate for trait marker-assisted selection.

The prolactin receptor (PRLR) gene is a representative gene affecting milk production. PRLR plays an important role in signal transduction for the regulation of pituitary PRL and milk protein genes. The binding of two prolactin receptor molecules with a single prolactin molecule activates the JAK2/STAT5 signaling pathway, ultimately activating the trans-acting factor STAT5, which acts on the milk protein gene promoter region, thereby initiating or enhancing milk protein gene expression (Bin et al. 2009). Many studies have shown that PRLR polymorphisms have a significant effect on milk performance. Zhang et al. (2008), using PCR-single strand conformation polymorphism (SSCP) and sequencing, revealed the presence of two and three single nucleotide polymorphisms (SNPs) in exons 3 and 7, respectively. A correlation analysis showed that the SNPs were significantly associated with milk yield and milk fat. In exon 3, the AB genotype was associated with high milk production and the AA genotype with high milk fat; in exon 7, the BB genotype was associated with high milk yield and milk fat percentage. Viitala et al. (2006), using multi-marker regression, showed that two QTL on BTA20 were located at GHR and PRLR; furthermore, they proved that the F279Y and S18N substitutions in GHR and PRLR are significantly associated with milk production traits, the PRLR S18N substitution significantly affects milk fat and milk protein production, and F279Y significantly affects fat and protein levels. Brym et al. (2005) used the PCR-SSCP method to detect nine major
PRLR functional domain fragment polymorphisms and found a SNP in intron 9 at position 205 (A → C). Although the milk yield and milk protein content for individuals with the CC genotype at first lactation are high, a preliminary analysis showed that the PRLR genotype and milk production traits were not significantly associated.

In the present study, the PCR-SSCP method was used to analyze alleles of the prolactin receptor gene in three buffalo breeds, i.e. crossbred buffalo, Murrah river buffalo, and Nili-Ravi river buffalo and in Holstein dairy cows for comparison. The effects of variation in the prolactin receptor gene on milk production traits were analyzed to provide a theoretical basis for marker-assisted selection.

MATERIALS AND METHODS

Sample Collection

Blood samples were collected from 185 dairy cattle, including 128 buffaloes and 57 Holstein–Friesian dairy cows (from the Guangxi Jingguang Nongken Holstein–Friesian dairy cow farm). Buffaloes included 65 crossbred buffaloes (from the Guangxi Jingguang Nongken buffalo breeding farm), 32 Murrah river buffaloes, and 31 Nili-Ravi river buffaloes (Guangxi Buffalo Breeding Institute). Ten milliliters of venous blood from non-consanguineous individuals was sampled and placed in anticoagulant tubes. Samples were brought back to the laboratory under low-temperature conditions and cryopreserved at -20 °C.

PCR-SSCP Analysis

Genomic DNA was extracted from the blood using an extraction kit obtained from Tiangen Biotech (catalog number DP318; Beijing, China) dissolved in TE buffer and preserved at -20 °C.

Based on the PRLR gene sequence in GenBank (AJ966356.4), specific primers for exons 3, 5, 7, and
10 were designed using Primer5.0. Primer sequences are shown in Table 1.

To reduce non-specific amplification, the annealing temperature and reaction conditions for each pair of primers were screened before PCR amplification. The reaction volume was 25 µL, including 3.0 µL of DNA template, 1.0 µL of dNTPs (2.5 mmol.L-1), 0.5 µL of each mixed primer (25 µmol.L-1), 2.5 µL of 10× Buffer, 0.5 µL of Taq DNA polymerase, and 17.0 µL of ddH2O.

PCR amplification was performed using a T1 Thermo Cycler/Gradient (Biometra, Jena, Germany) programmed for initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 59.7 °C, 62.8 °C, 62 °C, and 64 °C (for exons 3, 5, 7, and 10, respectively) for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 5 min. Reaction tubes were maintained at 10 °C before PCR products were visualized on a 2 % agarose gel stained with ethidium bromide. Then, 3 µL of PCR products were mixed with 10 µL of denaturing solution (98 % formamide, 0.025 % bromophenol blue, 0.025 % xylene-cyanole, and 10 mmol.L-1 EDTA (pH 8.0)), heated for 10 min at 98 °C, and chilled on ice. Denatured DNA was subjected to 10–12 % PAGE in 1× TBE buffer at a constant voltage (160 V) for 18–24 h. The gel was stained with 0.1 % silver nitrate and the bands were analyzed and photographed using the Tanon 1600 Gel Image System (Shanghai, China).

After the detection of polymorphisms, PCR products with different electrophoresis patterns were sent to Sangon (Shanghai Biological Engineering Company, Shanghai, China) for sequencing in both directions, and the sequences were analyzed using DNAstar 5.0.

Statistical Analysis

Genotype and allele frequencies and Hardy–Weinberg equilibrium were determined using PopGene32. Associations between genotype and milk production traits were determined using the least-squares
method (GLM procedure, SPSS 17.0, Chicago, IL, USA).

RESULTS

PCR Amplification

PRLR exons 3, 5, 7, and 10 were amplified with annealing temperatures of 59.7 °C, 62.5 °C, 62 °C, and 64 °C, respectively. Fragments were approximately 262 bp, 305 bp, 242 bp, and 259 bp. Amplified products were detected via electrophoresis on a 2 % agarose gel. These results, summarized in Fig. 1, 2, 3, and 4, indicated that PCR products could be used for subsequent SSCP analyses.

PCR-SSCP Analysis and Sequencing Results

After PCR-SSCP, polymorphisms were observed in PRLR exons 3, 5, and 10. No polymorphism was detected in PRLR exon 7 (Fig. 5). In PRLR exon 3, an A/G mutation was observed at 1177 bp in all buffalo and cow populations. When compared to the sequence in GenBank, a TTT/AAG mutation was revealed to be present in all buffalo and cow populations at 1218–1220 bp. At 1269 bp, a T/G mutation occurred in the crossbred, Murrah, and Nili-Ravi buffaloes, and the GG, GT, and TT genotypes were observed (Fig. 6).

In PRLR, exon 5 locus, a C/A mutation was detected in all buffalo and cow populations at 2984 bp. When compared to the reference sequence (AJ966356.4), all experimental cattle were found to carry G/T, CC/AA, A/C, AA/TG, and T/G mutations at 2990, 3005–6, 3012, 3053–4, and 3097 bp, respectively. The G/A mutation detected at 3151 bp in the tested cattle led to AA, GG, and AG genotypes (Fig. 7).

In PRLR exon 10, upon comparison with the reference sequence (AJ966356.4), G/T, C/T, T/G, G/A, G/A, G/T, A/G, and G/A mutations were detected at 9564 bp, 9610 bp, 9674 bp, 9689 bp, 9717 bp,
9733 bp, 9742 bp, and 9668 bp. The T/C mutation at 9637 bp in the buffalo population led to the genotypes TT, TC, and CC (Fig. 8).

**Genetic analysis of PRLR polymorphisms in buffalo populations**

In PRLR exon 3, the G allele and GG genotype were dominant in crossbred buffaloes, Murrah buffaloes, and Holstein cows, but the T allele and TT genotype were dominant in Nili-Ravi buffaloes. In the crossbred buffalo population, genetic homozygosity (Ho) was greater than 0.5, and the genetic heterozygosity (He), effective number of alleles (Ne), and polymorphism information content (PIC) were relatively low, indicating little polymorphism in this locus in the crossbred buffalo population. In Murrah and Nili-Ravi buffaloes, genetic homozygosity and heterozygosity were close to 0.5, and the effective number of alleles was approximately 2, suggesting that in these three groups, the G and T alleles were fairly evenly distributed. PIC values in crossbred, Murrah, and Nili buffaloes were 0.30–0.38, within $0.25 < \text{PIC} < 0.50$, indicating moderate polymorphism.

In PRLR exon 5, unlike in crossbred buffaloes, the G allele was dominant in Murrah, Nili buffaloes, and Holstein cows.

In PRLR exon 10, the C allele was dominant in the three buffalo breeds. Genetic homozygosity and heterozygosity in crossbred buffaloes were both 0.5 and the effective number of alleles was 2, indicating that the T and C allele distributions were uniform. PIC was 0.375, within the range 0.25–0.50, indicating moderate polymorphism. Murrah and Nili had high genetic homozygosity, but low genetic heterozygosity, with effective allele numbers of 1.67 and 1.14, respectively, indicating that the T and C alleles in both groups were not very uniformly distributed. PIC values in Murrah buffaloes and Nili-Ravi buffaloes were 0.32 and 0.11, respectively, suggesting moderate polymorphism in Murrah buffaloes and a low degree of polymorphism in Nili-Ravi buffaloes.
Hardy–Weinberg equilibrium of buffalo PRLR polymorphisms

In a Hardy–Weinberg equilibrium test, at the PRLR exon 3 locus, the $X^2$ value was 0.132 for the crossbred buffalo population ($P > 0.01$) but was 11.16 ($P < 0.01$) and 18.61 ($P < 0.01$) for the Murrah buffalo and Nili buffalo populations, respectively. These results indicate that equilibrium was only reached in crossbred buffaloes. In PRLR exon 5, the $X^2$ value for crossbred buffaloes was 0.028 ($P > 0.01$), indicating that the population was in Hardy–Weinberg equilibrium. In PRLR exon 10, $X^2$ values for crossbred, Murrah, and Nili buffalo populations were 5.853, 0.885, and 1.079 ($P > 0.01$); at this locus, Hardy–Weinberg equilibrium was reached in all three buffalo populations.

Correlations between PRLR polymorphisms and milk production traits

*LSM and least significant differences test for polymorphic loci*

A variance method was used to analyze the effect of PRLR genotypes on buffalo population milk production indices. With respect to PRLR exon 3, milk yield, milk protein percentage, and milk fat percentage were not significantly related to the three genotypes GG, GT, and TT in crossbred buffalo, Murrah buffalo, and Nili buffalo populations. For PRLR exon 5, there were also no significant differences in milk yield, milk protein percentage, and milk fat percentage among the detected genotypes AA, AG, and GG in the three buffalo varieties. In PRLR exon 10, only the average milk yields for the Murrah TT and CC genotypes were significantly different from those of other individuals ($P < 0.05$) (Table 2), and no significant differences were found with respect to genotype in the other populations.

*Effect of polymorphisms in PRLR exons 3, 5, and 10 on milk production traits*

The variance in milk production traits was analyzed using a fixed model considering species and polymorphic loci (Table 3). The results showed that species had a significant impact on buffalo milk
yield and milk protein percentage, but no significant impact on milk fat percentage. In PRLR exon 10, genotype had a significant impact on milk yield, while for the other loci, genotype did not have a significant impact on milk production traits.

**DISCUSSION**

**Characterization of PRLR variation in buffalo populations**

The genotype and allele frequencies in crossbred, Murrah, and Nili buffalo populations at PRLR exon 3 and PRLR exon 5 were closed. Analysis of homozygosity, heterozygosity, the effective number of alleles, and PIC showed that other than in crossbred buffalo, where homozygosity and heterozygosity were equal at the PRLR exon 10 locus, the three groups of buffalo at the three loci exhibited relatively high homozygosity and effective numbers of alleles and low heterozygosity. At PRLR exon 3, the PIC was high, and at PRLR exon 5 in the crossbred buffalo population, PIC was intermediate. At PRLR exon 10, other than in crossbred buffalo, where it was at an intermediate level, the PIC values were lower in Murrah and Nili buffalo. These genetic patterns were probably related to the breeding history.

The $X^2$ independence test results showed that between the three buffalo breeds, there were only significant genotypic differences at PRLR exon 3 in Murrah and Nili ($P < 0.01$). There were no significant genotypic differences ($P > 0.01$) at these three polymorphic loci in crossbred buffalo. For Murrah and Nili buffalo, the polymorphism at PRLR exon 10 was in Hardy–Weinberg equilibrium, and for crossbred buffalo, all three polymorphic loci were in Hardy–Weinberg equilibrium.

**Relationships between buffalo PRLR polymorphisms and milk performance**

Many recent studies have shown that PRLR is a candidate gene affecting mammalian lactation performance. In this study, polymorphic loci were detected in exons 3, 5, and 10 using PCR-SSCP. In
PRLR exon 3 and 5, SNPs at 1269 (G/T) and 3152 (A/G) were detected. However, these SNPs sites had no significant effect on lactation performance. These results are consistent with those of a previous study of the correlation of PRLR polymorphisms with traits in dairy cows by Zhang et al. (2008). In PRLR exon 10, we detected a SNP at 9637 (T/C); there was a significant difference in milk yield between the TT and CC genotypes. This result was consistent with those of Jun et al. (2011). Genetic polymorphism in PRLR in buffalo differed significantly between species; large population samples are needed for further studies.

References


Zhu, J.J., He, J.Z., Wang, X.P., Jing, Q.Y., Feng, X.P., Guo, Y.F., Lan, G.Q., Wei, Y.M., and Jiang,
H.S. 2008. Cloning and sequence analysis of swamp buffalo prolactin receptor partial cDNA.

**Table 1.** Primer sequences for four loci of the bovine *PRLR* gene

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primers (5′ →3′)</th>
<th>Length (bp)</th>
<th>Temperature (°C)</th>
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</table>
| PRLR EXON3 | 5′ ATAAAGTGGTGATGTGCTGCTCCT 3′  
           | 5′ GGACTGTGATGGATTCTCCC 3′                                                   | 262         | 59.7            |
| PRLR EXON5 | 5′ GATCAACCCCATGTCTCTATTGAT 3′  
           | 5′ CATTCTTCACCTGTCACGCTTCC 3′                                                | 305         | 62.8            |
| PRLR EXON7 | 5′ CCAATGGAGAAGTAGAAAACCCT 3′  
           | 5′ TTAGCAATAAAACAATGGCAGAT 3′                                                | 238         | 62              |
| PRLR EXON10 | 5′ AGGAAAGGCAAACCAACGAG 3′  
             | 5′ CGGCACCAACAACCGAGATA 3′                                                   | 259         | 64              |
Table 2. Summary of 305-day milk production, milk protein, and milk fat for various genotypes at *PRLR* exon 10 based on a least squares analysis

<table>
<thead>
<tr>
<th>Population</th>
<th>Genotype</th>
<th>Milk production</th>
<th>Milk protein</th>
<th>Milk fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crossbred buffalo</td>
<td>TT (10/65)</td>
<td>1114.60 ± 72.650</td>
<td>4.40 ± 0.051</td>
<td>7.99 ± 0.094</td>
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<tr>
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<td>TC (44/65)</td>
<td>1084.34 ± 26.060</td>
<td>4.38 ± 0.020</td>
<td>8.00 ± 0.049</td>
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<tr>
<td></td>
<td>CC (11/65)</td>
<td>1023.90 ± 38.490</td>
<td>4.38 ± 0.059</td>
<td>7.94 ± 0.083</td>
</tr>
<tr>
<td></td>
<td>P = 0.204</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murrah buffalo</td>
<td>TT (4/32)</td>
<td>2284.83 ± 502.335</td>
<td>4.37 ± 0.076</td>
<td>8.77 ± 0.757</td>
</tr>
<tr>
<td></td>
<td>TC (10/32)</td>
<td>1190.46 ± 181.628</td>
<td>4.69 ± 0.154</td>
<td>8.42 ± 1.158</td>
</tr>
<tr>
<td></td>
<td>CC (18/32)</td>
<td>1461.5 ± 254.446</td>
<td>4.50 ± 0.131</td>
<td>7.86 ± 1.194</td>
</tr>
<tr>
<td></td>
<td>P = 0.219</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nili-Ravi buffalo</td>
<td>TC (4/31)</td>
<td>2123.98 ± 372.388</td>
<td>4.08 ± 0.240</td>
<td>7.19 ± 0.961</td>
</tr>
<tr>
<td></td>
<td>CC (27/31)</td>
<td>1770.27 ± 200.291</td>
<td>4.14 ± 0.105</td>
<td>7.83 ± 0.286</td>
</tr>
<tr>
<td></td>
<td>P = 0.403</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holstein cow</td>
<td>TT (57/57)</td>
<td>5224.18 ± 104.782</td>
<td>3.12 ± 0.006</td>
<td>3.49 ± 0.017</td>
</tr>
</tbody>
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Note: Different letters indicate significantly different least square means in a column (P < 0.05)
Table 3. Variance analysis of milk production traits with respect to the three polymorphic loci of PRLR in buffalo

<table>
<thead>
<tr>
<th>LOCUS</th>
<th>Milk production</th>
<th>Milk protein</th>
<th>Milk fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>2496615.607***</td>
<td>0.673***</td>
<td>1.147</td>
</tr>
<tr>
<td>EXON3</td>
<td>Marked genotypes</td>
<td>73642.007</td>
<td>0.075</td>
</tr>
<tr>
<td>Species</td>
<td>683705.805***</td>
<td>0.721***</td>
<td>0.837</td>
</tr>
<tr>
<td>EXON5</td>
<td>Marked genotypes</td>
<td>11143.664</td>
<td>0.008</td>
</tr>
<tr>
<td>Species</td>
<td>5848476.489***</td>
<td>0.736***</td>
<td>1.617</td>
</tr>
<tr>
<td>EXON10</td>
<td>Marked genotypes</td>
<td>850325.490***</td>
<td>0.058</td>
</tr>
</tbody>
</table>

Note: *** indicates a significant difference (P < 0.01)
Figure 1. Amplified fragments of exon 3 of the buffalo PRLR gene
Figure 2. Amplified fragments of exon 5 of the buffalo *PRLR* gene
Figure 3. Amplified fragments of exon 7 of the buffalo *PRLR* gene
Figure 4. Amplified fragments of exon 10 of the buffalo PRLR gene
Figure 5. PCR-SSCP patterns for the PRLR exon 7 polymorphism in buffalo
Figure 6. PCR-SSCP patterns for the PRLR exon 3 polymorphism in buffalo
Figure 7. PCR-SSCP patterns for the PRLR exon 5 polymorphism in buffalo
**Figure 8.** PCR-SSCP patterns for the *PRLR* exon 10 polymorphism in buffalo