Gene 626 (2017) 367–375

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

# The complete mitochondrial genome sequence of the Sichuan Digging Frog, *Kaloula rugifera* (Anura: Microhylidae) and its phylogenetic implications

Lichun Jiang[a,b], Li Zhao[b], Dongmei Cheng[b], Lilan Zhu[b], Min Zhang[b], Qiping Ruan[b,*], Wei Chen[a,**]

[a] Ecological Security and Protection Key Laboratory of Sichuan Province, Mianyang Normal University, Mianyang, Sichuan 621000, PR China
[b] Key Laboratory for Molecular Biology and Biopharmaceutics, School of Life Science and Technology, Mianyang Normal University, Mianyang, Sichuan 621000, PR China

ARTICLE INFO

*Keywords:*
*Kaloula rugifera*
Microhylidae
Mitochondrial genome
Proteincoding genes
Control region
Phylogeny

ABSTRACT

The Sichuan Digging Frog (*Kaloula rugifera*) belongs to the family Dicroglossidae, which is endemic to northeastern Sichuan and southernmost Gansu provinces, in southwestern China. In this study, the complete mitochondrial genome of *K. rugifera* was sequenced. The mitogenome was 17,074 bp in length, consisting of 13 protein-coding genes, 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, and a non-coding control region. As in other vertebrates, most mitochondrial genes are encoded on the heavy strand, except for ND6 and eight tRNA genes which are encoded on the light strand. The overall base composition of the *K. rugifera* is 30.32% A, 25.76% C, 29.72% T, and 14.20% G, which is consistent with the lowest frequency for G content in typical amphibian animals' mitochondrial genomes. The alignment of the Kaloula species control regions exhibited high genetic variability and rich A + T content. Besides, 3 types of tandem repeat units were also identified in the control region. Phylogenetic tree demonstrated that *K. rugifera* was clustered together with *K. borealis* and *K. verrucosa* and they had a close relationship with each other. The complete mitogenome of *K. rugifera* can provide an important data for the studies on phylogenetic relationship to further explore the taxonomic status of Kaloula species.

## 1. Introduction

The near threatened Sichuan Digging Frog (*Kaloula rugifera*) belongs to the family Microhylidae which is endemic to northeastern Sichuan and southernmost Gansu provinces, in southwestern China (Frost, 2014; Fei and Xie, 2004). It is found in hilly areas near villages and tree-holes. It breeds in rain-filled, temporary pools and ponds. Its population has been decreased significantly due to habitat loss associated with infrastructure development (Fei and Xie, 2004). The genus *Kaloula* Gray, 1831 (Anura: Microhylidae) is pan Asian in distribution which range from Korea and northern China to the Lesser Sundas and the Philippines, Bangladesh, India, and Sri Lanka and currently contains 15 recognized species (Das et al., 2004, Diesmos et al., 2014; Iskadar and Colijn, 2000; Sengupta et al., 2009). Presently, only four species of *Kaloula* (*K. borealis* Barbour, 1908, *K. pulchra* Gray, 1831, *K. rugifera* Stejneger, 1924 and *K. verrucosa* Boulenger, 1904) have been reported in China (Fei et al., 2009). Among them, *K. rugifera* and *K. verrucosa* are endemic species, the former mainly restricted to Gansu and Sichuan Provinces, the latter restricted to Yunnan, Guizhou and Sichuan Provinces (Mo et al., 2013).

In the last decade, mitochondrial genomes have been studied increasingly because of the ease of recovering genetic information that may be useful for investigating molecular evolution as well as for phylogenetic and biogeographic studies (Bossuyt et al., 2006; Jiang et al., 2005; Matsui et al., 2011). Because of the extensive use of its individual genes across a wide range of studies, mitogenomes had been great impacts on vertebrate phylogenetics and molecular genetics. Recently, with the development of sequencing technologies, such as the next generation sequencing technology, mitogenomes can be sequenced reliably, cheaply and rapidly for almost all organisms (Sahoo et al., 2015; Li et al., 2015; Mu et al., 2015). In parallel to these general developments, there has been a gradual increase in available

*Abbreviations:* PCR, Polymerase chain reaction; ATP6 and ATP8, Subunits 6 and 8 of the F0 ATPase; Cyt*b*, Cytochrome *b*; COI–COIII, Cytochrome c oxidase subunits 1–3; Ile, Isoleucine; Leu, Leucine; ND1–ND6 and ND4L, NADH dehydrogenase subunit 1–6 and 4 L; 16S rRNA and 12S rRNA, Large and small subunit of ribosomal RNA genes; tRNAXXX, Genes encoding for transfer RNA molecules with corresponding amino acids denoted with a three-letter code and anticodon indicated in parentheses (XXX) when necessary; A + T rich region, Adenine + thymine-rich region; PCG, Protein coding gene; CR, Control region; TAS, Termination-associated sequences; CSB1–3, Conserved sequence blocks 1–3; $O_L$, Light-strand replication; H strand, Heavy strand; L strand, Light strand

* Corresponding author at: Key Laboratory for Molecular Biology and Biopharmaceutics, Mianyang Normal University, Mianyang, Sichuan 621000, PR China.
** Corresponding author.
*E-mail addresses:* qpruan20141230@163.com (Q. Ruan), wchen1949@gmail.com (W. Chen).

http://dx.doi.org/10.1016/j.gene.2017.05.039
Received 2 February 2017; Received in revised form 30 April 2017; Accepted 19 May 2017
Available online 20 May 2017
0378-1119/ 

mitochondrial genome data for amphibian animal (Zhang et al., 2005; Kurabayashi et al., 2011). The Digging Frog research mainly focuses on the study of morphology (Sengupta et al., 2009). In recent years, the use of mitochondrial DNA as a molecular marker has been widely used in the development of amphibian systems (Cannatella et al., 1998; Köhler and Günther, 2008; Kurabayashi et al., 2010). At present, the system development of genus *Kaloula* mentioned only the phylogenetic study in Microhylidae family or subfamily (van der Meijden et al., 2007; Matsui et al., 2011). In these studies, the monophyletic group of genus *Kaloula* is still controversial. Therefore the taxonomic and systematic relationships of *Kaloula* species still need further studies.

To further identify and protect the *Kaloula* species, we determined the complete mitogenome sequence of *K. rugifera*. In this study, the complete mitogenome of *K. rugifera* was determined, and its genome organization, gene arrangement and characterization were described. Moreover, *K. rugifera* was compared with *K. borealis* (NC_020044) and *K. pulchra* (NC_006405) based on different mitogenomic aspects. In addition, 12 complete mitochondrial genomes of the Microhyloidea were collected. Using the maximum likelihood (ML) and Bayesian inference (BI) methods, 13 protein-coding gene regions of the mtDNAs were used to separately perform molecular phylogenetic analyses to provide more information on the relationships among the genus *Kaloula*.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

The Sichuan Digging Frog, *Kaloula rugifera* were collected from the Youxian district, Mianyang city, Sichuan province, China in September 2015. The muscle was preserved in 95% ethanol and stored at − 80 °C until DNA extraction was performed. Whole genomic DNA (i.e. genomic and mitochondrial) was extracted from two adults using the protocol of Genomic DNA Extraction Kit (Tiangen, China) according to the manufacturer's instruction. Total DNA was diluted to approximately 20 ng/μL for polymerase chain reaction (PCR).

### 2.2. PCR amplifications and DNA sequencing

The entire mitochondrial genome was amplified in 12 overlapping segments by PCR with Taq DNA Polymerase (TaKaRa, China), using 10 ng of total genomic DNA from the sample as template. Complete mtDNA was amplified as concatenated sequences using selectively amplified mtDNA template and nine primer pairs derived from the literature (Kurabayashi and Sumida, 2009). Partial PCR primers were designed based on the alignments of the relatively conserved regions of congeneric *K. borealis* (NC_020044) and *K. pulchra* (NC_006405). 12 sets of primers were used for determining the mitochondrial DNA sequence of the *K. rugifera*. PCR amplification was performed in a total volume of 25 μL with 0.2 μL of rTaq or LA Taq (TaKaRa Co., Dalian, China), 1.0 μL of DNA, 2.5 μL 10 × rTaq buffer ($Mg^{2+}$ free), 2.5 μL 25 mM $MgCl_2$, 2.0 μL dNTPs, and 0.5 μL each primer. The PCR reaction protocol were initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C (30 s), annealing at 52–60 °C (30 s) and extension at 72 °C (90–180 s) followed by a final extension at 72 °C for 10 min. The PCR products were electrophoresed on a 1.0% agarose gel. Each amplicon was then purified using the E.Z.N.A Gel Extraction Kit (Omega Bio-tek, USA) and subjected to automated sequencing using an ABI 3730 sequencer, either directly or following sub-cloning into the pMD19-T vector (TaKaRa, China). To ensure maximum accuracy, each amplicon was sequenced twice independently and a third PCR product was sequenced to avoid discrepancies.

### 2.3. Assembly, annotation and analysis of the mitochondrial genome

DNA sequences were aligned using the software MEGA 6.0 (Tamura et al., 2013). Twelve mtDNA fragments were assembled by DNA BASER 3.2.4 software (http://www. DNABaser.com). Locations of protein-coding and rRNA genes were annotated by comparisons with genes from *K. borealis* (NC_020044) and *K. pulchra* (NC_006405). Protein coding gene (PCG) boundaries were identified by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). In addition, identification of tRNA genes was performed using the program tRNAscan-SE 1.21 (Lowe and Eddy, 1997; Laslett and Canback, 2008). The potential cloverleaf secondary structures within these tRNA gene sequences were calculated using the tRNAscan-SE Search Server available online (http://lowelab.ucsc.edu/tRNAscan-SE/). Undefined tRNAs were compared with the nucleotide sequences of *K. borealis* (NC_020044), *K. pulchra* (NC_006405) and *K. rugifera* (KP682314) (Zhang et al., 2005; Deng et al., 2016), and identified by proposed secondary structures (Kumazawa and Nishida, 1993) and investigation of the anti-codon.

The A + T-content of each gene and the whole genome were calculated via DNA frequency analysis (http://kd.lab.nig.ac.jp/mishima/nucfreq1.html). Nucleotide composition at each codon position of the protein coding genes (PCGs) was calculated using PAUP ver. 4.0b10 (Swofford, 2002). Gene overlap and intergenic-space sequences were hand-counted. Nucleotide composition, termed "compositional skew" was calculated for the PCGs between two strands and the whole genome with the EditSeq program included in the Lasergene software package (www.dnastar.com) using the following formula proposed by Perna and Kocher (1995): GC-skew = (G − C) / (G + C) and AT-skew = (A − T) / (A + T), where C, G, A and T are the frequencies of the four bases. The putative origin of light-strand replication ($O_L$) and control region were identified by comparison with the homologous sequences of other Digging Frog and proposed secondary structures. The secondary structure of the putative $O_L$ was analyzed with the program Mfold v.3.2 (http://mfold.bioinfo.rpi.edu/) with default settings (Zuker, 2003) and visualized using RNAViz (De Rijk and De Wachter, 1997).

### 2.4. Phylogenetic analysis

Phylogenetic analyses were performed with the 13 complete mitochondrial genomes of the Microhyloidea species from GenBank (Table 5). To determine the systematic status of *K. rugifera*, all currently available and complete mitochondrial genomes of Microhyloidea were used in the phylogenetic analysis, with setting the *Rhacophorus schlegelii* (AB202078) and *Buergeria buergeri* (AB127977) as outgroups. The concatenated sequences of the 13 protein-coding genes of the complete mitochondrial genomes were used for phylogenetic analysis. The 13 mitochondrial protein-coding genes sequences were aligned by Clustal X 1.83 (Thompson et al., 1997) with the default settings. Base composition was examined using MEGA 6.0 (Tamura et al., 2013). The optimal nucleotide substitution model was selected using jModeltest v.0.1.1 (Posada, 2008) under the Akaike Information Criterion (Posada and Buckley, 2004). ML analysis was performed using PhyML v.3.0 (Guindon and Gascuel, 2003). The confidence level (Felsenstein, 1985) at each branch was evaluated by allowing four substitution rate categories and performing bootstrapping with 1000 replicates in ML analysis. BI analysis was carried out using MrBayes v.3.1.2 (Huelsenback and Ronquist, 2001). Bayesian posterior probabilities were estimated using the Markov chain Monte Carlo (MCMC) sampling approach. The program initiated with randomly generated trees and ran for $3 \times 10^6$ generations in which a total of $3 \times 10^4$ trees were sampled at the intervals of every 100 generations, and then the first 25% of these sampled trees were discarded as burn-in. Tracer v.1.4 (Rambaut and Drummond, 2007) was used to verify that sampled values of log likelihood plotted against generation time reached stationarity. Finally, a majority-rule consensus tree was generated from the remaining trees. For ML and BI analysis, an optimum model of GTR + I + G (nst = 6; rates = gamma) was selected.

To further explore the evolutionary relationships within *Kaloula*,

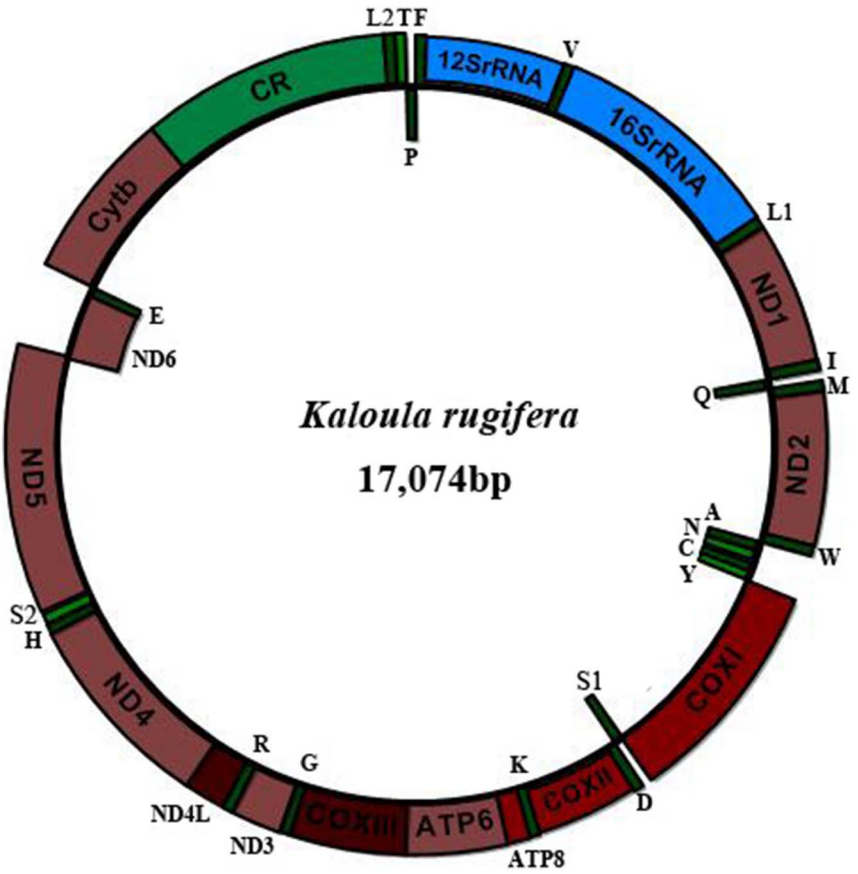


**Fig. 1.** Complete mitochondrial genome organization and gene arrangement of *Kaloula rugifera*. The asterisk indicates L-stand encoding genes. Genes coded on the H strand are directed to the outer ring, while the genes coded on the L strand are indicated in the interior of the ring. Genes are abbreviated as follows: ATP6 and ATP8 (subunits 6 and 8 of ATPase), COXI-COXIII (cytochrome *c* oxidase subunits 1–3), Cyt*b* (cytochrome *b*), ND1-ND6 and ND4L (NADH dehydrogenase subunits 1–6 and 4L), 12S rRNA and 16S rRNA (ribosomal RNA of 12S and 16S), CR (control region; noncoding region). One-letter amino acid abbreviations were used to label the corresponding tRNA genes.

12S rRNA and 16S rRNA concatenated sequences were used to construct phylogenetic trees with BI and ML. The methods are the same to complete mitochondrial genomes (13 protein-coding genes). The optimum ML and BI model of 12S rRNA and 16S rRNA sequences are TIM1 + G (nst = 6; rates = gamma). The 35 12S rRNA and 16S rRNA concatenated sequences were used to construct a phylogenetic tree within genus *Kaloula*.

## 3. Results and discussion

### 3.1. Genome content and organization

The *K. rugifera* mitogenome was a 17,074 bp circular DNA molecule (GenBank accession no. KT878719; Fig. 1 and Table 1), which was longer than *K. pulchra* (16,815 bp) but shorter than *K. borealis* (17,174 bp). The AT content of all three species was below 60.1%, and ranged from 59.1% (*K. pulchra*) to 60.0% (*K. rugifera*) (Table 2). All three species contained the 37 typical mitochondrial genes (two rRNA genes, 22 tRNA genes and 13 PCGs) and a variety of noncoding regions as that described earlier in other animal mitogenomes. As in other vertebrates, most mitochondrial genes are encoded on the heavy strand (H strand), except for ND6 and eight tRNA genes, which are encoded on the light strand (L strand) (Fig. 1, Table 1). The arrangement of the genes within amphibian animal mitogenomes is highly conserved. The total base composition of *K. rugifera* mitochondrial genome is 30.32% A, 25.76% C, 29.72% T, and 14.20% G, which is consistent with the lowest frequency for G content in typical amphibian animals' mitochondrial genomes (Table 1). Such an A-T rich pattern reflects the typical sequence feature of the vertebrate mitochondrial genome (Mayfield and McKenna, 1978).

The *K. rugifera* mitogenome contained 50 overlapping nucleotides. These were located in 8 pairs of neighboring genes and varied in length from 1 to 17 bp; the longest overlap (33 bp) was located between ND5 and ND6. A total of 58 intergenic nucleotides were dispersed in 12 locations and ranged in size from 1 to 33 bp; the longest intergenic spacer located between $tRNA^{Ser}$ and ND5 (Table 1). The 58-bp intergenic spacer in the *K. rugifera* mitogenome was same in length to the *K. pulchra* mitogenome (58 bp). Among the three digging frog species, the intergenic spacer was longest in *K. borealis* (59 bp) and intergenic spacer were A + T-rich in all three digging frog species. Among the three digging frog species, the length of overlapping nucleotides in *K. pulchra* was shortest (40 bp) and longest (50 bp) in *K. rugifera*.

The gene order of the *K. rugifera* mitogenome was congruent with the other two digging frogs and other amphibians (Zhang et al., 2005). In addition to the control region, the length and content of each region in *K. rugifera* were greater than the other digging frog. The AT content of the *K. rugifera* control region was (65.07%), which was higher than *K. pulchra* (61.94%) and lower than *K. borealis* (65.54%). The length of the control region was smallest (1411 bp) in *K. pulchra* and longest in *Kaloula borealis* (1769 bp). Despite its length, the A + T content of *K. pulchra* was lower than the other two species (Table 2).

### 3.2. Protein-coding genes

The A + T content of nine *K. rugifera* PCGs (ATP8, COX1, COX3, Cyt*b*, ND2, ND4, ND4L, ND5 and ND6) was the highest among the three digging frogs. Among the 13 PCGs, the A + T content of two ATP genes was higher than three COX genes and one Cyt*b* gene, with ATP8 having the highest A + T content (Table S1). In all three mitogenomes, AT-skews and GC-skews were both negative, indicating that all three mitogenomes contain a higher percentage of AT vs. GC nucleotides. Furthermore, AT-skews at the first and third positions of each codon were positive, but negative at the second position in the three digging frogs. The C content was higher than G for all three mitogenomes. In the PCGs, the AT bias in *K. borealis* was highest in and lowest in *K. rugifera* and *K. pulchra* (Table 3). Generally, amphibian mitochondrial genomes

**Table 1**
Characteristics of the mitochondrial genome of *Kaloula rugifera*.

| Gene | Position | | Sizes | Codon | | Intergenic | Strand[c] | A + T% |
|---|---|---|---|---|---|---|---|---|
| | From | To | Nudeotide (bp) | Start | Stop[a] | Nudeotide[b] | | |
| tRNA-Phe | 1 | 70 | 70 | | | 0 | H | 58.57 |
| 12S ribosomal RNA | 71 | 1008 | 938 | | | 0 | H | 54.06 |
| tRNA-Val | 1009 | 1078 | 70 | | | 0 | H | 64.29 |
| 16S ribosomal RNA | 1079 | 2668 | 1590 | | | 0 | H | 61.57 |
| tRNA-Leu | 2669 | 2741 | 73 | | | 2 | H | 47.95 |
| ND1 | 2744 | 3699 | 956 | GTG | TA- | 0 | H | 62.13 |
| tRNA-Ile | 3700 | 3770 | 71 | | | 0 | H | 45.07 |
| tRNA-Gln | 3771 | 3840 | 70 | | | − 1 | L | 54.29 |
| tRNA-Met | 3840 | 3908 | 69 | | | 1 | H | 60.87 |
| ND2 | 3910 | 4947 | 1038 | ATG | T– | − 2 | H | 63.97 |
| tRNA-Trp | 4946 | 5014 | 69 | | | 0 | H | 60.87 |
| tRNA-Ala | 5015 | 5083 | 69 | | | 7 | L | 63.77 |
| tRNA-Asn | 5091 | 5163 | 73 | | | 0 | L | 54.79 |
| rep_origin L-strand | 5164 | 5192 | 29 | | | − 3 | L | 55.17 |
| tRNA-Cys | 5190 | 5254 | 65 | | | 0 | L | 41.54 |
| tRNA-Tyr | 5255 | 5321 | 67 | | | 4 | L | 46.27 |
| COXI | 5326 | 6876 | 1551 | ATA | AGG | − 9 | H | 59.45 |
| tRNA-Ser | 6868 | 6938 | 71 | | | 1 | L | 52.11 |
| tRNA-Asp | 6940 | 7008 | 69 | | | 2 | H | 68.12 |
| COXII | 7011 | 7698 | 688 | ATG | T– | 0 | H | 60.61 |
| tRNA-Lys | 7699 | 7767 | 69 | | | 0 | H | 57.97 |
| ATP8 | 7768 | 7932 | 165 | ATG | TAA | − 10 | H | 66.06 |
| ATP6 | 7923 | 8604 | 682 | ATG | T– | 0 | H | 61.29 |
| COXIII | 8605 | 9388 | 784 | ATG | T– | 0 | H | 56.51 |
| tRNA-Gly | 9389 | 9456 | 68 | | | 0 | H | 57.35 |
| ND3 | 9457 | 9796 | 340 | ATG | T– | 0 | H | 55.59 |
| tRNA-Arg | 9797 | 9866 | 70 | | | 3 | H | 61.43 |
| ND4L | 9870 | 10,154 | 285 | ATG | TAA | − 7 | H | 61.40 |
| ND4 | 10,148 | 11,507 | 1360 | ATG | T– | 0 | H | 61.40 |
| tRNA-His | 11,508 | 11,576 | 69 | | | − 1 | H | 65.22 |
| tRNA-Ser | 11,576 | 11,644 | 69 | | | 33 | H | 47.83 |
| ND5 | 11,678 | 13,483 | 1806 | ATG | AGG | − 17 | H | 60.85 |
| ND6 | 13,467 | 13,970 | 504 | ATG | AGA | 1 | L | 57.54 |
| tRNA-Glu | 13,972 | 14,040 | 69 | | | 2 | L | 60.87 |
| Cyt*b* | 14,043 | 15,185 | 1143 | ATG | TAA | 0 | H | 54.77 |
| control region | 15,186 | 16,860 | 1675 | | | 0 | H | 65.07 |
| tRNA-Leu | 16,861 | 16,933 | 73 | | | 1 | H | 54.79 |
| tRNA-Thr | 16,935 | 17,004 | 70 | | | 0 | H | 55.71 |
| tRNA-Pro | 17,005 | 17,073 | 69 | | | 1 | L | 63.77 |

[a] T– and TA- represent incomplete stop codons.
[b] Numbers correspond to the nucleotides separating adjacent genes, negative numbers indicate overlapping nucleotides.
[c] H and L indicate genes transcribed on the heavy and light strands, respectively.

show a strong bias in nucleotide composition and third codon positions are the most strongly affected by nucleotide compositional bias and skew (Liu et al., 2005; Wu et al., 2016).

In this study, most codons ended in A or T. In the *K. rugifera* mitogenome, the start codon in all 13 PCGs was the standard ATG, ATA or GTG. Among the three digging frogs, many PCGs used ATG as a start codon (e.g., ATP6, ATP8, COX2, COX3, Cyt*b*, ND2, ND3, ND4, ND4L, ND5 and ND6). However, ND1 genes initiated with GTG in *K. rugifera* and *K. borealis*, but TTG in *K. pulchra*. However, COX1 in all three digging frogs used ATA as start codon (Table S2). Most genes had complete termination codons; e.g. TAA in ATP8, Cyt*b* and ND4L; AGG in COX1 and ND5 (*K. rugifera* and *K. borealis*); AGA in ND6. Genes that terminated with TAG included ND2 in *K. rugifera* and *K. pulchra* and ND1 in *K. pulchra*. Genes that use incomplete termination codons (e.g., T–) include ATP6, COX2, COX3, ND3 and ND4 and ND2 in *K. borealis* and ND5 in *K. pulchra*; (e.g., TA-) include ND1 in *K. rugifera* and *K. borealis* (Table S2); which may be presumably completed by post-transcriptional polyadenylation with poly A tail (Ojala et al., 1981). Moreover, overlaps of open reading frames (ORF) occurred in two pairs of contiguous genes encoded on the H strand: ATP8-ATP6 (10 bp) and ND4L-ND4 (7 bp), and one pair of contiguous genes encoded on the opposite strands: ND5-ND6 (17 bp). In addition, some protein-coding genes share two or nine nucleotides in common with adjacent tRNA genes. This common feature shows that the size of the mitochondrial DNA is very compact and economical (Curole and Kocher, 1999).

The Relative Synonymous Codon Usage (RSCU) of 13 protein-

**Table 2**
Nucleotide compositions in regions of three *Kaloula* species mitogenomes.

| Species | Whole | | 13 PCGs | | 2 rRNAs | | 22 tRNAs | | D-loop | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Length (bp) | AT% | Length (bp) | AT% | Length (bp) | AT% | Length (bp) | AT% | Length (bp) | AT% |
| *Kaloula rugifera* | 17,074 | 60.04 | 11,295 | 60.00 | 2526 | 58.79 | 1532 | 56.53 | 1675 | 65.07 |
| *Kaloula borealis* | 17,174 | 59.16 | 11,292 | 58.71 | 2530 | 57.94 | 1535 | 56.42 | 1769 | 65.52 |
| *Kaloula pulchra* | 16,815 | 59.05 | 11,285 | 59.33 | 2533 | 57.92 | 1533 | 56.23 | 1411 | 61.94 |

**Table 3**
Base composition for the 13 PCGs in the three *Kaloula* species' mitogenomes.

| Species | A(%) | T(%) | C(%) | G(%) | A + T(%) | C + G(%) | AT-skew | GC-skew |
|---|---|---|---|---|---|---|---|---|
| *Kaloula rugifera* | 29.11 | 30.93 | 25.04 | 14.92 | 60.04 | 39.96 | − 0.03 | − 0.25 |
| 1st | 26.61 | 24.060 | 23.72 | 23.61 | 50.67 | 47.33 | 0.05 | − 0.002 |
| 2nd | 17.50 | 41.57 | 28.02 | 12.91 | 59.07 | 40.93 | − 0.41 | − 0.37 |
| 3rd | 36.79 | 31.47 | 25.58 | 6.16 | 68.26 | 31.74 | 0.08 | − 0.61 |
| *Kaloula borealis* | 31.06 | 27.06 | 27.66 | 14.22 | 58.71 | 41.29 | − 0.06 | − 0.31 |
| 1st | 23.54 | 24.26 | 28.51 | 23.70 | 52.05 | 47.95 | 0.10 | − 0.01 |
| 2nd | 41.37 | 28.13 | 17.59 | 12.91 | 58.95 | 41.05 | − 0.40 | − 0.37 |
| 3rd | 28.27 | 28.80 | 36.88 | 6.06 | 65.14 | 34.86 | 0.13 | − 0.65 |
| *Kaloula pulchra* | 30.47 | 26.51 | 28.86 | 14.16 | 59.33 | 40.67 | − 0.03 | − 0.30 |
| 1st | 23.69 | 23.85 | 28.85 | 23.61 | 52.54 | 47.46 | 0.10 | − 0.01 |
| 2nd | 41.20 | 28.36 | 17.76 | 12.68 | 58.96 | 41.04 | − 0.40 | − 0.38 |
| 3rd | 26.50 | 27.33 | 39.98 | 6.19 | 66.48 | 33.52 | 0.20 | − 0.63 |

**Table 4**
Codon usage in *Kaloula rugifera* mitochondrial protein-coding genes.

| Codon | Count | RSCU | % | Codon | Count | RSCU | % | Codon | Count | RSCU | % | Codon | Count | RSCU | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UUU(F) | **188** | 1.41 | 4.99 | UCU(S) | 59 | 1.22 | 1.57 | UAU(Y) | 59 | 1.13 | 1.57 | UGU(C) | 17 | 1.26 | 0.45 |
| UUC(F) | 79 | 0.59 | 2.10 | UCC(S) | 41 | 0.85 | 1.09 | UAC(Y) | 45 | 0.87 | 1.20 | UGC(C) | 10 | 0.74 | 0.27 |
| UUA(L) | 137 | 1.4 | 3.64 | UCA(S) | 137 | 2.83 | 3.64 | UAA(*) | 3 | 1.71 | 0.08 | UGA(W) | 95 | 1.76 | 2.52 |
| UUG(L) | 14 | 0.14 | 0.37 | UCG(S) | **8** | 0.17 | 0.21 | UAG(*) | 1 | 0.57 | 0.03 | UGG(W) | 13 | 0.24 | 0.35 |
| CUU(L) | **169** | 1.73 | 4.49 | CCU(P) | 20 | 0.4 | 0.53 | CAU(H) | 27 | 0.57 | 0.72 | CGU(R) | 13 | 0.7 | 0.35 |
| CUC(L) | 92 | 0.94 | 2.44 | CCC(P) | 54 | 1.08 | 1.43 | CAC(H) | 68 | 1.43 | 1.81 | CGC(R) | 19 | 1.03 | 0.50 |
| CUA(L) | **144** | 1.47 | 3.82 | CCA(P) | 117 | 2.34 | 3.11 | CAA(Q) | 79 | 1.8 | 2.10 | CGA(R) | 38 | 2.05 | 1.01 |
| CUG(L) | 31 | 0.32 | 0.82 | CCG(P) | 9 | 0.18 | 0.24 | CAG(Q) | 9 | 0.2 | 0.24 | CGG(R) | **4** | 0.22 | 0.11 |
| AUU(I) | **269** | 1.53 | 7.14 | ACU(T) | 69 | 0.95 | 1.83 | AAU(N) | 64 | 1.01 | 1.70 | AGU(S) | 21 | 0.43 | 0.56 |
| AUC(I) | 83 | 0.47 | 2.20 | ACC(T) | 86 | 1.19 | 2.28 | AAC(N) | 63 | 0.99 | 1.67 | AGC(S) | 24 | 0.5 | 0.64 |
| AUA(M) | 139 | 1.56 | 3.69 | ACA(T) | 127 | 1.75 | 3.37 | AAA(K) | 74 | 1.8 | 1.97 | AGA(*) | 1 | 0.57 | 0.03 |
| AUG(M) | 39 | 0.44 | 1.04 | ACG(T) | **8** | 0.11 | 0.21 | AAG(K) | 8 | 0.2 | 0.21 | AGG(*) | 2 | 1.14 | 0.05 |
| GUU(V) | 63 | 1.39 | 1.67 | GCU(A) | 73 | 0.91 | 1.94 | GAU(D) | 29 | 0.77 | 0.77 | GGU(G) | 45 | 0.79 | 1.20 |
| GUC(V) | 37 | 0.82 | 0.98 | GCC(A) | 147 | 1.84 | 3.90 | GAC(D) | 46 | 1.23 | 1.22 | GGC(G) | 69 | 1.21 | 1.83 |
| GUA(V) | 63 | 1.39 | 1.67 | GCA(A) | 94 | 1.18 | 2.50 | GAA(E) | 64 | 1.52 | 1.70 | GGA(G) | 73 | 1.28 | 1.94 |
| GUG(V) | 18 | 0.4 | 0.48 | GCG(A) | **6** | 0.07 | 0.16 | GAG(E) | 20 | 0.48 | 0.53 | GGG(G) | 42 | 0.73 | 1.12 |

Boldface represents high frequency or very low frequency.

coding genes of the *K. rugifera* mitogenome was summarized in Table 4. Among the 13 protein-coding genes of the three digging frogs, the codons CCG, ACG, CAG and CGG, are seldom represented, accounting for 0.70% to 0.73%. The genome-wide A + T bias is also reflected in the codon usage of the three digging frogs mitogenome. The frequencies of UUU(F), AUU(I) and CUA(L) are higher than those of other codons, accounting for 13.83% to 15.18% (Table 4). Furthermore, similar to other vertebrates (Lopez et al., 1996; Zhang et al., 2003), a strong bias against G at the third codon position was observed in the 13 protein-coding genes of the three digging frogs mitochondrial DNA (Tables S3–S4). In the present study, two pairs of protein-coding genes (ATP8 and ATP6, ND4L and ND4), which are located on the same strand, overlap in reading-frames. ND5 and ND6 also overlap with each other, but encode on opposite strands.

### 3.3. Transfer RNA genes

The three digging frog genomes each contained 22 tRNAs with a typical cloverleaf secondary structure composed of four arms. The tRNAs in *K. rugifera* had a 56.53% A + T content, ranged from 65 to 73 bp in length, and comprised 1532 bp of the total mitogenome. The length of the tRNA region in *K. borealis* and *K. pulchra* was 1535 and 1533 bp, respectively, and individual genes ranged from 65 to 73 or 74 bp. The A + T content of the tRNA genes ranged from 56.23% (*K. pulchra*) to 56.53% (*K. pulchra*) (Table S5). The anticodons of the 22 tRNAs in *K. pulchra* were identical with other digging frogs. The $tRNA^{Asp}$ gene had the highest A + T content except for *K. pulchra*, where A + T content was highest in $tRNA^{Ala}$ (Table S5). Twenty of the 22 tRNAs were predicted and determined using tRNAscan-SE (Lowe and Eddy, 1997), and the other tRNA genes ($tRNA^{Cys}$ and $tRNA^{Ser2}$ (AGN)) were determined using visual inspection and alignment with published Microhylidae mitogenomes (Yong et al., 2016; de Sá et al., 2012).

A total of 26 mismatched base pairs were identified in the tRNAs of *K. rugifera*, and these contained G-U pairs in the AA (10 bp), DHU (4 bp), A-C (5 bp), and TψC stems (7 bp). Stem mismatches seemed to be a common phenomenon for mitochondrial tRNA genes and were probably repaired via a post-transcriptional editing process (Lavrov et al., 2000). Compared with other digging frogs, most of the mismatched nucleotides were G-U pairs, which can form a weak bond in tRNAs and non-canonical pairs in tRNA secondary structures (Gutell et al., 2002).

### 3.4. Ribosomal RNA genes

In the three digging frog species, the length of 12S rRNA ranged from 938 (*K. rugifera*) to 941 bp (*K. borealis* and *K. pulchra*), while the length of 16S rRNA ranged from 1589 (*K. borealis*) to 1592 bp (*K. pulchra*). The large ribosomal gene (16S rRNA) of *K. rugifera* was 1590 bp, had an A + T content of 61.6%, and mapped between $tRNA^{Val}$ and $tRNA^{Leu}$. The small ribosomal gene (12S rRNA) of *K. rugifera* was 938 bp, had an A + T content of 54.1%, and mapped between $tRNA^{Phe}$ and $tRNA^{Val}$ region (Table 1). The putative origin of L-strand replication ($O_L$) with a length of 29 bp between tRNA-Asn and tRNA-Cys had the potential to fold into a stem loop of secondary structure, similar to most vertebrates (Boore, 1999; Liu et al., 2005; Su et al., 2007). Such motif has been demonstrated to be involved in human in the transition from RNA to DNA synthesis (Hixson and Brown, 1986).

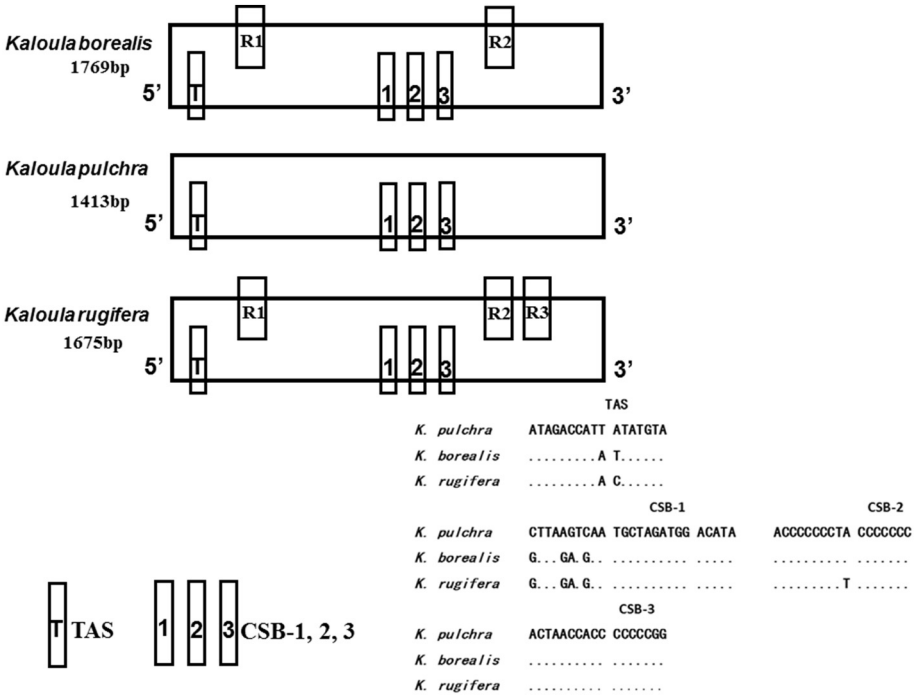


**Fig. 2.** Structures of control region and alignments of identified putative termination-associated sequences (TAS), conserved sequence blocks (CSB 1–3), and repeat motifs "R1, R2 and R3"are shown.

### 3.5. The control region

We identified conserved structural elements in the control region of three digging frog species. The largest non-coding region in the *K. rugifera* mitogenome was the control region (CR, 1675 bp), which was A + T-rich (65.1%) and located at the conserved position between Cyt*b* and $tRNA^{Leu}$ (Table 1). The control region of *K. pulchra* had the lowest A + T content (61.9%), while the A + T content of the other two digging frog was above 65%. The control region was shortest in *K. pulchra* (1141 bp) and longest in *K. borealis* (1769 bp) (Table 2).

When compared with the CRs of the three digging frog species, one termination-associated sequence (TAS) and three conserved sequence blocks (CSB) were identified in *K. rugifera*: TAS (376–392 bp), CSB1 (1299–1323 bp), CSB2 (1377–1393 bp) and CSB3 (1418–1434 bp) (Fig. 2). Variations in nucleotides are present within *Kaloula* in TAS, CSB1 and CSB2 of the control region (Fig. 2) (Zhang et al., 2003). The nucleotide sequences for CSB3 are invariant within the genus *Kaloula*. Nucleotides at positions 11 of TAS appear to be highly variable in *Kaloula*. Nucleotides at positions 10 for CSB2 appear to be "A" in *K. borealis* and *K. pulchra* which is represented by "G" in *K. rugifera*. The function of these conserved sequence blocks is unclear. Further study on the mechanistic basis of mtDNA replication is warranted for *K. rugifera* and other digging frog species. A sequence block similar to the H-strand origin of replication ($O_H$) in frogs (Sumida et al., 2001) is located in the central domain. CSB-1 of *K. rugifera* shares high similarity to the consensus in other vertebrates and is not reduced to a truncated pentamotif (5′-GACAT-3′) as in *Typhlonectes natans* (Zardoya and Meyer, 2000; San Mauro et al., 2004). However, in *Xenopus laevis*, a truncated CSB-1 had been reported (Roe et al., 1985). Two of the three conserved sequence blocks (CSB-2 and -3) were very conservative in *K. rugifera*, *K. borealis* and *K. pulchra* and involved in the initiation of the mtDNA synthesis (Walberg and Clayton, 1981; Zardoya and Meyer, 2000), which can be identified in the right domain, upstream of the tandem repeats (Fig. 2).

The control region possesses distinct repeat regions at both 5′ and 3′-sides (Fig. 2). Tandem repeat units were also observed in the control regions of *Paa spinosa*, *Bufo japonicus* and *Hyla japonica* (Igawa et al., 2008; Zhou et al., 2009). The 5′-side repeat region consists of 2 tandem repeat units of 23 bp (5′-AAAATGAAATGGTAACCGCCCAT-3′). Unlike the 5′-side repeat region, the 3′-side repeat region consists of 3 tandem repeat units of 12 bp (5′-CTTTTTTTTATT-3′) and 2 tandem repeat units of 22 bp (5′-TTTTTTATCCTTTTTTTTACT C′) (Fig. 2). Compared with corresponding regions of other *Kaloula* species, we found that the control region of *K. borealis* included 2 tandem repeat units of 24 bp (5′-ATAAAAATGAAATGG CAACCGCCC-3′) in the 5′-side repeat region and 9 tandem repeat units of 11 bp (5′-TTTTTTT GTGT′) in the 3′-side repeat region (Fig. 2), while no tandem repeats were found in *K. pulchra* which is unusual for a mitogenome control region.

### 3.6. Mitogenomic arrangement

The mitogenomic organizations of Microhylidae species are different from the vertebrate-type mt gene arrangement in LTPF cluster and CR, while are consistent with neobatrachian-type arrangement (Kurabayashi and Sumida, 2013). In Microhylidae (*Kaloula* and *Microhyla*) mitochondrial genome, the tRNA-Leu2 (CUN), tRNA-Thr, tRNA-Pro, and tRNA-Phe genes were translocated from their original position and formed a LTPF tRNA gene cluster at the upstream of 12S rRNA gene (Fig. 1), while CR which located between tRNAPro and tRNAPhe is in front of tRNA-Leu2 (CUN). The gene arrangement of the *K. rugifera* tRNA cluster (LTPF) is completely consistent with that of the typical neobatrachian type (e.g., *R. nigromaculata*), while differs from TPLF tRNA-gene cluster of *F. cancrivora* and *Rh. plancyi* (Ren et al., 2009). Though the members and arrangement of this tRNA-gene cluster are slightly modified in some taxa. Consequently, the tRNA-gene cluster supports the common ancestry of neobatrachians (Kurabayashi et al., 2006). We also found the same tRNA gene cluster in *K. rugifera*, supporting the viewpoint of Kurabayashi et al. (2006) and Ren et al. (2009).

### 3.7. Phylogenetic analyses

In terms of phylogenetic analyses, the nucleotide data contained 11,271 nucleotide sites from 13 protein-encoding genes, 4528 sites were conservative, and 4877 sites were parsimoniously informative. Both BI and ML trees based on complete mito-genome sequences (14 species, Table 5) showed identical topology, and major internal nodes were well supported by bootstrap values and posterior probabilities

**Table 5**
Mitogenomes of the Microhyloidea used in this study.

| Superfamily | Family | Genus | Species | Size (bp) | Accession no. | Reference |
|---|---|---|---|---|---|---|
| Microhyloidea | Microhylidae | *Kaloula* | *Kaloula rugifera* | 17,074 | NC_029409 | This study |
| | Microhylidae | *Kaloula* | *Kaloula borealis* | 17,174 | NC_020044 | Hwang and Lee (2012) |
| | Microhylidae | *Kaloula* | *Kaloula pulchra* | 16,818 | NC_006405 | Zhang et al. (2005) |
| | Microhylidae | *Microhyla* | *Microhyla okinavensis* | 16,717 | NC_010233 | Igawa et al. (2008) |
| | Microhylidae | *Microhyla* | *Microhyla pulchra* | 16,744 | KF798195 | Wu et al. (2016) |
| | Microhylidae | *Microhyla* | *Microhyla ornata* | 16,730 | NC_009422 | GenBank |
| | Microhylidae | *Microhyla* | *Microhyla heymonsi* | 16,707 | NC_006406 | Zhang et al. (2005) |
| | Microhylidae | *Microhyla* | *Microhyla butleri* | 16,722 | NC_030049 | Wang et al. (2016) |
| | Arthroleptidae | *Trichobatrachus* | *Trichobatrachus robustus* | 21,418 | AB777219 | Kurabayashi and Sumida (2013) |
| | Hyperoliidae | *Hyperolius* | *Hyperolius marmoratus* | 22,595 | AB777218 | Kurabayashi and Sumida (2013) |
| | Brevicipitidae | *Breviceps* | *Breviceps adspersus* | 28,757 | NC_023379 | Kurabayashi and Sumida (2013) |
| | Hemisotidae | *Hemisus* | *Hemisus marmoratus* | 20,093 | AB777217 | Kurabayashi and Sumida (2013) |
| Ranoidea | Rhacophoridae | *Rhacophorus* | *Rhacophorus schlegelii* | 21,359 | AB202078 | Sano et al. (2005) |
| | Rhacophoridae | *Buergeria* | *Buergeria buergeri* | 19,959 | AB127977 | Sano et al. (2004) |

(Fig. 3). In BI tree given in Fig. 3, the phylogenetic relationship of the Microhyloidea, including *Kaloula*, *Microhyla*, *Trichobatrachus*, *Hyperolius*, *Breviceps* and *Hemisus*, corresponds well with the traditional taxonomy (Kurabayashi and Sumida, 2013). In this tree, *Kaloula* and *Microhyla* formed a monophyletic group, respectively, which were a sister group of the branch indicated a closer genetic relationship (Fig. 3). In this study, *K. rugifera*, *K. borealis* and *K. pulchra* form a sister clade to the genus *Microhyla*, which is consistent with previous studies (Zhang et al., 2005; Igawa et al., 2008; Ren et al., 2009; Zhang et al., 2005; Zhang et al., 2009).

For the phylogeny of *Microhyla* based on 13 PCGs, they form such a lineage (*M. butleri* - (*M. pulchra* - (*M. okinavensis* - (*M. heymonsi* - *M. ornata*)))) (Fig. 3), which was concordant with the results of Matsui et al. (2005, 2011) and Yong et al. (2016) and distinct from the lineage comprising (((*M. heymonsi* - *M. okinavensis*) - *M. pulchra*) - *M. ornata*) (Matsui et al., 2011). In contrast, based on 12S rRNA, BDNF (brain-derived neurotrophic factor), tyrosinase, and 28S sequences, *M. butleri* is distant from *M. ornata* which belongs to the lineage (*M. okinavensis* - (*M. ornata* - *M. pulchra*)) (de Sá et al., 2012). This is also reflected by the findings based on 3 mitochondrial and 9 nuclear genes which indicate *M. butleri* to be distant from the lineage comprising (*M. pulchra* - (*M. heymonsi* - (*M. okinavensis* - *M. ornata*))) (Pyron and Wiens, 2011), and 12S rRNA sequences which indicate *M. butleri* to be distant to (*M. okinavensis* - (*M. pulchra* - *M. ornata*)) (Howlader et al., 2015). In the present study, the molecular phylogeny indicated *M. butleri* to be basal to other congeners (Fig. 3), which is consistent with the findings of van der Meijden et al. (2007), Pyron and Wiens (2011) and Howlader et al. (2015). In genus *Kaloula*, the phylogenetic tree demonstrated that *K. pulchra* was clustered together with *K. rugifera* and *K. borealis* and they had a close relationship with each other (Fig. 3), which was consistent with the results of previous studies (Yong et al., 2016). Genetic variation among species within the genus *Kaloula* are relatively large, and *K. rugifera* and *K. borealis* are located in the same branch and k2p genetic distance between them is 0.125, while genetic distance between *K. pulchra* and *K. borealis* is 0.204, indicating *K. pulchra* is deeply divergent with others.

To further investigate the phylogenetic relationships of *K. rugifera*, the phylogenetic relationships were reconstructed based on 12S rRNA and 16S rRNA genes (2289 bp) and *Me. pollicaris*, *Me. sundana* and *Mi. ornata* as outgroup. 12S rRNA and 16S rRNA genes of 19 samples in genus *Kaloula* were derived from GenBank. Among 2289 characters of 12S rRNA and 16S rRNA genes, 1652 were conserved, 435 of 646 variable sites are informative for parsimony. Previous research suggests that genus *Kaloula* appear to be paraphyletic (de Sá et al., 2012), while our results are monophyletic which is consistent with previous findings (Matsui et al., 2011). The consistent topology given by BI and ML trees suggested that *Kaloula* species formed three completely separated clades (Fig. 4). Clades 1 includes *K. rugifera*, *K. borealis* and *K. verrucosa*.

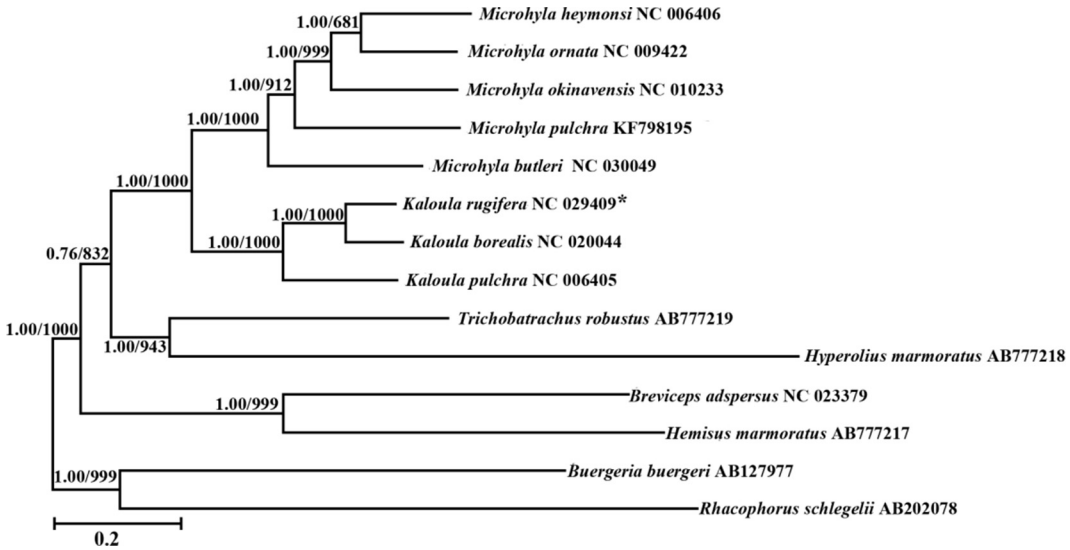


**Fig. 3.** Results of phylogenetic analyses by using Bayesian inferences (BI) and maximum likelihood (ML) analysis indicated evolutionary relationships among 14 individuals based on 13 PCGs sequences. *Rhacophorus schlegelii* (AB202078) and *Buergeria buergeri* (AB127977) were used as outgroups. Tree topologies produced by Bayesian inferences (BI) and maximum likelihood (ML) analyses were equivalent. Bayesian posterior probability and bootstrap support values for ML analyses are shown orderly on the nodes. The asterisks indicate new sequences generated in this study.

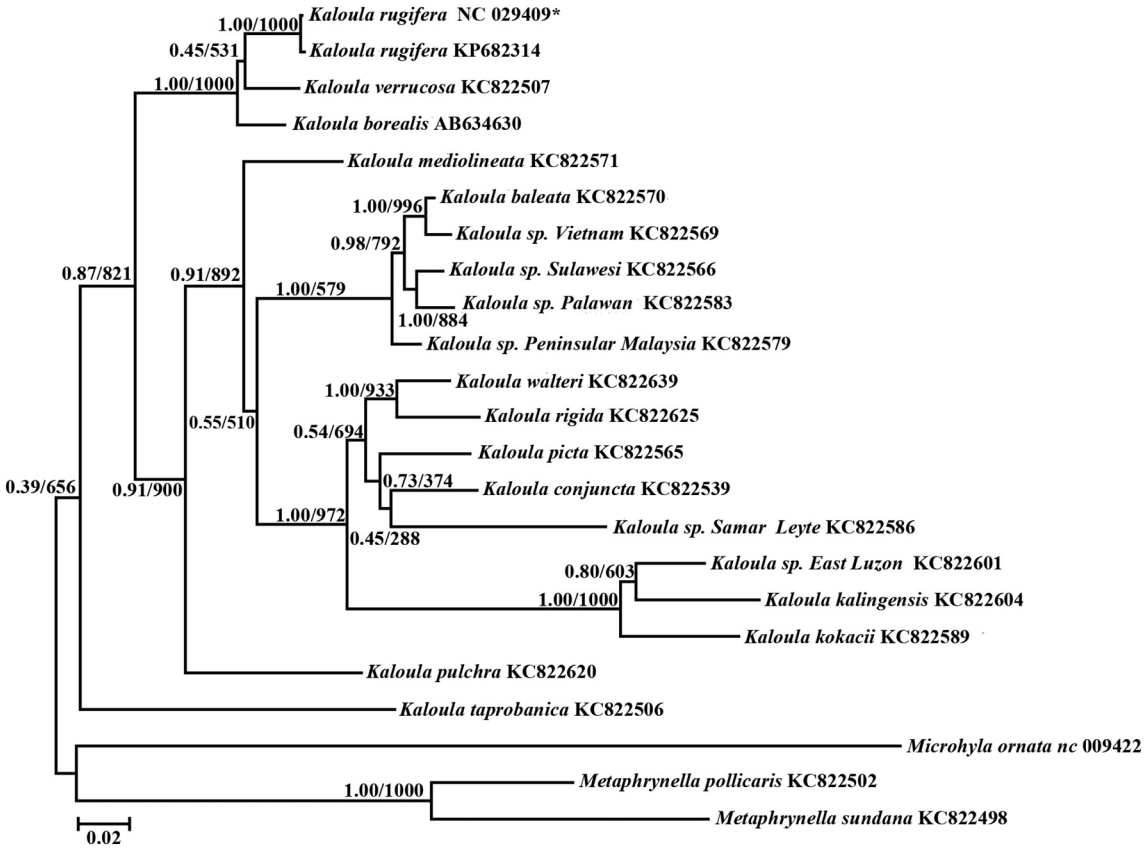


**Fig. 4.** Phylogenetic relationships of *Kaloula* species reconstructed from 23 12S rRNA + 16S rRNA sequences. *Metaphrynella pollicaris* (KC822502), *Metaphrynella sundana* (KC822498) and *Microhyla ornata* (NC_009422) were used as outgroups. The asterisks indicate new sequences generated in this study.

*K. rugifera* are closely related to *K. borealis* and *K. verrucosa* form sister group which is concordant with results of Li et al. (2012). Though we did not have the biogeographical and monophyletic data on the three species, the morphology of *K. rugifera* and *K. verrucosa* is more similar basing on the description of Fei et al. (2009), and the distribution of the two species overlaps partially in Sichuan province in China (Fei and Xie, 2004; Fei et al., 2009). Thus our results, in our opinion, is reasonable. Clades 2 includes *K. pulchra*, *K. mediolineata*, *K. kokacii*, *K. conjuncta*, *K. kalingensis*, *K. baleata*, *K. rigida*, *K. walteri*, *K. picta* and *K.* sp. species (Fig. 4). In the clade, *K. pulchra* is the earliest differentiation which is consistent with the findings of Matsui et al. (2011) and de Sá et al. (2012). In addition, phylogenetic relationships within this taxon are in conformity to that of de Sá et al. (2012) (*K. pulchra* - (*K. picta* - *K. baleata*)), while are different from the results of Li et al. (2012) (*K. pulchra* - (*K. baleata* - *K. mediolineata* - (*K. picta* - *K. conjuncta*))) and Matsui et al. (2011) (*K. pulchra* - (*K. picta* - (*K. mediolineata* - *K. baleata*))). Clade 3 includes *K. taprobanica*, and it is the earliest diverging lineage of the *Kaloula* (Fig. 4), which is consistent with previous studies (de Sá et al., 2012; Li et al., 2012; Blackburn et al., 2013).

## 4. Conclusion

The sequence structure of the *K. rugifera* mitochondrial genome is typical and shares high similarities with other *Kaloula* species. The *K. rugifera* mito-genome exhibits pronounced strand-specific asymmetry in nucleotide composition, which was also reflected in the codon usage. Besides, 3 types of tandem repeat units were also identified in the control region. In addition, phylogenetic analyses suggested that *K. rugifera* was clustered in the clade of *K. borealis* and *K. pulchra* which they had closer genetic relationship. Further analysis on the phylogeny of the genus *Kaloula* using monophyletic taxon and extra molecular information is necessary.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2017.05.039.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

## Acknowledgements

This research was supported by the National Natural Science Foundation of China (No. 31670392), the Research Project of Ecological Security and Protection Key Laboratory of Sichuan Province (No. ESP1410), Scientific Research Foundation Projects of Mianyang Normal University (No. QD2015A003), Education Office Project of Sichuan Province (No. 15ZB0279) and the Scientific Research Fund of Mianyang Normal University (No. 2014A05 and Mnu-JY1420). We thank Scientific Editor and the two anonymous reviewers for valuable comments on the manuscript.

## References

Blackburn, D.C., Siler, C.D., Diesmos, A.C., Mcguire, J.A., Cannatella, D.C., Brown, R.M., 2013. An adaptive radiation of frogs in a southeast Asian island archipelago. Evolution 67, 2631–2646.
Boore, J.L., 1999. Animal mitochondrial genomes. Nucleic Acids Res. 27, 1767–1780.
Bossuyt, F., Brown, R.M., Hillis, D.M., Cannatella, D.C., Milinkovitch, M.C., 2006. Phylogeny and biogeography of a cosmopolitan frog radiation: Late Cretaceous diversification resulted in continent-scale endemism in the family Ranidae. Syst. Biol. 55, 579–594.
Cannatella, D.C., Hillis, D.M., Chippindale, P.T., Weigt, L., Rand, A.S., Ryan, M.J., 1998. Phylogeny of frogs of the *Physalaemus pustulosus* species group, with an examination of data incongruence. Syst. Biol. 47, 311–335.
Curole, J.P., Kocher, T.D., 1999. Mitogenomics: digging deeper with complete mitochondrial genomes. Trends Ecol. Evol. 14, 394–398.
Das, I., Sengupta, S., Ahmed, M., Firoz, Dutta, S.K., 2004. A new species of Kaloula (Microhylidae) from Assam State, north-eastern India. Hamadryad 29, 101–109.

De Rijk, P., De Wachter, R., 1997. RnaViz, a program for the visualisation of RNA secondary structures. Nucleic Acids Res. 25, 4679–4684.
Deng, X., Wang, S., Liang, X., Jiang, J., Wang, B., Deng, L., 2016. The complete mitochondrial genome of *Kaloula rugifera* (Amphibia, Anura, Microhylidae). Mitochondrial DNA 5, 1–2.
Diesmos, A.C., Brown, R.M., Alcala, A.C., 2014. New species of narrow-mouthed frog (Amphibia: Anura: Microhylidae; genus *Kaloula*) from the mountains of southern Luzon and Polillo Islands, Philippines. Copeia 2002, 1037–1051.
Fei, L., Xie, F., 2004. *Kaloula rugifera*. The IUCN red list of threatened species. Version 2015.3. Available at: www.iucnredlist.org (Accessed 8 October 2015).
Fei, L., Hu, S.Q., Ye, C.Y., Huang, Y.Z., 2009. Fauna Sinica. Amphibia. Anura, vol. 2. Science Press, Beijing, pp. 957.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
Frost, D.R., 2014. "*Kaloula rugifera* Stejneger, 1924". Amphibian Species of the World: An Online Reference. Version 6.0. American Museum of Natural History Available at: http://research.amnh.org/herpetology/amphibia/index.html Accessed 6 October 2015.
Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.
Gutell, R.R., Lee, J.C., Cannone, J.J., 2002. The accuracy of ribosomal RNA comparative structure models. Curr. Opin. Struct. Biol. 12, 301–310.
Hixson, J.E., Brown, W.M., 1986. A comparison of the small ribosomal RNA genes from the mitochondrial DNA of the great apes and humans: sequence, structure, evolution and phylogenetic implications. Mol. Biol. Evol. 3, 1–18.
Howlader, M.S.A., Nair, A., Gopalan, S.V., Merilä, J., 2015. A new species of *Microhyla* (Anura: Microhylidae) from Nilphamari, Bangladesh. PLoS One 10, e0119825.
Huelsenback, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Hwang, D.S., Lee, J.S., 2012. Complete mitochondrial genome of the boreal digging frog *Kaloula borealis* (Anura, Microhylidae). Mitochondrial DNA 23, 301–302.
Igawa, T., Kurabayashi, A., Usuki, C., Fujii, T., Sumida, M., 2008. Complete mitochondrial genomes of three neobatrachian anurans: a case study of divergence time estimation using different data and calibration settings. Gene 407, 116–129.
Iskadar, D.T., Colijn, E., 2000. Preliminary checklist of southeast Asian and New Guinean herpetofauna. I. Amphibians. Treubia 31, 1–133.
Jiang, J., Dubois, A., Ohler, A., Tillier, A., Chen, X., Xie, F., Stöck, M., 2005. Phylogenetic relationships of the tribe Paini (Amphibia, Anura, Ranidae) based on partial sequences of mitochondrial 12S and 16S rRNA genes. Zool. Sci. 22, 353–362.
Köhler, F., Günther, R., 2008. The radiation of microhylid frogs (Amphibia: Anura) on New Guinea: a mitochondrial phylogeny reveals parallel evolution of morphological and life history traits and disproves the current morphology-based classification. Mol. Phylogenet. Evol. 47, 353–365.
Kumazawa, Y., Nishida, M., 1993. Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. J. Mol. Evol. 37, 380–398.
Kurabayashi, A., Sumida, M., 2009. PCR primers for the Neobatrachian mitochondrial genome. Curr. Herpetol. 28, 1–11.
Kurabayashi, A., Sumida, M., 2013. Afrobatrachian mitochondrial genomes: genome reorganization, gene rearrangement mechanisms, and evolutionary trends of duplicated and rearranged genes. BMC Genomics 14, 633.
Kurabayashi, A., Usuki, C., Mikami, N., Fujii, T., Yonekawa, H., Sumida, M., et al., 2006. Complete nucleotide sequence of the mitochondrial genome of a Malagasy poison frog *Mantella madagascariensis*: evolutionary implications on mitochondrial genomes of higher anuran groups. Mol. Phylogenet. Evol. 39, 223–236.
Kurabayashi, A., Yoshikawa, N., Sato, N., Hayashi, Y., Oumi, S., Fujii, T., Sumida, M., 2010. Complete mitochondrial DNA sequence of the endangered frog *Odorrana ishikawae* (family Ranidae) and unexpected diversity of mt gene arrangements in ranids. Mol. Phylogenet. Evol. 56, 543–553.
Kurabayashi, A., Matsui, M., Belabut, D.M., Yong, H.S., Ahmad, N., Sudin, A., Sumida, M., 2011. From Antarctica or Asia? New colonization scenario for Australian-New Guinean narrow mouth toads suggested from the findings on a mysterious genus *Gastrophrynoides*. BMC Evol. Biol. 11, 175.
Laslett, D., Canback, B., 2008. ARWEN: a program to detect tRNA genes in metazoan mitochondrial nucleotide sequences. Bioinformatics 24, 172–175.
Lavrov, D.V., Brown, W.M., Boore, J.L., 2000. A novel type of RNA editing occurs in the mitochondrial tRNAs of the centipede *Lithobius forficatus*. Proc. Natl. Acad. Sci. 97, 13738–13742.
Li, B., Wang, J., Yuan, S., Liu, N., Rao, D., Liang, H., Wang, X., 2012. Study on the phylogeny of *Kaloula* (Microhylidae, Amphibia) based on the mitochondrial gene. J. West China For. Sci. 41, 51–55.
Li, W., Zhang, X.C., Zhao, J., Shi, Y., Zhu, X.P., 2015. Complete mitochondrial genome of *Cuora trifasciata* (Chinese three-striped box turtle), and a comparative analysis with other box turtles. Gene 555, 169–177.
Liu, Z.Q., Wang, Y.Q., Bing, S., 2005. The mitochondrial genome organization of the rice frog, *Fejervarya limnocharis*, (Amphibia: Anura): a new gene order in the vertebrate mtDNA. Gene 346, 145–151.
Lopez, J., Cevario, S., O'Brien, S.J., 1996. Complete nucleotide sequence of the domestic cat (*Felis catus*) mitochondrial genome and a transposed mtDNA tandem repeat (Numt) in the nuclear genome. Genomics 33, 229–246.
Lowe, T.M., Eddy, S.R., 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25, 955–964.
Matsui, M., Ito, H., Shimada, T., Ota, H., Saidapur, S.K., Khonsue, W., Tanaka-Ueno, T., Wu, G.F., 2005. Taxonomic relationships within the Pan-Oriental narrow-mouth toad Microhyla ornata as revealed by mt-DNA analysis (Amphibia, Anura, Microhylidae). Zool. Sci. 22, 489–495.
Matsui, M., Hamidy, A., Belabut, D.M., Ahmad, N., Panha, S., Sudin, A., Khonsue, W., Oh, H., Yong, H., Jiang, J., Nishikawa, K., 2011. Systematic relationships of Oriental tiny frogs of the family Microhylidae (Amphibia, Anura) as revealed by mtDNA genealogy. Mol. Phylogenet. Evol. 61, 167–176.
Mayfield, J.E., McKenna, J.F., 1978. A-T rich sequences in vertebrate DNA. A possible explanation of q-banding in metaphase chromosomes. Chromosoma 67, 157–163.
van der Meijden, A., Vences, M., Hoegg, S., Boistel, R., Channing, A., Meyer, A., 2007. Nuclear gene phylogeny of narrow-mouthed toads (Family: Microhylidae) and a discussion of competing hypotheses concerning their biogeographical origins. Mol. Phylogenet. Evol. 44, 1017–1030.
Mo, Y., Zhang, W., Zhou, S., Chen, T., Tang, H., Meng, Y., Chen, W., 2013. A new species of *Kaloula* (Amphibia: Anura: Microhylidae) from southern Guangxi, China. Zootaxa 3710, 165–178.
Mu, X., Liu, Y., Lai, M., Song, H., Wang, X., Hu, Y., Luo, J., 2015. Characterization of the *Macropodus opercularis* complete mitochondrial genome and family Channidae taxonomy using Illumina-based de novo transcriptome sequencing. Gene 559, 189–195.
Ojala, D., Montoya, J., Attardi, G., 1981. tRNA punctuation model of RNA processing in human mitochondria. Nature 290, 470–474.
Perna, N.T., Kocher, T.D., 1995. Patterns of nucleotide composition at four fold degenerate sites of animal mitochondrial genomes. J. Mol. Evol. 41, 353–358.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.
Pyron, R.A., Wiens, J.J., 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol. Phylogenet. Evol. 61, 543–583.
Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. Available from. http://beast.bio.ed.ac.uk/Tracer.
Ren, Z., et al., 2009. Complete nucleotide sequence and gene arrangement of the mitochondrial genome of the crab-eating frog *Fejervarya cancrivora* and evolutionary implications. Gene 441, 148–155.
Roe, B.A., Ma, D.P., Wilson, R.K., Wong, J.F., 1985. The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J. Biol. Chem. 260, 9759–9774 Aug 15.
de Sá, R.O., Streicher, J.W., Sekonyela, R., Forlani, M.C., Loader, S.P., Greenbaum, E., Haddad, C.F., 2012. Molecular phylogeny of microhylid frogs (Anura: Microhylidae) with emphasis on relationships among New World genera. BMC Evol. Biol. 12, 241.
Sahoo, P.K., Goel, C., Kumar, R., Dhama, N., Ali, S., Sarma, D., ... Barat, A., 2015. The complete mitochondrial genome of threatened chocolate mahseer (*Neolissochilus hexagonolepis*) and its phylogeny. Gene 570, 299–303.
San Mauro, D., Gower, D.J., Oommen, O.V., Wilkinson, M., Zardoya, R., 2004. Phylogeny of caecilian amphibians (Gymnophiona) based on complete mitochondrial genomes and nuclear RAG1. Mol. Phylogenet. Evol. 33, 413–427.
Sano, N., Kurabayashi, A., Fujii, T., Yonekawa, H., Sumidam, M., 2004. Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the bell-ring frog, *Buergeria buergeri* (family Rhacophoridae). Genes Genet. Syst. 79, 151–163.
Sano, N., Kurabayashi, A., Fujii, T., Yonekawa, H., Sumida, M., 2005. Complete nucleotide sequence of the mitochondrial genome of Schlegel's tree frog *Rhacophorus schlegelii* (family Rhacophoridae): duplicated control regions and gene rearrangements. Genes Genet. Syst. 80, 213–224.
Sengupta, S., Das, A., Das, S., Hussain, B., Choudhury, N.K., Dutta, S.K., 2009. Taxonomy and biogeography of *Kaloula* species of Eastern India. Nat. Hist. J. Chulalongkorn Univ. 9, 209–222.
Su, X., Wu, X.B., Yan, P., 2007. Rearrangement of a mitochondrial tRNA gene of the concave-eared torrent frog, *Amolops tormotus*. Gene 394, 25–34.
Sumida, M., Kanamori, Y., Kaneda, H., Kato, Y., Nishioka, M., Yonekawa, H., 2001. Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. Genes Genet. Syst. 76, 311–325.
Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other Methods). Version 4.0b10. Sinauer Associates, Sunderland, M.A.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Walberg, M.W., Clayton, D.A., 1981. Sequence and properties of the human KB cell and mouse L cell d-loop regions of mitochondrial DNA. Nucleic Acids Res. 9, 5411–5421.
Wang, S., Liu, L., Jiang, J., 2016. The complete mitochondrial genome of *Microhyla butleri* (Amphibia, Anura, Microhylidae). Mitochondrial DNA A DNA Mapp. Seq. Anal. 1, 154–155.
Wu, X., Li, Y., Zhang, H., Yan, L., Wu, X.B., 2016. The complete mitochondrial genome of *Microhyla pulchra* (Amphidia, Anura, Microhylidae). Mitochondrial DNA A DNA Mapp. Seq. Anal. 27, 40–41.
Yong, H.S., Song, S.L., Lim, P.E., Eamsobhana, P., Tan, J., 2016. Complete mitochondrial genome and phylogeny of *Microhyla butleri* (Amphibia: Anura: Microhylidae). Biochem. Syst. Ecol. 66, 243–253.
Zardoya, R., Meyer, A., 2000. Mitochondrial evidence on the phylogenetic position of caecilians (Amphibia: Gymnophiona). Genetics 155, 765–775.
Zhang, P., Chen, Y.Q., Liu, Y.F., Zhou, H., Qu, L.H., 2003. The complete mitochondrial genome of the Chinese giant salamander, *Andrias davidianus* (Amphibia: Caudata). Gene 311, 93–98.
Zhang, P., Zhou, H., Chen, Y.Q., Liu, Y.F., Qu, L.H., 2005. Mitogenomic perspectives on the origin and phylogeny of living amphibians. Syst. Biol. 54, 391–400.
Zhang, J.F., Nie, L.W., Wang, Y., Hu, L.L., 2009. The complete mitochondrial genome of the large-headed frog, *Limnonectes bannaensis* (Amphibia: Anura), and a novel gene organization in the vertebrate mtdna. Gene 442 (1–2), 119–127.
Zhou, Y., Zhang, J.Y., Zheng, R.Q., Yu, B.G., Yang, G., 2009. Complete nucleotide sequence and gene organization of the mitochondrial genome of *Paa spinosa* (Anura: Ranoidae). Gene 447, 86–96.
Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.