Complete mitochondrial genomes of *Nanorana taihangnica* and *N. yunnanensis* (Anura: Dicroglossidae) with novel gene arrangements and phylogenetic relationship of Dicroglossidae

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Abstract

**Background:** Complete mitochondrial (mt) genomes have been used extensively to test hypotheses about microevolution and to study population structure, phylogeography, and phylogenetic relationships of Anura at various taxonomic levels. Large-scale mt genomic reorganizations have been observed among many fork-tongued frogs (family Dicroglossidae). The relationships among Dicroglossidae and validation of the genus *Feirana* are still problematic. Hence, we sequenced the complete mt genomes of *Nanorana taihangnica* (= *F. taihangnica*) and *N. yunnanensis* as well as partial mt genomes of six *Quasipaa* species (dicroglossid taxa), two *Odorrana* and two *Amolops* species (Ranidae), and one *Rhacophorus* species (Rhacophoridae) in order to identify unknown mt gene rearrangements, to investigate the validity of the genus *Feirana*, and to test the phylogenetic relationship of Dicroglossidae.

**Results:** In the mt genome of *N. taihangnica* two *trnM* genes, two *trnP* genes and two control regions were found. In addition, the *trnA*, *trnN*, *trnC*, and *trnQ* genes were translocated from their typical positions. In the mt genome of *N. yunnanensis*, three control regions were found and eight genes (*ND6, trnP, trnQ, trnA, trnN, trnC, trnY* and *trnS* genes) in the L-stand were translocated from their typical position and grouped together. We also found intraspecific rearrangement of the mitochondrial genomes in *N. taihangnica* and *Quasipaa boulengeri*. In phylogenetic trees, the genus *Feirana* nested deeply within the clade of genus *Nanorana*, indicating that the genus *Feirana* may be a synonym to *Nanorana*. Ranidae as a sister clade to Dicroglossidae and the clade of (Ranidae + Dicroglossidae) as a sister clade to (Mantellidae + Rhacophoridae) were well supported in BI analysis but low bootstrap in ML analysis.

**Conclusions:** We found that the gene arrangements of *N. taihangnica* and *N. yunnanensis* differed from other published dicroglossid mt genomes. The gene arrangements in *N. taihangnica* and *N. yunnanensis* could be explained by the Tandem Duplication and Random Loss (TDRL) and the Dimer-Mitogenome and Non-Random Loss (DMNR) models, respectively. The invalidation of the genus *Feirana* is supported in this study.

**Keywords:** Dicroglossidae, *Feirana*, Mitochondrial genome, Gene rearrangement, Phylogeny
Background

Vertebrate mitochondrial (mt) genomes are closed circular molecules that generally have lengths varying from 15 to 27 kb [1]. They typically encode 37 genes including two ribosomal RNAs (12S and 16S rRNAs), 22 transfer RNAs (tRNAs), 13 protein-coding genes, and one long non-coding region (NCR) called the control region (CR; also referred to as the D-loop region) [2, 3]. The mt genome has several valuable characteristics including small size, rapid evolutionary rate, relatively conserved gene content and organization, maternal inheritance, and limited recombination [4]. Complete mt genomes have been extensively used to test hypotheses about microevolution, to study population structure, phylogeography, and phylogenetic relationships at various taxonomic levels, and to identify cryptic species [2, 5, 6]. The mitochondrial DNA (mtDNA) of many neobatrachian anurans shows gene rearrangement of the relative position of NADH dehydrogenase subunit 5 (ND5); this has been reported in Ranidae, Dicroglossidae, Mantellidae and Rhacophoridae [7–11]. Rearrangements of two transfer ribonucleic acid M (trnM) genes were also reported in dicroglossid and mantellid mt genomes [5, 8–10, 12–17]. Other mt genomic rearrangements can also be found in some species of neobatrachians. For example, Rhacophorus schlegelii [11], Mantella madagascariensis [9], and Rana kunyuenensis [17] possessed duplicated control regions. By contrast, Nanorana taihangnica [13] lost the trnT gene and Polyedเทพades megacephalus [18] lost the ATPase subunit 8 (ATP8) and ND5 genes. Gene rearrangements in the mitochondrial genome can be mainly explained by six available models: the recombination model [8, 19], the Tandem Duplication and Random Loss model (TDLR) [20], the Tandem Duplication and Non-Random Loss model (TDNL) [21], the tRNA miss-priming model [22], the Dimer-Mitogenome and Non-Random Loss model (DMNR) [23] and/or the Double Replications and Random Loss model (DRRL) [24].

Within the Neobatrachia, the monophyly of the combined Mantellidae and Rhacophoridae has been generally accepted, but the relationships of Ranidae, Dicroglossidae and (Mantellidae + Rhacophoridae) have been in controversy. The relationships of (Dicroglossidae + (Ranidae + (Rhacophoridae + Mantellidae))) was supported by Frost et al. [25], Kakehashi et al. [1], Kurabayashi and Sumida [26], Kurabayashi et al. [27], Li et al. [7], Xia et al. [28], Pyron and Wiens [29] and Yuan et al. [30]. However, Chen et al. [31], Ren et al. [32], Zhang et al. [33] and Zhou et al. [12] supported the relationship of ((Ranidae + Dicroglossidae) + (Rhacophoridae + Mantellidae)). Furthermore, the relationships within Dicroglossidae are extremely problematic, and have received much attention. The dicroglossids are divided into two subfamilies and four tribes: Dicroglossinae (Dicroglossini, Limnonectini, and Painii) and Occidozyginae (Occidozygini) with the classification of spiny frogs and non-spiny frogs belonging to the tribe Paini (Dicroglossidae) remaining obscure [25, 34–36]. The taxonomy of this group has been revised numerous times [37–40]. The genus Feirana of tribe Paini including three species (F. taihangnica, F. quadratus and F. kangsianensis) is widely distributed in China [36, 41, 42] and was considered to be a synonym to Nanorana by Frost et al. [25, 43] and Che et al. [35, 44]. Although Dubois transferred Feirana species to the subgenus Rana (Paa) [38], Fei et al. [39] assigned them to the newly created subgenus Paa (Quadranera). Dubois [38] and Fei et al. [40] placed the subgenus Quadranera as genus Feirana. So, the validity of genus Feirana is still unknown.

Large-scale mt genomic rearrangements in many Dicroglossidae species have been observed. However, complete information on Nanorana and Quasipaa mt genomes is still lacking except for Nanorana parkeri [15], Nanorana pleskei [31], Quasipaa boulengeri [16], Quasipaa spinosa [12], Yerana yei [14] and Nanorana taihangnica [13]. Compared with neobatrachian families, the dicroglossid mt genomes investigated thus far feature differences in gene arrangements, which gave us more chances to discuss the potential reasons for gene rearrangements in the mitochondrial genome.

In the present study, we determined the complete mt genomes of N. taihangnica and N. yunnanensis as well as the partial mt genomes of six Quasipaa species (Dicroglossidae), two Odorrana and two Amolops species (Ranidae), and one Rhacophorus species (Rhacophoridae). In this paper, we follow the system of anuran taxonomy published by Fei et al. [40] and Frost et al. [43] to prevent unnecessary confusion in taxonomy. The data was used to determine unknown mt gene rearrangements, to investigate the validity of the genus Feirana, and to test the phylogenetic relationships of Ranidae and Dicroglossidae.

Methods

Ethical statement

The thirteen species studied (N. taihangnica, N. yunnanensis, Q. boulengeri, Q. exilispinosa, Q. jiulongensis, Q. robertingeri, Q. shiini, Q. verrucospinosa, Odorrana livida, O. schmackeri, Amolops hongkongensis, A. wuyiensis, Rhacophorus dennysi) are not protected by the provisions of the laws of People’s Republic of China on the protection of wildlife. Thus, the experiments in this study were performed with toe-clip tissue samples collected from all frog specimens and stored in 100% ethanol. Sample acquisition was reviewed, approved and carried out in accordance with the relevant guidelines of the Committee of Animal Research Ethics of Zhejiang Normal University.
Sample collection
Specimens included two species of Nanorana (*N. taihangnica = F. taihangnica, N. yunnanensis*), seven samples belonging to six species of Quasipaa (*Q. boulengeri, Q. exilispinosa, Q. jiulongensis, Q. robertingeri, Q. shini, Q. verrucospinosa*) (Dicroglossidae) including two *Q. boulengeri* samples from two different sites, two species of *Odorrana* (*O. livida, O. schmackeri*) (Ranidae), two species of *Amolops* (*A. hongkongensis, A. wuyiensis*) (Ranidae), and *R. dennysi* (Rhacophoridae). Information on all of the sequenced samples is shown in Table 1. We were unable to successfully sequence the displacement loop (D-loop) region of these samples except for *N. taihangnica* and *N. yunnanensis* because of highly repetitive regions in the D-loop or other unknown reasons despite many optimization efforts; this is similar to the report of Zhang et al. [45].

PCR and sequencing
Total DNA was extracted from the clipped toe of each frog specimen using a DNeasy Tissue Kit (Qiagen, Germany). We amplified overlapping fragments that covered the entire mt genome of *N. taihangnica* and *N. yunnanensis* by normal PCR and long-and-accurate polymerase chain reaction (LA PCR) methods slightly modified from Yu et al. [5, 46] and Zhang et al. [45]. All PCR procedures were performed using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). TaKaRa Ex-Taq and LA-Taq kits (Takara Biomedical, Dalian, China) were used for the normal and LA-PCR reactions. The resulting PCR fragments were electrophoresed on 1% agarose gels, and all target DNAs were purified from excised pieces of gel using a SanPrep DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China) prior to sequencing. The sequences for each fragment were obtained in an automated DNA sequencer (ABI 3730) from both strands. The long fragments were sequenced using specific primer walking of both strands.

Sequence assembly and analysis
Sequences were checked and assembled using SeqMan (Lasergene version 5.0) [47]. The locations of the 13 protein coding genes and two rRNA genes were determined by comparison with the available RefSeq sequences of closely related anurans downloaded from GenBank using ClustalW in Mega 5.0 [48, 49]. All tRNA genes were identified by their cloverleaf secondary structure using tRNA-scan SE 1.21 [50] or determined by comparison with the homologous sequences of other anurans. The mt genomes (see Fig. 1) of all taxa were analyzed to determine the corresponding mt gene arrangements. The resultant sequences were deposited in GenBank with accession numbers KF199146-KF199152, KX233864-KX233869 and KM282625 (see Table 1).

Molecular phylogenetic analysis
With the recently increased number of mitochondrial genomes available for Anura, phylogenetic analyses were performed with 83 anurans for which complete or partial mt genomes were available including 14 samples of the 13 species from this study. In total this included the ingroup of 33 species from Ranidae [1, 27, 28, 45, 46, 51–63], 28 species from Dicroglossidae [5, 10, 12–14, 16, 17, 31–33, 64], 13 species from Rhacophoridae [11, 18, 65], one species from Mantellidae [9], one species from Petropedetidae [45], one species from Pyxicephalidae [45], one species from Phrynobatrachidae [45], one species from Ptychadenidae [45], one species from Brevicipitidae [65], one species from Mantis 

| Species               | Collection Locality | Collection Date | Specimen No. | Accession No. |
|-----------------------|---------------------|-----------------|--------------|---------------|
| *N. taihangnica*      | Luanchuan, Henan    | 17-July-2010    | LGW-LC-001   | KF199146      |
| *N. yunnanensis*      | Luoping, Yunnan     | 22-Oct-2010     | STJW-LP-001  | KF199150      |
| *Q. boulengeri*       | TONGLH, Hubei       | 7-Jun-2009      | JFW-TS-002   | KF199152      |
| *Q. exilispinosa*     | Wuyishan, Fujian    | 8-Jun-2010      | XJW-WYS-001  | KF199151      |
| *Q. jiulongensis*     | Wuyishan, Fujian    | 8-Jun-2010      | JLVW-WYS-002 | KF199149      |
| *Q. shini*            | Longsheng, Guangxi  | 10-Oct-2011     | XJW-LS-001   | KF199148      |
| *Q. verrucospinosa*   | Pingbían, Yunnan    | 7-July-2007     | DLYW-PB-003  | KF199147      |
| *Q. boulengeri*       | Luoping, Yunnan     | 22-Oct-2010     | JFW-LPS-002  | KX233867      |
| *Q. robertingeri*     | Hejiang, Sichuan    | 7-July-2012     | HJXW-HJ-001  | KX233868      |
| *O. livida*           | Wenzhou, Zhejiang   | 7-Aug-2013      | DLW-WZ-004   | KX233865      |
| *O. schmackeri*       | Wenzhou, Zhejiang   | 7-Aug-2013      | HCW-WZ-001   | KX233866      |
| *A. hongkongensis*    | Wuyishan, Fujian    | 13-July-2013    | DYTW-WYS-001 | KX233864      |
| *A. wuyiensis*        | Wenzhou, Zhejiang   | 7-Aug-2013      | WYTW-WZ-001  | KM282625      |
| *R. dennysi*          | Yangjiang, Guangdong| 1-Sept-2011     | DFSW-YJ-002  | KX233869      |
In order to discuss the phylogenetic relationship of Anura, we used the amino acid data and the nucleotides data to compare the identical topology or not according to the methods of Zhang et al. [6] and Zhou et al. [12]. The amino acid sequences of 10 mt protein-coding genes were separately aligned in Mega 5.0 [48] excluding the ATP8, ND5 and ND6 genes for the following reasons: (a) the ATP8 sequence was too short in length and had too little good information (only 18 nucleotides or <0.5% of the total nucleotides of combined PCGs) after G-Block analysis, (b) the loss of the ND5 gene in some species [18], and (c) the heterogeneous base composition and poor phylogenetic performance for ND6 which failed to support the consistency analysis with other PCGs [45]. The alignments were revised using Gblocks 0.91b software with the default parameters [68] to select conserved regions of the putative amino acids. We concatenated the alignments of the 10 other mitochondrial protein-coding genes and got an alignment consisting of 2497 amino acid residues as 10Paa dataset. An alignment of 7491 nucleotides sites with 4919 variable informative sites was converted from 2497 amino acids data directly using the amino acid alignment as the backbone. Saturation analysis was performed for subsets with first, second, and third codon positions using DAMBE 4.2.13 [69]. The results showed that the third codon positions were saturated. Thus, we excluded the third codon positions from further phylogenetic analyses and obtained a dataset called 10P consisting of 4994 nucleotide sites from the 1st and 2nd codon positions of the 10 protein-coding genes according to the methods of Cameron et al. [70], Zhang et al. [6] and Zhou et al. [12].

The phylogeny was analyzed using the combined datasets 10P (nucleotides dataset) and 10Paa (amino acid dataset) by the maximum likelihood (ML) and Bayesian inference (BI) methods. To improve the fit of the substitution model to the datasets of 10P and 10Paa, we compared data partitioning schemes according to the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) using the program PartitionFinder v1.0 and PartitionFinderProtein [71]. We set the 10 coding-
The genome organization of mtDNA with ten partitions was chosen for ML by the RaxML with MTMAM. So, the optimal model for 10P with ten-partitions were optimal: 1) first codon positions of the 10 protein-coding genes; 2) second codon positions of the 10 protein-coding genes. The best substitution model of twenty-partitions in ten different genes of dataset 10P is always GTR + I + G. For the dataset 10Paa, model of twenty-partitions in ten different genes of data-the 10 protein-coding genes. The best substitution 10 protein-coding genes; 2) second codon positions of dataset 10Paa, respectively. For the dataset 10P, twenty-genes as 20 partitions in dataset 10P and 10 partitions in

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Results

Genome organization of mtDNA

The N. taihangnica mt genome is 21,322 base pairs (bp) in length and contains 13 protein coding genes, two rRNA genes, 24 tRNA genes (including extra trnM and trnP genes), and 10 NCRs including two control regions (CRs). The two CRs were located between the cytochrome b (Cyt b) and trnL genes (CR1 2014 bp) and between the trnC and trnT genes (CR2 2698 bp). Remarkably, CR1 and CR2 have nearly identical nucleotide sequences (99.9% similarity with only 1 substitution in 2014 alignment sites) excluding the 5'–635 bases and 3'–49 bases in CR2. Tandem duplication of the trnM gene and an additional trnP gene were found (Fig. 1). The trnT-trnP-trnF tRNA cluster moved from the typical neobatrachian LTPF tRNA cluster to a position between the CR1 and NADH dehydrogenase subunit 1 (ND1) genes. The typical LTPF tRNA cluster was replaced by a trnL-trnP-pseudo trnF tRNA cluster. The pseudo-trnF showed 89.9% nucleotide similarity with the corresponding trnF gene in the trnT-trnP-trnF tRNA cluster. This pseudo-trnF contained the same anticodon nucleotides (Fig. 2) compared to trnF. The trnA, trnN, trnC, and trnQ genes were translocated from their typical positions and replaced by a 40–138 bp NCR (Fig. 1). The trnQ gene moved from the typical dicroglossid IQMM tRNA cluster to a location between a 209 bp NCR and a 208 bp NCR (Fig. 1) and within the former IQMM tRNA cluster the trnQ gene was replaced by a 40 bp NCR between the trnl and tandem trnM genes. The trnA, trnN, and trnC genes also moved from the WANCY tRNA cluster to a position between a 208 bp NCR and CR1 (Fig. 1). The positions of the Light-strand replication origin (O_L) are located between a 138 bp NCR (non-coding region) and a 52 bp NCR for the translocations of trnA, trnN, and trnC genes, and a W-NCR (138 bp)-O_L-NCR (52 bp)-Y gene cluster was formed in the position of the typical WANOQCY gene cluster. Furthermore, a new cluster consisting of a L-NCR (209 bp)-Q-NCR (208 bp)-A-N-C gene arrangement was observed (Fig. 1).

The two trnP genes contained the same anticodon nucleotides as in trnF whereas trnA and trnN contained different anticodon nucleotides (Fig. 2).

The N. yunnanensis mt genome is 23,685 bp in length and contains 13 protein coding genes, two rRNA genes, 23 tRNA genes (including an extra trnM gene), and nine non-coding regions (including three control regions) (Fig. 1). Eight genes (ND6, trnP, trnQ, trnA, trnN, trnC, trnY and trnS) genes in the L-stand were translocated from the typical position to CR regions or near to CR regions and grouped together. CR1, CR2 and CR3 with lengths of 1635 bp, 1581 bp and 1560 bp, respectively, were found between Cyt b and trnQ, between ND6 and trnP, and between trnS and trnL, respectively (Fig. 1). The three CRs have a similar sequence with a length of 1372 bp. The typical WAN(OL)CY tRNA cluster was replaced by a modified W-NCR (139 bp)-O_L-NCR (84 bp) arrangement. Through trnP translocation, the LTF tRNA cluster replaced the LTPF tRNA cluster. Through trnQ translocation, the J-NCR (40 bp)-MM tRNA cluster replaced the IQMM tRNA cluster. Through ND6 gene translocation, a 231 bp NCR replaced the ND6 gene in the original region. Through translocation of the trnS gene to between cytochrome c oxidase subunit 1 (COI) and trnD, the 51 bp NCR replaced trnS. A 47 bp NCR was found between the trnS and ND5 genes. The detailed gene rearrangements of other known dicroglossids, ranids and rhacophorids in this study are described below.

Quasipaa boulengeri, Q. jiulongensis, Q. verrucospinosa

The typical WANOQCY tRNA cluster was replaced by a W-A-NCR-O_L-NCR-N-C-Y tRNA cluster, the NCR of which ranged from 20 bp to 201 bp. The typical IQMM tRNA cluster was found. A 41–56 bp NCR was also found between the trnS and ND5 genes.

Quasipaa robertingeri

The typical WANOQCY tRNA cluster was replaced by a W-A-N-310 bp NCR-O_L-C-Y tRNA cluster. The typical
IQMM tRNA cluster was found. A 46 bp NCR was also found between the \textit{trnS} and \textit{ND5} genes.

\textit{Quasipaa exilispinosa}, \textit{Q. shini}

The typical \textit{WANO}_{2}CY tRNA clusters and IQMM tRNA clusters were also found in \textit{Q. spinosa} and \textit{Q. yei}. A 32–48 bp NCR was found between the \textit{trnS} and \textit{ND5} genes.

\textit{Odorrana livida}

The typical \textit{WANO}_{2}CY tRNA cluster and a 52 bp NCR between the \textit{ND5} and \textit{ND6} gene were found.

\textit{Odorrana schmackeri}

The typical \textit{WANO}_{2}CY tRNA cluster was replaced by a \textit{W}-\textit{A}-\text{NCR-\textit{O}_{2}-NCR-C-Y} tRNA cluster. A 296 bp NCR...
with tandem sequence between the ND5 and ND6 genes was found.

**Amolops hongkongensis, A. wuyiensis**
The typical WANO/CY tRNA cluster and no NCR between the ND5 and ND6 gene were found.

**Rhacophorus dennysi**
The typical WANO/CY tRNA cluster was found and the ND5 gene between trnS and ND6 was translocated to the region between CR and trnT.

**Phylogenetic analysis**
All BI and ML phylogenetic analyses performed in this study showed similar topologies (Figs. 3 and 4). In the phylogeny of Dicroglossidae, Ranidae, Mantellidae and Rhacophoridae, the monophyly of Dicroglossidae, Ranidae and Rhacophoridae are well supported. Dicroglossidae is a sister clade of Ranidae (1.00 in posterior probability for nucleotides and amino acids datasets; 64% and 69% bootstrap frequencies for nucleotides and amino acids, respectively), and Mantellidae is a sister clade of Rhacophoridae (1.00 in posterior probability for nucleotides and amino acids datasets; 58% and 75% bootstrap frequencies for nucleotides and amino acids, respectively). Then the clade of (Ranidae + Dicroglossidae) is a sister clade of (Mantellidae + Rhacophoridae) (1.00 in posterior probability for nucleotides and amino acids datasets; 46% and 59% bootstrap frequencies for nucleotides and amino acids, respectively).

In the dicroglossid clade, Dicroglossidae was divided into two clades: Occidozyginae and Dicroglossinae (Figs. 3 and 4).
and 4). Among the dicroglossid frogs in this study, O. martensii (Occidozyginae: Occidozygini) occupied the basal phylogenetic position (1.00 in BI of both datasets; 88% in ML of nucleotide and 85% in ML of amino acids). The monophyly of Quasipaa and Feirana was supported but the monophyly of Nanorana was not supported because the clade of (N. taihangnica (=F. taihangnica) + N. quadrans (≡F. quadrans)) was not supported within the clade of Nanorana (Figs. 3 and 4). In Quasipaa, the relationship of Q. delacouri + (Q. yei + (((Q. spinosa + Q. exilispinosa) + Q. jiulongensis) + Q. shini) + ((Q. boulengeri + Q. robertingeri) + Q. verrucospinosa))) was supported in BI and ML of nucleotide datasets (most nodes: 1.00 posterior probability, >68% bootstrap frequencies). In the ranid clade, the monophyly of the Genera Odorrana, Pelophylax, Anolops and Glandirana was well supported, but the monophyly of Babina and Hylarana was not supported because Hylarana guentheri (KM035413) clustered into the clade of Genus Babina. Using the Blast function in NCBI, we found that the mt genome of Hylarana guentheri (KM035413) [57] was the most similar to Hylarana in NCBI, we found that the mt genome of Babina into the clade of Genus Babina (DQ283117) [46] with 98% identity, which suggests that Hylarana guentheri (KM035413) was misidentified and possibly corresponds to Babina adeopleura.

**Discussion**

The mtDNA arrangement

In Dicroglossidae, a 32–85 bp NCR between the trnS (AGY) and ND5 genes was observed in Paini and
The N. taihangnica tRNA cluster was lost in the previously sequenced LTPFWANCY, the trnA different gene rearrangements of the N. taihangnica previously sequenced LTPFWANCY. Comparing the tRNA gene rearrangements of mitochondrial genome (mt) gene arrangements among species of the genus Nanorana and discussions on mitochondrial gene arrangements among same species, N. taihangnica, we found different gene rearrangements in the N. taihangnica of this study because of the translocation of trnI and trnP of this study because the trnP gene was found in N. taihangnica of this study. The trnQ gene between CR1 and the 289 bp NCR occurred in N. taihangnica of this study but an extra 40 bp W- NCR (W- NCR) was found in N. taihangnica of this study. The trnQ gene was found in N. taihangnica of this study. The WANO3CY tRNA cluster was lost in previously sequenced N. taihangnica [13], whereas the trnT gene between CR1 and trnP gene was found in N. taihangnica of this study. The L-NCR (35 bp) -P tRNA cluster in the previously sequenced N. taihangnica [13] was also found in N. taihangnica of this study but an extra TPF tRNA cluster between CR1 and a 289 bp NCR occurred in N. taihangnica of this study. The IQMM tRNA cluster and the WANCY tRNA cluster existed. The trnT gene of the LTPF tRNA cluster was lost in the previously sequenced N. taihangnica [13], whereas the trnT gene between CR1 and trnP gene was found in N. taihangnica of this study. The WANO3CY tRNA cluster was lost in previously sequenced N. taihangnica while W- NCR (138 bp) -O2-NCR (52 bp) -Y was found in N. taihangnica of this study because of the translocation of trnA, trnN and trnC. Comparing mt genomes of Q. boulengeri between this study and other known sequences [16, 30], we found different gene rearrangements in the WANO3CY tRNA cluster as also found by Xia et al. [77]. In species of the genus Nanorana and Quasipaa, two types of tRNA clusters (L-NCR-MM or IQMM, WANO3CY or WAO3NCY) were found. Even in the same species, N. taihangnica and Q. boulengeri, different tRNA clusters were found, which may motivate future discussions on mitochondrial gene arrangements among Nanorana and Quasipaa species. This suggests that more mt genomes of Nanorana and Quasipaa species need to be sequenced to further determine how these different gene arrangements formed.

In N. yunnanensis, seven tRNA genes (trnQ, trnA, trnC, trnY, trnS, trnN and trnP) and the ND6 gene on the L-strand were translocated into or near to control regions and grouped together. We did not find any other species of Anura where these gene arrangements existed, but in a fish Crossorhombus azureus (Pleurostomidae) [23] seven tRNA genes (trnQ, trnA, trnC, trnY, trnS1, trnE, trnP) and the ND6 gene encoded by the Light-strand (L-strand) were translocated to a position between trnT and trnF, which is very similar to our results.

Possible gene rearrangement mechanisms

In N. taihangnica, we observed several gene rearrangements (extra CR, trnP and trnM genes as well as translocation of trnQ, trnA, trnN, and trnC) in the region between CR and the cox1 gene. We propose that the gene rearrangements may be explained by the TDRL model. Although long tandem duplication is a very rare event in mtDNA duplication, the duplication can happen between the origin for H-strand replication (O_H) in the CR and the origin for L-strand replication (O_L) in the WANCY tRNA cluster, which is a distance of about two-thirds of the genomic length. The mechanism for duplication between the CR and the WANO3CY tRNA genes in N. taihangnica could be caused by the O_H and O_L structures and be explained by the TDRL model [20], which is similar to the research of Shi et al. [24]. The hypothesized intermediate steps are as follows. Firstly, the above-mentioned O_H and O_L structures initiated DNA synthesis twice during mitochondrial replication, causing tandem duplication of the genes located between the CR and the WANCY region in the ancestral mitogenome (Fig. 5). Secondly, one of each of the duplicated gene pairs was randomly deleted completely or partially and then lost its function or became a pseudogene (Fig. 5).

In N. yunnanensis the genes of the mitogenome are extensively rearranged with clustering of eight genes on the L-strand in the same polarity and three control regions in an unexpected gene order. These special features of eight genes in the same polarity on the L-strand and two noncoding regions were reported in Crossorhombus azureus which proposed a new mechanism for gene rearrangement [23]. We can use this gene rearrangement mechanism to explain the polarity of gene rearrangement in N. yunnanensis. The hypothesized intermediate steps are as follows. Firstly, the inferred “dimer-mitogenome” intermediate of the N. yunnanensis mtDNA (Fig. 6) could be formed by two entire mitogenomes if the two mt genomes were linked by the head-to-tail method. Secondly, some duplicated genes were non-randomly deleted completely except that all ten genes on the L-strand of one mt monomer were retained; some duplicated genes were also non-randomly deleted completely or partially from the other mt monomer (Fig. 6). Thirdly, the region of CR-trnP-trnQ-trnA-trnN-trnC-trnY-trnS-trnS-ND6-trnE was duplicated. Fourthly,
Phylogenetic analyses of Dicroglossidae

The evolutionary relationships of dicroglossid taxa indicated by the phylogenetic trees were mostly similar to previously reported molecular phylogeny [5]. Roelants et al. [78] suggested that Occidozygini is a sister clade to (Dicroglossini + Paini) + Limnonectini), whereas van der Meijden et al. [79] found that Occidozyga (Occidozygini) is located within Dicroglossinae. Dubois [37] returned Occidozygini to Dicroglossinae as a tribe based on the strength of evidence produced by van der Meijden et al. [79]. In the present study, Occidozygini was found to be a sister clade to (Dicroglossini + (Paini + Limnonectini)), and Occidozygini (Occidozyginae) was observed to be a basal clade to Dicroglossinae.

In phylogenetic trees, the clade of (N. quadranus + (N. taihangnica + N. taihangnica (KJ569109)) was clustered into the Nanorana. Although N. taihangnica and N. quadranus belong to the genus Feirana according to Fei et al. [42], we draw the conclusion that genus
**Feirana** is not valid according to the phylogenetic relationship of *Nanorana* and *Feirana*, which was also supported by Frost et al. [25, 43] and Che et al. [35, 44]

**Invalidation of Q. robertingeri as a species**

The validity of *Quasipaa robertingeri* is also heatedly debated. Che et al. [35] found that *Quasipaa robertingeri* nested deeply within *Q. boulengeri* and suggested that *Q. robertingeri* should be synonymous with *Q. boulengeri*, which is supported by Frost et al. [25]. However, Fei et al. [36, 42] insisted on the validity of *Q. robertingeri* as a species. The data of Pyron and Wiens [29] supported the proposal that *Q. robertingeri* was a sister clade to *Q. shini*, not to *Q. boulengeri*. To compare the genetic divergence we analyzed the complete mt genomes and 16S RNA gene of *Q. boulengeri* and *Q. robertingeri* in Mega 5.0 with the parameter *p*-distance model. The average genetic distance between *Q. boulengeri* and *Q. robertingeri* using mt genomes and 16S RNA was determined to be 4.3% and 1.1%, respectively, which is lower than the lowest interspecies mt genomes between *Q. spinosa* and *Q. exilispinosa* (6.8%) and 16S RNA diversity as a species threshold (3%) [80], respectively. Although *Q. boulengeri* is as a sister clade to *Q. robertingeri* in phylogenetic relationship, the genetic distance between *Q. boulengeri* and *Q. robertingeri* is lower than the genetic distance between interspecies of *Quasipaa*. The different gene arrangement of *Q. boulengeri* and *Q. robertingeri* cannot be used as a species delimitation method because the gene rearrangement can also happened within intraspecies. So we deduce that *Q. robertingeri* may not be a valid species.

**Conclusion**

The characteristics of mt genomes and gene arrangements provide novel insights into the phylogenetic relationships among several major lineages of Dicroglossidae. The phylogenetic relationship of ((Ranidae + Dicroglossidae) + (Mantellidae + Rhacophoridae)) is supported in BI analyses. *Feirana* is not a valid genus according to the phylogenetic relationship with *Nanorana*. *Quasipaa robertingeri* may be an invalid species according to genetic divergence. The gene arrangements of *N. taihangnica* and *N. yunnanensis* differed from those of other published dicroglossid mt genomes. The mt genomes are promising markers for discussing the reasons for intraspecies gene rearrangements, and the current results broadens our knowledge of the evolution of anuran mt genomes.

**Abbreviations**

AIC: Akaike Information Criterion; ATP6/8: ATPase subunit 6/8; Bi: Bayesian inference; BIC: Bayesian Information Criterion; BPP: Bayesian posterior probability; CR: Control region; Cyt b: cytochrome b; DMNR: The Dimer-Mitogenome and Non-Random Loss model; DRRL: Double Replications and Random Loss model; MCMC: Markov chain Monte Carlo; NCR: Non-coding region; ND1–6: NADH dehydrogenase subunit 1–6; rRNAs: Ribosomal ribonucleic acid; TDRL: The Tandem Duplication and Random Loss model; tRNA: Transfer RNA acid X

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**Availability of data and materials**

The sequences of frogs in this study were deposited in GenBank with accession numbers: KF199146-KF199152, KX233864-KX233869 and KM282652. The alignment data used for the phylogenetic analyses are available from the corresponding authors upon request on reseachgate.

**Authors’ contributions**

Conceived and designed the experiments: YDN ZJY ZLP. Performed the experiments: YDN ZJY ZLP. Analyzed the data: ZJY YDN. Contributed reagents/materials/analysis tools: YJY ZDN ZRQ. Wrote the paper: YDN ZJY KBS ZLP ZRQ. All the authors read and approved the final version of the manuscript. The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

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