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Molecular characterization of the encoding regions and tissue expression analyses for 3 novel buffalo *AKT* genes, *AKT1*, *AKT2*, and *AKT3*

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Abstract: The objective of this study was to obtain the complete coding sequences (CDSs) of 3 buffalo genes (*AKT1*, *AKT2*, and *AKT3*) using reverse-transcriptase polymerase chain reaction and to depict their molecular characterizations and tissue expression patterns in buffalo. The buffalo *AKT1*, *AKT2*, and *AKT3* CDSs were 1443 bp, 1446 bp, and 1440 bp in length and encoded 480, 481, and 479 amino acids, respectively. Nine, 13, and 3 nucleotide differences were found in the CDSs between buffalo and other bovine species. Phylogenetic analyses showed that buffalo AKT1 and AKT2 have close genetic relationships with other species in the family Bovidae, while AKT3 is highly conserved in mammals. Buffalo AKT1, AKT2, and AKT3 all have a pleckstrin homology domain, a Ser/Thr-specific kinase domain, and a C-terminal hydrophobic regulatory domain. The tissue expression profiles were tested by real-time quantitative PCR and revealed that the buffalo *AKT*s were expressed in 10 tissues. Among these tissues, mammary glands showed high expression levels, which indicated that *AKT* genes might be important in the regulation of mammary gland functions in buffalo.

Key words: Buffalo, *AKT* genes, polymorphism, characterization, tissue expression profile

1. Introduction

The serine/threonine protein kinase AKT, also known as protein kinase B (PKB), was identified as the v-akt protooncogene homolog in 1991 (Bellacosa et al., 1991). In mammals, 3 subtypes of AKT exist: AKT1, AKT2, and AKT3. These 3 subtypes are encoded by 3 independent genes. However, the amino acid sequence identity among the 3 subtypes is greater than 80%. AKT proteins contain 3 conserved domains: a pleckstrin homology (PH) domain in the N-terminus, a Ser/Thr-specific kinase domain (catalytic domain), and a C-terminal hydrophobic regulatory domain (Kumar and Madison, 2005).

AKT is a downstream effector of the PI3K signaling pathway (Burgering and Coffer, 1995; Franke et al., 1995). The PI3K-AKT signal pathway plays a crucial role in regulating the balance of glucose metabolism (Hers et al., 2011). Previous studies have shown that AKT1 silenced alone, or simultaneously with AKT2, could significantly reduce ATP levels in a mouse fibroblast cell line (Gottlob et al., 2001; Hahn-Windgassen et al., 2005). Proper development of bovine mammary tissue is one of the important factors affecting milk production traits. The appropriate energy supply is essential for mammary gland development, lactation, and degradation. Even though the specific mechanism by which AKT regulates mammary gland development, lactation, and degradation remains unclear, the expression level of *AKT* was significantly upregulated during late pregnancy and the lactation period in mice (Chodosh et al., 2000).

Although the 3 AKT isoforms have similar structural domains and show a certain degree of consistency in function, some differences in specific functions are known. Through constructing *AKT1*-, *AKT2*-, and *AKT3*-deficient animal models, researchers found that perinatal mortality increased and weight decreased by 20%–30% in *AKT1*-deficient mice (Chen et al., 2001; Yang et al., 2003). In contrast, *AKT2* knockout mice showed normal growth. However, these mice exhibited similar diabetic syndrome symptoms, such as increased fasting blood glucose levels and insulin resistance (Cho et al., 2001; Garofalo et al., 2003). Due to the reduction in the volume and number of brain cells, the *AKT3* gene-deficient mice showed a decrease in brain weight but were able to maintain normal body weight and blood sugar balance (Easton et al., 2005;

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Tschopp et al., 2005). In general, only AKT1 performs the basic AKT functions, such as glucose homeostasis, cell proliferation, differentiation, and early development, while the growth and development of *AKT2*$^{-/-}$*AKT3*$^{-/-}$ mice and even *AKT1*$^{+/-}$*AKT2*$^{-/-}$*AKT3*$^{-/-}$ mice were nearly normal, despite AKT levels being significantly lower in the mutant tissues (Dummler et al., 2006).

Despite differences in the specific functions of AKT isoforms, the 3 subtypes are all potential candidate genes associated with milk production. A recent study showed that the expression levels of *AKT1* and *AKT3* were markedly increased during lactation periods in cattle (Bionaz and Loor, 2011). Adipose tissue atrophy was observed in *AKT2* gene knockout female mice (Garofalo et al., 2003). In addition, AKT could affect the synthesis of breast milk fat and cholesterol by regulating activity of the sterol regulatory element binding protein (SREBP) in humans and mice (Porstmann et al., 2005; Du et al., 2006; Bengoechea-Alonso and Ericsson, 2007; Yecies et al., 2011). In mice mammary glands, AKT regulates the activity of SREBP through 2 possible pathways that both lead to an increase in nuclear SREBP: 1) AKT could promote the coat protein II migration of immature SREBPs from the endoplasmic reticulum to the Golgi and/or 2) AKT could inhibit the phosphorylation of glycogen synthase kinase-3β (Rudolph et al., 2007).

Buffalo is an important domestic animal in subtropical and tropical areas, but the genes related to their lipid metabolism and mammary gland development, lactation, and degradation are unclear. *AKT* genes are important genes that have many biological functions in the mammary gland. To date, *AKT* genes have been reported in humans, mice, cattle, and other animals, but buffalo *AKT* genes have not been reported. In this study, we first obtained the full-length complete coding sequences (CDSs) of buffalo *AKT1*, *AKT2*, and *AKT3* genes using reverse-transcriptase polymerase chain reaction (RT-PCR). Next, we performed a bioinformatics analysis based on the data obtained. Finally, we examined the gene expression patterns in 10 tissues by real-time quantitative PCR (qPCR). This study establishes the primary foundation for understanding the mechanisms of lipid synthesis and mammary gland development, lactation, and degradation in buffalo.

2. Materials and methods

2.1. Animals and sample collection

Ten types of tissue samples, including the abomasum, kidney, heart, liver, brain, lung, mammary gland, pituitary, intestine, and muscle tissues, were collected from 12 local dairy buffalo in peak lactation stage at the age of about 54 months in Yunnan Province, China. The buffalo included 6 Binlangjiang buffalo (river type), 3 Dehong buffalo (swamp type), and 3 Diandongnan buffalo (swamp type). The sampled individuals had no blood relationships. The samples were snap-frozen in liquid nitrogen immediately after the buffalo were slaughtered.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted using the RNAiso Plus kit (TaKaRa, China) according to the manufacturer's instructions. To remove genomic DNA contamination, the total RNA was digested with RNase-free DNase I (TaKaRa). The RNA (3 μg) was reverse-transcribed with an oligo$(dT)_{18}$ primer and M-MLV reverse transcriptase (Invitrogen, USA). The efficiency of reverse transcription was confirmed by 2% agarose gel electrophoresis containing ethidium bromide, and the cDNA concentrations from the different types of tissues were measured using a UV-Vis spectrophotometer (NanoDrop 2000).

2.3. Gene isolation

The *AKT* gene sequences for cattle and sheep were used to design primer pairs to amplify the complete buffalo *AKT* CDSs using Primer Premier 5.0 software. Detailed primer information is described in Table 1.

The cDNAs obtained from the 12 buffalo were used to isolate the buffalo *AKT1*, *AKT2*, and *AKT3* genes using RT-PCR methods. PCR reactions were performed in a final volume of 25 μL containing the following components: 2.5 μL of cDNA (50 ng/μL), 1.25 μL of 10 mM mixed dNTPs (TaKaRa), 12.5 μL of 2X GC buffer I

Table 1. RT-PCR primer pairs for amplifying buffalo *AKT1*, *AKT2*, and *AKT3* genes.

Gene	Primer sequences	Temp.	Product size
AKT1	Forward: 5'-CTGAGAGGCACGAGAGTGAGGT-3'	55 °C	1633 bp
	Reverse: 5'-GGTTCCACTCAAACGCATCCAG-3'		
AKT2	Forward: 5'-ACGCTGCCACCATGAACGAG-3'	55 °C	1695 bp
	Reverse: 5'-CCCGAGTCCTGGTCTGTAACA-3'		
AKT3	Forward: 5'-TCATGAGCGATGTTACCATTGTG-3'	55 °C	1629 bp
	Reverse: 5'-GTCGAGTGTGTCTGCGTATATGTG-3'		

(TaKaRa), 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 0.25 μL of EX Taq DNA polymerase (5 U/μL, TaKaRa), and 7.5 μL of sterile water. The PCR program used to amplify the *AKT* genes was the following: denaturation at 95 °C for 2 min; 34 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min 45 s; a final extension at 72 °C for 10 min; and a 4 °C incubation to terminate the reaction. The PCR products from the buffalo *AKT* cDNAs were then sequenced bidirectionally using the commercial fluorometric method. The PCR primers were also used for sequencing. The sequences from the buffalo *AKT1*, *AKT2*, and *AKT3* genes were deposited in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) (accession numbers: KC138892, KC493631, and JX678290).

2.4. Sequence analysis

Nucleotide sequences were examined and edited using DNASTAR software (DNASTAR, Inc., USA). Sequence alignments were performed using online software from the NCBI. A base composition analysis was performed by employing MEGA version 4.0 (Kumar et al., 2008). The protein-conserved domains and functional sites were analyzed using the Conserved Domain Architecture Retrieval Tool in BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/) and SMART (http://smart.embl-heidelberg.de/). The molecular weights and theoretical isoelectric points (pI) were calculated using Compute pI/Mw (http://us.expasy.org/tools/pi_tool.html) (Wilkins et al., 1999). Signal peptides were predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). PSort II (http://psort.hgc.jp/) was used to predict protein sorting signals and intracellular localization. A protein hydrophobic analysis was conducted using the online software ProtScale (http://web.expasy.org/protscale/). Secondary structures of deduced amino acid sequences were predicted by SOPMA (http://npsa-pbil.ibcp.fr/) (Geourjon and Deleage, 1995). Transmembrane helices in proteins were also predicted by TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Sonnhammer et al., 1998). The position and number of single-nucleotide polymorphisms and the corresponding haplotypes were exported with MEGA version 4.0 (Kumar et al., 2008). NetPhos2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was employed to predict protein phosphorylation sites.

2.5. Phylogenetic analysis

Neighbor-joining phylogenetic trees were constructed based on the AKT1, AKT2, and AKT3 amino acid sequences by employing CLUSTAL X 2.0 and MEGA version 4.0 (Kumar et al., 2008). Statistical significance of the groups within phylogenetic trees was evaluated using the bootstrap method with 10,000 replications.

2.6. Expression profile analysis by qPCR

qPCR was performed using an ABI 7500 Fast System (Applied Biosystems, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Considering that the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) is stably expressed in most tissues of the body, we selected the housekeeping gene *GAPDH* as the endogenous control. Relative transcript quantification was performed using standard curves generated for the *AKT1*, *AKT2*, *AKT3*, and *GAPDH* genes from a 5-fold serial dilution of cDNA. In this experiment, the efficiencies of the *AKT1*, *AKT2*, *AKT3*, and *GAPDH* primers were all in the ideal range from 90% to 105% in each sample. The 20-μL reaction included 2 μL of cDNA, 10 μL of Power SYBR Green PCR Master Mix, 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, and 7 μL of sterile water. The amplification conditions used the default settings, which were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. A melting curve stage of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, and 60 °C for 15 s followed the amplification. Optical data were collected at the end of each extension step, and the relative expression of PCR products was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Detailed qPCR primer information is listed in Table 2.

Table 2. qPCR primers for buffalo *AKT1*, *AKT2*, *AKT3*, and *GAPDH*.

Gene	Primer sequences	Temp.	Product size
AKT1	Forward: 5'-TACCTGCACTCGGAGAAGAAC-3'	60 °C	147 bp
	Reverse: 5'-CCCGCAGAAAGTCTTCATGG-3'		
AKT2	Forward: 5'-TCAGGTCACGTTTGAGGTCG-3'	60 °C	140 bp
	Reverse: 5'-AGGAAAACTGGGGGAAGTGC-3'		
AKT3	Forward: 5'-ACCGCACACGTTTCTATGGT-3'	60 °C	175 bp
	Reverse: 5'-TTCATGGTGGCTGCATCAGT-3'		
GAPDH	Forward: 5'-ATCAAGAAGGTGGTGAAGCAG-3'	60 °C	111 bp
	Reverse: 5'-GGTAGAAGAGTGAGTGTCGCTG-3'		

3. Results

3.1. RT-PCR results for *AKT1*, *AKT2*, and *AKT3*

The *AKT1*, *AKT2*, and *AKT3* PCR products amplified from different buffalo tissue cDNAs were 1633 bp, 1695 bp, and 1629 bp, respectively (Figure 1).

3.2. Nucleotide sequence characterization

Sequence analysis revealed that the *AKT1*, *AKT2*, and *AKT3* gene sequences obtained in this study were not homologous to any of the known buffalo genes. The CDSs for the *AKT1*, *AKT2*, and *AKT3* genes were 1443 bp, 1446 bp, and 1440 bp, which encoded 480, 481, and 479 amino acids, respectively. The *AKT1* CDS had an overall base composition of 21.87% A, 31.96% G, 16.29% T, and 29.88% C. The *AKT2* CDS had a composition of 21.85% A, 29.74% G, 18.46% T, and 29.95% C. The *AKT3* CDS had a composition of 33.13% A, 23.26% G, 26.32% T, and 17.29% C. The complete CDSs of the *AKT* genes and their deduced amino acids are presented in Figures 2–4.

Further sequence alignment among the family Bovidae revealed that the buffalo *AKT1* CDS in this study had 98%, 97%, and 97% identity with the cattle, goat, and sheep *AKT1* genes, respectively. The *AKT2* CDS had 98% and 98% identity with the cattle and yak *AKT2* genes, respectively. The *AKT3* CDS had 99% and 99% identity with the cattle and sheep *AKT3* genes, respectively. No polymorphisms were found in the CDSs of the 3 *AKT* genes among the buffalo. However, 9 nucleotide differences in the *AKT1* gene were found between buffalo and the other bovine species. Among the differences, only the nucleotide change at c.376 (G>A) was nonsynonymous, and this difference changes a glycine (G) at p.126 in other bovine species to a serine (S) in the buffalo protein. The c.96C, c.153T, c.222T, c.376A, c.393T, c.771T, c.844T, c.1020T, and c.1194T differences in the *AKT1* CDS were unique to buffalo in the present study. Differences in 13 nucleotides in the *AKT2* gene were found between buffalo

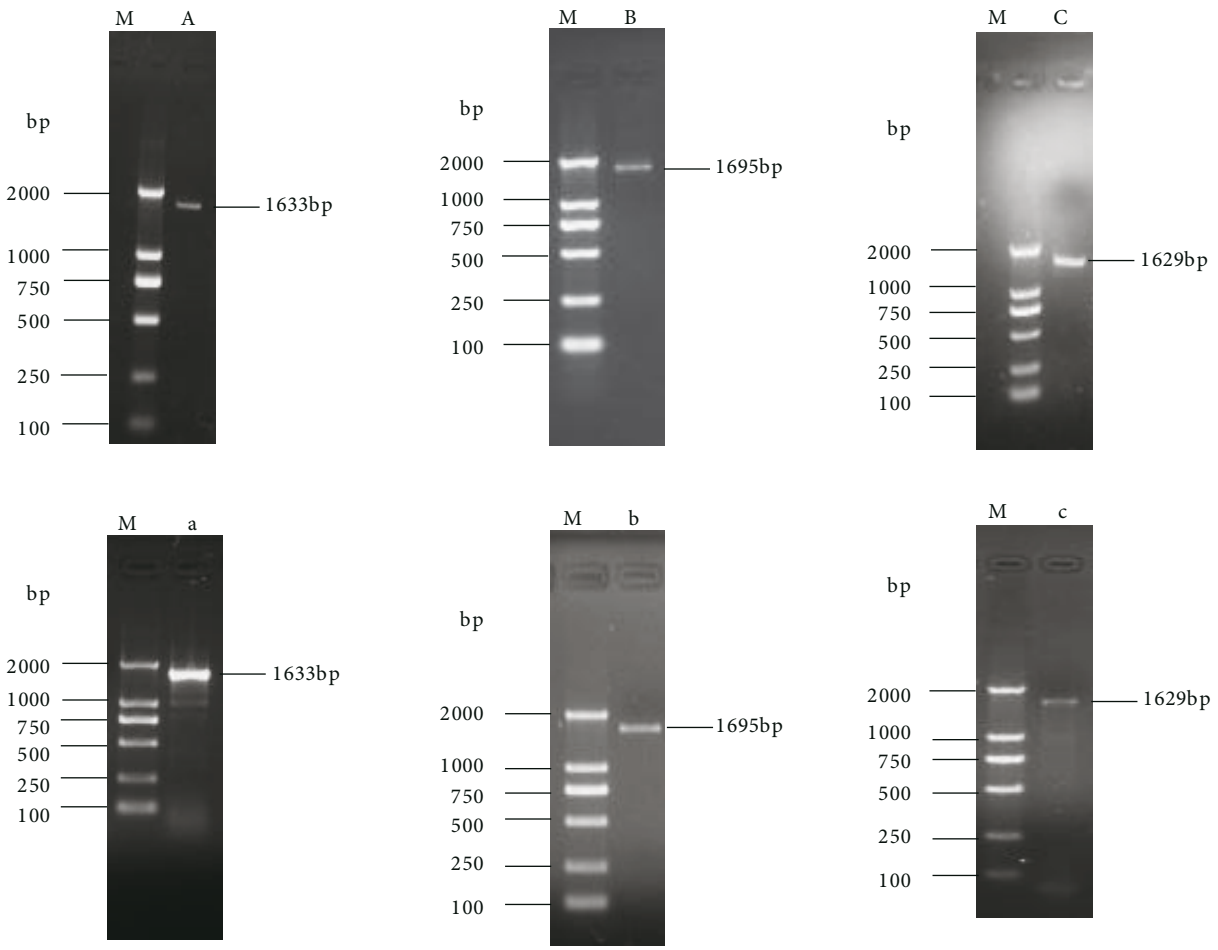

Figure 1. PCR results for the buffalo *AKT* genes. M, DL2000 DNA Marker; A, PCR product for the river buffalo *AKT1* gene; a, PCR product for the swamp buffalo *AKT1* gene; B, PCR product for the river buffalo *AKT2* gene; b, PCR product for the swamp buffalo *AKT2* gene; C, PCR product for the river buffalo *AKT3* gene; c, PCR product for the swamp buffalo *AKT3* gene.

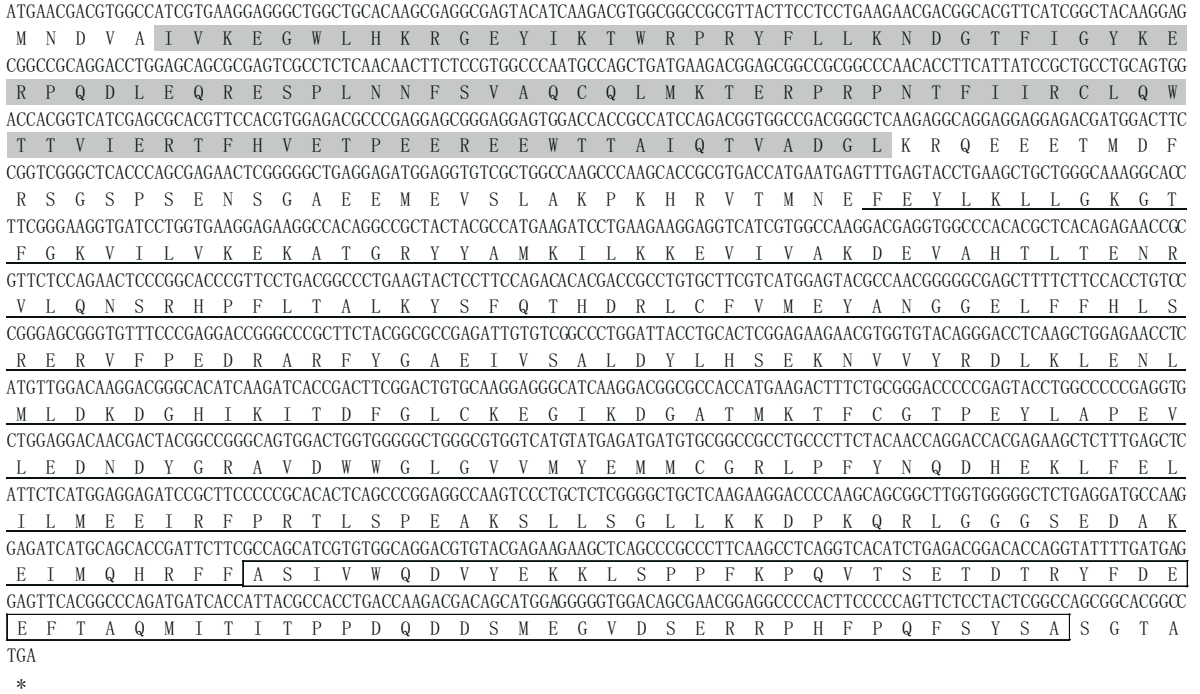

Figure 2. The complete CDS of the buffalo *AKT1* and its deduced amino acids. An asterisk denotes the stop codon. The PH domain (6–110AA) is shaded. The Ser/Thr-specific kinase domain (150–408AA) is underlined, and the C-terminal hydrophobic regulatory domain (409–476AA) is boxed.

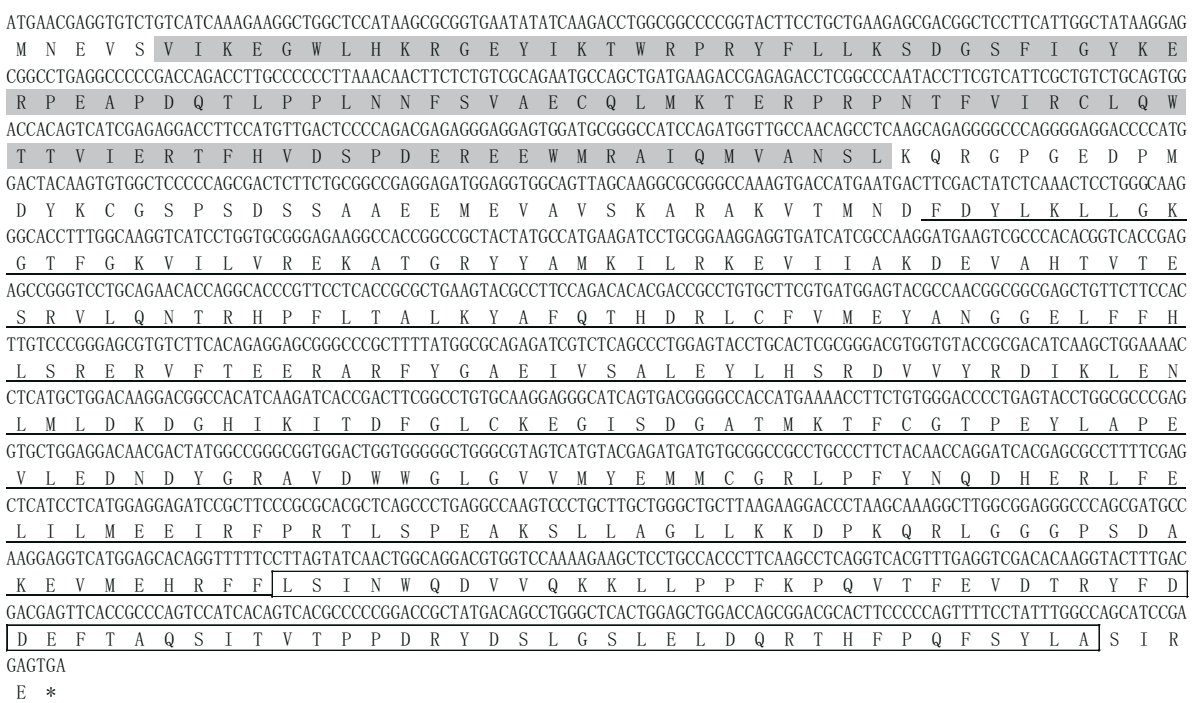

Figure 3. The complete CDS of the buffalo *AKT2* and its deduced amino acids. An asterisk denotes the stop codon. The PH domain (6–110AA) is shaded. The Ser/Thr-specific kinase domain (152–409AA) is underlined, and the C-terminal hydrophobic regulatory domain (410–477AA) is boxed.

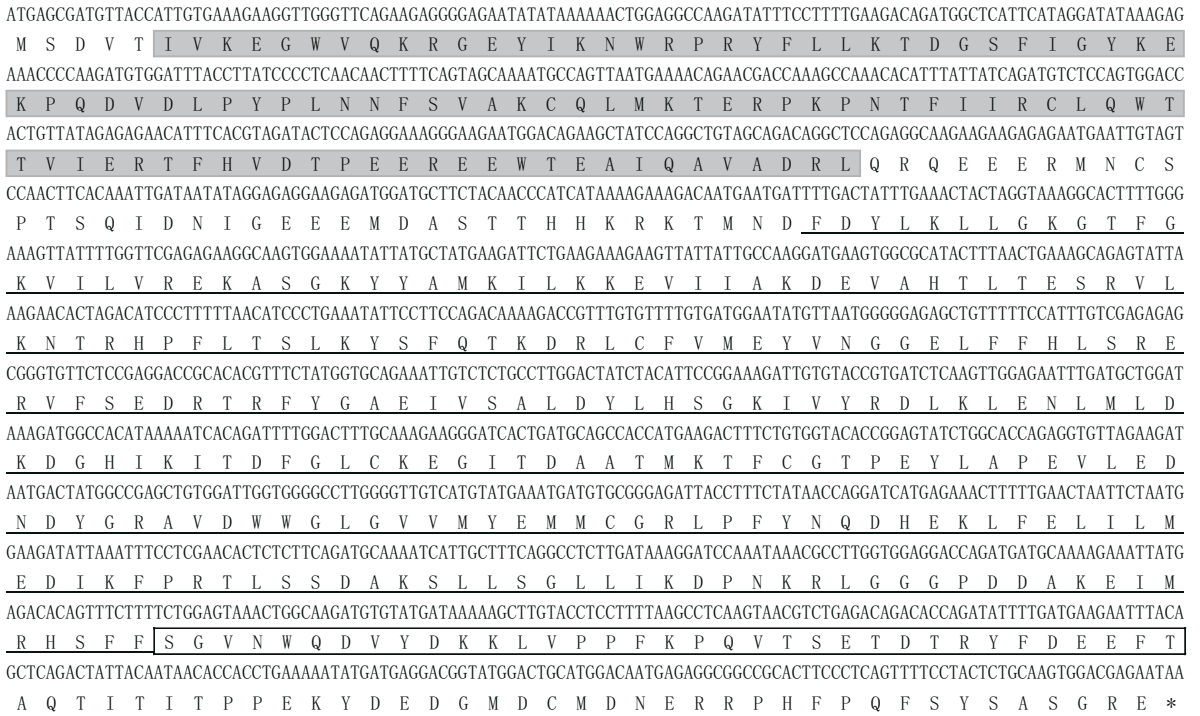

Figure 4. The complete CDS of the buffalo *AKT3* and its deduced amino acids. An asterisk denotes the stop codon. The PH domain (6–109AA) is shaded. The Ser/Thr-specific kinase domain (148–405AA) is underlined, and the C-terminal hydrophobic regulatory domain (406–475AA) is boxed.

and the other bovine species. The nucleotide changes c.1295C>T and c.1427C>T were nonsynonymous, and these differences lead to p.432 S>F and p.476 S>L amino acid changes from the bovine species sequences. The c.145T, c.438A, c.522C, c.924A, c.1014A, c.1074T, c.1179T, c.1197T, c.1224T, c.1257A, c.1295T, c.1419T, and c.1427T differences in the *AKT2* gene were unique to buffalo. In the *AKT3* gene, only 3 unique nucleotide differences were found between buffalo and the other bovine species. The nucleotide changes c.324A>G, c.576C>T, and c.930A>G were synonymous. The sequence variations between the haplotypes of the *AKT* genes in buffalo and other bovine species are shown in Figures 5–7.

3.3. Protein sequence analyses and functional site prediction

The pIs of buffalo AKT1, AKT2, and AKT3 were 5.83, 6.18, and 5.83, respectively. Their respective molecular weights were 55.77 kDa, 55.74 kDa, and 55.77 kDa. Sequence analysis indicated that buffalo AKT is a hydrophilic protein without a transmembrane region. In addition, AKT had no N-terminal signal peptide sequence, which indicated that AKTs are nonsecretory proteins. The analysis of cytoplasmic/nuclear localization suggested that AKT1, AKT2, and AKT3 function in the cytoplasm with high reliability (89.0%, 94.1%, and 89.0%, respectively).

Three conservative functional domains (a PH domain, Ser/Thr-specific kinase domain, and C-terminal hydrophobic regulatory domain) were found in the buffalo AKT proteins and displayed in Figures 2, 8, and 9. A prediction of secondary structures showed that buffalo AKT1 contains 162AA alpha helices, 84AA extended strands, 31AA beta turns, and 203AA random coils; AKT2 contains 179AA alpha helices, 82AA extended strands, 28AA beta turns, and 192AA random coils; and AKT3 contains 174AA alpha helices, 73AA extended strands, 29AA beta turns, and 203AA random coils (Figure 10). A prediction of protein phosphorylation sites showed that buffalo AKT1, AKT2, and AKT3 contained 25, 23, and 26 phosphorylation sites, respectively. Detailed information is shown in Table 3.

3.4. Phylogenetic analysis

To evaluate the evolutionary relationship of buffalo AKTs with other species, we constructed phylogenetic trees based on AKT amino acid sequences using neighbor-joining methods (Figure 11). The phylogenetic trees showed that buffalo AKT1 and AKT2 have close genetic relationships with other Bovidae species. Buffalo AKT3 was very conserved and clustered into one group with cattle, pigs, humans, and other large mammals.

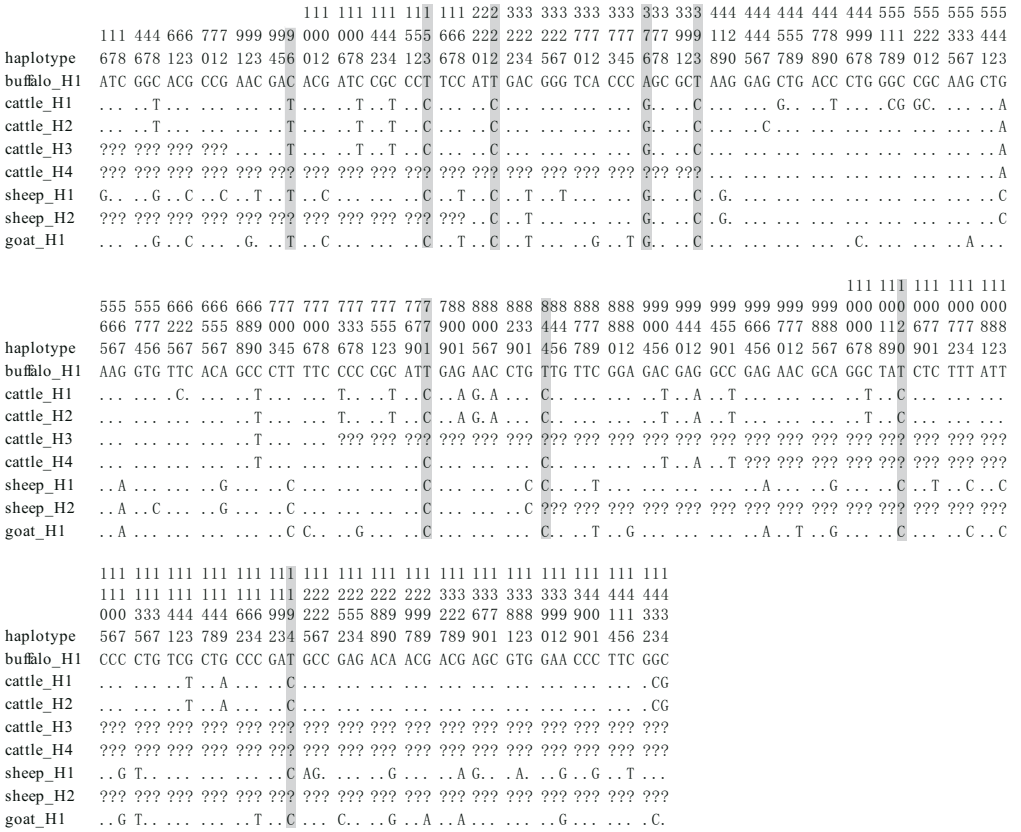

Figure 5. Alignment of the coding region of the *AKT1* gene in the family Bovidae. Numbering is scored relative to the first nucleotide in the coding sequence of buffalo *AKT1*. Dots (·) denote similarity with the buffalo reference sequence. Missing nucleotides in the sequence are demonstrated by question marks. The variable nucleotide sites between buffalo and other bovine species are shaded (the same as in Figures 6–7). The sequences cited are NM_173986 (cattle), AY781100 (cattle), EE892279 (cattle), CK832550 (cattle), EE217486 (cattle), NM_001161857 (sheep), GO717451 (sheep), GO774958 (sheep), and HM130679 (goat).

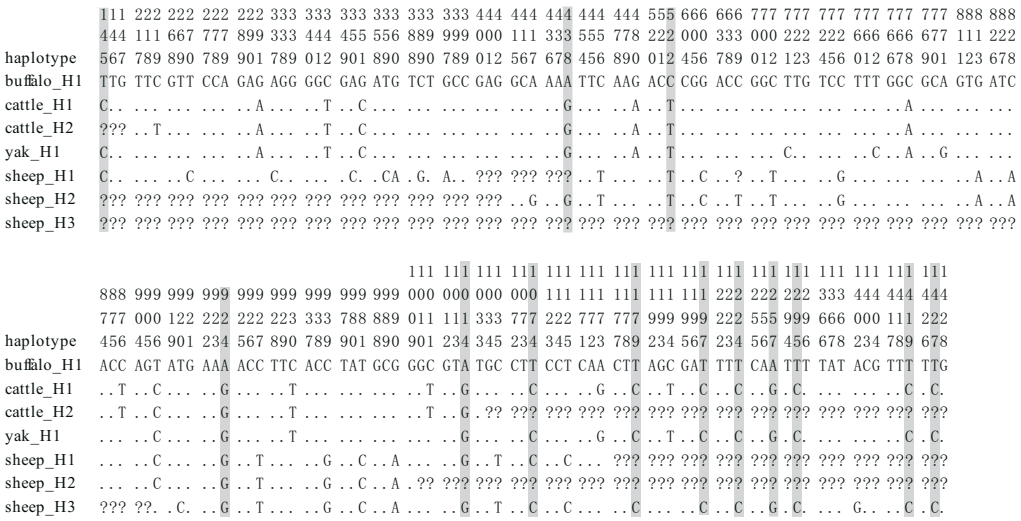

Figure 6. Alignment of the coding region of the *AKT2* gene in the family Bovidae. The cited sequences are NM_001206146 (cattle), NW_001493618 (cattle), NC_007316 (cattle), DV931534 (cattle), DT809346 (cattle), CK960221 (cattle), DT809336 (cattle), EH180020 (cattle), CK848210 (cattle), CK774813 (cattle), NW_004080177 (sheep), GO691333 (sheep), EE805331 (sheep), DY492136 (sheep), EE805697 (sheep), EE819603 (sheep), GO736338 (sheep), EE818361 (sheep), EE812777 (sheep), and JH880628 (yak).

```
                                                                   111 111 111 111 111
              222 222 333 444 555 555 555 666 666 777 999 000 111 222 333 444
              444 888 222 222 566 777 777 000 666 999 223 222 333 788 555 001
haplotype     456 678 234 123 901 123 456 456 123 012 890 123 567 901 456 890
buffalo_H1    GTT GAA AGG AAA AAG GCG CAT AAC TTG TCC CCG TGC GGC ACG TAT CAG
cattle_H1     ... ... ..A ... ... ... ..C ... ..A ... ..A ... ... ..A ... ...
cattle_H2     ... T.. ..A ... ... ... ..C ... ..A ... ..A ... ... ..A ... ...
cattle_H3     ... ... ..A ... C.. ... ..C ... ??? ??? ??? ??? ??? ??? ??? ???
indicus_H1    ??? ??? ??? ??? ... ... ..C ... ..A ... ..A ..? ??? ??? ??? ???
yak_H1        ... T.. ..A ... ... ... ..C ..T ..A ... ..A ... ... ... ... ..A
sheep_H1      ..C ... ..A ..G ... ..A ..C ... ... ..T ..A ..T ..G ..A ... ..A
sheep_H2      ..C ... ..A ..G ... ..A ..C ... ... ..T ..A ..T ..G ..A .G. ???
sheep_H3      ..C T.. ..A ..G ... ..A ..C ... ... ..T ..A ..T ..G ..A ... ..A
```

Figure 7. Alignment of the coding region of the *AKT3* gene in the family Bovidae. The cited sequences are NM_001191309 (cattle), NC_007314 (cattle), NW_001493406 (cattle), AC_000173 (cattle), EH372879 (cattle), XM_004013655 (sheep), XM_004013656 (sheep), NW_004080175 (sheep), AM027388 (Indian cattle: *Bos indicus*), and JH881275 (yak).

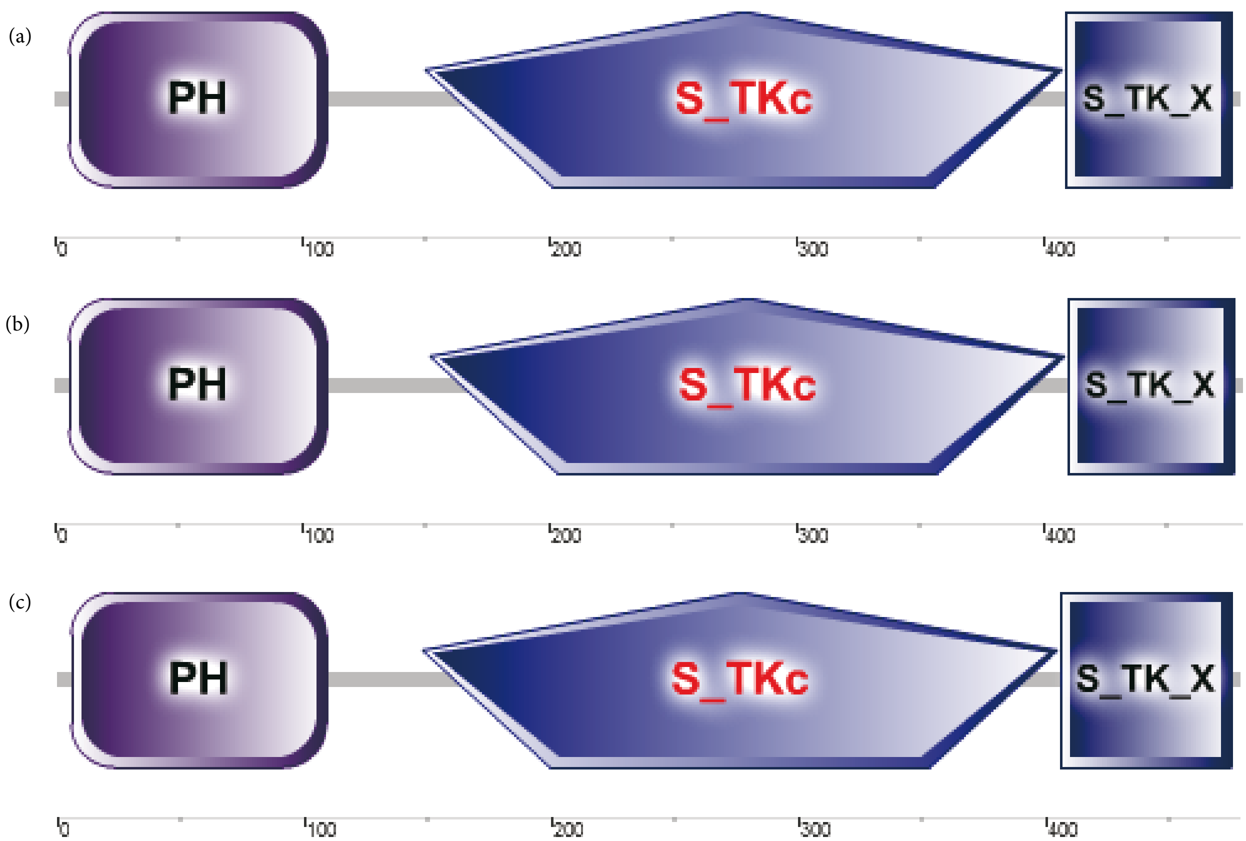

Figure 8. Predicted functional domains of buffalo AKT1 (a), AKT2 (b), and AKT3 (c) by SMART. PH: pleckstrin homology domain. S_TKc: Ser/Thr-specific kinase domain. S_TK_X: C-terminal hydrophobic regulatory domain.

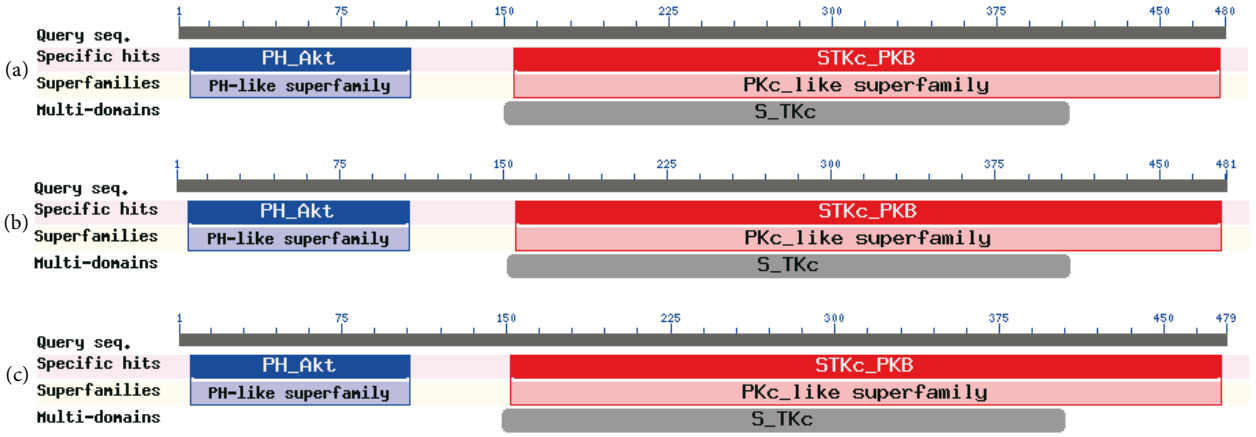

Figure 9. The putative conserved domains in the proteins encoded by water buffalo AKT1 (a), AKT2 (b), and AKT3 (c).

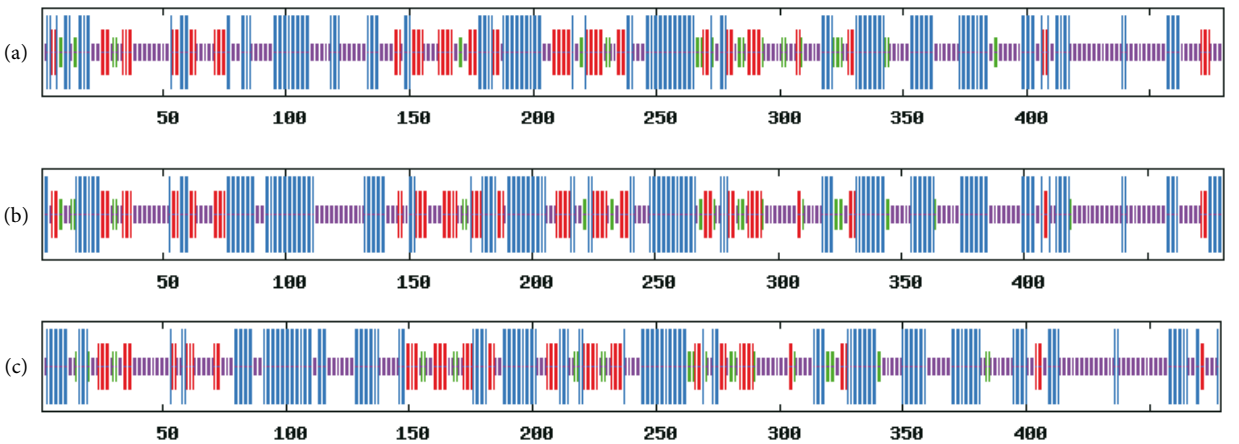

Figure 10. Predicted secondary structures of the buffalo AKT1 (a), AKT2 (b), and AKT3 (c) proteins by SOPMA. Alpha helices, extended strands, beta turns, and random coils are indicated with the longest, second longest, third longest, and shortest vertical lines, respectively.

3.5. Tissue expression profile analysis

To examine the differential distributions of the *AKT*s in buffalo tissues, we evaluated their relative mRNA expression levels by qPCR (Figure 12). The *AKT* genes were widely expressed in the tissues examined with different expression patterns. *AKT1* was highly expressed in the mammary gland; moderately expressed in the abomasum and intestine; weakly expressed in the pituitary, brain, heart, and kidney; and minimally expressed in the muscles and liver. *AKT2* was also highly expressed in the mammary gland; moderately expressed in the abomasum, intestine, and pituitary; weakly expressed in the brain, lung, kidney, and liver; and minimally expressed in the heart and muscles. For *AKT3*, the highest expression level was in the intestine, followed by the mammary gland, abomasum, pituitary, kidney, lung, heart, and brain; the lowest levels were in the muscles and liver.

4. Discussion

In this study, the full-length CDSs of the *AKT1*, *AKT2*, and *AKT3* genes were isolated from buffalo cDNAs and the CDSs of these 3 genes and their encoding proteins were characterized. The *AKT* genes have been reported to be involved in mammary gland development, lactation, degradation, and lipid synthesis (Chodosh et al., 2000; Schwertfeger et al., 2001; Porstmann et al., 2005; Du et al., 2006; Bengoechea-Alonso and Ericsson, 2007; Rudolph et al., 2007; Bionaz and Loor, 2011; Yecies et al., 2011). Thus, this study provides a molecular basis for unfolding the genetic variation characteristics of the *AKT* genes and the

Table 3. Predicted phosphorylated sites in the buffalo AKT proteins.

Gene	Phosphorylated amino acids	Site	Score	Site	Score	Site	Score	Site	Score
AKT1	Serine	50	0.988	126	0.952	396	0.526	457	0.981
		122	0.930	129	0.901	422	0.915	463	0.992
		124	0.997	373	0.995	431	0.996	475	0.834
	Threonine	21	0.898	92	0.930	146	0.610	371	0.514
		65	0.614	105	0.542	219	0.869		
	Tyrosine	152	0.830	263	0.625	326	0.923	437	0.704
		229	0.521	315	0.506	417	0.710		
AKT2	Serine	92	0.995	128	0.830	131	0.952	374	0.995
		126	0.997	130	0.879	141	0.965	458	0.990
	Threonine	21	0.898	65	0.614	221	0.916	372	0.514
		48	0.511	72	0.658	248	0.873		
	Tyrosine	38	0.588	231	0.521	316	0.506	438	0.571
		154	0.756	273	0.746	327	0.923	456	0.951
AKT3	Serine	123	0.992	244	0.997	403	0.904	476	0.914
		136	0.569	370	0.592	428	0.996		
		170	0.840	375	0.837	474	0.511		
	Threonine	64	0.652	138	0.631	217	0.919		
		91	0.969	144	0.744	443	0.637		
	Tyrosine	18	0.575	173	0.635	312	0.506	452	0.936
		38	0.801	227	0.760	323	0.923		
		150	0.756	261	0.868	434	0.704		

primary foundation for understanding the mechanisms of mammary gland development, lactation, degradation, and lipid synthesis in buffalo.

A significantly higher synonymous than nonsynonymous rate in 3 *AKT* genes was found in bovine species. This result indicated the functional conservation of *AKT*s within the family Bovidae. Nine, 13, and 3 variable sites were found in the *AKT1*, *AKT2*, and *AKT3* genes, respectively, between buffalo and other bovine species. However, only 3 of these variable sites led to nonsynonymous substitutions between bovine species (c.376G>A in *AKT1* and c.1295C>T and c.1427C>T in *AKT2*). The deduced amino acids shared the same properties as corresponding amino acids in other bovine species (p.126 G>S, p.432 S>F, and p.476 S>L), which indicates functional conservation of the AKTs within the family Bovidae.

Phylogenetic analysis revealed that buffalo AKT1 and AKT2 have close genetic relationships with other Bovidae species. This result implied that buffalo AKT1 and AKT2 had a minor functional divergence from other Bovidae species. Therefore, the study of buffalo AKT1 and AKT2 could be used as a reference for understanding the possible functions of AKT1 and AKT2 in other Bovidae species. Buffalo AKT3 clustered into one group with cattle, pigs, humans, and other large mammals, which indicates that AKT3 is highly conserved among mammals compared to AKT1 and AKT2. This conservation suggests that the function of AKT3 might be very important in mammals, although few related features of AKT3 have been reported.

Bioinformatics analyses suggested that AKTs are hydrophilic proteins located in the cytoplasm, with no N-terminal signal peptide sequence or transmembrane region. These results indicated that AKT mainly functions in

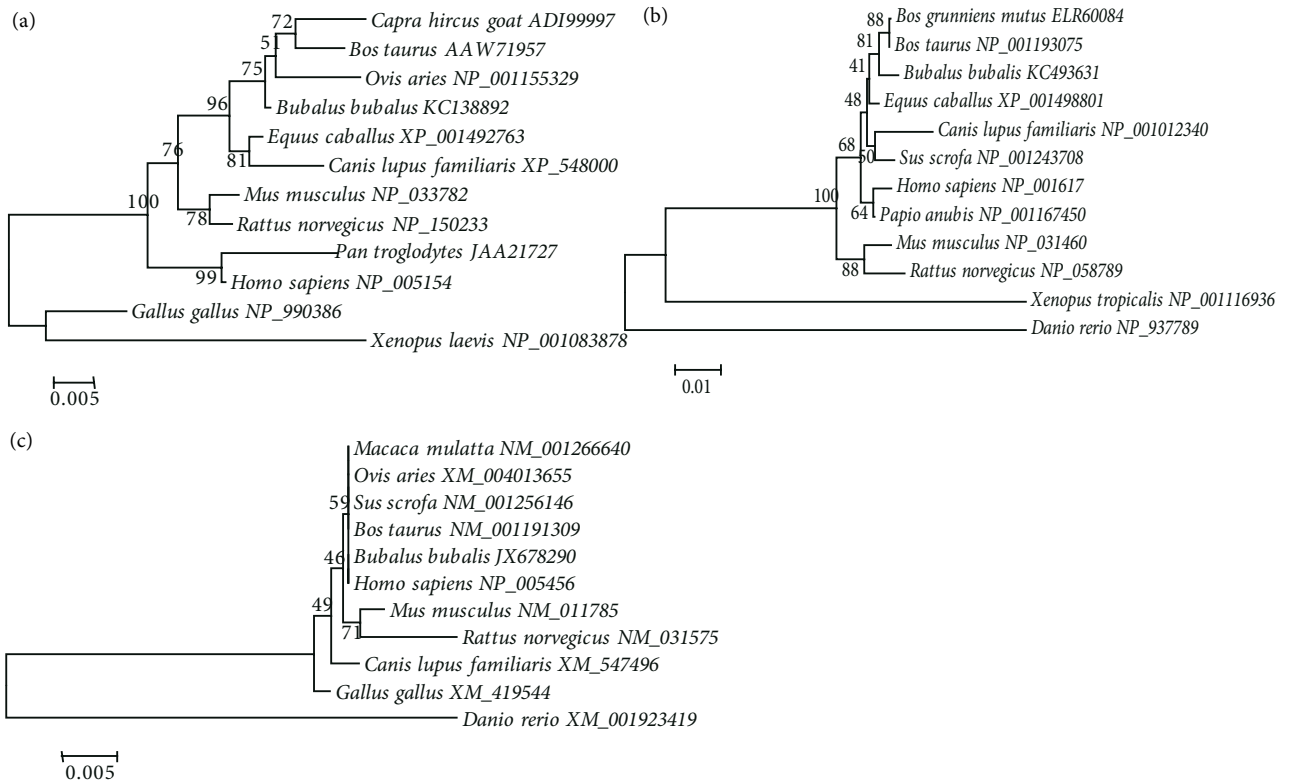

Figure 11. Phylogenetic trees based on the sequences of AKT1 (a), AKT2 (b), and AKT3 (c) between buffalo and other species. The trees were constructed using the neighbor-joining method; the numbers on the branches represent bootstrap values for 10,000 replications.

the cytoplasm. In addition, 3 conserved functional domains (a PH domain, a Ser/Thr-specific kinase domain, and a C-terminal hydrophobic regulatory domain) were found in the 3 buffalo AKT proteins. This result was consistent with previous research in humans (Kumar and Madison, 2005). The PH domain plays a critical role in activating AKT. Through an interaction between the PH domain and PIP3, AKT is recruited to the plasma membrane (Lasserre et al., 2008). Once activated, AKT can dissociate from the plasma membrane and interact with other downstream factors by the Ser/Thr-specific kinase domain (Lasserre et al., 2008). Thus, we can infer that the buffalo AKTs perform functions similar to those of other species.

Tissue expression profile analysis showed that the *AKT1*, *AKT2*, and *AKT3* genes had different expression patterns in buffalo. The 3 *AKT* genes were widely expressed in the tissues examined, but the genes were expressed at different levels. *AKT1* and *AKT2* had similar expression patterns. The 2 genes shared high expression in the mammary gland. One major difference was that *AKT1* had low expression in the heart and minimal expression in the muscles and liver, whereas *AKT2* had low expression in the liver and minimal expression in the heart and muscles. Compared with *AKT1* and *AKT2*, *AKT3* showed high expression not only in the mammary gland but also in the abomasum, and especially very high expression in the intestine, which indicates that *AKT3* may be associated with the gastrointestinal function of dairy buffalo due to more energy and nutritional requirements in lactating stage**.** Features of tissue expression are always closely related to the function of the gene. A previous study in humans suggested that the primary function of *AKT* genes is not insulin signaling, because of their low expression in the muscles and liver (Liu et al., 2006). Similar expression levels in buffalo muscle and liver tissues suggested that the principal function of AKT may also not be involved in regulating insulin signaling. Furthermore, the high expression of *AKT* in mammary glands implied that the 3 genes might be important in regulating mammary gland functions in buffalo. A previous study found that *AKT* expression was remarkably downregulated in the early stages of breast degradation and that the activation of AKT could effectively delay the arrival of breast degradation (Radisky and Hartmann, 2009). In addition, accumulating evidence has confirmed that AKT could affect the synthesis of breast milk fat and cholesterol by regulating the activity of SREBP (Porstmann et al., 2005; Du et al., 2006; Bengoechea-Alonso and Ericsson, 2007; Yecies et

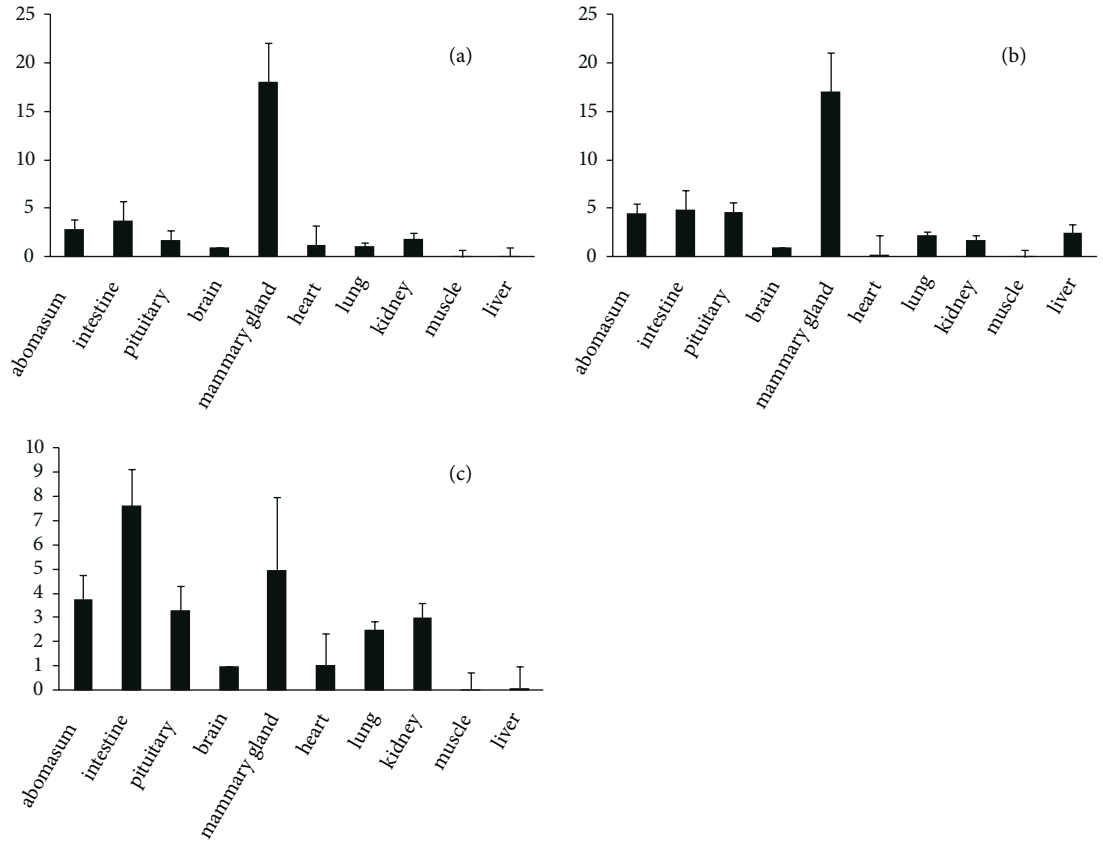

Figure 12. Tissue expression profiles of buffalo *AKT1* (a), *AKT2* (b), and *AKT3* (c). The horizontal axis and vertical axis indicate different tissues and the $2^{-\Delta\Delta CT}$ value (mean ± SE), respectively. Each sample was repeated 3 times, and the brain was set as the reference tissue.

al., 2011). We speculate that AKT serves analogous roles in water buffalo mammary glands. In future studies, the specific function of AKT in the mammary gland and the relationships between *AKT* and milk production traits in buffalo should be investigated.

In summary, we isolated water buffalo *AKT* genes and performed the necessary bioinformatics analysis and tissue expression profile analysis. This study provides a valuable foundation for further insights into the water buffalo *AKT* genes.

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