

Original Research Articles

Screening of microsatellite loci and analysis of polymorphism in *Triplophysa hsutschouensis* based on RAD sequencing

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To assess the genetic diversity of *Triplophysa hsutschouensis* population in the Shule River basin, we employed restriction site-associated DNA sequencing (RAD-Seq) to sequence *T. hsutschouensis* genome. Using this technology, we identified suitable microsatellite (SSR) markers specific to *T. hsutschouensis* population and synthesized 67 primer pairs, of which 18 were found to be polymorphic. Our analysis of 46 *T. hsutschouensis* individuals revealed that the number of alleles (Na) ranged from 2 to 16, with the effective number of alleles (Ne) varying between 1.293 and 8.672. Observed heterozygosity (Ho) ranged from 0.261 to 0.957, expected heterozygosity (He) from 0.227 to 0.885, and polymorphism information content (PIC) from 0.201 to 0.874. The average values obtained were Na = 7.056, Ne = 3.540, Ho = 0.609, He = 0.639, and PIC = 0.595. Hardy-Weinberg equilibrium testing showed that five of the 18 polymorphic SSR loci deviated significantly from expectations after Bonferroni correction, while the remaining 13 loci conformed to expectations, indicating no significant genetic disturbances in the population. These newly identified polymorphic SSR markers will be valuable for future genetic diversity analysis and conservation efforts regarding *T. hsutschouensis* populations.

INTRODUCTION

Triplophysa hsutschouensis, a freshwater fish endemic to China, is classified under the order Cypriniformes, family Cobitidae, and genus *Triplophysa*. It is a small benthic fish species found uniquely in the northeastern Tibetan Plateau,¹ primarily inhabiting the Hexi Corridor region of Gansu Province, including areas such as Jiuquan and the Heihe River basin. Notably, it is the only species within its genus characterized by a relatively simple evolutionary history.¹ Additionally, it serves as an economically significant fish in the region.² However, research on *T. hsutschouensis* has been relatively limited. Previous studies have mainly focused on taxonomy, morphology, resources assessment, geographical distribution,²⁻⁴ and mitochondrial DNA (mtDNA) genetic markers.⁵ Feng et al.¹ explored the evolutionary history of the *Triplophysa robueta* (Cobitoidea) complex, which encompasses *T. hsutschouensis*, using multilocus analyses and coalescent simulations. Chen et al.³ evaluated the current suitable habitats of *T. hsutschouensis* and predicted changes in habitat distribution by 2050 and 2070.³ Despite these efforts, no studies have reported on microsatellite molecular markers for this species to date. Further research into this area is therefore necessary.

Microsatellites, or simple sequence repeats (SSR),⁶ are molecular markers that emerged in the late 1980s. Based on the polymerase chain reaction (PCR) technique, SSR markers offer several advantages, including high polymorphism, abundant repeat sequences, ease of operation, and high co-dominance.^{7,8} These characteristics have made SSRs a widely favored tool among researchers for studying genetic diversity of species. As theoretical research has deepened, experimental techniques have improved, and data analysis software has become more refined, SSR technology has matured and found extensively application in genetic diversity and genetic structure studies of various species, such as *Triplophysa yarkandensis*,⁹ *Schizothorax biddulphi*,¹⁰ *Pelodiscus sinensis*,¹¹ and *Lateolabrax maculatus*.¹²

Restriction site-associated DNA sequencing (RAD-Seq) is a method where genomic DNA is digested using restriction enzymes, followed by high-throughput sequencing of the resulting fragments. RAD-Seq offers several advantages over other sequencing technologies. It simplifies the genome by focusing on restriction enzyme-digested fragments and does not require a reference genome, making it suitable for genotyping. Due to these benefits, RAD-Seq has been widely used in various fields, including molecular marker development, genetic diversity analysis, functional

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gene identification, and germplasm evaluation across different species.¹³⁻¹⁸

Assessing species' genetic diversity is crucial for managing and conserving fishery resources and advancing genetic theory. Higher genetic diversity enhances a species' evolutionary potential and adaptation to environmental changes.¹⁹ Our previous study examined *T. hsutschouensis* single nucleotide polymorphism (SNP) markers via RAD-Seq.²⁰ However, SSR and SNP marker development has systematic differences across multiple levels. Using the same biological samples, this study developed SSR markers through RAD-Seq to evaluate *T. hsutschouensis* genetic resources. Specifically, we analyzed the genetic diversity of *T. hsutschouensis* population in the Gansu Province's Shule River basin (Ganqing section). The goal was to offer scientific guidance for conserving and developing wild *T. hsutschouensis* resources and to effectively assess its germplasm status.

MATERIALS AND METHODS

INFORMATION ON THE SEQUENCED SAMPLES

The Shule River, part of the Hexi River system, has likely undergone multiple exchanges with formerly separated rivers due to the Tibetan Plateau's uplift, which has complicated local river systems.¹ Between October 2019 and September 2021, 46 *T. hsutschouensis* individuals were collected from the Ganqing section of the Shule River basin in Gansu Province (96°57'42.62" E, 39°33'16.55" N). Results therefore represent within-population diversity; geographic generalization awaits multi-locality sampling. Sample collection details are shown in [Figure 1a](#). After morphological identification ([Fig. 1b](#)), muscle and fin tissues from *T. hsutschouensis* were collected and preserved in 95% alcohol for later use.

DNA EXTRACTION AND PRESERVATION

We used the Ezup Column Animal Genomic DNA Extraction Kit from Sangon Biotech (Shanghai) Co., Ltd., following the manual's instruction to extract DNA from the samples. The integrity of the extracted DNA was verified using 1% agarose gel electrophoresis. DNA samples with an A260/280 ratio between 1.8 to 2.0 were considered acceptable. The extracted DNA was stored at -20°C for subsequent use.

RAD-SEQ OF DNA SAMPLES

We randomly selected six samples (denoted as J1, J2, J3, J4, J5 and J6) from all *T. hsutschouensis* samples for SSR development via RAD-Seq. Genomic DNA from each sample was digested with EcoRI, and an adapter (P1) containing forward amplification and Illumina sequencing primer sites, as well as a 6 bp nucleotide barcode for sample identification, was ligated to the fragment's compatible ends. The adapter-ligated fragments were pooled, randomly sheared, and size-selected. DNA was then ligated to a second adapter (P2), a Y adapter with divergent ends. For detailed RAD sequencing methods, refer to Emerson et al.²¹ Finally, fragments of

200 bp to 400 bp and 400 bp to 600 bp were collected for library construction. We used a Qubit2.0 kit to analyze library quality. Agilent 2100 to check the insert size after dilution the library to 1 ng/μL, and Q-PCR to detect library effective concentration (aim for > 2nM) when the insert size was appropriate. All steps ensured quality.

DEVELOPMENT OF SSR MARKERS

Using the Illumina HiSeq PE150 platform, we employed paired-end sequencing to develop SSR markers for *T. hsutschouensis*. The SSR Search software was employed to identify microsatellite loci. In this process, a minimum sequencing volume of 1.5 Gb was set, and the search was conducted according to specific criteria. Specifically, the search criteria required repeat units of 2-6 bases with ≥ 5 repetitions, and PCR product lengths of 100-400 bp. Primer Premier 5.0 software²² designed primers with lengths of 18 bp to 35 bp and Tm values of 46°C to 60°C, ensuring a maximum Tm difference of 5°C between forward and reverse primers. All primer synthesis and Short Tandem Repeat (STR) genotyping were completed by Sangon Biotech (Shanghai) Co., Ltd. synthesized the primers and conducted STR genotyping to enable subsequent sample detection.

PRELIMINARY SCREENING OF SSR LOCI AND PCR AMPLIFICATION

We collected 46 *T. hsutschouensis* individuals from the Ganqing section of the Shule River basin in Gansu Province and conducted PCR amplification using 67 synthesized primer pairs. Through gradient temperature experiments, we optimized conditions to select SSR primers that could stably amplify target fragments visible via agarose gel electrophoresis. The 30 μL PCR reaction included 14 μL Premix Taq (2 × Taq Plus MasterMix, CWBIO), 1 μL DNA template, 1 μL forward primer (10 pmol/μL), 1 μL reverse primer (10 pmol/μL), and 13 μL sterile deionized water. The PCR program involved initial denaturation at 94°C for 5 min; denaturation at 94°C for 45 s, annealing for 30 s at gradient temperatures, and extension at 72°C for 45 s over 32 cycles; final extension at 72°C for 7 min. Capillary electrophoresis (Seq100) was used to analyze the PCR products and identify polymorphic primers.

GENETIC DIVERSITY ANALYSIS

Fluorescently labeled primers, marked with FAM at the 5' end of the forward primer, were synthesized for stable amplification and polymorphism detection. These primers were used to conduct PCR amplification of *T. hsutschouensis* individuals. The amplified products were sequenced by Sangon Biotech (Shanghai) Co., Ltd., and the resulting sequences were analyzed to assess the genetic polymorphism of *T. hsutschouensis* in the Shule River basin of Gansu Province.

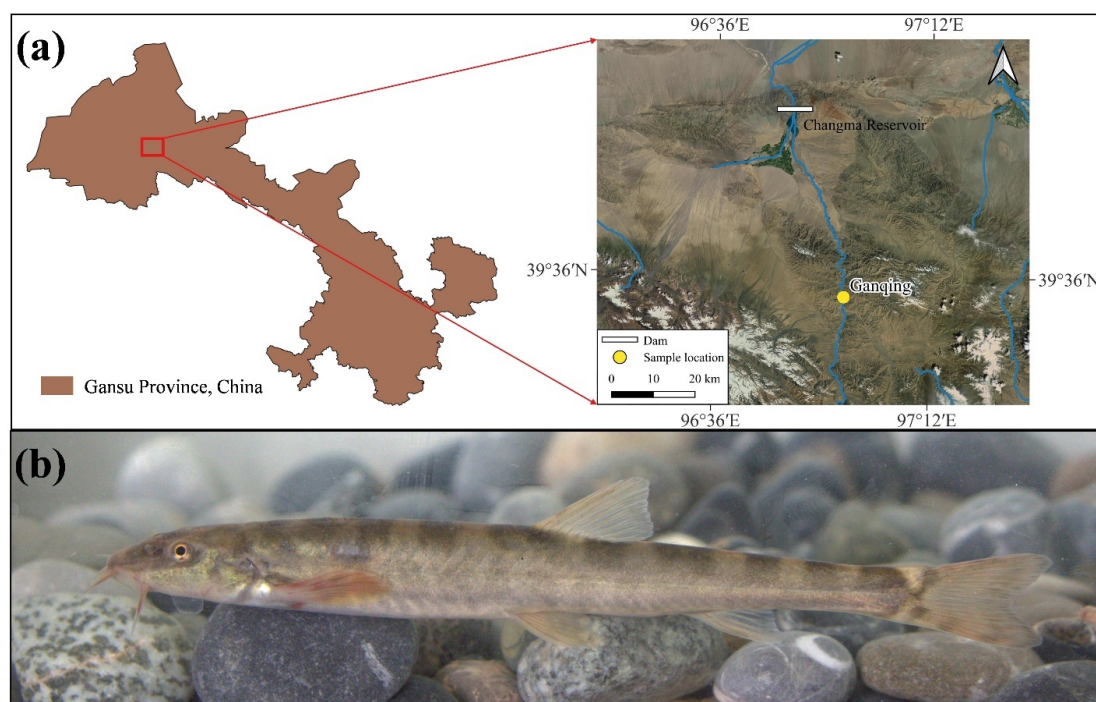


Figure 1. (a) Sampling site of *T. hsutschouensis*. The sampling site map was created in QGIS (version 3.40.5) by overlaying Tianditu imagery data and vector shapefile data of the Shule River Basin (National Basic Geographic Information 2021). (b) *T. hsutschouensis*.

DATA ANALYSIS

Data were converted to software-compatible formats using CONVERT 1.31. Preliminary data processing and genotyping were done with Genemarker® (v1.95 Demo). Genotyping relied on SSR locus fragment lengths. The number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC), and the P-values representing deviations from Hardy-Weinberg equilibrium (HWE) were calculated using Cervus 3.0.²³

RESULTS

SEQUENCING RESULTS ANALYSIS

After removing the low-quality reads, we obtained 14,610,309,600 bp of clean base data. Each sample had over 1,909,844,100 bp of clean base. Q20 values ranged from 96.98 to 97.78%, Q30 values from 91.51 to 93.26%, and GC content from 39.51 to 40.28% (Table 1), indicating the data were suitable for subsequent biological information analysis. Enzymatic digestion validation showed that while all six samples (J1–J6) were acceptable, only J3 was free of gel pore impurities, with the other five samples showing slight contamination (Table S1). Thus, we only utilized RAD-seq data from J3 for SSR development.

The histogram depicting SSR mutation type distribution reveals a notable pattern (Fig. 2). SSRs with repeat units of two bases are the most predominant, number over 2000. Those with a repeat type of 3 are the second most abundant, with just under 1000. There is a sharp decline in num-

bers from repeat types 4 to 6, indicating that SSR mutation types with lower repeat types are significantly more common in the dataset.

MICROSATELLITE LOCI AND PRIMER INFORMATION

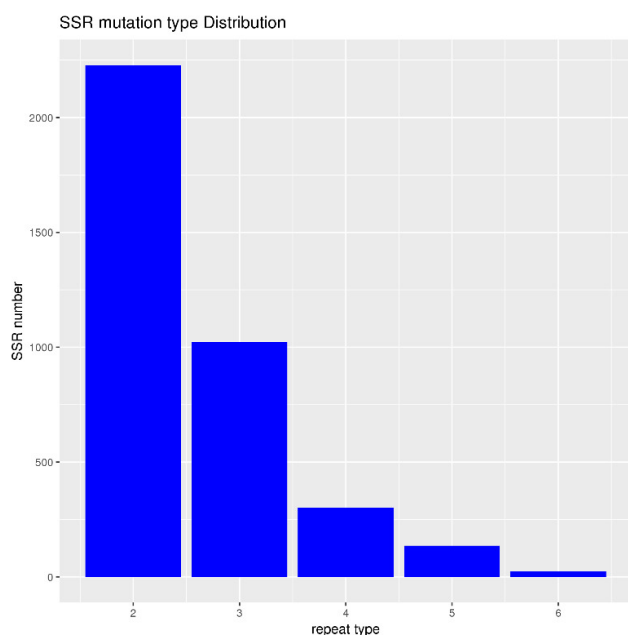
Based on the settings, 67 pairs of primers were successfully designed and synthesized (Table S2). Using the genomic DNA of *T. hsutschouensis* as the template, 18 polymorphic primer pairs (Table 2) were selected from the 67 SSR primers designed in this study. Specifically, after an initial analysis via capillary electrophoresis, we identified 18 primers with high polymorphism information content.

SSR LOCUS DIVERSITY ANALYSIS

Eighteen highly polymorphic SSR primers were used to amplify DNA from 46 individuals of *T. hsutschouensis*, and the amplification results were analyzed to obtain information on SSR genetic diversity (Table 3). The number of alleles (N_a) in *T. hsutschouensis* ranged from 2 to 16, with the most polymorphic locus, SSRJ65, possessing 16 alleles. Among the 18 SSR loci, the effective number of alleles (N_e) varied from 1.293 to 8.672, the observed heterozygosity (H_o) ranged from 0.261 to 0.957, the expected heterozygosity (H_e) ranged from 0.227 to 0.885, and the polymorphism information content (PIC) ranged from 0.201 to 0.874. The average N_a and N_e were 7.056 and 3.540, respectively, with an average H_o of 0.609 and an average H_e of 0.639. The average PIC value was 0.595. Among the 18 polymorphic SSR loci developed, five loci (27.8%) showed significant deviations from HWE after Bonferroni correction ($P < 0.001$).

Table 1. RAD-Seq data for *T. hsutschouensis*

Sample	Raw Base (bp)	Clean Base (bp)	Q20 (%)	Q30 (%)	GC Content (%)
J1	3118428300	3093978900	96.98	91.51	39.97
J2	2760924600	2739976200	97.12	91.77	39.88
J3	2458324800	2440187400	97.07	91.69	39.51
J4	2185130100	2172553500	97.75	93.13	39.61
J5	2268936900	2253769500	97.72	93.09	40.06
J6	1923942000	1909844100	97.78	93.26	40.28

**Figure 2. Distribution of SSR repeat types in the genome of *T. hsutschouensis*.**

The remaining 13 loci (72.2%) conformed to *HWE* expectations ($P > 0.05$), indicating no significant population-level genetic disturbances at these markers.

DISCUSSION

Genetic diversity is crucial for the evolution and adaptation of organisms. Population genetic diversity enhances adaptability to environmental changes, while reduced diversity threatens animal populations in fluctuating environments.²⁴ To assess genetic diversity, key indicators like *Na*, *Ho*, and *PIC* are used.²⁵ High values of *Na*, *Ho*, *He*, and *PIC* suggest rich allelic resources and greater genetic variation. Typically, a closer effective-to-observed allele count ratio indicates a more even allele distribution.²⁶ *PIC* is crucial for evaluating SSR locus diversity, with values above 0.5 indicating high polymorphism, values between 0.25 and 0.5 indicating moderate polymorphism, and values below 0.25 indicating low polymorphism.²⁷ *PIC* evaluation offers precise insights into SSR locus diversity levels.

High-throughput sequencing has proven to be an effective strategy for developing genetic studies, microsatellite

markers, and conducting genetic studies in aquatic species. For instance, Zhang et al.²⁸ sequenced the genome of *Sinogastromyzon sichangensis* and identified 29 polymorphic SSR loci, which showed high polymorphism with an average *PIC* value of 0.859. Similarly, Li et al.²⁹ 25 SSR loci were isolated from *Acipenser dabryanus* using the same technology, with an average *PIC* of 0.727, highlighting their utility in population genetic analyses. These studies demonstrate that high-throughput sequencing is a robust technique for developing SSR markers across divergent fish species. In this study, we used RAD-Seq technology to develop SSR markers for *T. hsutschouensis*. Among the 67 designed SSR primer pairs (for repeat units of 2–6 bases), 18 loci (26.9%) showed polymorphic amplification. The average *Na* and *PIC* values were 7.056 and 0.595, respectively, which are generally in line with the ranges reported for *A. dabryanus* (*Na* = 7.2, *PIC* > 0.5) and *S. sichangensis* (*Na* = 14.5, *PIC* = 0.859). The relatively lower average *PIC* values in our study may be due to differences in repeat motif composition or population genetic structure. Notably, locus SSRJ65 exhibited exceptional polymorphism with 16 alleles and a *PIC* value of 0.847, surpassing the maximum values reported in the aforementioned studies. The observed heterozygosity patterns (*Ho* = 0.609, *He* = 0.639) indicate moderate genetic diversity within the sampled population. This difference in heterozygosity levels compared to *S. sichangensis* (*Ho* = 0.620, *He* = 0.882), may be due to the specific ecological constraints of *T. hsutschouensis*. Compared to prior *T. hsutschouensis* studies using SNP markers,²⁰ this study's SSR markers provided comprehensive polymorphism parameters, including *Na* and *Ne*.

In our panel of 18 loci, the mean *PIC* was 0.595, indicating overall moderate informativeness. Fourteen loci met the conventional 'high polymorphism' threshold (*PIC* > 0.50), whereas four loci showed *PIC* ≤ 0.50 (Table 3). Accordingly, downstream applications should prioritize the higher-*PIC* markers, while the lower-*PIC* loci may still be helpful for diversity baselines or cross-population transferability tests. In addition, five of the 18 microsatellite loci (27.8%) showed deviations from the Hardy-Weinberg equilibrium. These deviations could be due to null alleles, population substructure, selective forces, or genotyping errors. It is essential to note that, despite these deviations, the loci in question still provide valuable insights into the genetic diversity and structure of *T. hsutschouensis* populations. Future research should investigate these deviations further, potentially using MICRO-CHECKER to assess null

Table 2. Eighteen microsatellite loci used for PCR amplification and locus diversity analysis

Locus	Forward (5'to 3')	Reverse (5'to 3')	Motif	Annealing T (°C)	Expected size (bp)
SSRJ2	CAGTATTACCAGCATTACACGGG	GATTTGTACAGACGGACTGAAGC	(TCA) 9	53.0	125~310
SSRJ8	CACATCAACGGAATTTAAAGAGC	GTTTCTGTGGATTCAATTTTCAGC	(AAC) 8	50.3	112~155
SSRJ9	GTGTAATGGTGTAACTCCCAAGC	GACCGGTTTTATGCGAGAATAAT	(TTC) 8	52.1	131~145
SSRJ10	TATGTATGCTTTGGCCCTTCATA	TTCGGGCTACAATCATCTAAAAA	(GAT) 8	49.4	129~257
SSRJ17	TGCGACTGCATTAGTAGAGTCAT	TCTTAAACAACCTGAACCATAAACAA	(TAAAA) 6	49.4	120~199
SSRJ18	CCTAGCGTGTGTTTTCTGTCTCT	GAAGAACAATGCGTTTCAATTTTC	(GTTCT) 6	52.1	147~228
SSRJ19	ACCACAACTCTGAATCTCTCCA	TACCATCTGTCATCCTCTCGTCT	(ATCC) 8	52.6	154~180
SSRJ20	GAGAAGACAGATTGTTGAGGTGG	CAATCATTGAGTTTCACTTTTGA	(TTGT) 7	52.6	126~366
SSRJ21	GTTATTTTGAACGGCACTCTG	TCGCAAATAGAAAAACACAGACA	(GTCT) 7	49.4	141~156
SSRJ24	TTGGTCTTGAGCTCTAAATGTC	ACGAATGAAGTCGCAATTAGAAA	(ATTC) 6	49.4	122~136
SSRJ25	GAGATTACTGAGTTGGCCAGT	ACGAAATGCCATTAATAAACAC	(TCCA) 6	52.1	98~284
SSRJ26	AGGAAATGAGAATGGAGTAGAAGC	TGGAGTCTCAACACATCTCTGAA	(CAGA) 6	52.1	116~128
SSRJ30	CATTGGAAAGAACTGGGTGAATA	GACATGATGAATGAATTTGCTGA	(AATA) 6	49.4	113~114
SSRJ49	CATTTAGCAGATGCTTTTATTCACA	CCGATGATGTGTTTGAGTAATGA	(TC) 16	50.0	143~147
SSRJ61	TTTAATTGTCTGTCTGTCGGTCC	GAATGATAATGAAGCAAACCTGGG	(TC) 15	50.0	141~164
SSRJ64	AATGATTGGATGTTTTTCACAGC	AACAATGTTGGATGCTTCATTT	(TG) 15	48.0	146~171
SSRJ65	ATAAGATCTCAGAACCACACCGA	CTTTTGCTGTGCATCACTGTGTC	(AG) 15	52.0	113~144
SSRJ66	CATGGCAAAAAGTACACACACAT	CGAAAGAACTTTACCTCTTTGATT	(AT) 16	50.0	159~179

Table 3. Genetic diversity information of SSR markers in *T. hsutschouensis*

Pop	Locus	N	Na	Ne	Ho	He	PIC	PHWE
Pop1 (Ganqing)	SSRJ2	46	2.000	1.293	0.261	0.227	0.201	NS
	SSRJ8	45	3.000	1.922	0.289	0.480	0.392	***
	SSRJ9	46	11.000	5.330	0.957	0.812	0.790	***
	SSRJ10	46	5.000	1.858	0.478	0.462	0.397	NS
	SSRJ17	45	5.000	3.603	0.533	0.722	0.672	***
	SSRJ18	46	3.000	2.816	0.674	0.645	0.571	NS
	SSRJ19	46	5.000	2.301	0.565	0.565	0.502	NS
	SSRJ20	46	4.000	2.546	0.522	0.607	0.525	NS
	SSRJ21	45	5.000	3.263	0.644	0.694	0.635	NS
	SSRJ24	46	6.000	2.383	0.587	0.580	0.543	NS
	SSRJ25	46	7.000	2.129	0.500	0.530	0.503	NS
	SSRJ26	46	5.000	2.108	0.478	0.526	0.473	***
	SSRJ30	46	8.000	2.943	0.478	0.660	0.618	***
	SSRJ49	46	6.000	2.744	0.609	0.636	0.599	NS
	SSRJ61	46	12.000	6.571	0.957	0.848	0.832	NS
	SSRJ64	46	14.000	8.672	0.826	0.885	0.874	NS
	SSRJ65	46	16.000	7.173	0.848	0.861	0.847	NS
	SSRJ66	45	10.000	4.066	0.756	0.754	0.728	NS
Average		46	7.056	3.540	0.609	0.639	0.595	/

Note: *N* represents the total number of samples. *Ne* represents the number of effective alleles. *Na* represents the number of alleles. *Ho* represents the observed heterozygosity. *He* represents the expected heterozygosity. *PIC* represents the polymorphism information content. *P_{HWE}* represents the Hardy-Weinberg equilibrium test (with Bonferroni correction). Significant values (*** means $P < 0.001$) indicate deviance from Hardy-Weinberg expected proportions at these values. NS: no significant difference.

alleles and calculating FIS to gain a better understanding of population genetics. Moreover, we recommend that these loci be validated in future research before they are applied to specific applications such as population genetics or conservation management.

This study confirms that RAD-Seq technology is effective for developing polymorphic markers via SSRs, complementing SNP marker research and advancing genetic studies. These markers highlight RAD-Seq's efficiency in SSR development and offer essential tools for future conservation genomics of this ecologically vulnerable species. To enhance

transregional conservation efforts, future studies should focus on genome-wide validation of these markers and extend their application to nearby populations.

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AUTHORS' CONTRIBUTION

Conceptualization: Ya Liu, Qiang Li; *Data curation:* Hangyu Lin; *Formal Analysis:* Ya Liu; *Funding acquisition:* Qiang Li; *Investigation:* Ya Liu, Qiang Li; *Methodology:* Ya Liu, Zhongmeng Zhao; *Project administration:* Qiang Li; *Resources:* Zhongmeng Zhao, Qiang Li;

Software: Han Zhao, Hongyu Ke; *Supervision:* Jue Lin; *Validation:* Ya Liu; *Visualization:* Hangyu Lin; *Writing – original draft:* Ya Liu; *Writing – review & editing:* Hangyu Lin, Shengqi Su.

COMPETING INTEREST – COPE

No competing interests were disclosed.

ETHICAL CONDUCT APPROVAL – IACUC

All animal handling procedures in the current study were approved by the Institutional Animal Care and Use Committee of the Fisheries Research Institute, Sichuan Academy of Agricultural Sciences in Chengdu, China (Approval No.: 20190416001A). All experiments were conducted in accordance with the committee's approved guidelines.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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SUPPLEMENTARY MATERIALS

Supplementary for Screening of microsatellite loci and analysis of polymorphism in *Triplophysa hsutschouensis* based on RAD sequencing

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