



Genetic Decline and Conservation Challenges of the Yiwu Salamander (*Hynobius yiwuensis*)

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Abstract Maintaining genetic variability is widely regarded as essential for ensuring the adaptive capacity and long-term persistence of threatened species in changing environments. Native to Zhejiang Province in China, *Hynobius yiwuensis* is a threatened amphibian species that has undergone severe population reductions, primarily attributed to environmental degradation and the loss of suitable habitats. In this study, we applied transcriptome-derived microsatellite markers (SSRs) to evaluate genetic diversity and population structure in five *H. yiwuensis* populations from Zhejiang Province. We identified 9 817 SSR loci in total, of which mononucleotide repeats occurred most frequently. Genetic diversity analyses revealed moderate polymorphism, with an average observed heterozygosity (H_o) of 0.4080 and expected heterozygosity (H_e) of 0.5020 across the populations. Among the examined populations, genetic diversity was highest in Lihuang ($H_e = 0.5802$) and lowest in

Huantan ($H_e = 0.4000$), indicating regional differences in heterozygosity levels. Population bottleneck tests indicated genetic constriction, particularly in the Huantan population, significantly increasing extinction risks. The study also found substantial genetic differentiation between island and mainland populations, with the Mahuangshan population being highly isolated. These results underscore the urgency of implementing targeted conservation efforts to preserve the genetic integrity of *H. yiwuensis* and provide insights for the management of other endangered amphibians.

Keywords conservation genetics, genetic diversity, *Hynobius yiwuensis*, microsatellite markers, population structure

1. Introduction

Insight into the drivers of genetic diversity in threatened taxa is vital, as such variation determines both ecological plasticity and the evolutionary trajectory of populations, informing conservation decision-making (Kosch *et al.*, 2022; Zhou *et al.*, 2024). Genetic diversity is not only indicative of a population's ability to respond to environmental changes but also impacts its long-term survival and evolutionary prospects. High genetic diversity provides populations with greater adaptive variability, enabling them to better cope with environmental challenges; in contrast, low genetic diversity can weaken resilience, making populations more vulnerable to environmental changes and disease threats (DeWoody *et al.*, 2021; Pröhl *et al.*, 2021).

Hynobius yiwuensis, commonly known as the Yiwu salamander, is an amphibian species endemic to China, primarily found in Jinhua, Zhoushan, Ningbo, and Hangzhou

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in Zhejiang Province. Due to habitat destruction and human activities, its population size has drastically declined, and it is currently listed as a vulnerable species under national protection (Shan *et al.*, 2022). Fragmentation of its habitat has further exacerbated the risks to the species, including reduced genetic diversity, diminished adaptability, and increased extinction risk (Figure 1).

Population bottlenecks often lead to fragmentation into small, geographically or reproductively isolated groups, which in turn diminishes genetic variability and restricts the emergence of novel adaptive variants (Miyu *et al.*, 2022; Femerling *et al.*, 2023). In reduced populations, the effects of genetic drift and inbreeding become more pronounced, leading to a further decline in genetic diversity and diminished resilience to environmental stressors, thereby heightening extinction risk (Robinson *et al.*, 2023). Therefore, studying the genetic diversity and population structure of *H. yiwuensis* is essential for evaluating its genetic health and developing effective conservation strategies.

This study developed 14 polymorphic SSRs for *H. yiwuensis* and conducted population genetic analyses across five geographic populations in Zhejiang Province. Owing to their high allelic variability and experimental simplicity, simple sequence repeat markers remain a standard tool in population-level genetic analyses. In this study, we utilized molecular markers to analyze patterns and levels of genetic variation within *H. yiwuensis*, with a particular focus on identifying signals of population differentiation that may inform species-specific conservation strategies. Our findings aim to clarify the genetic mechanisms contributing to its endangered status, and offer foundational information to support both species recovery efforts and broader amphibian conservation initiatives.

2. Materials and Methods

2.1. Sample collection and preparation *H. yiwuensis*



Figure 1 Adult male of *H. yiwuensis*.

samples were collected from April 2023 to April 2024 across five locations in Zhejiang Province, encompassing most of the species' distribution range. A total of 108 samples were obtained from these five sites: four mainland locations (Limei [LM], Wangdao [WD], Lihuang [LH], and Huantan [HT]) and one insular site on Zhoushan Island (Mahuangshan [MHS]) (Table 1). Muscle tissue was collected from the tail tip or toe of each individual using minimally invasive methods. Individuals were first anesthetized with 0.1% MS-222 (tricaine methanesulfonate), and tissue was collected using sterile surgical scissors. All individuals were allowed to recover in clean, oxygenated water before being released back to their original habitat. To ensure RNA integrity, tissue samples were promptly immersed in stabilization buffer and subsequently kept at -80°C until processing.

2.2. Total RNA isolation and cDNA library preparation Total RNA was extracted from muscle samples using a guanidinium isothiocyanate-based method (RNA Extraction Kit, Tiangen Biotech, Beijing, China), following the manufacturer's instructions. After tissue homogenization, chloroform was added to induce phase separation. The aqueous phase containing RNA was collected and precipitated with isopropanol, followed by washing in 75% ethanol. The RNA pellet was then air-dried and redissolved in RNase-free water. For mRNA purification, poly(A)+ RNA was isolated using oligo(dT)-attached magnetic beads (Vazyme Biotech, Nanjing, China) and used for downstream cDNA library construction.

The synthesized cDNA was subsequently purified and subjected to end-repair and adapter ligation. Fragment size selection was performed by agarose gel electrophoresis using a standard gel extraction system (Tanon Science & Technology, Shanghai, China). Three biological replicates per group across five populations were used to construct cDNA libraries.

2.3. Sequencing data quality control and SSR analysis We first implemented quality filtering of raw sequencing reads by removing adapter sequences and discarding reads of low quality. *De novo* transcriptome assembly was performed using Trinity v2.8.4 (Gardenalli, 2023), and its completeness was assessed through BUSCO v4.1.4 (Wang and Wang, 2023).

Table 1 Sampling information for *H. yiwuensis* in this study.

Sampling Site	Abbreviation	Coordinates (E, N)	Sample Size
Limei	LM	119°53', 29°41'	26
Wangdao	WD	119°57', 29°30'	22
Lihuang	LH	119°41', 29°17'	25
Huantan	HT	120°32', 30°08'	11
Mahuangshan	MHS	122°06', 30°05'	24
Total			108

SSR loci were identified using MISA v2.1 (Mokhtar *et al.*, 2023). The dataset was screened for six SSR motif types: mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats. Detection parameters were set as follows: mononucleotide repeats ≥ 10 units, dinucleotide repeats ≥ 6 units, and tri- to hexanucleotide repeats ≥ 5 units.

2.4. DNA extraction Genomic DNA was extracted from 108 muscle tissue samples using the TIANamp Genomic DNA kit (TIANamp Genomic DNA kit, Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. Genomic DNA was first examined for integrity on 0.8% agarose gels. Concentration measurements were obtained using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were then normalized to 50 ng/ μ L and kept at -20°C prior to downstream applications.

2.5. SSR primer design and screening Microsatellite loci were identified from the transcriptome sequences of *H. yiwuensis*, and SSR primers were designed using Primer 5.0 software. The designed SSR primers were further evaluated using Oligo7 software to identify and exclude primers with potential hairpin structures, primer dimers, or mismatches. The primers were synthesized by Sangon Biotech (Shanghai, China). Based on melting temperature (T_m) value differences and expected product sizes, 140 primer pairs were synthesized. Initial PCR amplifications were conducted using pooled DNA samples of *H. yiwuensis* to evaluate the amplification efficiency of each primer pair. The PCR reaction system consisted of 0.8 U Taq DNA polymerase, 2 mmol·L⁻¹ buffer, 1.5 mmol·L⁻¹ dNTPs, 0.5 μ mol·L⁻¹ of each primer, 25 ng of template DNA, and ddH₂O to a final volume of 20 μ L. The amplification process started with a 5-min denaturation at 95 $^{\circ}\text{C}$, followed by 30 cycles of denaturation at 94 $^{\circ}\text{C}$, annealing at 48–55 $^{\circ}\text{C}$, and extension at 72 $^{\circ}\text{C}$, each lasting 30 s. A final extension step was applied at 72 $^{\circ}\text{C}$ for 8 min to complete the reaction. The PCR products were analyzed using 8% polyacrylamide gel electrophoresis (PAGE), and SSR primers that produced clear and specific amplification bands were selected for further analysis. The selected SSR primers were subsequently synthesized with fluorescent labels (6-FAM, HEX, and TAMRA) by Sangon Biotech (Shanghai, China).

2.6. Detection of PCR amplification products of polymorphic primers and data analysis Fourteen pairs of SSR primers were selected for marker analysis of 108 samples from five populations of *H. yiwuensis*. These fluorescent-labeled primers were used to amplify genomic DNA from all collected *H. yiwuensis* samples, and the PCR products were analyzed by capillary electrophoresis for

genotyping. The raw data were collected using GeneMarker software and subsequently organized in Excel for further analysis (Murphy and Greenhouse, 2024). We assessed genetic diversity using several standard indices, including the observed number of alleles (N_a), effective alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and the inbreeding coefficient (F_{is}), calculated with Genepop v4.1.4. Tests for Hardy-Weinberg equilibrium and pairwise linkage disequilibrium were also performed via Genepop v4.3, applying Bonferroni corrections for multiple comparisons at a significance threshold of $P = 0.05$ (Hu *et al.*, 2023).

To explore population genetic structure, we used Popgen v1.32 to estimate genetic differentiation coefficient (F_{st}) values, gene flow (N_m), and genetic distances between population pairs (Wu *et al.*, 2020). A neighbor-joining (NJ) tree was constructed to represent genetic relationships among populations based on genetic distances, with bootstrap values set to 1000 replicates. Bayesian clustering analysis was performed using Structure 2.2 to determine the optimal number of clusters (K value) using a mixed model. The CLUMPP 1.1.2 software was used for repeated clustering analysis to determine consistent K values (Liu *et al.*, 2024). We conducted principal coordinate analysis (PCoA) in GenALEx v6.51 to visualize genetic relationships among populations based on pairwise genetic distances (Alcalá-Gómez *et al.*, 2022). To further partition genetic variation, we used Arlequin v3.5 to perform an analysis of molecular variance (AMOVA), which quantified the proportion of variation attributable to differences within and among populations. The relationship between genetic and geographic distances was evaluated using the Mantel test. To assess whether the populations experienced genetic bottlenecks, Bottleneck v1.2 (Shults *et al.*, 2023) software was used in conjunction with three models as well as the mode-shift test. Each analysis was performed with 1000 replications to determine if *H. yiwuensis* populations underwent recent bottleneck events.

2.7. Ethics approval and consent to participate All animal procedures were performed in accordance with protocols approved by the Animal Care and Use Committee of Jinhua University of Vocational Technology (Jinhua, China; Approval Number: ZSDW2022081; Approval Date: November 2022).

3. Results

3.1. Transcriptome sequencing quality control and assembly The transcriptomes of *H. yiwuensis* from 15 samples across five geographic populations underwent *de novo* assembly using Trinity, resulting in a total of 37 638 unigenes. The total clean data volume across all samples

reached 150.97 Gb, with each sample providing over 6.22 Gb of clean data, and a Q30 base percentage consistently above 94.06%, indicating high-quality sequencing results. The average unigene sequence length was 1 590.95 bp, and the N50 length was 2 833 bp, reflecting the completeness and quality of the assembly (Figure 2A). We assessed sample-to-sample expression consistency through correlation analysis. For each group, the three biological replicates showed pairwise correlation coefficients exceeding 0.99, reflecting high within-group consistency and reliable data reproducibility (Figure 2B and C). More than 80% of clean reads were successfully aligned to the reference sequences generated through Trinity-based transcriptome assembly, with mapping efficiencies varying from 78.71% to 83.01% (Supplementary Table S1). These results suggest that the sequencing and assembly provided a reliable representation of the *H. yiwuensis* transcriptome, enabling further functional annotation and downstream analyses.

3.2. Unigene functional annotation and expression analysis

Functional characterization of unigenes was

achieved by sequence similarity searches against public repositories such as NR, KEGG, GO, Swiss-Prot, and others. Among the annotated transcripts, 17 727 matched at least one of these databases. The KEGG and NR databases contributed significantly to the annotations, with 13 540 (35.97%) and 16 526 (43.91%) unigenes matched, respectively. Swiss-Prot provided annotations for 8 109 unigenes (21.55%), while COG annotations were available for 3 759 unigenes (9.98%) (Supplementary Table S2). Further species distribution analysis based on NR annotations revealed the highest sequence homology with *Rhinatrema bivittatum*, accounting for 21.18% of all matched sequences, followed by *Microcaecilia unicolor* (10.01%) and *Geotrypetes seraphini* (6.42%). Other notable homologous species included *Xenopus tropicalis* (3.82%), *Xenopus laevis* (3.60%), and *Chelonia mydas* (2.43%) (Figure 2D). These findings indicate significant phylogenetic concordance between *H. yiwuensis* and other amphibian species, especially with *R. bivittatum* and *M. unicolor*.

3.3. Mining and development of EST-SSR markers from the transcriptome of *H. yiwuensis*

The MISA

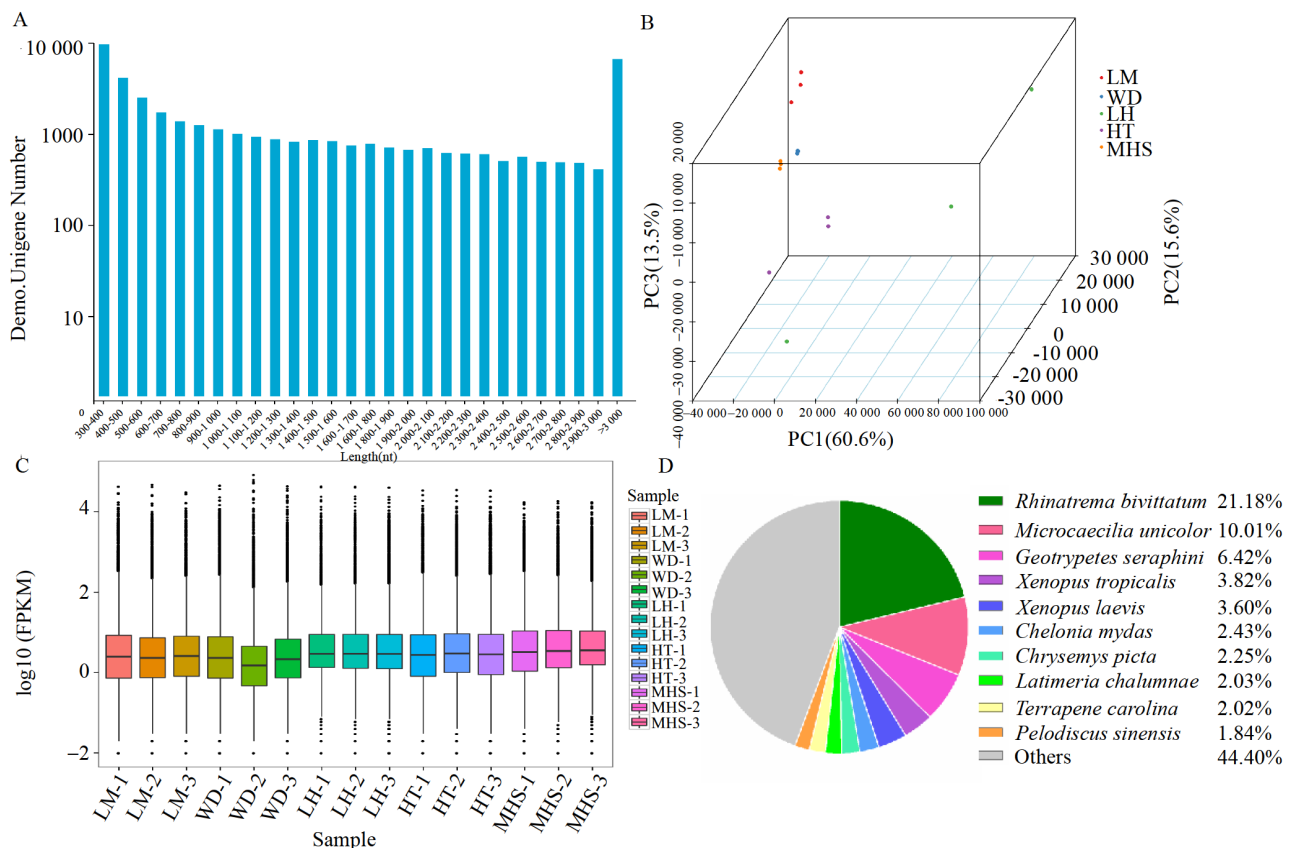


Figure 2 Assembly and basic annotation of unigenes in *H. yiwuensis*, including unigene length distribution (A), principal component analysis (PCA) of expression profiles (B), sample correlation heatmap based on FPKM values (C), and species distribution of annotated unigenes based on NR database similarity (D); abbreviations: nt = nucleotide, FPKM = fragments per kilobase of transcript per million mapped reads.

analysis identified 9 817 SSR loci across 32 709 sequences within the *H. yiwuensis* transcriptome, which spans 154 297 bp. Among these, 7 148 sequences contained a single SSR locus. SSR motifs varied in length from one to six nucleotides, with mononucleotide repeats being the most frequent (74.56%), followed by dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide types (Supplementary Table S3; Figure 3A).

Trinucleotide motifs exhibited the greatest structural diversity, with 53 distinct types detected. Overall, SSRs were predominantly short in length, and most were mononucleotide, dinucleotide, or trinucleotide repeats. Only a small proportion displayed extended repeat units, contributing to moderate polymorphism levels (Figure 3B).

3.4. Polymorphic primer screening A total of 140 SSR primer pairs were randomly chosen and designed using Primer 5.0 software. Primer synthesis was carried out by Sangon Biotech (Shanghai, China). Using an optimized SSR amplification system, these primers were tested on *H. yiwuensis* samples. Ultimately, 20 primer pairs produced clear amplification bands. After further screening by PAGE, 14 polymorphic primer pairs were identified (Figure 4; Table 2).

3.5. Genetic diversity analysis of *H. yiwuensis* Using 14 polymorphic SSR loci, we assessed genetic diversity across five geographic populations of *H. yiwuensis*. Analysis of 14 polymorphic SSR loci revealed that *H. yiwuensis* populations displayed a moderate level of genetic diversity. The observed N_a per locus averaged 4.1143, while N_e was 2.4190. Mean observed and expected heterozygosity values ($H_o = 0.4080$, $H_e = 0.5026$) indicated relatively limited allelic variation. F_{is} exhibited a broad range from -0.1607 to 0.3519 , reflecting differences in inbreeding intensity among populations. A

subset of loci significantly deviated from Hardy-Weinberg expectations ($P < 0.05$), though no evidence of linkage disequilibrium was found (Supplementary Table S4). Among the five populations, Lihuang exhibited the highest genetic diversity ($H_e = 0.5802$; $N_e = 2.6341$), whereas Huantan displayed the lowest ($H_e = 0.4000$; $N_e = 1.8500$). Despite observable differences in H_e values among populations, the overall range of genetic diversity was relatively narrow, suggesting a moderate level of consistency (Table 3).

3.6. Genetic differentiation analysis Pairwise genetic differentiation based on Weir-Cockerham's F_{st} ranged from 0.046 (LM-WD) to 0.248 (LM-MHS), and seven of the ten comparisons were significant in Genepop exact tests ($P < 0.05$; Table 4). Jost's D , calculated independently in Arlequin, was smaller in magnitude (0.001–0.018) but showed the same ranking: contrasts involving the Mahuangshan island population gave the highest D values (0.014–0.018), whereas Limei – Wangdao was the lowest (0.001) (Supplementary Table S5). An AMOVA attributed 17.96% of the total genetic variance to differences among populations ($\phi_{st} = 0.179$, $P < 0.001$; Supplementary Table S6). Collectively, F_{st} , Jost's D , and ϕ_{st} indicate moderate genetic differentiation, with the Mahuangshan island population showing the greatest divergence from the mainland populations, consistent with reduced gene flow between island and mainland groups.

3.7. Genetic structure analysis of *H. yiwuensis* Structure analysis was performed twice, first with the complete panel of 14 SSR loci and then with the four loci (W18, W30, W49, and W82) that conformed to Hardy-Weinberg equilibrium in all populations. In both runs, the evanno ΔK method identified $K = 2$ as the most likely number of genetic clusters (Figure 5). The Mahuangshan island population consistently formed one

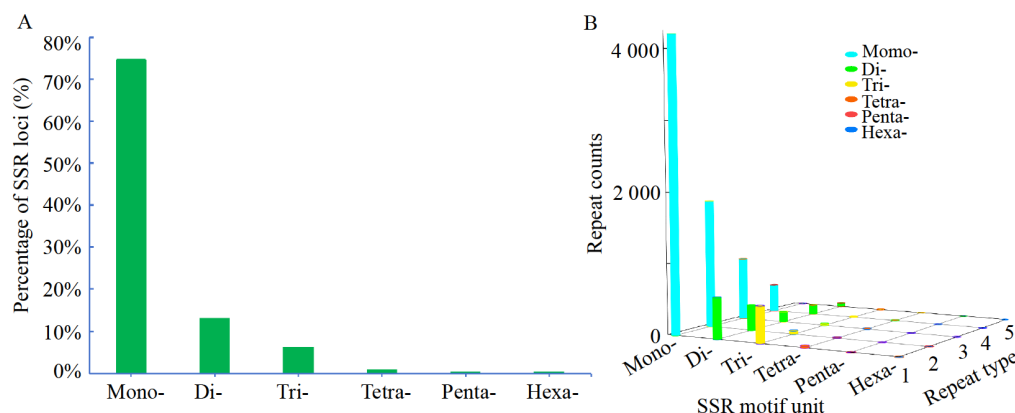


Figure 3 Characterization of SSRs identified in the transcriptome of *H. yiwuensis*. Panel A shows the relative contribution of each motif category, while panel B depicts the distribution of SSRs based on repeat unit length.

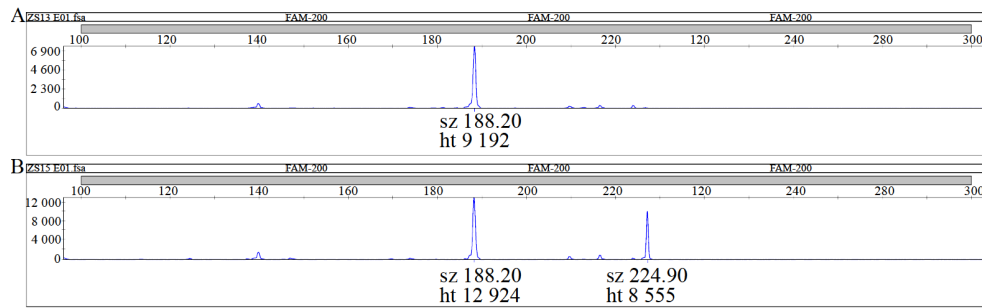


Figure 4 Capillary electrophoresis profile of *H. yiwuensis* genotyping results. Using primer W30 as an example, this figure displays the genotyping results of 108 *H. yiwuensis* samples. Panel A shows a heterozygote, while panel B shows a homozygote.

Table 2 Information on 14 SSR loci developed based on transcriptome sequencing.

Loci	Repeat motif	Primer sequences	Amplicon size	Tm (°C)
W1	(GAGCAG)5	F: AGAGCAGGAGCAGGAGCAG R: AGTCAGTGTTGAAGGCCACC	152	59.1
W13	(TGGT)5	F: AGTATTTGCTTGGTGGTGGC R: AACCTCACAACCGACAAACC	173	55.7
W18	(TGAA)5	F: GCATGTAACCTCCCTTTCCA R: CAAATCCTGGTTCTAGGGCA	258	54.7
W21	(TCAA)5	F: GCACCATATAGCCGGAGAAA R: CACCCATCCTATTCAACATCTG	167	53.8
W30	(GATG)5	F: CACCGAACCCTCAGGACTT R: TGAGGATTCCCAGGACAGAC	188	56.9
W49	(GTT)7	F: ATGACTTGGTCTGGAGTGGG R: AAGAGGAGCCGAAGGAAGAG	239	56.8
W56	(AATG)5	F: TAGAGCAATGCAACACCACC R: CGTACACAGGGTGAGAGCAA	165	56.4
W60	(AAAC)5	F: GATTTTCAATGATGGCCTCG R: ATTGACTGAGTGGGTCCAGG	162	54.3
W69	(CTAT)5	F: AGCAGCATATCATTTGGCCTT R: CAAGTCTGACCCAAACGGTAA	256	54.6
W70	(CATT)5	F: CATATTCCATCAACGCATCG R: TCCGCATTCTTAGGTTACCG	241	52.9
W82	(ATA)7	F: CTGCATCCCTGAAGGACAAT R: CGTAGAGGCGCTATATAAGTGT	270	55.3
W98	(GAT)5	F: CATTTTCAGTTCCCCAGCAT R: GTGGTCACACCCACAGTCAG	158	55.9
W111	(TTG)6	F: AAGCCATAAAACGCTGCACT R: GCTGCTGTGTAGCTGAAACG	120	56.1
W115	(TTA)6	F: ATGGCAAACAAGCACAACA R: TTAGCCTTTACGCTGGTGG	211	54.2

Table 3 Genetic diversity parameters of the five populations of *H. yiwuensis*.

Pop	Na	Ne	Ho	He	Fis
LM	4.4286	2.2453	0.3048	0.4703	0.3519
WD	5.7857	2.9026	0.4135	0.5648	0.2679
LH	4.5000	2.6341	0.4670	0.5802	0.1951
MHS	3.8571	2.4652	0.3905	0.4979	0.2157
HT	2.0000	1.8476	0.4643	0.4000	-0.1607
Average	4.1143	2.4190	0.4080	0.5026	0.1740

Note: Na refers to the count of alleles, Ne indicates their effective number, Ho and He represent observed and expected heterozygosity, respectively, while Fis denotes the coefficient of inbreeding.

cluster, whereas the four mainland populations (LM, WD, LH, and HT) grouped into the other, mirroring the geographical separation between island and mainland regions.

3.8. PCoA and cluster analysis PCoA based on pairwise Nei distances was performed in GenAlEx 6.51b2 (Figure 6A). The first three axes explained 93.96 % of the total variance (66.69%, 18.00%, and 8.67%). Wangdao and Limei clustered tightly, indicating little differentiation, whereas the Mahuangshan samples were displaced along the first axis and showed moderate separation from the mainland populations. Rerunning the PCoA with the four HWE

Table 4 Estimates of gene flow (Nm, above diagonal) and pairwise population differentiation (Fst, below diagonal) across *H. yiwuensis* populations.

	LM	WD	LH	MHS	HT
LM	—	5.185	1.353	0.758	1.498
WD	0.046	—	1.462	0.866	2.691
LH	0.156*	0.146*	—	1.406	2.559
MHS	0.248**	0.224*	0.151*	—	1.406
HT	0.143*	0.085	0.089	0.151*	—

Note: Fst values below the diagonal; Nm values above. Statistical significance of Fst values was assessed using pairwise population differentiation tests in Genepop v4.3. $P < 0.05$ (*); $P < 0.01$ (**).

conforming loci produced an almost identical ordination (Procrustes $r = 0.981$, $P < 0.001$), confirming that the pattern is robust and consistent with the two-cluster grouping recovered by structure.

Mantel test results ($r = 0.8419$, $P = 0.090$) revealed no measurable correlation between population genetics and geographic separation. Nei genetic distances calculated in PopGene were used to build a neighbour-joining tree (Figure 6B), which also separated Mahuangshan from the four mainland populations, in agreement with both structure and PCoA results.

3.9. Genetic bottleneck analysis Bottleneck analysis was performed using three mutation models (IAM, SMM, and TPM). Among them, the two-phase model (TPM) is widely considered the most appropriate for SSR data, as it reflects both stepwise mutations and occasional multistep changes, better approximating the mutation dynamics of microsatellites (Kabasakal *et al.*, 2023). Genetic bottleneck analysis of *H. yiwuensis* was conducted using Bottleneck v1.2 software under three models (Table 5). The results showed that under the IAM, TPM, and SMM models, the Huantan population experienced a highly significant bottleneck effect ($P < 0.01$). Overall, the analyses suggest that the Huantan population of *H. yiwuensis* experienced a genetic bottleneck.

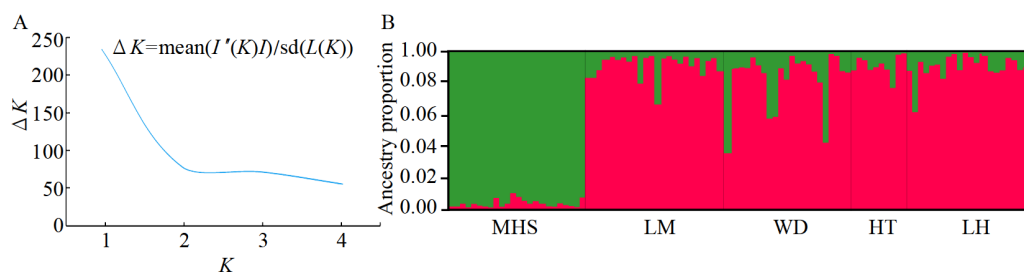


Figure 5 Genetic distance and clustering analysis. (A) Relationship between K values and ΔK for the five geographic populations. (B) Classification of five *H. yiwuensis* geographic populations based on structure analysis ($K = 2$).

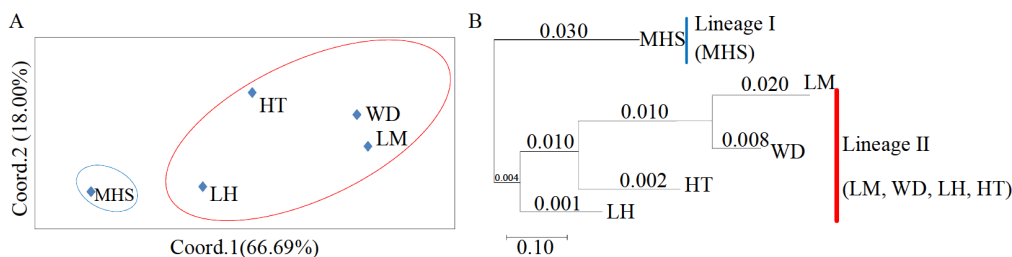


Figure 6 Genetic distance analysis of the five geographic populations. (A) PCoA based on pairwise genetic distances among populations. (B) NJ phylogenetic tree.

Table 5 Bottleneck analysis of five *H. yiwuensis* populations based on 14 SSR loci.

Pop	P-value		
	IAM	TPM	SMM
LM	0.380371	0.233398	0.996948
WD	0.855225	0.153076	0.998322
LH	0.295776	0.903198	0.104004
MHS	0.129395	0.733398	0.733398
HT	0.000977**	0.000977**	0.000977**

Note: $P < 0.01$ (**).

4. Discussion

4.1. Transcriptome-derived SSR markers for analyzing genetic diversity in *H. yiwuensis* Molecular research on *H. yiwuensis* remains limited, particularly in the development of SSR markers in public databases. In our study, we identified 9 817 SSR sequences in *H. yiwuensis*, the majority of which were mononucleotide repeats (7 320 loci, 74.56%), predominantly A/T repeats (80.89%). Analysis of SSR markers in *H. yiwuensis* revealed a substantial proportion of loci with moderate to high polymorphism, with 58.26% of SSRs exceeding 12 bp in length. This observation aligns with findings from Wang *et al.* (2022) and Zhao *et al.* (2023), who emphasized the role of longer polymorphic loci in improving the resolution of genetic diversity analyses. Although the loci are polymorphic, EST-SSRs alone cannot capture genome-wide diversity and should be regarded as a first-pass tool rather than a definitive measure.

While transcriptome-derived SSR markers provide valuable insights, there are certain limitations that need to be addressed. First, these markers primarily represent expressed regions of the genome, which may not fully capture the total genetic diversity of *H. yiwuensis*. This limitation is due to the fact that non-expressed regions, such as those found in introns or intergenic regions, may harbor genetic variation that is not represented in the transcriptome. As such, the genetic diversity inferred from transcriptome-derived markers may be an underestimate of the total genetic variation present in the species.

Second, sampling bias in transcriptome sequencing can overrepresent certain genes or loci that are more highly expressed, leading to skewed results. The A/T-rich SSRs identified in this study, while informative, may not be fully representative of the genome's overall genetic diversity.

4.2. Low genetic diversity and population decline risk in *H. yiwuensis* Genetic diversity is essential for understanding species evolution and taxonomy, particularly

in endangered species, where it informs conservation efforts (Hohenlohe *et al.*, 2021; Hu *et al.*, 2021). Using 14 polymorphic SSR loci, we obtained mean diversity metrics for the five populations ($N_a = 4.11$; $N_e = 2.42$; $H_o = 0.41$; $H_e = 0.50$), values that slightly exceed those reported by Liu *et al.* (2024; $N_a = 3.43$) and point to a moderate, rather than high, level of genetic diversity. These results align with Liu's findings for the Zhoushan *H. yiwuensis* population, where cross-species SSR markers yielded a H_o of 0.39 and an H_e of 0.66, further confirming the species' low genetic diversity. By contrast, Wang *et al.* (2021) reported a higher N_a (ranging from 6 to 9, with an average of 7.4) for *Andrias davidianus*, whereas Zhang *et al.* (2022) found elevated N_a , H_o , and H_e values in *Pseudohynobius flavomaculatus*, underscoring the relatively low genetic diversity of *H. yiwuensis*. Furthermore, using comparable EST-SSR marker panels, Kabasakal *et al.* (2023) showed that other amphibians, such as *H. amjiensis*, also display relatively low genetic diversity in small, isolated populations. The observed genetic diversity in *H. amjiensis* populations, as measured by H_e (0.2772) and H_o (0.3303), aligns closely with that of *H. yiwuensis*. This further underscores the conservation risks associated with low genetic diversity in species with restricted geographic ranges and small population sizes.

F_{is} ranged from -0.1607 to 0.3519 , indicating inbreeding within the populations; positive F_{is} values may in part reflect the EST-SSR dataset, which can exaggerate heterozygote deficiency. In the HT population, the H_o slightly exceeded the H_e , resulting in a negative F_{is} value. This may reflect sampling variance due to the small sample size ($n = 11$), or a genuinely low local population size, as few individuals were observed during field surveys. Similar patterns have been observed in amphibians, such as *Pelophylax esculentus* (Mezhzherin *et al.*, 2023) and *Bufo torrenticola* (Iwaoka *et al.*, 2021), where population isolation presents inbreeding challenges. Additionally, ten of the fourteen loci deviated from HWE, indicating restricted gene flow, habitat fragmentation, or genetic bottlenecks (Guo *et al.*, 2020). Moderate F_{st} values ($0.046 - 0.248$), seven of ten pair-wise comparisons being significant in Genepop exact tests ($P < 0.05$), indicate that geographic isolation has contributed to genetic differentiation, most notably between the Mahuangshan island population and the mainland groups. These results underscore the potential risks of population decline, particularly in isolated populations such as those on the island of Mahuangshan, which are vulnerable to genetic drift and inbreeding.

Given the EST-SSR-based estimates of relatively low genetic diversity and the potential for genetic erosion, it is critical to implement tailored conservation strategies. For island populations, habitat restoration and the establishment of gene flow corridors are essential to facilitate connectivity

with mainland populations and mitigate the effects of isolation (Lucena-Perez *et al.*, 2021; DiLeo *et al.*, 2024). For mainland populations, management efforts should focus on enhancing genetic diversity through breeding programs and the restoration of habitats to support long-term population stability. Additionally, monitoring and genetic assessments should be continuously carried out to track the success of these conservation measures (Becker *et al.*, 2023; Ochs *et al.*, 2024). Future work that integrates genomic SSRs and, especially, genome-wide SNP panels will provide a more complete view of genetic variation in *H. yiwuensis* and enable a statistically robust test of the provisional bottleneck signal reported here.

Abbreviations Na: Number of alleles; Ne: Effective number of alleles; Ho: Observed heterozygosity; He: Expected heterozygosity; Fis: Inbreeding coefficient; Nm: Gene flow; Fst: Genetic differentiation coefficient; HWE: Hardy-Weinberg Equilibrium; PCoA: Principal Coordinate Analysis; LM: Limei population; WD: Wangdao population; LH: Lihuang population; HT: Huantan population; MHS: Mahuangshan population

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Data accessibility The RNA-Seq data obtained in this study are available from the NCBI (<https://www.ncbi.nlm.nih.gov/>) under the accession number PRJNA1279763

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References

- Alcalá-Gómez G., Pérez-Alquicira J., Cabrera-Toledo D., Cortés-Cruz M., Zamora-Tavares M. P., Vargas-Ponce O. 2022. Genetic diversity and structure in husk tomato (*Physalis philadelphica* Lam.) based on SNPs: A case of diffuse domestication. *Genet Resour Crop Evol*, 69: 443–459
- Becker C. G., Greenspan S. E., Martins R. A., Lyra M. L., Prist P., Metzger J. P., São Pedro V., Haddad C. F., Le Sage E. H., Woodhams D. C. 2023. Habitat split as a driver of disease in amphibians. *Biol Rev*, 98(3): 727–746
- DeWoody J. A., Harder A. M., Mathur S., Willoughby J. R. 2021. The long-standing significance of genetic diversity in conservation. *Mol Ecol*, 30(17): 4147–4154
- DiLeo M. F., Nair A., Kardos M., Husby A., Saastamoinen M. 2024. Demography and environment modulate the effects of genetic diversity on extinction risk in a butterfly metapopulation. *Proc Natl Acad Sci USA*, 121(33): e2309455121
- Femerling G., Van Oosterhout C., Feng S., Bristol R. M., Zhang G., Groombridge J., Gilbert M. T. P., Morales H. E. 2023. Genetic load and adaptive potential of a recovered avian species that narrowly avoided extinction. *Mol Biol Evol*, 40(12): msad256
- Gardenalli L. 2023. Evaluating Transcriptome Assembly Potential by Different de Novo Sequence Assembler Types. Diploma Thesis. University of Skövde
- Guo Y., Chang J., Han L., Liu T., Li G., Garber P. A., Xiao N., Zhou J. 2020. The genetic status of the critically endangered Hainan gibbon (*Nomascus hainanus*): A species moving toward extinction. *Front Genet*, 11: 608633
- Hohenlohe P. A., Funk W. C., Rajora O. P. 2021. Population genomics for wildlife conservation and management. *Mol Ecol*, 30(1): 62–82
- Hu Y., Fan H., Chen Y., Chang J., Zhan X., Wu H., Zhang B., Wang M., Zhang W., Yang L. 2021. Spatial patterns and conservation of genetic and phylogenetic diversity of wildlife in China. *Sci Adv*, 7(4): eabd5725
- Hu Z., Yang F., Zhang D., Zhang S., Yu X., Yang M. 2023. Genetic diversity and fine-scale genetic structure of *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) in southern China based on microsatellite markers. *Animals*, 13(4): 560
- Iwaoka Y., Watanabe T., Satoh S. S., Nambu H., Yamazaki Y. 2021. Hybridization of two species of Japanese toads, *Bufo torrenticola* and *Bufo japonicus formosus*, in the central part of Japan. *Zool Sci*, 38(6): 506–512
- Kabasakal A. 2023. Intra-breed genetic diversity and genetic bottleneck tests in a Karacabey Merino sheep breeding farm using microsatellite markers. *Kafkas Univ Vet Fak Derg*, 29(3): 231–238
- Kosch T. A., Waddle A. W., Cooper C. A., Zenger K. R., Garrick D. J., Berger L., Skerratt L. F. 2022. Genetic approaches for increasing fitness in endangered species. *Trends Ecol Evol*, 37(4): 332–345
- Liu X., Kopelman N. M., Rosenberg N. A. 2024. Clumppling: Cluster matching and permutation program with integer linear programming. *Bioinformatics*, 40: btad751
- Lucena-Perez M., Kleinman-Ruiz D., Marmesat E., Saveljev A. P., Schmidt K., Godoy J. A. 2021. Bottleneck-associated changes in the genomic landscape of genetic diversity in wild *Lynx* populations. *Evol Appl*, 14(11): 2664–2679
- Mezhzhherin S., Morozov-Leonov S., Nekrasova O., Rostovskaya O. 2023. Geographic peculiarities of structure and hemiclone reproduction of *Pelophylax esculentus* water frog complex (Anura, Ranidae) populations in the East European Plain within Ukraine. *Amphibia-Reptilia*, 44(3): 263–275
- Miyu T. R., Soga M., Saito Y., Evans M. J., Kobayashi R., Sekiya K., Tanikawa A., Miyashita T., Yoshida K. T. 2022. Conservation genetics of the endangered Sado wrinkled frog *Glandirana susurra* reveals strong regional endemism and population bottlenecks. *Wetlands*, 42 (7): 66
- Mokhtar M. M., Alsamman A. M., El Allali A. 2023. MegaSSR: A web server for large scale microsatellite identification, classification, and marker development. *Front Plant Sci*, 14: 1219055
- Murphy M., Greenhouse B. 2024. MOIRE: A software package for the estimation of allele frequencies and effective multiplicity of infection from polyallelic data. *Bioinformatics*, 40(10): btae619
- Ochs A. E., Swihart R. K., Saunders M. R. 2024. A comprehensive review of the effects of roads on salamanders. *Landsc Ecol*, 39(4): 77

- Pröhl H., Auffarth J., Bergmann T., Buschmann H., Balkenhol N. 2021. Conservation genetics of the yellow-bellied toad (*Bombina variegata*): Population structure, genetic diversity and landscape effects in an endangered amphibian. *Conserv Genet*, 22(3): 513–529
- Robinson J., Kyriazis C. C., Yuan S. C., Lohmueller K. E. 2023. Deleterious variation in natural populations and implications for conservation genetics. *Annu Rev Anim Biosci*, 11: 93–114
- Shan S., Kan X., Gong J., Shao C., Wang Y. 2022. Development and characterization of 36 SNP markers for *Hynobius yiwuensis*. *Conserv Genet Resour*, 14(1): 15–18
- Shults P., Zhang X., Moran M., Cohnstaedt L. W., Gerry A. C., Vargo E. L., Eyer P.-A. 2023. Immigration and seasonal bottlenecks: high inbreeding despite high genetic diversity in an oscillating population of *Culicoides sonorensis* (Diptera: Ceratopogonidae). *J Med Entomol*, 60 (5): 987–997
- Wang P., Wang F. 2023. A proposed metric set for evaluation of genome assembly quality. *Trends Genet*, 39(3): 175–186
- Wang Q., Cheng L., Tian H., Dong X., Xiao H., Hu Q., Chang Y. 2021. Characterization of single nucleotide polymorphism marker in the Chinese giant salamander *Andrias davidianus*. *Agric Sci Dig*, 41: 476–483
- Wang X., Xie Y., Hu W., Wei Z., Wei X., Yuan H., Yao H., Dunxue C. 2022. Transcriptome characterization and SSR discovery in the giant spiny frog *Quasipaa spinosa*. *Gene*, 842: 146793
- Wu Q., Zang F., Ma Y., Zheng Y., Zang D. 2020. Analysis of genetic diversity and population structure in endangered *Populus wulianensis* based on 18 newly developed EST-SSR markers. *Glob Ecol Conserv*, 24: e01329
- Zhang L., Sechi P., Xie J., Dong R., Zheng R. 2024. Genetic diversity and population structure of the endangered salamander *Hynobius yiwuensis* inferred from mitochondrial DNA sequences. *Life*, 14(6): 769
- Zhang Y., Wang M., Cheng R., Luo Y., Li Y., Liu Z., Chen Q., Shen Y. 2022. Mitochondrial characteristics of *Pseudohynobius flavomaculatus*, a protected salamander in China, and biogeographical implications for the family Hynobiidae (Amphibia, Caudata). *Zoosyst Evol*, 98(2): 263–274
- Zhao M., Shu G., Hu Y., Cao G., Wang Y. 2023. Pattern and variation in simple sequence repeat (SSR) at different genomic regions and its implications to maize evolution and breeding. *BMC Genomics*, 24: 136
- Zhou Y., Li N., Zhou H., Zhou R., Cui S., Zheng G. 2024. Mitogenomics reveals extremely low genetic diversity in the endangered Jilin clawed salamander: Implications for its conservation. *Ecol Evol*, 14(3): e11132

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