Mitochondrial DNA phylogeography of the Guizhou odorous frog: limited population genetic structure and evidence for recent population size expansion

Shize Li a,b,c, Gang Wei a,*, Jing Liu d and Houqiang Xu a,b

aLaboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, Collaborative Innovation Center for Mountain Ecology and Agro-Bioengineering (CICMEAB), College of Life Sciences, Guizhou University, Guiyang, China; bKey Laboratory of Animal Genetics Breeding of Guizhou Province, Guizhou University, Guiyang, China; cDepartment of Food Science and Engineering, Moutai Institute, Renhuai, China; dBiodiversity Conservation Key Laboratory, Guiyang College, Guiyang, China

ABSTRACT
The Guizhou odorous frog *Odorrana kweichowensis* is endemic to Guizhou Province, China. In this study, a comparative analysis of the mitochondrial COI and ND2 gene sequences was performed to examine genetic diversity in 109 individuals from ten localities across the geographic range of the species. Haplotype diversity and nucleotide diversity were 0.576 and 0.00055, respectively. Phylogenetic analyses almost nested all haplotypes into one lineage. AMOVA indicated that total variation was mainly derived from variation within individual populations. Neutral tests indicated that a recent expansion occurred in the total population. Fst estimations indicated that genetic divergence was not correlated with geographic distance. Accordingly, the species probably experienced a recent population expansion, and there no obvious population genetic structure is apparent. The findings provide useful information for the conservation of this species.

Introduction
The odorous frog *Odorrana kweichowensis* Li et al. 2018 (Amphibia, Anura, Ranidae) was recently described to science from mountainous streams in Guizhou Province, China (Li et al. 2018). Although Li et al. (2018) reported three populations of *O. kweichowensis* in the northern part of Guizhou Province, China, the species was suggested to occur in parapatry with its phylogenetically closest species, *O. schmackeri*, and to have a wider distributional range in Guizhou Province and perhaps into northern Guanxi Province, China (Li et al. 2015, 2018; Zhu 2016). The karst and mountainous topography (e.g., Dalou Mountain and Wuling Mountain series), including some deep valleys (e.g., Wujiang River and Chishui River), in this region would be expected to impede gene flow between populations of amphibians restricted to mountainous streams (e.g., Che et al. 2010; Yan et al. 2013).

We conducted comprehensive surveys of *O. kweichowensis* and collected a series of specimens from the northern part to the southern edge of Guizhou Province, China. These surveys showed that a variety of human-caused threats, such as habitat destruction, overharvesting, and pollution, have likely caused a recent decline in the number of *O. kweichowensis*, as also reported in Li et al. (2018). Based on this information, the species was classified as Vulnerable in the IUCN Red List of Threatened Species (IUCN 2021). Understanding population history and genetic diversity is fundamental for conserving species (reference), but until now there has been limited attention toward *O. kweichowensis*.

Mitochondrial DNA (mtDNA) markers are frequently used for inferring levels of population genetic divergence and structure (Liu et al. 2015; Wang et al. 2017) due to their rapid rates of evolution and maternal inheritance (Sun et al. 2012). In this study, the mitochondrial Cytochrome c oxidase subunit I (COI) and NADH dehydrogenase subunit 2 (ND2) genes were used to reveal the population genetic structure and diversification history of *O. kweichowensis* to provide information that will assist in the conservation of the species.

Materials and methods
A total of 109 specimens were collected from ten localities (P1–P10) in Guizhou Province, China that span the known geographical range of *O. kweichowensis* (Figure 1(A); Table 1). The Animal Care and Use Committee of Guizhou College provided full approval for this study (Number: GYU2018040002). Field work was approved by the Management Office of the Kuankuoshui Nature Reserve (project number: KKS201504003). Specimens were euthanized before taking
muscle tissue. Muscle tissue samples were taken and preserved separately in 99% ethanol prior to fixation of the voucher specimen in 10% formalin. Preserved specimens were deposited in the Moutai Institute (voucher numbers in Table 1). Total DNA was extracted using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). Two fragments of the mitochondrial COI and ND2 genes were amplified. For COI, Chmf4 (5'-TYTCWACWAAYCAYAAAGAY
Table 1. Genetic diversity and neutrality tests of *O. kweichowensis*.

| ID   | Locality                  | Longitude (E)  | Latitude (N) | Population  | Altitude (m) | Haplotype (voucher No.)       | H0                                      | F0                                      | Fu's Fs                                | Tajima's D | Fu's s D (Tajima 1989) | Fu's s (Fu 1997) | F (Fst) | p-variance | p-values          |
|------|---------------------------|----------------|--------------|-------------|--------------|-----------------------------|-----------------------------------------|-----------------------------------------|----------------------------------------|------------|-----------------------|---------------------|---------|------------|----------------------|
| P1   | Libo Co., Guizhou Prov.   | 108.070361     | 25.28836     | 15          | 512          | H2 (ML2018082002-03, 04)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P2   | Dayong Co., Guizhou Prov. | 107.37692      | 26.53997     | 12          | 1055         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P3   | Guiding Co., Guizhou Prov. | 107.239431     | 26.34663     | 7           | 1078         | H2 (ML2018082002-01, 02)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P4   | Guizhou City, Guizhou Prov. | 107.644305     | 26.26815     | 15          | 1111         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P5   | Gufeng Co., Guizhou Prov. | 107.239431     | 26.53997     | 12          | 1055         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P6   | Kayang Co., Guizhou Prov. | 107.37692      | 26.53997     | 12          | 1055         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P7   | Xileng Co., Guizhou Prov. | 107.644305     | 26.26815     | 15          | 1111         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P8   | Dayong Co., Guizhou Prov. | 107.239431     | 26.53997     | 12          | 1055         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P9   | Xijiang Co., Guizhou Prov. | 107.644305     | 26.26815     | 15          | 1111         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| P10  | Jinghui Co., Guizhou Prov. | 107.239431     | 26.53997     | 12          | 1055         | H2 (ML2018082002-02, 03)    | 0.000                                   | 0.000                                   | 0.000                                  | 0.000       | 0.000                 | 0.000               | 0.286  | 0.000      | 0.000               |
| Total|                           |                |              |             |              | H0                                    |                                         |                                      |                                        | 0.576       | 0.000               | 0.000               | 0.095   | 1.087      | 0.000               |
Table 2. F-statistics (Fst) among the populations of O. kweichowensis.

|     | P1  | P2  | P3  | P4  | P5  | P6  | P7  | P8  | P9  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| P1  | 0.125 |     |     |     |     |     |     |     |     |
| P2  | 0.000 | 0.125 |     |     |     |     |     |     |     |
| P3  | 0.545 | 0.433 | 0.545 |     |     |     |     |     |     |
| P4  | 0.469 | 0.384 | 0.469 | 0.506 |     |     |     |     |     |
| P5  | 0.020 | 0.079 | 0.020 | 0.430 | 0.361 |     |     |     |     |
| P6  | 0.000 | 0.125 | 0.000 | 0.545 | 0.469 | 0.020 |     |     |     |
| P7  | 0.750 | 0.576 | 0.750 | 0.645 | 0.599 | 0.583 | 0.583 | 0.750 |     |
| P8  | 0.000 | 0.054 | 0.000 | 0.370 | 0.329 | 0.007 | 0.000 | 0.500 | 0.000 |
| P9  | 0.000 | 0.085 | 0.000 | 0.467 | 0.407 | 0.013 | 0.000 | 0.636 | 0.000 |

Discussion

The geographical topology within the distributional range of O. kweichowensis in southwestern China is complex and has been proposed to present mountain or river barriers for gene flow that promote speciation (Che et al. 2010; Yan et al. 2013). Mitochondrial genes have often been used to investigate population genetic structure within frog species in southwestern China and have often revealed considerable divergence between populations (e.g., Wang et al. 2012, 2013, 2017). However, our results based on the mitochondrial COI and ND2 gene sequences found low genetic diversity and did not find obvious population genetic structure in the frog O. kweichowensis. In this study, only 16 haplotypes were found among 109 specimens from 10 populations across the range of the species, and one haplotype (H2) occurred in all populations, indicating that all examined populations of the species might have a recent common origin. The genetic distances between populations was very low (average of 0.2% between samples). Further, AMOVA also suggested that more than 60% molecular variance was attributed to differentiation within populations rather than between populations. This point was also evidenced by neutrality tests that indicated a significant recent population size expansion in the total population of the species. Obviously, the recent population expansion was mainly derived from the ubiquitous distribution of haplotype H2. However, most populations of the frog have been experienced some minimal divergence, for example, the haplotype network indicated that in P4, haplotypes H12 and H13 were probably derived from H11 rather than the common H2. These results indicate that the frogs of the species have probably experienced shallow population divergences only very recently. Nevertheless, based on current results, we could not deduce the ancestral region or expansion center due to seven populations exhibiting unique haplotypes with low genetic diversity.

In summary, genetic diversity and structure in O. kweichowensis were relatively low, indicating that it might be a young species or has experienced bottleneck effects (Miracle and Campton 1995). Future work with more markers might clarify the cause of this pattern of relatively low genetic diversity and structure. However, the existing genetic diversity within this Guizhou-endemic frog species should be protected in light of threats from human activities. Populations P4, P5, P6 and P9 harbor most of the genetic diversity, and so should be the highest priority for conservation.

Acknowledgments

We would like to express our gratitude to the editors and the anonymous reviewers for their critical comments on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).
**Funding**

This work was supported by National Natural Sciences Foundation of China (NSFC31960099 and 32070426), Basic research project of science and technology department of Guizhou Province (No. [2020] 1Y083), Science and technology support project of science and technology department of Guizhou Provincial (No. [2020] 4Y029) and Guizhou Provincial Department of Education Youth Science and Technology Talents Growth Project (Nos. KY[2018]455 and KY[2018]468).

**Data availability statement**

The data of this study are openly available in figshare at http://doi.org/10.6084/m9.figshare.13152869. All haplotype sequences were submitted to GenBank under accession numbers MW067106–MW067120 for COI and MW071122–MW071136 for ND2.

**References**

Che J, Zhou WW, Hu JS, Yan F, Papenfuss TJ, Wake DB, Zhang YP. 2010. Spiny frogs (Paini) illuminate the history of the Himalayan region and Southeast Asia. Proc Natl Acad Sci U S A. 107(31):13765–13770.

Che J, Chen HM, Yang JX, Jin JQ, Jiang K, Yuan ZY, Murphy RW, Zhang YP. 2012. Universal COI primers for DNA barcoding amphibians. Mol Ecol Resour. 12(2):247–258.

Clement M, Posada D, Crandall K. 2000. TCS: a computer program to estimate gene genealogies. Mol Ecol. 9(10):1657–1660.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 9(8):772.

Excoffier L, Laval G, Schneider S. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform. 1:47–50.

Fu YX. 1997. Statistical test of neutrality of mutations against population growth, hitchhiking and background selection. Genetics. 147(2):915–925.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 59(3):307–321.

Hall TA. 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 41(41):95–98.

IUCN. 2021. The IUCN Red List of Threatened Species. Version 2020-3. https://www.iucnredlist.org.

Li SZ, Xu N, LV JC, Jiang JP, Wei G, Wang B. 2018. A new species of the odorous frog genus *Odorrana* (Amphibia, Anura, Ranidae) from southwestern China. PeerJ. 6(e5695):e5695–28.

Li YM, Wu XY, Zhang HB, Yan P, Xue H, Wu XB. 2015. Vicariance and its impact on the molecular ecology of a Chinese Ranid frog species-complex (*Odorrana schmackeri*, Ranidae). PLoS One. 10(9):e0138757.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25(11):1451–1452.

Liu J, Wang CM, Fu DL, Hu XJ, Xie XM, Liu PF, Zhang Q, Li MH. 2015. Phylogeography of *Nanorana parkeri* (Anura: Ranidae) and multiple refugia on the Tibetan Plateau revealed by mitochondrial and nuclear DNA. Sci Rep. 5(1):9857.

Miracle AL, Campton DE. 1995. Tandem repeat sequence variation and length heteroplasy in the mitochondrial DNA D-loop of the threatened Gulf of Mexico sturgeon, *Acipenser oxyrhynchos* desotoi. J Hered. 86(1):22–27.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. 123(3):585–595.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 30(12):2725–2729.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Sun Y, Wang A, Wan D, Wang Q, Liu J. 2012. Rapid radiation of *Rheum* (Polygonaceae) and parallel evolution of morphological traits. Mol Phylogenet Evol. 63(1):150–158.

Wang B, Jiang J, Xie F, Li C. 2012. Postglacial colonization of the Qinling Mountains: phylogeography of the swelled vent frog (*Feirana quadranus*). PLoS One. 7(7):e41579.

Wang B, Jiang J, Xie F, Li C. 2013. Phylogeographic patterns of mtDNA variation revealed multiple glacial refugia for the frog species *Feirana taihangnica* endemic to the Qinling Mountains. J Mol Evol. 76(3):112–128.

Wang B, Xie F, Li JN, Wang G, Li C, Jiang JP. 2017. Phylogeographic investigation and ecological niche modelling of the endemic frog species *Nanorana pleskei* revealed multiple refugia in the eastern Tibetan Plateau. PeerJ. 5:e3770.

Yan F, Zhou W, Zhao H, Yuan Z, Wang Y, Jiang K, Jin J, Murphy RW, Che J, Zhang Y. 2013. Geological events play a larger role than Pleistocene climatic fluctuations in driving the genetic structure of *Quasipaa boulengeri* (Anura: Dicroglossidae). Mol Ecol. 22(4):1120–1133.

Zhu YJ. 2016. Genetic differentiation of *Odorrana schmackeri* species complex. Henan: Henan Normal University.