ORIGINAL RESEARCH

The first complete mitochondrial genome sequence of *Nanorana parkeri* and *Nanorana ventripunctata* (Amphibia: Anura: Dicroglossidae), with related phylogenetic analyses

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**Abstract**
Members of the *Nanorana* genus (family Dicroglossidae) are often referred to as excellent model species with which to study amphibian adaptations to extreme environments and also as excellent keystone taxa for providing insights into the evolution of the Dicroglossidae. However, a complete mitochondrial genome is currently only available for *Nanorana pleskei*. Thus, we analyzed the complete mitochondrial genomes of *Nanorana parkeri* and *Nanorana ventripunctata* to investigate their evolutionary relationships within *Nanorana* and their phylogenetic position in the family Dicroglossidae. Our results showed that the genomes of *N. parkeri* (17,837 bp) and *N. ventripunctata* (18,373 bp) encode 13 protein-coding genes (PCGs), two ribosomal RNA genes, 23 transfer RNA (tRNA) genes, and a noncoding control region. Overall sequences and genome structure of the two species showed high degree of similarity with *N. pleskei*, although the motif structures and repeat sequences of the putative control region showed clear differences among these three *Nanorana* species. In addition, a tandem repeat of the tRNA-Met gene was found located between the tRNA-Gln and ND2 genes. On both the 5′ and 3′-sides, the control region possessed distinct repeat regions; however, the CSB-2 motif was not found in *N. pleskei*. Based on the nucleotide sequences of 13 PCGs, our phylogenetic analyses, using Bayesian inference and maximum-likelihood methods, illustrate the taxonomic status of *Nanorana* with robust support showing that *N. ventripunctata* and *N. pleskei* are more closely related than they are to *N. parkeri*. In conclusion, our analyses provide a more robust and reliable perspective on the evolutionary history of Dicroglossidae than earlier analyses, which used only a single species (*N. pleskei*).

**KEYWORDS**
control region, mitochondrial genome, *Nanorana parkeri*, *Nanorana pleskei*, *Nanorana ventripunctata*, phylogenetic analyses
1 | INTRODUCTION

The Nanorana (Amphibia: Anura: Dicroglossidae) are a genus of dicroglossid frogs found over much of Asia including Pakistan, India, Nepal, China, Myanmar, Thailand, Laos, and Vietnam (Frost, 2018). The Nanorana genus comprises about 28 species (Frost, 2018), including three species (Nanorana parkeri, Nanorana ventripunctata, and Nanorana pleskei) that are endemic to the Tibetan Plateau in China (Che et al., 2010; Chen, Liu, Jiang, Xie, & Zheng, 2005; Fei, Hu, Ye, & Huang, 2009; Fei, Ye, Huang, Jiang, & Xie, 2005; Fei et al., 2004; Lu & Yang, 2004; Wang, Annemarie, Muhammad, & Xie, 2004). Nanorana parkeri is known from southern and eastern Xizang, China, and the Tibetan Plateau of Nepal at elevations of 2,850–5,000 m asl. It has recently been reported from Kashmir in northern India, but the distribution within this region needs further confirmation. Nanorana ventripunctata is endemic to northwestern Yunnan province in China, ranging in elevation from 3,120 to 4,100 m asl, while Nanorana pleskei is known from Qinghai, Gansu, and Sichuan provinces in China, at elevations of 3,300–4,500 m asl (Fei et al., 2004). Similar to most anurans, Nanorana species have a terrestrial adult life history. However, due to their high-elevation habitats, Nanorana species experience extremely harsh abiotic factors, including hypoxia, high UV radiation, and dramatic temperature changes on a daily basis. Consequently, Nanorana is an excellent model species for studying the adaptations of frogs to extreme environmental conditions (Sun et al., 2015). Does the unique high-elevation environment of Nanorana have a greater impact on species differentiation and gene sequences characteristics? Our study aimed to clarify the mitochondrial genome sequence characteristics and phylogenetic relationship and the taxonomic status of the three species in the genus Nanorana.

The phylogenetic relationships of Nanorana have been studied previously (Che et al., 2009; Chen, Wang, Liu, Xie, & Jiang, 2011; Lu, 1995; Zhou et al., 2014); however, debates on the taxonomic status of the three species that are the focus of this study are still ongoing. The taxonomy of Nanorana species is not yet fully settled because of numerous changes during the last decade. Previous phylogenetic analyses support N. pleskei and Quasipaa spinosa as having a close relationship (Chen et al., 2011), as well as N. ventripunctata and N. parkeri (N. pleskei + (N. parkeri + N. ventripunctata)) (Lu, 1995). In other literature, however, N. pleskei and N. ventripunctata are reported to have a closer relationship (N. parkeri + (N. pleskei + N. ventripunctata)) (Che et al., 2009; Zhou et al., 2014), while Pyron and Wiens (2011) thought that N. pleskei and N. parkeri had a closer evolutionary relationship (N. ventripunctata + (N. pleskei + N. parkeri)). Thus, complete sequencing of the mtDNA in Nanorana can help clarify the phylogenetic relationships and genetic diversity within the genus. With that information, we can then better understand the phylogenetic status and intraspecific relationships among the three species within this group (Che et al., 2009; Chen et al., 2011; Jiang & Zhou, 2001, 2005; Jiang et al., 2005; Roelants, Jiang, & Bossuyt, 2004).

Mitochondrial genomes have been widely used as molecular markers in phylogenetic and phylogeographic studies of amphibians because of their high mutation and substitution rates, rare gene recombination, maternal transmission pathway, high copy number, and easy accessibility (Bossuyt, Brown, Hills, Cannatella, & Milinkovitch, 2006; Howlader, Nair, Gopal, & Merlí, 2015; Jiang et al., 2005; Matsui et al., 2011; de Sá et al., 2012). Moreover, complete mitochondrial genomes are effectively used as molecular markers in studies of population genetics and conservation biology (Ren et al., 2009; Sahoo et al., 2015; San Mauro, Gower, Oommen, Wilkinson, & Zardoya, 2004; Kurbayashi, Fuji, Yonekawa, & Sumidam, 2004; Yong, Song, Lin, Eamsobhana, & Tan, 2016). For example, complete mitochondrial genomes have been used to elucidate many evolutionary questions regarding amphibians (Liu, Wang, & Bing, 2005; Yuan, Xia, Zheng, & Zeng, 2016; Zhang, Nie, Wang, & Hu, 2009), as well as to investigate the evolutionary relationships of endangered species, such as Odorrana ishikawai, Mantella madagascariensis, Andrias davidianus, and Paa spinosa (Kurbayashi et al., 2006, 2010; Zhang, Chen, Liu, Zhou, & Qu, 2003; Zhou, Zhang, Zheng, Yu, & Yang, 2009).

Mitochondrial genes such as the COX I, Cytochrome b (Cytb), D-loop, tRNA, and NADH have been used for previous phylogenetic and phylogeographic studies on the genetic divergence of Nanorana (Che et al., 2010; Liu et al., 2015; Wang et al., 2013; Zhang et al., 2010; Zhou et al., 2014). Here, we use complete mitochondrial genomes to analyze the phylogenetic relationships of the three Nanorana species (N. parkeri, N. ventripunctata, and N. pleskei) and other related species. Moreover, in order to reconstruct a robust evolutionary relationship among the three species, we need additional mitochondrial genomic information from Nanorana species. Therefore, we sequenced the complete mitochondrial genome of N. parkeri and N. ventripunctata and summarized the structural variations of 40 mitochondrial genome sequences in the Family Dicroglossidae. We reconstructed the phylogenetic relationships of Dicroglossidae using the concatenated sequences of 13 protein-coding genes from Dicroglossidae mitochondrial genomes, based on which the evolutionary characteristics of the mitochondrial genomes in Dicroglossidae were evaluated. Furthermore, we analyzed the mitochondrial genomic sequence and phylogenetic relationships within N. pleskei, N. ventripunctata and N. parkeri to assess the evolutionary status of the three species within the Nanorana genus. Additionally, the complete mitochondrial genomes of two Nanorana species (N. ventripunctata and N. parkeri) were analyzed to find novel data with which to investigate the placement of the three Nanorana species in the phylogenetic tree of Dicroglossidae and to provide molecular data for further study on the taxonomic status and adaptive evolutionary mechanisms of these high-altitude species.

2 | MATERIALS AND METHODS

2.1 | Sampling and DNA extraction

The Xizang Plateau frog (N. parkeri, Figure 1) was sampled from Dangxiong County (4,300 m asl), the Tibet Autonomous Region, China, in September 2015. The Yunnan slow frog (N. ventripunctata)
was sampled from Xianggelila County (4,200 m asl), Yunnan province, China, in July 2016. All collections were initially preserved in 95% ethanol and stored at −70°C until DNA extraction was performed. According to the protocol adopted by Zhang, Chao, Lai, Li, and Zhao (2000) and Xia, Liu, and Lu (2002), total mtDNA of two Nanorana species was extracted from skin tissue for the following PCR amplification.

2.2 Mitochondrial DNA amplification and sequencing

The entire mitochondrial genome was amplified in twelve overlapping segments by PCR with LA-Taq DNA Polymerase (TaKaRa, China), using 20 ng of total genomic DNA from the sample as a template. Complete mtDNA was amplified as concatenated sequences by adopting selectively amplified mtDNA templates and 10 primer pairs, as published by Kurabayashi and Sumida (2009). Partial PCR primers were also designed based on the alignments of the relatively conserved regions of congeneric N. pleskei (NC_016119) and N. taihangnica (NC_024272). The PCR amplification was performed as follows: 2.5 min at 94°C, followed by 30 cycles of 0.5 min at 94°C, 0.5 min at 50–59°C, 3–5 min at 60°C, and a 9 min final extension at 72°C. PCR reactants were loaded on 0.8%–1.0% agarose gels, stained with ethidium bromide and photographed under ultraviolet light. PCR products were purified with Gel Extract Purification Kits (V-gene) and automated sequencing using an ABI 3730 sequencer, either directly or following subcloning into the pMD18-T vector (TaKaRa, China). To ensure maximum accuracy, each amplification product was sequenced twice independently, followed by a third PCR amplification.

2.3 Sequence assembly and analysis

Sequences were assembled manually and aligned, and each gene was then translated into an amino acid sequence using MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The amino acid sequence alignments of each of the protein-coding genes (PCGs) were generated using the computer program Clustal X 1.83 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Based on sequence similarity results from BLAST searches, ribosomal RNA (rRNA) genes were recognized, and tRNA genes were identified using tRNAscan-SE 1.21 (Schattner, Brooks, & Lowe, 2005). Base composition and codon usage were analyzed in MEGA 6.0 (Tamura et al., 2013). The mitochondrial genome sequences have been submitted to NCBI GenBank with the accession number NC_026789 (N. parkeri) and KY594708 (N. ventripunctata). Features of the base composition of nucleotide sequences were detected using the AT-skew and GC-skew in the mitochondrial genome. We then calculated the AT-skew and GC-skew using the following formulae from Perna and Kocher (1995): AT-skew = (A − T)/(A + T) and GC-skew = (G − C)/(G + C).

2.4 Phylogenetic analysis

Combined with 38 other Dicroglossidae mitochondrial genomes from NCBI GenBank (Supporting information Table S1), the mitochondrial genomes of N. parkeri and N. ventripunctata were analyzed using the phylogenetic tree method, with the concatenated sequences of the 13 protein-coding genes and the two species Babina subaspera (NC_022871) and Hylarana guentheri (NC_024748) as out-groups. First, we aligned the 13 mitochondrial protein-coding gene sequences in Clustal X 1.83 (Schattner et al., 2005) with the default settings, and then we concatenated individual genes excluding the stop codon. We selected the optimal nucleotide substitution model in jModeltest v0.1.1 (Posada, 2008) and used the Akaike Information Criterion (AIC: Posada & Buckley, 2004). Maximum likelihood (ML) and Bayesian inference (BI) were used for phylogenetic analyses in MrBayes 3.2.2 (Ronquist et al., 2012), and BI of nucleotide acid datasets was performed using the GTR+I+G model (Lanave, Preparata, Saccone, & Serio, 1984). A ML tree was constructed using RAxML, and the robustness of the phylogenetic results was tested through bootstrap analysis with 1,000 replicates (Stamatakis, 2014).

3 RESULTS AND DISCUSSION

3.1 Genome content and organization

The mitochondrial genomes of N. parkeri (17,837 bp), N. ventripunctata (18,373 bp) and N. pleskei (17,660 bp) included 13 PCGs (ND1-5, ND4L, COX1-3, Cyt b, ATP6 and ATP8), two ribosomal RNA genes (12S and 16S rRNA), 23 tRNA genes and one large noncoding region (putative control region; CR) (Table 1; Figure 2). In the three genomes, 12 protein-coding genes (ND1-5, ND4L, COX1-3, ATP8, ATP6, and Cyt b) and two rRNAs (12S and 16S rRNA) were encoded on the heavy (H) strand along with 15 tRNAs (tRNA-Leu1, tRNA-Thr, tRNA-Phe, tRNA-Val, tRNA-Leu2, tRNA-Ile, tRNA-Met1, tRNA-Met2, tRNA-Trp, tRNA-Asp, tRNA-Lys, tRNA-Gly, tRNA-Arg,
tRNA-His, and tRNA-Ser2) and CR, while the remaining one protein- coding gene (ND6) and eight tRNAs (tRNA-Pro, tRNA-Gln, tRNA- Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser1, and tRNA-Glu) were encoded on the light (L) strand (Table 1; Figure 2). Overall we found that there were no differences in the gene arrangement of mitochondrial genomes among these Nanorana and Quasipaa species (Chen, Zhai, Zhu, & Chen, 2015; Simon et al., 1994; Zhou et al., 2009), but there were some differences between these two frog species and the typical neobatrachian type (e.g., Rana nigromaculata) in the position of tRNA-Met, with formation of a tandem duplication of tRNA-Met gene between tRNA-Gln and ND2.

The gene components were very loosely juxtaposed with 134/42 (N. parkeri) and 63/39 (N. ventripunctata) of gap/overlapping nucleotides, compared to that of N. pleskei (71/49; Table 1) (Simon et al., 1994). Although the overall A + T contents of 57.87% in N. parkeri and 59.1% in N. ventripunctata were relatively higher than that of N. pleskei (57.5%), those values are within the range (52.8%–62.74%) of Dicroglossidae (Supporting information Table S1). The nucleotide skew was highly similar among these mitochondrial genomes including that of N. pleskei, with only some exceptions found on COX2, ATP6, ATP8 and the putative control region (Table 2).

3.2 | Protein-coding genes (PCGs) and codon usage patterns

The inferred start/stop codons for protein-coding genes of N. parkeri, N. ventripunctata, and N. pleskei are listed in Table 1. In three mitochondrial genomes, the protein-coding genes were initiated by ATG, with the exceptions of COX1, ND1, ND2, and ND3 (Table 1). The open reading frame of ND1 and ND3 started with GTG, while that of COX1 and ND2 started with ATA and ATT, respectively. The canonical stop codon (TAA or TAG) can be found in four protein-coding genes (ATP8, Cytb, ND4L, and NDS; Table 1), while COX1 and ND6 use AGG and AGA as the termination codon, respectively. The remaining seven (ATP6, COX2-3, and ND1-4) had incomplete T-stop codons (Table 1), completed (TAA) by polyadenylation after transcription (Boore, 2001).

The relative synonymous codon usage (RSCU) values of the three species of Nanorana mitochondrial genomes are shown in Table 3. Supporting information Tables S2–S4. The results demonstrate that synonymous codon usage has a distinct bias toward A or T for 13 PCGs. The codons AUU (5.03%–5.62%), UUU (3.92%–4.37%), GCC (3.84%–4.03%), and CUU (3.63%–3.79%) were the most frequently used codons in the mitogenomes of our three species of Nanorana, accounting for 16.42%–17.81%. In addition to GCC codon, these codons were mainly composed of A or U nucleotides, indicating the highly biased usage of A and T nucleotides in the three species of Nanorana PCGs. Meanwhile, the most frequently represented amino acids in the three species of Nanorana mitochondrial proteins were Leu (16.27%–16.38%), Ala (8.24%–8.32%), Ile (7.95%–8.11%), and Phe (6.62%–6.78%), accounting for 39.08%–39.59%. The least frequently represented amino acid was Cys (0.74%–0.77%). Codon usage of PCGs showed a major bias of A + T content, which played a major role in the A + T bias of the entire mitogenome. Similar patterns with a strong T- or A-bias in the wobble position have been found among other Nanorana species also. The RSCU analysis showed that codons with A or T (U) at the third position are more over- used compared to other synonymous codons. Therefore, the codon usage can reveal nucleotide bias too. These data imply a high A + T content in the three Nanorana species. The bias toward the use of Ts over As, to the 13 PCGs, is more obvious in these three Nanorana mitogenomes with −0.080 to −0.100 AT skewness. Moreover, negative AT-skew and GC-skew were found in the third position, whereas both the first and second positions showed positive AT-skew and negative GC-skew in N. parkeri and N. ventripunctata. In contrast, the first, second and third positions showed negative AT-skew and GC-skew in N. pleskei (Supporting information Table S5).

3.3 | Transfer and ribosomal RNA genes

A total of 23 tRNA genes (including an extra copy of tRNA-Met gene) with 65 to 73 bp in length were identified in mitochondrial genomes of both N. parkeri and N. ventripunctata (Figure 3), including an extra copy of the tRNA-Met genes. All tRNA genes can fold into the canonical cloverleaf secondary structure with the same anticodon usage as reported in other vertebrates. The sequences, anticodon nucleotides, and secondary structures of tRNA genes in N. parkeri and N. ventripunctata were very similar to those in N. pleskei (Chen et al., 2011) (Figure 3). In addition, a tandem repeat of tRNA-Met gene was easy to find in the three Nanorana species, located between the tRNA-Gln and ND2 genes. Extra tRNA-Met was also found in Quasipaa boulengeri, Fejervarya cancrivora, Hoplobatrachus rugulosus, Euphlyctis hexadactylus, Limnonectes bannaensis, and Occidozyga martensii (Alam et al., 2010; Chen et al., 2011; Li et al., 2014a, 2014b; Ren et al., 2009; Shan, Xia, Zheng, Zou, & Zeng, 2014; Zhang et al., 2009). But this phenomenon is different to that seen in Amolops tormotus and other typical vertebrates (Su, Wu, Yan, Cao, & Hu, 2007). Two tRNA-Met genes in each lineage may come from different origins (Kurabayashi et al., 2006), and the tandem duplication of the tRNA-Met gene can be seen as a synapomorphic feature of Dicroglossidae. A tandem duplication of the mitochondrial tRNA-Pro and tRNA-Thr genes in Bipes biporus has been reported from previous research (Macey, Schulte, Larson, & Papenfuss, 1998). Based on tandem duplication/deletion models, pseudogene formation in tandemly duplicated sequences might result from mtDNA rearrangement. Our results found two tandem tRNA-Met genes in N. parkeri and N. ventripunctata which supports this view.

12S and 16S rRNAs were encoded on the H strand and were separated by tRNA-Val in both of the mitochondrial genomes that we analyzed. The length of 12S and 16S rRNA genes was estimated to be 936 and 1,587 bp for N. parkeri, and 937 and 1,593 bp for N. ventripunctata, respectively.
| Gene          | Strand | Position | Codon | Intergenic bp^b | Position |
|--------------|--------|----------|-------|-----------------|----------|
| tRNA-Leu1    | H      | 1–72     | 72    | −3              | 1–72     |
| tRNA-Thr     | H      | 70–142   | 73    | 0               | 73–142   |
| tRNA-Pro     | L      | 143–211  | 69    | −1              | 143–211  |
| tRNA-Phe     | H      | 211–280  | 70    | 0               | 211–280  |
| 12S RNA      | H      | 281–1,216| 936   | 0               | 281–1,216|
| tRNA-Val     | H      | 1,217–1,286| 67    | 0               | 1,218–1,287|
| 16S RNA      | H      | 1,287–2,873| 1,587 | 0               | 1,288–2,880|
| tRNA-Leu2    | H      | 2,874–2,946| 73    | 0               | 2,881–2,953|
| ND1          | H      | 2,947–3,904| 958   | 0               | 2,954–3,911|
| tRNA-Ile     | H      | 3,905–3,975| 71    | 0               | 3,912–3,982|
| tRNA-Gln     | L      | 3,976–4,046| 71    | 0               | 3,983–4,053|
| tRNA-Met1    | H      | 4,047–4,115| 69    | 10              | 4,054–4,122|
| tRNA-Met2    | H      | 4,126–4,194| 69    | 0               | 4,132–4,200|
| ND2          | H      | 4,195–5,227| 1,033 | 0               | 4,201–5,233|
| tRNA-Trp     | H      | 5,228–5,297| 70    | 0               | 5,234–5,303|
| tRNA-Ala     | L      | 5,298–5,367| 70    | 2               | 5,304–5,373|
| tRNA-Asn     | L      | 5,370–5,442| 73    | 0               | 5,376–5,448|
| rep_origin   | L      | 5,443–5,471| 29    | 0               | 5,449–5,477|
| tRNA-Cys     | L      | 5,472–5,536| 65    | 0               | 5,478–5,542|
| tRNA-Tyr     | L      | 5,537–5,603| 67    | 4               | 5,543–5,609|
| COXI         | H      | 5,608–7,158| 1,551 | −9              | 5,614–7,164|
| tRNA-Ser1    | L      | 7,150–7,220| 71    | 0               | 7,156–7,226|
| tRNA-Asp     | H      | 7,221–7,289| 69    | 1               | 7,227–7,296|
| COXI1        | H      | 7,291–7,975| 685   | 1               | 7,299–7,983|
| tRNA-Lys     | L      | 7,976–8,045| 70    | 3               | 7,984–8,053|
| ATP8         | H      | 8,049–8,210| 162   | −7              | 8,055–8,216|
| ATP6         | H      | 8,204–8,885| 682   | 0               | 8,210–8,891|
| COXI11       | H      | 8,886–9,669| 784   | 0               | 8,892–9,675|
| tRNA-Gly     | H      | 9,670–9,738| 69    | 0               | 9,676–9,744|
| ND3          | H      | 9,739–10,096| 358   | 0               | 9,745–10,102|
| tRNA-Arg     | H      | 10,097–10,165| 69    | 1               | 10,103–10,171|
| ND4          | H      | 10,167–10,451| 285   | −7              | 10,173–10,457|
| ND4          | H      | 10,445–11,807| 1,363 | 0               | 10,451–11,813|
| tRNA-His     | H      | 11,808–11,875| 68    | 0               | 11,814–11,813|
| tRNA-Ser2    | H      | 11,876–11,943| 68    | 106             | 11,883–11,950|
| ND5          | H      | 12,050–13,873| 1824  | −15             | 11,988–13,811|
| ND6          | L      | 13,859–14,356| 498   | 0               | 13,797–14,294|
| tRNA-Glu     | L      | 14,357–14,425| 69    | 7               | 14,295–14,363|
| CYTB         | H      | 14,433–15,578| 1,146 | 0               | 14,371–15,516|
| Control region | H   | 15,579–17,837| 2,259 | 1               | 15,517–18,373|

Note. H and L indicate genes transcribed on the heavy and light strands, respectively. Numbers correspond to the nucleotides separating adjacent genes, negative numbers indicate overlapping nucleotides. T represents incomplete stop codons.
| Size | Codon | Position | Codon | Position |
|------|-------|----------|-------|----------|
|      | Start | Stop | From | To | Start | Stop | From | To |
| 72   | 0     | 81   | 81   | -9 |
| 70   | 0     | 140  | 68   | 0  |
| 69   | -1    | 209  | 69   | -1 |
| 70   | 0     | 278  | 70   | 0  |
| 937  | 0     | 1,212| 934  | 0  |
| 70   | 0     | 1,282| 70   | 0  |
| 1,593| 0     | 2,873| 1,591| 0  |
| 73   | 0     | 2,946| 73   | 0  |
| 958  | GTG   | 0    | 958  | GTG |
| 71   | 0     | 3,975| 71   | -1 |
| 71   | 0     | 4,045| 71   | 0  |
| 69   | 9     | 4,114| 69   | 9  |
| 69   | 0     | 4,192| 69   | 0  |
| 1,033| ATT   | 0    | 1,033| ATT |
| 70   | 0     | 5,251| 70   | 0  |
| 70   | 2     | 5,365| 70   | 2  |
| 73   | 0     | 5,440| 73   | 0  |
| 29   | 0     | 5,470| 30   | 0  |
| 65   | 0     | 5,536| 66   | 0  |
| 67   | 4     | 6    | 67   | 4  |
| 1,551| ATA   | AGG  | 1,551| ATA |
| 71   | 0     | 7,158| 71   | 0  |
| 70   | 2     | 7,289| 69   | 2  |
| 685  | ATG   | 0    | 685  | ATG |
| 70   | 1     | 7,977| 70   | 2  |
| 162  | ATG   | TAA  | 162  | ATG |
| 682  | ATG   | T–   | 682  | ATG |
| 784  | ATG   | T–   | 784  | ATG |
| 69   | 0     | 9,738| 69   | 0  |
| 385  | GTG   | T–   | 385  | GTG |
| 69   | 1     | 10,165| 69  | 1  |
| 285  | ATG   | TAA  | 285  | ATG |
| 1,363| ATG   | T–   | 1,363| ATG |
| 69   | 0     | 11,876| 69  | 0  |
| 68   | 37    | 11,944| 68  | 44 |
| 1824 | ATG   | TAA  | 1824 | ATG |
| 498  | ATG   | AGA  | 498  | ATG |
| 69   | 7     | 14,364| 69  | 7  |
| 1,146| ATG   | TAG  | 1,146| ATG |
| 2,857| 0     | 15,518| 2,143| 0  |
Noncoding regions

Putative control region, of 2,259 bp (N. parkeri) and 2,857 bp (N. ventripunctata) were found in Cytb and tRNA-Leu, which is longer than that of N. pleskei (2,143 bp) (Table 1). The size of control region variation demonstrated different lengths of the total mitogenomes for the three species. The A + T contents (65.96% in N. parkeri and 69.86% in N. ventripunctata) in control region were higher than in other regions (Table 1). Additionally, the A + T contents rated different lengths of the total mitogenomes for the three species. The A + T content in this region is higher than that in the coding regions (Boore, 1999; Simon et al., 1994). The L-strand origin of replication (OL) of the N. parkeri and N. ventripunctata mitogenome is located at the gene boundary of tRNA-Asn and tRNA-Cys in the WANCY tRNA gene cluster and has the same sequence as N. pleskei (Chen et al., 2011). The sequence, structure, and position of OL are well conserved in the anuran mitogenomes, and are also similar to those in other vertebrates (San Mauro, Gower, Zardoya, & Wilkinson, 2006), implying it is a common and important characteristic of this short intergenic spacer region.

On both 5′ and 3′-sides, the control region has distinct repeat regions (Figure 4) with the 5′-side repeat region consisting of 6.5 and 4.6 tandem repeat units of 124 bp in N. ventripunctata and N. parkeri, respectively. Dissimilar to the 5′-side repeat region, the 3′-side repeat region includes 10.8 tandem repeat units of 11 bp (5′-GCTCGTATTCT-3′), 10.9 tandem repeat units of 11 bp (5′-CTTCGCTTATC-3′), 29.6 tandem repeat units of 10 bp (5′- GTTTTTGTTA-3′), 8.7 tandem repeat units of 12 bp (5′-GCTCGTATATT-3′), and 8.9 tandem repeat units of 10 bp (5′-GTTTGTGGTTA-3′). The sequence, structure, and position of OL are well conserved in the anuran mitogenomes, and are also similar to those in other vertebrates (San Mauro, Gower, Zardoya, & Wilkinson, 2006), implying it is a common and important characteristic of this short intergenic spacer region.

| Gene   | AT-skew | CG-skew |
|--------|---------|---------|
|        | N. parkeri | N. ventripunctata | N. pleskei | N. parkeri | N. ventripunctata | N. pleskei |
| ND1    | −0.130  | −0.141  | −0.131     | −0.356  | −0.305  | −0.305     |
| ND2    | −0.042  | −0.072  | −0.069     | −0.510  | −0.407  | −0.404     |
| ND3    | −0.316  | −0.212  | −0.264     | −0.309  | −0.370  | −0.287     |
| ND4    | −0.122  | −0.119  | −0.139     | −0.362  | −0.354  | −0.336     |
| ND4L   | −0.124  | −0.148  | −0.157     | −0.362  | −0.328  | −0.308     |
| ND5    | −0.091  | −0.070  | −0.097     | −0.266  | −0.289  | −0.261     |
| ND6    | −0.261  | −0.289  | −0.300     | 0.453   | 0.490   | 0.453      |
| COX1   | −0.136  | −0.114  | −0.118     | −0.160  | −0.181  | −0.160     |
| COX2   | −0.005  | 0.028   | −0.022     | −0.223  | −0.236  | −0.175     |
| COX3   | −0.127  | −0.148  | −0.187     | −0.256  | −0.240  | −0.225     |
| ATP6   | 0.262   | −0.137  | −0.137     | 0.455   | −0.410  | −0.424     |
| ATP8   | 0.056   | 0.050   | 0.000      | −0.481  | −0.508  | −0.448     |
| Cytb   | −0.060  | −0.088  | −0.110     | −0.339  | −0.316  | −0.297     |
| 12S rRNA| 0.138   | 0.108   | 0.145      | −0.111  | −0.131  | −0.119     |
| 16S rRNA| 0.144   | 0.136   | 0.145      | −0.101  | −0.091  | −0.096     |
| CR     | −0.104  | −0.216  | −0.050     | −0.208  | −0.219  | −0.155     |
| 13PCG  | −0.107  | −0.110  | −0.120     | −0.277  | −0.270  | −0.248     |
| Overall| −0.042  | −0.066  | −0.043     | −0.261  | −0.259  | −0.155     |
| Amino acids | Codon | No. | Npar | Nven | Nple | Amino acids | Codon | No. | Npar | Nven | Nple | Amino acids | Codon | No. | Npar | Nven | Nple |
|-------------|-------|-----|------|------|------|-------------|-------|-----|------|------|------|-------------|-------|-----|------|------|------|
| Phe         | UUU   | 156 | 148  | 165  |      | CCA         | 58    | 61  | 62   |      |      | AAG         | 15    | 18  | 23   |      |      |
|             | UUC   | 103 | 102  | 91   |      | CCG         | 25    | 18  | 19   |      |      | GAU         | 30    | 31  | 33   |      |      |
| Leu         | UUA   | 118 | 123  | 117  |      | ACU         | 80    | 73  | 86   |      |      | GAC         | 44    | 40  | 37   |      |      |
|             | UUG   | 26  | 30   | 44   |      | ACC         | 97    | 95  | 82   |      |      | GAA         | 61    | 57  | 62   |      |      |
|             | CUU   | 143 | 142  | 137  |      | ACA         | 109   | 100 | 112  |      |      | GAG         | 29    | 32  | 29   |      |      |
|             | CUC   | 137 | 134  | 130  |      | ACG         | 13    | 18  | 8    |      |      | UGU         | 11    | 17  | 15   |      |      |
|             | CUA   | 140 | 128  | 132  | Ala  | GCU         | 72    | 69  | 78   |      |      | UGC         | 18    | 12  | 13   |      |      |
|             | CUG   | 50  | 58   | 58   |      | GCC         | 152   | 148 | 145  | Trp  |      | UGA         | 84    | 88  | 86   |      |      |
|             |       |     |      |      |      |             |       |     |      |      |      |             |       |     |      |      |      |
| Ile         | AUU   | 190 | 204  | 212  |      | GCA         | 69    | 69  | 73   |      |      | UGG         | 25    | 22  | 25   |      |      |
|             | AUC   | 112 | 102  | 88   |      | GCG         | 19    | 25  | 18   | Arg  |      | CGU         | 14    | 11  | 12   |      |      |
|             |       |     |      |      |      |             |       |     |      |      |      |             |       |     |      |      |      |
| Met         | AUA   | 121 | 125  | 119  |      | UAU         | 63    | 61  | 60   |      |      | CGC         | 18    | 23  | 20   |      |      |
|             | AUG   | 58  | 60   | 62   |      | UAC         | 43    | 47  | 50   |      |      | CGA         | 35    | 33  | 31   |      |      |
|             |       |     |      |      |      |             |       |     |      |      |      |             |       |     |      |      |      |
| Val         | GUU   | 71  | 71   | 84   |      | UAA*        | 3     | 3   | 3    |      |      | CGG         | 7     | 10  | 10   |      |      |
|             | GUC   | 43  | 52   | 47   |      | UAG*        | 1     | 1   | 1    | Ser  |      | AGU         | 25    | 23  | 25   |      |      |
|             | GUA   | 59  | 66   | 73   | His | CAU         | 34    | 28  | 34   |      |      | AGC         | 30    | 33  | 29   |      |      |
|             | GUG   | 38  | 24   | 21   |      | CAC         | 67    | 73  | 67   |      |      | AGA*        | 1     | 1   | 1    |      |      |
|             |       |     |      |      |      |             |       |     |      |      |      |             |       |     |      |      |      |
| Ser         | UCU   | 62  | 67   | 71   | Gln | CAA         | 75    | 78  | 77   |      |      | AGG*        | 1     | 1   | 1    |      |      |
|             | UCC   | 79  | 72   | 68   |      | CAG         | 18    | 13  | 14   | Gly  |      | GGU         | 36    | 42  | 47   |      |      |
|             | UCA   | 70  | 78   | 78   | Asn | AAU         | 63    | 69  | 63   |      |      | GGC         | 74    | 69  | 61   |      |      |
|             | UCG   | 16  | 7    | 9    |      | AAC         | 62    | 49  | 54   |      |      | GGA         | 50    | 52  | 48   |      |      |
|             |       |     |      |      |      |             |       |     |      |      |      |             |       |     |      |      |      |
| Pro         | CCU   | 33  | 34   | 40   | Lys | AAA         | 69    | 73  | 64   |      |      | GGG         | 58    | 63  | 67   |      |      |
|             | CCC   | 91  | 98   | 83   |      |             |       |     |      |      |      |             |       |     |      |      |      |
FIGURE 3  Putative tRNA secondary structures predicted from the 22 tRNA gene sequences found in the (a) Nanorana parkeri and (b) Nanorana ventripunctata mitochondrial genome
(b) *Nanorana ventripunctata*

![Diagram of *Nanorana ventripunctata* amino acid structures](image)

**FIGURE 3** (Continued)
of 11 bp (5′-ATACTTCGCTT-3′), 16.3 tandem repeat units of 8 bp (5′-TAATTGTA-3′), 12.8 tandem repeat units of 8 bp (5′-GCTGATCG-3′), respectively (Table 4). No tandem repeats in the 3′-side region were found in N. pleskei which is unusual for a mitogenome control region. Unlike the corresponding region in other anurans, the control region of N. ventripunctata and N. parkeri included TAS, CSB-1, CSB-2, CSB-3, and OH (Figure 5). The 5′-side tandem repeat units included 6.5 and 4.6 putative termination-associated sequences (TASs, 5′-TATAAGACATCTAT GTA-3′) of N. ventripunctata and N. parkeri, respectively (Table 4). Tandem repeat units including TASs were also detected in the control regions of Bufo japonicas, Paa spinosa and Hyla japonica (Igawa, Kurabayashi, Usuki, Fujiji, & Sumida, 2008; Zhou et al., 2009). Three conserved sequence blocks (CSBs) may be related to the initiation of the mtDNA synthesis and they (CSB-1, CSB-2, CSB-3) can be identified between the tandem repeat units at the 5′ and 3′-sides (Table 4; Figure 4). CSB-1, CSB-2 and CSB-3 of N. ventripunctata and N. parkeri showed high similarity to the consensus in other amphibians, while the variation in N. pleskei is slightly larger (Figure 5); moreover, CSB-1 is not reduced to a truncated penta motif (5′-GACAT-3′) as it is in the caecilians (San Mauro et al., 2004; Zardoya & Meyer, 2000). However, a truncated CSB-1 had been recorded in Xenopus laevis (Anura) (Roe, Ma, Wilson, & Wong, 1985). The CSB-2 motif was not found in N. pleskei (Figure 5). In addition, the multiple motifs of mtDNA control regions (CR) may be associated with the transcription and replication of the mitochondrial genome (Taanman, 1999). The function of these conserved sequence blocks is unclear. Further study on the mechanistic basis of mtDNA replication is warranted for Nanorana species.

3.5 | Phylogenetic relationships

The concatenated PCG data of the mitogenome sequences in our study contained 11,292 nucleotide positions, including 4,314 conserved sites, 6,978 variable sites and 6,505 potentially parsimony-informative sites. Phylogenetic trees were reconstructed using BI and ML analyses, based on the nucleotide dataset. The use of PCG sequences of the mitogenomes has become an informative strategy for inferring phylogenetic relationships (Boore, Macey, & Medina, 2005). Using the 13 PCG sequences to concatenate may achieve a more complete analysis. BI and ML methods consistently support similar tree topologies by strong node-supporting values.

So far, combined with the 38 mitochondrial genome sequences in GenBank database, our phylogenetic analyses revealed that the subfamily Dicroglossinae’s monophyly was well supported (Li et al., 2014a, 2014b; Roelants et al., 2007; Yuan et al., 2016). The subfamily Dicroglossinae’s monophyly was well supported (Li et al., 2016; Zhang, Xia, & Zeng, 2016). Dicroglossinae species was divided into two clades with one clade (Clade 1) containing Nanorana, Quasipaa, and Limnonectes, and the other (Clade 2) including Fejervarya, Euphlyctis, and Hoplobatrachus (Figure 6), as supported by previous studies (Lv, Bi, & Fu, 2014; Yuan et al., 2016; Zhang, Xia, & Zeng, 2016). Quasipaa and Nanorana belong to the sister genus. Clade Quasipaa comprised Q. yei as the sister taxon to the subclade (BP = 100%, PP = 1.00) containing ((Q. jiulongensis + (Q. spinosa + Q. exilispinosa)) + (Q. shini + (Q. boulengeri + Q. verrucospinosa))) (Figure 6). Within the genus Quasipaa, the phylogenetic inferences based on mtDNA sequences showed that all individuals of Q. boulengeri formed a monophyly with high support, sister to Q. verrucospinosa (KF199147). This result is similar to the results from Che et al. (2009), but different to those of Qing et al. (2012). Furthermore, we find that Q. verrucospinosa is paraphyletic in the genus Quasipaa, as one sample of Q. verrucospinosa (KF19917) was grouped with Q. boulengeri, while another sample of Q. verrucospinosa (NC_032333) was not (Figure 6). All the Nanorana species were clustered together. In the Nanorana clade, N. yunnanensis is the sister group of a clade composed of N. quadranus and N. taihangnica (Subclade 2). And the Subclade 2 composed of these 3 species is the sister group of a clade (Subclade 1) that includes N. pleskei, N. ventripunctata, N. parkeri and N. maculosa. So
the Nanorana species clustered in a single monophyly. Our molecular phylogeny indicates *N. ventripunctata* and *N. pleskei* are more closely related compared with *N. parkeri*, and strongly supports that *N. parkeri* is basal to *N. pleskei* and *N. ventripunctata* based on 13 PCG genes of the mitogenome (BP = 100%, PP = 1.00) (Figure 6), in agreement with the relationships inferred by the research report of Che et al. (2009, 2010). However the phylogeny of the three species (*N. parkeri*, *N. pleskei* and *N. ventripunctata*) based on 13 PCGs was not concordant with those reported earlier based on 12 genes (three mitochondrial and nine nuclear genes) (Pyron & Wiens, 2011). This difference may be caused by the use of different molecular markers, and their evolutionary relationships need further investigating and searching for more evidences from molecular markers and morphological characters. *Nanorana* and *Quasipaa* were resolved as the sister group of the genus *Limnonectes* (BP = 99%, PP = 1.00). The phylogenetic relationships supported the authenticity of the two obtained mitogenomes among *Nanorana*. And the phylogenetic reconstruction using the whole mitogenome, rather than single genes, provided more credible results. The mitogenomic approach, as previously demonstrated (Cai, Che, Pang, Zhao, & Zhang, 2007; Liyan, Xia, Zheng, & Zeng, 2012; Yan et al., 2013), is an excellent tool with which to infer phylogenetic relationships within Neobatrachia. In the present study, all clades were well resolved, with only a few exceptions less than 90%, while Bayesian posterior probabilities were 1.00. Despite their fast evolutionary rates, mitochondrial genomes

**Table 4** Location of features in the D-loop of three *Nanorana* species (*Nanorana parkeri*, *Nanorana ventripunctata*, and *Nanorana pleskei*)

| Species               | Sequence in D-loop | Start position | Stop position | Length (bp) |
|-----------------------|--------------------|----------------|---------------|-------------|
| *Nanorana ventripunctata* | 6.5 tandem repeat units | 21             | 830           | 5 × 124 + 66 |
|                       | TAS                | 119            | 135           | 17          |
|                       | TAS                | 243            | 259           | 17          |
|                       | TAS                | 367            | 383           | 17          |
|                       | TAS                | 491            | 507           | 17          |
|                       | TAS                | 615            | 631           | 17          |
|                       | TAS                | 739            | 755           | 17          |
|                       | OH                 | 1,552          | 1,623         | 72          |
|                       | CSB-1              | 1,713          | 1,740         | 28          |
|                       | CSB-2              | 1,809          | 1,827         | 19          |
|                       | CSB-3              | 1,814          | 1,831         | 18          |
|                       | 10.8 tandem repeat units | 1,917         | 2,035         | 10 × 11 + 9 |
|                       | 10.9 tandem repeat units | 2,042         | 2,161         | 10 × 11 + 10 |
|                       | 29.6 tandem repeat units | 2,196         | 2,492         | 29 × 10 + 6 |
| *Nanorana parkeri*      | 4.6 tandem repeat units | 42             | 610           | 4 × 124 + 72 |
|                       | TAS                | 151            | 167           | 17          |
|                       | TAS                | 275            | 291           | 17          |
|                       | TAS                | 399            | 415           | 17          |
|                       | TAS                | 523            | 539           | 17          |
|                       | OH                 | 1,389          | 1,460         | 72          |
|                       | CSB-1              | 1,495          | 1,522         | 28          |
|                       | CSB-2              | 1,595          | 1,613         | 19          |
|                       | CSB-3              | 1,640          | 1,657         | 18          |
|                       | 8.7 tandem repeat units | 1,702         | 1,796         | 8 × 11 + 8  |
|                       | 8.9 tandem repeat units | 1,801         | 1,898         | 8 × 11 + 10 |
|                       | 16.3 tandem repeat units | 1,898         | 2,027         | 16 × 8 + 10 |
|                       | 12.8 tandem repeat units | 2,028         | 2,120         | 12 × 8 + 8  |
| *Nanorana pleskei*       | 3.6 tandem repeat units | 22             | 467           | 3 × 124 + 74 |
|                       | TAS                | 130            | 146           | 17          |
|                       | TAS                | 254            | 270           | 17          |
|                       | TAS                | 378            | 394           | 17          |
|                       | OH                 | 1,554          | 1,627         | 74          |
|                       | CSB-1              | 514            | 541           | 28          |
|                       | CSB-3              | 1,203          | 1,220         | 18          |
contain species-specific evolutionary affinities, which can be efficiently recovered by improving taxon sampling (Rubinstein et al., 2013).

### 4 CONCLUSIONS

In summary, the complete mitochondrial genomes of two *Nanorana* species were determined in this study. Our mitogenome analyses, including gene content, gene order, strand asymmetry, base composition, rRNA and tRNA secondary structure and phylogenetic analysis, indicate several significant features: a tandem repeat of the tRNA-Met gene was detected in three *Nanorana* species, located between the tRNA-Gln and ND2 genes. The control region contains distinct repeat regions at both 5′ and 3′-sides, and the CSB-2 motif was not found in the *N. pleskei*. Based on nucleotide sequences of 13 PCGs, and using BI and maximum-likelihood

![FIGURE 5](image_url) Structures and alignments of identified putative termination-associated sequences (TAS) and, conserved sequence blocks (CSB 1-3). Alignment gaps and nucleotides identical to the first line are indicated by dashes (-) and a dot (·), respectively. Variable nucleotides are marked with corresponding nucleotides.

![FIGURE 6](image_url) Results of phylogenetic analyses using BI and ML analysis indicated evolutionary relationships among 38 individuals based on 13 PCGs sequences. *Babina subaspera* (NC_022871) and *Hylarana guentheri* (NC_024748) were used as outgroups. Tree topologies produced by BI and ML analyses were equivalent. Bayesian posterior probability (PP) and bootstrap support (BP) values for ML analyses are shown in order on the nodes. The asterisks indicate new sequences generated in this study.
analyses, the phylogenetic data illustrate the taxonomic status of *Nanorana* and provides robust support that *N. ventripunctata* and *N. pleskei* are more closely related than *N. parkeri*. Our study provides useful additional data for further phylogenetic analysis of the *Nanorana* genus. Expanding our knowledge of the phylogenetic relationships within the *Nanorana* genus will ultimately aid in future research to protect and maintain biodiversity within many other anuran species. However, the proposed evolutionary relationships among these three species based on the findings that emerged in the study should be accepted with caution due to limited taxon sampling. Many aspects of the phylogeny of the genus *Nanorana* remain to be resolved and further analysis based on more molecular information (including nuclear gene data) and extensive taxon sampling is necessary to elucidate the phylogenetic relationships among genus *Nanorana* or Dicroglossidae.

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CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

AUTHOR CONTRIBUTION

Jiang L., Ruan Q., and Chen W. designed the manuscript, You Z. and Yu P. analyzed the data, and Jiang L. and Chen W. wrote the manuscript.

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REFERENCES

Alam, M. S., Kurabayashi, A., Hayashi, Y., Sano, N., Khan, M. R., Fujii, T., & Sumida, M. (2010). Complete mitochondrial genomes and novel gene rearrangements in two dicroglossid frogs, *Hoplobatrachus tigrinus* and *Euphlyctis hexadactylus*, from Bangladesh. *Genes & Genetic Systems, 85*, 219–232. https://doi.org/10.1266/ggs.85.219

Boore, J. L. (1999). Animal mitochondrial genomes. *Nucleic Acids Research, 27*, 1767–1780. https://doi.org/10.1093/nar/27.8.1767

Boore, J. L. (2001). Complete mitochondrial genome sequence of the polychaete annelid *Platyneris dumerilii*. *Molecular Biology and Evolution, 18*, 1413–1416. https://doi.org/10.1093/oxfordjournals.molbev.a003925

Boore, J. L., Macey, J. R., & Medina, M. (2005). Sequencing and comparing whole mitochondrial genomes of animals. *Methods in Enzymology, 395*, 311–348. https://doi.org/10.1016/S0076-6879(05)95019-2

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. *Systematic Biology, 55*, 579–594. https://doi.org/10.1080/10635150600812551

Cai, H. X., Che, J., Pang, J. F., Zhao, E. M., & Zhang, Y. P. (2007). Paraphyly of Chinese *Amolops* (Anura: Ranidae) and phylogenetic position of the rare Chinese frog, *Amolops tormotus*. *Zootaxa*, 1531, 49–55.

Che, J., Hu, J. S., Zhou, W. W., Murphy, R. W., Papenfuss, T. J., Chen, M. Y., ... Zhang, Y. P. (2009). Phylogeny of the asian spiny frog tribe Paini (Family Dicroglossidae) sensu Dubois. *Molecular Phylogenetics and Evolution, 50*, 59–73. https://doi.org/10.1016/j.ympev.2008.10.007

Che, J., Zhou, W. W., Hu, J. S., Yan, F., Papenfuss, T. J., Wake, D. B., & Zhang, Y. P. (2010). Spiny frogs (paini) illuminate the history of the Himalayan region and Southeast Asia. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 13765–13770. https://doi.org/10.1073/pnas.1008415107

Chen, G. Y., Liu, J. Y., Jiang, J. P., Xie, F., & Zheng, Z. H. (2005). Systematics and distribution of the genus *Nanorana*. *Herpetologica Sinica, 10*, 47–51.

Chen, G., Wang, B., Liu, J., Xie, F., & Jiang, J. (2011). Complete mitochondrial genome of *Nanorana pleskei* (Amphibia: Dicroglossidae) and evolutionary characteristics. *Current Zoology, 57*, 785–805. https://doi.org/10.1093/czoolo/57.6.785

Chen, Z., Zhai, X., Zhu, Y., & Chen, X. (2015). Complete mitochondrial genome of the Ye’s spiny-vented frog *Yerana yei* (Dicroglossidae). *Mitochondrial DNA, 26*, 1–2.

de Sá, R. O., Streicher, J. W., Sekonyela, R., Fortlani, M. C., Loader, S. P., Greenbaum, E., & Haddad, C. F. (2012). Molecular phylogeny of microhyllid frogs (Anura: Microhyldae) with emphasis on relationships among New World genera. *BMC Evolutionary Biology, 12*, 241.

Fei, L., Hu, S. Q., Ye, C. Y., & Huang, Y. Z. (2009). *Fauna Sinica: Amphibia*, Vol. 3. Anura. Beijing: Science Press.

Fei, L., Lu, S., Yang, D., Sushil, D., Annemarie, O., & Tej, K. S. (2004). *Nanorana parkeri*. The IUCN Red List of Threatened Species e.T58392A11766426. https://doi.org/10.2305/iucn.uk.2004.rlts.t58392a11766426.en.2004 Downloaded on 03 February 2017.

Fei, L., Ye, C. Y., Huang, Y. Z., Jiang, J. P., & Xie, F. (2005). *An illustrated key to Chinese Amphibians*. Sichuan Chengdu: Sichuan Publishing House of Science and Technology.

Frost, D. R. (2018). Amphibian Species of the World: an Online Reference. Version 6.0 (Date of access). American Museum of Natural History, New York, USA. Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html.

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. https://doi.org/10.1371/journal.pone.0119825

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. https://doi.org/10.1016/j.gene.2007.10.001

Jiang, J. P., Dubois, A., Ohler, A., Tillier, A., Chen, X., Xie, F., & Stöck, M. (2005). Phylogenetic relationships of the tribe *Paini* (Ranidae, *Anura, Amphibia*) based on partial sequences of mitochondrial 12S
and 16S rRNA genes. Zoological Science, 22, 353–362. https://doi.org/10.2108/zsj.22.353
Jiang, J. P., & Zhou, K. Y. (2001). Evolutionary relationships among Chinese Ranid frogs inferred from mitochondrial DNA sequences of 12S rRNA genes. Acta Zoologica Sinica, 47, 38–44.
Jiang, J. P., & Zhou, K. Y. (2005). Phylogenetic relationships among Chinese ranids inferred from sequence data set of 125 and 16S rDNA. The Herpetological Journal, 15, 1–8.
Kurabayashi, A., & Sumida, M. (2009). PCR primers for the Neobatrachian mitochondrial genome. Current Herpetology, 28, 1–11.
Kurabayashi, A., Usuki, C., Mikami, N., Fujii, T., Yonekawa, H., Sumida, M., & Hasegawa, M. (2006). Complete nucleotide sequence of the mitochondrial genome of a Malagasy poison frog, Mantella madagascarensis: Evolutionary implications on mitochondrial genomes of higher anuran groups. Molecular Phylogenetics and Evolution, 39, 223–236. https://doi.org/10.1016/j.ympev.2005.11.021
Kurabayashi, A., Yoshikawa, N., Sato, N., Hayashi, Y., Oumi, S., Fujii, T., & Sumida, M. (2010). Complete mitochondrial DNA sequence of the endangered frog Odorrana ishikawaee (family Ranidae) and unexpected diversity of mt gene arrangements in ranids. Molecular Phylogenetics and Evolution, 56, 543–553. https://doi.org/10.1016/j.ympev.2010.01.022
Lanave, C., Preparata, G., Saccone, C., & Serio, G. (1984). A new method for calculating evolutionary substitution rates. Journal of Molecular Evolution, 20, 86–93. https://doi.org/10.1007/BF02101990
Li, E., Li, X., Wu, X., Ge, F., Zhang, M., Shi, H., ... Jiang, J. (2014a). Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of Ociddozyga martensi. Journal of Genetics, 93, 631–641. https://doi.org/10.1007/s12041-014-0418-4
Li, Y., Zhang, H., Wu, X., Xue, H., Yan, P., & Wu, X. (2014b). A novel mitogenomic rearrangement for Odorrana Schmackeri (Anura: Ranidae) and phylogeny of Ranidae inferred from thirteen mitochondrial protein-coding genes. Amphibia-Reptilia, 35, 331–343. https://doi.org/10.1163/15658381-00002958
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. https://doi.org/10.1016/j.gene.2004.10.013
Liu, J., Wang, C., Fu, D., Hu, X., Xie, X., Liu, P., ... Li, M. H. (2015). Phylogeography of Nanorana parkeri (Anura: Ranidae) and multiple refugia on the tibetan plateau revealed by mitochondrial and nuclear DNA. Scientific Reports UK, 5, 9857. https://doi.org/10.1038/srep09857
Lian, Q., Xia, Y., Zheng, Y., & Zeng, X. (2012). A de novo case of floating Chromosomal Polymorphisms by Translocation in Quasispa boulengeri (Anura, Dicroglossidae). PLoS ONE, 7, e46163.
Lu, S. (1995). A study of relationships among ranid frogs of the genera Nanorana and Altirana in the Transshimalaya Mountains of China. Asiatic Herpetological Research, 6, 73–77. https://doi.org/10.5962/bhl.part.7988
Lu, S., & Yang, D. (2004). Nanorana ventripunctata. The IUCN Red List of Threatened Species 2004c. T583941A11767025. https://doi.org/10.2305/iucn.uk.2004.rts.T583941a11767025.en/downloaded on 03 February 2017.
Lv, B., Bi, K., & Fu, J. Z. (2014). A phylogeographic evaluation of the Amolops mantzorum species group: Cryptic species and plateau uplift. Molecular Phylogenetics and Evolution, 73, 40–52.
Macey, J. R., Schulte, J. A., Larson, A., & Papenfuss, T. J. (1998). Tandem duplication via light-strand synthesis may provide a precursor for mitochondrial genome rearrangement. Molecular Biology and Evolution, 15, 71–75. https://doi.org/10.1093/oxfordjournals.molbev.a025849
Matsui, M., Hamidy, A., Belabut, D. M., Ahmad, N., Panha, S., Sudin, A., ... Nishikawa, K. (2011). Systematic relationships of Oriental tiny frogs of the family Microhylidae (Amphibia, Anura) as revealed by mtDNA genealogy. Molecular Phylogenetics and Evolution, 61, 167–176. https://doi.org/10.1016/j.ympev.2011.05.015
Perna, N. T., & Kocher, T. D. (1995). Patterns of nucleotide composition at four fold degenerate sites of animal mitochondrial genomes. Journal of Molecular Evolution, 41, 353–358. https://doi.org/10.1007/BF01215182
Posada, D. (2008). ModelTest: Phylogenetic model averaging. Molecular Biology and Evolution, 25, 1253–1256. https://doi.org/10.1093/molbev/msn083
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 Systematic Biology, 53, 793–808. https://doi.org/10.1006/jmot.2004.59223024
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. Molecular Phylogenetics and Evolution, 61, 543–583. https://doi.org/10.1016/j.ympev.2011.06.012
Qing, L., Xia, Y., Zheng, Y., & Zeng, X. (2012). A De Novo Case of Floating Chromosomal Polymorphisms by Translocation in Quasispa boulengeri (Anura, Dicroglossidae). The Herpetological Journal, 31, 227–234. https://doi.org/10.1093/oxfordjournals.molbev.a025849
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology, 61, 539–548. https://doi.org/10.1093/sysbio/sys029
Rubinstein, N. D., Feldstein, T., Shenkar, N., Botero-Castro, F., Griggio, F., Mastrototaro, F., ... Huchon, D. (2013). Deep sequencing of mixed total DNA without barcodes allows efficient assembly of highly plastic ascidian mitochondrial genomes. Genome Biology and Evolution, 5, 1185–1199. https://doi.org/10.1093/gbe/evt081
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. https://doi.org/10.1016/j.gene.2015.07.024
San Mauro, D., Gower, D. J., Oommen, O. V., Wilkinson, M., & Zardoya, R. (2004). Phylogeny of caecilian amphibians (Gymnophiona) based including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Molecular Phylogenetics and Evolution, 61, 543–583. https://doi.org/10.1016/j.ympev.2011.06.012
