Genome-wide identification and expression analysis of the Collagen family during lactation in water buffalo (*Bubalus Bubalis*)

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Abstract

Background: Collagens, as extracellular matrix molecules, support cells for structural integrity and a variety of other functions, thereby contribute to support mammary basic structure and development. However, little information on the identification and expression profiles in response to the mammary gland of the collagen family in buffalo (*Bubalus Bubalis*) has been reported. **Results:** A total of 128 buffalo collagen protein sequences corresponding to 45 collagen genes were identified and classified into six clusters based on their phylogenetic relationships, conserved motifs, and gene structure analyses. A transcription factor binding sites (TFBS) analysis inferred that a total of 142 TFBS were predicted within the buffalo collagens, suggesting that different collagen subfamilies harbored different TFBS and played a variety of functions involved in the mammary gland development and lactation. The identified collagen sequences were unequally distributed on 17 chromosomes, 103 of which were determined to be tandem duplicated genes. Transcriptome data and qRT-PCR analysis revealed the expression diversity of buffalo collagen genes in various tissues. Most of the identified collagen genes were significantly up-regulated at the early lactation, 6 collagens upregulated at the peak lactation, and only COL24A1 was up-regulated at the late lactation. **Conclusions:** The present study provides significant insights into the potential functions of collagen family in dairy buffalo and helps in the functional characterization for collagen genes in further research.

Background

The mammary gland is a dynamic organ in mammals that produce milk to meet nourishment requirements for the offspring [1, 2]. It is also a unique glandular secretory organ composed of different cell types, including epithelial, adipose, fibroblasts, immune, lymphatic and vascular cells [2, 3]. Mammary epithelium consists of two cell layers: a luminal layer of secretory epithelial cells and a basal layer of myoepithelial cells. Luminal epithelial cell is responsible for the synthesis and secretion of milk components, while the myoepithelial cell mainly participates in the process of milk ejection. The myoepithelial cells reside in the basement membrane of the extracellular matrix (ECM). Accumulating evidence has demonstrated that collagen is the major component of ECM that play a crucial regulatory role in the epithelial architecture and function [4-6]. Schedin and Keely [7] also highlighted ECM composition and organization are tightly regulated throughout the mammary gland development. Consequently, as the major component of ECM, the collagen family maybe play a vital role in the specific stages of mammary gland development.

Collagens represent the most abundant protein in mammals commonly possessing at least one triple-helical domain. Since collagen II was discovered by Miller and Matukas [8], twenty-eight members of the collagen family have recently been identified and characterized [9]. These identified collagen members are deposited in the ECM where most of them can form supramolecular aggregates. Notably, the knowledge about the molecular structure, biosynthesis, and function of the collagen family has emerged [10]. Twenty-eight collagen genes, for example, can be grouped into several clusters based on their structure [11]: Fibrillar collagens (types I, II, III, V, XI, XXIV, and XXVII), Fibril-associated collagens with interrupted triple helices (types IX, XII, XIV, XVI, XIX, XX, XXI, and XXII), Network-forming collagens (types IV, VIII, and X), Trans-membranous collagens (types XIII, XVII, XXIII, and XXV), Endostatin precursor collagens (types XV and XVIII), and others (types VI, VII, XXVI, and XXVIII). Moreover, evidence revealed that different collagen genes displayed different expression patterns and played the structural and functional roles [10]. Chen et al. [12] found the COL4A1 were significantly downregulated in the

inflammation-associated fibroblasts extracted from bovine mammary glands with clinical mastitis compared with normal fibroblasts from a slaughtered dairy cow, implying that it might involve in the ECM remodeling. Crisà et al. [13] reported that COL4A1 was highly expressed in the mature milk compared with that of the colostrum milk in goat. Furthermore, collagens are demonstrated to participate in cell-matrix interactions via several receptor families [14-16]. Although the expression profiles of collagens have been previously reported, expression patterns of the collagen gene family in buffalo (*Bubalus Bubalis*) are poorly characterized.

Water buffalo is the second largest source of milk production that mainly distributed in the tropical and subtropical areas, providing more than 5% of the world's milk supply [17]. Remarkably, buffalo milk is more abundant in fat and protein than cow's milk [18], which has attracted widespread attention from the dairy industry. In the past several decades, numerous high-throughput data were generated and utilized for identifying the candidate genes related to traits of interest in buffalo, such as the potential genes related to milk or productive traits [19, 20], transcriptome profiles of buffalo embryos with normal and retarded growth [21], and maternally-expressed proteins in buffalo oocyte [22]. All those data, along with the complete buffalo genome sequence [23], provide the possibility to perform the gene family analysis at a genome-wide level. In plants, numerous gene families have been identified and characterized, such as the sugar transporter gene family [24], KUP family [25], and heat shock factor gene family [26], which contribute to the understanding of gene structure, evolutionary patterns, and expression profiles. In animals, Körbes et al. [27] reported the molecular evolution of the lysophosphatidic acid acyltransferase (LPAAT) gene family, suggesting that the evolution of triacylglycerol biosynthesis shaped by diversification of the LPAAT genes. However, little information on the expression profiles of the collagen family in different tissues and developmental mammary gland of buffalo is available. Because of the vital functions of collagen in animals, it is of considerable importance to investigate the buffalo collagen family. In the present study, we identified collagen family from the buffalo genome and analyzed their evolutionary relationship, sequence features, chromosomal location, gene duplication, tissue-specific expression levels and dynamic expression patterns in response to different lactation points in mammary gland tissues. Our results provide some insight into the understanding of buffalo collagen family and present vital evidence for future functional studies.

Results

Identification and classification of the collagen family in buffalo

In the present study, an integrative analysis of BLAST and HMMER showed that a total of 128 putative collagen protein sequences were identified from the buffalo whole genome, belonging to the 45 buffalo collagen genes (**Table 1**). The open reading frames (ORFs) of the collagen proteins ranged from 1,317 to 9,654 bp in length encoding the protein of 438 to 3,217 residues, with the predicted MW from 107.46 to 786.60 kDa. The pI values of these proteins ranged from 4.54 to 4.92. Furthermore, the phylogenetic analysis revealed that the identified collagen genes could be classified into six clusters (**Figure 1**). The top 3 clusters contained 8, 7, and 4 collagen genes, corresponding to the Cluster II, Cluster I, as well as Cluster IV and VI, respectively. The smallest group is the Cluster V with only two collagen members (collagens XV and XVIII). The constructed dendrogram further showed that buffalo collagen family was generally most closely evolutionary relationship with other five representative mammals.

Gene Structure, Conserved Motif Analysis, and Promoter Analysis

As shown in **Figure 2A**, the exon numbers of buffalo collagen family ranged from 2 to 118, and most of them harbored 40 to 60 exons. The analysis of intron numbers in the studied species showed that the intron number in the collagen family varied widely, ranging from 1 to 117 (**Supplementary Figure S1**). Most collagen sequences contained more than forty introns, whereas only the COL8A1, COL8A2, and COL10A1 contained fewer than three introns. The collagen members in the same group had similar numbers of exon and intron among the studied species. These findings suggest that different groups in the collagen family have different patterns of intron number, which verify our previous classification process.

The MEME analysis showed that a total of 10 conserved motifs were identified in all collagens (**Figure 2B**). After the Pfam search, the motifs 3 and 5 were annotated as the collagen domain (**Table 2**), suggesting that at least collagen domain existed in the identified collagens. The results were also supported by the identified collages blasted against the conserved domain database (CDD) from NCBI (**Figure 2A**). Interestingly, the Fibrillar collagen C-terminal domain (COLFI), Fibronectin type 3 domain (FN3), C-terminal tandem repeated domain in type 4 procollagen (C4), von Willebrand factor (vWF) type A domain (VWA), von Willebrand factor type C domain (VWC), and EMI domain also was determined in some collagen genes.

All identified buffalo collagens with the 1500 bp upstream sequences of coding sequence were selected and used to predict the TFBS. The results exhibited a total of 142 TFBS were predicted within the buffalo collagen genes (**Figure 3A**). Among them, the largest TFBS detected in the identified collagen genes was the Myeloid Zinc Finger 1 (MZFI), with the numbers of 61 (7.96%), followed by the Homeobox protein Meis1 (MEIS1) (43; 5.61%), Zinc Finger Protein 354C (ZNF354C) (42; 5.48%), Rhox homeobox family member 1 (RHOXF1) (40; 5.22%), Krueppel-like factor 5 (KLF5) (33; 4.31%), Zinc finger protein 384 (ZNF384) (25; 3.26%), and Transcription factor Spi-B (SPIB) (21; 2.74%), while the remaining TFBS with the number of ≤ 20 (**Supplementary Figure S2**). For the buffalo collagen family, moreover, we found that the MZF1 element was mostly detected in the Clusters I, III, and V, the MEIS1 element was mostly predicted in the Clusters II and VI, and ZNF384 element was mainly discovered in the Cluster IV (**Supplementary Figure S3**).

Chromosomal location and Gene Duplication Analysis of buffalo collagen family

The 128 buffalo collagen sequences were distributed on 17 chromosomes randomly (**Figure 4**). Among them, chromosome 2, 4, 1, and 3 separately had 26, 18, 15, and 13 collagen each, chromosome 10 and X both contained 7 collagen each, chromosome 6 possessed 8 collagen each, chromosome 14 contained 6 collagen sequence, chromosome 7 and 15 both had 5 collagen each, while the remaining chromosome has less than 5 collagen each. Notably, the buffalo collagen genes were mainly located on the proximate or the distal ends of the chromosomes.

To investigate the evolution progress of putative gene family, we analyzed the duplication events of buffalo collagens. Here, a total of 103 (80.47%) collagen sequences were confirmed to be tandem duplicated genes. The majority of collagen tandem duplicated genes located on chromosome 1 ~ 4. Four separate pairs of collagen tandem duplicated genes located on chromosome 10 and X. Four groups of three tandem duplicated gene pairs located on chromosomes 6, 7, 14, and 15. Four chromosomes (9, 13, 23, and 24) had the tandem duplicated gene pairs with the numbers of ≤ 2 . However, only one segmental duplication event (COL1A1 and COL1A2) was discovered in the buffalo collagen family. These results suggested that the tandem duplication played a predominant role in the expansion of buffalo collagen family.

To further explore whether the positive selection was involved in buffalo collagen genes, we analyzed the Ka/Ks ratios for each duplicated collagens pair (**Table 3**). The results showed that the COL14A1_2/COL14A1_1 duplicated collagen pair was identified with the Ka/Ks ratios > 1, implying that this gene pair might involve in the positive selection. Moreover, a total of 23 duplicated collagen gene pairs with the Ka/Ks ratios < 1 were identified, 8 of which might experience strong purifying selection pressures because they had the Ka/Ks ratios were less than 0.5.

Expression profiling of buffalo collagen genes

To investigate the tissue expression distribution of the buffalo collagen family, we examined the expression patterns of 45 collagen genes using the public RNA-seq data. The results showed that a total of 19 collagen genes were not expressed in any tissues of buffalo, including the COL10A1, COL11A1, COL13A1, COL19A1, COL1A2, COL20A1, COL21A1, COL23A1, COL24A1, COL25A1, COL26A1, COL4A4, COL4A6, COL5A1, COL6A3, COL6A4, COL6A5, COL6A6, and COL8A2 (**Figure 5A**). A total of 26 collagen genes was detected from at least one tissue and displayed differential expression in various buffalo tissues. Among them, 5 (COL9A3, COL6A1, COL6A2, COL3A1, and COL5A2) genes showed highest expression levels in the testis, while 3 (COL14A1, COL4A1, and COL4A2) genes had highest expression levels in the ovary follicle, indicating that these genes might be related to the reproductive traits in buffalo. Interestingly, the COL16A1 gene exhibited the highest expression levels in the mammary gland, suggesting that it might involve in the biological function of milk production trait in buffalo. Moreover, the result of qRT-PCR also showed a similar trend with the RNA-seq data (**Figure 5B**).

Using the RNA-seq data, we further dissect the transcript levels of buffalo collagen family in mammary gland tissues at four lactation points. The results showed that 29 out of 45 collagen genes were mainly up-regulated at the D7, six collagens (COL6A6, COL3A1, COL1A1, COL27A1, COL8A1, and COL14A1) upregulated at the D50, two collagen genes (COL4A3 and COL10A1) were mainly expressed at the D50 and D140, and only the COL24A1 was up-regulated at the D280 (**Figure 6**). The results of qRT-PCR also demonstrated that four collagen genes in buffalo were up-regulated in the D50 and two collagens were highly expressed in the D7, implying that the RNA-seq data in the present study was available.

Discussions

Collagens, as extracellular matrix molecules, support cells for structural integrity and a variety of other functions [11], thus contribute to support mammary basic structure and development [28]. Currently, our understanding of the functional role of buffalo collagen family is limited. In the present study, we identified 128 collagen protein sequences in buffalo based on its complete genome sequence. The identified collagen protein sequences corresponded to the 45 collagen genes in buffalo, which was classified into six clusters based on their evolutionary relationships. The result was consistent with the previous classification of collagens described by S Ricardblum [9]. The conserved motif and gene structure analyses of buffalo collagen genes also supported this classification perspective. Conserved motif analysis indicated that all the identified collagens harbored at least collagen domain. Interestingly, the collagen cluster I genes contained a fibrillar collagen C-terminal domain (COLFI). For the gene structure analysis, most of the collagen genes contained 40 to 60 exons. The intron number analysis revealed that the majority of collagen genes contained more than forty introns, whereas only the COL8A1, COL8A2, and COL10A1 contained fewer than three introns. These results coincide with the exon-

intron structure of collagen genes from other representative mammals, suggesting that collagen family had a conserved gene structure.

For the identified buffalo collagens, we further predicted the TFBS in their promoter regions. The results exhibited a total of 142 TFBS were detected within the buffalo collagen family. We found that the largest TFBS with the numbers of 61 was the MZF1 (7.96%), followed by the MEIS1 (5.61%), ZNF354C (5.48%), RHOF1 (5.22%), KLF5 (4.31%), ZNF384 (3.26%), and SPIB (2.74%). Moreover importantly, we found that the MZF1 element was mostly detected in Clusters I, III, and V of buffalo collagen family, the MEIS1 element was mostly predicted in the Clusters II and VI, and ZNF384 element was mainly discovered in the Cluster IV. Previous studies have demonstrated that as an N-cadherin transcription factor, MZF1 could participate in the epithelial-mesenchymal transition that plays a critical role in cell invasion and metastasis [29]. It is noted that cell migration through the extracellular matrix was a key component of morphogenesis [30], suggesting that these genes in the Clusters I, III, and V of buffalo collagen family might be involved in the mammary gland morphogenesis. MEIS1 has been demonstrated to be a transcriptional regulator of PAX6 that is required for the normal development of the endocrine cells [31, 32]. This indicated that the MEIS1 along with PAX6 might be involved in the insulin secretion and synthesis pathway, thereby resulting in the changes of lactation function in mammary gland because the insulin plays a vital role for the mammary gland differentiation and subsequent lactation [33, 34]. Our data also revealed the MEIS1 element was mostly detected in Cluster II and VI, implying that the biological function of them might be related to the insulin secretion and synthesis. Moreover, evidence indicated that ZNF384 as a transcription factor could bind and regulate the promoters of the extracellular matrix genes that played critical roles in mammary gland development [35, 36]. Our finding predicted the ZNF384 element was mostly detected in the Cluster IV, indicating that these cluster genes had effects on the extracellular matrix remodeling in the mammary gland. These results indicated that the different collagen genes in different clusters play a variety of functions involved in the mammary gland development and lactation.

Four events during genetic evolution including the chromosome doubling, chromosome fragment insertion mutation, tandem duplication, and transposition provide a possibility for the novel gene function acquisition [37, 38]. Gene duplication including the tandem duplication and segmental duplication can mainly help to accelerate the gene family expansion and genome evolutionary mechanisms [39-41] (Vision et al., 2000, Liu et al., 2009). In buffalo, all identified buffalo collagen genes were unevenly distributed on 17 chromosomes. Here, a total of 103 (80.47%) buffalo collagen sequences were confirmed to be tandem duplicated genes, but only one segmental duplication event to be discovered, which revealed that the tandem duplication had a predominant role in the expansion of buffalo collagen family. This finding was supported by Liu et al. [39] who highlighted that segmental duplications in most mammalian lineages are organized in a tandem configuration. For the duplicated collagen pairs, interestingly, we found that the COL14A1_2/COL14A1_1 duplicated collagen pair underwent the positive selection during the evolution of buffalo collagen family. Furthermore, twenty-three pairs of duplicated collagens with the Ka/Ks ratios < 1 were identified, eight of them with the Ka/Ks ratios were less than 0.5 that might experience strong purifying selection pressures.

Tissue expression profiles from the RNA-seq data analysis were presented in this study, showing that a total of 26 collagen genes was detected from at least one tissue and displayed differential expression in various buffalo tissues. In particular, buffalo collagen genes in the same clusters had no consistent expression. For example, a total of five (COL9A3, COL6A1, COL6A2, COL3A1, and COL5A2) genes showed highest expression levels in the testis, while three (COL14A1, COL4A1, and COL4A2) genes had highest expression levels in the ovary follicle,

indicating that these genes might be related to the reproductive traits in buffalo. The reason for the difference could be the limitation of available expression data for buffalo collagen. Importantly, some of the collagen genes exhibited tissue-specific expression, such as COL16A1 was mainly expressed in the mammary gland, while the COL2A1, COL8A1, and COL27A1 were explicitly expressed in the thymus, corpus luteum, and cerebellum, respectively. Apparently, the collagen family has been demonstrated to have various functions in multiple tissues because they contribute to maintaining the structural integrity and stability of tissues and organs [10]. Compared with the expression pattern represented by RNA-seq data, the expression profile generated by qRT-PCR was not wholly equal to that. We assumed that there existed multiple reasons to result in a discrepancy of expression profiles. For example, highly expressed genes can significantly affect the FPKM values, thereby resulting in the bias of FPKM [42]. In this regard, Zhao et al. [41] reported that the expression pattern of Hsp20 gene family in potato from the RNA-seq data and qRT-PCR was a little different. Previously studies showed that the members of the collagen family are the most abundant proteins in ECM that are tightly regulated throughout the development of the mammary gland [28, 43, 44]. Therefore, to explore the expression pattern of collagen genes in the buffalo mammary gland at different lactation points is necessary, which contribute to dissect the potential roles of these genes in the mammary gland morphogenesis. In the present study, our data revealed that most of the collagen genes are mainly expressed in the mammary gland at early lactation. It is noted that a total of 6 buffalo collagens (COL6A6, COL3A1, COL1A1, COL27A1, COL8A1, and COL14A1) was upregulated at the peak lactation, two collagen genes (COL4A3 and COL10A1) were mainly expressed at the peak and mid-lactation, and only the COL24A1 was up-regulated at the late lactation. These results were also supported by the qRT-PCR. Interestingly, Dai et al. [45] found that COL8A1 and COL1A2 both were upregulated in the bovine mammary gland during lactation compared to the dry period. Our findings suggested that these buffalo collagen genes displayed the specific biology function at different stages of mammary gland development. However, these findings remain to be confirmed.

Conclusion

In this work, we performed a genome-wide identification of buffalo collagen family, with 128 collagen protein sequences confirmed. Next, we performed the analyses of buffalo collagen genes, including the phylogeny, gene structures, chromosomal location, gene duplication, and expression patterns in different tissues. Most of the collagen genes were expressed in the different buffalo tissues and were up-regulated in the mammary gland at the early lactation, indicating that these genes play vital roles in the milk production of buffalo. The study provides valuable information on the collagen family in buffalo and will helps in determining the collagen gene functions.

Methods

Animal source

A total of eleven Murrah buffalo was used as the animal material in the present study, which was kept at the Buffalo Research Institute, Chinese Academy of Agricultural Sciences (BRI-CAAS). A total of three buffaloes were slaughtered and used for the fresh tissue collection, including brain, heart, liver, spleen, lung, kidney, stomach, large intestine, ovary, oviduct, pituitary, uterus, hypothalamus, lymph, and mammary gland. The biopsy samples of mammary gland tissue from eight buffaloes were collected on days 7 (D7), 50 (D50), 140 (D140), and 280 (D280) after calving, which was further used for the real-time quantitative PCR (qRT-PCR). The biopsy procedure

was performed based on the method reported by Schmitz et al. [46]. All fresh samples were immediately preserved in the liquid nitrogen until use.

Identification of the buffalo collagen genes

The whole-genome data of six representative mammals including the human (*GRCh38.p12*), cattle (*ARS-UCD1.2*), buffalo (*UOA_WB_1*), goat (*ARST*), sheep (*Oar_rambouillet_v1.0*), and horse (*EquCab3.0*) were downloaded from the NCBI Genome database (<https://www.ncbi.nlm.nih.gov/genome/>), aiming to identify the buffalo collagen genes. The HMM profile of the collagen domain (PF01391) from the Pfam database (<http://pfam.xfam.org/family/Collagen>) was employed to search the buffalo dataset using the HMMER [47, 48] software with an E-value cut-off of $1E-5$. The identified buffalo collagen protein sequences were also validated by the BLAST with collagens from other five mammals as queries. Further, the ClustalW algorithm was used for the multiple sequence alignment of collagen with the full-length protein sequences. The aligned sequences were used for constructing the neighbor-joining (NJ) phylogenetic tree of collagen family using the MEGA7 [49] software with the Poisson model, pairwise deletion, and 1000 bootstrap resamplings.

Sequence Analysis

ExPASy proteomics server was utilized for the prediction of theoretical molecular weight (MW) and isoelectric points (pI) of buffalo collagen family (Gasteiger et al., 2003). The gene structure was assessed and visualized using the TBtools version. 0.6657 [50]. Exon/intron structure analysis was performed by the buffalo genome annotation file using the in-house scripts. We analyzed the conserved protein motifs of collagen proteins in buffalo using the MEME programs (<http://meme-suite.org/tools/meme>) at a maximum number of motifs, 10. Moreover, the transcription factor binding sites (TFBS) in the identified collagen promoter region with 1500 bp in length were predicted using the TFBSTools [51] R package. The main parameters of TFBS analysis were employed as the following: JASPAR2018 was used to retrieve data from JASPAR database, the Homo sapiens was as the reference species, the minimum score for the hit was more than 0.98, and only the positive strand was considered.

Chromosome locations of each collagen gene were obtained from their genome resources. Collagen duplication events were identified using the Multiple Collinearity Scan Toolkit (MCScanX) previously described by Wang et al. [52]. Overall, a total of 58,532 buffalo protein sequences were analyzed using the BLAST search with E-value $< 1E-5$, and then the corresponding gene positions file were obtained from the buffalo genome annotation file with the GFF format, which can be set as the input files of MCScanX program.

For the duplicated collagen genes, we further performed the divergence estimates and diversity analysis. In brief, MEGA7 software with the MUSCLE algorithm was firstly utilized for the pairwise alignment of the homologous collagen gene pairs. Subsequently, the DnaSP v6.0 [53] software was employed to calculate the pairwise synonymous (Ks) and nonsynonymous (Ka) numbers of substitutions corrected for multiple hits.

Expression pattern Analyses

In order to determine the tissue expression profiles of collagen family, the RNA sequencing (RNA-seq) data (BioProject: PRJNA207334) from 29 different Italian Mediterranean buffalo tissues was used to calculate the fragments-per-kilobase-per-million fragments mapped (FPKM) values of buffalo collagens. Briefly, FASTQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) program was used to remove the adapter

sequence from the raw sequence reads. The clean reads were aligned to the buffalo reference genome using Hisat2 [54] with default parameters. FPKM values were calculated using the StringTie [55].

To explore the expression profiles of the buffalo collagen family in mammary gland tissues at different lactation, including the early lactation (D7), peak lactation (D50), mid-lactation (D140), and late lactation (D280), the raw data (BioProject ID: PRJNA480718) from buffalo mammary gland tissues submitted by our group was used. Hierarchical clustering was performed using the Pheatmap [56] R package with the normalized scale method.

qRT-PCR Analysis

Total RNA for the different buffalo tissues was isolated using the RNA Plus reagent (Tiangen, China). For the 2 µg total RNA, first-strand cDNA was synthesized using a reverse transcriptase kit (Takara, Dalian, China). SYBR® Premix Ex Taq™ (Takara, Dalian, China) was used for the qRT-PCR analysis, which was monitored on the LightCycler 480 (Roche, CH), and each reaction was performed in triplicate. The expression levels of buffalo collagen family were analyzed by the $2^{-\Delta\Delta C_t}$ method [57] and normalized using the *GAPDH* expression analysis. The collagens primer pairs used for the qRT-PCR analysis are shown in the **Supplementary Table S1**.

Abbreviations

NCBI: National Center for Biotechnology Information; FPKM: fragments-per-kilobase-per-million fragments mapped; D7: day 7 after calving; D50: day 50 after calving; D140: day 140 after calving; and D280: day 280 after calving.

Declarations

Ethics approval and consent to participate

All experimental design and animal care were approved by the Animal Ethics Committee of the BRI-CAAS (approval code GXBRI-01-2019).

Consent for publication

Not applicable.

Availability of data and material

The data supporting our findings can be found in the additional supporting files.

Competing interests

The authors declare no competing financial interests

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Authors' contributions

TXD designed the study and analyzed the data; TXD, XRL, and AQD wrote the drafted manuscript; SSL, XRL, XYM, and CYP performed the experiments and qRT-PCR analysis; WXL helped to draft the manuscript; All authors read and approved this paper.

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Additional File Legends

- Additional file 1: Table S1. Primers of qRT-PCR for collagen genes in buffalo
- Additional file 2: Figure S1. Information on intron numbers of the collagen family in studied species.
- Additional file 3: Figure S2. Summary of the TFBS detected in the buffalo collagen genes.
- Additional file 4: Figure S3. Summary of the TFBS detected in the buffalo collagen subfamilies.

Tables

Table 1. Features of the identified collagens protein sequences in buffalo

Symbol	Name	GeneID	ProteinID	Protein(aa)	CDS(bp)	MW(kDa)	pI
COL10A1	COL10A1	102389861	XP_025150531.1	2067	688	167.822	4.87
COL11A1	COL11A1	102411572	XP_025143983.1	5415	1804	435.856	4.71
COL11A2	COL11A2_1	102395601	XP_025127671.1	4029	1342	320.1372	4.71
	COL11A2_2	102395601	XP_025127672.1	4029	1342	320.1372	4.71
	COL11A2_3	102395601	XP_025127673.1	4029	1342	320.1372	4.71
	COL11A2_4	102395601	XP_025127670.1	4968	1655	397.8534	4.66
	COL11A2_5	102395601	XP_006050365.1	4890	1629	391.0736	4.66
	COL11A2_6	102395601	XP_006050364.1	4953	1650	396.1965	4.66
	COL11A2_7	102395601	XP_025127669.1	5031	1676	402.9763	4.66
	COL11A2_8	102395601	XP_006050363.1	5211	1736	417.553	4.65
	COL11A2_9	102395601	XP_025127668.1	5133	1710	410.7732	4.65
COL12A1	COL12A1_1	102397550	XP_006062066.1	5871	1956	481.867	4.66
	COL12A1_2	102397550	XP_006062065.1	9198	3065	753.4744	4.58
	COL12A1_3	102397550	XP_006062064.1	9363	3120	766.9291	4.58
COL13A1	COL13A1_1	102391074	XP_025140452.1	2163	720	169.9635	4.86
	COL13A1_10	102391074	XP_025140449.1	2349	782	187.4364	4.83
	COL13A1_11	102391074	XP_025140454.1	2076	691	163.4114	4.87
	COL13A1_12	102391074	XP_025140460.1	2091	696	164.273	4.87
	COL13A1_13	102391074	XP_025140459.1	2100	699	165.2032	4.87
	COL13A1_14	102391074	XP_025140450.1	2331	776	186.1869	4.83
	COL13A1_15	102391074	XP_025140461.1	2031	676	159.6319	4.87
	COL13A1_16	102391074	XP_025140456.1	2040	679	160.5622	4.87
	COL13A1_2	102391074	XP_025140447.1	2376	791	189.3553	4.83
	COL13A1_3	102391074	XP_025140446.1	2385	794	190.2855	4.83
	COL13A1_4	102391074	XP_025140451.1	2319	772	184.5388	4.84
	COL13A1_5	102391074	XP_025140458.1	2127	708	167.1222	4.87
	COL13A1_6	102391074	XP_025140457.1	2193	730	172.8689	4.86
	COL13A1_7	102391074	XP_025140453.1	2127	708	167.1143	4.86
	COL13A1_8	102391074	XP_025140448.1	2358	785	188.3744	4.83
	COL13A1_9	102391074	XP_025140455.1	2061	686	161.3676	4.87

COL14A1	COL14A1_1	102389728	XP_006074754.1	5370	1789	438.6139	4.7
	COL14A1_2	102389728	XP_006074753.1	5418	1805	442.4683	4.69
COL15A1	COL15A1_1	102398398	XP_025137574.1	4035	1344	331.1	4.72
	COL15A1_2	102398398	XP_025137571.1	4119	1372	337.7977	4.72
	COL15A1_3	102398398	XP_025137569.1	4131	1376	338.917	4.72
	COL15A1_4	102398398	XP_025137570.1	4131	1376	338.917	4.72
	COL15A1_5	102398398	XP_025137572.1	4065	1354	333.4969	4.72
	COL15A1_6	102398398	XP_025137573.1	4065	1354	333.1664	4.72
COL16A1	COL16A1_1	102397619	XP_025134331.1	4878	1625	390.0826	4.68
	COL16A1_2	102397619	XP_025134330.1	4899	1632	391.9759	4.68
	COL16A1_3	102397619	XP_025134334.1	4851	1616	388.1235	4.68
	COL16A1_4	102397619	XP_025134336.1	4833	1610	386.131	4.68
	COL16A1_5	102397619	XP_025134332.1	4854	1617	388.0243	4.68
	COL16A1_6	102397619	XP_025134335.1	4809	1602	384.2089	4.68
COL17A1	COL17A1_1	102415719	XP_006067794.2	4458	1485	365.6887	4.69
	COL17A1_2	102415719	XP_006067796.2	4347	1448	356.3409	4.69
COL18A1	COL18A1_1	102395494	XP_025148666.1	3990	1329	323.4921	4.69
	COL18A1_2	102395494	XP_006052291.1	4614	1537	376.1224	4.65
	COL18A1_3	102395494	XP_025148650.1	4641	1546	378.3079	4.65
	COL18A1_4	102395494	XP_025148645.1	5355	1784	439.2372	4.61
	COL18A1_5	102395494	XP_025148643.1	5358	1785	439.4684	4.61
	COL18A1_6	102395494	XP_025148641.1	5382	1793	441.4226	4.61
COL19A1	COL19A1_1	102390934	XP_025150732.1	3396	1131	271.4768	4.8
	COL19A1_2	102390934	XP_025150733.1	3342	1113	267.3664	4.8
COL1A1	COL1A1	102391895	XP_006041214.2	4392	1463	363.0366	4.67
COL1A2	COL1A2	102414428	XP_006054012.1	4095	1364	335.3795	4.73
COL20A1	COL20A1	102416355	XP_025119622.1	3918	1305	321.4133	4.7
COL21A1	COL21A1	102389834	XP_025128099.1	2871	956	231.0769	4.86
COL22A1	COL22A1_1	102396959	XP_025121358.1	4845	1614	387.2009	4.67
	COL22A1_2	102396959	XP_025121359.1	4476	1491	357.0108	4.69
	COL22A1_3	102396959	XP_025121360.1	4350	1449	346.9775	4.69

COL23A1	COL23A1	102394574	XP_025148653.1	1557	518	122.9447	4.92
COL24A1	COL24A1_1	102406262	XP_025144145.1	5130	1709	412.8481	4.74
	COL24A1_2	102406262	XP_025144143.1	5277	1758	425.2749	4.74
	COL24A1_3	102406262	XP_025144144.1	5277	1758	425.2749	4.74
COL25A1	COL25A1_1	102390242	XP_025146095.1	2001	666	157.8101	4.91
	COL25A1_2	102390242	XP_025146096.1	1974	657	155.769	4.91
	COL25A1_3	102390242	XP_025146097.1	1965	654	154.995	4.91
	COL25A1_4	102390242	XP_025146098.1	1956	651	154.1831	4.91
	COL25A1_5	102390242	XP_025146099.1	1938	645	152.9538	4.91
COL26A1	COL26A1_1	102389806	XP_006045472.1	1317	438	107.4612	4.92
	COL26A1_2	102389806	XP_006045471.1	1323	440	107.9657	4.92
COL27A1	COL27A1_1	102415572	XP_025138105.1	5469	1822	443.7718	4.63
	COL27A1_2	102415572	XP_006061277.1	5523	1840	448.1909	4.63
	COL27A1_3	102415572	XP_025138102.1	5526	1841	447.8854	4.63
	COL27A1_4	102415572	XP_025138099.1	5529	1842	448.1026	4.63
	COL27A1_5	102415572	XP_025138104.1	5475	1824	443.9739	4.63
	COL27A1_6	102415572	XP_025138103.1	5475	1824	443.8918	4.63
COL28A1	COL28A1	102407731	XP_006074647.1	3444	1147	276.3516	4.81
COL2A1	COL2A1_1	102403678	XP_006059500.1	4464	1487	364.1477	4.68
	COL2A1_2	102403678	XP_006059501.1	4257	1418	347.1815	4.69
COL3A1	COL3A1	102394969	XP_006073767.2	4401	1466	357.2321	4.72
COL4A1	COL4A1	102405654	XP_025118865.1	5010	1669	401.5158	4.66
COL4A2	COL4A2	102403266	XP_025118864.1	5133	1710	411.3817	4.65
COL4A3	COL4A3_1	102416276	XP_006047187.1	5013	1670	401.8	4.7
	COL4A3_2	102416276	XP_025117915.1	4296	1431	342.0154	4.73
	COL4A3_3	102416276	XP_006047188.1	4614	1537	369.6487	4.72
COL4A4	COL4A4_1	102389047	XP_025117873.1	4950	1649	397.2704	4.69
	COL4A4_2	102389047	XP_006047189.1	5070	1689	406.8914	4.69
	COL4A4_3	102389047	XP_006047190.1	5070	1689	406.8914	4.69
	COL4A4_4	102389047	XP_006047191.1	5070	1689	406.8914	4.69
	COL4A4_5	102389047	XP_006047192.1	5070	1689	406.8914	4.69

	COL4A4_6	102389047	XP_006047193.1	5070	1689	406.8914	4.69
	COL4A4_7	102389047	XP_006047197.1	4254	1417	339.8506	4.73
	COL4A4_8	102389047	XP_006047198.1	4110	1369	328.1391	4.74
COL4A5	COL4A5_1	102399306	XP_006072403.1	5085	1694	414.9557	4.69
	COL4A5_2	102399306	XP_006072404.1	5076	1691	414.1878	4.69
	COL4A5_3	102399306	XP_006072408.1	5058	1685	412.6561	4.69
	COL4A5_4	102399306	XP_006072405.1	5076	1691	414.1878	4.69
	COL4A5_5	102399306	XP_025131953.1	5076	1691	414.1918	4.69
	COL4A5_6	102399306	XP_006072407.1	5067	1688	413.424	4.69
COL4A6	COL4A6	102410702	XP_025131955.1	5085	1694	412.8158	4.68
COL5A1	COL5A1	102414254	XP_025118027.1	5541	1846	447.3157	4.62
COL5A2	COL5A2	102395275	XP_006073768.1	4500	1499	361.1947	4.74
COL5A3	COL5A3_1	102407916	XP_006044492.1	5241	1746	420.8161	4.66
	COL5A3_2	102407916	XP_006044493.1	5238	1745	420.5848	4.66
	COL5A3_3	102407916	XP_025149345.1	4881	1626	392.9009	4.68
COL6A1	COL6A1	102389816	NP_001277797.1	3084	1027	248.0209	4.77
COL6A2	COL6A2_1	102397245	XP_025148825.1	2781	926	224.0028	4.79
	COL6A2_2	102397245	XP_006047111.1	3084	1027	248.74	4.77
	COL6A2_3	102397245	XP_025148817.1	3084	1027	248.74	4.77
COL6A3	COL6A3	102392465	XP_025144989.1	9654	3217	786.5997	4.54
COL6A4	COL6A4	102391810	XP_025127254.1	6504	2167	526.7032	4.66
COL6A5	COL6A5	102394387	XP_025149876.1	7821	2606	636.0357	4.64
COL6A6	COL6A6_1	102393741	XP_006043497.1	6813	2270	553.3681	4.64
	COL6A6_2	102393741	XP_025149867.1	6813	2270	553.3681	4.64
	COL6A6_3	102393741	XP_006043498.1	6801	2266	552.4591	4.64
COL7A1	COL7A1	102405985	XP_006065225.2	8793	2930	703.4311	4.54
COL8A1	COL8A1	102398637	XP_006047857.1	2238	745	181.1666	4.84
COL8A2	COL8A2	102415548	XP_025144875.1	2115	704	171.0488	4.83
COL9A1	COL9A1	102391279	XP_025150021.1	3216	1071	259.2767	4.79
	COL9A2_1	102400772	XP_006061989.2	2109	702	166.8184	4.86
	COL9A2_2	102400772	XP_006061990.2	2067	688	163.2783	4.86

COL9A3	COL9A3_1	102411321	XP_025119609.1	2055	684	164.345	4.84
	COL9A3_2	102411321	XP_025119605.1	2271	756	182.1856	4.82
	COL9A3_3	102411321	XP_025119606.1	2271	756	182.1856	4.82
	COL9A3_4	102411321	XP_025119607.1	2271	756	182.1856	4.82
	COL9A3_5	102411321	XP_025119608.1	2271	756	182.1856	4.82

Table 2. Ten different motifs commonly observed in buffalo collagen family

Motif	Protein sequence	Length	Pfam domain
MEME-1	GLPGLKGEKGEAGLPGFKGEKGVKGEKGE	29	-
MEME-2	KGEDGLPGLPGEKGEKGEKGDPPGPPG	29	-
MEME-3	GEKGERGLPGLPGKKGAKGEPGIPGAKGEKGPPGPPGPPGE	41	Collagen
MEME-4	GPPGPPGPPGPPGPPGLPGPPGPPGLPGPP	30	-
MEME-5	PGPPGPKGPRGEKGDPGSTGPPGEPGLPGLQGPPGEKGDKG	41	Collagen
MEME-6	GPKGGERGPKGQKGEKGQPGEP	21	-
MEME-7	TGPPGPIGLPGLPGPKGEKGE	21	-
MEME-8	GEPGJPGEKGEPGLPGPPGLPGEKGPKGK	29	-
MEME-9	GEQGERGPKGGEKGEA	15	-
MEME-10	RGEPGLPGPPGPPGP	15	-

Table 3. Analysis of the Ka/Ks ratios for each pair of duplicated collagen genes in buffalo

Gene pairs	CHR	Ka	Ks	Ka/Ks ratio
COL11A2_4/COL11A2_9	2	0.0093	0.0095	0.9805
COL12A1_2/COL12A1_1	10	0.0005	0.0014	0.3362
COL12A1_3/COL12A1_2	10	0.0003	0.0009	0.3327
COL13A1_2/COL13A1_3	4	0.0158	0.0182	0.8665
COL13A1_3/COL13A1_10	4	0	0.0017	0
COL13A1_10/COL13A1_14	4	0	0.0017	0
COL14A1_2/COL14A1_1	15	0.0035	0.0008	4.3743
COL17A1_2/COL17A1_1	23	0.0003	0.0018	0.1825
COL22A1_1/COL22A1_2	15	0.0112	0.0131	0.8518
COL22A1_2/COL22A1_3	15	0.0033	0.0038	0.8753
COL27A1_5/COL27A1_4	3	0	0.0021	0
COL4A2/COL4A1	X	0.5336	1.9459	0.2742
COL4A3_1/COL4A3_2	X	0.0029	0.0086	0.3357
COL4A3_2/COL4A3_3	X	0.0339	0.0586	0.5781
COL4A4_2/COL4A4_7	X	0.0061	0.0073	0.8406
COL5A3_1/COL5A3_2	9	0	0.0007	0
COL5A3_2/COL5A3_3	9	0	0.0007	0
COL6A1/COL6A2_2	1	0.7068	1.0659	0.6631
COL6A2_1/COL6A2_3	1	0.0547	0.0696	0.7861
COL6A2_2/COL6A2_1	1	0.0547	0.0696	0.7861
COL6A6_2/COL6A5	1	0.3759	1.4319	0.2625
COL6A6_3/COL6A6_1	1	0.0002	0.0006	0.3146
COL9A1/COL19A1_2	6	0.6218	2.6177	0.2375
COL9A2_2/COL9A2_1	6	0.0239	0.0263	0.9098

Note. CHR=Chromosome of buffalo

Figures

Figure 1

Phylogenetic relationship of collagen proteins in 6 mammals. Different capitals represent different species: B means buffalo, C means cattle, G means goat, S means sheep, H means horse, and Hu mean human; Line with different colors indicates different subfamilies.

Figure 2

Phylogenetic relationships (A), gene structure (B), and conserved protein motifs (C) of buffalo collagen family. The different-colored boxes indicate different domains or motifs of the collagen family in buffalo.

Figure 3

TFBS analysis of the promoter region in buffalo collagen. (A) The phylogenetic tree of buffalo collagen genes was constructed, and different colors indicated different clusters. (B) The predicted TFBS was displayed in the promoter region of buffalo collagen genes. The different-colored boxes indicate different TFBS.

Figure 4

Chromosome location and gene duplication of buffalo collagen sequences. The tandem duplicated genes were marked by the red line, and segmentally duplicated genes are indicated by symbol #

Figure 5

Tissue expression distribution of buffalo collagen using RNA-seq data and qRT-PCR. (A) tissue expression distribution of buffalo collagen genes based on the transcriptomic data. (B) tissue expression pattern of buffalo collagen genes based on the qRT-PCR.

Figure 6

Cluster analysis of buffalo collagen in mammary gland tissue at different lactation points.

Supplementary Files

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- [Supplementarymaterial.pdf](#)
- [NC3RsARRIVEGuidelinesChecklist2014.docx](#)