Identification of the full-length β-actin sequence and expression profiles in the tree shrew (*Tupaia belangeri*)

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Abstract. The tree shrew (*Tupaia belangeri*) diverges from the primate order (Primates) and is classified as a separate taxonomic group of mammals - Scandentia. It has been suggested that the tree shrew can be used as an animal model for studying human diseases; however, the genomic sequence of the tree shrew is largely unidentified. In the present study, we reported the full-length cDNA sequence of the housekeeping gene, β-actin, in the tree shrew. The amino acid sequence of β-actin in the tree shrew was compared to that of humans and other species; a simple phylogenetic relationship was discovered. Quantitative polymerase chain reaction (qPCR) and western blot analysis further demonstrated that the expression profiles of β-actin, as a general conservative housekeeping gene, in the tree shrew were similar to those in humans, although the expression levels varied among different types of tissue in the tree shrew. Our data provide evidence that the tree shrew has a close phylogenetic association with humans. These findings further enhance the potential that the tree shrew, as a species, may be used as an animal model for studying human disorders.

Introduction

The tree shrew (*Tupaia belangeri*, family Tupaiidae) is a small mammal native to tropical forests, broadly distributed across Southeast Asia (1). It is classified as Scandentia, a separate taxonomic group of mammals and which probably diverged from the primate order (Primates) approximately 85 million years ago (2,3). It has been suggested that tree shrews can

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potentially be used as an animal model for the study of some human diseases (2). Over the past decades, tree shrew breeding has been on the increase in laboratories (4-7) and tree shrews have been used as experimental animal models for the study of diseases of the nervous system, visual system and viral infection diseases, such as the human hepatitis virus, herpes simplex virus and influenza virus (7-12).

However, the genetic background of the tree shrew is largely unidentified as only few tree shrew genes have been fully sequenced. In the GenBank database, there are less than 200 molecules with coding information (partial or complete). To date, there is no record available for the full-length sequence of the tree shrew β-actin (tsACTB) gene. In addition, few antibodies and detecting kits are available for characterizing the tree shrew genome. Due to the lack of genetic and immune background information, the wide application of the tree shrew as a model for the study of human diseases has been greatly impeded.

Housekeeping genes are a group of typically constitutive genes that are required for the maintenance of basic cellular function. When examining human samples, some housekeeping genes are frequently used as an internal reference, such as β-actin (*ACTB*), 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), heat-shock protein-90 (*Hsp90*), β-tubulin, β-2-microglobulin and others (13-15). However, as regards the tree shrew, most of the sequences of these genes remain unrevealed.

The actin family is an essential component of the cytoskeleton involved in a wide range of cellular functions, such as cell migration, division, junction formation, cell shape regulation, vesicle trafficking, transcriptional regulation and chromatin remodeling. The family is composed of six isoforms, four of which are muscle-specific, including one γ-isoform for smooth muscle and three α-isoforms for skeletal, cardiac and smooth muscles, respectively; the other two are ubiquitously expressed in the cytoplasm, including the β- and γ-isoform (16-18). All isoforms are remarkably similar to each other, with over 93% identity in the amino acid sequence between them (16). Among them, β-actin is a member of the conserved cytoskeleton structural proteins, which are abundant and widely distributed in all eukaryotic cells and play critical roles in cell migration, cell division, wound healing, embryonic development and the immune response (18,19).

In this study, we reported the full-length cDNA sequence of β-actin in the tree shrew and performed phylogenetic analysis of *ACTB* by constructing a cladogram, further verifying the ts*ACTB* sequence by measuring its expression profiles with quantitative polymerase chain reaction (qPCR) and western blot analysis. Our data provide novel proteomic information on the genetic background of the tree shrew, and enhance the potential of the future use of the tree shrew as a model for the study of human diseases.

Materials and methods

Ethics statement. All procedures related to the use of animals were reviewed and approved by the Review Committee of Guangxi University of Chinese Medicine, Nanning, China in accordance with the Institutional Animal Care and Use regulations and rules.

Tree shrew sampling. Four tree shrews (subfamily of *Tupaia belangeri Chinensis*) including one male and three females, purchased from Kunming Medical University, Kunming, China were used in this study. All procedures, including animal care, blood collection and tissue collection protocols were conducted in accordance with the Institutional Animal Care and Use regulations and rules.

RACE and sequencing. The 3'- and 5'-rapid amplification of cDNA ends (RACE) was used to clone the ts*ACTB* gene sequence. In brief, total RNA was isolated from the blood samples using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). The concentration of the RNA was calculated according to the absorbance at 260 and 280 nm which was measured using a Take3 model plate with an Epoch microplate reader (BioTek, Winooski, VT, USA). Based on the published nucleotide sequences of the partial tree shrew *ACTB* gene (GenBank accession no. AF110103.1), we designed two gene-specific primers (GSP) for the 5'-RACE and 3'-RACE reaction, respectively (Table I). First-strand cDNA was synthesized using the SMARTer RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA); while the amplifications were performed with LA Taq DNA Polymerase (Takara, Dalian, China). The PCR products was electrophoresed on a 1% agarose gel, and then purified using the Gel Extraction kit (CWBio, Beijing, China). Subsequently, the RACE products were linked to the pMD-18T vector (Takara) in accordance with the established protocols, and the constructed plasmids were then sent for sequencing (Life Technologies, Guangzhou, China).

Phylogenetic analysis. Alignment was performed using the Clustalw2 program. The phylogenetic tree was computed and constructed by the neighbor-joining method with 5,000 bootstrap replicates, as previously described (20). Values >50% were indicated. All ACTB sequences used in this study are available in GenBank (National Institutes of Health, Bethesda, MD, USA). Their accession numbers are as follows: NM_001101.3, KC215183, NM_001009945.1, AB188274.1, NM_001033084.1, NM_007393.3, NM_031144.3, NM_001172909.1, NM_001281595.1, NM_173979.3, U39357.1, NM_001081838.1, NM_205518.1, NM_001133354.1, NM_001195845.1, NM_001244575.1, NM_001101683.1, NM_213719.1, NM_001088953.1, AF025305.1 and AF012125.1.

Organ and tissue collection. The animals were anesthetized with an injection of 10% chloral hydrate (0.2 ml/100 g) in the abdominal cavity. Warm physiological saline solution was injected through the abdominal vein to expel blood from the organs. When paled, the organ was isolated, weighed and then cut into small tissue sections. These tissue sections were sorted and kept in RNAstore Reagent for the extraction of total RNA, and in Protease Inhibitor Cocktail Buffer (both from CWBio) for protein extraction processes.

Cell culture and collection. According to the datasheet of anti-ACTB antibody (GTX109639; GeneTex, Irvine, CA, USA), the human cell lines, 293T (GNHu17) and HeLa (TCHu187; both from Cell Bank of Shanghai Institutes for Biological Sciences), were used as positive controls. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). The cells were collected following a general protocol. In brief, cells were detached from the vessel with Trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) and then washed with phosphate buffer solution.

Construction of standard plasmid for absolute qPCR. Primers (Table I) were designed based on the sequence (GenBank accession no. KC215183) and then synthesized (Life Technologies, Guangzhou, China). First-strand cDNA was synthesized using the ProtoScript First Strand cDNA Synthesis kit (New England Biolabs, Beijing, China). The fragment for the standard plasmid was amplified using LA Taq DNA Polymerase (Takara). PCR products were electrophoresed on a 1% agarose gel, and purified using the Gel Extraction kit (CWBio). Subsequently, the purified PCR products were cloned into the pMD-18T Vector (Takara). The constructed plasmid was transformed into DH5α *E. coli* cells and multiplied through propagation of the DH5α *E. coli* bacteria, and then extracted and purified from bacteria using the TIANprep Mini Plasmid kit (Tiangen, Beijing, China). The purified plasmid was measured by the absorbance method of 260/280 nm, and diluted to a series of concentration gradients: 10^{10}, 10^9, 10^8, 10^7, 10^6 and 10^5 copies.

Reverse transcription and qPCR. The mRNA expression of ts*ACTB* in various types of tissue was detected using the absolute qPCR method following the MIQE guidelines, as previously described (21). In brief, total RNA was isolated using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) from the isolated tissues and measured using the absorbance method of 260/280 nm. The PrimeScript II 1st Strand cDNA Synthesis kit (Takara) was used to synthesize the first-strand cDNA from total RNA. The qPCR reactions were carried out using SYBR Premix Ex Taq II (Takara) in the MasterCycler Ep Realplex⁴ (Eppendorf, Hamburg, Germany). For each reaction, 2 µl cDNA and a final concentration of 50 nmol/l of each primer were used. The cycling profile consisted of an initial denaturation at 95℃ for 5 min followed by 40 cycles of 95℃ for 20 sec, 55℃ for 30 sec, 72℃ for 20 sec, followed by melt curve analysis.

Protein extraction and western blot analysis. Tissue protein extraction was carried out using the Tissue Protein Extraction kit,

Table I. Primers used in this study.

Primer	Sequence (5'→3')	Application
tsACTB-GSP3RACE	CCTGAAGTACCCCATCGAGCACGG	3'-RACE amplification
tsACTB-GSP5RACE	ATTTGCGCTCAGGGGGAGCGATG	5'-RACE amplification
tsACTB-STDf	CTGACCCTGAAGTACCCCATCGAG	Standard plasmid construction
tsACTB-STDr	CGTACTCCTGCTTGCTGATCCAC	Standard plasmid construction
tsACTB01 (efficiency, 105%)	Forward: CACGGCATTGTCACCAACT Reverse: TCAGTCAGCAGCACAGGAT	RT-qPCR
tsACTB02 (efficiency, 101%)	Forward: CCTGTATGCCTCTGGTCGTA Reverse: CCACATAGCACAACTTCTCCTT	RT-qPCR
tsACTB03 (efficiency, 105%)	Forward: CACTCTTCCAGCCATCTT Reverse: TGTCCACATCACACTTCAT	RT-qPCR
tsACTB04 (efficiency, 99%)	Forward: AGGAGAAGTTGTGCTATGT Reverse: ATGGAGTTGAAGGTGGTT	RT-qPCR

tsACTB, tree shrew β-actin; GSP, gene-specific primers; RACE, rapid amplification of cDNA ends; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

and cellular protein was extracted using the Mammalian Protein Extraction kit (both from CWBio). The total protein concentration was measured using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA) with an Epoch microplate reader (BioTek). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were performed following the standard protocol. The sample was loaded with total protein of 10 μg/lane. The anti-ACTB antibody (GTX109639) and secondary HRP-conjugated IgG antibody (GTX213110-01) (both from GeneTex) were applied. Images of the blots were obtained using the Pierce ECL Plus Western Blotting Substrate (Thermo Scientific) in a 4000MM Pro Image Station (Carestream Health, Inc., Rochester, NY, USA).

Data analysis, software and statistical analysis. The primers used in this study were designed by the Primer3 online program, as previously described (22,23). The Clustalw2 online program (24) was used to align and score gene/protein sequences. BioEdit (25) was used to build the alignment graph. MEGA5 (20) was used to compute and construct the phylogenetic tree. The nucleic acid concentration was measured and calculated using Gen5 software (BioTek). Realplex software (Eppendorf, Hamburg, Germany) was used to analyze the results from qPCR. From the results of western blot analysis, graphs were created and analyzed using Molecular Imaging Software (Carestream Health, Inc.). A statistical comparison was carried out with One-Way Analysis of Variance (ANOVA) using the agricolae package (26) in R (27). Charts were built using ggplot2 (28), scales (29) and gridExtra (30) packages in R (27). Charts and graphs were created using Inkscape (31) and the GNU Image Manipulation Program (GIMP) (32).

Results

Full-length cDNA sequence of tsACTB. We identified full-length cDNA sequences of ts*ACTB* from three individual tree shrew specimens. The three sequences were the same. The full-length amino acid sequence of ts*ACTB* was deduced based on its full-length cDNA sequence. The sequence is a cDNA 1,866 bp in length and encodes a protein with 375 amino acids. This is similar to other animals. We deposited the ts*ACTB* cDNA and uploaded the amino acid sequences into GenBank with the accession number: KC215183. To the best of our knowledge, ours is the first study to identify the full-length sequence of ts*ACTB*.

Phylogenetic analysis. To further examine the association between the tree shrew and other species, we compared the ts*ACTB* with *ACTB* of other animals which can be found in GenBank. The multiple sequence alignment (MSA) results (Fig. 1) revealed that there was no difference in the ACTB amino acid sequence between the tree shrew and humans. Among the different species examined, the ACTB amino acid sequences were exactly the same or differed only in one or two amino acid sequences. With the full-length cDNA sequence of ts*ACTB* from our study, we were able to construct a phylogenetic tree (Fig. 2) using the genetic database from GenBank. Due to the extremely conserved amino acid sequence of ACTB, we constructed this phylogenetic tree with the open reading frame (ORF) of *ACTB*.

Expression profile analysis of tsACTB in tree shrew tissue. To further determine the ts*ACTB* expression pattern in the tree shrew, qPCR was used to measure the mRNA expression levels of *ACTB* in various types of tissue from the tree shrew (Fig. 3). We designed four pairs of primers (Table I) to detect ts*ACTB* expression patterns based on the full-length cDNA sequence. The amplification efficiencies of the four pairs of primers are shown in Table I. These pairs of primers are similar in their amplification efficiencies (99-105%). The levels of *ACTB* expression calculated by the tsACTB03 primer pair were higher than the other three primer pairs, but were

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                                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Human(NM_001101.3)                 MDDDIAALVV DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
Chimpanzee(NM_001009945.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
W. Chimpanzee (AB188274.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
R. macaque(NM_001033084.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Tree shrew(KC215183)               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Mouse(NM_007393.3)                 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Rat(NM_031144.3)                   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Guinea pig(NM_001172909.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
G. hamster(NM_001281595.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Cattle (NM_173979.3)               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Sheep (U39357.1)                   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Horse (NM_001081838.1)             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Junglefowl(NM_205518.1)            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S. orangutan(NM_001133354.1)       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Gray wolf (NM_001195845.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. hamster (NM_001244575.1)        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Rabbit (NM_001101683.1)            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
W. clawed frog (NM_213719.1)       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Clawed frog (NM_001088953.1)       .E........ .......... .......... .......... .......... .......... .......... .......... .......... ..........
Zebrafish (AF025305.1)             ...E...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
A. salmon (AF012125.1)             .E.E...... .......... .......... .......... .......... .......... .......... .......... .......... ..........

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Human(NM_001101.3)                 HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
Chimpanzee(NM_001009945.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
W. Chimpanzee (AB188274.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
R. macaque(NM_001033084.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Tree shrew(KC215183)               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Mouse(NM_007393.3)                 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Rat(NM_031144.3)                   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Guinea pig(NM_001172909.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
G. hamster(NM_001281595.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Cattle (NM_173979.3)               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Sheep (U39357.1)                   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Horse (NM_001081838.1)             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Junglefowl(NM_205518.1)            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S. orangutan(NM_001133354.1)       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Gray wolf (NM_001195845.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. hamster (NM_001244575.1)        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Rabbit (NM_001101683.1)            R......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
W. clawed frog (NM_213719.1)       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Clawed frog (NM_001088953.1)       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Zebrafish (AF025305.1)             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
A. salmon (AF012125.1)             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

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Human(NM_001101.3)                 TTTAEREIVR DIKEKLCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
Chimpanzee(NM_001009945.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
W. Chimpanzee (AB188274.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
R. macaque(NM_001033084.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Tree shrew(KC215183)               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Mouse(NM_007393.3)                 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Rat(NM_031144.3)                   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Guinea pig(NM_001172909.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
G. hamster(NM_001281595.1)         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Cattle (NM_173979.3)               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Sheep (U39357.1)                   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Horse (NM_001081838.1)             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Junglefowl(NM_205518.1)            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S. orangutan(NM_001133354.1)       .......... .......... .......... .......... .......... .......... .......... .......... .N........ ..........
Gray wolf (NM_001195845.1)         .......... .......... .......... .......... .......... .......... .......... .S........ .......... ..........
C. hamster (NM_001244575.1)        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Rabbit (NM_001101683.1)            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
W. clawed frog (NM_213719.1)       .......... .......... .......... .......... .......... .......... .......... .......... .Y........ ..........
Clawed frog (NM_001088953.1)       .......... .......... .......... .......... .......... .......... .......... .......... .Y........ ..........
Zebrafish (AF025305.1)             .......... .......... .......G.. .......... .......... .......... .......... .......... .......... ..........
A. salmon (AF012125.1)             .......... .......... .......G.. .......... .......... .......... .......... .......... .Y........ ..........

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Human(NM_001101.3)                 GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSILASLS TFQQMWISKQ EYDESGPSIV HRKCF        375 aa
Chimpanzee(NM_001009945.1)         .......... .......... .......... .......... .......... .......... .......... .....        375 aa
W. Chimpanzee (AB188274.1)         .......... .......... .......... .......... .......... .......... .......... .....        375 aa
R. macaque(NM_001033084.1)         .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Tree shrew(KC215183)               .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Mouse(NM_007393.3)                 .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Rat(NM_031144.3)                   .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Guinea pig(NM_001172909.1)         .......... .......... .......... .......... .......... .......... .......... .....        375 aa
G. hamster(NM_001281595.1)         .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Cattle (NM_173979.3)               .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Sheep (U39357.1)                   .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Horse (NM_001081838.1)             .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Junglefowl(NM_205518.1)            .......... .......... .......... .......... .......... .......... .......... .....        375 aa
S. orangutan(NM_001133354.1)       .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Gray wolf (NM_001195845.1)         .......... .......... .......... .......... .......... .......... .......... .....        375 aa
C. hamster (NM_001244575.1)        .......... .......... .......... .......... .......... ...E...... .......... .....        375 aa
Rabbit (NM_001101683.1)            .......... .......... .......... .......... .......... .......... .......... .....        375 aa
W. clawed frog (NM_213719.1)       .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Clawed frog (NM_001088953.1)       .......... .......... .......... .......... .......... .......... .......... .....        375 aa
Zebrafish (AF025305.1)             .......... ......S... .......... .......... .......... .......... .......... .....        375 aa
A. salmon (AF012125.1)             .......... ......S... .......... .......... .......... .......... .......... .....        375 aa
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Figure 1. Alignment of β-actin. Comparison of the deduced full-length amino acid sequences of β-actin and other animals. Identical amino acids are shown by dots.

similar to those of the other three primer pairs. The expression levels of ts*ACTB* in various types of tissue were compared by One-Way ANOVA followed by Duncan's multiple range test, with a significance level of $P=0.05$. According to this statis-tical analysis, the expression of *ACTB* can be grouped into two cross groups, marked as 'a' and 'b', and the crossed parts are marked as 'ab' (Fig. 3). The levels of *ACTB* in the same group did not differ significantly.
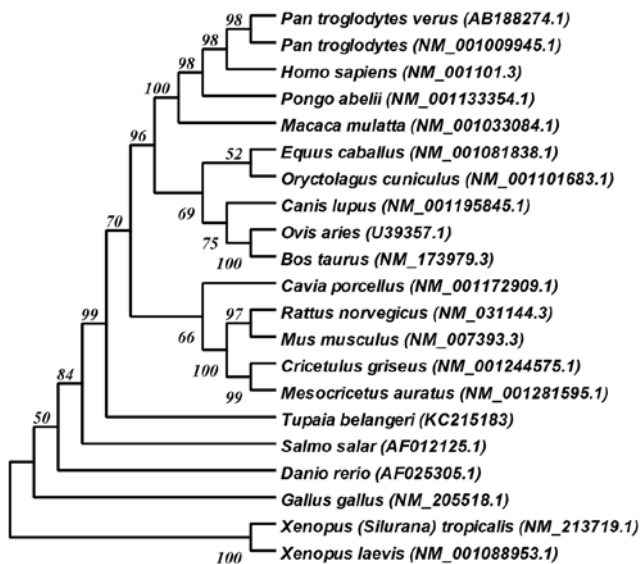
Figure 2. Phylogenetic analysis of β-actin. The phylogenetic tree is based on open reading fram (ORF) of β-actin and constructed using the neighbor-joining method. Only bootstrap values >50% are shown.

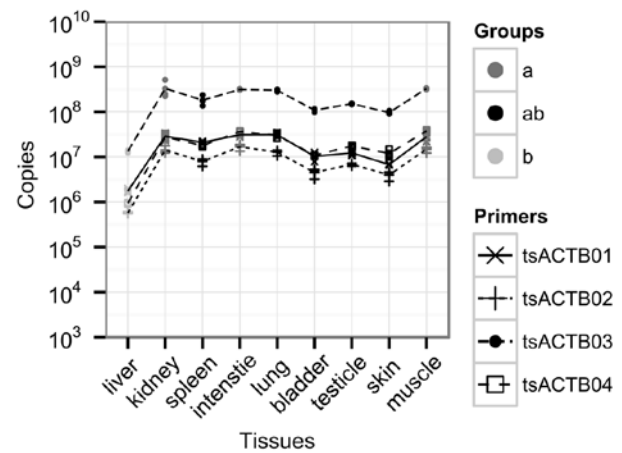

Figure 3. Analysis of mRNA expression profiles of tree shrew β-actin. Four pair of primers for quantitative polymerase chain reaction (qPCR) were designed to measure the mRNA expression of tree shrew β-actin (ts*ACTB*). The amount of each sample was calculated according to its equation. Data are shown in a point chart and grouped according to ANOVA. Lines are the means of copies of each primer's product.

Expression pattern of ACTB in the tree shrew. The total identity of ACTB in the amino acid sequence suggests the possibility of the identification of the tsACTB molecule with anti-ACTB antibody which reacts with human ACTB. To assess the ACTB protein expression, western blot analysis was performed to detect tsACTB using an anti-ACTB antibody for humans. All examined tissues expressed the ACTB protein (Fig. 4). The highest level of tsACTB expression in tree shrew tissue was observed in the spleen, bladder and testicles. The lowest levels of tsACTB expression in tree shrew tissue were observed in the liver and muscle (Fig. 4).

Discussion

In this study, we reported the full-length cDNA sequence of ACTB in the tree shrew and performed evolution and sequence analysis. We then identified the sequence using qPCR and verified the protein expression of tsACTB by western blot analysis. Phylogenetic analysis revealed that the tree shrew is classified as Scandentia between the order of Primates and Insectivora. The tsACTB amino acid sequence was found to be completely equal to the ACTB sequence of several species (Fig. 1), including the human, chimpanzee, macaque, mouse, rat guinea pig, hamster cattle, sheep, horse and jungle fowl species. Due to the fact the molecule of ACTB is highly conserved in all species, we constructed a phylogeny tree with the ORF of the *ACTB* cDNA sequence instead of its amino acid sequence, even though phylogenetic associations are usually analyzed with the amino acid sequence of the molecule. As shown in the cladogram (Fig. 2), the closest phylogenetic relatives to the tree shrew, not belonging to the order of Primates, are the rabbit (*Oryctolagus cuniculus*) and horse (*Equus caballus*), which is consistent with the results of previous studies on the tree shrew MHC gene (33,34). Our results support the current classification of the tree shrew, namely that the tree shrew is a small-sized mammal phylogenetically closer to humans than other small-sized experimental animals, such as the mouse, rat, rabbit and guinea pig, but phylogenetically farther than non-human primates, such as the chimpanzee and monkey.

ACTB, a housekeeping gene in humans, mice, rats and other experimental animals, is widely used as an internal reference in quantitative methods, including qPCR and western blot analysis, which are two popularly methods used for the quantification of the mRNA and protein content, respectively. In this study, tsACTB expression was verified to widely and richly exist in each examined tissue of the tree shrew by both qPCR and western blot analysis, although the expression levels varied among the different types of tissue from the tree shrews. Moreover, tsACTB mRNA and protein expression levels were
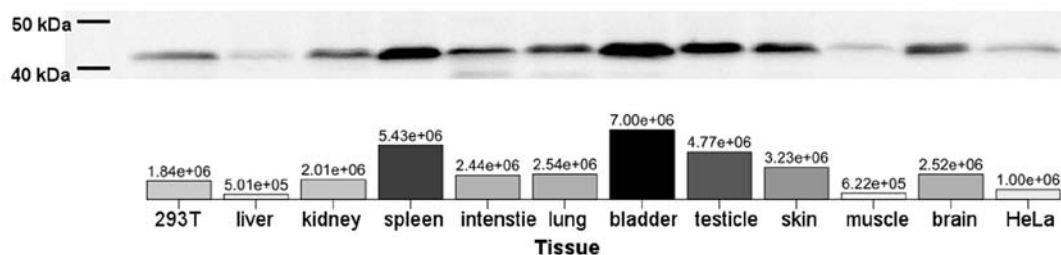
Figure 4. Analysis of protein expression of β-actin in tree shrew by western blot analysis using anti-β-actin antibody. Upper panel, western blot. Each lane was loaded with 10 μg total protein. Lower panel, each band intensity value is shown below itself as a bar and numerical value.

not completely parallel. For instance, our data indicated that the muscle tissue from the tree shrew had significantly high mRNA levels of ts*ACTB* expression and significantly low protein expression levels (Figs. 3 and 4). We also observed that the levels of tsACTB detected by the tsACTB03 primer pair were higher than those detected by the other three qPCR primer pairs (Fig. 3). A possible reason for this is that the product length of the tsACTB primer pair was only 87 bp, which was shorter than the products of the tsACTB01 (104 bp), tsACTB02 (236 bp), and tsACTB04 (208 bp) primer pairs (data not shown). Another possible reason is the high homology of the six isoforms of the actin family and this primer pair may amplify other non-specific isoforms of the actin family. However, as the majority of the cDNA sequences of the tree shrew, including other isoforms of actin family members, are still unknown, the actual reason remains to be revealed.

One of the most crucial steps in experimental design is selecting an internal control gene to normalize the gene expression data according to the objective of the experiment. Our data may prove to be a helpful reference and may aid in the selection of an internal reference in future studies using tree shrews. If experiments are carried out using samples, the reliability of ts*ACTB* expression before and after treatment must be verified in advance. According to our data, ts*ACTB* is a suitable candidate only for comparing expression levels in different types of tissue (the tissue types verified to express similar levels). The primers for used for qPCR and anti-ACTB antibody (human) were good tools for the detection of the mRNA and protein epxression of ACTB in the tree shrew.

Acknowledgements

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