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Cloning of *Xenopus laevis* TRPV2 by Gene Prediction

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Abstract

TRPV2 is a non-specific cation channel expressed in sensory neurons, and activated by noxious heat. Particularly, TRPV2 has six transmembrane domains and three ankyrin repeats. TRPV2 has been cloned from various species such as human, rat, and mouse. Oocytes of *Xenopus laevis* - an African clawed frog - have been widely used for decades in characterization of various receptors and ion channels. The functional property of rat TRPV2 was also identified by this oocyte expression system. However, no TRPV2 orthologue of *Xenopus laevis* has been reported so far. Hence, we have focused to clone a TRPV2 orthologue of *Xenopus laevis* with the aid of bioinformatic tools. Because the genome sequence of *Xenopus laevis* is not available until now, a genome sequence of *Xenopus tropicalis* - a close relative species of *Xenopus laevis* – was used. After a number of bioinformatic searches *in silico*, a predicted full-length sequence of TRPV2 orthologue of *Xenopus tropicalis* was found. Based on this predicted sequence, various approaches such as RT-PCR and 5'-RACE technique were applied to clone a full length of *Xenopus laevis* TRV2. Consequently, a full-length *Xenopus laevis* TRPV2 was cloned from heart cDNA.

Keywords: BLAST, Multiple alignment, TRPV2, *Xenopus laevis, Xenopus tropicalis*

Introduction

Among the plethora of ion channels, transient receptor potential (TRP) channel family has gained its popularity, as a large amount of TRP channels are involved in physiological sensory responses such as thermal, mechanical and chemical sensations (Harteneck *et al.*, 2000; Koltzenburg, 2004; Montell *et al.*, 2002a; Montell *et al.*, 2002b). TRP channel was first discovered in *Drosophila melanogaster* (Montell, 1989; Montell and Rubin, 1989). Since its first identification, at least 22 genes were discovered as a member of TRP ion channel family (Clapham *et al.*, 2003).

TRP family can be divided into a number of sub-families, such as TRPC, TRPM and TRPV. Among them, TRPM sub-family plays various roles in cell division, cell migration, calcium signaling and even thermal sensing (TRPM8) (Fleig and Penner, 2004; Peier *et al.*, 2002). TRPV families are relatively well-characterized ion channels in terms of thermosensation. TRPV1 through TRPV4 channels are known to respond to change in temperature, serving as molecular sensors of heat sensations (Gunthorpe *et al.*, 2002).

TRPV2 (formerly known as VRL1) was cloned from rat dorsal root ganglia (Caterina *et al.*, 1999). TRPV2 is a non-selective cation channel activated by noxious heat (> 52°C). Like other TRPV channels, TRPV2 contains six transmembrane domains with a putative pore-loop region between fifth and sixth transmembrane domains and holds three ankyrin repeats at the cytoplasmic amino terminus, where protein-protein interactions are supposed to occur (Ferrer-Montiel *et al.*, 2004). Up until now, a number of TRPV2 orthologues have been cloned from various species such as human, rat, mouse and cow.

An African clawed frog, *Xenopus laevis,* has been the choice of the heterologous expression system for testing cloned ion channels because of the ease of mRNA injection into oocytes. Still, genetic approach to this frog is not possible to date. However, the genome project of *Xenopus tropicalis*, a close relative frog of *Xenopus laevis,* is under way. One main reason for the lack of the genome seqeunce of *Xenopus laevis* is because *Xenopus laevis* has some drawbacks compared to *Xenopus tropicalis*: *Xenopus laevis* takes 1~2 years to reach sexually maturity, whereas *Xenopus tropicalis* takes 3 or 4 months (Ishii *et al.*, 2004). Moreover, *Xenopus tropicalis* has a smaller diploid genome (20 chromosomes) than that of *Xenopus laevis* (36 chromosomes).

BLAST (Basic Local Alignment Search Tool) is one of the most widely used similarity search tools in data analysis, which is based on a heuristic method to find the optimal alignments between genes from the highest scoring (Korf, 2003). BLAST algorithm depends on the

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statistics of ungapped sequence alignment called maximal segment pair (MSP). The mathematical results of MSP scores generate the statistical significance of alignment between selected sequences.

Here in this study, we focused to clone a TRPV2 orthologue of *Xenopus laevis* with the aid of bioinformatics tools such as BLAST based on the genome sequence of *Xenopus tropicalis.*

Methods

BLAST Search from Xenopus tropicalis Protein Sequences

A *Xenopus tropicalis* protein sequence file (*Xenopus tropicalis* genome assembly 2.0, "xenopus.040430.proteins.gz") was downloaded from Xenbase web site (http://www.xenbase.org/). The sequence file was composed of total 116058 computer-predicted protein sequences. BLAST program was downloaded from National Center for Biotechnology Information (NCBI, ftp://ftp.ncbi.nih.gov/blast) and installed on Linux-based computer. All sequence data were converted to support BLAST format. During the BLAST search, default parameters were used. A human TRPV2 protein sequence is obtained from NCBI Genbank (gi: 8394534).

Primer design and RT-PCR

First-strand cDNA was synthesized from *Xenopus laevis* heart, brain and oocytes and reverse-transcribed with SuperScriptTM (Life Technology, Inc.) following the manufacturer's protocol. Based on the predicted Xenopus tropicalis TRPV2 sequence (xtTRPV), primer was designed manually for reverse transcription-polymerase chain reaction (RT-PCR). The forward primer was 5'-ATGTCG GGTACAGACTCAGA TG-3' (Primer 1) and reverse primer was 5'-CTACACCGCTGGGCTGCTT-3' (Primer 2). Using these primers, RT-PCR was carried out under following conditions : 94°C X 5 min, 94°C X 30 sec, 55°C X 30 sec, 72°C X 2 min at 30 cycles/ 72°C X 10 min using PCR thermal cycler (TAKARA).

RT-PCR primers of highly-conserved region between TRPV2 orthologues were designed as following: The forward primer was 5'-CTACTACCGAGGCCAGAC-3' (Primer 3) and reverse primer was 5'-CAGCAGGAGGA TGTAGGTC-3' (Primer 4). RT-PCR was carried out as following conditions: 94°C X 5 min, 94°C X 30 sec, 55°C X 30 sec, 72°C X 1 min 30 sec at 30 cycles/ 72°C X 10 min using PCR thermal cycler (TAKARA).

Based on the conserved 3'-region of Xenopus laevis EST (expressed sequence tag) fragments, a new reverse primer (Primer 5) was designed as 5'-CTACA CGTCCGGAGTGCT-3'. These three EST fragments were acquired from NCBI (gi : 14185141, 33627140 and 34884469). RT-PCR was performed under the same previous conditions.

5'-RACE

First-strand cDNA for 5'-RACE (Rapid-Amplification of cDNA Ends) of xlTRPV2 was synthesized from *Xenopus laevis* heart with TAKARA 5'-Full RACE Core set following manufacturer's protocol (Takara, Osaka, Japan). In briefly, first-strand cDNA was amplified by PCR (polymerase chain reaction) in a volume of 15 $\mu\ell$ using total RNA 5 μg, 10x RT Buffer, RNase Inhibitor (40units/ $\mu\ell$), AMV Reverse Transcriptase and 5' end-phosphorylated RT-Primer 5'-ATGCTGCATCCA-3'. The amplification program was 30°C X 10 min / 50°C X 60 min / 80°C X 2 min using a thermal cycler (TAKARA). PCR product was stored at 4°C.

Hybrid RNA from the synthesized first-strand cDNA was selectively degraded by RNase H. 5X Hybrid RNA Degradation Buffer and RNase H was added in first-strand cDNA, and then incubated at 30 °C for 1 hour.

Following step for ligation reaction (circularization of single-stranded cDNA, or formation of concatemers) was performed by adding the reagents in volume of 40$\mu\ell$ using 5x RNA (ssDNA) Ligation Buffer, 40% PEG #6000, 1$\mu\ell$ single-stranded cDNA collected by ethanol precipitation, and 1$\mu\ell$ of T4 RNA Ligase and incubating at 15°C for overnight. Then, this product was used as a template for 5'RACE PCR and stored at -20°C.

5'RACE PCR was done by 2 step PCR reactions. Primers were prepared for 1st PCR reaction. 1st PCR S1 Primer was 5'-GACATGTCTGCTGAAGGC-3' and 1st PCR A1 Primer was 5'-CTGGTTGCTTTCCATTGCC-3'. 5'RACE template was amplified by PCR using *TAKARA LA Taq*™. The 5'RACE 1st PCR condition was 94°C X 3 min / 94°C X 30 sec, 55°C X 30 sec , 72°C X 2 min at 25 cycles.

The 2nd PCR template was obtained from product of 1st PCR. To assure a clear PCR product, 2nd PCR templates were prepared by 3 dilutions (no dilution, 10 fold, and 100 fold) of the 1st PCR product. The 2nd PCR primers were also ready for experiment. 2nd PCR S2 Primer was 5'-CCGATACTGCTGGAAGCT-3' and 2nd PCR A2 Primer was 5'-CAGGTGAGTAGTCGTCACT-3'. The 2nd 5'RACE PCR was also amplified by using *TAKARA LA Taq*™. The 5'RACE 2nd PCR amplification program was 94°C X 30 sec, 55°C X 30 sec, 72°C X 2 min at 25 cycles.

Multiple Alignments and Phylogenetic Trees

ClustalW was downloaded from European Bioinformatics

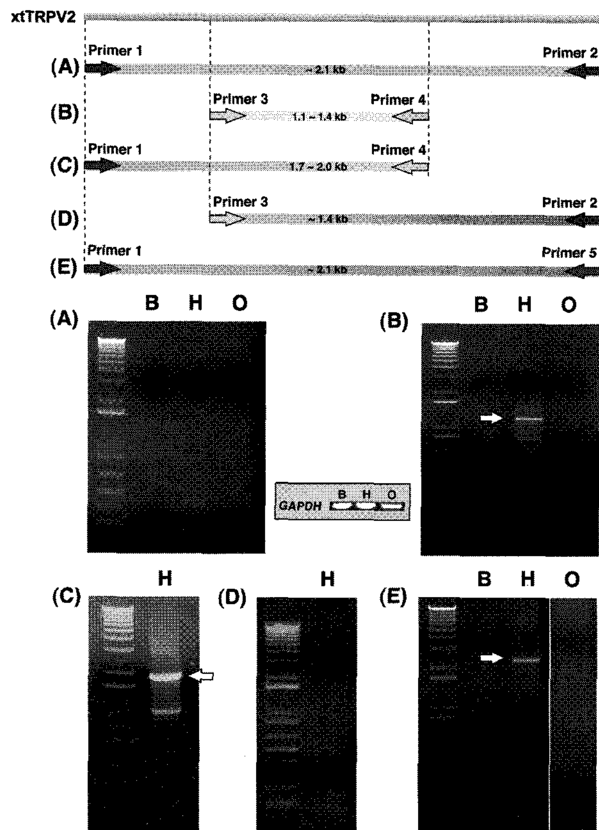


Fig. 1. Schematic diagram of primer design and RT-PCR products in Xenopus laevis.

(A) Based on xtTRPV2 mRNA sequence, primer 1 and primer 2 were designed and amplified by RT-PCR. RT-PCR product was not appeared in each lane (B; brain, H; heart, O; oocytes.). Note that gray box in the middle of the figure is GAPDH for positive control.

(B) RT-PCR product by using primer 3 and primer 4 was appeared in the heart, but not in the brain and oocytes. The arrow indicates the expected band.

(C) RT-PCR performed by primer 1 and primer 4. Note that the PCR product was matched with the predicted size in the heart (arrow).

(D) Primer 3 and Primer 2 were used for RT-PCR. Bands were weakly present in RT-PCR product.

(E) RT-PCR with Primer 1 and re-designed reverse primer (Primer 5). The arrow indicates the expected product in the heart

Institute (EMBL-EBI, ftp.ebi.ac.uk/software) and installed locally on the Linux-based operating system. Default parameters were used during the process. Phylogenic tree was made by TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results

BLASTP search was done to identify a TRPV2 orthologue of *Xenopus tropicalis* among its computer-predicted 116,058 protein sequences. As a result, the most probable TRPV2 orthologue sequence was found and named as xtTRPV2. In order to make clear that this xtTRPV2 sequence was a real orthologue of TRPV2, additional BLASTP search was done with xtTRPV2 as a query sequence against NCBI non-redundant (nr) database. Indeed, the search result clarified that the xtTRPV2 was a true TRPV2 orthologue of *Xenopus tropicalis.* With this predicted full-length xtTRPV2 sequence, custom primers were designed. RT-PCR was carried out over *Xenopus laevis* heart, brain and oocyte cDNA. As shown in Fig. 1, size of the RT-PCR product with Primer 1 and Primer 2 should be around 2,100 base pair long. However, no PCR product was obtained (Fig. 1A). Although various temperature conditions of PCR program were attempted (45, 50, 55 and 60• C as annealing temperatures), no apparent bands were detected. These results indicate that TRPV2 orthologue of *Xenopus laevis* is not identical to that of xtTRPV2.

We thus searched a highly-conserved region in *Xenopus laevis* TRPV2. To confirm this, new primer pairs (Primer 3 and Primer 4) that match to highly-conserved region were constructed using multiple alignments with xtTRPV2 and other TRPV2 orthologs. RT-PCR with Primer 3 and Primer 4 pairs should amplify the product within a range of 1,100bp through 1,400bp. Indeed, as depicted in Fig. 1B, a strong PCR band was found in *Xenopus laevis* heart cDNA. However, any PCR product was found in the brain and oocytes. These results show that TRPV2 orthologue of *Xenopus laevis* is present in the heart, but rarely in the brain and oocytes. We then swapped primers alternatively as shown in Fig. 1. RT-PCR with Primer 1 and Primer 4 detected a PCR product within a range of 1,700 ~ 2,000 bp (Fig. 1C). However, RT-PCR with Primer 3 and Primer 2 detected a dim and weak band (Fig. 1D). These data indicate that Primer 1, not Primer 2, matches to TRPV2 orthologue of *Xenopus laevis.* Thus, it was concluded that a new 3' reverse primer is required to clone a full-length TRPV2 orthologue from *Xenopus laevis.*

Fortunately, 3'-EST fragments of *Xenopus laevis* (Genbank Index number 14185141, 33627140 and 34884469) were found in BLAST, which facilitated the designing of a new 3' reverse primer (Primer 5). However, this new reverse primer (Primer 5) has a few different bases compared to Primer 2. RT-PCR with Primer 1 and Primer 5 was done against *Xenopus laevis* heart, brain, and oocytes. We then found a PCR product with expected size of 2.1 Kb in the heart, but not in the brain and oocytes (Fig. 1E). After confirmation by sequencing analysis, it was found that this product is conisted of total 2319 nucleotides.

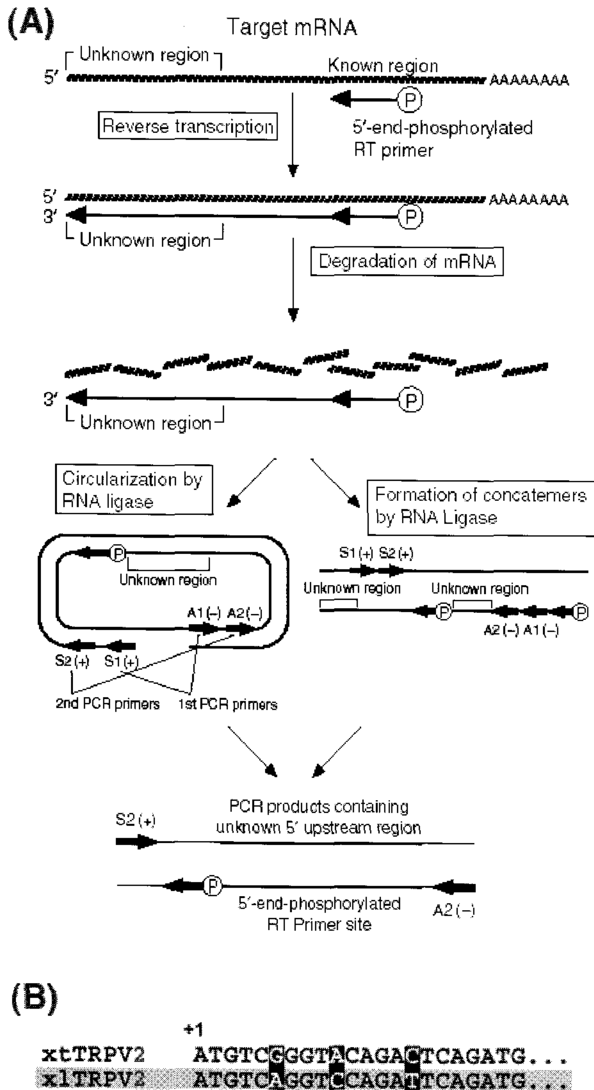


Fig. 2. 5'RACE PCR of xlTRPV2

(A) Schematic diagram of 5'-RACE of xlTRPV2 using the Takara 5'-full RACE core set

(1) The 1st strand cDNA was synthesized by reverse transcription from *Xenopus laevis* heart total RNA using 5' end-phosphorylated RT Primer, which is specific to the target RNA. 5' end RT Primer was designed in xlTRPV2 sequence and should be phosphorylated in 5' end.

(2) Hybrid RNA was selectively degraded by treatment with RNase H from the synthesized 1st strand cDNA

(3) Circular single-strand cDNA or concatemers was formed by treatment with T4-RNA Ligase, and then two-step DNA amplification was performed. (4) PCR products containing unknown 5' upstream region was obtained.

(B) Difference between 5'-RACE product of xlTRPV2 sequence and predicted xtTRPV2 sequence. 5'-RACE PCR product sequence was compared with xtTRPV2 sequence. Three nucleotides were mismatched between 5'-RACE product sequence and predicted xtTRPV2 sequence.

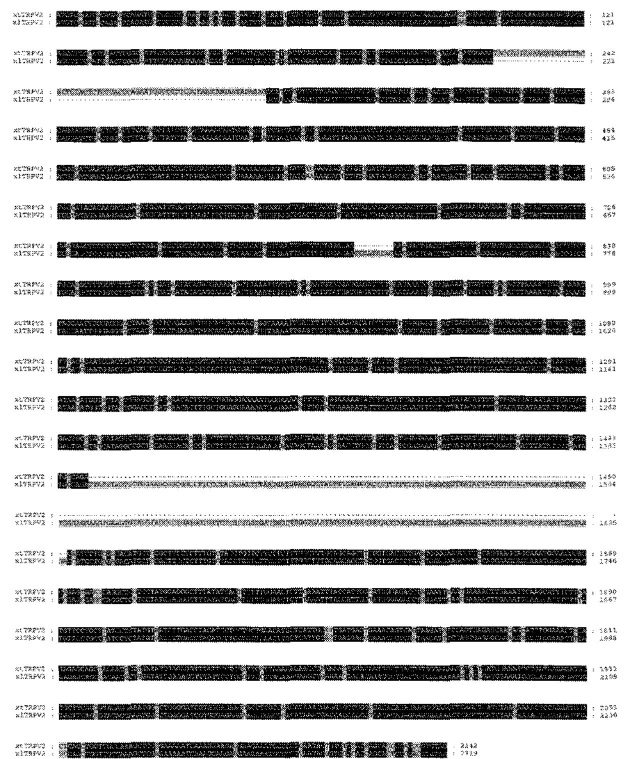


Fig. 3. Alignment of xtTRPV2 and xlTRPV2 sequence.
Black box indicates that both sequences are identical; dark gray box indicates that both sequences are 80 percent homology. Light gray means 60 percent homology and white for non-match. Note that "-" refers to gap alignment.

In order to confirm the sequence in the 5' region, 5'-RACE was carried out as shown in Fig. 2A. As a result, we confirmed the full length of TRPV2 orthologue from *Xenopus laevis*. As shown in Fig. 2B, there were 3 mismatches in the 5' start region. We named it as 'xlTRPV2' hereafter.

As represented in Fig. 3, xlTRPV2 sequence was aligned with xtTRPV2. xtTRPV2 was 2142 base pair long, whereas xlTRPV2 was 2319 base pair long. Similarity between xlTRPV2 and xtTRPV2 was very high (about 90%), although two regions were notably different.

Based on the xlTRPV2 DNA sequence, xlTRPV2 protein sequence was obtained by translation *in silico* (Fig. 4). It is composed of 773 amino acids and holds six transmembrane domains and three ankyrin repeats at N-terminal. This indicates that xlTRPV2 shares a common characteristic of other TRPV2 in terms of predicted protein topologies.

To compare the similarity between xlTRPV2 and other TRPV2 orthologues, multiple alignment was done by ClustalW (Fig. 5A) (Li, 2003). xlTRPV2 has 50%

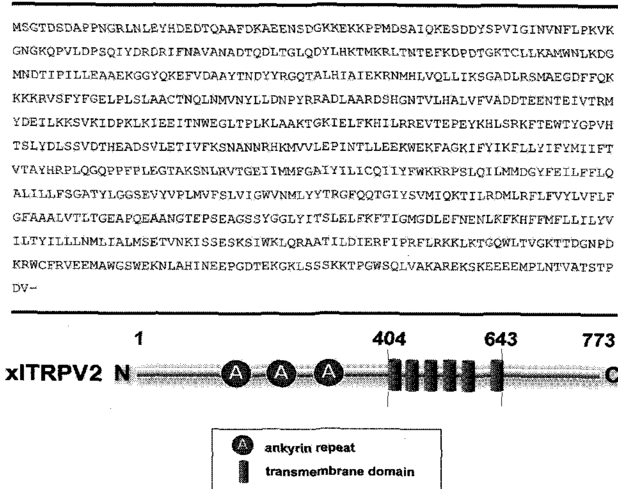


Fig. 4. Protein sequence and topology of xlTRPV2
Upper panel: xlTRPV2 amino acid sequence (Upper panel). The length of xlTRPV2 amino acid is 773. Lower Panel: xlTRPV2 topology. A putative xlTRPV2 protein topology was predicted by Simple Modular Architecture Research Tool (SMART, http://smart. embl-heidelberg.de) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM). The xlTRPV2 has six transmembrane domains and three ankyrin repeats at N-terminal.

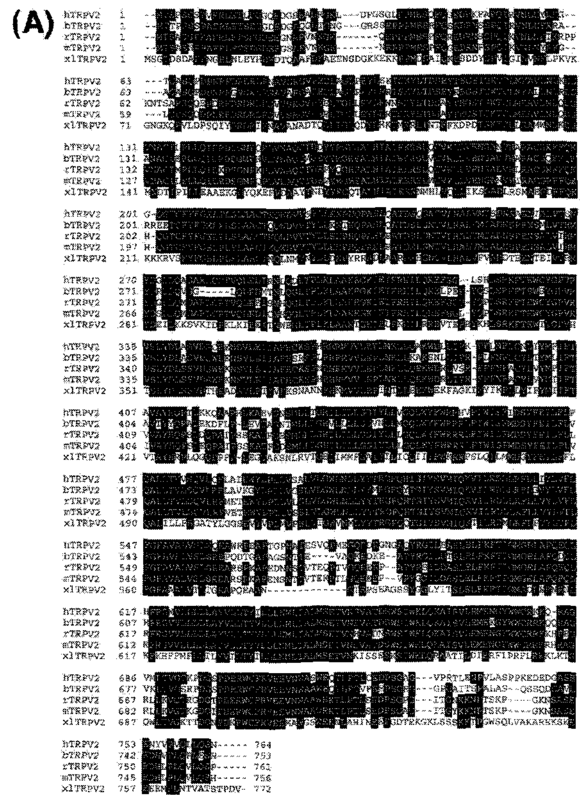
homology with human TRPV2. Using the result obtained from multiple alignments, phylogenetic tree was made by TreeView program (Fig. 5B) (Page, 1996).

Discussion

TRPV2 is heat-sensitive, non-selective cation channel found in various species including mammals. Even though mammalian TRPV2 is known, the presence of a TRPV2 orthologue in *Xenopus laevis* is not known even though frequent use of the amphibian oocytes as heterologous expression system. We thus cloned and identified a full length of TRPV2 orthologue in *Xenopus laevis*.

We initially tried to clone xlTRPV2 using the sequence of xtTRPV2. But, RT-PCR with Primer 1-2 pairs that were based on the orthologue of *Xenopus tropicalis* was unable to detect any product from selected tissues under various PCR conditions. This result was rather anticipated because there was a species difference between the predicted sequence which was xtTRPV2 and the source of PCR template DNA (*Xenopus laevis*). In addition, the reason for the RT-PCR failure would stem from an imperfect prediction of the full genome sequence of *Xenopus tropicalis*, which is often the case in prediction of exons *in silico*.

In summary, a full sequence of TRPV2 orthologue of



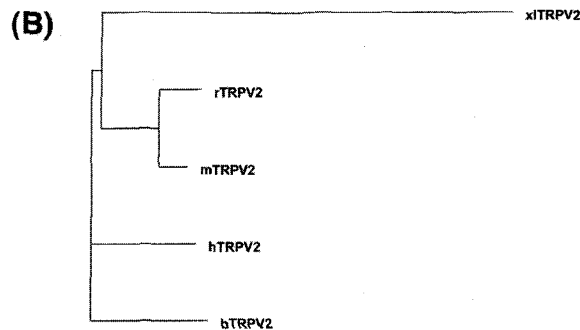


Fig. 5. Multiple alignments and Phylogenetic tree of xlTRPV2 and other TRPV2 orthologues.
(A) Multiple alignments of various TRPV2 orthologues. hTRPV2 : human, bTRPV2 : cow, rTRPV2 : rat, mTRPV2 : mouse.
(B) Based on multiple alignment result, phylogenetic tree was obtained by TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Xenopus laevis was identified. Yet, further experiments are required to characterize the function of xlTRPV2.

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