The Complete Mitochondrial Genome of *Rhacophorus dennysi* (Anura: Rhacophoridae) with Novel Gene Arrangements and its Phylogenetic Implications

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**ABSTRACT**

We determined the complete mitochondrial (mt) genome of *Rhacophorus dennysi* (family Rhacophoridae). The *R. dennysi* mitogenome (18,052 bp) contained the 37 genes and a single control region (CR) typically found in neobatrachian mtDNAs. In the new mt genome, the ND5 gene and a TLPF tRNA cluster (tRNAThr, tRNALeu(CUN), tRNAPro and tRNA Phe) were located between the CR and the 12S rRNA gene. *R. dennysi* mitochondrial gene rearrangements observed here could be explained by the Tandem Duplication and Random Loss (TDRL) model. We used twelve mitochondrial protein-coding genes of the newly sequenced and other reported species to assess phylogenetic relationships of Ranoidea. Phylogenetic analyses using maximum likelihood (ML) and Bayesian inference (BI) methods supported the sister-group relationship between ((Rhacophoridae + Mantellidae) + Ranidae) and Dicroglossidae. Within Rhacophoridae, two species of the genus *Rhacophorus* (*R. schlegelii* and *R. dennysi*) were clustered together with the representative of the genus *Polypedates* (*P. megacephalus*), meanwhile, the representative of the genus *Buergeria* (*B. buergeri*) occupied the basal position in the clade of Rhacophoridae.

**INTRODUCTION**

Vertebrate mitochondrial (mt) DNAs form closed circular molecules which have lengths varying from 15 to 21 Kb (Boore, 1999; Sano et al., 2005; Chen et al., 2011; Zhang et al., 2015). These mt genomes typically contain 13 protein-coding genes (PCGs), 2 ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes and a control region (CR) (Boore, 1999). The CR is a long non-coding region (approximately 0.5 Kb–9 Kb) (Kurabayashi et al., 2008), which includes signals for regulating and initiating mitochondrial genome replication and transcription and a short non-coding sequence referred to as the light-strand replication origin (OL) (Boore, 1999).

Mitochondrial genes organization is usually conserved in nearly all vertebrates (Boore, 1999). However, the mt gene arrangements of the neobatrachians are different, and a variety of reorganizations have occurred (Kurabayashi and Sumida, 2013; Zhang et al., 2013, 2018; Li et al., 2014; Yuan et al., 2016).

For instance, there is a lack of the tRNA<sup>His</sup> gene in the *Odorrana schnackeri* mitogenome (Li et al., 2014); a tandem duplication of tRNA<sup>Met</sup> gene has been found in the mtDNA of *Quasipaa boulengeri* (Yuan et al., 2016); ND5 gene between tRNA<sup>Ser</sup> and ND6 has been translocated to the region between the CR and the LTPF tRNA cluster in *Buergeria buergeri* (Sano et al., 2004). The phenomena of mt gene reorganizations have generally been interpreted by the Tandem Duplication and Random Loss model (San Mauro et al., 2006).

In the Neobatrachia, the phylogenetic relationships among Rhacophoridae, Mantellidae, Dicroglossidae and Ranidae remain controversial. Some phylogenetic studies supported the relationship of ((Rhacophoridae + Mantellidae) + (Dicroglossidae + Ranidae))

**Abbreviations**

PCR, polymerase chain reaction; tRNA, Ribosomal RNA; rRNA, transfer RNA; ATP6, ATPase subunit 6; ATP8, ATPase subunit 8; bp, base pairs; COI-II, cytochrome c oxidase subunit I-II; Cyt b, cytochrome b; CR, control region; H strand, heavy strand; L strand, light strand; mtDNA, mitochondrial DNA; ND1-6, and ND4L, NADH dehydrogenase subunit 1-6, and 4L; OL, L-strand replication; ML, maximum likelihood; BI, Bayesian inference.
(Zhang et al., 2009, 2018; Chen et al., 2011), but others supported a sister-taxon relationship between ((Rhacophoridae+Mantellidae) + Ranidae) and Dicroglossidae (Frost et al., 2006; Pyron and Wiens, 2011; Kakehashi et al., 2013; Kurabayashi and Sumida, 2013; Kurabayashi et al., 2010; Zhang et al., 2013; Li et al., 2014; Xia et al., 2014; Yuan et al., 2016; Chen et al., 2017).

mtDNA is an important molecular marker and has been widely used in the studies of genetics, phylogenetics and phylogeography. In the present study, we determined the complete mitochondrial genome of Rhacophorus dennysi. We performed phylogenetic analyses based on complete mt genomes of the newly sequenced and other reported species of Ranoida to assess the taxonomic position of Rhacophorus dennysi, and to test the phylogenetic relationship of Rhacophoridae and Ranidae.

MATERIALS AND METHODS

Sample collection and PCR

The R. dennysi sample was collected from Qifeng, Guniujiang, Anhui province in China. This frog sample used was stored at -40°C (Sample No. AM12026) in the Conservation Biology Laboratory, College of Life Sciences of Anhui Normal University. Total DNA was extracted from a piece of muscle tissue by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (Sambrook et al., 2001).

To determine the complete mitochondrial genomic sequence of R. dennysi, polymerase chain reaction (PCR) was carried out with the primers for the mtDNAs of frogs described in the literatures (Kurabayashi and Sumida, 2009; Zhang et al., 2013). Furthermore, based on the complete mtDNA sequences of R. schlegelii (AB202078) and P. megacephalus (AY458598), we also designed two pairs of primers to amplify mt fragments from the Cytb gene to the ND5 gene. PCR reaction volume of 30 μl contained 21μl sterile double distilled water, 3 μl 10× reaction buffer (with Mg2+), 2.5 μl (2.5 mmol/l) dNTPs, 1 μl each primer (10μmol/l), 0.5μl Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan) and 1 μl template DNA. Amplification was performed using Applied Biosystems 2720 Thermal Cycler with the following conditions: initial denaturation at 94°C for 4 min, 32 cycles of denaturation at 94°C for 40 s, annealing at 52-58°C for 40 s and elongation at 72°C for 60 s, and a final extension at 72°C for 10 min. The resulting PCR fragments were separated by electrophoresis in 1.0% agarose gels, then PCR products were purified using TIANQuick Midi Purification Kit (TIANGEN Bio Inc., Beijing, China), and then directly sequenced on an automated sequencer (ABI 3730) from both strands.

Sequence assembly and analysis

Nucleotide sequences were checked and assembled using the program SeqMan (DNASTAR Inc., Madison, WI, USA). The 13 protein-coding and two rRNA genes were annotated by comparison with the known complete mtDNA sequences of Rhacophorus schlegelii (Sano et al., 2005), Polypedates megacephalus (Zhang et al., 2005) and Buergeria buergeri (Sano et al., 2004). The 22 tRNA genes were identified by their cloverleaf secondary structure and anticodon sequences using tRNA Scan-SE v.2.0.2 (http://lowelab.ucsc.edu/tRNAscan-SE; Lowe and Chan, 2016). The complete mtDNA sequence of R. dennysi was deposited in GenBank with the accession number KM035412.

Phylogenetic reconstruction

In order to address the phylogenetic relationships among Rhacophoridae, 3 additional, previously published Rhacophoridae mitogenomes were included in the analysis. In addition, mitochondrial genomes from one species in the family Mantellidae, twenty-two species in Ranidae, and thirteen species in Dicroglossidae were retrieved from GenBank to further confirm the phylogenetic position of the family Rhacophoridae among Ranoida. Additionally, three Microhylidae species were used as the outgroups based on Pyron and Wiens (2011) (Table I).

We constructed the phylogenies using the concatenated 12 mt protein-coding genes and partitioned these genes by codon position. The best fitted substitution model for each partition was estimated using Akaike Information Criterion (AIC) implemented in jModeltest v.2.1.7 (Darriba et al., 2012). The GTR+I+G model was chosen for ML and Bayesian inference (BI) analyses, which were separately performed using RaxML (Kozlov et al., 2019) with 1000 bootstrap replications and MrBayes v.3.2.7 (Ronquist et al., 2012). Besides, the following settings were applied in the BI analysis: 10 million Markov chain Monte Carlo (MCMC) generations, a sampling frequency of 1000, burn-in = 1000.

RESULTS

Genome organization of R. dennysi mtDNA

The R. dennysi mt genome was 18,052 bp in length, containing 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes and a control region (Table II). The base composition of the light strand (L-strand) was 31.5.9% A, 31.0% T, 23.2% C, and 14.3% G, which is similar to other vertebrates (Zhang et al., 2015; Li et al., 2016).
Table I. Species in phylogenetic analyses.

| Family         | Species                | GenBank accession no. |
|----------------|------------------------|-----------------------|
| Microhylidae   | Kaloula pulchra        | NC_006405             |
|                | Microhyla okinavensis  | NC_010233             |
|                | M. heymonsi            | NC_006406             |
| Mantellidae    | Mantella madagascariensis | NC_007888          |
| Rhacophoridae  | Buergeria buergeri     | NC_008975             |
|                | Polypedates megacephalus | NC_006408          |
|                | Rhacophorus schlegeli  | NC_007178             |
|                | R. dennysi             | KM035412              |
| Ranidae        | Pelophylax ridibundus  | JN627421              |
|                | P. lessonae            | JN627426              |
|                | P. esculenta           | JN627424              |
|                | P. chosena             | NC_016059             |
|                | P. nigromaculata       | NC_002805             |
|                | P. planci             | NC_009264             |
|                | Amolops mantzorum      | KJ546429              |
|                | A. ricketti            | KF956111              |
|                | A. wayiensis           | KJ933509              |
|                | Odorrana schmackeri    | KJ149452              |
|                | O. tornoos             | NC_009423             |
|                | O. ishikawa            | NC_015305             |
|                | Rana catesbeiana       | NC_022696             |
|                | R. dybowskii           | NC_023528             |
|                | R. cf. chensisinensis  | NC_023529             |
|                | Babina holsti          | NC_022870             |
|                | B. subaspera           | NC_022871             |
|                | B. okinavana           | NC_022872             |
|                | B. adenopleura         | JX033120              |
|                | Hylarana albolabialis  | JX564871              |
|                | H. guentheri           | KM033541             |
|                | Rugosa tientiansiens   | KJ941041              |
| Dicroglossidae | Hoplobatrachus rugulosus | NC_019615           |
|                | H. tigrinus            | NC_014851             |
|                | Euphlyctis hexadactylus| NC_014854             |
|                | Fejervarya cancrivora  | NC_012647             |
|                | Limnonectes banaensis  | NC_012837             |
|                | L. fijianensis         | NC_007440             |
|                | L. fragilis            | NC_021937             |
|                | Quasipaa boulengeri    | NC_013270             |
|                | Paa spinosa            | NC_016119             |
|                | Nanorana pleskei       | KJ569109              |
|                | N. taihangnica         | NC_014685             |

Table II. Location of features in the mitochondrial DNA of Rhacophorus dennysi.

| Region | Nucleotide No. | Size (bp) | Codon | Spacer (+)/ Overlap (-) | strand |
|--------|----------------|-----------|-------|--------------------------|---------|
| CR     |                |           |       |                          |         |
| ND5    | 2604           | 4376      | 1773  | ATG TAA                  | H       |
| tRNA7p | 4472           | 4541      | 70    |                          | H       |
| tRNA7a | 4556           | 4627      | 72    |                          | H       |
| tRNA7m | 4642           | 4704      | 63    |                          | L       |
| tRNA7u | 4705           | 4774      | 70    |                          | H       |
| 12S rRNA | 4775       | 5070      | 928   |                          | H       |
| tRNA8k | 5703           | 5771      | 69    |                          | H       |
| 16S rRNA | 5772      | 5732      | 1581  |                          | H       |
| tRNA4k | 7353           | 7425      | 73    |                          | H       |
| ND1    | 7426           | 8386      | 961   | ATA T-                   | H       |
| tRNA5k | 8387           | 8457      | 71    |                          | L       |
| tRNA9k | 8457           | 8527      | 71    |                          | L       |
| tRNA10k| 8527           | 8595      | 69    |                          | L       |
| ND2    | 8596           | 9633      | 1038  | ATT TAG                  | H       |
| tRNA2p | 9637           | 9707      | 71    |                          | H       |
| tRNA3k | 9708           | 9777      | 70    |                          | L       |
| tRNA4k | 9779           | 9851      | 73    |                          | L       |
| OL     | 9852           | 9881      | 30    |                          | L       |
| tRNA6k | 9879           | 9943      | 65    |                          | L       |
| tRNA7p | 9944           | 10010     | 67    |                          | L       |
| tRNA1p | 11005          | 11568     | 1554  | ATA AGG                  | H       |
| tRNA2p | 11556          | 11626     | 71    | -13                      | L       |
| tRNA3k | 11629          | 11697     | 69    |                          | H       |
| COI1   | 11698          | 12381     | 684   | ATG TAA                  | H       |
| tRNA2p | 12393          | 12463     | 71    |                          | L       |
| ATP8   | 12464          | 12628     | 165   | ATG TAA                  | H       |
| ATP6   | 12607          | 13300     | 694   | ATG T-                   | H       |
| COI2   | 13301          | 14084     | 784   | ATG T-                   | H       |
| tRNA5k | 14085          | 14152     | 68    |                          | H       |
| ND3    | 14153          | 14492     | 340   | ATG T-                   | H       |
| tRNA6k | 14493          | 14561     | 69    |                          | H       |
| ND4L   | 14564          | 14848     | 285   | ATG TAA                  | H       |
| ND4    | 14842          | 16204     | 1363  | ATG T-                   | H       |
| tRNA7k | 16205          | 16273     | 69    |                          | H       |
| ND6    | 16345          | 16836     | 492   | ATG TAA                  | H       |
| tRNA8k | 16837          | 16904     | 68    |                          | L       |
| Cyt b  | 16907          | 18052     | 1146  | ATG TAA                  | H       |
Remarkably, the tree frog *R. dennysi* possessed a novel mitogenomic gene organization much different from other neobatrachians. In the *R. dennysi* mt genome, the *ND5* gene between tRNA<sub>Ser</sub> (AGY) and *ND6* was translocated to a position between the CR and tRNA<sub>Thr</sub>. In this new mitogenome, four tRNA genes (tRNA<sub>Thr</sub>, tRNA<sub>Leu(CUN)</sub>, tRNA<sub>Pro</sub> and tRNA<sub>Phe</sub>) formed a TLPF tRNA cluster, different from the neobatrachian-type arrangement (Sumida et al., 2001; Irisarri et al., 2012; Li et al., 2014).

Among the 13 protein-encoding genes in the *R. dennysi* mitogenome, most of these protein-coding genes started with the common initiation codon ATG except two genes (*ND1* and *COI*) beginning with ATA, one gene (*ND2*) beginning with ATT. Stop codons were variable for all protein-coding genes. Seven protein genes (*ND2*, *COII*, *ATP8*, *ND4L*, *ND5*, *ND6* and *Cytb*) used complete stop codon TAR, and *COI* ended with AGG, whereas other genes (*ND1*, *ATP6*, *COIII*, *ND3* and *ND4*) ended with incomplete stop codon T.

The noncoding regions in the *R. dennysi* mtDNA contained the control region and some spacers. The control region was located between the *Cytb* and *ND5* genes with the length of 2,603 bp. The length of the CR in this study is obviously longer than that of *R. Dennysi* (2,122 bp) in the literature of Huang et al. (2016).

**Phylogenetic analyses**

The BI and ML analyses of the molecular dataset produced the identical topologies and very similar branch support (Fig. 1). In the phylogeny of Rhacophoridae, Ranidae, Dicroglossidae and Mantellidae, the monophyly of Dicroglossidae, Ranidae and Rhacophoridae are well supported.

![Fig. 1](image)

**Fig. 1.** The phylogeny of Ranidae sensu lato (Dubois, 2005) inferred from the combined sequences of 12 protein-coding and two rRNA genes. The Bayesian tree was shown here, the ML had an identical tree topology. Numbers of nodes were support values from ML (bootstrap proportions; left) and BI (posterior probabilities; right).
In our tree, the 40 in group species referred in this study were divided into four major clades: Mantellidae, Rhacophoridae, Ranidae and Dicroglossidae, which strongly supported the monophyly of Dicroglossidae, Ranidae and Rhacophoridae. Rhacophoridae was a sister clade to Mantellidae with strong supports (BP = 100, PP = 1.00) and the clade of (Rhacophoridae + Mantellidae) appeared as the sister taxon to Ranidae (BP = 100, PP = 0.92), then together as a sister group of the Dicroglossidae (BP = 100, PP = 1.00). The Rhacophoridae clade included 4 species R. schlegelii, R. dennysi, P. megacephalus, B. buergeri. R. schlegelii and R. dennysi were grouped as the sister clade of P. megacephalus with high supports (BP = 100, PP = 1.00), then together as a sister taxon of B. buergeri (BP = 100, PP = 1.00).

**DISCUSSION**

*Gene rearrangement and the significance for the phylogeny*

In present study, we discovered a novel gene arrangement of *R. dennysi* mt genome. The ND5 gene and four tRNA genes (tRNA\[^{Thr}\], tRNA\[^{Leu(CUN)}\], tRNA\[^{Pro}\] and tRNA\[^{Phe}\]) forming TLPF tRNA cluster) were located between the CR and the 12S tRNA gene, which differed from the neobatrachian-type arrangement (LTFF tRNA cluster) but shared similarities to those of *R. schlegelii* (Sano et al., 2005) and *P. megacephalus* (Zhang et al., 2005), two other species from the same family. Gene rearrangement in animal mtDNA is generally believed to take place through the Tandem Duplication and Random Loss (TDRL) model (San Mauro et al., 2006). According to the TDRL model, a multigene portion of the genome is duplicated, then one copy becomes nonfunctional and is subsequently deleted from the genome.

Generally, gene rearrangements have been considered as useful indicators to resolve some phylogenetic relationships (San Mauro et al., 2006; Zhang et al., 2018). For example, the duplication of tRNA\[^{Met}\] likely appeared in all the descendants of Dicroglossidae, indicating that the duplicated tRNA\[^{Met}\] genes can be regarded as a synapomorphic character of Dicroglossidae (Alam et al., 2010; Chen et al., 2011, 2017).

Within neobatrachians, the translocation of ND5 was discovered in two distinct lineages: Rhacophoroida and Mantellidae, and a part of Dicroglossidae (Oecidozyga, Fejervarya, Euphlyctis and Hoplobatrachus) (Sano et al., 2004, 2005; Kurabayashi et al., 2008; Alam et al., 2010; Chen et al., 2017). Thus, convergent gene rearrangements occur frequently in non-sister lineages (Kurabayashi and Sumida, 2013). We should require careful consideration when genomic features are employed for phylogenetic relationship.

**Phylogeny of Ranoidea (Dubois 2005)**

Phylogeny of Ranoidea (i.e., Rhacophoridae, Mantellidae, Ranidae and Dicroglossidae) has not reached a consensus (Chen et al., 2011; Yuan et al., 2016). In our analyses, the sister-group relationship between ((Rhacophoridae + Mantellidae) + Ranidae) and Dicroglossidae has been well supported (BP = 100, PP = 1.00), consistent with the molecular studies of Kakehashi et al. (2013), Kurabayashi and Sumida (2013), Zhang et al. (2013), Xia et al. (2014), Yuan et al. (2016) and Chen et al. (2017).

**Phylogenetic analyses of Rhacophoridae**

In our phylogenetic trees, two species of the genus *Rhacophorus* (*R. schlegelii* and *R. dennysi*) were clustered together with the representative of the genus *Polypedates* (*P. megacephalus*), indicating a very close phylogenetic relationship, meanwhile, the representative of the genus *Buergeria* (*B. buergeri*) occupied the basal position in the clade of Rhacophoridae. The phylogenetic relationship among the representatives of the family Rhacophoridae revealed here is congruent with the results of most phylogenetic analyses (Yu et al., 2009; Pyron and Wiens, 2011; Zhang et al., 2013, 2018; Chen et al., 2017; Chan et al., 2018).

**CONCLUSIONS**

As a whole, this study provides some evidence for the generic classification of Rhacophoridae proposed by Pyron and Wiens (2011), and our phylogenetic analyses supported the sister-group relationship between ((Rhacophoridae + Mantellidae) + Ranidae) and Dicroglossidae.

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**Statement of conflict of interest**

The authors have declared no conflict of interest.

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