Polish Journal of Veterinary Sciences Vol. 21, No. 1 (2018), 55–64

DOI 10.24425/119022

Original article

Molecular characterization and expression patterns of ghrelin in the reindeer (*Rangifer tarandus*)

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Abstract

Ghrelin is a novel growth hormone (GH)-releasing peptide, which has been identified as an endogenous ligand for the GH-sretagogue receptor. The sequence and expression of ghrelin has been determined in many species. In this study, to reveal the molecular characterization and expression patterns of ghrelin in the reindeer (Rangifer tarandus), the full-length DNA and cDNA encoding ghrelin were cloned from reindeer stomach using genome walking and rapid amplification of complementary deoxyribonucleic acid ends (RACE). The expression of ghrelin in almost all tissues was examined by real-time quantitative PCR (RT-qPCR). The 4076 bp amplicon of the ghrelin gene consisting of 4 exons and 3 introns was cloned from reindeer. Results of cDNA cloning and sequence analysis revealed that the full-length ghrelin cDNA was composed of 539 bp that included a 5'-untranslated region (46 bp), an open reading frame (ORF) (351 bp), and a 3'-untranslated region (142 bp). In addition, ghrelin was expressed in the all tissues examined, with the expression in the abomasum significantly higher than that in other tissues ($p<0.05$), followed by the pancreas, duodenum, testis and oesophagus. The results show that the expression of ghrelin in the reindeer gastrointestinal tract is extensive, suggesting its may have a role in regulating the digestive function.

Key words: ghrelin, reindeer (*Rangifer tarandus*), cDNA cloning, structural characterization, tissue expression

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Introduction

Ghrelin is a novel endogenous growth hormone (GH) sretagogue as an endogenous ligand for the GH sretagogue receptor (GHS-R) (Kojima et al. 1999). Ghrelin has two main physiological functions, GH-releasing activity and appetite-stimulating activity (Kojima and Kangawa 2005). Ghrelin has been implicated in other functions involving gastric acid secretion and motility, pancreatic activity, carbohydrate metabolism, prolactin secretion, cardiovascular actions, reproduction, fetal growth and development, apoptosis and cell proliferation (Barreiro et al. 2004, Van et al. 2004, Gualillo et al. 2006, Sato et al. 2006, Baatar et al. 2011), and also has a therapeutic potential for intestinal failure-associated liver disease (IFALD) and acetic acid-induced colitis (Matuszyk et al. 2016, Onishi et al. 2016).

Ghrelin was originally isolated from rat stomach tissue as a prepro-peptide hormone of 117 amino acids, which is processed to an active 28-amino acid peptide with an n-octanoylated serine at position 3 (Kojima et al. 1999). The other form of ghrelin found in rat and humans is a 27-amino acid peptide lacking Gln14 or Arg28 (Hosada et al. 2000, Hosada et al. 2003). In goats, the 27-amino acid peptide is the major ghrelin form (Ida et al. 2010). This structural divergence in peptide length and fatty acid modification has also been reported in the ghrelin of non-mammalian vertebrates, such as rainbow trout, chicken, and bullfrog (Kaiya et al. 2001, Kaiya et al. 2002, Kaiya et al. 2003).

The sequence of ghrelin has been identified in many species including human (Kojima et al. 1999), rodent (Kojima et al. 1999), bird (Nie et al. 2009), dog (Tomasetto et al. 2001), pig (Vitari et al. 2012), fish (Xu and Volkoff 2009), panda (Xu et al. 2010) and ruminant (Ida et al. 2010, Zhang et al. 2010). Accumulating evidence in many mammals suggests that ghrelin mainly exists in the stomach and is also expressed widely in other tissues, such as the pituitary, hypothalamus, intestine, kidney, liver, placenta, pancreas, testicle, and longissimus dorsi muscle (Murakami et al. 2002, Korbonits et al. 2004). Expression of ghrelin and GHS-R1a immunoreactive cells were detected in the abomasum, anterior pituitary gland, testis, ovary, hypothalamic and hindbrain regions of the brain in the adult sheep (Miller et al. 2005). A recent reports show that ghrelin mRNA and ghrelin O-Acyltransferase (GOAT) co-expressed in the porcine stomach, pancreas, and duodenum at high levels (Lin et al. 2011). In addition, a study found that ghrelin is also present in plants, and the fruit from Prunus x domestica L. and Marus alba had higher levels than the vegetative parts (Aydin et al. 2006). From the above-mentioned studies, ghrelin is widely distributed in plants and animals, and may have an important regulatory role in various tissues of the biological world.

Reindeer (Rangifer tarandus), one of the important economic animals and a rare animal protected by law in China, belong to a wild species and their antler, meat, milk, whip, gonads, skin and fetal blood have high economic value. The reindeers' main food in winter and spring is wild lichens with low protein content requiring a gastrointestinal function capable of adaptive variations. During summer and autumn, various highly nutritious plant species and mushrooms are their main food, accumulating body protein and fat. In winter, even a moderate protein ration can significantly increase free water intake and its thermal energy cost as compared to the dominant natural feed (lichens) (Soppela et al. 1992). Feeding lichens was related to a significant decline in plasma total protein, albumin and globulin, but not in the albumin/globulin ratio (Säkkinen et al. 2005). The survival rate of this species is mainly affected by environment and food. However, the primary causes responsible for gastrointestinal function capable of adaptive variations are still unknown. Taking into account the gastric acid secretion and motility functions of ghrelin, we have cloned the full-length ghrelin DNA and cDNA and examined the expression and distribution to reveal the molecular characteristics of reindeer ghrelin, which will establish the basis for studying the relationship between gastrointestinal function and the structure of reindeer ghrelin.

Materials and Methods

Animal and sample preparation

Three male young reindeers (2 years old), which were sacrificed in winter because of broken hind limbs, were supplied by the Aoluguya District of the Inner Mongolia Municipality in China. All experimental animals in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Inner Mongolia Agricultural University. Animal handling, euthanasia and experimental procedures were approved by the Tecon Group Regulation (License No. SYXK, Inner Mongolia, 2010-0006) approved by the Animal Ethics Committee of the Inner Mongolia Agricultural University. Euthanasia was carried out by intravenous injection of barbiturate overdose, followed by bleeding, the tissue samples were obtained and epithelial tissues were scraped from the mucous membranes of the tongue, oesophagus, rumen, reticulum, omasum, abomasum, duo-

denum, jejunum, ileum, colon and bladder, tissue masses were cut from the pancreas, testis, thyroid gland, adrenal gland, hypophysis, hypothalamus, heart, thymus, spleen, lymphatic node, liver, kidney, ureter, lung, skeletal muscle, seminal vesicle gland, epididymis, ductus deferens, and all tissues were stored at -80°C for RNA isolation. Total RNA was extracted from 500 ng of the tissues using TRI Reagent (Promega, Madison, WI) following the manufacturer's instructions, and assessed for quality and purity by spectrophotometry.

Cloning of Ghrelin Genomic DNA

Genomic DNA was extracted using the MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 (Takara, Dalian, China) from reindeer abomasum. Three pairs of PCR primers P1 and P2, P3 and P4, P5 and P6 which were in the ghrelin DNA exons 1-2, 2-3 and 3-4, were designed on the basis of reindeer ghrelin DNA exons 1-4 sequences (GenBank: AH013722.1) available at GenBank (www.ncbi.nlm.nih.gov). Amplification of the reindeer ghrelin gene was performed with TransStart®FastPfu DNA Polymerase (TransGen, Beijing, China), whereby the reaction mixture consisted of genomic DNA 100 ng, 5×TransStart® FastPfu Buffer (10 µL), 10 pM of each primer, 2.5 U of TransStart®FastPfu DNA Polymerase, 2.5 mM dNTPs, and quantum-sufficient nuclease-free water. A negative control with no template DNA was also included. The amplification conditions were 95°C for 2 min, and 35 cycles of 98°C for 20 sec, 60°C for 20 sec, and 72°C for 1 min, and a final extension for 5 min at 72°C. The product was visualized after electrophoresis with ethidium bromide using a 1% agarose gel. The amplicon of ghrelin DNA exons 1-2, 2-3 and 3-4 gene was purified by using MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (Takara, Dalian, China), and the purified PCR product was subcloned using the pEASY®-Blunt Cloning Kit (TransGen, Beijing, China).

The full-length DNA sequence was obtained using the Genome Walking Kit (Takara, Dalian, China) with high temperature annealing of specific primers SP1, SP2, SP3 and DP1, DP2, DP3 based on the sequences of reindeer ghrelin DNA exons 1-2, 2-3 and 3-4, and the AP (AP1, AP2, AP3, AP4) primers were supplied in the kit. The 5'-end of genomic DNA was amplified by thermal asymmetric PCR reaction as follows: Genomic DNA (1 ng), 2.5 mM of dNTP Mixture, 10 × LA PCR Buffer II (Mg^{2+}plus) (5 µL), 5 U of TaKaRa LA Taq, 100 pmol of AP3 primer, 10 pmol of SP1 primer, nuclease-free water (32 µL). The 1st PCR program was 94°C for 1 min, 98°C for 1 min, 5 cycles of 94°C for 30 sec, 65°C for 1 min, 72°C for 2 min, 15 cycles of 94°C for 30 sec, 25°C for 3 min, 72°C for 2 min; 94°C for 30 sec, 65°C for 1 min, 72°C for 2 min; 94°C for 30 sec, 25°C for 1 min, 72°C for 2 min; 94°C for 30 sec, 44°C for 1 min, 72°C for 2 min, and a final extension for 10 min at 72°C. A 1 µL aliquot of the 1st PCR product was amplified in a 2nd PCR with the same components except for the second pair of gene-specific outer nested primers (AP3 and SP2). The 2nd PCR program was 15 cycles of 94°C for 30 sec, 25°C for 3 min, 72°C for 2 min; 94°C for 30 sec, 65°C for 1 min, 72°C for 2 min; 94°C for 30 sec, 25°C for 1 min, 72°C for 2 min; 94°C for 30 sec, 44°C for 1 min, 72°C for 2 min, and 72°C for 10 min. A 1 µL aliquot of the 2nd PCR product was amplified in a 3rd PCR using the same components and program except for the third pair of gene-specific outer nested primers (AP3 and SP3). A single 5'-end product was visualized after electrophoresis with ethidium bromide using a 1% agarose gel.

The 3'-end was amplified using the same components and program with the 5'-end except for the specific primers DP1, DP2, DP3 as sense primer and the low annealing temperature degenerate primer AP4 (Genome Walking Kit supply) as antisense primer. A single 3'-end product was visualized after electrophoresis with ethidium bromide using a 1% agarose gel. The sequence of primers for 5'-end and 3'-end are shown in Table 1.

Cloning of Ghrelin cDNA

To obtain the ghrelin cDNA sequence, we designed a pair of PCR primers according to the known reindeer partial ghrelin gene CDS sequence (GenBank: AH013722). The sequences of primers were: sense 5'-GCGTGCTCTGGCTGGACTTG-3' (reghP1) and antisense 5'-CCAGGGTTTCGTCG GCTTCTTC-3' (reghP2). RT-PCR was conducted using an Access Quick RT-PCR System kit (Promega, Madison, WI) in a 50 µL reaction volume containing 0.2 mM of each deoxy-NTP, AMV/TfI Reaction Buffer, 1 mM $MgSO_4$, 0.1 U AMV Reverse Transcriptase, 0.1 U TfI Taq DNA polymerase, 1 µM of reghP1 and reghP2, and 500 ng abomasum mucosa total RNA. The one-step RT-PCR profile was: 45°C reverse transcription for 45 min, 94°C denaturation for 2 min, followed by 45 cycles of 94°C denaturation for 30 sec, 60°C annealing for 1 min, and 68°C extension for 2 min, followed by a final extension at 68°C for 5 min. A negative control (without reverse transcriptase) was also included to test for genomic DNA contamination. A single, approximately 300 bp RT-PCR product, was visualized after electrophoresis using a 1% agarose gel with ethidium bromide.

Table 1. Primers designed for cloning and expression of Ghrelin.

Primer name	Sequence	Application
P1	5'-TCCGTCTGCCTCCAGCCAGGGAAGCCAT-3'	Ghrein DNA cxtron 1-2 cloning
P2	5'-GCCCGCTGTTGTCCTGCTGTTTGATGTC-3'	
P3	5'-GAAAGGAACCTAAGAAGCCATCAG-3'	Ghrein DNA cxtron 2-3 cloning
P4	5'-GAAACTTCCCCAGCGTC-3'	
P5	5'-CCAGGTAAAGAGGGCATAAGG-3'	Ghrein DNA cxtron 3-4 cloning
P6	5'-GCAAGAAGGCAAGTCAAAGAG-3'	
SP1	5'-ATGTGAATACTTGCTGGCACGCCC-3'	Ghrein DNA 5'-end cloning
SP2	5'-TGTTGATGGCGAAGACCTGGCAGA-3'	
SP3	5'-GTTTGATGCCCCTTGAGTAGCTGC-3'	
DP1	5'-GGTGGlCCICCICCTAGTTCAA-3'	Ghrein DNA 3'-end cloning
DP2	5'-TGAGAACCCAGGCCTCTTCTGA-3'	
DP3	5'-TGGGAAGAAGCCGACGGTAAGT-3'	
Oligo $(dT)_{15}$-3 sites adaptor primer	5'-CTGATCTAGAGGTACCGGATCC(T)15-3'	Adaptor primers for 3'-RACE and 5'-RACE
RT primer for 5'-RACE	5'-(P)CCATTTGAGCATTTA-3'	
regsP	5'-GCGCGCTCTGGCTGGACTTG-3'	3'-RACE cloning
3 sites adaptor primer	5'-CTGATCTAGAGGTACCGGATCC-3'	
S1	5'-AACGCCCCCITTGACATTGG-3'	
A1	5'-CTGCACCTTCCGCCTGACTTC-3'	5'-RACE cloning
S2	5'-CCGACGAAACCCTGGCTGATG-3'	
A2	5'-ATGGCTTCTTAGGTTCCTTTCTCTG-3'	
A1-1	5'-TGAGGGTGGGGAACGGACAG-3'	Real-time PCR for ghrelin
S3	5'-CCCGGAGAAGGAGCCTGAGAAAC-3'	Real-time PCR lor 18SrRNA
A3	5'-TGATGCCCCCGACTGTCCCTATTA-3'	

The full-length cDNA sequence was obtained using a 5' and 3'-RACE (rapid amplification of cDNA ends) kit (Takara Shuzo, Dalian, China) with specific primers based on the reindeer ghrelin cDNA sequence obtained from RT-PCR.

For 3'-RACE, 500 ng of reindeer abomasum mucosa total RNA was reverse-transcribed in a total volume of 20 μL containing 20 U of RNasin, 2.5 μM of Oligo (dT) 18 -3 sites adaptor primer (3'-RACE kit supply), 5 U of AMV reverse transcriptase XL, 10 mM dNTP, 25 mM $MgCl_2$, and 10×RT buffer. The RT profile was: 30°C for 10 min, 50°C for 30 min, and 95°C for 5 min. The 3' cDNA ends were then amplified by PCR, using the reindeer gene-specific primer (regsP) as sense primer and the 3 sites adaptor primer (3'-RACE kit supply) as antisense primer. The PCR component was 1 μL aliquot of the RT products, 20 μM of 3 sites adaptor primer and regsP, 10 × Ex PCR buffer, 2.5 mM dNTP, and 2.5 U of Ex Taq polymerase. The PCR program was 30 cycles of 94°C for 1 min, 98°C for 10 sec, 55°C for 30 sec, and 72°C for 1 min. A single 3'-RACE product was visualized after electrophoresis with ethidium bromide using a 1% agarose gel.

The 5'-RACE procedure was modified from the traditional anchor PCR as follows: Total RNA (1 μg) from reindeer abomasum mucosa was reverse transcribed in a total volume of 15 μL containing 200 μM of gene-specific RT primer which was phosphated at the 5' site, 5 U of AMV reverse transcriptase XL, 20 U of RNasin, and 10 × RT buffer (including dNTP mixture). The RT profile was: 30°C for 10 min, 50°C for 30 min, and 80°C for 2 min. The RT products were looped with T4 RNA ligase and used for templates of nested PCR. The first PCR product was amplified by using a pair of gene-specific inner nested primers (S1 and A1). The first PCR component and program were the same as for the PCR of 3' RACE except for the primers. A 1 μL aliquot of the first PCR product was amplified in a second PCR with the same components and program except for the second pair of gene-specific outer nested primers (S2 and A2). A single 5'-RACE product was visualized after electrophoresis with ethidium bromide using a 1% agarose gel. The sequence of primers for 3'-RACE and 5'-RACE are shown in Table 1.

cDNA sequencing

The RT-PCR, 3'-RACE and 5'-RACE products were purified and cloned into PMD18-T vector (Takara Shuzo, Dalian, China), followed by transformation into *E.coli* (JM109; Takara Shuzo, Dalian, China) and colony screening with PCR. The sense and antisense gene-specific primers were used for PCR clone screening. From the bacterial culture, plasmids were extracted derived from identified single colonies, and the inserts were sequenced with T7/SP6 promoter primers using a T7 Sequenase version 2.0 DNA

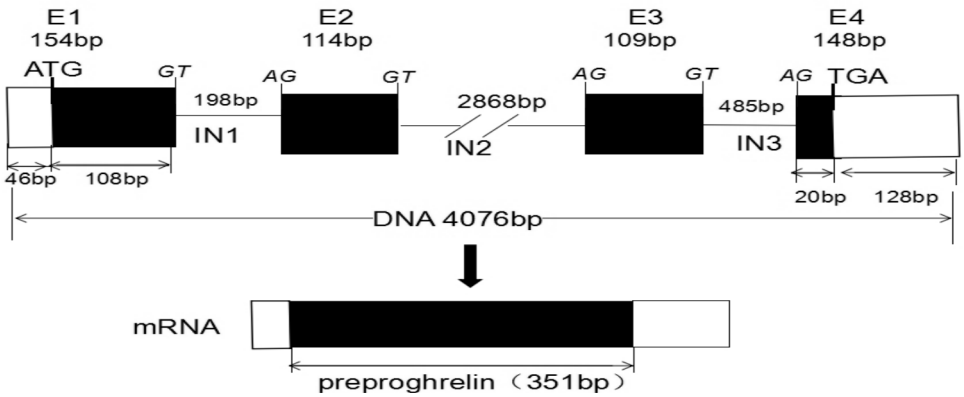

Fig. 1. Schematic diagram showing exons and introns organization in reindeer Ghrelin genomic DNA.

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CCTCCGCCCGGAACCCCAGGTCCGTCTGCCTCCAGCCAGGGAAGCCATGCCTGCCCCATGGACCATCTGC      70
                                                M  P  A  P  W  T  I  C    8
AGCCTGCTGCTGCTCAGCGTGCTCTGGCTGGACTTGGCCATGGCGGGCTCCAGCTTTCTGAGCCCCGAACAT  142
S  L  L  L  L  S  V  L  W  L  D  L  A  M  A  G  S  S  F  L  S  P  E  H   32
CAGAAACTGCAGAGAAAGGAACCTAAGAAGCCATCAGGCAGACTGAAGCCCCGGGCCCTGGAAGGCCAG   211
Q  K  L  Q  R  K  E  P  K  K  P  S  G  R  L  K  P  R  A  L  E  G  Q     55
TTTGACCCGGAGGTGGGAAGTCAGGCGGAAGGTGCAGAGGACGAGCTGGAAATCCGGTTCAACGCCCCC  280
F  D  P  E  V  G  S  Q  A  E  G  A  E  D  E  L  E  I  R  F  N  A  P    78
TTTGACATTGGGATCAAGCTGTCAGGGGCTCAGTCCCTCCAGCATGGCCAGACGCTGGGGAAGTTTCTTCAG  352
F  D  I  G  I  K  L  S  G  A  Q  S  L  Q  H  G  Q  T  L  G  K  F  L  Q   102
GACATCCTTTGGGAAGAAGCCGACGAAACCCTGGCTGATGAGTGACCAGCCCTGGGACCAACCCCCGTCCGT  424
D  I  L  W  E  E  A  D  E  T  L  A  D  E  *                             116
TCTCCACCCTCAGAAGCTCTCACGTGGCTTCCAGGACACTTCCGAGACCACATGTAGCTCTGAGGGGTGCTA  496
GCCTAGGAGATGAATAAATGCTCAAATGGAAAAAAAAAAAAA                                539
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Fig. 2. Nucleotide of reindeer ghrelin and predicted 116 amnio acid sequenes of prepro-ghrelin. ATG: start code. Double underline represents the putative signal peptide, single underline represents putative mature ghrelin peptide, moire underline represents the putative C-terminal peptide, and one asterisks indicate the stop codon.

sequencing kit (Amersham Life Science, Cleveland, OH). All sequence reactions were performed in both directions. In order to obtain the partial and full-length reindeer cDNA sequences, at least ten independent cDNA clones were sequenced in each case.

RT-qPCR

Quantitative real-time PCR (RT-qPCR) was used to quantify the expression of ghrelin. Total RNA was extracted from the different tissues, and the first-strand cDNA was derived from the total RNA. Subsequently, we designed a pair of specific primers with DNAStar 5.01 on the basis of cloned reindeer ghrelin cDNA sequence. The specific primer pairs S1 and A1-1 (Table 1) were used to amplify the 165 bp amplicon. A pair of primers S3 and A3 (Table 1) was used to amplify 18S rRNA as an endogenous control. The RT-qPCR was performed using a VIIA™7 Real-Time PCR System. All reactions were performed using SYBR®Premix Ex TaqTMII (Tli RNase Plus; TaKaRa Shuzo, Dalian, China) in a 20 µL reaction volume containing SYBR Premin Ex Taq (2×) 10 µL, 0.8 µL of each gene-specific primer, 2 µL of cDNA diluted 1/10 in nuclease-free water and quantum-sufficient nuclease-free water. RT-qPCR thermal cycling was 95°C initial denaturation for 30 sec, 45 cycles of 95°C denaturation for 5 sec, 62°C annealing for 30 sec. A dissociation curve was generated to determine the specificity of the PCR reaction after completion of amplification. The PCR efficiency for ghrelin and 18S rRNA were 92 and 98, respectively.

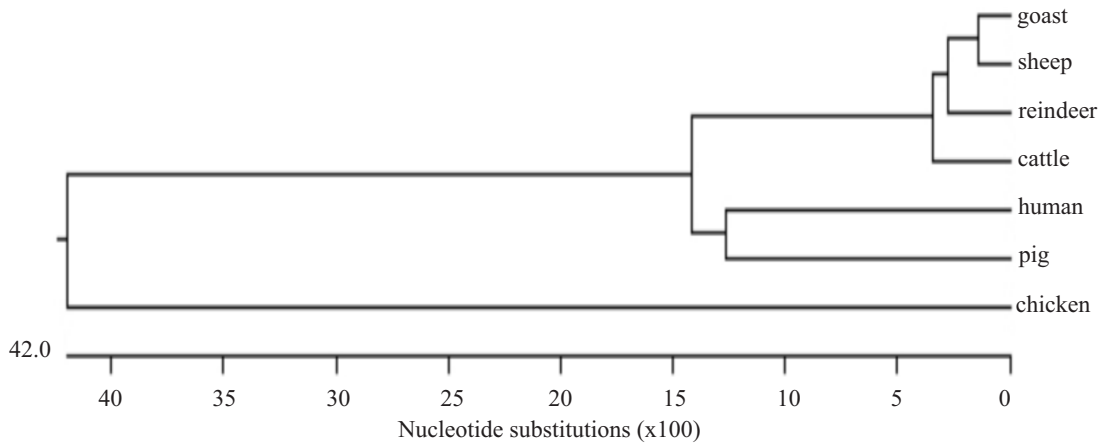

Fig. 3. Phylogenetic tree of the cDNA sequences of reindeer ghrelin and other species animal ghrelin. GenBank Accession: reindeer: EU099031; goat: BAD34669; sheep: ABC00742; cattle: AAK18612; pig: BAB19048; human: AB029434; chicken: AB075215.

Statistical analysis

The relative expression levels of Ghrein mRNA were presented as ratios of the copy numbers of ghrelin gene to 18S rRNA, and all values were expressed as means+SEM. A one-way analysis of variance (ANOVA) was used to compare the differences of relative ghrelin mRNA expression in various tissues using spss 20.0. Duncan's multiple range test was used to assess the differences after checking for data normality and homogeneity of variances. Differences were considered significant at $p<0.05$.

Results

Reindeer ghrelin genomic DNA was 4076 bp in length and consisted of 4 exons and 3 introns (data not shown, GenBank accession KX857495). The alternative splicing of mRNA conformed to the typical GT-AG rule. Figure 1 summarizes the structural characteristics of exon and intron sizes.

The full-length cDNA of ghrelin was cloned from the reindeer stomach total RNA (Fig. 2). Firstly, a single-strand approximately 300 bp cDNA product was detected by RT-PCR in the abomasum tissue. After sequencing ten independent clones, we found that all their sequences were the same. To obtain the full-length cDNA sequence, 5'- and 3'-RACE techniques were carried out with some primer pairs designed according to the above 300 bp partial cDNA sequence. After sequencing ten unattached clones from each of the 5'- and 3'-RACE products, the full-length cDNA was obtained by connection of these sequences and deletion of the overlapping region (Fig. 2).

The reindeer ghrelin cDNA was 539 bp in length and comprised a 46 bp 5' -terminal untranslated region (UTR), a 351 bp open reading frame (ORF) and a 142 bp 3' UTR. The ghrelin ORF (351bp) encodes a prepro-ghrelin of 116 amino acids. The predicted prepro-ghrelin was composed of an N-terminal signal peptide of 23 amino acid residues, a 27 residues mature ghrelin peptide and a 66 residues C-terminal peptide (Fig. 2).

The nucleotide sequence of the ghrelin cDNA has been deposited in GenBank with accession number EU099031.

Using DNAstar Analysis Software (MegAlign ClustelV), the phylogenetic tree analysis showed that the cDNA sequence of reindeer ghrelin exhibited a closer relationship with reported goat, sheep and cattle than pig, human and chicken ghrelin cDNA sequences (Fig. 3). Alignment of the deduced amino acid sequence of grass carp ghrelin with those of other animals (Fig. 4A) revealed that reindeer had a higher sequence homology of ghrelin with goat, sheep, cattle than with other animals, and simultaneously the reindeer mature ghrelin peptide showed high identity with that of goat (100%), sheep (100%), cattle (96.3%) and correspondingly low identity with that of pig (63.0%), human (63.0%) and chicken (38.5%) mature ghrelin peptide (Fig. 4B).

RT-qPCR results showed that the ghrelin mRNA could be found in all tissues examined with significant differences in expression, predominantly in the abomasum (Fig. 5). The expressions in the pancreas, duodenum, testis and oesophagus were remarkably lower than in the abomasum but were higher compared with other tissues, and the expressions were low in the liver, lung, omasum, ileum, colon, seminal vesicle gland, ductus deferens, spleen, thyroid gland, skeletal muscle, heart, epididymis, hypothalamus, bladder and lymphatic node, and were lowest in the jejunum, hypophysis, thymus, kidney, ureter, tongue, adrenal gland, rumen, and reticulum.

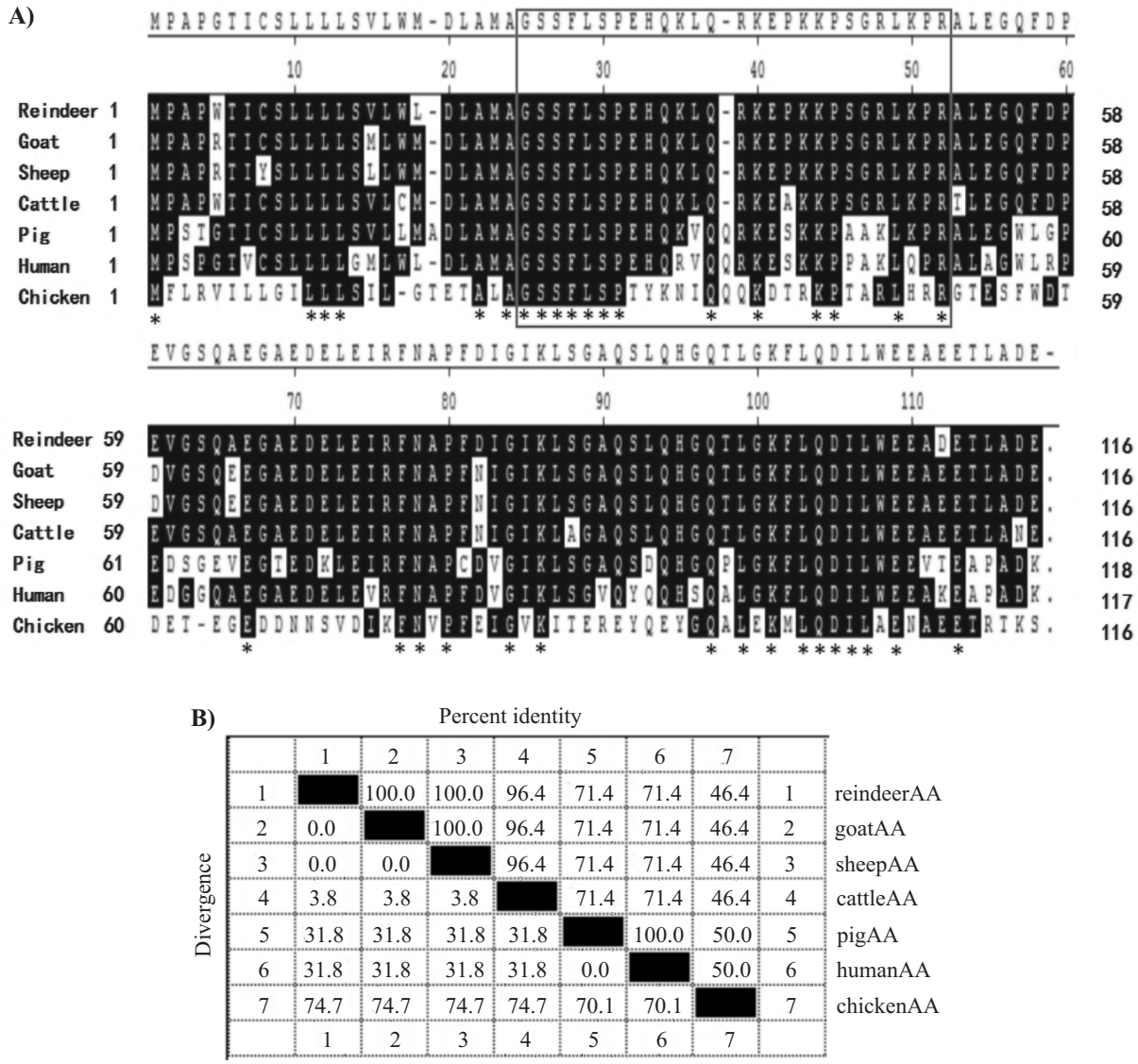

Fig. 4. (A): Comparison of the prepro-ghrelin amino acid sequence between the reindeer and other animals. Asterisk means same amino acid between different animals. The region of the mature ghrelin peptide is red boxed. (B): Sequence distance showing the percent identity of the deduced amino acid sequence of reindeer mature ghrelin with those of other species. GenBank Accession: reindeer: EU099031; goat: BAD34669; sheep: ABC00742; cattle: AAK18612; pig: BAB19048; human: AB029434; chicken: AB075215.

Discussion

Mammalian ghrelin is consistently found in stomach and intestinal tissues, but is also produced in a variety of other tissues including the hypothalamus, pituitary, pancreas, and reproductive tissues (Van et al. 2004). Gastric production is the primary source of ghrelin in the blood (Barreiro and Tena-Sempere 2004). In the present study, we cloned the genomic DNA and cDNA sequence from the reindeer stomach and identified the characteristics of reindeer ghrelin.

The reindeer ghrelin gene shares its highest identity with that of bubaline (Kandasamy et al. 2013) and cattle (Ishida et al. 2009) at both the nucleotide and amino acid levels. The predicted reindeer mature ghrelin was 27 amino acids in length. In general, mammalian ghrelin was chiefly composed of 28 amino acids, but the reindeer ghrelin lacked Gln14 that was present in pig, human and chicken ghrelin. The Gln14 deletion was previously seen in other mammals such as goat, sheep, cattle, house musk and cat (Ida et al. 2007, Kojima et al. 2008, Ishida et al. 2009, Ida et al. 2010). Des-Gln14-ghrelin was known to be a second ligand for GHS-R1a in rat and human (Hosoda et al. 2000), and it was generated by alternative splicing of the ghrelin gene. Similar alternative splicing of the ghrelin gene was seen in ruminants (Ida et al. 2010), and genomic analysis of the intron and exon boundary

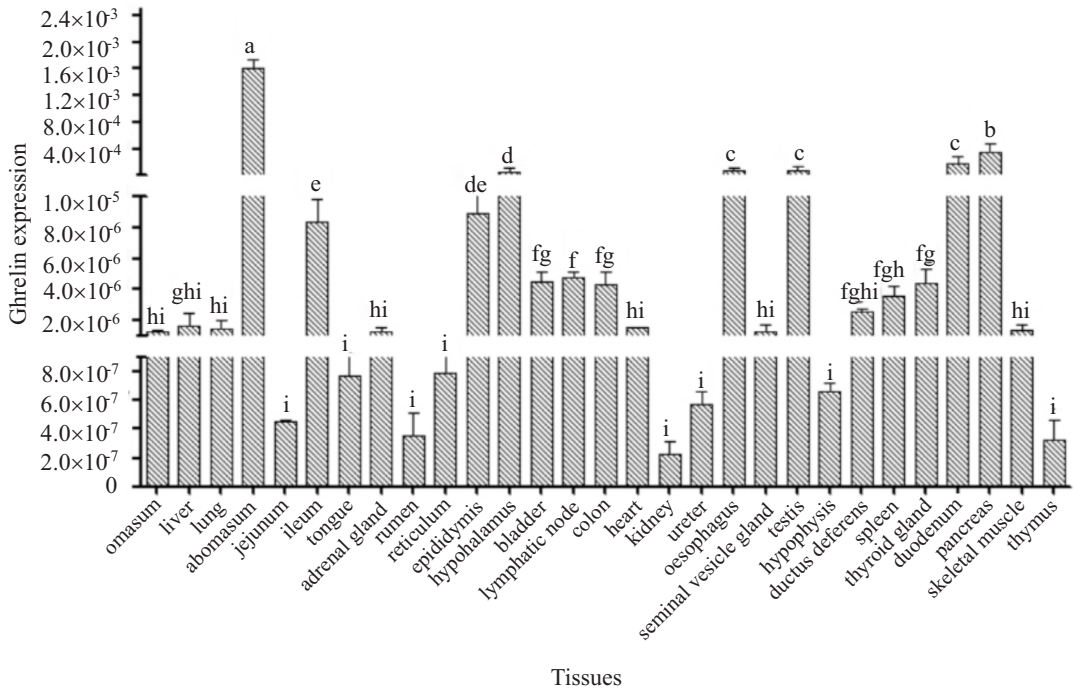

Fig. 5. RT-qPCR analysis of ghrelin mRNA in the different tissues of reindeer. Values are presented as the mean fold change+SEM (n=3). The expression level is normalized against 18S rRNA as an internal standard. Different letters above the bars indicate significant difference at $p<0.05$.

of mature ghrelin revealed that the last nucleotide triplet in the second intron does not code for Gln. These two peptides had not only similar chemical structure but also pharmacologic activity (Rindi et al. 2004).

The reindeer ghrelin cDNA had a closer relationship with other ruminants, especially that of goat and sheep. The results indicated that the ruminants had a species specificity in the expression of ghrelin. Also, the sequence comparisons indicated that the seven amino acids at the N-terminal of reindeer mature ghrelin sequence is completely preserved in other mammals. Among these preserved amino acids, the first, third and fourth amino acids (Gly/Ser/Phe) have a pivotal role in ghrelin functions (Bednarek et al. 2000, Matsumoto et al. 2001). Thus, it can be seen that both nucleotide and amino acid sequence homology of the reindeer ghrelin is close to goat, sheep and cattle ghrelin.

Ghrelin was detected in various reindeer tissues, which was consistent with results found in humans and chickens (Gnanapavan et al. 2002, Kaiya et al. 2002). As previously, ghrelin was found to be mainly expressed in the abomasum of lambs (Wang et al. 2014), Kazak sheep and Xinjiangfine wool sheep by RT-PCR (Huang et al. 2006). We also found that ghrelin was most abundantly expressed in the abomasum. Thus, it can be seen that ghrelin was expressed at high levels in the abomasum of ruminants. Our results suggest that reindeer ghrelin plays a possible role in regulating digestive function. In Lin's experiment (Lin et al. 2011), the RT-qPCR results showed that expressions of ghrelin in pig abomasum and duodenum occured at very high levels, followed by the pancreas. However, the present study found that ghrelin in the pancreas of reindeer was higher than in the duodenum; the difference may be due to differences in the species or ages (Vitari et al. 2012).

Acknowledgements

We thank the Aoluguya District of the Inner Mongolia Municipality in China for supplying all tissues samples. Thanks are also due to the innovative team project of the Inner Mongolia Agricultural University for providing financial assistance (NDPYTD2010-6).

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