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A Comparative Assessment Study on the *Prolactin* Gene as a Genetic Marker for Milk Production in High Milk-Producing Mammals through Phylogenetic Analysis

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Abstract

The prolactin (PRL) gene is a focus in milk-related studies for high-yielding ruminants like dairy cows, water buffaloes, dairy goats, and sheep. Despite its recognition as a genetic marker for milk traits, little is known about the specific genetic features enabling heightened milk production. The PRL nucleotide sequence of the ruminants and the human outgroup were obtained from the GenBank database. Using the MEGA 7 software, amino acid translation was conducted and multisequence analysis was done, allowing for the construction of phylogenetic trees. In both trees, ruminants were consistently clustered together forming pairings of the dairy cow: water buffalo and the dairy goat: sheep. In the nucleotide sequences of all the involved taxa, cytosine and guanine were the unanimous most and least comprised nucleotide. In the amino acid composition, the unanimous most and least amino acid percentages were leucine and tryptophan, respectively. Leucine, being the highest comprised, was substantiated by related studies stating that such amino acid is highly involved in milk production and yield. Glycine percentages were found to be higher in ruminants than in humans, providing a possible reasoning for such milk-yield disparity in the involved taxa.

***Keywords**:* amino acid, dairy, glycine, milk-producing ruminants, *prolactin*

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1. Introduction

The ability to produce milk is an inherent trait to animals belonging to a vertebrate group called mammals (Reece *et al.*, 2019). Organs called mammary glands allow females of mammalian species to secrete such nutrient-rich food (Akers, 2016). Although all mammals have the capacity for milk production, commercially-produced dairy milk is primarily sourced from a single group of animals: ruminants (Abubakar, 2018). Cows, water buffaloes, goats, and sheep are the primarily used animals for commercial dairy farms, with cows accounting for 81% of the world milk production, 15% for buffalo milk, and 4% for goat, sheep, and camel milk combined (Food and Agriculture Organization [FAO] and Organization for Economic Cooperation and Development [OECD], 2019). Commercial dairy remains to be one of the faster growing agricultural commodities of today, generating a high demand for fresh dairy products as well as dairy derivatives (i.e., dairy fat) (FAO and OECD, 2019). Hence, it is not surprising that much attention is given to maximizing commercial milk production by dairy farms.

Milk production is largely affected by factors such as genetics, environment, and managerial practices. In particular, molecular genetics techniques are utilized to elucidate genes that are able to determine the milk production potential of an animals before they are expressed (Kiplagat *et al.*, 2012). Such genes function as genetic markers, associating livestock-related production traits to the genetic makeup of an animal (Teneva and Petrović, 2010). Additionally, techniques such as phylogenetic analyses could be utilized, providing relative comparison to the DNA and protein sequences of the gene markers to other closely-related dairy animals.

The *prolactin* (*PRL*) gene has been on the forefront of milk quality and production study on dairy animals (Singh *et al.*, 2014). It encodes the *PRL* protein that is a single chain polypeptide hormone secreted from specialized lactotroph cells of the anterior pituitary gland (Freeman *et al.*, 2000). The protein has its role in lactogenesis as well as galactopoiesis (Lazebnaya *et al.*, 2013). Although *PRL* is present in all vertebrate species (i.e., mammals), the protein is primarily involved in the stimulation of lactogenesis of the mammary glands (Horseman *et al.*, 1997). It is in this encoded protein's function that allowed scientists to establish a connection between genetics and milk productivity.

Ruminants such as cows, water buffaloes, sheep, and goats have all been subjected to milk production research due to their ubiquity in dairy milk production as well as high milk yield capabilities. One common factor in such studies is the influence of the *PRL* gene with regards to the milk output. In cows, the polymorphism of the *PRL* gene has been proven to influence the milk production capabilities of different cattle (Alipanah *et al.*, 2007; Alfonso *et al.*, 2012; Dong *et al.*, 2013; Nadeem and Maryam, 2016). The *prolactin*-

related researches of water buffaloes involves the use of *PRL* as a potential milk production marker with varying results (Shi *et al.*, 2012; Magd *et al.*, 2015). The relationship of milk traits and the *PRL* gene in sheep have been studied extensively in the studies of Ramos *et al.* (2009), Padilla *et al.* (2018), and Jawasreh *et al.* (2019). In goats, *PRL* has been observed to have lactogenic and galactopoietic properties (Lacasse *et al.*, 2016).

The intricate regulation of milk production in high-yielding mammals involves a myriad of factors, including hormonal influences and nutritional inputs. While existing research acknowledges the multifaceted nature of these contributors, a critical gap persists in understanding the genetic markers associated with efficient lactation. The *PRL* gene has been identified as a potential biomarker for milk production, but a comprehensive comparative assessment, especially through phylogenetic analysis, is lacking. This study sought to address this gap by investigating the role of the *PRL* gene as a genetic marker for milk production in high-yielding mammals. The elucidation of the specificities of the ruminant *PRL* gene could provide insights on the high milk-producing capabilities of animals and further improve the economic efficiency of dairy production.

The general objective of the study was to compare the *PRL* gene DNA and protein sequences of the dairy cow, water buffalo, sheep, and dairy goat using phylogenetic analysis and evaluate the *PRL* gene as a gene marker for milk production. The specific objectives were to (1) obtain the *PRL* nucleic acid and amino acid sequences of dairy cows, water buffaloes, sheep, dairy goats, and the human outgroup, (2) analyze the nucleotide and amino acid frequencies of the aligned multisequence, (3) compare and analyze the DNA and protein sequences using a phylogenetic analysis software, and (4) identify the specificities that enable the milk capabilities of involved ruminant species.

2. Methodology

2.1 Species of Interest

The involved ruminants of the study are the dairy cows (*Bos taurus*), water buffaloes (*Bubalus bubalis*), sheep (*Ovis aries*) and dairy goats (*Capra aegagrus hircus*). These animals were specifically chosen due to their high milk-producing capabilities and are widely studied in milk-related research. Their inclusion is based on their importance in agriculture and global milk production, playing crucial roles in the dairy industry and economies of various regions. Humans (*Homo sapiens*) was used as the outgroup for this current study.

2.2 Acquisition of PRL Genetic Sequences

The *PRL* gene of the dairy goat, sheep, dairy cows and the water buffaloes were procured through the GenBank genetic sequence database of the National Center for Biotechnology Information (NCBI). In order to determine the gene sequence specificities unique to high milk-yielding ruminants, a human *PRL* gene was used as the outgroup. This gene was procured through GenBank database.

2.3 Sequence Analysis and Construction of Phylogenetic Tree

The *PRL* nucleic acid sequences of the dairy goat, sheep, dairy cow, water buffalo, and the human outgroup were subjected to a multiple sequence alignment using ClustalW in the MEGA7 software (Kumar *et al*., 2016) and Clustal Omega (ClustalO v1.2.4) in EMBL-EBI.

The frequency statistics for the nucleotide sequences were exported using the in-program tools of the MEGA software into a spreadsheet-compatible format. Windows Excel 2019 was used to view and analyze the nucleotide frequencies. The consensus nucleotide sequences and reference sequences were translated into peptide sequences using the EMBOSS Transeq tool. Afterwards, the BLASTx search tool was used to select the optimal amino acid reading frames for the protein sequences. The peptide sequences were subjected to multiple sequence alignment using MEGA7 or the Clustal Omega (ClustalO v1.2.4) in EMBL-EBI. The aligned protein sequences were analyzed using the MEGA7's appropriate parameters (statistical method: maximum likelihood, test of phylogeny: bootstrap method, bootstrap number: 1000, model/method: Kimura 2-parameter model) to construct a phylogenetic tree. Sequence and phylogenetic analysis was then conducted on the nucleotide and amino acid sequences.

Similar to the nucleotide sequences, the frequency statistics for the amino acid sequences were exported using the in-program tools of the MEGA software into a spreadsheet-compatible format. Windows Excel 2019 was then used to view and analyze the amino acid frequencies.

3. Results and Discussion

3.1 Sequence Analysis

The comparative analysis of *PRL* nucleotide sequences from diverse species provides valuable insights into genetic relationships and evolutionary patterns. In this study, five *PRL* nucleotide sequences were acquired from the NCBI GenBank database, namely: the dairy cows *(B. taurus)*, water buffaloes *(B. bubalis)*, sheep *(O. aries)*, dairy goats *(C. aegagrus hircus)*, and the human outgroup *(Homo sapiens)*. The base pair (bp) sequence lengths were as follows: dairy cows with 907 bp, water buffaloes with 862 bp, sheep with 926 bp, dairy goats with 845 bp, and the humans with 894 bp. After aligning and trimming the sequences, what remained were five sequences consisting of an 830 bp length. There were 637 conserved sites, indicating that the nucleotides at these places were congruent. Conversely, there were 193 variable sites where nucleotides were not all the same.

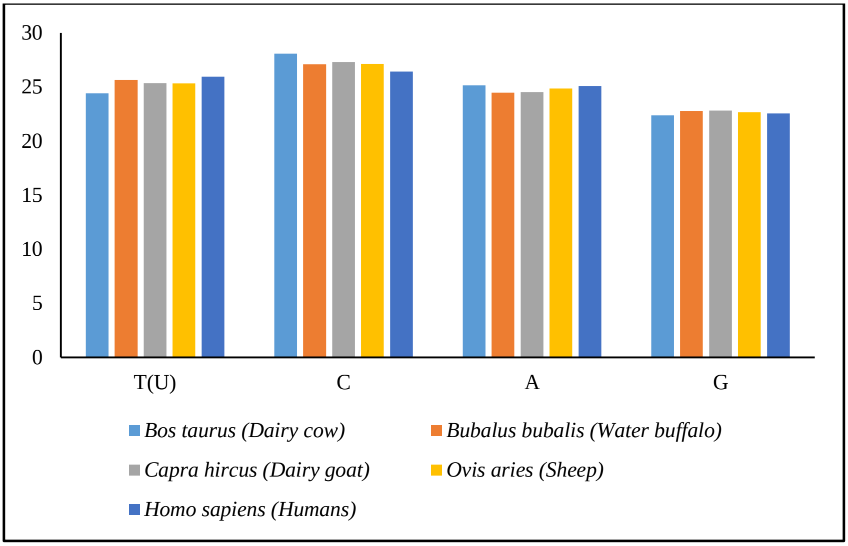
Among ruminants, the dairy cow and water buffalo exhibited the highest similarity, evidenced by 817 identical pairs in their prolactin gene. Further exploration within the ruminant group highlights 805 conserved sites shared among cow, buffalo, and goat. With the inclusion of the dairy sheep, 782 out of 830 sites remained conserved, leaving 48 incongruent nucleotide sites. Notably, the dairy sheep displayed the highest number of incongruent sites, suggesting distinctive features in its prolactin gene.

The conserved sites identified in the study indicated regions essential for *PRL* gene functionality across diverse species, offering potential targets for further research, genetic manipulation, and applications in the field of animal/livestock breeding (Kiplagat *et al.*, 2012). Identifying conserved sites allows targeted selection of individuals with desirable traits which increases animal productivity, leading to the development of high-yielding livestock.

3.2 Nucleotide Frequency Composition

In the examined *PRL* sequences, a consistent pattern emerged with cytosine (C) being the dominant nucleotide, averaging 27.20%, while guanine (G) was at a lesser percentage with an average of 22.64% (Figure 1). Exploring this trend across species, water buffaloes exhibited a similar composition, predominantly featuring C at 27.30%, while G at 22.82%. In dairy cows, C was most abundant at 27.11%, and G was minimally represented at 22.80%.

In the *PRL* of dairy goats, C maintains prominence at 27.14%, with G being the minority at 22.67%. A comparable pattern was observed in dairy sheep, where C dominates with 26.42% and G at 22.56%.



T – thymine, U – uracil, C – cytosine, A – adenine, and G – guanine

Figure 1. Nucleotide distribution according to percentage

It was evident that although the prevalence of C remained a common thread, there were subtle variations in the proportions of G among different species. Interestingly, this consistent trend extended to the human outgroup, where C was predominant at 28.07%, and G was the minority at 22.36%. The analysis allowed for a more comprehensive understanding of the nucleotide composition across diverse species.

3.3 Amino Acid Frequency Composition

Upon close analysis with the involved taxa individually, it was observed that leucine (Leu) was most abundant, while tryptophan (Trp) was the least (Figure 2). On average, the majority of the amino acids in the multisequence was composed of leucine (Leu) (13.73%), followed by serine (Ser) (9.23%), glutamic acid (Glu) (6.5%), isoleucine (Ile) (5.68%), valine (Val) (5.39%), alanine (Ala) (5.02%), arginine (Arg) (4.94%), asparagine (Asn) (4.87%), glycine (Gly) (4.79%), proline (Pro) (4.58%), lysine (Lys) (4.13%), histidine (His) (4.06%), phenylalanine (Phe) (3.98%), threonine (Thr) (3.98%), aspartic acid (Asp) (3.91%), glutamine (Gln) (3.54%), tyrosine (Tyr) (3.47%), methionine (Met) (3.39%), cysteine (Cys) (2.58%), and the least was tryptophan (Trp) (2.21%).

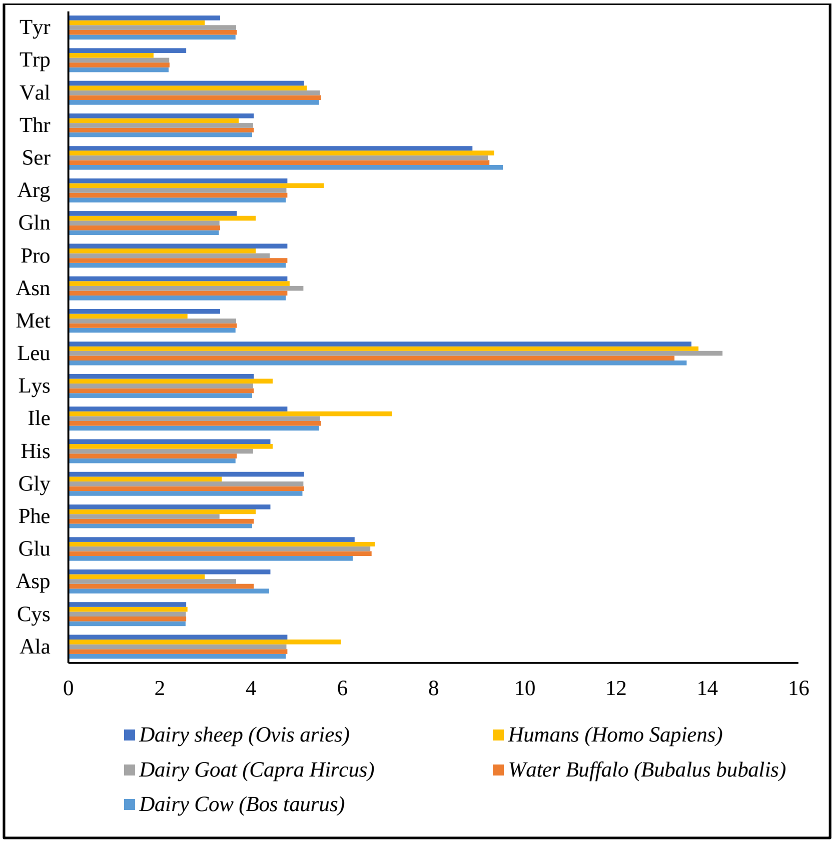


Figure 2. Amino acid distribution according to percentage

The graphic representation in Figure 2 depicts that in water buffaloes, Leu was most abundant (13.28%), and Trp was least (2.21%). Dairy cows had a composition primarily of Leu (13.55%), the least was Trp (2.19%). In dairy goats, the majority of the amino acids was Leu (14.34%), and the least was Trp (2.20%). In dairy sheep, Leu was most abundant at 13.65%, while its minority percentages were equivalent in two amino acids: Trp and Cys at 2.58%. In the human outgroup, Leu garnered the majority with 13.81% while Trp was least (1.87%).

The amino acid Leu is recognized as a functional amino acid that is absorbed by the mammary gland is essential for milk protein and fluid synthesis (Li *et* *al.*, 2009). It is heavily involved with activating signal factors, as well as promote and regulate certain proteins in mammary milk production (Bequette *et al.*, 1998; Kimball and Jefferson, 2004). Leucine even has effects on the suppression of hormone secretion, affecting protein production and in turn milk production (Sener *et al.*, 1982). With regards to its relationship with milk production capabilities, the abundance of Leu in amino acid composition has been tackled in previous studies such as that of Lucy *et al.* (1993) and Tian *et al.* (2017). In such studies, the deletion of Leu had negative effects on the milk yield. Perhaps the *PRL* gene's unanimously high percentage of leucine contributes to its function as a genetic marker for milk production traits as previously discussed in the studies of Shi *et al.* (2012), Dong *et al.* (2013), and Magd *et al.* (2015). Hence, this observation of leucine dominance in the involved taxa of the current study is supported by the findings of the related studies.

3.4 Nucleotide Phylogenetic Tree

Maximum likelihood with 1,000 bootstrap replications yielded a tree that clustered all the ruminants together. Two pairings within the ruminant clusters were created. The first being the dairy cow and water buffalo; and the second, dairy goat and dairy sheep. There is a bootstrap value of 62 between the dairy cow and water buffalo, and between the dairy goat and sheep: a value of 95 (Figure 3). Both these values imply that that the groupings and clusters produced are sufficiently supported. In the dairy goat and sheep's case, a bootstrap value of 95 indicates that the pairing is highly supported by numerous replications.

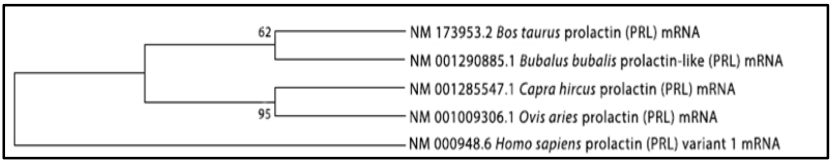


Figure 3. Genetic relationships of ruminant species through phylogenetic tree based on nucleotide sequences

3.5 Amino Acid Phylogenetic Tree

Maximum likelihood with 1,000 bootstrap replications depicted a tree that also clustered all ruminants together, producing two pairings with the dairy cow: water buffalo and dairy goat: dairy sheep (Figure 4).

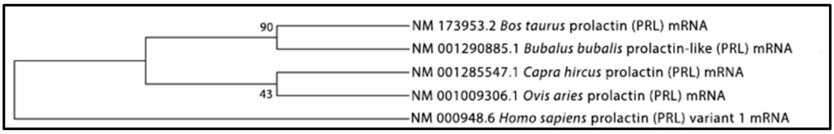


Figure 4. Genetic relationships of ruminant species through phylogenetic tree based on amino acid sequences

Between the dairy cow and water buffalo, there was a bootstrap value of 90; and between the dairy goat and dairy sheep, there was a bootstrap value of 43. The high value of the cow and buffalo pairing indicates that there was a relatively high confidence value for this particular branch, and it was well supported. The goat and sheep branch, however, was very weakly supported.

Such phylogenetic trees concurred with the initial sequence analysis of the involved taxa. The ruminants were grouped together and presented with their expected branches. The dairy cow and water buffalo, which had a high percentage of conserved sites, were paired together. This was the same way with the goat and sheep pairing; as dairy sheep had a large number of incongruent sites if aligned the other ruminants, which indicated minimum nucleotide sequence identity. This clustering and sub-grouping in both trees is substantiated in related studies by Faith *et al.* (2019) and Mukesh *et al.* (2006). In Faith *et al.*'s (2019) case, using UPGMA tree construction and Neighbor Joining tree estimating methods for casein study produced phylogenetic trees that were identical to those of the current study. The ruminant clustering of the nucleotide and amino acid phylogenetic trees of Mukesh *et al.*'s (2006) kappa-casein study was also found to be identical to the phylogenetic trees of the current study.

3.6 Specificities of the Human Outgroup

With regard to specificities found in the human outgroup in particular, despite following the same pattern of the most and least abundant amino acids (that being leucine and tryptophan, respectively), there were still variances with the other amino acids in the composition. In the human outgroup, there was a notable lack of glycine compared with the average percentage by the involved ruminants. On average, 5.15% of the *PRL* gene of ruminants was comprised of glycine, but for the human outgroup, only 3.36% was of the same amino acid. This means that ruminants had 1.79% more glycine than the outgroup. This is significant as previous studies observed that glycine composition had a direct relationship with the milk yield of certain ruminants (Shibano *et al.*, 2005). Perhaps, this provides reason for the higher milk output of ruminants compared to other mammals.

4. Conclusion and Recommendation

In analyzing ruminant nucleotide sequences, water buffaloes and dairy cows exhibited the highest congruence, evidenced by the greatest number of conserved sites, while sheep displayed the least characteristic resemblance with the fewest conserved sites. Notably, cytosine emerged as the unanimously most prevalent nucleotide across all taxa, contrasting with guanine as the least. A parallel pattern was observed in amino acid composition, with leucine dominating and tryptophan being the least present. This consistency aligned with the nucleotide and amino acid phylogenetic tree, grouping ruminants together and forming distinct branches. Regarding the specificities with ruminants that allow them to have a higher milk yield than other mammals, it was observed that there was a notable higher composition of the glycine amino acid in ruminants compared to the human outgroup. Considering that glycine was also involved in the milk yield process, this could provide reason for such a difference in milk yield capabilities.

In conclusion, the study suggested that glycine’s abundance, coupled with conserved sites, could underpin ruminants’ superior milk yield. Future research could delve into single nucleotide polymorphism for a more comprehensive multisequence analysis, providing additional insights into ruminant specificities.

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