



Intraspecific Diversity Analysis of Rice Frogs, *Fejervarya multistriata* (Anura: Ranidae), Based on mtDNA D-Loop Sequences, in Tongren, Guizhou Province, China

Zhen-Yang Wu¹, Guang-Xin E^{2,*}, Hui Ran¹, Da-Hui Wang¹ and Tian-You Yang¹

¹Tongren University, 238 Qihang Road, Tongren Guizhou, 554300, China

²College of Animal Science and Technology, 2 Tiansheng Road, Southwest University, Chongqing 400715, China

ABSTRACT

The rice frog, *Fejervarya multistriata*, is an amphibian that is widely distributed in Guizhou Province, China. In this study, we determined the diversity and phylogeography of 10 geographic populations (252 individuals) from Tongren, Guizhou Province using 516-bp sequences of the mitochondrial D-Loop region. In total, 63 polymorphic sites, including four single-nucleotide polymorphisms and 59 parsimony-informative sites, and 18 haplotypes were detected. The number of haplotypes within populations ranged from one (WS and DJ) to six (JK). Haplotype diversity ranged from 0.000 (DJ and WS) to 0.793 (JK). The largest amount of nucleotide diversity was found in the SQ population (0.03111), whereas the smallest amount was in the DJ and WS (0.00000) populations. Phylogenetic analysis revealed that these 10 populations could be separated into two clusters: the first included CD, WS, YP, JK, and ST, and the second included DJ, YJ, YH, SQ, and SN. However, there was no significant divergence between populations based on pair-wise differences (F_{ST}). Overall, the 10 populations were divided into two subgroups and two D-Loop haplotype clusters. This study showed that the different rice frog populations in Tongren had low genetic diversity and little genetic flow, which has led to substantial differences in genetic divergence between western and eastern populations. These results show that geographical isolation, especially by mountains, has played an important role in limiting rice frog migration.

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Authors' Contribution

TTY and HR conceived and designed the study. ZYW analyzed the data and prepared the manuscript. GXE helped in data analysis and produces some figures. DHW did sample collection.

Key words

Fejervarya multistriata, Mitochondrial DNA, D-Loop region, Haplotype, Genetic diversity.

INTRODUCTION

Tongren City is located in northeastern Guizhou Province, from the slope of the Yungui Plateau to the Xiangxi Hills. The rivers in this territory belong to the Yuan and Wu River systems, and amphibians are widely distributed in these river systems. The migration ability of amphibians is limited and they are easy to sample, which makes them ideal candidates for molecular biogeographic research (Kurabayashi *et al.*, 2010; Zhang *et al.*, 2013). Additionally, their distributions may reflect the ecological conditions of Tongren and therefore be informative for constructing environmental protection plans.

The rice frog is a dominant amphibian and common prey for many animals. This predator pressure is thus considered an evolutionary dynamic that promotes and maintains *F. multistriata* polymorphisms (Yang *et al.*, 2017). Moreover, several stressors, such as habitat destruction, environmental pollution, human hunger

killing, and travel industry development, have caused amphibian populations to rapidly decline. Meanwhile, Increasing urbanization has a significant impact on natural ecosystems and presents a major threat to anuran populations (Li *et al.*, 2016). Therefore, it is necessary to use molecular genetic data to study the genetic structure and differentiation of *F. multistriata* populations.

Mitochondrial genome (mtDNA) analysis is a popular tool for estimating phylogenetic patterns and migration of wild (Gong *et al.*, 2018; Liu and Zhou, 2017; Niedziałkowska, 2017) and domestic animals (Paramasivam *et al.*, 2017; Li *et al.*, 2018). However, little research has been conducted on the genetic diversity of *F. multistriata*. Sumida *et al.* (2002) were the first to examine the phylogenetic structure of the rice frog from Japanese main islands by using molecular methods. Thereafter, they carried out similar research on *F. multistriata* and elucidated genetic relationships with extensive sampling sites across most countries except Chinese mainland (Sumida *et al.*, 2007). In order to make up this shortcoming, Zhong *et al.* (2008) characterized the genetic structure of the rice frog which covered in most parts of China. However, the complete mitochondrial DNA of *F. multistriata* has been

* Corresponding author: eguangxin@126.com
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sequenced until 2016 (Huang and Tu, 2016). Therefore, it is important to study the genetic structure and differentiation of Tongren rice frog populations and estimate gene flow among different rice frog populations in this area by using techniques such as mtDNA analysis, which would be helpful to further access environment of the local area.

MATERIALS AND METHODS

Muscle samples from 252 individual rice frogs of 10 populations were obtained from the area around Tongren City, Guizhou Province; geographic information is presented in Table I and Figure 1. Muscle samples were stored in absolute ethyl alcohol and frozen at -20°C until extraction. Genomic DNA was isolated using a DNA extraction kit (Tiagen Biotech Co., LTD., China) and checked for DNA quality on a 1% agarose gel.

The highly variable region of the mtDNA control region (D-Loop) was amplified using primers mtDNA-F (5'-CTG TCC ATA TCA TGA CTA CTT G-3') and mtDNA-R (5'-GGT CTT AGC TTG TAG AGA GGT C-3'), which were referenced from a rice frog sequence. PCR amplification was conducted in a total reaction volume of 60 μL , with 30 μL 2 \times PCR standard reaction buffer mix, and 10 $\mu\text{mol/L}$ each forward and reverse primer. The PCR cycle was adjusted at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The PCR product was subjected to electrophoresis for detection amplified DNA. PCR products were directly sequenced using mtDNA-F and mtDNA-R to ensure sequencing accuracy with Genetic Analyzer 3130 xl (AB Applied Bio Systems, USA).

The D-Loop sequence alignments were constructed using Clustal X 1.83. To screen for haplotypes and polymorphisms, estimation of the average number of nucleotide differences between populations was conducted in DnaSP 5.10 (Rozas and Rozas, 1995). The best-fit model of DNA substitution for BI (Bayesian Inference) was obtained using jModelTest 0.1.1 (Posada,

2008). The maximum likelihood phylogenetic network of D-Loop sequences among all individuals was constructed with MEGA 7.0 (Tamura *et al.*, 2011), and the bootstrap values to support the nodes of the tree were based on 1000 iterations of a heuristic search. In addition, a visual haplotype phylogenetic network were constructed using median-joining method and subjected to analysis following the protocols described by Bandelt *et al.* (1999) and Lyimo *et al.* (2014) using Network 4.1 (<http://www.fluxus-engineering.com/sharenet.htm>).

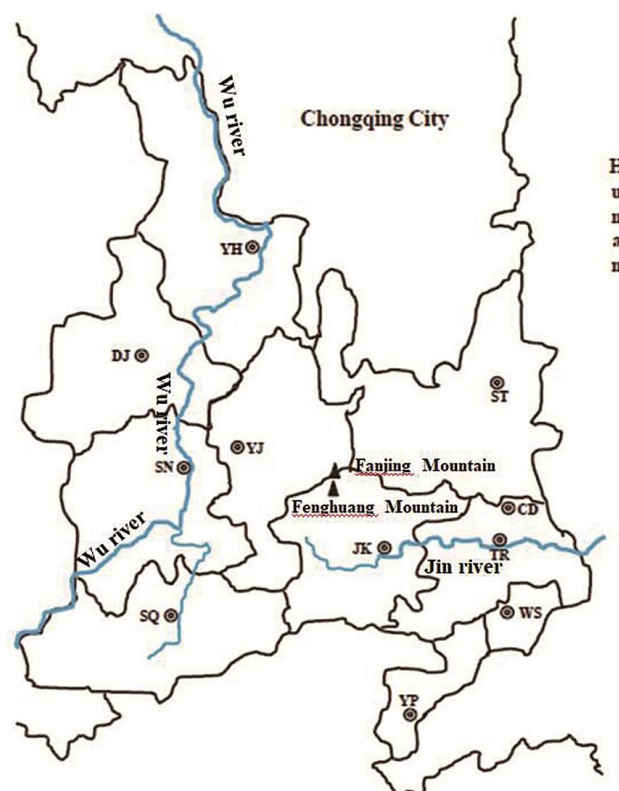


Fig. 1. Geographic information of sampling location of ten Tongren rice frogs populations.

Table I.- Geographic information of sampling location of ten Tongren rice frogs populations.

Population	Code	Sample size	Altitude (m)	Latitude (N)	Longitude (E)	Location
YuPing Strain	YP	29	340	27°16'18"	108°57'22"	Nanning village, Tongren, Guizhou, China
YingJiang Strain	YJ	22	610	27°56'12"	108°28'29"	Qinghe village, Tongren, Guizhou, China
YanHe Strain	YH	16	540	28°35'11"	108°28'53"	Mazhuxi village, Tongren, Guizhou, China
WanShan Strain	WS	30	720	27°33'39"	109°8'24"	Dashan village, Tongren, Guizhou, China
SongTao Strain	ST	10	350	28°6'31"	109°8'40"	Hongyan village, Tongren, Guizhou, China
ShiQian Strain	SQ	30	560	27°29'56"	108°12'9"	Gaotang village, Tongren, Guizhou, China
SiNan Strain	SN	31	450	27°58'55"	108°15'15"	Changjiang village, Tongren, Guizhou, China
JiangKou Strain	JK	30	320	27°47'59"	108°53'24"	Guakou village, Tongren, Guizhou, China
DeJiang Strain	DJ	24	580	28°14'31"	108°8'60"	Dejing country, Tongren, Guizhou, China
ChuanDong Strain	CD	30	500	27°47'59"	109°12'43"	Chuandong town, Tongren, Guizhou, China

Table II.- Nucleotide and haplotype polymorphisms of mtDNA D-Loop within ongren rice frogs populations.

Population	Nucleotide polymorphism				Haplotype polymorphism	
	Sample size	Nucleotide diversity (π)	Tajima's D	Tajima's D P-value	No of haplotype	Haplotype diversity
YP	29	0.00169	-1.53817	$P > 0.10$	3	0.1970
YJ	22	0.00052	-1.17515	$P > 0.10$	3	0.2550
YH	16	0.00049	-1.49796	$P > 0.10$	3	0.2420
WS	30	0.00000	N.A	N.A	1	0.0000
ST	10	0.00244	0.62422	$P > 0.10$	3	0.7110
SQ	30	0.03111	0.51819	$P > 0.10$	2	0.0287
SN	31	0.03064	0.33920	$P > 0.10$	4	0.4320
JK	30	0.00643	1.90751	$0.10 > P > 0.05$	6	0.7930
DJ	24	0.00000	N.A	N.A	1	0.0000
CD	30	0.00013	-1.14700	$P > 0.10$	2	0.0670

NA means no data.

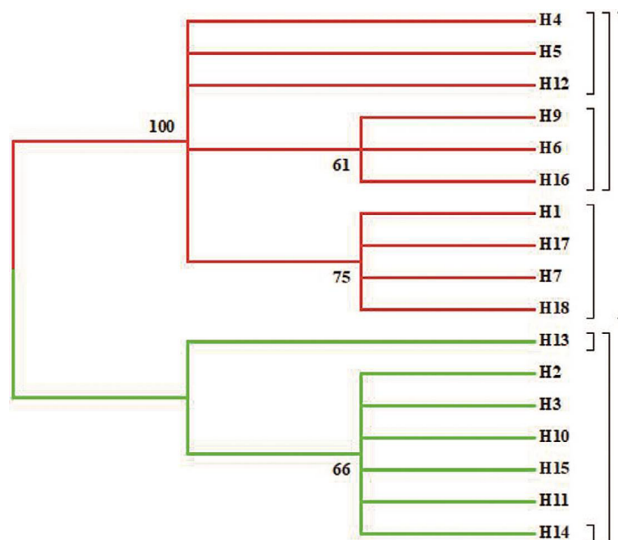


Fig. 2. Molecular phylogenetic analysis of 18 rice frogs mtDNA D-Loop haplotype by maximum likelihood method.

RESULTS

Sixty-three polymorphic sites, including four single-nucleotide polymorphisms and 59 parsimony-informative sites, were identified in the D-Loop region of these 252 individuals. Nucleotide polymorphisms ranged from 0.00000 (DJ and WS) to 0.03111 (SQ). Tajima's D ranged from -1.53817 (YP) to 4.83465 (YH), and the Tajima's D P-values were not significant in any populations based on Chi-Square test ($P > 0.10$) (Table II).

A total of 18 haplotypes were identified in 252 individuals. The JK population had the most haplotypes (six), whereas the DJ and WS populations each only had one haplotype. Haplotype diversity of the 10 populations

ranged from 0.000 (DJ and WS) to 0.793 (JK; Table II). The maximum likelihood-based phylogenetic analysis of the 18 haplotypes revealed two clusters (Fig. 2). Haplotype 2 was the most frequent haplotype and shared by five populations, and haplotypes 1, 5, 3, and 9 were shared by four, three, two, and two populations, respectively. The other haplotypes were only found in one population each (Fig. 3).

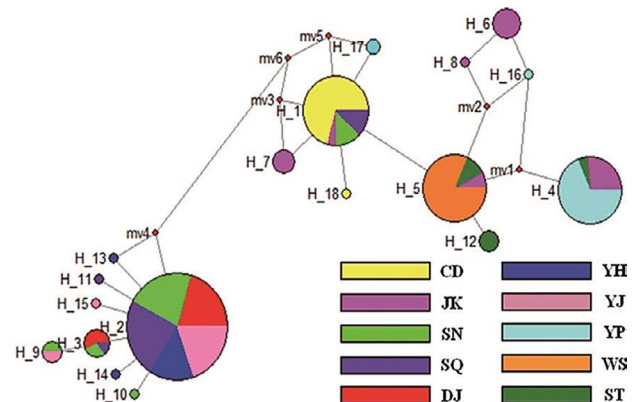


Fig. 3. Network and frequency profile of the 18 rice frogs haplotypes with mtDNA D-loop.

F_{ST} analysis of the Tongren rice frog populations revealed that the largest difference was between WS and YH ($F_{ST} = 0.99795$), and the smallest was between SN and SQ ($F_{ST} = -0.03285$ (Table III)). Moreover, the F_{ST} distribution indicated that these 10 populations were separated into two groups: one included JK, CD, YP, WS, and ST, and the other included SN, DJ, YH, YJ, and SQ. However, there was no significant divergence between the two groups, which was consistent with the phylogenetic network constructed from these 10 populations (Fig. 4).

Table III.- Genetic divergence between populations with D_{ST} and matrix of pairwise F_{ST}

	CD	DJ	JK	SN	SQ	ST	WS	YH	YJ	YP
CD		60.20000	3.56667	50.58172	50.10000	2.833330	2.033330	60.09583	60.26060	3.860920
DJ	0.99704		60.90000	10.02688	10.22222	60.76670	61.16667	0.291670	0.363640	61.13218
JK	0.52981	0.97063		51.7333	51.26667	2.893330	2.466670	60.79583	60.96061	2.749430
SN	0.82936	0.12807	0.80203		16.73548	51.50323	51.70968	10.01815	10.09531	51.97553
SQ	0.82593	0.13597	0.79848	-0.03285		51.03333	51.23333	10.18125	10.28788	51.50920
ST	0.76863	0.98738	0.21685	0.82098	0.81758		0.80000	60.66250	60.82727	2.172410
WS	0.98361	0.99763	0.33364	0.83373	0.83044	0.22222		61.06250	61.22727	2.103450
YH	0.99737	0.07453	0.97091	0.1293	0.13445	0.98768	0.99795		0.352270	61.02802
YJ	0.99582	0.00026	0.96945	0.12668	0.13432	0.98618	0.99643	0.02458		61.19279
YP	0.87973	0.99058	0.2454	0.82629	0.82298	0.51517	0.79508	0.99089	0.98938	

Above diagonal is K_{xy} , below diagonal is F_{ST}

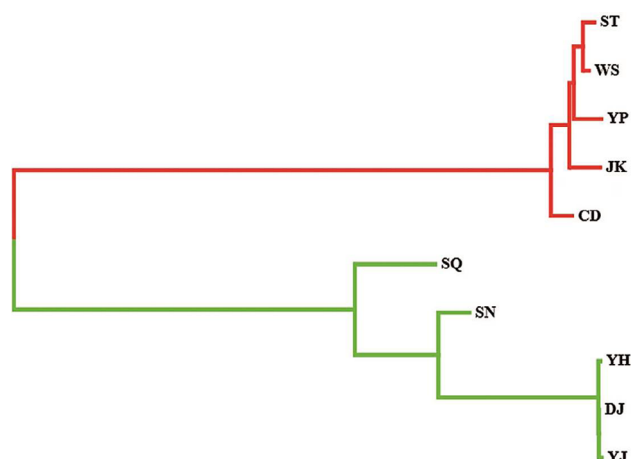


Fig. 4. Average number of nucleotide differences between populations (K_{xy}) among ten rice frogs populations.

DISCUSSION

Recently, mitochondrial DNA polymorphisms have been widely used to estimate gene flow and phylogenetic relationships of maternal lineages in domestic and wild animals. Although there have been some studies on rice frogs, they have mainly focused on population dynamics (Wang *et al.*, 2013; Li *et al.*, 2016), ecology (Xiong *et al.*, 2010), and morphology and histology (Xiong *et al.*, 2011; Chen and Liu, 2012). Little work has been done on *F. multistriata* genetic diversity (Huang and Tu, 2016). Amphibians are very sensitive to environmental and climatic changes; thus, the genetic diversity of their populations can provide useful information for tracking environmental variation.

The nucleotide polymorphisms of the D-Loop region in this study ranged from 0.00000 to 0.03111, which was greater than that described by Zhong *et al.* (2008), who

collected 95 samples from a wide range of locations across China and had polymorphisms that ranged from 0.000 to 0.013. However, the haplotype diversity of the 10 Tongren frog populations (0.0000 to 0.7930) was lower than that reported by Zhong *et al.* (2008) (0.000 to 1.000). Additionally, the number of haplotypes of 10 populations ranged from one to six, and a total of 18 haplotypes was found. Although Zhong *et al.* (2008) collected samples from across China, they only found 38 haplotypes. These results indicate that our results are consistent with the migration ability of the rice frog. Although WS and JK were relatively close, JK found six haplotypes, while WS found only one. Because the topography of Tongren City is very complex and the topography of each place is different. The number of haplotypes is related to geographic isolation, not distance. It further shows that the geographical isolation of mountains plays a decisive role.

However, there was no significant divergence in F_{ST} between the 10 Tongren rice frog populations, and their genetic distance reflected the geographic distance between the populations. This finding is consistent with the amount of gene exchange among partial subgroups that would be expected based on the ecological environment in which *F. multistriata* is found. In addition, two D-Loop haplotype clusters, according phylogenetic network from K_{xy} , were identified from the 252 Tongren frogs. The 10 populations were divided into two subgroups, one of which included ST, WS, YP, CD, and JK, the other included YH, YJ, DJ, SN, and SQ. Kurniawan *et al.* (2010) constructed molecular phylogenetic trees based on nucleotide sequences of the 16S rRNA and Cyt b genes, the results showed that the individuals of *F. cancrivora* analyzed comprised two clades and one clade further split into two subclades. These results are consistent with the geographic distribution of rice frogs.

As shown in Figure 1, the central part of Tongren is

blocked by three mountains. There is a river that flows from north to south in western Tongren and a river that flows from west to east in eastern Tongren. Tongren City is mainly mountainous, followed by hilly. The highest is 2,572 meters above sea level and the lowest is 205 meters. Fanjing mountain is a watershed in the east and west of Tongren City. The results show that the geographical isolation by mountains was important in shaping the genetic diversity of these rice frogs. Network and frequency profiles of the 18 rice frogs haplotypes based on the mtDNA D-Loop region (Fig. 3) showed that most western populations of Tongren (DJ, YJ, YH, SQ, and SN) shared haplotype 2, whereas JK, ST, WS, and YP populations shared haplotypes 4 and 5. The majority of the CD population had haplotype 1. This finding can also be explained by the results shown in Figure 4. No significant differences based on Tajima's D ($P > 0.10$ or $0.10 > P > 0.05$) were found in the analyzed populations, which indicated that there was no historical population expansion in Tongren.

CONCLUSION

The phylogenetic relationship of the 18 haplotypes was constructed by maximum likelihood method, and two clusters were identified. The distribution of F_{ST} indicated that these ten populations were separated into two groups. These result indicate that mountains can have a significant impact on the migration of rice frogs, and also imply that urbanization and human activity may affect the genetic structure of rice frogs.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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