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ORIGINAL ARTICLE

Molecular Cloning, Characterization and Sequence Analysis of *KCNQ4* in Large Odorous Frog, *Odorrana graminea*

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Abstract Acoustic communication is essential for anuran survival and reproduction, and masking background noise can affect the effective acoustic communication. The larger odorous frog (*Odorrana graminea*) inhabits noise montane streams, and it has shown an ultrasound communication adaptation. However, the molecular mechanism underlying their ultrasonic hearing adaptation remains unknown. To characterize and investigate the molecular characteristics and evolution of the high-frequency hearing-sensitive gene (*KCNQ4*) in *O. graminea*, termed as *OgKCNQ4*, the rapid amplification of cDNA ends (RACE) was performed to amplify the cDNA of *OgKCNQ4*. Different bioinformatics analyses were used to investigate the molecular characteristics. Multiple nucleotide and amino acid sequence alignment were conducted, and phylogenies were reconstructed under the maximum likelihood and Bayesian approaches. The full-length cDNA of *OgKCNQ4* was 2065 bp, and the open reading frame (ORF) was 2046 bp encoding for a putative protein with 681 amino acids. The relative molecular weight of OgKCNQ4 was 76.453 kD and the putative PI was 9.69. Secondary structure prediction analyses suggested 42.29% alpha helixes and 43.76% random coils in OgKCNQ4. Gene homology and Phylogenetic analyses revealed the closest relationship between *OgKCNQ4* and *KCNQ4* of *Nanorana parkeri* with 96.9% similarity and 95.0% identity. We first determined the full-length cDNA of *OgKCNQ4* and the results here could provide foundations for further study on the evolution of *KCNQ4* and its relationship to ultrasonic communication in amphibians.

Keywords *Odorrana graminea*, *KCNQ4*, cDNA, bioinformatics analyses

1. Introduction

Acoustic communication plays an important role in the survival, reproduction and evolution of most animals, and masking background noise always affect the detection and discrimination among signals (Velez *et al*., 2013). In order to minimal masking background noise, some animals evolved ultrasonic communication, and researches on ultrasonic communication were mainly focused on mammals (e.g., whales, dolphins, bats and rodents) (reviewed in Velez *et al*., 2013). Amphibians are significant in the evolutionary history of vertebrate due to their transition from water to land, and their hearing system underwent many important morphological and functional adaptations (Webster *et al*., 1992). The large odorous frog (*Odorrana graminea*) belongs to the family Ranidae and inhabits cold swift boulder-strewn, montane streams at elevations from about 450–1200 m throughout Southern China and Southeast Asia (Fei *et al*., 2009). Previous electrophysiological studies have shown that the calls of the large odorous frog contains ultrasonic components (≥20 kHz), and they have an ultrasonic communication adaptation to the intense, predominately low-frequency ambient noise from nearby streams and waterfalls (Shen *et al*., 2011). In addition, the other two torrential frogs (i.e., *O. tormota* and *Huia Cavitympanum*) were also shown to have ultrasonic communication adaptation (Feng *et al*., 2006; Arch *et al*., 2008). However, the molecular mechanisms underlying their high-sensitive hearing adaptation is poorly documented to date.

Many genes and signaling pathways are involved in acoustic communication, the voltage-gated potassium

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channel subfamily KQT member 4 (*KCNQ4*) gene is significantly expressed in the inner ear and the central auditory pathway, and it encodes a potassium channel protein (Kharkovets *et al*., 2000). Interestingly, previous studies have suggested that the *KCNQ4* gene was associated with high-frequency hearing, and mutations of *KCNQ4* gene in humans and mice could cause non-syndromic DFNA2 hereditary deafness (Kubisch *et al*., 1999; Kharkovets *et al*., 2006). In addition, previous molecular studies on bat echolocation revealed that the *KCNQ4* gene underwent parallel evolution in echolocating bats (Liu *et al*., 2011; Liu *et al*., 2012), suggesting the important role of KCNQ4 in high-frequency hearing.

We want to determine whether the genetic variations of *KCNQ4* in amphibians are associated with their high-frequency hearing based on the evolutionary analysis of amphibian *KCNQ4* genes. However, to date the *KCNQ4* gene were only identified and characterized in three amphibian species (e.g., *Nanorana parkeri*, *Xenopus laevis* and *Xenopus tropicalis*) based on the genomic sequencing. In the present study, we first determined and analyzed the full-length cDNA of *KCNQ4* in one of the three high-frequency hearing frogs (*O. graminea*). The results here could provide foundations for further study on the evolution of *KCNQ4* and its relationship to ultrasonic communication in amphibians.

2. Materials and Methods

2.1. Sample collection and ethics statement Samples of *O. graminea* were collected from the Huangshan mountain, Anhui province (30°04' N, 118°08' E). Sampling was conducted according to all the ethical guidelines and legal requirements in China. The animal-use protocols of this study were approved by the Institutional Care and Ethics Committee of Henan Normal University.

2.2. RNA isolation and cDNA synthesis Total RNA was isolated from the brain tissue of *O. graminea* using RNeasy Mini Kit (QIAGEN, Germany) according to manufacturer's instructions. The RNA integrity was determined by electrophoresis on 1% agarose gel electrophoresis, and the concentrations were assessed spectrophotometrically by measuring their absorbance at 260 nm and 280 nm using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The first-strand cDNAs were synthesized using the PrimeScriptTM II 1ST Strand cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's instructions, and then stored at –80°C for further use.

2.3. Amplification and sequencing of *OgKCNQ4* gene Degenerate primers of the intermediate fragments of *KCNQ4* gene were first designed with Primer Premier 5.0 (Premier Biosoft International, CA, USA) based on an alignment of *KCNQ4* sequences from *N. parkeri*, *X. laevis* and *X. tropicalis*, and then the 5'/3' RACE primers were designed according to the intermediate fragments of Og*KCNQ4* amplified. The primer information is shown in Table 1. We first amplified partial intermediate sequences of the conserved region. The cycling protocol was one cycle of 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 40 s, followed by one cycle of 72°C for 10 min. The 5' and 3' RACE were then performed using a SMARTer RACE cDNA amplification kit and SMARTer RACE kit (TaKaRa) according to the manufacturer's instructions. The amplified PCR products were purified with a MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, China), cloned into Pmd18-T vectors, and then sequenced in both directions using an ABI 3730

Table 1 Primers used to amplify *OgKCNQ4* in the present study.

Primer name	Primer sequence (5'-3')
OGKINTERF	GTGCCVTCSYTGTCCYT
OGKINTERR	TCRTCTCCWGSWCCCTC
OGK5' RACE1	AGCACCCAGCCGACCAGAT
OGK5' RACE2	ATGCCAAACACGACAATCA
OGK3' RACEF1	GAACCCGCAGCCGAAATGG
OGK3' RACEF2	GCAGAGGGAGCAGGAGATG
3'RACEOlig(T)-Adaptor	CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTT
3'RACE Adaptor	CTGATCTAGAGGTACCGGATCC
5'RACEOlig(T)-Adaptor	GACTCGAGTCGACATCGATTTTTTTTTTTTTTT
5'RACE Adaptor	GACTCGAGTCGACATCG

Note: R = A/G; Y = C/T; V = A/C/G; S = C/G; W = A/T.

automated genetic analyzer (Applied Biosystems) by Shanghai Sangon Biological Engineering Techonology and Service Co., Ltd. Five to six repeated amplifications were conducted and sequenced to confirm its sequence. The same PCR primers were used for sequencing. The newly *KCNQ4* sequence was deposited in GenBank under accession number: MK956830.

2.4. Sequence analysis of *OgKCNQ4* and protein structure prediction The chromatograms of each sequence were proofread and assembled with the program DNASTAR SeqMan v7.21 (DNASTAR Inc., Madison, WI, USA), and Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was also performed to confirm the sequence. The open reading frame (ORF) was determined using the program DNASTAR EditSeq (DNASTAR Inc., Madison, WI, USA) based on the full-length cDNA sequence of the verified *OgKCNQ4* gene. The putative pI and molecular weight of the predicted OgKCNQ4 protein were estimated using the Online software Compute pI/Mw (http://web.Expasy.org/tool/pi_tool.html) (Gasteiger *et al*., 2005). The ProtScale (http://web.Expasy.org//protscale/) was used to estimate the hydrophilic and hydrophobic properties of OgKCNQ4. The transmembrane structure of OgKCNQ4 was analyzed by TMpred Server. The secondary structure of OgKCNQ4 protein was predicted with PORTER (http://distill.ucd.i.e./porter). Three-dimensional domain structure of OgKCNQ4 protein was predicted using the SWISS-MODEL Server (http://swissmodel.Expasy.Org/swissmod/ SWISS-MODEL.html) (Schwede *et al*., 2003).

2.5. Evolutionary analysis of *OgKCNQ4* To reveal the evolutionary history of *OgKCNQ4* gene, we searched and downloaded *KCNQ4* gene sequences of other thirteen vertebrate species from NCBI (http://www.ncbi.nlm.nih.gov/). The taxonomic and sequence information are shown in Table 2. All nucleotide sequences from the ORFs of *KCNQ4* and their deduced amino acid sequences were first aligned separately using MUSCLE v3.8 (Edgar, 2004) implemented in MEGA v5.0 (Tamura *et al*., 2011) under default settings, and then manually adjusted with GeneDoc. The nucleotide sequence alignment was generated based on the protein sequence alignment. The similarity and identity of the *OgKCNQ4* with other vertebrate KCNQ4 genes was calculated using the MatGAT program with default parameters (Campanella *et al*., 2003). The evolutionary relationship of *KCNQ4* was determined using the Bayesian inference (BI) in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) and maximum likelihood (ML) algorithms in MetaPIGA v2.0 (Helaers and Milinkovitch, 2010) based on both of the nucleotide and amino acids alignment. *Latimeria chalumnae* was utilized as outgroup for the phylogenetic analyses based on Anderson and Wiens (2017). Modeltest v3.7 (Posada and Crandall, 1998) was used to select the optimal nucleotide substitution models based on the Akaike Information Criterion (AIC), and the best-fit substitution model selected for mtDNA dataset is GTR+I+G model. Maximum likelihood analyses were conducted using MetaPIGA v2.0 (Helaers and Milinkovitch, 2010) with 1000 metaGA replicate searches. The Bayesian analyses were conducted with four (one cold and three heated) Metropolis-coupled Markov chain Monte Carlo iterations for twenty million generations with default heating values and trees were sampled every 1000 generation. The first 10% of trees were deleted as the "burn-in" stage and the remaining trees were used to generate the consensus tree

Table 2 Sequences used in the present study and results of homology analysis between OgKCNQ4 protein and 13 other vertebrates.

Class	Order	Family	Scientific name	Accession number	Similarity (%)	Identity (%)
Amphibian	Anura	Dicroglossidae	*Nanorana parkeri*	XM_018576753.1	96.9	95.0
		Pipidae	*Xenopus tropicalis*	XM_012957340.2	93.7	89.6
Reptilia	Squamata	Elapidae	*Pseudonaja textilis*	XM_026702686.1	69.5	61.2
			Notechis scutatus	XM_026673682.1	69.5	61.0
Aves	Columbiformes	Columbidae	*Columba livia*	XM_021297453.1	67.3	59.7
Mammalia	Primates	Hominidae	*Homo sapiens*	AF105202.1	78.9	69.5
			Pan troglodytes	XM_513360.4	79.1	69.5
	Rodentia	Muridae	*Mus musculus*	NM_001081142.2	79.1	69.7
	Cetartiodactyla	Delphinidae	*Tursiops truncatus*	XM_019938027.1	56.7	50.7
			Lagenorhynchus obliquidens	XM_027108941.1	78.8	69.1
Actinopterygii	Salmoniformes	Esocidae	*Esox lucius*	XM_010873558.2	78.3	69.2
	Tetrodontiformes	Tetraodontidae	*Takifugu rubripes*	XM_003968897.2	77.3	69.2
Sarcopterygii	Coelacanthiformes	Latimeriidae	*Latimeria chalumnae*	XM_014489102.1	78.5	71.3

and calculate Bayesian posterior probabilities (PP). The stationarity of the likelihood scores of sampled trees was determined using Tracer v1.4 (Rambaut, 2007).

3. Results

3.1. Characterization of the cDNA sequence of *OgKCNQ4* The full-length cDNA of *OgKCNQ4* was 2065 bp, including a 7 bp 5'-terminal untranslated region (UTR), a 12bp 3'-UTR, and a 2046 bp open reading frame (ORF) region. The ORF encodes a putative OgKCNQ4 protein with 681 amino acids (Figure 1). The estimated molecular weight and theoretical pI of the putative OgKCNQ4 protein were 76.453 kD and 9.69, respectively. The putative OgKCNQ4 protein consisted with 299 hydrophobic amino acids (A, I, L, F, W, V, M, P), 382 polar amino acids (G, S, Y, C, T, N, Q, K, R, H, D, E), 61 strongly acidic amino acids (D, E), and 90 strongly basic amino acids (K, R).

As shown in Table 3, the secondary structures of OgKCNQ4 protein are mainly alpha helix (42.29%) and random coils (43.76%), whereas the proportion of Beta turn and extended strand structure is low (3.82%

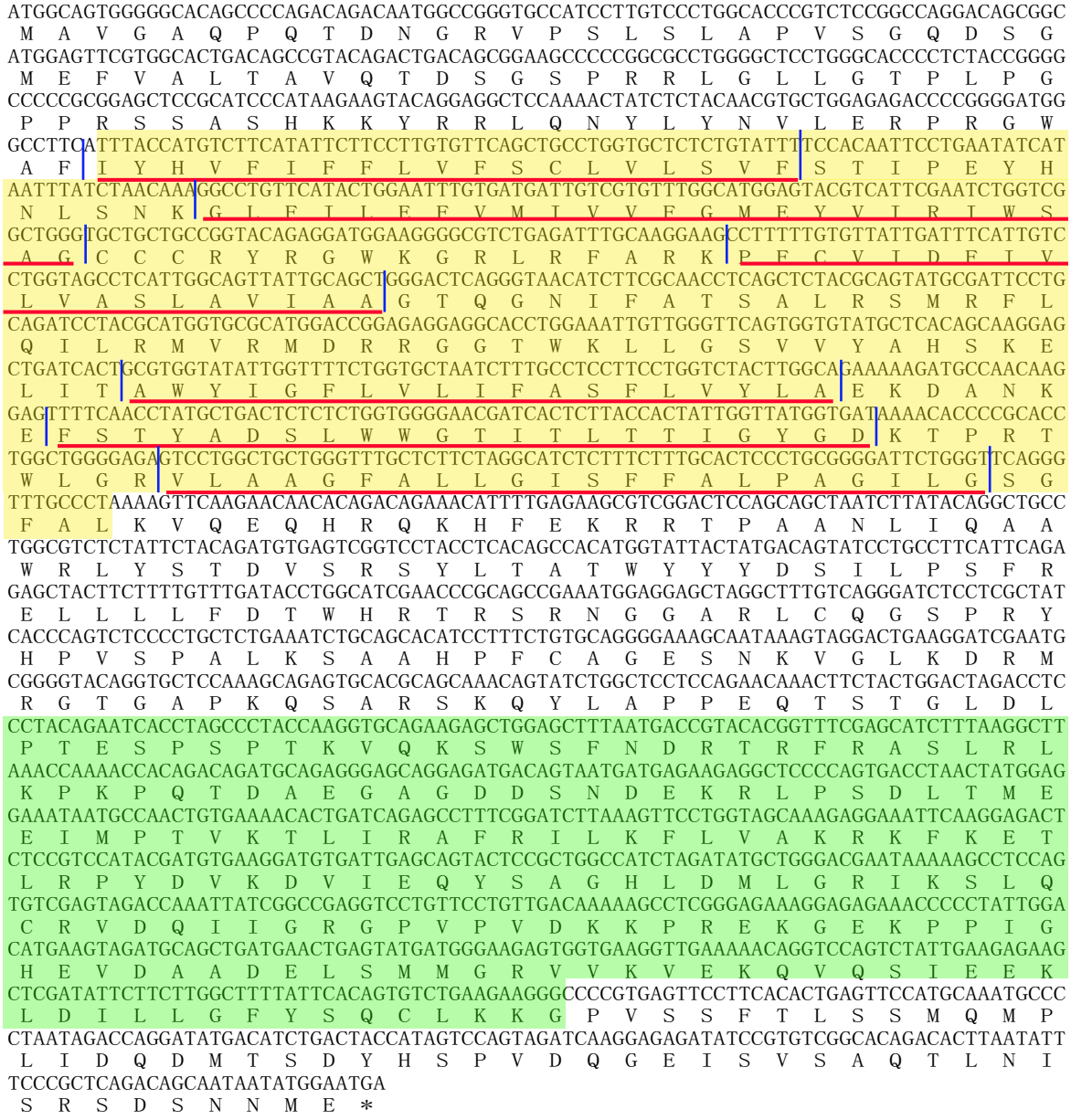

Figure 1 Full-length open reading frame (ORF), deduced amino acid sequence and domain structure of OgKCNQ4. The six transmembrane structures are highlighted with red line under the related amino acids. The ion transport domain and potassium ion channel domain are highlighted by yellow and green boxes, respectively.

Table 3 The proportion of secondary structure of OgKCNQ4 protein in *O. graminea.*

Structure	Proportion
Alpha helix	42.29%
Extended strand	10.13%
Beta turn	3.82%
Random coil	43.76%

and 10.13%, respectively). Six transmembrane domains (each is composed of 19–25 amino acids) were identified within OgKCNQ4 protein, and the OgKCNQ4 protein also possessed the characteristic N-terminal ion transport functional domain (87–311) and the potassium ion channel functional domain (449–631) (Figure 1). The tertiary structure of putative OgKCNQ4 protein is mainly consisted of alpha helixes and random coils (Figure 2).

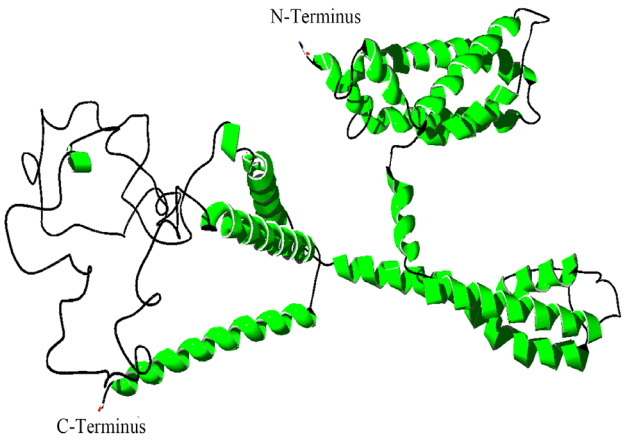

Figure 2 Three-dimensional structure of OgKCNQ4. Backbone ribbon and the secondary structure topology are shown: alpha helixes are shown in green. Amino and carboxy terminal ends are indicated.

3.2. Alignments and evolutionary analysis of *OgKCNQ4* As shown in Figure 3, the homology analysis of OgKCNQ4 protein sequence with other vertebrate KCNQ4s revealed strong conservation in the ion transport domain and the potassium ion channel domain. The OgKCNQ4 protein had the highest identity (95.0%) and similarity (96.9%) to the KCNQ4 protein in *Nanorana parkeri*, and shared 67.3%–93.7% similarity and 59.7%–89.6% with the other vertebrate KCNQ4 homologues (Table 2). To explore the evolutionary relationship between the *OgKCNQ4* and other KCNQ4 genes, thirteen other representative vertebrate KCNQ4 sequences were used to construct the phylogenetic tree using MrBayes method with *Latimeria chalumnae* as outgroup. The Bayesian and ML analyses of nucleotide and amino acids datasets produced smiliar topologies, and both Bayesian posterior probability (PP) and bootstrp support (BP) are repesented on the BI tree (Figure 4 and Figure S1). The monophyly of the tetrapods (i.e., amphbians, reptiles, birds and mammals), and each of the four major groups were strongly supported (PP = 1.0, BP = 100). The *OgKCNQ4* showed closest relationship with *N. parkeri*, and they together formed a sister-group relationship with *Xenopus tropicalis*. However, the phylogenetic position of the representative bird was inconsistent between the amino acids dataset and nucleotide dataset (Figures 4 and S1). Representatives of birds and reptiles were grouped as the sister taxa to mammals with strong supports based on the analyses of the amino acids dataset, whereas the nucleotide analyses revealed a sister group relationship of bird with mammals (Figures 4 and S1).

4. Discussion

To date, although *KCNQ4* genes have cloned and sequenced in many vertebrates (reviewed at NCBI: http://www.ncbi.nlm.nih.gov/), full-length cDNA sequences were available only in three amphibians (i.e., *N. parkeri*, *X. laevis* and *X. tropicalis*) (Hellsten *et al*., 2010; Sun *et al*., 2015). In the present study, we first cloned and characterized the full-length cDNA of *OgKCNQ4* in one of the three high-frequency hearing frogs (*O. graminea*) (Feng *et al*., 2006; Arch *et al*., 2008; Shen *et al*., 2011). Comparison of the deduced amino acid sequence with those of other amphibians and mouse showed that these secondary structural features of OgKCNQ4 in *O. graminea* were similar to those previously reported vertebrate (Kubisch *et al*., 1999; Kharkovets *et al*., 2006; Heidenreich *et al*., 2012). The six transmembrane domains, the two characteristic ion transport domain and potassium ion channel domain in OgKCNQ4 predicted protein were also found in humans, mouse and other mammals (Kubisch *et al*., 1999; Kharkovets *et al*., 2006; Xu *et al*., 2013).

KCNQ4 were shown to play important roles in the basolateral K^+ conductance, which contributes to the modulation of electrical excitation and the removal of intracellular K^+ from the hair cells (Kubisch *et al*., 1999). Mutations in KCNQ4 were found to be associated with an autosomal dominant progressive hearing loss in humans (DFNA2) (Kubisch *et al*., 1999). Hearing and acoustic communication is important for land vertebrates, and many genes involved in the sound signal transduction were conserved and under purifying selection (Liu *et al*.,

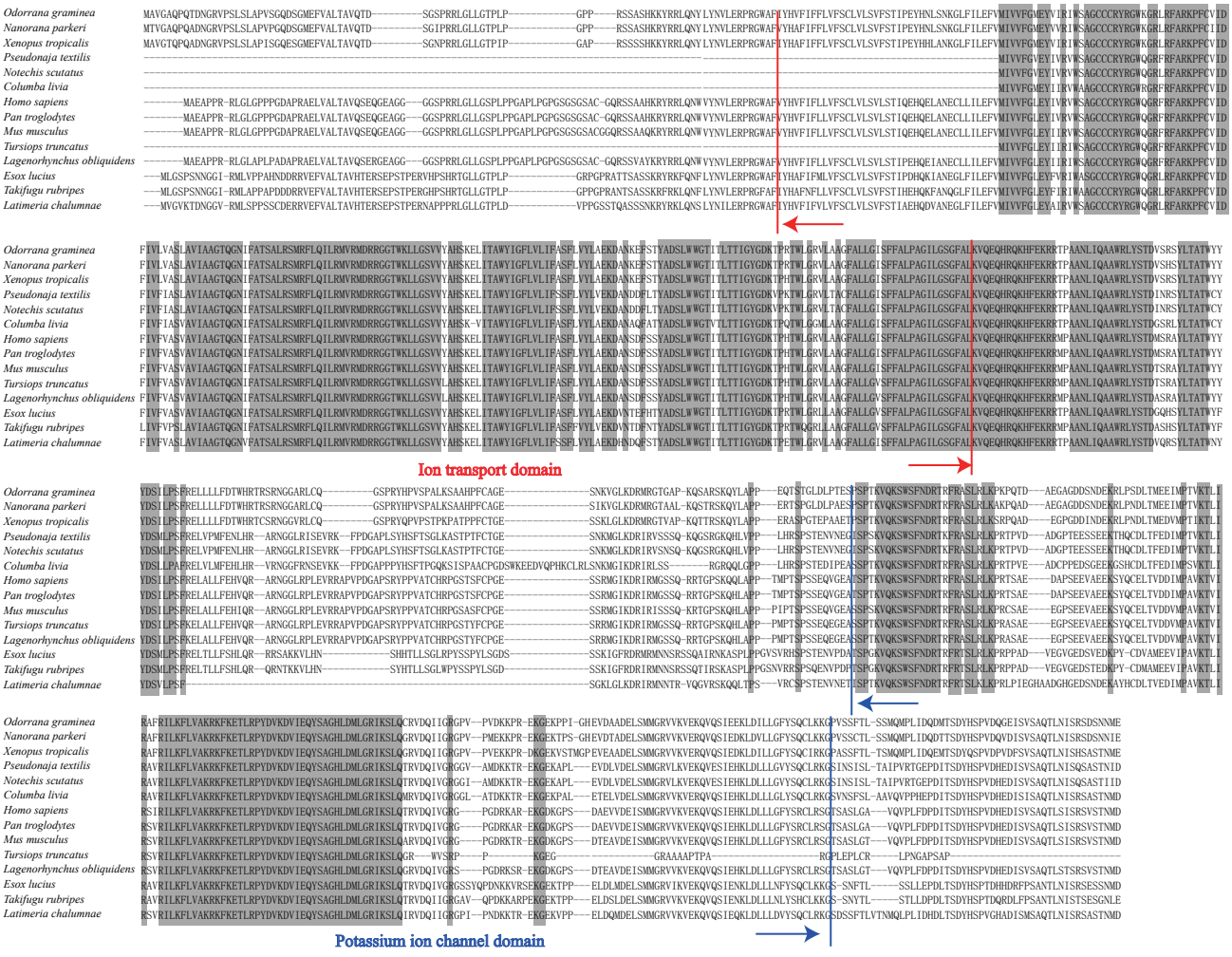

Figure 3 Multiple alignments of the deduced amino acid sequences of OgKCNQ4 with other 13 representative KCNQ4 sequences. Similar amino acids are highlighted by gray boxes. The positions of the ion transport domain and potassium ion channel domain are indicated.

2011; Shen *et al*., 2012). The strong conservatism in the ion transport domain and the potassium ion channel domain between OgKCNQ4 and other vertebrate KCNQ4 proteins found in the present study further suggested the important function of KCNQ4 protein among vertebrates (Kubisch *et al*., 1999; Kharkovets *et al*., 2006). In addition, the phylogenetic tree of KCNQ4 from representative vertebrate species shows that the OgKCNQ4 was nested within amphibian, and relationships among the higher taxonomic level based on the amino acids dataset were in general accordance with the taxonomy of Anderson and Wiens (2017).

The *KCNQ4* were shown to exclusively express in the outer hair cells in mouse cochlea (Kubisch *et al*., 1999), whereas Kharkovets *et al*. (2000) found the KCNQ4 was also expressed in the mouse auditory system (inner ear and brain). Whether the KCNQ4 is expressed in the brain of other animals is still unclear. Interestingly, we cloned and characterized the KCNQ4 gene from the brain tissue of the *O. graminea*, which further suggesting the important role of *KCNQ4* in the maturation of the auditory function. Whether adaptive evolution occurred on *KCNQ4* in amphibians and its relationship to the high-frequency hearing in amphibians should be further investigated with more amphibian *KCNQ4* characterized. In conclusion, the present study first characterized the *OgKCNQ4* in one of the three high-frequency hearing amphibians, and the results here could also provide foundations for further study on the evolution of *KCNQ4* and its relationship to ultrasonic communication in amphibians.

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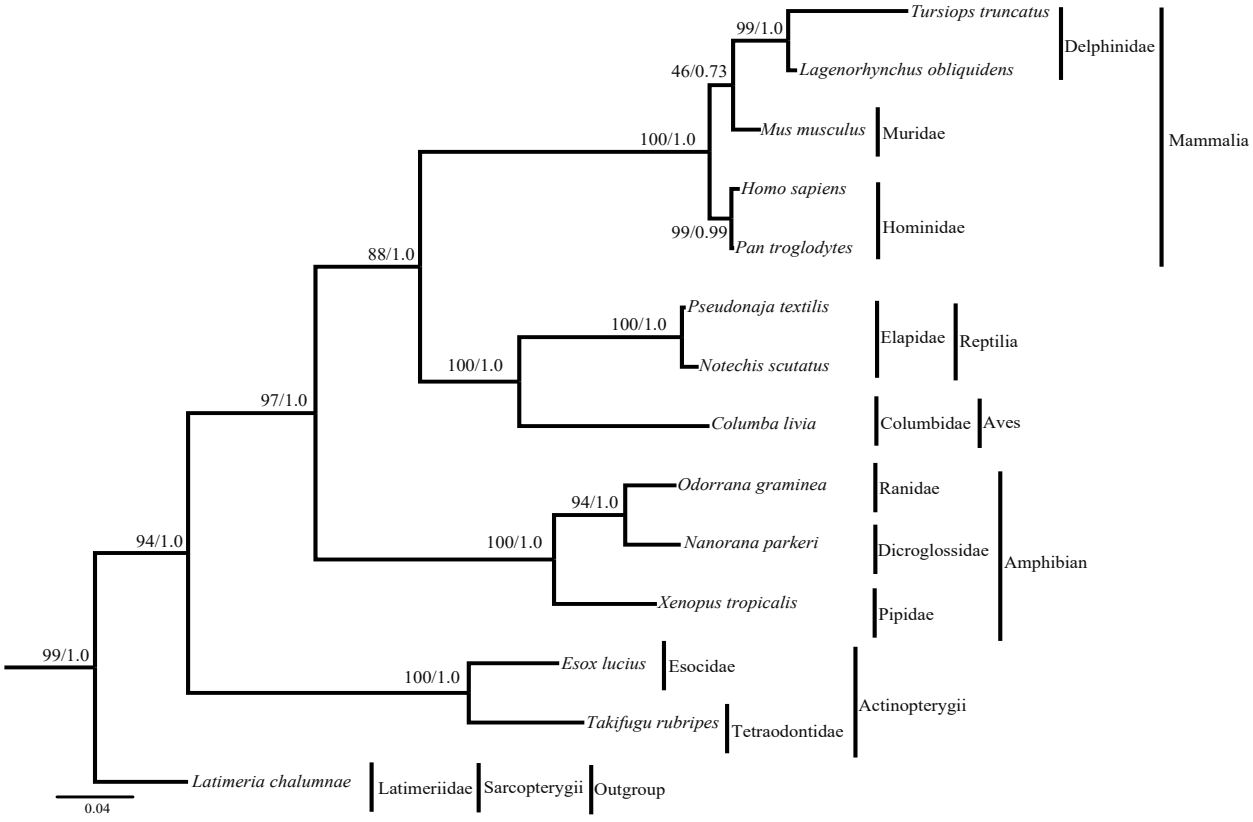

Figure 4 Phylogenetic relationship of *OgKCNQ4* with other 13 representative vertebrate *KCNQ4* constructed based on amino acid sequences by MrBayes method. Integers associated with branches are bootstrap support values for ML inference whereas values of 1 or less are Bayesian posterior probabilities. Representative members are delimited by vertical lines to the right of the tree.

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Appendix

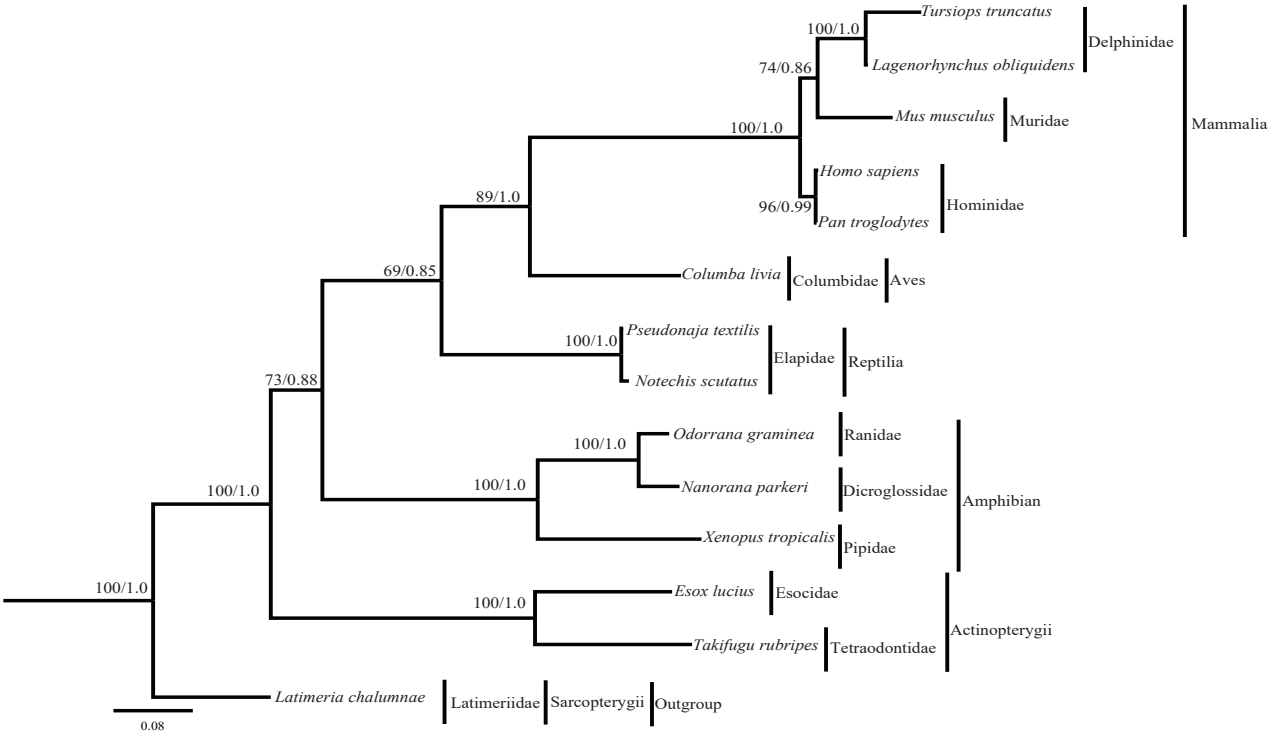

Figure S1 Phylogenetic relationship of *OgKCNQ4* with other 13 representative vertebrate *KCNQ4* constructed based on nucleotide sequences by MrBayes method. Integers associated with branches are bootstrap support values for ML inference whereas values of 1 or less are Bayesian posterior probabilities. Representative members are delimited by vertical lines to the right of the tree.