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# Genetic diversity of *Rana hanluica* based on mitochondrial cytb and nuclear rag2

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**Abstract**

*Rana hanluica* is an endemic amphibian in China that is distributed in the hills and mountains south of the Yangtze River. In this study, 162 samples (comprising six groups) from 19 localities were collected, and the genetic diversity of *R. hanluica* groups was studied using mitochondrial cytb and nuclear rag2. The results showed that the genetic diversity of *R. hanluica* groups as a whole is high in haplotype diversity and low in nucleotide diversity. All haplotypes clustered into one branch and showed inconsistencies with the geographic structure. The levels of gene flow between the NL group and the other five groups as well as between the LXS group and two groups (WYS and NL) were all greater than 1, indicating that there was no barrier to gene flow between the above groups. Analysis of molecular variance also showed that genetic variation primarily occurred within groups, but the higher genetic differentiation reflected differentiation between groups of *R. hanluica* that may have been caused by genetic drift. Among the six groups of *R. hanluica*, only the LXS and NL groups have expanded. In conclusion, the degree of genetic diversity in each group of *R. hanluica* was not very high, while the level of genetic diversity varied significantly among groups. It is recommended that priority should be given to protecting groups with a large number of unique haplotypes (e.g., NL and LXS); the NL group is distributed in the South Ridge, as an important biological corridor.

**Key Words**

*Rana hanluica*, genetic diversity, mitochondrial DNA, nuclear DNA, phylogeny

## Introduction

The protection of the genetic diversity of species is one of the core contents of biodiversity conservation. Research on genetic diversity helps understand the evolutionary history and potential as well as population development trends of species. Thus, genetic diversity is significant for biodiversity conservation and sustainable development (Jiang and Ma 2014). Amphibians as an important branch of vertebrates are experiencing severe population decline globally due to their special life history characteristics and body structures that require a very specific environment (Fu

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et al. 2021; Houlahan et al. 2000). Currently, habitat degradation or loss, illegal capture, and environmental pollution are the most serious threats to amphibians in China (Jiang et al. 2016). In this context, it is particularly important to study the genetic diversity of amphibians and determine their spatial distribution patterns for amphibian conservation (Fu et al. 2021). The genetic variation in the genus *Rana* has received extensive attention. However, there are few studies on the genetic diversity of individual species (Chen et al. 2022; Kim et al. 1999; Zhan et al. 2009; Zhou et al. 2012). *R. hanluica* is an endemic species in China that is largely distributed in the hills and mountains south of the Yangtze River (Jiang et al. 2021; Shen et al. 2007). Studies on this species have generally focused on species validity (Wang et al. 2009) and population ecology (Xia et al. 2021; Xia et al. 2022). It is necessary to determine the status of the genetic diversity of this species to provide more scientifically based guidance for the rational formulation of conservation and management measures.

Mitochondrial DNA (mtDNA) has unique features, including maternal inheritance and lack of recombination, that provide significant advantages in analyzing the evolutionary history and phylogenetic status of amphibians (Wilkinson et al. 1996). The cytochrome b (cytb) sequence in mtDNA is commonly used in molecular research and has been widely applied in the evolutionary analysis of amphibians. However, the evolutionary information from mtDNA cannot fully represent the evolutionary history of both parents. Therefore, nuclear DNA (nuDNA) must be used in combination with mtDNA to comprehensively explore genetic diversity and population genetic structure (Behura 2006). The recombinase activating 2 protein (rag2) gene in nuDNA, which belongs to the family of recombination activating genes (rags), plays a crucial role in

vertebrate-specific immune responses and has been frequently used in phylogenetic studies when combined with mtDNA (Agrawal et al. 1998).

In this study, we analyzed the genetic diversity of *R. hanluica* by collecting samples within its known distribution range and using mtDNA and nuDNA. We conducted this study in four aspects: (1) analysis of the genetic diversity of *R. hanluica* based on DNA sequences; (2) understanding the genetic structure of populations by constructing phylogenetic analysis and haplotype networks; (3) determination of the degree of genetic variation among *R. hanluica* populations by estimating genetic differentiation and gene flow; and (4) exploration of the historical demography of *R. hanluica* populations using neutrality tests and mismatch distribution.

# Methods

## Sampling

A total of 162 individuals from 19 localities covering nearly the entire range of *R. hanluica* were collected between 2019 and 2021 (Figure 1 and Table S1). These samples were divided into six groups based on mountain ranges (Table S1). A small piece of muscle tissue or liver tissue was clipped and stored in 95% ethanol. Voucher specimens for populations were deposited in the Zoology Specimen Room, Institute of Wildlife Conservation, Central South University of Forestry and Technology (China); College of Ecology, Lishui University (China); College of Life Sciences, Guizhou Normal University (China); the Museum of Biology, Sun Yat-sen University (China); and College of Life Science, Guizhou University (China).

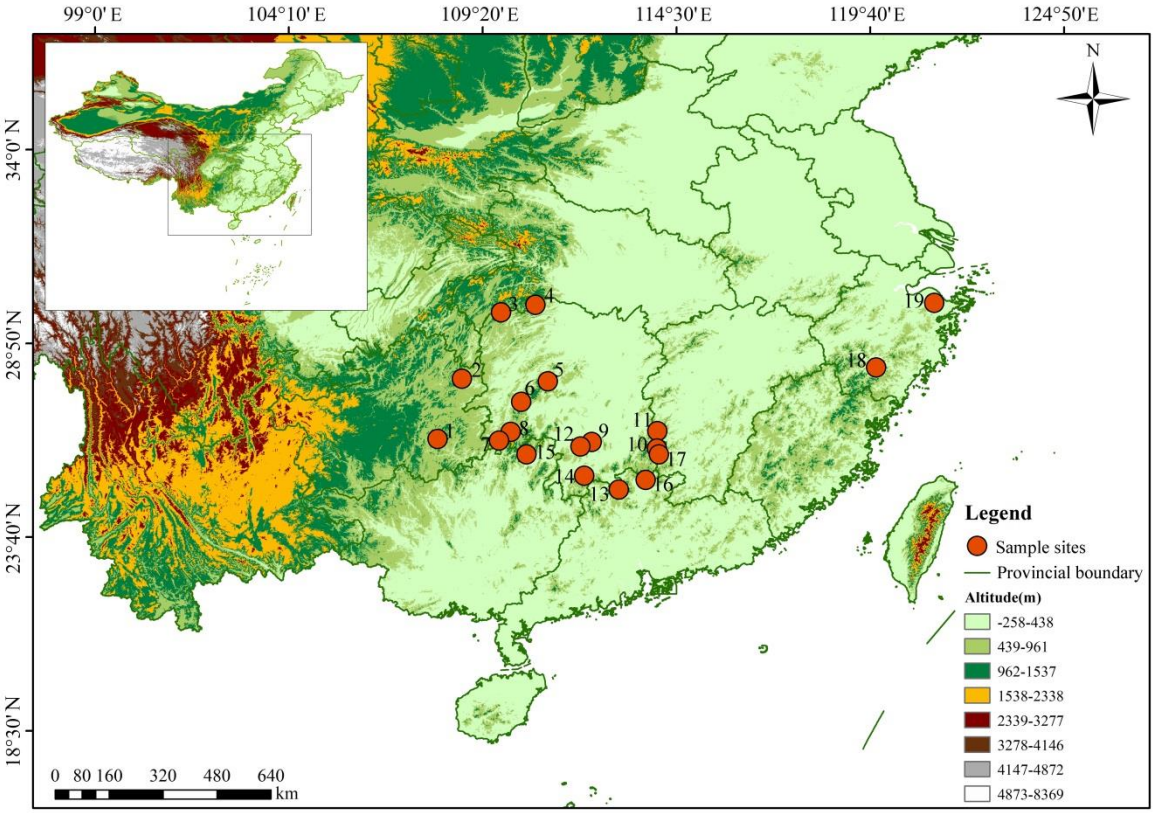



**Figure 1.** Sampling sites of *R. hanluica*

## DNA extraction and sequencing

Genomic DNA was extracted using a Tsingke TSP201-200 (https://www.tsingke.net) DNA extraction kit. A partial fragment of the mitochondrial gene encoding cytb was amplified for 162 individuals, and partial sequences of the nuclear gene encoding rag2 were amplified for 143 individuals. Primers used for cytb were Cytbs and Cytba following the study of Zhou et al. (2012) and L14850 and H15502 following the study of Tanaka-Ueno et al. (1998), and for rag2, primers used were RAG2s and RAG2a following the study of Zhou et al. (2012). Standard polymerase chain reactions (PCR) were performed in a 50 µl volume with the following cycling conditions: an initial denaturing step at 98°C for 2 min, 30 cycles of denaturing at 98°C for 10 s, annealing at 50–57°C for 10 s, and extension at 72°C for 10 s, followed by a final extension step of 72°C for 5 min.

PCR purification and sequencing were performed by Biomarker Technologies Co. Ltd (China).

## Sequence analyses and haplotype network

For molecular analyses, we also retrieved cytb and rag2 sequences of other *R. hanluica* individuals from GenBank (https://www.ncbi.nlm.nih.gov/) (Table 1) (Yan et al. 2011; Yuan et al. 2016; Zhou et al. 2017). All sequences were checked and assembled using the SeqMan II program included in the LASERGENE 7.0 software package (DNAStR Inc., Madison, WI, USA). Sequence alignment was performed using MEGA v.7.0 with default settings (Kumar et al. 2016). The concatenation of the two genes was conducted in PhyloSuite v.1.2.2 (Zhang et al. 2020). The nucleotide diversity ($\pi$) and haplotype diversity (hd) of each group were analyzed using DnaSP v.5 (Librado and Rozas 2009). To infer allelic phases from polymorphic sites in the nuDNA, the program PHASE v.2.1 (Stephens et al. 2001) was used, with input files created using seqPHASE (Flot 2010). Haplotype networks under the median-joining algorithm were produced to display intraspecific variation for *R. hanluica* for the cytb and rag2 using PopART v.1.7 (Bandelt et al. 1999; Leigh and Bryant 2015).

**Table 1.** Localities, voucher information, and GenBank numbers of the sequences downloaded from NCBI

| No. | Species | Voucher | Locality | GenBank No. | | References |
|---|---|---|---|---|---|---|
| | | | | cytb | rag2 | |
| 1 | *R. chensinensis* | KIZ-RD05SHX01 | Huxian, Shaanxi, China | KX269333 | KX269626 | Yuan[19] |
| 2 | *R. dybowskii* | tissue ID: MSUZP-IVM-1d | Khasanskii District, Primorye region, Russia | KX269335 | KX269628 | Yuan[19] |
| 3 | *R. hanluica* | KIZ03477 | Mt. Mangshan, Hunan, China | JF939135 | — | Yan[20] |
| 4 | | KIZYPX1172 | Mt. Yangmingshan, Hunan, China | JF939136 | — | Yan[20] |
| 5 | | KIZYPX1177 | Mt. Yangmingshan, Hunan, China | JF939137 | — | Yan[20] |
| 6 | | KIZYPX1182 | Mt. Yangmingshan, Hunan, China | JF939139 | — | Yan[20] |
| 7 | | KIZHuN024 | Jiemuxi National Nature Reserve, Hunan, China | JF939114 | — | Yan[20] |
| 8 | | KIZHuN01 | Jiemuxi National Nature Reserve, Hunan, China | JF939113 | — | Yan[20] |
| 9 | | KIZHuN06 | Jiemuxi National Nature Reserve, Hunan, China | JF939115 | — | Yan[20] |
| 10 | | KIZGX84 | Mt. Maoer'shan, Guangxi, China | JF939112 | — | Yan[20] |
| 11 | | KIZYPX10654 | Mt. Maoer'shan, Guangxi, China | JF939123 | — | Yan[20] |
| 12 | | KIZYPX10656 | Mt. Maoer'shan, Guangxi, China | JF939124 | — | Yan[20] |

| No. | Species | Voucher | Locality | GenBank No. | | References |
|---|---|---|---|---|---|---|
| | | | | cytb | rag2 | |
| 13 | | SYNU07100490 | Mt. Yangmingshan, Hunan, China | KF020629 | KJ371968 | Zhou[21] |
| 14 | | KIZGX07112915 | Mt. Maoer'shan, Guangxi, China | KX269338 | KX269631 | Yuan[19] |

## Phylogenetic analyses

Phylogenetic analyses of the cytb and rag2 sequences were conducted using Bayesian inference (BI). Two species (*R. chensinensis* and *R. dybowskii*) were selected as outgroups. BI analyses were performed using MrBayes 3.2.7 (Ronquist et al. 2012). The best-fitting substitution model for each gene was selected using ModelFinder (Kalyaanamoorthy et al. 2017). The Bayesian information criterion was used to select a model because of its high accuracy and precision. Two independent runs were conducted in the BI analysis using four Markov Chain Monte Carlo chains starting with a random tree; each chain was run for 1,000,000 generations and sampled every 100 generations. The first 25% of the samples were discarded as a burn-in, resulting in a potential scale reduction factor (PSRF) of $< 0.01$. Nodes in the trees were considered well supported when Bayesian posterior probabilities (BPP) were $\geqslant 0.95$.

## Genetic variation

A total of 145 sequences of concatenated cytb and rag2 with a total length of 1062 bp were used to analyze genetic variation and historical demography. Analysis of molecular variance (AMOVA) was completed using Arlequin v.3.11 to detect the partitions of genetic diversity within and among populations (Excoffier and Lischer 2010). The pairwise fixation index (*F*st) values were estimated to measure the genetic differentiation between the groups using Arlequin v.3.11. Gene flow (Nm) was calculated according to the formula: $Nm = (1 - Fst)/4Fst$, ignoring *F*st values that were not statistically significant (Slatkin and Barton 1989).

## Historical demography

Neutrality tests and Mismatch distributions were calculated using Arlequin v.3.11 (Excoffier and Lischer 2010). Multimodal distributions are expected in populations at demographic equilibrium or in decline, and unimodal distributions are anticipated in populations having experienced a recent demographic expansion (Rogers and Harpending 1992). The expected distributions were generated by bootstrap resampling (1000 replicates) using a model of sudden demographic expansion. The sum of squared deviations (SSD) and raggedness index (RI) between the observed and the expected mismatch were used as test statistics. *P*-values were calculated as the probability of simulations producing a greater value than the observed value.

The time of population expansion ($t$, time in generations) was calculated through the relationships $\tau = 2ut$, where $\tau$ is the mode of the mismatch distribution; $u$ is the substitution rate per generation for the whole sequence under study considering that $u = \mu k$, where $k$ is the number of nucleotides (Rogers and Harpending 1992). Due to the lack of fossil records, we adopted a substitution rate of the cytb gene in most amphibians ($\mu = 0.65–1.00\%$ per Ma) (Li et al. 2015).

# Results

## Sequence information

We obtained cytb sequences for 174 individuals of *R. hanluica*, comprising 162 that were newly sequenced and 12 retrieved from previous studies (Yan et al. 2011; Yuan et al. 2016; Zhou et al. 2017). Rag2 sequences for 145 individuals of *R. hanluica* were obtained from a subset of 143 newly obtained sequences and two sequences retrieved from previous studies (Yuan et al. 2016; Zhou et al. 2017). All newly obtained sequences were deposited in GenBank (Table S1).

## Genetic diversity

Cytb sequences consisted of 635 base pairs (bp), of which 35 positions exhibited variation and 14 were parsimony informative, resulting in 20 haplotypes for the ingroup (Table 2). For cytb, the overall π value was 0.00215, and the hd value was 0.653. For individual populations, the LXS group showed the highest genetic diversity (π = 0.00337 and hd = 0.827), while WYS had the lowest (π = 0.00036 and hd = 0.216). The NL group had seven endemic haplotypes, followed by the LXS, WLS, WYS, and HS groups, while the XFS group had no endemic haplotypes.

Rag2 sequences consisted of 429 bp containing 15 variable sites and nine parsimony-informative sites for a total of 15 haplotypes (Table 2). The overall π value was 0.00254, and the hd value was 0.659. The LXS group showed the highest genetic diversity (π = 0.00310 and hd = 0.779), while WYS had the lowest level (π = 0.00052 and hd = 0.209). The HS group only contained one haplotype, indicating no nucleotide or hd. Among the endemic haplotypes, the NL group had the largest number, followed by LXS, WLS, WYS, HS, and XFS groups that did not have any endemic haplotypes.

**Table 2.** Genetic diversity parameters and haplotype distribution of *R. hanluica*

| Groups | Sample size | Genes | Number of haplotype | Distribution of haplotypes | Nucleotide diversity | Haplotype diversity |
|---|---|---|---|---|---|---|
| HS | 5 | Cytb | 2 | C-H1,H2 | 0.00131 | 0.400 |
| | 5 | Rag2 | 1 | R-H1 | 0.00000 | 0.000 |
| LXS | 25 | Cytb | 7 | C-H3,H4,H5,H6,H12,H15,H18 | 0.00337 | 0.827 |
| | 23 | Rag2 | 7 | R-H1,H2,H4,H5,H6,H14,H15 | 0.00310 | 0.779 |
| NL | 74 | Cytb | 11 | C-H1,H3,H4,H8,H9,H10,H11,H12,H13,H14,H19 | 0.00295 | 0.743 |

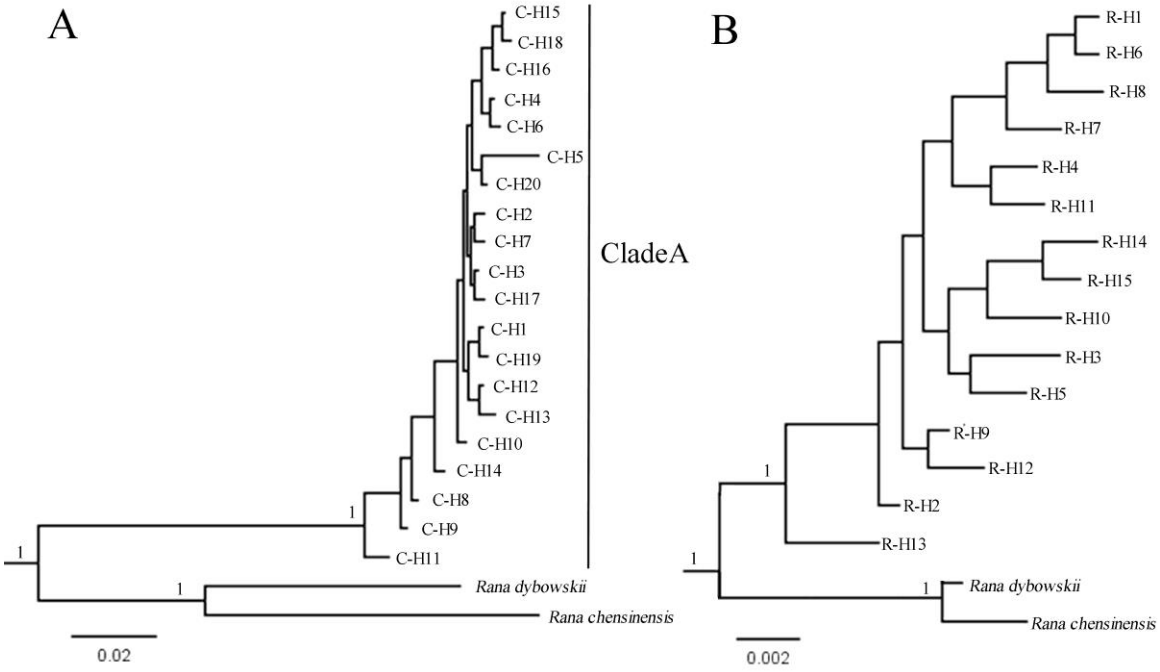
| Groups | Sample size | Genes | Number of haplotype | Distribution of haplotypes | Nucleotide diversity | Haplotype diversity |
|---|---|---|---|---|---|---|
| | 55 | Rag2 | 8 | R-H1,H2,H5,H7,H8,H10,H11,H12 | 0.00257 | 0.681 |
| WLS | 29 | Cytb | 4 | C-H3,H15,H16,H17 | 0.00065 | 0.259 |
| | 23 | Rag2 | 3 | R-H1,H3,H13 | 0.00248 | 0.245 |
| XFS | 23 | Cytb | 1 | C-H3 | 0.00000 | 0.000 |
| | 21 | Rag2 | 2 | R-H1,H2 | 0.00063 | 0.257 |
| WYS | 18 | Cytb | 3 | C-H3,H7,H20 | 0.00036 | 0.216 |
| | 18 | Rag2 | 2 | R-H2,H9 | 0.00052 | 0.209 |
| ALL | 174 | Cytb | 20 | C-H1,H2,H3…H18,H19,H20 | 0.00215 | 0.653 |
| | 145 | Rag2 | 15 | R-H1,H2,H3…H13,H14,H15 | 0.00254 | 0.659 |

## Phylogeny and haplotype network

For cytb, the topology of the phylogenetic tree showed inconsistencies with the geographic structure (Figure 2A). Samples of *R. hanluica* from different localities were included in Clade A, showing high support (BPP = 1). The haplotype network constructed based on the cytb sequence was consistent with the phylogenetic analysis (Figure 3A). The C-H3 haplotype had the highest frequency and was in the central position, and there was almost no intersection between the endemic haplotypes (C-H8, C-H9, C-H11, and C-H14) of the NL group and other haplotypes. There may be a certain number of missing haplotypes in this population.

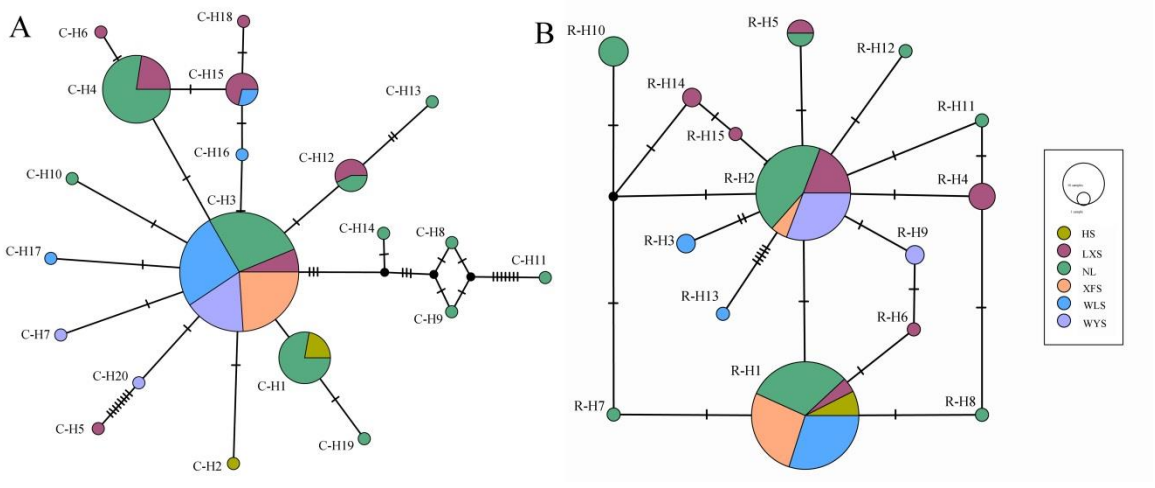
BI analyses of rag2 data supported the results of cytb (Figure 2B). Due to the limited number of potential parsimony-informative sites, rag2 sequences did not form geographic units but rather were nested together. The haplotype network constructed based on the rag2 sequences showed that

168 the population of *R. hanluica* presented a network structure, with the highest frequency of R-H1
169 and R-H2 in the center of the entire population, from which the largest number of differentiated
170 haplotypes was derived (Figure 3B).




171

172 **Figure 2.** Phylogenetic tree of *R. hanluica* haplotypes from Bayesian analysis. (**A**): cytb. (**B**): rag2. Numbers
173 beside nodes are BPP, and values above 0.95 are shown.




174

175 **Figure 3.** Haplotype network of *R. hanluica* based on Median-joining method. (**A**): cytb. (**B**): rag2. The area of a
176 circle represents numbers of individuals sharing a haplotype and black dotes represent missing haplotypes.

## Genetic variation

From the AMOVA, the genetic variation within the groups accounted for 78.07%, while the variation among groups accounted for only 21.93% (Table 3). For the concatenated gene, 15 *F*st values were obtained, of which 14 were statistically significant (Table 4). The value of *F*st between the HS and WYS groups was the largest (0.7429), while *F*st between the NL and LXS groups was the lowest (0.0856). The results for gene flow were consistent with those of genetic differentiation (Table 4), with the lowest level of gene flow observed between the HS and WYS groups (0.0865) and the highest level of gene flow observed between the NL and LXS groups (2.6695).

**Table 3.** Analysis of molecular variances among *R. hanluica* groups

| Gene | Source of variation | Degree of freedom | Sum of squares | Variance components | Percentage of variation | Fixation index |
|---|---|---|---|---|---|---|
| concatena | Among populations | 5 | 36.533 | 0.28277 | 21.93 | 0.21928** |
| ted gene | Within populations | 139 | 139.943 | 1.00678 | 78.07 | |

Note：**extremely significant, $P<0.01$

**Table 4.** Gene flow (below the diagonal) and genetic differentiation (above the diagonal) among *R. hanluica* groups

| **Groups** | **HS** | **LXS** | **NL** | **WLS** | **XFS** | **WYS** |
|---|---|---|---|---|---|---|
| HS | — | 0.3274* | 0.2156* | 0.2421* | 0.4149* | 0.7429* |
| LXS | 0.5135 | — | 0.0856* | 0.2794* | 0.3336* | 0.1729* |
| NL | 0.9094 | 2.6695 | — | 0.1595* | 0.1802* | 0.1890* |
| WLS | 0.7826 | 0.6446 | 1.3176 | — | −0.0080 | 0.4502* |
| XFS | 0.3526 | 0.4994 | 1.1377 | — | — | 0.6301* |
| WYS | 0.0865 | 1.1957 | 1.0726 | 0.3053 | 0.1468 | — |

Note: *significant, $P < 0.05$

## Historical demography

In the neutrality tests, the value of Tajima's D of the LXS group was significantly negative (−1.5616, $P < 0.05$), and Fu's FS values for the LXS, NL, and WYS groups were all significantly negative (−9.1281, $P < 0.05$; −9.9068, $P < 0.05$; and −2.0034, $P < 0.05$, respectively) (Table 5). The mismatch distribution (Figure 4) showed that the HS, LXS, and NL groups all exhibited a unimodal pattern, and the SSD and RI did not reach significant levels (SSD = 0.0129, $P_{SSD} > 0.05$, RI = 0.1200, $P_{RI} > 0.05$; SSD = 0.0034, $P_{SSD} > 0.05$, RI = 0.0389, $P_{RI} > 0.05$; SSD = 0.0066, $P_{SSD} > 0.05$, RI = 0.0348, $P_{RI} > 0.05$) (Table 5). Mismatch distribution analyses accepted the hypothesis of sudden expansion. The $\tau$ values of the LXS and NL groups in the mismatch distribution were 1.500 and 1.189, respectively. From this, the expansion times of the LXS and NL groups were estimated to be 0.365–0.156 Ma and 0.388–0.169 Ma, respectively.

**Table 5.** Neutrality test and mismatch distribution parameters for the *R. hanluica* groups

| Groups | Neutrality test | | | | Mismatch distribution | | | |
|---|---|---|---|---|---|---|---|---|
| | Tajima's D test | Tajima's D *p*-value | Fu's FS test | FS *p*-value | SSD | $P_{SSD}$ | RI | $P_{RI}$ |
| HS | −0.1748 | 0.48 | 0.0607 | 0.30 | 0.0129 | 0.86 | 0.1200 | 0.84 |
| LXS | −1.5616* | 0.04 | −9.1281* | 0.00 | 0.0034 | 0.51 | 0.0389 | 0.64 |
| NL | 0.1066 | 0.61 | −9.9068* | 0.00 | 0.0066 | 0.16 | 0.0348 | 0.53 |
| XFS | 0.8930 | 0.79 | −0.5486 | 0.30 | 0.0281 | 0.10 | 0.2047 | 0.06 |
| WLS | −1.4797 | 0.07 | 0.4404 | 0.63 | 0.0301 | 0.05 | 0.1371 | 0.20 |
| WYS | −1.4014 | 0.07 | −2.0034* | 0.01 | 0.0055 | 0.54 | 0.1675 | 0.45 |

Note:*significant, $P < 0.05$

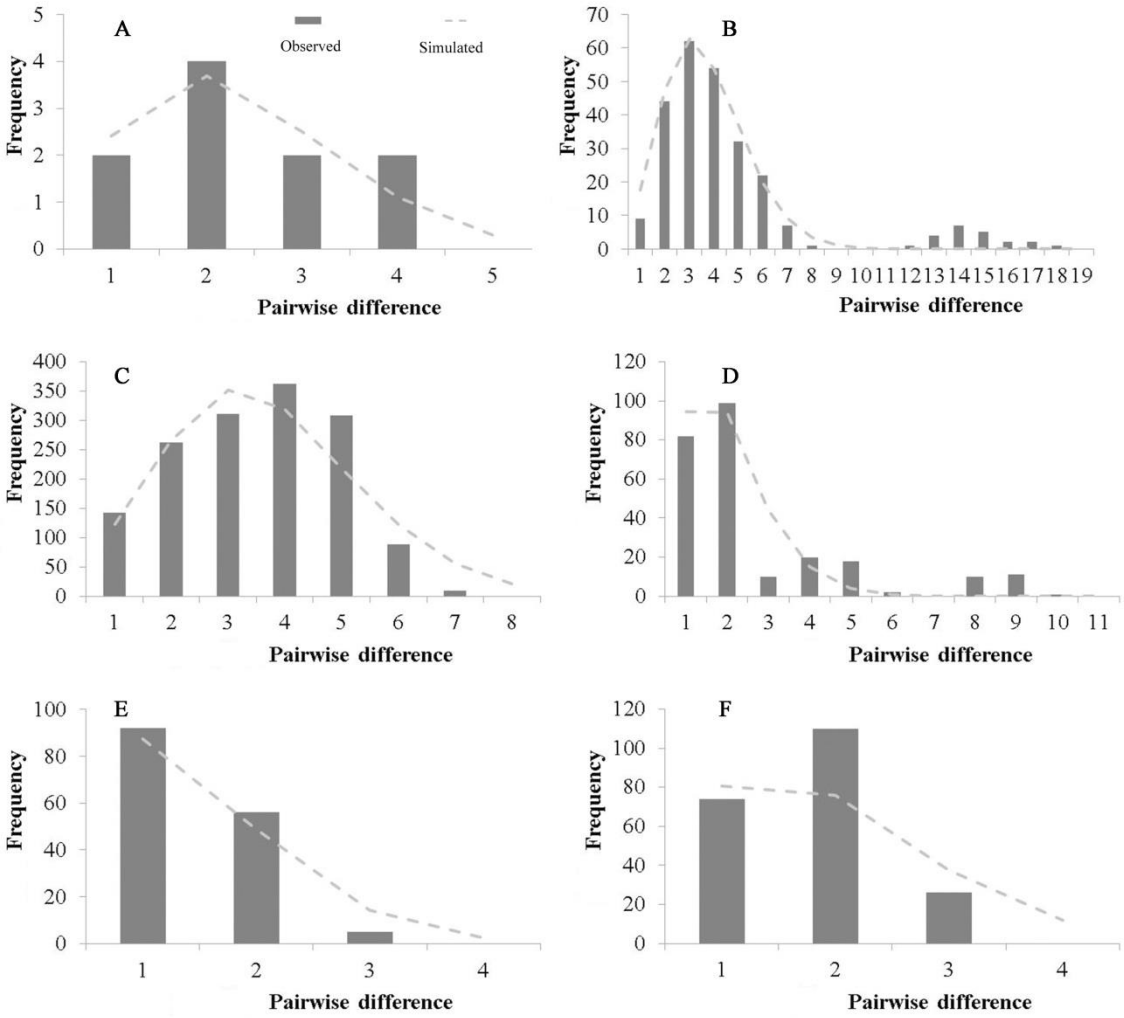



**Figure 4.** Mismatch distribution of *R. hanluica* groups. (**A**) HS group; (**B**) LXS group; (**C**) NL group; (**D**) WLS group; (**E**) WYS group; (**F**) XFS group.

## Discussion

Genetic diversity is the foundation of a species' ability to adapt to a changing environment and thereby maintain a viable population (Allentoft and O'Brien 2010). Due to different selection pressures, mitochondrial genes and nuclear genes have different rates of evolution and therefore provide different genetic information. The dataset using concatenated mtDNA and nuDNA provides a theoretical basis for genetic research. Cytb and rag2 are protein-coding genes that contain important genetic information, and they have been widely used in analyses of systematics and genetic variation (Yuan et al. 2016). The frequently occurring and widely distributed shared

haplotypes in a population are often considered ancestral haplotypes, and the areas that contain ancestral haplotypes with the most abundant genetic diversity may be biological refugia (Crandall and Templeton 1993; Hewitt 2000). The haplotype distribution showed that both the C-H3 and R-H1 haplotypes were ancestral haplotypes, and the NL population not only contained the ancestral haplotypes but also had the greatest number of unique haplotypes, indicating that the Nanling Mountains may be a biological refugium for *R. hanluica*. Multiple studies have also confirmed that the Nanling Mountains in China have a high level of species diversity and harbor a large number of biological species (Li et al. 2015; Lyu et al. 2020; Tian et al. 2018). In addition, the absence of shared haplotypes between the HS and WYS group indicates that there may be some restrictions on gene flow between the two populations. However, due to the limited sample size for the Hengshan Mountains, increasing the sample size is necessary for further comparison and analysis of the reasons for the differences.

Hd and π are two important indicators of the level of genetic variation within a population (Brooks et al. 2015). According to Grant and Bowen (1998), the entire population of *R. hanluica* exhibits high hd and low π (hd > 0.5, π < 0.005), indicating rapid population expansion and mutation after experiencing a bottleneck. Similar genetic diversity patterns have been reported in other species of *Rana*, including *R. kukunoris* (Zhou et al. 2012), *R. kunyuensis* (Cui 2018), and *R. dybowskii* (Li 2014). In terms of mountain grouping, the π values of all populations of *R. hanluica* were below 0.005, and only two groups (LXS and NL) showed high hd (> 0.5). This result suggests that the entire population of *R. hanluica* may have experienced a genetic bottleneck and that the LXS and NL populations underwent rapid expansion and accumulated many mutations after the

239 bottleneck effect.

240 The phylogenetic analysis was consistent with the haplotype network. These results suggested
241 that the genetic structure of *R. hanluica* did not exhibit clear geographic patterns, and the spatial
242 distribution of genetic diversity conformed to the "allopatric distribution, spatial isolation, and
243 lineage continuity" model proposed by Avise et al. (1987). Similar patterns have also been
244 observed in other amphibians such as *R. chensinensis* (Zhan et al. 2009) and *Bufo gargarizans* (Wu
245 and Hu 2009).

246 The results from mtDNA and nuDNA were not entirely consistent. The haplotype network
247 based on the cytb gene showed a star-like structure, and some haplotypes were not detected in the
248 NL group, indicating a recent population expansion. However, the haplotype network based on the
249 rag2 gene showed a reticulate distribution, with multiple connections between haplotypes from
250 different populations, suggesting high levels of gene flow among *R. hanluica* groups. This may be
251 related to the different rates of evolution of these two genes (Brown et al. 1979).

252 Genetic variation is an important factor in the formation of new species, and determining the
253 genetic differentiation among populations and analyzing its causes is essential for understanding
254 the evolutionary history of a species. Some studies suggest that when $F$st < 0.05, there is almost no
255 differentiation between populations; when 0.05 < $F$st < 0.15, there is mild differentiation; when
256 0.15 < $F$st < 0.25, there is moderate differentiation; and when $F$st > 0.25, there is high
257 differentiation (Weir and Cockerham 1984). Regarding gene flow between populations, Wright
258 (1949) believed that if Nm is < 1, genetic differentiation between populations may be detected; if
259 Nm is > 1, higher gene flow between populations may inhibit genetic differentiation caused by

genetic drift. The Nm values between groups of *R. hanluica* ranged from 0.0865 to 2.6695, and the gene flow between the NL group and the other five populations, as well as between the LXS group and two groups (WYS and NL), was greater than 1, indicating that there are no barriers to gene flow between these populations. The results of the AMOVA also indicated that genetic variation primarily occurs within populations of *R. hanluica*. However, higher genetic differentiation (0.0856–0.7429) reflected the existence of differentiation between populations of *R. hanluica*, possibly due to genetic drift (Zhang et al. 2018). Previous studies have shown that the causes of genetic differentiation in amphibians in southern China are mainly geological history (Li et al. 2022; Tian et al. 2018), climate fluctuations (Li et al. 2018), and sky islands (Pan et al. 2019; Shepard and Burbrink 2009). The distribution range of *R. hanluica* is in the middle and low altitudes, and its breeding environment is limited by stagnant waters such as ponds and paddy fields. High-altitude areas such as the Nanling and Luoxiao Mountains and large rivers such as the Xiangjiang and Ganjiang may also restrict the migration and diffusion of *R. hanluica*. However, the high level of gene flow and single phylogenetic branch both indicate gene exchange between populations of *R. hanluica*. Therefore, the authors believe that, on the one hand, these patterns may be due to the short time since the species differentiated; combined with the influence of genetic drift, the populations have not accumulated enough variation during the evolutionary process. On the other hand, multiple studies have shown that the Nanling Mountains are an important biological corridor, and after the expansion of the *R. hanluica* population, different subpopulations may have migrated and spread through the Nanling Mountains, resulting in secondary contact (Li et al. 2022; Li et al. 2015).

Neutrality tests and mismatch distribution analysis are commonly used to assess the historical demography of populations. A positive statistic in neutrality tests indicates the presence of a large number of alleles under neutral selection, while a significantly negative statistic suggests the existence of low-frequency alleles, reflecting population expansion and directional selection (Fu 1997; Ramos-Onsins and Rozas 2002). When the population is stable for a long time, the mismatch distribution presents a multimodal form, whereas a single peak represents recent population expansion. SSD and RI are used to determine whether the observed mismatch distribution matches the expected model. A significant SSD indicates that the population does not conform to the population expansion model, whereas a small and insignificant RI value indicates that the observed values of the mismatch distribution are smoother than expected, suggesting consistency between population dynamics and in agreement with the expansion model (Harpending 1994; Slatkin and Hudson 1991). From the results of the neutrality tests and mismatch distribution analysis, only the LXS and NL groups of the six populations of *R. hanluica* exhibited significant and consistent expansion patterns. Furthermore, the expansion times of the LXS and NL groups were 0.365–0.156 Ma and 0.388–0.169 Ma, respectively, both during the Pleistocene, which may be related to climate fluctuations during the Pleistocene. Population expansion after the ice age is a common evolutionary pattern for amphibians in China (Li et al. 2018). Although large-scale glaciers did not develop in southern China during the Quaternary, the climate oscillations during the ice age still had an effect on the distribution and evolution of amphibians in southern China (Jiang et al. 2022). For example, after the climate warmed following the ice age, *Leptobrachium liui* that inhabited refugia underwent expansion, leading to secondary contact among populations (Li et al. 2022).

## Conclusions

In summary, the genetic diversity of the entire population of *R. hanluica* was not very high, while the genetic diversity varied significantly among populations, a pattern that may indicate local population extinctions. The results suggested that the protection of populations with a high number of unique haplotypes, such as the NL and LXS groups, should be given high priority. Meanwhile, as the Nanling Mountains serve as an important corridor for gene flow among populations, they should be given special attention in conservation efforts. Additionally, as this study only used two genes and thus reflected limited evolutionary information, and some populations had small sample sizes, the results may be subject to error. It is recommended that future studies should increase the number and types of molecular markers and conduct in-depth social surveys and population ecology studies to accumulate more basic data for the conservation of the genetic resources of *R. hanluica*. At the same time, residents should be encouraged to participate in wildlife conservation education, and protective artificial breeding should be promoted to preserve high-quality genetic resources and prevent the loss of genetic diversity in this species.

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