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

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XBW designed the study. XYW and YML performed fieldwork. XYW, YML, PY and HBZ analysed the data. YML, XYW, HBZ and DWL wrote the article.

Key words
***Rhacophorus dennysi*, Mitochondrial genome, Phlyogenetic relationship, Rhacophoridae**

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 (tRNA^{Thr}, tRNA$^{Leu(CUN)}$, tRNA^{Pro} 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^{His} gene in the *Odorrana schmackeri* mitogenome (Li *et al*., 2014); a tandem duplication of tRNA^{Met} gene has been found in the mtDNA of *Quasipaa boulengeri* (Yuan *et al*., 2016); *ND5* gene between tRNA^{Ser} 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))

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Abbreviations
PCR, polymerase chain reaction; rRNA, Ribosomal RNA; tRNA, transfer RNA; ATP6, ATPase subunit 6; ATP8, ATPase subunit 8; bp, base pairs; COI-III, cytochrome c oxidase subunit I-III; 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 nucleotide sequence of the mitochondrial genome of *Rhacophorus dennysi*. We performed phylogenetic analyses based on complete mt genomes of the newly sequenced and other reported species of Ranoidea 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 Mg^{2+}), 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 Ranoidea. 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 schlegelii	NC_007178
	R. dennysi	KM035412
Ranidae	*Pelophylax ridibundus*	JN627421
	P. lessonae	JN627426
	P. esculenta	JN627424
	P. chosenica	NC_016059
	P. nigromaculata	NC_002805
	P. plancyi	NC_009264
	Amolops mantzorum	KJ546429
	A. ricketti	KF956111
	A. wuyiensis	KJ933509
	Odorrana schmackeri	KJ149452
	O. tormotus	NC_009423
	O. ishikawae	NC_015305
	Rana catesbeiana	NC_022696
	R. dybowskii	NC_023528
	R. cf. chensinensis	NC_023529
	Babina holsti	NC_022870
	B. subaspera	NC_022871
	B. okinavana	NC_022872
	B. adenopleura	JX033120
	Hylarana albolabris	JX564871
	H._guentheri	KM035413
	Rugosa tientaiensis	KJ941041
Dicroglossidae	*Hoplobatrachus rugulosus*	NC_019615
	H. tigerinus	NC_014581
	Euphlyctis hexadactylus	NC_014584
	Fejervarya cancrivora	NC_012647
	F. limnocharis	NC_005055
	Limnonectes bannaensis	NC_012837
	L. fujianensis	NC_007440
	L. fragilis	AY899241
	Quasipaa boulengeri	NC_021937
	Paa spinosa	NC_013270
	Nanorana pleskei	NC_016119
	N. taihangnica	KJ569109
	Occidozyga martensii	NC_014685

Table II. Location of features in the mitochondrial DNA of *Rhacophus dennysi.*

Region	Nucleotide No.		Size (bp)	Codon		Spacer (+)/ Over-lap (-)	strand
	From	To		Start	Stop		
CR	1	2603	2603				
ND5	2604	4376	1773	ATG	TAA		H
$tRNA^{Thr}$	4472	4541	70			95	H
$tRNA^{Leu(CUN)}$	4556	4627	72			14	H
$tRNA^{Pro}$	4642	4704	63			14	L
$tRNA^{Phe}$	4705	4774	70				H
12S rRNA	4775	5702	928				H
$tRNA^{Val}$	5703	5771	69				H
16S rRNA	5772	7352	1581				H
$tRNA^{Leu(UUR)}$	7353	7425	73				H
ND1	7426	8386	961	ATA	T-		H
$tRNA^{Ile}$	8387	8457	71				H
$tRNA^{Gln}$	8457	8527	71			-1	L
$tRNA^{Met(AUN)}$	8527	8595	69			-1	H
ND2	8596	9633	1038	ATT	TAG		H
$tRNA^{Trp}$	9637	9707	71			3	H
$tRNA^{Ala}$	9708	9777	70				L
$tRNA^{Asn}$	9779	9851	73			1	L
OL	9852	9881	30				–
$tRNA^{Cys}$	9879	9943	65			-3	L
$tRNA^{Tyr}$	9944	10010	67				L
COI	10015	11568	1554	ATA	AGG	4	H
$tRNA^{Ser(UCN)}$	11556	11626	71			-13	L
$tRNA^{Asp}$	11629	11697	69			2	H
COII	11698	12381	684	ATG	TAA		H
$tRNA^{Lys}$	12393	12463	71			11	H
ATP8	12464	12628	165	ATG	TAA		H
ATP6	12607	13300	694	ATG	T-	-22	H
COIII	13301	14084	784	ATG	T-		H
$tRNA^{Gly}$	14085	14152	68				H
ND3	14153	14492	340	ATG	T-		H
$tRNA^{Arg}$	14493	14561	69				H
ND4L	14564	14848	285	ATG	TAA		H
ND4	14842	16204	1363	ATG	T-		H
$tRNA^{His}$	16205	16273	69				H
$tRNA^{Ser(AGY)}$	16274	16340	67				H
ND6	16345	16836	492	ATG	TAA		L
$tRNA^{Glu}$	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 $tRNA^{Ser\ (AGY)}$ and *ND6* was translocated to a position between the CR and $tRNA^{Thr}$. In this new mitogenome, four tRNA genes ($tRNA^{Thr}$, $tRNA^{Leu(CUN)}$, $tRNA^{Pro}$ and $tRNA^{Phe}$) 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.

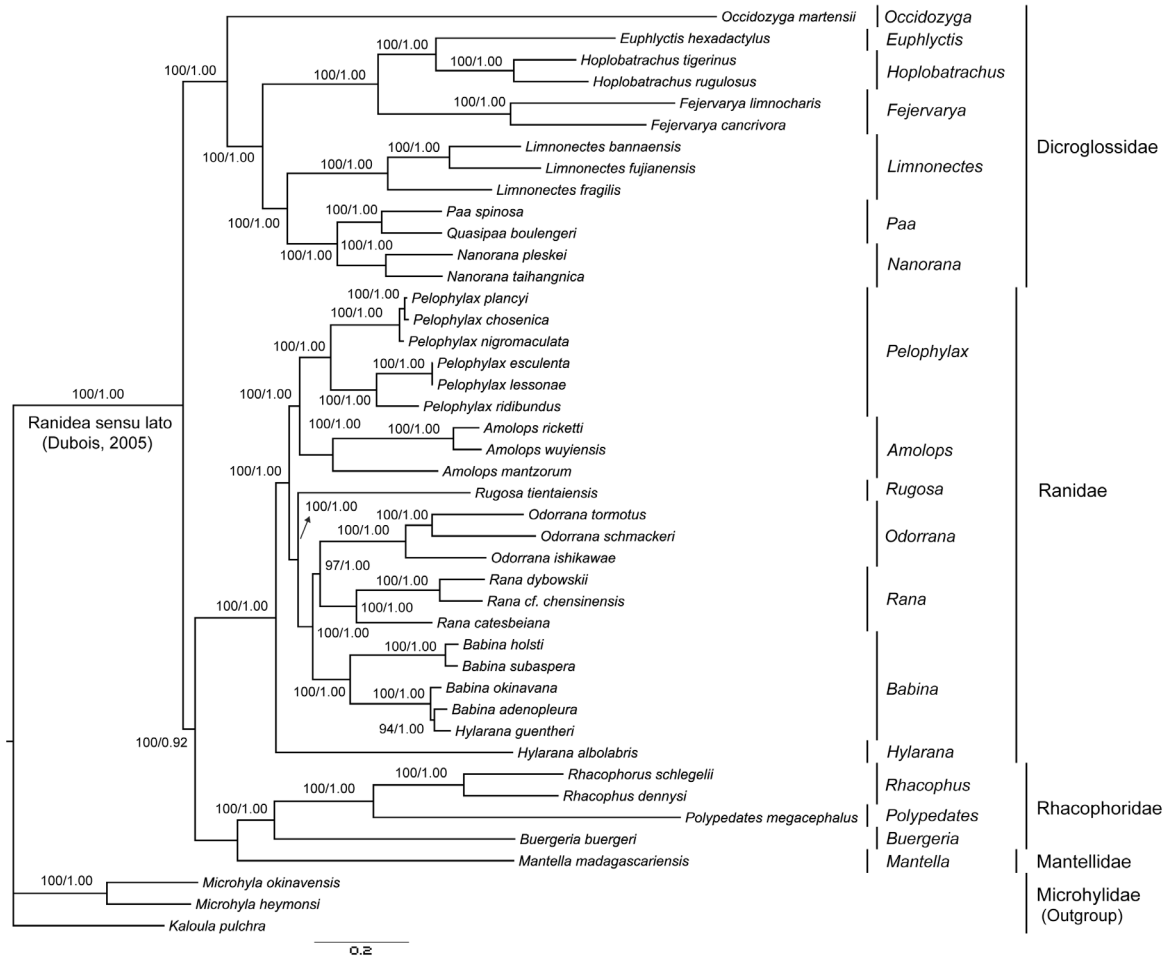

Fig. 1. The phylogeny of Ranidae sensu lato (Dubois, 2005) inferred from the combined sequences of 12 protein-coding and two rRNA genes. The Bayesian tree was shown here, the ML had an identical tree topology. Numbers of nodes were support values from ML (bootstrap proportions; left) and BI (posterior 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 rRNA gene, which differed from the neobatrachian-type arrangement (*LTPF* 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: Rhacophoroidea and Mantellidae, and a part of Dicroglossidae (*Occidozyga*, *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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