Mitogenome of *Fejervarya multistriata*: a novel gene arrangement and its evolutionary implications

Z.H. Huang[1] and F.Y. Tu[2]

[1]School of Life Sciences, Jinggangshan University, Ji'an, Jiangxi Province, China
[2]Jiangxi Academy of Forestry, Nanchang, Jiangxi Province, China

Corresponding author: Z.H. Huang
E-mail: hzhow@163.com

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ABSTRACT. In this study, we determined the complete nucleotide sequence of the mitochondrial (mt) DNA of the paddy frog *Fejervarya multistriata*. mtDNA is 17,750-bp long and contains 13 protein-coding regions, 2 ribosomal RNA, non-coding genes, and 23 tRNA because of the presence of an extra copy of tRNA-Met. The gene arrangements among two related species of *Fejervarya* were compared, and the combined mtDNA data were subjected to a phylogenetic analysis. Interestingly, we observed a unique translocation of the tRNA-Leu gene, similar to that reported in previous studies on two *Fejervarya* species. Phylogenetic analyses supported the classification into two evolutionary clades, Ranidae and Dicroglossidae, as well as placement of *Hylarana guentheri* in the genus *Babina*. Our results suggested that *Fejervarya limnocharis* and *Fejervarya multistriata* may be conspecific,

because of its low pairwise genetic distance. However, these results must be further validated with additional analyses.

Key words: *Fejervarya multistriata*; Mitogenome; Gene arrangement; Phylogeny

INTRODUCTION

The paddy frog *Fejervarya multistriata*, is a species of frog belonging to the Dicroglossidae family. It is mainly found in southern China; however, it is also seen in Vietnam, Laos, Thailand, and Myanmar (Frost, 2009). The taxonomy of *F. multistriata* and related frogs in China is complex and not yet definitive: although formerly referred to as *Fejervarya limnocharis*, this species is now regarded as a complex species (Djong et al., 2011).

Ranidae (Anura: Neobatrachian) is a systematically neglected larger frog family that comprises a quarter of all extant frog species (Ford and Cannatella, 1993). The taxonomy of this family has also been revised several times (Zhou et al., 2009): Dubois (1992, 2005) proposed major taxonomic revisions of Ranidae, while Frost et al. (2006) proposed a new taxonomy across all living amphibians, with the Ranidae family being partitioned to avoid paraphyly with other monophyletic groups. Moreover, the subfamily Dicroglossinae, which was formerly placed in Ranidae, was elevated to Dicroglossidae (Scott, 2005; Frost, 2009). Frost et al. (2006) released an online platform for the classification of extant amphibians. In fact, Frost (2009) has made several revisions to the naming of this family, which have been employed in this study. Che et al. (2007) used combined mitochondrial and nuclear genetic data to conclude that the monophyly of *Amolops* and *Rana* was not supported. Recently, Huang et al. (2014) reported that some recognized genera (*Amolops*, *Rana*, *Babina*, and *Hylarana*) of Ranidae may not be monophyly, based on COI barcode information. Despite the utilization of different methods or markers to analyze the phylogeny of Ranidae in these studies (Scott, 2005; Che et al., 2007; Zhang et al., 2013), its phylogenetic relationships remain obscure (Scott, 2005; Zhang et al., 2013).

The mitochondrial (mt) DNA serves as a good molecular marker for the identification of phylogenetic inference in amphibians (Kurabayashi et al., 2010; Zhang et al., 2013). In this study, mtDNA of 39 anuran species excluding *F. multistriata* (consisting of 25 species of Ranidae and 14 species of Dicroglossidae) was used to amplify the complete mitochondrial genome of *F. multistriata*, analyze its composition and structure, and infer its evolutionary relationships.

MATERIAL AND METHODS

Sample collection

F. multistriata were collected from Ji'an city, Jiangxi Province, China; muscle samples from these specimens were associated with the voucher specimen (zjbj3) available at Jinggangshan University.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from muscle tissues using a standard phenol-

chloroform extraction protocol (Sambrook et al., 1989). Long-and-accurate and normal polymerase chain reaction (PCR) methods were combined to amplify the complete mtDNA sequence of *F. multistriata*. Eight overlapping PCR products, 1256-3978 bp in length, were amplified to obtain the entire mitochondrial genome. Primers used to generate PCR products, and the sizes of obtained fragments, are summarized in **Table S1**.

The PCR protocol was set as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 48°-60°C for 50 s, and elongation at 72°C for 70-240 s; and a final extension at 72°C for 7 min. The PCR products were then stored at 4°C. The amplification was performed in 25 μL reaction mixtures, containing 2.5 μL 10X PCR buffer (Mg^{2+} free; TaKaRa Bio Inc., Dalian, China), 2.5 μL $MgCl_2$ (25 mM), 1.0-4.0 μL deoxyribonucleoside triphosphates (dNTP, 2.5 mM), 1.0 μL of each primer (10 μM), 0.2 μL EX Taq polymerase (5 U/μL), and approximately 200 ng total genomic DNA (template). The obtained PCR products (N = 11) were electrophoresed on a 1.0% agarose gel, purified using the DNA Agarose Gel Extraction Kit (Omega, Norcross, GA, USA), and directly sequenced using the primer walking method in an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA).

Analysis of obtained sequences

All sequences were assembled and edited using the SeqMan (DNASTAR 7.1.0) software package (Swindell and Plasterer, 1997). The boundaries of protein-coding genes were predicted by comparing with homologous sequences obtained from other related frogs using the MEGA 6.0 (Tamura et al., 2013) software platform. Transfer RNA (tRNA) genes were identified using tRNA-scan SE 1.21 (Lowe and Eddy, 1997). tRNA-Lys and tRNA-Ser (AGY) genes, which could not be identified using tRNA-scan SE, were identified by observing previously proposed secondary rRNA structures (Kumazawa and Nishida, 1993) and investigating the anti-codons.

Phylogenetic analyses

The phylogenetic position of *F. multistriata* in the subfamily Dicroglossinae and its relationship to other anurans was confirmed by retrieving the complete mtDNA sequences of 40 anurans and 2 salamander (*Ranodon sibiricus* and *Rhinatrema bivittatum*) from GenBank (Table 1). The sequence datasets, including 2 rRNA (12S and 16S) and 11 protein-coding genes (PCGs) were compared. This led to the exclusion of *ND5* and *ND6* genes; the former because of the translocation of ND5 from the neobatrachian gene order (Ren et al., 2009), and the latter, as being encoded in a light strand made it more mutable compared to the sequences in the heavy strand (Waddell et al., 1999). Transitions and transversions were plotted against a pairwise sequence divergence using the datasets in DAMBE (Xia and Xie, 2001), in order to detect the possible bias of substitution saturation. Phylogenetic analyses were performed using the maximum likelihood (ML) and Bayesian inference (BI) methods. A best-fit partitioning scheme was determined for the ML analysis using Partitionfinder v.1.0.0 (Lanfear et al., 2012) under the Bayesian information criterion (Luo et al., 2010) (**Table S2**). An ML tree was constructed with the RAXML HPC software (Stamatakis et al., 2008) using a GTR + gamma model with 1000 bootstrap replicates. The datasets were partitioned by different genes (12S

RNA, 16S RNA, and 12 PCGs) for the BI method, and the concatenated PCGs were partitioned based on their codon position (**Table S3**). The best fit models for each partition were obtained using MRMODEL Test2.2 (Nylander, 2004) under the Akaike Information Criterion (Akaike, 1974). BI analysis was carried out using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Posterior distributions were obtained by the Markov chain Monte Carlo (MCMC) method with one cold and three heated chains for 10,000,000 generations, sampled one tree per 100 generations. The first 25% posterior samples were discarded as a conservative burn-in and the remaining samples were used to generate a 50% majority-rule consensus tree. MCMC runs were repeated twice to confirm a consistent approximation of the posterior parameter distributions.

Table 1. Various species analyzed in this study, their taxa, and GenBank accession numbers.

Family	Species	GenBank No.
Dicroglossidae	*Fejervarya multistriata*	KR071859
	Fejervarya cancrivora	NC012647
	Fejervarya limnocharis	NC005055
	Euphlyctis hexadactylus	NC014584
	Hoplobatrachus rugulosus	NC019615
	Hoplobatrvachus tigerinus	NC014581
	Licmnonectes bannaensis	NC012837
	Limnonectes fragilis	AY899241
	Limnonectes fujianensis	NC007440
	Nanorana parkeri	NC026789
	Nanorana pleskei	NC016119
	Nanorana taihangnica	NC024272
	Quasipaa boulengeri	NC021937
	Quasipaa spinosa	NC013270
	Quasipaa yei	NC024843
Ranidae	*Amolops mantzorum*	NC024180
	Amolops ricketti	NC023949
	Amolops wuyiensis	NC025591
	Babina adenopleura	NC018771
	Babina holsti	NC022870
	Babina okinavana	NC022872
	Babina subaspera	NC022871
	Glandirana tientaiensis	NC025226
	Lithobates catesbeianus	NC022696
	Lithobates sylvaticus	KP222281
	Odorrana ishikawae	NC015305
	Odorrana margaretae	NC024603
	Odorrana tormota	NC009423
	Pelophylax chosenicus	NC016059
	Pelophylax cretensis	NC025575
	Pelophylax cypriensis	NC026893
	Pelophylax epeiroticus	NC026894
	Pelophylax kurtmuelleri	NC026895
	Pelophylax nigromaculatus	AB043889
	Pelophylax plancyi	NC009264
	Pelophylax shqipericus	NC026896
	Rana chensinensis	NC023529
	Rana dybowskii	NC023528
	Rana kunyuensis	NC024548
	Hylarana guentheri	NC024748
Hynobiinae	*Ranodon sibiricus*	NC004021
Rhinatrematidae	*Rhinatrema bivittatum*	NC006303

Gene arrangement

The gene arrangement of the species was better explained by identifying the same as

explained by Kurabayashi et al. (2010). The obtained arrangement was then compared to that seen in its related species, *F. limnocharis* and *F. cancrivora*.

RESULTS

Sequence information

The *F. multistriata* mtDNA is 17,750-bp long and contains 13 PCGs, 2 ribosomal RNA, 23 tRNA genes, as well as noncoding regions (**Table S4**). Additionally, the *F. multistriata* mtDNA possesses an extra copy of tRNA-Met, similar to the mtDNA of the related species, *F. limnocharis* and *Fejervarya cancrivora*, in the subfamily Dicroglossinae (Liu et al., 2005; Ren et al., 2009). The overall base composition of the *F. multistriata* mtDNA is as follows: A = 28.0%, T = 29.9%, C = 26.9%, and G = 15.2%, which was similar to that of other anurans.

Phylogenetic analyses

The alignment sequences (9666 bp) were combined to build phylogenetic trees. We detected no nucleotide substitution saturation based on the combined data using DAMBE. Phylogenetic trees constructed using a combination of data obtained for the 13 genes in 43 anurans (including two outgroup species) using the ML and BI methods were robust and similar (Figure 1). The 41 species sampled in this study belong to two sister families Ranidae and Dicroglossidae (Figure 1). These results indicated that Dicroglossidae is not a monophyly. The phylogenetic relationships of the Dicroglossidae family [(*Euphlyctis*, *Hoplobatrachus*) *Fejervarya*, (*Nanorana*, *Quasipaa*) *Limnonectes*] were recovered with strong support values. The species *F. multistriata* and *F. limnocharis* were recovered; moreover, *F. cancrivora* was clustered with the common ancestor of *F. multistriata* and *F. limnocharis* (Figure 1). Most species of the family Ranidae were grouped in different clades, except for *Hylarana guentheri*, which was nested in the genus *Babina* (Figure 1).

Gene arrangement

The gene arrangement of *F. multistriata* was carefully analyzed. The mitochondrial genome orders of *F. multistriata* indicated the unique position of the *ND5* gene, similar to the gene arrangement of *F. limnocharis* and *F. cancrivora* (Liu et al., 2005; Ren et al., 2009). The gene order ND5-Thr-Pro-Leu was observed in *F. multistriata*.

DISCUSSION

Anurans are a well-studied group of amphibians. The complete mitochondrial DNA of several Anuran species has been sequenced, allowing researchers to infer the phylogenomics of Anurans. However, there remain considerable disagreements regarding the phylogenetic relationships of these amphibians. The mitochondrial phylogenetic tree shows that all species are grouped into two deeply divergent clades, corresponding to Ranidae and Dicroglossidae (Figure 1).

The generic placement of *Hylarana* within Ranidae is still under debate (Frost, 2011).

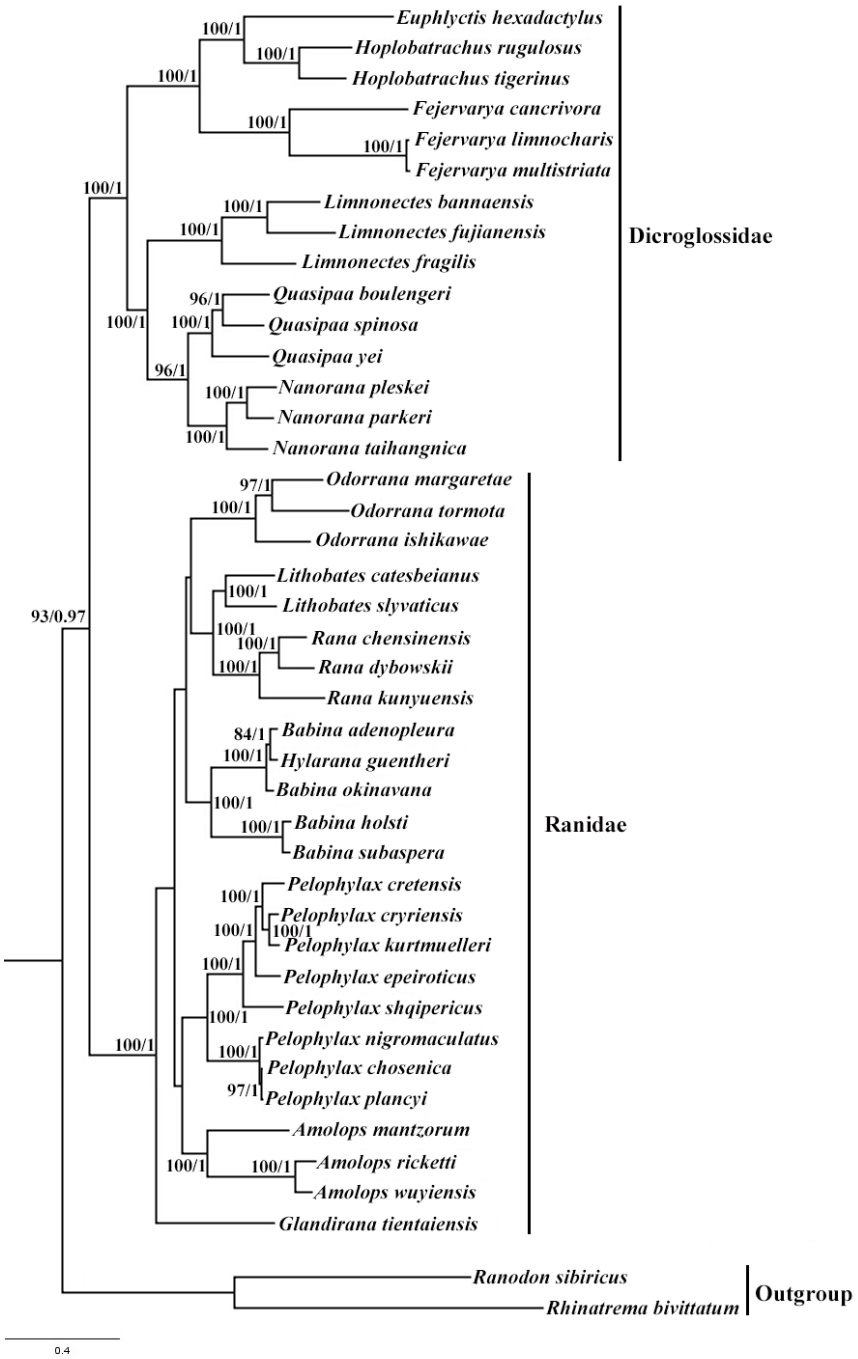

Figure 1. Partition phylogenetic tree of 42 species was constructed using the dataset comprising 14 concatenated mitochondrial PCGs. The numbers on the inter-node branches are ML and BI support values.

In fact, the species *H. guentheri* has often been recognized as the monotypic genus *Sylvirana*. Dubois (1992) included *H. guentheri*, *Hylarana maosonensis*, and *Hylarana milleti* in the genus *Rana*. COI barcode analysis, on the other hand, suggested *H. guentheri* to be a sister taxa to members of *Babina* (Huang et al., 2014). The molecular data obtained in our study supported the placement of *H. guentheri* in the genus *Babina* (Figure 1).

The analysis conducted in this study succeeded in producing a completely dichotomous phylogenetic tree within the Dicroglossidae family. *Euphlyctis* is a sister genus of *Hoplobatrachus*; together, these two genus form a sister group of *Fejervarya* (Figure 1). Despite the large number of morphological and molecular analyses conducted, a consensus on the taxonomic system and phylogenetic relationships of the genus *Fejervarya* has not been established (Kotaki et al., 2010). *Fejervarya* was first introduced as a subgenus of *Rana*, and subsequently re-classified as a subgenus of *Limnonectes*. Our results indicated that *Fejervarya* is not a sister genus of *Limnonectes.* Kotaki et al. (2010) reported *F. multistriata* to be a problematic taxa among the various species. The taxonomy of *F. multistriata* and related frogs in China is complicated and not fully settled. Djong et al. (2007) contended that the name "*F. multistriata*" could be applied to frog populations in China that were formerly referred to as *F. limnocharis*, while Kotaki et al. (2010) suggested the name to be a junior synonym of *F. limnocharis*, based on the 16S gene sequence. Analysis using Cytb or Dloop showed the low pairwise genetic distance between the two taxa (0.44 and 1.45%, respectively). Our results indicated that *F. limnocharis* and *F. multistriata* could be conspecific. However, further taxon sampling and loci is required to support these results.

Four tRNA genes [tRNA-Leu (CUN), tRNA-Thr, tRNA-Pro, and tRNA-Phe] are commonly rearranged in neobatrachian groups (Sumida et al., 2001; Zhang et al., 2005). Kurabayashi et al. (2010) depicted a ND5-Leu-Pro-Thr gene arrangement among two *Fejervarya* species, *F. limnocharis* and *F. cancrivora*. However, our results indicated that another gene arrangement (ND5-Thr-Pro-Leu) supported by other previous studies (Liu et al., 2005; Ren et al., 2009) occurred among the species. A careful reexamination of the gene orders of *F. limnocharis* and *F. cancrivora* revealed the erroneous gene arrangement (ND5-Leu-Pro-Thr) proposed by Kurabayashi et al. (2010) in these two species. The conserved block Leu-Pro-Thr has been reported in many ranid taxa. A unique translocation of the tRNA-Leu (*CUN*) gene has been observed in the *Fejervarya* species. However, the mechanism of tRNA-Leu (CUN) translocation can only be explained by further research.

Conflict of interest

The authors declare no conflict of interest.

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REFERENCES

Akaike H (1974). A new look at the statistical model identification. *IEEE Trans. Automat. Contr.* 19: 716-723. http://dx.doi.org/10.1109/TAC.1974.1100705

Che J, Pang J, Zhao H, Wu GF, et al. (2007). Phylogeny of Raninae (Anura: Ranidae) inferred from mitochondrial and nuclear sequences. *Mol. Phylogenet. Evol.* 43: 1-13. http://dx.doi.org/10.1016/j.ympev.2006.11.032

Djong HT, Matsui M, Kuramoto M, Nishioka M, et al. (2011). A new species of the *Fejervarya limnocharis* complex from Japan (Anura, Dicroglossidae). *Zoolog. Sci.* 28: 922-929. http://dx.doi.org/10.2108/zsj.28.922

Djong TH, Matsui M, Kuramoto M, Belabut DM, et al. (2007). Morphological divergence, reproductive isolating mechanism, and molecular phylogenetic relationships among Indonesia, Malaysia, and Japan populations of the Fejervarya limnocharis complex (Anura, Ranidae). *Zoolog. Sci.* 24: 1197-1212. http://dx.doi.org/10.2108/zsj.24.1197

Dubois A (1992). Notes sur la classification des Ranidae (Amphibian, Anoures). *B. Mens. Soc. Linn. Lyon.* 61: 305-352.

Dubois A (2005). Amphibia Mundi.1.1. An ergotaxonomy of recent amphibians. *Alytes* 23: 1-24.

Ford LS and Cannatella DC (1993). The major clades of frogs. *Herpetological Monogr.* 7: 94-117. http://dx.doi.org/10.2307/1466954

Frost DR (2009). Amphibian Species of the World: an Online Reference (v.5.3). American Museum of Natural History, New York, USA. http://research.amnh.org/herpetology/amphibia/index.php. Accessed December 4, 2009.

Frost DR (2011). Amphibian Species of the World: an Online Reference (v.5.5). American Museum of Natural History, New York, USA. http://research.amnh.org/vz/herpetology/amphibia/. Accessed January 31, 2011.

Frost DR, Grant T, Faivovich J, Bain RH, et al. (2006). The amphibian tree of life. *Bull. Am. Mus. Nat. Hist.* 297: 1-370. http://dx.doi.org/10.1206/0003-0090(2006)297[0001:TATOL]2.0.CO;2

Huang Z, Yang C and Ke D (2014). DNA barcoding and molecular phylogeny in Ranidae. *Mitochondrial DNA* 29: 1-5.

Kotaki M, Kurabayashi A, Matsui M, Kuramoto M, et al. (2010). Molecular phylogeny of the diversified frogs of genus *Fejervarya* (Anura: Dicroglossidae). *Zoolog. Sci.* 27: 386-395. http://dx.doi.org/10.2108/zsj.27.386

Kumazawa Y and Nishida M (1993). Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. *J. Mol. Evol.* 37: 380-398. http://dx.doi.org/10.1007/BF00178868

Kurabayashi A, Yoshikawa N, Sato N, Hayashi Y, et al. (2010). Complete mitochondrial DNA sequence of the endangered frog *Odorrana ishikawae* (family Ranidae) and unexpected diversity of mt gene arrangements in ranids. *Mol. Phylogenet. Evol.* 56: 543-553. http://dx.doi.org/10.1016/j.ympev.2010.01.022

Lanfear R, Calcott B, Ho SYW and Guindon S (2012). PartitionFinder: combined selection of partitioning schemes and substitution considering the relative likelihood of nucleotide and condon changes. *Mol. Biol. Evol.* 2: 150-174.

Liu ZQ, Wang YQ and Su B (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. http://dx.doi.org/10.1016/j.gene.2004.10.013

Lowe TM and Eddy SR (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* 25: 955-964. http://dx.doi.org/10.1093/nar/25.5.0955

Luo A, Qiao H, Zhang Y, Shi W, et al. (2010). Performance of criteria for selecting evolutionary models in phylogenetics: a comprehensive study based on simulated datasets. *BMC Evol. Biol.* 10: 242. http://dx.doi.org/10.1186/1471-2148-10-242

Nylander JAA (2004). MrModeltest V2. Program distributed by the author Computer Program, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

Ren Z, Zhu B, Ma E, Wen J, et al. (2009). Complete nucleotide sequence and gene arrangement of the mitochondrial genome of the crab-eating frog Fejervarya cancrivora and evolutionary implications. *Gene* 441: 148-155. http://dx.doi.org/10.1016/j.gene.2008.09.010

Ronquist FR and Huelsenbeck JP (2003). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 19: 1572-1574. http://dx.doi.org/10.1093/bioinformatics/btg180

Sambrook J, Fristsch EF and Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, USA.

Scott E (2005). A phylogeny of ranid frogs (Anura: Ranoidea: Ranidae), based on a simultaneous analysis of morphological and molecular data. *Cladistics* 21: 507-574. http://dx.doi.org/10.1111/j.1096-0031.2005.00079.x

Stamatakis A, Hoover P and Rougemont J (2008). A rapid bootstrap algorithm for the RAxML Web servers. *Syst. Biol.* 57: 758-771. http://dx.doi.org/10.1080/10635150802429642

Sumida M, Kanamori Y, Kaneda H, Kato Y, et al. (2001). Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata. Genes Genet. Syst.* 76: 311-325. http://dx.doi.org/10.1266/ggs.76.311

Swindell SR and Plasterer TN (1997). SEQMAN. Sequence data analysis guidebook. Springer, NY.

Tamura K, Stecher G, Peterson D, Filipski A, et al. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729. http://dx.doi.org/10.1093/molbev/mst197

Waddell PJ, Cao Y, Hauf J and Hasegawa M (1999). Using novel phylogenetic methods to evaluate mammalian mtDNA, including amino acid-invariant sites-LogDet plus site stripping, to detect internal conflicts in the data, with special reference to the positions of hedgehog, armadillo, and elephant. *Syst. Biol.* 48: 31-53. http://dx.doi.org/10.1080/106351599260427

Xia X and Xie Z (2001). DAMBE: software package for data analysis in molecular biology and evolution. *J. Hered.* 92: 371-373. http://dx.doi.org/10.1093/jhered/92.4.371

Zhang P, Zhou H, Liang D, Liu YF, et al. (2005). The complete mitochondrial genome of a tree frog, Polypedates megacephalus (Amphibia: Anura: Rhacophoridae), and a novel gene organization in living amphibians. *Gene* 346: 133-143. http://dx.doi.org/10.1016/j.gene.2004.10.012

Zhang P, Liang D, Mao RL, Hillis DM, et al. (2013). Efficient sequencing of Anuran mtDNAs and a mitogenomic exploration of the phylogeny and evolution of frogs. *Mol. Biol. Evol.* 30: 1899-1915. http://dx.doi.org/10.1093/molbev/mst091

Zhou Y, Zhang JY, Zheng RQ, Yu BG, et al. (2009). Complete nucleotide sequence and gene organization of the mitochondrial genome of *Paa spinosa* (Anura: Ranoidae). *Gene* 447: 86-96. http://dx.doi.org/10.1016/j.gene.2009.07.009

Supplementary material

Table S1. Eight primer combinations used to amplify the complete mtDNA of Fejervarya multistriata.

Table S2. Partition scheme for concatenated datasets.

Table S3. Parameter data from MrModeltest for different partition genes.

Table S4. Characteristics of the mitochondrial genome of Fejervarya multistriata.