Cloning and sequence analysis of the coding sequence of *β-actin* cDNA from the Chinese alligator and suitable internal reference primers from the *β-actin* gene

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ABSTRACT. *β-Actin* is an essential component of the cytoskeleton and is stably expressed in various tissues of animals, thus, it is commonly used as an internal reference for gene expression studies. In this study, a 1731-bp fragment of *β-actin* cDNA from *Alligator sinensis* was obtained using the homology cloning technique. Sequence analysis showed that this fragment contained the complete coding sequence of the *β-actin* gene (1128 bp), encoding 375 amino acids. The amino acid sequence of *β-actin* is highly conserved and its nucleotide sequence is slightly variable. Multiple alignment analyses showed that the nucleotide sequence of the *β-actin* gene from *A. sinensis* is very similar to sequences from birds, with 94-95% identity. Ten pairs of primers with different product sizes and different annealing temperatures were screened by PCR amplification, agarose gel electrophoresis, and DNA sequencing, and could be used as internal reference primers in gene expression studies. This study expands our knowledge of *β-actin* gene

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phylogenetic evolution and provides a basis for quantitative gene expression studies in *A. sinensis*.

Key words: *Alligator sinensis*; β-actin; cDNA cloning; Sequence analysis; Internal reference

INTRODUCTION

Actin is the most abundant protein in most eukaryotic cells. It is an essential component of the cytoskeleton, with critical roles in a wide range of cellular processes, including cell migration, cell division, and the regulation of gene expression (Sehring et al., 2007; Bunnell et al., 2011). Vertebrates express three main actin isoforms, including three α-isoforms of skeletal, cardiac, and smooth muscles, and the β- and γ-isoforms expressed in non-muscle and muscle cells (Rubenstein 1990; Sehring et al., 2007; Bunnell et al., 2011). Due to its high and invariable expression in various tissues of animals, *β-actin* is widely used as an internal reference for gene expression studies (Lee et al., 2009; Kim et al., 2011; Wu et al., 2011; Perez-Perez et al., 2012). To date, although *β-actin* cDNA from more than 30 species of vertebrates has been released in GenBank (until May 31, 2014 at NCBI), only two are from reptiles. No complete coding sequence of *β-actin* cDNA from crocodilians has been reported.

Alligator sinensis, belonging to Crocodilia Alligatoridae, is an endemic species in China, and was listed by the Chinese government as a first level state protected species in 1972 (Yan et al., 2005). Nature reserves and artificial farms of *A. sinensis* were set up in Anhui and Zhejiang Province. In order to expand our knowledge of actin phylogenetic evolution and find a normalization gene for further studies of functional genes, the complete coding sequence (CDS) of *β-actin* cDNA of *A. sinensis* was obtained using the homology cloning technique in this study.

MATERIAL AND METHODS

Extraction of total RNA

Two adult alligators, more than 10 years old, were collected in Xuancheng Alligator Culturing Centre of Anhui Province in late November 2011 and late February 2012. After sacrifice, their liver, intestine, stomach, and pancreas tissues were removed and immediately placed in an RNA-locker (Sangon Biotec, Shanghai, China) and stored at -80°C. Total RNA was extracted from liver, intestine, stomach, and pancreas tissues using Total RNA Extractor (Sangon Biotec). After a sufficient amount of tissue was ground quickly in liquid nitrogen, total RNA was extracted according to the manufacturer protocol and maintained at -80°C until required.

Development and synthesis of polymerase chain reaction (PCR) primers

Complete *β-actin* cDNA sequences of fishes, amphibians, reptiles, birds, and mammals were obtained from GenBank and the species names and accession numbers are shown in Table 1. The conserved regions were determined after comparing the above-mentioned sequences using the Clustal X software (Larkin et al, 2007). Two pairs of primers, having an overlap, with degenerate bases were designed using Primer Premier 5.0 and they were both located in the CDS of the *β-actin*

cDNA. Primer pair 1, 1F: 5'-GCCCATCTAYGAAGGYTA-3' 1R: 5'-RTCCAGACRGAGTAYTTRCG-3'; Primer pair 2, 2F: 5'-YTKAAYCCCAAAGCCAACAG-3', 2R: 5'-ATACCRCARGACTCCATACC-3'.

After PCR amplification using primer pairs 1 and 2 and the cDNA reversely transcribed from pancreas and liver RNA, anticipated *β-actin* cDNA fragments were obtained and sequenced. The obtained partial sequences were run through BLAST on NCBI and were identified as partial cDNA sequences of *β-actin*. The two fragments were spliced into one longer fragment. On the basis of this longer fragment and the BLAST results of non-CDS areas of *β-actin* from different species, we tried to design primer pairs with one located in the obtained sequence region and the other located upstream or downstream of this region. Finally, four pairs of primers, one in the CDS region and the others in the 5'- and 3'-untranslated regions of the *β-actin* sequence, were successfully generated to amplify the anticipated fragments. The details of the four primer pairs are as follows: Primer pair 3, 3F: 5'-GACCGCGTTACTCCCACAGC-3', 3R: 5'-CACCAGAGTCCATCACAATACCAGT-3'. Primer pair 4, 4F: 5'-TGATATTGCTGCGCTCGTTGTTGAC-3', 4R: 5'-TGGTAACAGTCCGGTTTAGAAGCAT-3'. Primer pair 5, 5F: 5'-CAGCCATGGATGATGATATTGC-3', 5R: 5'-CACCAGAGTCCATCACAATACCA GT-3'. Primer pair 6, 6F: 5'-GAGTACGACGAATCTGGACC-3', 6R: 5'-TGCACTTTTATTTGAACTG GTC-3'. All primers mentioned in this paper were synthesized by Sangon Biotec.

Table 1. Amino acid and cDNA nucleotide identities among *β-actin* cDNA sequences of *Alligator sinensis* and other representative vertebrates.

Taxonomy	Species name	GenBank accession No.	Nucleotide identity (%)	Amino acid identity (%)	No. amino acid residues
Mammals	*Homo sapiens*	NM_001101	89	100	375
	Spermophilus citellus	AY646115	90	100	375
	Macaca mulatta	NM_001033084	89	100	375
	Mus musculus	NM_007393.3	89	100	375
Birds	*Gallus gallus*	NM_205518 XM_429312	95	100	375
	Meleagris gallopavo	AY942620	94	100	375
	Anas platyrhynchos	EF667345	94	99	375
Reptiles	*Mauremys mutica*	HQ244396	87	98	376
	Anolis carolinensis	AF199487	89	99	334 (partial)
Amphibians	*Andrias davidianus*	HQ822274	90	99	374
	Cynops ensicauda	AB117093	89	99	374
	Xenopus laevis	NM_001088953NM_001088954	85	99	375
Fishes	*Cirrhinus molitorella*	DQ007446	90	99	375
	Danio rerio	BC067566	90	99	374
	Elopichthys bambusa	JN102135	90	99	375

Synthesis of first strand cDNA

A PrimerScript 1st strand cDNA synthesis kit (Takara, Dalian, China) was used to synthesize the first cDNA strand. RT-PCR was undertaken using a 20-μL reaction volume containing 150 ng RNA template, which was extracted from the tissues of liver, intestine, stomach, and pancreas, 4 μL 5X PrimerScript buffer, 1 μL PrimerScript RTase, 1 μL 50 μM Oligo dT primer, 0.5 μL 40 U/μL RNase inhibitor, 1 μL 10 mM of each dNTP mixture, and RNase free ddH_2O up to 20 μL. The RT-PCR cycling conditions were as follows: 40 min at 42°C and 5 min at 95°C. Products were refrigerated at -20°C.

PCR, cloning, and sequencing

A 15-μL PCR mixture contained 30-50 ng cDNA template, 0.25 μM primer, 0.3 mM dNTPs, 3 μL 5X prime STAR buffer (Mg^{2+} plus) (TaKaRa), and 0.65 U Prime STAR HS DNA polymerase

(TaKaRa). The PCR protocol was as follows: an initial denaturation step at 94°C for 3 min, 30 cycles of 94°C for 30 s, annealing at the correct temperature for 30 s, and extension at 72°C for 30 s, followed by a final extension step at 72°C for 10 min. PCR products were purified using a DNA gel extraction kit (Axygen, Hangzhou, China). Purification products were then sub-cloned into a pMD-18T vector (TaKaRa) and transformed recombinant DNA into DH5α *Escherichia coli* cells (TransGen, Beijing, China). Three to four positive clones were sequenced by Sangon Biotech.

Primers for quantitative PCR assay

A pair of internal primers (R10 in Table 2) was designed for quantitative PCR (qPCR) assay and synthesized by Sangon Biotech. The primer pair was verified on a Bio-RAD iO5 system using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara), following the manufacturer protocol. The 20-μL qPCR reaction volume contained 0.8 μL RNA template, 10 μL 2X SYBR® Premix Ex Taq™ (Tli RNaseH Plus), 0.4 μL forward primer, 0.4 μL reverse primer, 0.5 μL 40 U/μL RNase Inhibitor, and ddH_2O up to 20 μL. The RNA templates used were extracted from liver, intestine, stomach, and pancreas tissues. The qPCR conditions were as follows: after an initial denaturation step at 95°C for 30 s, 40 cycles of 95°C for 30 s and annealing at the correct temperature for 30 s, were conducted. All data were representative of experiments performed at least three times in duplicate and were analyzed using the Bio-RAD iO5 real time PCR system.

Table 2. Internal reference primers developed for the *Alligator sinensis β-actin* gene.

Primer pair code	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
R1	F: TGATATTGCTGCGCTCGTTGTTGAC R: TGGTAACAGTCCGGTTTAGAAGCAT	1135	62
R2	F: ATATTGCTGCGCTCGTTGTTGAC R: AACAGTCCGGTTTAGAAGCATTTGC	1129	61
R3	F: GAGTACGACGAATCTGGACC R: TGCACTTTTATTTGAACTGGTC	632	55
R4	F: GAGTACGACGAATCTGGACC R: TGGTCTTAAGTCAGTGTACAGG	614	55
R5	F: GCCCATCTATGAAGGCTA R: TCCAGACTGAGTACTTGCG	533	55
R6	F: CTGAACCCCAAAGCCAACAG R: ATACCACAGGACTCCATACC	494	55
R7	F: CAGCCATGGATGATGATATTGC R: CACCAGAGTCCATCACAATACCAGT	474	58
R8	F: CTTAGGTATGGAATCCTGTGGT R: CATTCCAGTTTTTTAATCCTGAGTC	452	55
R9	F: GGCACCACACCTTCTACAATGAGC R: TGATATCACGCACGATTTCCCTCT	381	59
R10	F: ACCGAAACAAGAACCCAT R: CCGACACGCTAAGACTGC	133	55

F = forward primer; R = reverse primer. Annealing temperature may vary according to PCR conditions.

Phylogenetic analysis

DNA sequences were run in BLAST on NCBI, aligned using ClustalX, and integrated in the DNASTAR Lasergene software package (http:www.dnastar.com). The open reading frame (ORF), CDS, and amino acid sequence of the last integration were confirmed using the ORF finder on NCBI. ClustalX and Mega 6.00 Beta (Tamura et al., 2013) were used to construct a phylogenetic tree. The maximum likelihood statistical method and nucleotide substitution model were used to

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identify the best model, and the neighbor-joining method was used to construct the final tree. Partial deletion was chosen as the treatment for gap/missing data. Among 24 models, general time reversible and gamma distributed with invariant sites were selected to reconstruct the phylogenetic tree on the basis of the most likelihood method with 1000 bootstrap replications.

RESULTS

Amplification of partial fragments of *β-actin* cDNA

RNA gel electrophoresis showed that the RNA extraction products had two distinct bands of 18S and 28S, which were suitable for follow-up experiments. Total RNA was reversely transcribed to synthesize cDNA, which was used as a template for PCR. After PCR amplification using primer pairs 1 and 2, 1.5% agarose gel electrophoresis showed that there were distinct bands in the location of anticipated fragments (Figure 1, P1, P2). Two DNA fragments of 533 and 494 bp were obtained following sequencing. The results of a BLAST on NCBI indicated that these fragments were partial fragments of *β-actin* cDNA.

In the same way, four other fragments with lengths of 530, 1135, 474, and 632 bp were acquired (Figure 1, P3, P4, P5, P6). These fragments were also confirmed as partial fragments of *β-actin* cDNA using BLAST on NCBI.

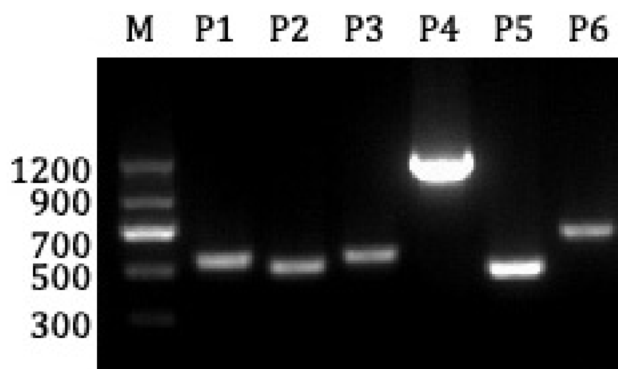

Figure 1. Agarose gel electrophoresis of PCR products following amplification of *β-actin* cDNA in *Alligator sinensis*. *Lane M*, DNA marker; *lane P1*, PCR amplification product with primer pair 1, 533 bp; *lane P2*, primer pair 2 product, 494 bp; *lane P3*, primer pair 3 product, 530 bp; *lane P4*, primer pair 4 product, 1135 bp; *lane P5*, primer pair 5 product, 474 bp; *lane P6*, primerpair 6, 632 bp.

CDS of *β-actin* cDNA

The four fragments mentioned above were spliced using the software DNAMAN and a 1731-bp fragment was obtained. Sequence analyses showed that the sequence contained the complete CDS of *β-actin* cDNA (1128 bp), encoding 375 amino acids whose initiation and termination codons are ATG and TAA, respectively (Figure 2). This long fragment of *β-actin* cDNA has been deposited in the GenBank database under accession No. KC286488.

Phylogenetic analysis

The amino acid and nucleotide sequences of *A. sinensis β*-actin and 15 other species were

compared using Clustal X. As shown in Table 1, the amino acid sequence length of *β*-actin varied from 374 to 376 amino acid residues among the species investigated. The amino acid sequence of *β-actin* is highly conserved and the identity of *A. sinensis β-actin* with mammals and birds was 100%, and ranged from 98-99% with reptiles, amphibians, and fishes. The nucleotide sequence of *A. sinensis β*-actin is slightly variable and the nucleotide sequence identity was generally above 85%, with a maximum of 95% among the fifteen species. The highest identity of approximately 94-95% was with birds, followed by that with reptiles of approximately 87-90%.

The phylogenetic tree of *β-actin* cDNA sequences shows that fishes are clustered together on the same branch, amphibians are clustered together on one branch, mammals are converged on one branch, and *A. sinensis*, *Anolis carolinensis*, and birds are clustered together on a branch (Figure 3). This phylogenetic relationship is roughly consistent with traditional classification. Here, it indicates that *A. sinensis* and *A. carolinensis* have a close relationship with birds.

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CCCCAGCCCAGCTCGACGCCTGCAGCCGCCCGTTAGTTCGCCATGGATGATGATATTGCTGCGCTCGTTGTGGACAATGGCTCCGGTATG   90
                                          M  D  D  D  I  A  A  L  V  V  D  N  G  S  G  M      16
TGCAAGGCCGGCTTCGCTGGGGACGACGCCCCTCGTGCCGTCTTCCCATCCATCGTCGGGCGCCCCAGACACCAGGGTGTGATGGTTGGC  180
 C  K  A  G  F  A  G  D  D  A  P  R  A  V  F  P  S  I  V  G  R  P  R  H  Q  G  V  M  V  G    46
ATGGGCCAGAAAGACAGCTACGTTGGTGATGAGGCCCAAAGCAAAAGAGGTATCCTGACCCTGAAGTACCCCATTGAACACGGCATTGTC  270
 M  G  Q  K  D  S  Y  V  G  D  E  A  Q  S  K  R  G  I  L  T  L  K  Y  P  I  E  H  G  I  V    76
ACCAACTGGGATGACATGGAGAAGATCTGGCACCACACCTTCTACAACGAGCTCAGAGTTGCCCCTGAGGAGCACCCTGTGCTGCTCACA  360
 T  N  W  D  D  M  E  K  I  W  H  H  T  F  Y  N  E  L  R  V  A  P  E  E  H  P  V  L  L  T   106
GAGGCTCCCCTGAACCCCAAAGCCAACAGGGAAAAGATGACACAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTAGCCATT  450
 E  A  P  L  N  P  K  A  N  R  E  K  M  T  Q  I  M  F  E  T  F  N  T  P  A  M  Y  V  A  I   136
CAGGCTGTCCTGTCCCTGTACGCCTCTGGTCGTACCACTGGTATTGTGATGGACTCTGGTGATGGTGTCACCCACACTGTGCCCATCTAT  540
 Q  A  V  L  S  L  Y  A  S  G  R  T  T  G  I  V  M  D  S  G  D  G  V  T  H  T  V  P  I  Y   166
GAAGGCTATGCCCTTCCCCATGCCATTCTCCGTCTGGATCTGGCTGGCCGTGATCTGACGGACTACCTCATGAAGATCCTGACCGAGAGA  630
 E  G  Y  A  L  P  H  A  I  L  R  L  D  L  A  G  R  D  L  T  D  Y  L  M  K  I  L  T  E  R   196
GGCTACAGCTTCACCACCACAGCCGAGAGGGAAATCGTGCGTGACATCAAGGAGAAGCTGTGCTATGTCGCCCTGGACTTCGAACAGGAG  720
 G  Y  S  F  T  T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y  V  A  L  D  F  E  Q  E   226
ATGGCCACCGCAGCCTCCAGCTCTTCCCTGGAGAAGAGCTACGAACTCCCTGATGGTCAGGTGATCACCATTGGCAATGAGAGGTTCAGG  810
 M  A  T  A  A  S  S  S  S  L  E  K  S  Y  E  L  P  D  G  Q  V  I  T  I  G  N  E  R  F  R   256
TGCCCTGAAGCTCTCTTCCAGCCTTCTTTCTTAGGTATGGAATCCTGTGGTATCCATGAAACTACCTTCAACTCCATCATGAAGTGCGAT  900
 C  P  E  A  L  F  Q  P  S  F  L  G  M  E  S  C  G  I  H  E  T  T  F  N  S  I  M  K  C  D   286
GTTGATATCCGTAAAGATCTGTATGCCAACACTGTGCTGTCTGGTGGTACCACCATGTACCCAGGCATTGCTGACAGAATGCAGAAGGAG  990
 V  D  I  R  K  D  L  Y  A  N  T  V  L  S  G  G  T  T  M  Y  P  G  I  A  D  R  M  Q  K  E   316
ATCACAGCCCTGGCACCCAGCACAATGAAAATCAAGATCATTGCCCCGCCTGAGCGCAAGTACTCTGTCTGGATTGGTGGCTCCATCCTG 1080
 I  T  A  L  A  P  S  T  M  K  I  K  I  I  A  P  P  E  R  K  Y  S  V  W  I  G  G  S  I  L   346
GCCTCCCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGACGAATCTGGACCCTCCATTGTCCACCGCAAATGCTTCTAA 1170
 A  S  L  S  T  F  Q  Q  M  W  I  S  K  Q     E  Y  D  E  S  G  P  S  I  V  H  R  K  C  F  ★   375
ACCGGACTGTTACCACGTCACACCGAAACAAGAACCCATATCTGCGCATCAACCAAGACAAGATTGGCATGGCTTTATTTGTTTTTTTTG 1260

GCGCTTGACTCAGGATTTAAAAACTGGAATGGTGAAGGTGACAGCAGCAGTCTTAGCGTGTCGGAGCGATGGTCCCCAAAGTTCTACAAT 1350

GCATCTGAGGACTTTGATTGTACATTTGTTTGTTTCTTTTTTTTTGGTTTTGTTTTTTTAATAGTCATTCCAAATATTGTATAATGCATT 1440

GTTACAGGAAGTTCTCGCCTCTGCGAAGGCCGCAGCCCACCTCGAAGGAGCCTGTACCTAAAGAGGGTTTTAGGTGTAACTGCTTGTCTG 1530

TAAATTATGTAACCCAACAAGTGTCTTTTGTATCTTCCGCCTTAAAAACAAACACTTGATTCCTCTTTTTGATTTGTCAAGCAAGCAGGG 1620

CTGTGTTTCCCAGTGGTAGATGTGAATGAAGGCTTTAGTCCCCCTGGAGTGTCCAGTGAAAAGCGGTGCCAGTATGTGGGGGAGGGAGGG 1710

GCTACCTGTACACTGACTTAA                                                                      1731
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Figure 2. *β-actin* cDNA of *Alligator sinensis* and deduced amino acid sequence. ATG shows initiation codon; upper sequence indicates the nucleotide and the lower sequence shows the deduced amino acids; TAA shows termination codon; the numbers on the right refer to the position of the nucleotides and the amino acids.

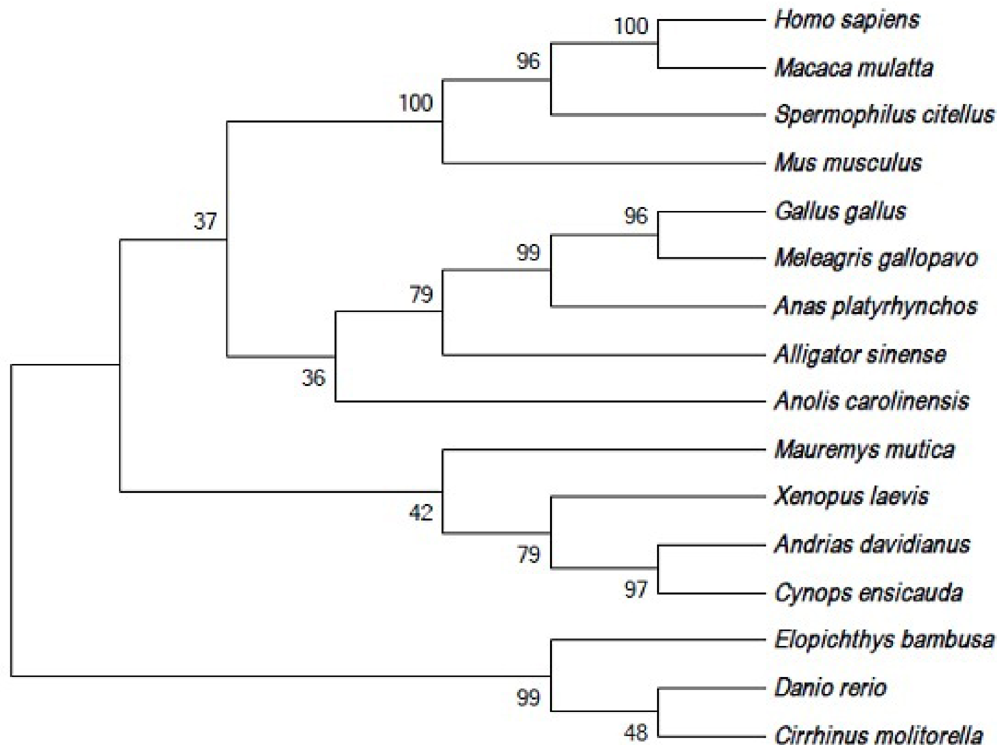

Figure 3. Phylogenetic tree of *β-actin* cDNA sequences from *Alligator sinensis* and other representative vertebrates reconstructed by Mega 6.00 Beta.

Internal reference primers from the *β*-actin gene

β-actin is commonly used as a reference gene for mRNA normalization in gene expression studies. Here, we designed primer sequences at different positions along the *β*-actin CDS and screened ten pairs of primers with different product sizes and different annealing temperatures (Table 2). The agarose gel electrophoresis of PCR products generated from primer pairs R1-R9 were all unique bands of anticipated length (Figure 4). The melt peak chart of qPCR amplification with primer pair R10 was a single peak (Figure 5), indicating no contamination, mis-priming, primer-dimer artifacts, etc. Thus, these primer pairs could be used as internal reference primers for semi-quantitative or quantitative PCR gene expression studies.

DISCUSSION

There are three different but highly conserved actin isoforms in muscle cells of vertebrates (Khaitlina, 2001; Sehring et al., 2007; Bergeron et al., 2010), and each isoform is remarkably similar to every other isoform, with only slight variations in amino acid sequences (Sehring et al., 2007; Perrin and Ervasti, 2010). To avoid the disturbance of other isoforms, the non-muscular tissues, pancreas and liver, were chosen as the material to clone the *A. sinensis β-actin* cDNA in this study.

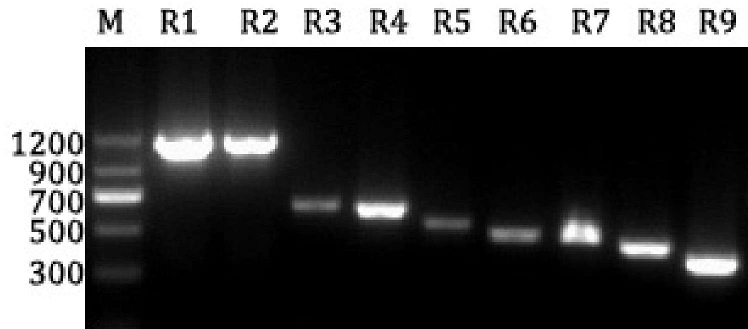

Figure 4. Agarose gel electrophoresis of PCR products amplified using the reference primer pairs 1-9 developed for the *Alligator sinensis β-actin* gene. *Lane M* = DNA marker; *lane R1*, PCR amplification product with reference primer R1, 533 bp; *lane R2*, primer R2 product, 494 bp; *lane R3*, primer R3 product, 530 bp; *lane R4*, primer R4 product, 1135 bp; *lane R5*, primer R5 product, 452 bp; *lane R6*, primer R6 product, 632 bp; *lane R7*, primer R7 product, 474 bp; *lane R8*, primer R8 product, 452 bp; *lane R9*, primer R9 product, 381 bp.

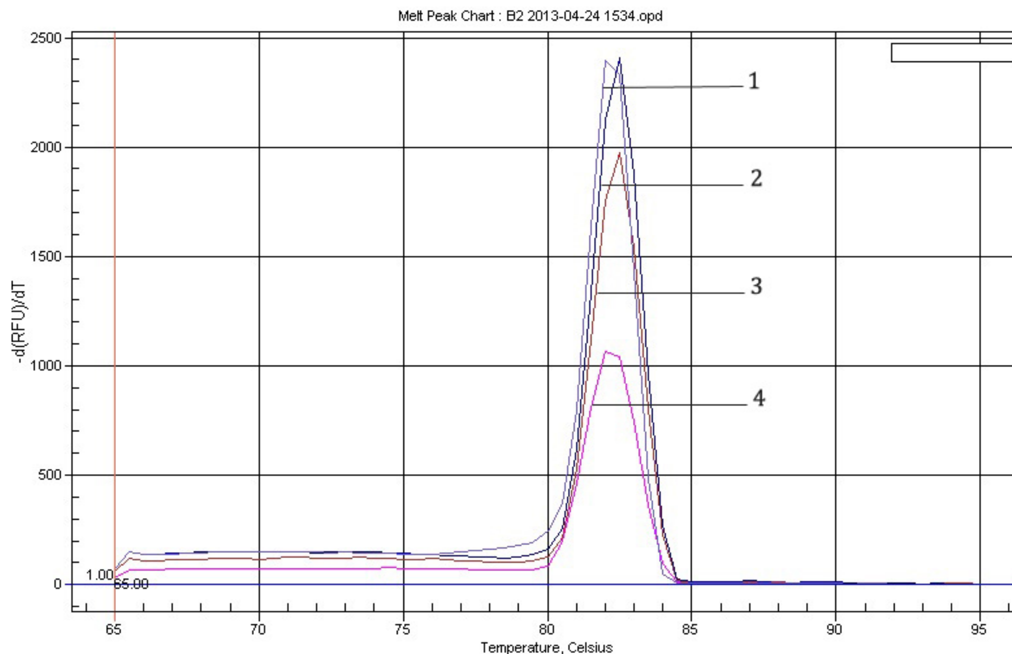

Figure 5. Melt peak chart of qPCR amplification of the *β-actin* gene in *Alligator sinensis* using reference primer R10. The rate of change (first derivative) of the relative fluorescence units with time (T) (d(RF)/dT) on the y-axis versus the temperature on the x-axis, which peaks at the melting temperature (Tm). Similar peaks indicate no contamination, mis-priming, primer-dimer artifacts, etc. Curves 1-4 were the results of different RNA templates extracted from stomach, intestine, pancreas, and liver tissues, respectively.

Previous studies and the identified full length cDNA sequences of *β-actin* from vertebrates in GenBank indicated that the 5'- and 3'-untranslated regions of the sequence of the actins are also conserved in evolution (Ponte et al., 1984), thus, it is feasible to get the complete CDS using the homology cloning technique. In this study, the complete CDS of the *β-actin* gene from *A. sinensis*

was successfully obtained with the use of primer design, specific amplification, and sequence splicing methods on the basis of conservation in the *β-actin* gene. DNA sequence analysis showed that the complete CDS of *β-actin* cDNA was 1128 bp long, encoding 375 amino acids.

Multiple alignment analyses showed that the *β-actin* gene of *A. sinensis* shared high identity with those of other vertebrates, with generally 85% identity in nucleotide sequence and 98% in amino sequence. Generally, the closely related species clustered together and the phylogenetic tree branches were supported by high statistical values. The phylogenetic tree showed that the *β-actin* gene of *A. sinensis* is much closer to those of birds, which is consistent with the hypothesis that crocodiles and birds have a close phylogenetic relationship.

The amino acid sequence of *β-actin* is highly conserved, and its nucleotide sequence is slightly variable, so it is necessary to clone its cDNA for gene expression research. Ten pairs of primers with different product sizes and different annealing temperatures were screened by PCR amplification, agarose gel electrophoresis, and DNA sequencing and could be used as internal reference primers for subsequent possible quantitative gene expression studies.

Conflicts of interest

The authors declare no conflict of interest.

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