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Study on the characteristics of genetic diversity and population structure of a rare and endangered species of *Rhododendron nymphaeoides* (Ericaceae) based on microsatellite markers

Jun Luo[1,4], Xiaoyong Dai[1,3*], Jin Chen[1], He Shuang[1], Congjun Yuan[1,2] and Dali Luo[1,4]

Abstract

Background *Rhododendron nymphaeoides* is explicitly listed as an endangered species in the "*the International Union for Conservation of Nature's Red List* (IUCN)", "*The Red List of Rhododendrons*", "*Red List of China's Higher Plants*" and "*Threatened Species List of China's Higher Plants*". It is also listed as a provincial-level key protected wild plant in Sichuan, with few individuals in the wild and significant conservation value. The genetic diversity and population structure have never been described, making it difficult to plan conservation strategies for this plant.

Results This study utilized 15 pairs of microsatellite markers to examine the genetic diversity of 79 samples of *R. nymphaeoides* sourced from five different geographic populations. A total of 214 alleles were detected, with the average effective number of alleles (N_e) of 7.0324. The averages for the polymorphism information index (*PIC*) and expected heterozygosity (H_e) were 0.7832 and 0.8102, respectively, indicating that the *R. nymphaeoides* populations harbor a rich genetic information content, the genetic differentiation coefficients (F_{ST}) average was 1.2607. There was high genetic diversity among populations, with average observed heterozygosity (H_o) and expected heterozygosity (H_e) values of 0.6375 and 0.6663, respectively, suggesting a degree of inbreeding within populations. Mantel test results showed a significant positive correlation between geographic distance and genetic distance amongst populations ($r = 0.8456$, $P = 0.0021$), which conforms to the isolation-by-distance (IBD) model. Due to geographical barriers, there is also a high level of genetic differentiation among populations, with an average genetic differentiation coefficient (F_{ST}) of 0.2685. Analysis of molecular variance (AMOVA) indicated that the main source of molecular variance exists within populations (73%), rather than between populations (27%). There was higher historical gene flow (average = 1.0850) and lower contemporary gene flow (average = 1.2849), with seed and pollen dispersal being impeded. Under the Two-Phase Model (TPM) assumption, findings are consistent with the mutation-migration model, suggesting that there has been no genetic bottleneck. STRUCTURE analysis, principal coordinate analysis (PCoA), and UPGMA analysis all support the division of the five natural populations into three genetic clusters.

*Correspondence:
Xiaoyong Dai
dxy5198@163.com

Full list of author information is available at the end of the article

Conclusions This is the first comprehensive analysis of the genetic diversity and population structure of the endangered plant *R. nymphaeoides* using microsatellite markers. The study results indicate that this endangered plant's natural populations maintain a high level of genetic diversity. Due to geographical barriers, there is also a high level of genetic differentiation, with the primary source of genetic variation originating within populations. There is higher historical gene flow and lower contemporary gene flow, with seed and pollen dispersal being obstructed. The five populations can be divided into three evolutionary units, for which corresponding conservation management units should be established. These findings will benefit the conservation and development of the species and provide a theoretical basis for further studies on its evolution and biogeography.

Keywords *Rhododendron nymphaeoides*, Genetic diversity, Populations structure, Microsatellite, Endangered species protection

Background

Rhododendron plants, known for their lush flowers and leaves and vibrant beauty, are ranked among the world's four major flowers and China's top ten famous flowers, earning the reputation as the "king of woody flowers" [1]. Since their extensive discovery and introduction to Europe in the 19th century, they have held a significant position in horticulture [2].

Rhododendron nymphaeoides W. K. Hu is a perennial tree in the genus Rhododendron, characterized by ovate leaves with auriculate bases, and ovaries and styles entirely covered with reddish-brown short-stalked glands. After nearly a decade of research, we have discovered five wild populations of *R. nymphaeoides*, including the Longzhaohe population (LZH) and the Hutoushan population (HTS) located in Gulin County, Sichuan Province; the Huagaoxi population (HGX) in Xuyong County, Sichuan Province; the Luosike population (LSK) in Duyun City, Guizhou Province; and the Heshancun population (HSC) in Dafang County, Guizhou Province. These are currently the only five known wild populations of *R. nymphaeoides*, These are the only five known wild populations of *R. nymphaeoides*, consisting of approximately 150 mature individuals. This species is found exclusively in limestone mountains at elevations above 1500 m, covering a total area of approximately 2 hectares, with an extremely small population size [3, 4]. Based on its distribution, biological characteristics, and survival status, the "*Red List of Threatened Species*" published by the International Union for Conservation of Nature (IUCN), the "*Red List of Rhododendrons*" co-issued by the International Plant Protection Alliance, World Wildlife Fund, Global Trees Campaign, and Royal Botanic Garden Edinburgh, as well as the "*Red List of China's Higher Plants*" published by the Ministry of Ecology and Environment of China, and the "*Threatened Species List of China's Higher Plants*" published by the Chinese Academy of Sciences, *R. nymphaeoides* is explicitly listed as an endangered species (EN), and is one of the 19 endangered Rhododendron species globally [5, 6]. It is listed as a provincial-level key protected plant in Sichuan Province. Conservation of genetic resources and plant breeding programs require an assessment of the genetic diversity and structure of endangered species [7]. However, current research on *R. nymphaeoides* is very limited, and studies focusing on population structure and genetic diversity are notably absent. Due to the lack of genetic background knowledge, it is difficult to plan conservation strategies for this plant, and there is an urgent need to accurately understand the population structure and genetic diversity of *R. nymphaeoides*.

Molecular markers are tools for accurately assessing genetic diversity. With the advancement of technology, researchers have developed various molecular markers, including simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), restriction fragment length polymorphism (RFLP) and single nucleotide polymorphism (SNP) to analyze the genetic diversity of plants [8, 9, 10, 11, 12, 13, 14, 15, 16]. Among these molecular marker techniques, microsatellite markers have been widely applied to many plant species for assessing genetic diversity, constructing genetic maps, and determining species phylogenies because: (1) they are abundant, cover the whole genome, and exhibit high levels of polymorphism; (2) they follow Mendelian inheritance and are co-dominant; (3) they provide high information content due to multiple alleles per locus; (4) primers designed for each locus facilitate collaboration and exchange between different laboratories. Microsatellite markers have been extensively used in genetic mapping, marker-assisted selection, variety identification, pedigree analysis, estimation of genetic distances between populations, and studies of evolution and genetic diversity [17, 18]. This study utilized microsatellite markers to analyze the genetic diversity indices, genetic differentiation, gene flow, and population structure of the currently identified five only remaining wild populations of *R. nymphaeoides*. Our results aimed: (1) to systematically reveal the genetic diversity and population structure of the endangered *R. nymphaeoides*; (2) to analyze the causes of the current genetic structure of the species; (3) to provide key information to establish appropriate conservation and management strategies.

Results

Genetic characteristic of 15 microsatellite markers

In this study, 15 pairs of primers were selected to analyze microsatellite markers in 79 samples from five natural populations of *R. nymphaeoides* [19, 20, 21, 22] (Table 1), including 17 samples from the Luosike population (LSK), 10 from the Heshancun population (HSC), 17 from the Longzhaohe population (LZH), 25 from the Hutoushan population (HTS), and 10 from the Huagaoxi population (HGX). Using these 15 pairs of primers, a total of 214 alleles were detected (Table 2). The number of observed alleles (N_a) ranged from 6 (RDW38) to 24 (R-432) with an average of 14.2667. The effective number of alleles (N_e) across all loci in this study was 105.4866, and the number of effective alleles per locus ranged from 2.5196 (N84) to 14.8772 (N8) with an average of 7.0324. No loci show evidence for a null allele (N_{null}). The shannon information index (I) ranged from 1.2202 (RDW38) to 2.8616 (N8) with an average of 2.0692. The polymorphic information content polymorphism information index (*PIC*) ranged from 0.5753 (RDW38) to 0.9288 (N8), with an average of 0.7832, suggested that each locus exhibited high polymorphism ($PIC \geq 0.5$). From the perspective of loci, except for RDE11, the remaining 14 pairs of primers deviated from Hardy-Weinberg equilibrium (HWE), which could be associated with limited population size, natural selection pressures, and genetic drift. *R. nymphaeoides*, being a endangered species with a small number of populations, is likely to experience genetic drift. Moreover, it only grows in rocky habitats at altitudes above 1500 m, which intensifies the natural selection, allowing only individuals with strong adaptability to survive and causing changes in genotype frequencies. Research suggests that these loci should not be discarded simply because they do not conform to Hardy-Weinberg equilibrium (HWE), especially if the sample size is moderate and the allelic diversity is high [23, 24]. Therefore, we believe that the deviations from HWE will not significantly

Table 1 Information of 15 pairs of polymorphic microsatellite primers

Locus	forward sequence(5′—3′)	Repetit motif	Fluorescence	BL(bp)	TBN	PBN	PR(%)
N8	F: CGGAGAGTGATGAAACAGAA	$(AG)_{19}(TG)_8$	HEX	116–183	22	22	100
	R: GCAAAAGCGAAATACAGAAC						
N73	F: GCAACCTACATTCTCAACAT	$(AC)_3$ C $(CA)_6$	ROX	153–187	15	15	100
	R: ACTACAAGCCTGAACCACAT						
N84	F: GTGAAGCCATGAGTACCAACT	$(TG)_8$	ROX	168–186	9	9	100
	R: ACATCCACTAGACAGAGAGCA						
R-172	F: GCTGTCGTATTTACCCTAA	$(CT)_6$ / $(CT)_7$	FAM	220–240	9	9	100
	R: AGATGAAGAAGTGCTGGA						
R-210	F: TTTCTGGCTCAAAGAGGG	$(AG)_{21}$	TAMRA	77–118	17	17	100
	R: ATTTGGCAGTGTTTCTCA						
R-335	F: TCAGGCAACCGTAACAAC	$(CT)_{11}$ / $(CT)_8$	TAMRA	66–112	19	19	100
	R: AAACGCAACAAACAGCAA						
R-432	F: CCGTTTGAGTATCTTCCC	$(CT)_{14}$	TAMRA	111–178	24	24	100
	R: CTGGTCCATTCTCCAAGTA						
R-557	F: CGAAACTCAGAACCTCCG	$(CT)_9$ / $(TG)_6$	FAM	204–229	12	12	100
	R: TTCCGAACTCCTTCACCAG						
RD5	F: GAGGCTCCCATCAAAACACT	$(CT)_9$	HEX	104–142	17	17	100
	R: GGGTTGGCGACCTTGATCTT						
RDE1	F: CAAAAGTTTTTCAGCGGGTA	$(AG)_{14}$	FAM	227–243	9	9	100
	R: AAGAAACAGCCGTATGCGAT						
RDE11	F: TAATCCAGACTATCCAGTGC	$(CT)_7$	HEX	142–157	7	7	100
	R: CAAGAGCTTGATGGACTGAA						
RDE12	F: GTGATCTCCAGTAAAAACGC	$(AG)_{16}$	ROX	185–218	17	17	100
	R: CATAGATGAGAGATTCCCTG						
RDW6	F: AATGAAGCCCCTAAAAGTAA	$(GA)_{10}$	ROX	161–187	13	13	100
	R: CTAGCAGTATGGTGGGTTGT						
RDW35	F: TAAGGTTGGTGTAGCGTGTA	$(TC)_5(CT)_6(ATA)_3$	FAM	205–262	18	18	100
	R: GTGACTTCGGATTCGTGGAG						
RDW38	F: GTGTTTGAAATTGTCGGC	$(TAGAG)_4(AG)_7(AGAGAT)_3$	HEX	126–143	6	6	100
	R: AACAGCGACGAGAAAAGC						
Mean	-	-	-	-	14.27	14.27	100
Range	-	-	-	66–262	6–24	6–24	100

BL: Band length; TBN: Total band number; PBN: Polymorphic band number; PR: Polymorphism rate

Table 2 Genetic charateristics of 15 pairs of microsatellite markers

Locus	N_a	N_e	N_{null}	I	PIC	P_{HWE}	H_o	H_e	F_{is}	F_{it}	F_{ST}	N_m
N8	22	14.8772	0.0000	2.8616	0.9288	0.0027**	0.7722	0.9387	0.0590	0.1964	0.1460	1.4625
N73	15	6.7911	0.0000	2.2604	0.8408	0.0004***	0.6835	0.8582	0.0883	0.1512	0.0690	3.3744
N84	9	2.5196	0.0000	1.3921	0.5821	0.0000***	0.2532	0.6069	0.3291	0.5161	0.2788	0.6467
R-172	9	2.9204	0.0000	1.3277	0.5981	0.0000***	0.6456	0.6618	-0.1390	0.0290	0.1475	1.4453
R-210	17	7.1818	0.0000	2.2898	0.8486	0.0000***	0.6835	0.8662	0.1347	0.2446	0.1271	1.7176
R-335	19	8.0737	0.0000	2.4121	0.8656	0.0009***	0.7215	0.8817	0.0173	0.1933	0.1791	1.1457
R-432	24	13.9308	0.0000	2.8322	0.9237	0.0000***	0.8608	0.9341	0.0120	0.0789	0.0677	3.4430
R-557	12	4.5655	0.0000	1.8954	0.7607	0.0000***	0.6962	0.7859	0.0405	0.1599	0.1245	1.7585
RD5	17	11.3991	0.0000	2.5500	0.9055	0.0000***	0.8481	0.9181	-0.0828	0.0694	0.1406	1.5287
RDE1	9	4.7460	0.0000	1.7858	0.7618	0.0002***	0.6329	0.7943	-0.0158	0.1885	0.2011	0.9932
RDE11	7	2.9411	0.0000	1.3087	0.6109	0.5243ns	0.5696	0.6642	-0.0114	0.1230	0.1329	1.6315
RDE12	17	6.4208	0.0000	2.2509	0.8307	0.0000***	0.5949	0.8496	0.0866	0.3687	0.3088	0.5596
RDW6	13	6.1548	0.0000	2.1228	0.8220	0.0004***	0.6329	0.8429	0.1859	0.3219	0.1670	1.2468
RDW35	18	10.1977	0.0000	2.5280	0.8942	0.0087**	0.8734	0.9077	-0.0629	0.0339	0.0911	2.4939
RDW38	6	2.7670	0.0000	1.2202	0.5753	0.0000***	0.2658	0.6427	0.1636	0.6147	0.5394	0.2135
Mean	14.2667	7.0324	0.0000	2.0692	0.7832	0.0358	0.6489	0.8102	0.0430	0.2085	0.1729	1.1957
Range	6–24	2.7670-14.8772	0.0000	1.2202-2.8616	0.5821-0.9288	-	0.2532-0.8734	0.6069-0.9387	-0.1390-0.3291	0.0339-0.6147	0.0911-0.5394	0.2135-3.4430

N_a: The number of observed alleles; N_e: The effective number of alleles; N_{null}: The number of null alleles; *I*: Shannon' Information Index; *PIC*: Polymorphism information index; P_{HWE}: Loci showing a significant departure from Hardy-Weinberg equilibrium (*: $P<0.05$. **: $P<0.01$. ***: $P<0.001$. ns: $P>0.05$.); H_o: Observed heterozygosity; H_e: Expected heterozygosity; F_{is}: The within-population inbreeding coefficient; F_{it}: The total inbreeding coefficient; F_{ST}: Genetic differentiation coefficient; N_m: gene flow

impact subsequent data analysis. In the linkage disequilibrium (LD) tests, no significant linkage disequilibrium was found between any pair of loci, indicating that the loci are minimally affected by selection, mutation, and migration factors. Using RStudio to perform the "Genotype Accumulation Curve" test, Fig. 1 shows that the curve rises rapidly before leveling off, indicating that the current sample size has covered most of the genotypes. Therefore, the 15 pairs of SSR primers used in this study exhibit high polymorphism and cover the vast majority of genotypes, meeting the requirements for analyzing the genetic diversity of *R. nymphaeoides*. The observed heterozygosity (H_o) ranged from 0.2532 (N84) to 0.8734 (RDW35) with an average of 0.6489. The expected heterozygosity (H_e) ranged from 0.6069 (N84) to 0.9387 (N8) with an average of 0.8102.

At the locus level, the within-population inbreeding coefficient (F_{is}) ranged from −0.1390 (R-172) to 0.3291 (N84), with an average of 0.0430. The total inbreeding coefficient (F_{it}) ranged from 0.0290 (R-172) to 0.6147 (RDW38), with an average of 0.2085. The genetic differentiation coefficient (F_{ST}) ranged from 0.0677 (R-432) to 0.5394 (RDW38), with an average of 0.1729, indicating that 17.29% of the genetic variation occurred between populations, while the remaining 82.71% occurred within populations, suggesting the presence of inbreeding among the selected genetic loci. The gene flow (N_m) ranged from 0.2135 (RDW38) to 3.4430 (R-432), with an average of 1.1957. Among them, N84 (N_m = 0.6467) and RDE1 (N_m = 0.9932) and RDE12 (N_m = 0.5596) and RDW38 (N_m = 0.2135) was less than 1, indicating that populations at these four loci are more susceptible to genetic drift, leading to significant differences in allele frequency among populations (Table 2).

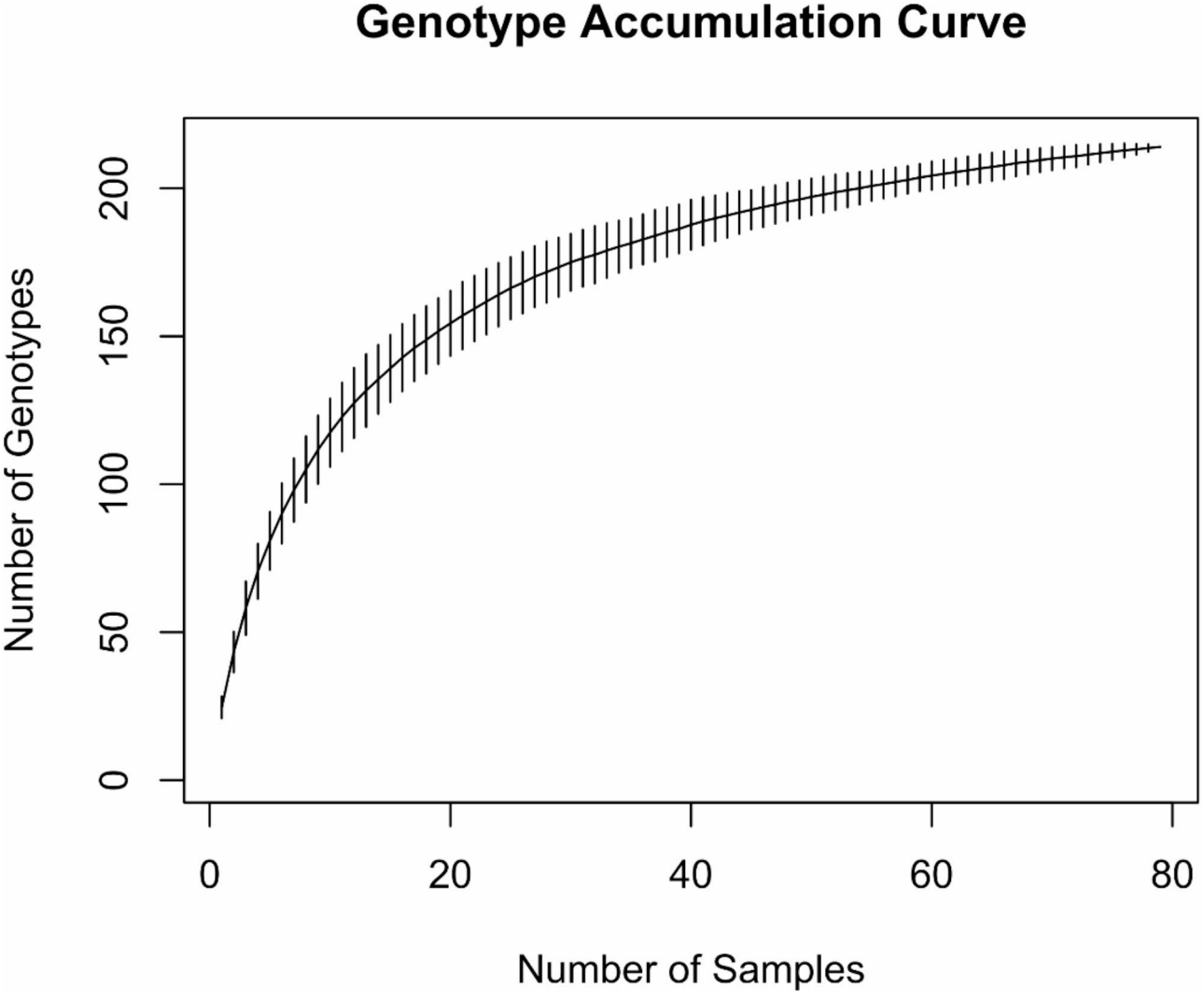

Fig. 1 Genotype accumulation curve of 15 pairs of microsatellite markers

Table 3 Genetic diversity of 5 populations of *R. nymphaeoides*

Populaition	N_a	N_e	N_{null}	I	H_o	H_e	F	HWE
LSK	5.0000	3.0979	0.0000	1.1832	0.5961	0.6015	0.0081	RDE1*
HSC	4.7333	3.2858	0.0000	1.1680	0.5733	0.5783	0.0258	R-210**, R-557**
LZH	8.1333	5.1802	0.0000	1.6581	0.6314	0.7115	0.1233	N73***, R-210***, RDE1*
HTS	8.6000	4.9008	0.0000	1.6774	0.7200	0.7373	0.0515	N73***, N84***
HGX	6.2000	4.4891	0.0000	1.4909	0.6667	0.7027	0.0678	RDW6**
Mean	6.5333	4.1908	0.0000	1.4355	0.6375	0.6663	0.0553	

N_a: The number of observed alleles; N_e: The effective number of alleles; N_{null}: The number of null alleles; *I*: Shannon' information index; H_o: Observed heterozygosity; H_e: Expected heterozygosity; *F*: Fixed index; HWE: loci showing a significant departure from Hardy-Weinberg equilibrium with a global test at 5% level and after a sequential Bonferroni correction. (*$P<0.05$. ** $P<0.01$. *** $P<0.001$. indicates loci with heterozygote deficit); LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

Genetic diversity of populations in *R. nymphaeoides*

We conducted an analysis of five populations based on microsatellite markers. (Table 3). The results showed that The number of observed alleles (N_a) in the five populations ranged from 4.7333 (HSC) to 8.6000 (HTS) with an average of 6.5333. The effective number of alleles (N_e) ranged from 3.0979 (LSK) to 5.1802 (LZH) with an average of 4.1908. No loci show evidence for a null allele (N_{null}). The values of The shannon information index (*I*) range from 1.1680 (HSC) to 1.6774 (HTS), with an average of 1.4355, and the differences between the populations are small, indicating relatively similar levels of genetic diversity among the populations. The observed heterozygosity (H_o) ranges from 0.5733 (HSC) to 0.7200 (HTS), with an average of 0.375. The expected heterozygosity (H_e) ranges from 0.5783 (HSC) to 0.7373 (HTS), with an average of 0.6663. Moreover, The observed heterozygosity (H_o) is lower than expected heterozygosity (H_e) in all populations, indicating a certain degree of heterozygote deficiency within the populations. Combining the expected heterozygosity (H_e) and Shannon diversity index, HTS population has the highest genetic diversity, while HSC has the lowest genetic diversity. The fixed index (*F*) values range from 0.0081 (LSK) to 0.1233 (LZH), with an average of 0.0553, all of which are greater than 0. This indicates a certain degree of inbreeding among the five populations, which may lead to genetic homogeneity between the populations. In addition, we also tested whether 15 pairs of microsatellite loci followed the Hardy-Weinberg equilibrium (HWE) in the central distribution of the *R. nymphaeoides*. The results showed that majority of loci were in accord with the Hardy-Weinberg equilibrium (HWE), but several populations did not fully satisfy the HWE, especially populations LZH in which part loci were found to deviate from the Hardy-Weinberg equilibrium (HWE) (3loci). This deviation could be due to the small size of the LZH population, which leads to random fluctuations in allele frequencies caused by genetic drift. Additionally, inbreeding may lead to a reduction in heterozygotes ($H_o < H_e$) and an excess of homozygotes ($F=0.1233$), ultimately causing this deviation.

Table 4 Analysis of molecular variance (AMOVA) for 5 populations of *R. nymphaeoides*

Source of variation	Degree of freedom	Sum of Squares	Mean Square	Variance components	Percentage of variance(%)
Among Populations	4	301.091	75.273	4.177	27%
Within Populations	74	837.947	11.324	11.324	73%
Total	78	1139.038		15.501	100%

Table 5 Result of historical gene flow N_m (upper triangle) and genetic differentiation coeffcient (F_{ST}) results (lower triangle) between populations. Bold character indicates the highest value, while italic bold character displays the lowest value

Population	LSK	LZH	HTS	HGX	HSC
LSK	-	0.4389	0.4824	***0.3834***	0.3909
LZH	0.3629	-	3.3725	2.3684	0.5327
HTS	0.3414	0.0690	-	**3.5915**	0.5093
HGX	**0.3947**	0.0955	***0.0651***	-	0.5370
HSC	0.3901	0.3194	0.3292	0.3176	-

LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

Genetic differentiation and gene flow among populations in *R. nymphaeoides*

Analysis of molecular variance (AMOVA) showed that that 73% of the genetic variation mainly come from between-individual variations within the population variation, while only 12% is due to between-population variation (Table 4).

The values of the genetic differentiation coefficients (F_{ST}) between different populations ranges from 0.0651 (between HGX and HTS) to 0.3947 (between HGX and LSK), with an average of 0.2685 (Table 5), which is consistent with the result of Analysis of molecular variance (AMOVA), indicating that there is a clear, higher level of genetic differentiation between populations, with most genetic variation primarily existing within the populations of *R. nymphaeoides*.

Based on the genetic differentiation coefficients (F_{ST}), the historical gene flow (N_m) among the five populations was derived. The historical gene flow associated with

genetic differentiation ranges from 0.3834 (between HGX and LSK) to 3.5915 (between HGX and HTS) among different populations, with an average of 1.2607 (Table 5), indicating that there is significant, higher level historical gene flow between *R. nymphaeoides* populations. Simultaneously, the historical migration rates among the five populations were inferred using the software Migrate-n v3.6.4. As shown in Table 6, the average historical migration rate for the five populations is 1.0850. The historical migration rates between the HTS, LZH, and HGX populations are relatively high, with the highest historical migration rate from the HTS population to the HGX population (4.3567), and a significantly high rate from the HGX population to the HTS population (3.3163). Conversely, the historical migration rates between these three populations and the LSK and HSC populations are comparatively low. Additionally, recent migration rates among the five populations were estimated using BayesAss v3.0.0 software. According to Table 7, while the recent migration rates between HTS, LZH, and HGX are higher than the overall recent migration rates, they are still lower compared to the historical migration rates, with both the Recent migration rates among the five populations being relatively small (Average value = 0.2849). These results indicate that *R. nymphaeoides* has a higher historical gene flow and a lower contemporary gene flow, particularly the gene flow between the HTS and HGX populations is notably high.

Table 6 Historical migration rate (Migrate-n) for 5 populations of *R. Nymphaeoides*. The upper triangle represents the immigration rate, and the lower triangle represents the emigration rate

Population	LSK	LZH	HTS	HGX	HSC
LSK	-	0.5428	0.7287	0.6135	0.4927
LZH	0.3248	-	1.4254	2.0342	0.5472
HTS	0.2906	3.8547	-	4.3567	0.3154
HGX	0.3546	1.1345	3.2163	-	0.4265
HSC	0.2106	0.3247	0.2043	0.3026	-

LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

Table 7 Recent migration rate (BayesAss) for 5 populations of *R. Nymphaeoides*. The upper triangle represents the immigration rate, and the lower triangle represents the emigration rate

Population	LSK	LZH	HTS	HGX	HSC
LSK	-	0.0486	0.0357	0.0427	0.1385
LZH	0.0325	-	0.3965	0.4068	0.0894
HTS	0.0969	1.2849	-	1.2483	0.0631
HGX	0.0482	0.3782	1.0721	-	0.0853
HSC	0.0702	0.0352	0.0681	0.0524	-

LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

The results of the bottleneck effect analysis are presented in Table 8. The mutation-drift equilibrium of the five populations of *R. nymphaeoides* was evaluated using different models and detection methods. Under the Two-Phase Model (TPM) assumption, both detection methods indicated that the heterozygosity excess in all five populations was not significant ($P > 0.05$), which is consistent with the mutation-migration model. However, under the Stepwise Mutation Model (SMM) assumption, the HTS population exhibited a significant deficiency in heterozygosity ($P < 0.05$), deviating from the mutation-migration equilibrium, while the remaining four populations conformed to the mutation-migration model ($P > 0.05$). The model migration graphs showed a normal L-shaped distribution across all five populations. These results suggest that under the Two-Phase Model (TPM) assumption, none of the five populations experienced a genetic bottleneck effect, but the HTS population did experience a genetic bottleneck under the Stepwise Mutation Model (SMM) assumption.

Genetic distances between the five populations of *R. nymphaeoides* were calculated using the Popgen32 software (Table 9). The genetic distance ranges from 0.1988 (between HTS and LZH) to 1.3535 (between HGX and LSK), with an average of 0.7659, and the coefficient of genetic similarity ranges from 0.2583 (between HGX and LSK) to 0.8197 (between HTS and LZH), with an average of 0.4995, indicating that the farthest genetic relationships with HGX and LSK, while between HTS

Table 8 Bottleneck analysis for 5 populations of *R. nymphaeoides*

Population	TPM			SMM			Mode shift
	Sign test	Wilcoxon test		Sign test	Wilcoxon test		
		Heterozygosity deficiency	Heterozygosity excess		Heterozygosity deficiency	Heterozygosity excess	
LSK	0.5169	0.5980	0.4235	0.1431	0.1796	0.8349	L
LZH	0.3925	0.4235	0.5980	0.3991	0.2444	0.7729	L
HTS	0.2265	0.2106	0.8053	0.0122	0.0206	0.9823	L
HGX	0.2035	0.8486	0.1651	0.3318	0.7003	0.3193	L
HSC	0.3192	0.5000	0.5242	0.1535	0.2708	0.7492	L
All	1.6586	2.5807	2.5161	1.0397	1.4157	3.6586	L

TPM: Two-Phase Model; SMM: Stepwise Mutation Model; L: normal L-shaped distribution; LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

Table 9 Result of genetic identity between populations (upper triangle) and genetic distance (lower triangle) between populations. Bold character indicates the highest value, while italic bold character displays the lowest value

Population	LSK	LZH	HTS	HGX	HSC
LSK	-	0.3245	0.3817	***0.2583***	0.4338
LZH	1.1253	-	**0.8197**	0.7368	0.4110
HTS	0.9631	***0.1988***	-	0.8032	0.3974
HGX	**1.3535**	0.3054	0.2191	-	0.4288
HSC	0.8351	0.8892	0.9227	0.8468	-

LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

and LZH are the closest. A Mantel test conducted for *R. nymphaeoides* indicated correlation between genetic distance and geographic distance among populations was a positive significant ($r = 0.8456$, $P = 0.0021$) (Fig. 2), in line with the isolation-by-distance (IBD) model. this suggests that genetic differentiation among *R. nymphaeoides* was caused by geographic distance.

Population structure and genetic relationships

The result of the STRUCTURE analysis revealed that maximum value of delta K was at K = 2, suggesting that the population can be divided into two genetic clusters (Fig. 3a; Table. S1). However, the genetic differentiation coefficient (F_{ST}) (0.3901) between the HSC and LSK populations is relatively high, indicating that it would be more appropriate to divide them into different genetic clusters. Therefore, we believe that K = 3 is more consistent with reality. Based on this, the five populations in this study are divided into three genetic clusters: Genetic cluster 1 (blue square) contained 52 individual plants collected from three populations (LZH, HTS and HGX) in Sichuan province, genetic cluster 2 (orange square) consisted of 17 individual plants sampled from LSK population in Guizhou province and genetic cluster 3 (purple square) consisted of 10 individual plants sampled from HSC population in Guizhou province, indicating that these populations are heterogeneous and have distinct genetic structures (Fig. 3b). This classification is supported by the principal coordinate analysis (PCoA).

To further assess the genetic relationships among five *R. nymphaeoides* populations, principal coordinate analysis (PCoA) was performed based on Nei's genetic distance for the 5 populations (Figs. 4) and 79 plant samples (Fig. S1). It was apparent that the clusters identified in principal coordinate analysis (PCoA) were identical to the three genetic clusters, representing the natural distribution of *R. nymphaeoides*. The cumulative variance percentage of the first three axes was 96.63% (Axis

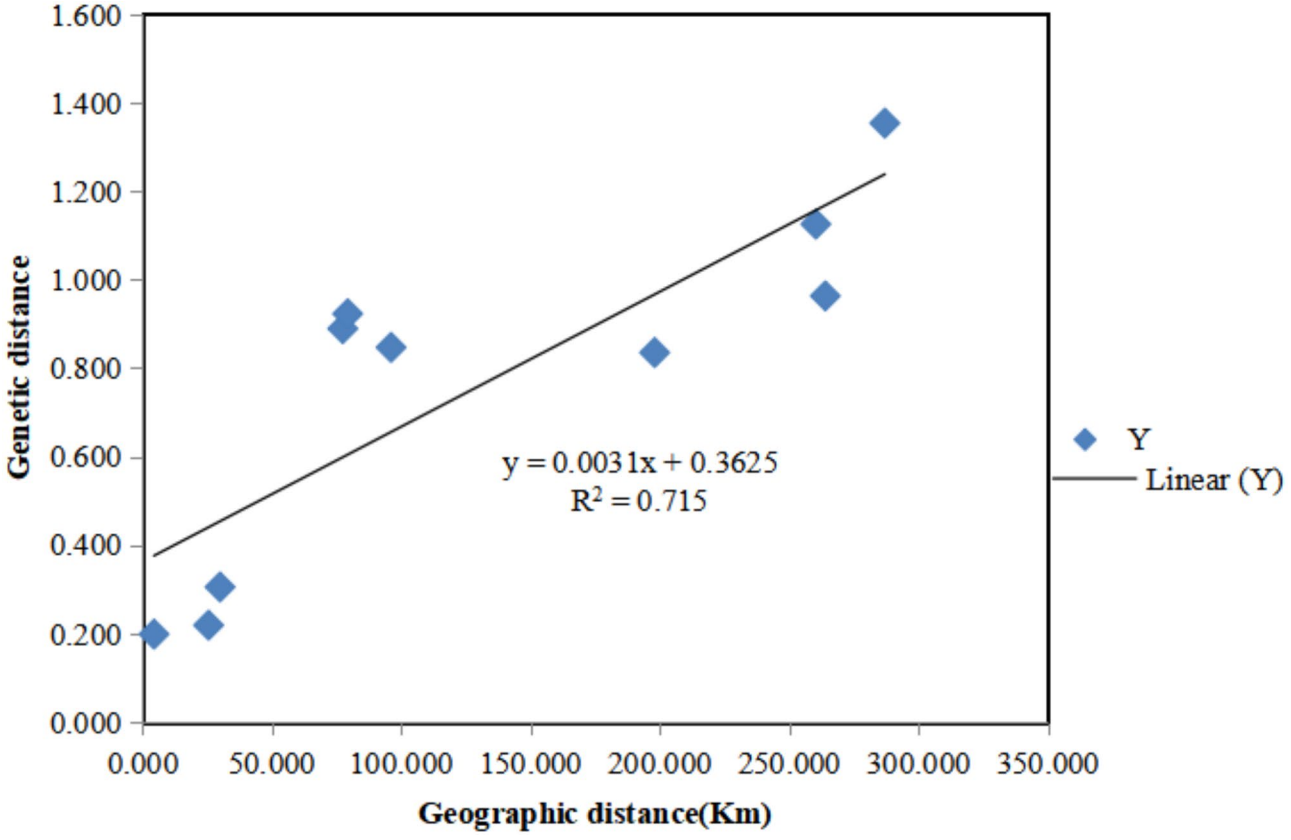

Fig. 2 Correlation test of Genetic distance and Geographic distance for 5 populations of *R. nymphaeoides*

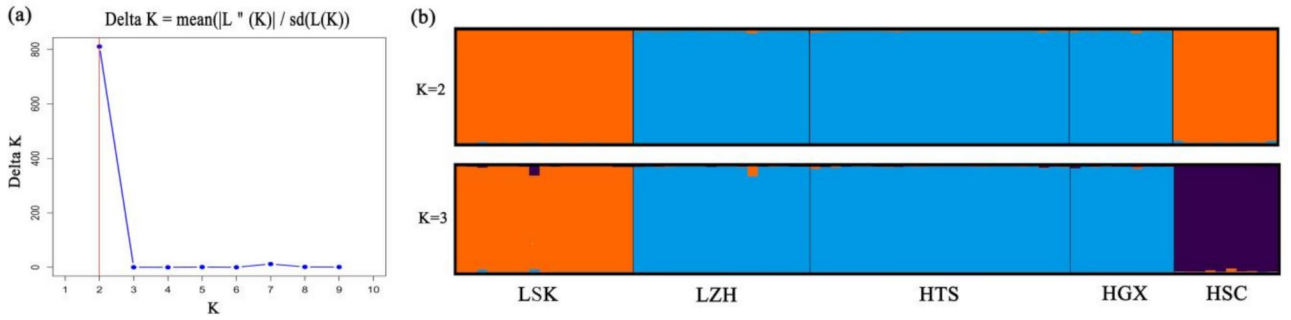

Fig. 3 **(a)** The ΔK method of STRUCTURE analysis plots the change of K values; **(b)** Genetic structure of 5 populations as inferred with STRUCTURE at K = 2 and K = 3; LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

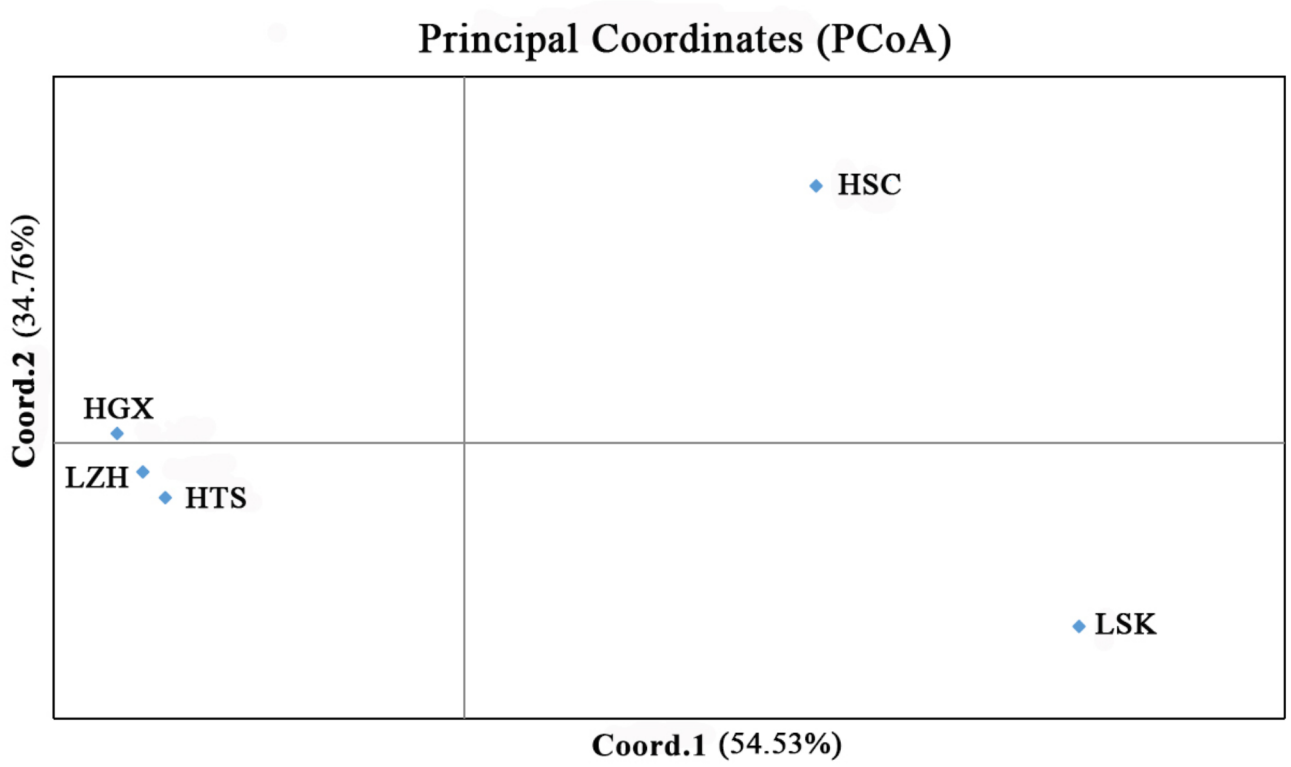

Fig. 4 Principal coordinate analysis (PCoA) for 5 populations of *R. nymphaeoides*. Coord.1 represents the first principal component (54.53%), and Coord.2 represents the second principal component (34.76%); LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

1–54.53%, Axis 2–34.76%, Axis 3–7.34%) (Fig. 3). The closer the distance between two populations in the graph, the smaller genetic background differences between them. And the results of the principal coordinate analysis (PCoA) were consistent with those of the STRUCTURE analysis and supported the UPGMA clustered tree, as described below.

The UPGMA dendrogram was constructed from Nei's genetic distance values and is an accurate reflection of the genetic relationships among and within populations (Fig. 5). The UPGMA dendrogram indicated that the five *R. nymphaeoides* populations could be divided into three major clusters (Fig. 6). This clustering result is consistent with the results of genetic similarity and genetic distance between populations.

Discussion

Studying the genetic diversity within natural populations is crucial for ensuring the ongoing survival, adaptability, and evolutionary potential of endangered plants [25, 26]. Among the various DNA molecular marker techniques, microsatellite marker technology is widely considered one of the most practical methods in population genetics research because it measures codominant alleles and

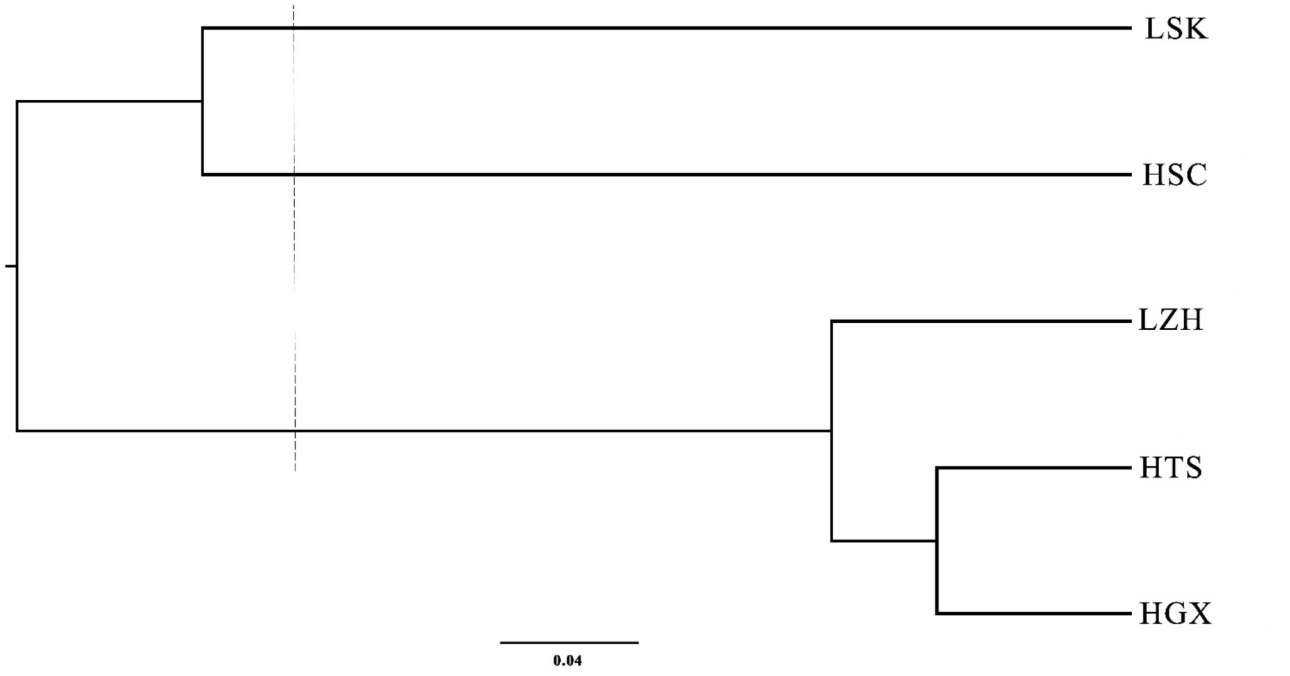

Fig. 5 UPGMA clustering results for five populations. The branch length represents genetic distance, LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

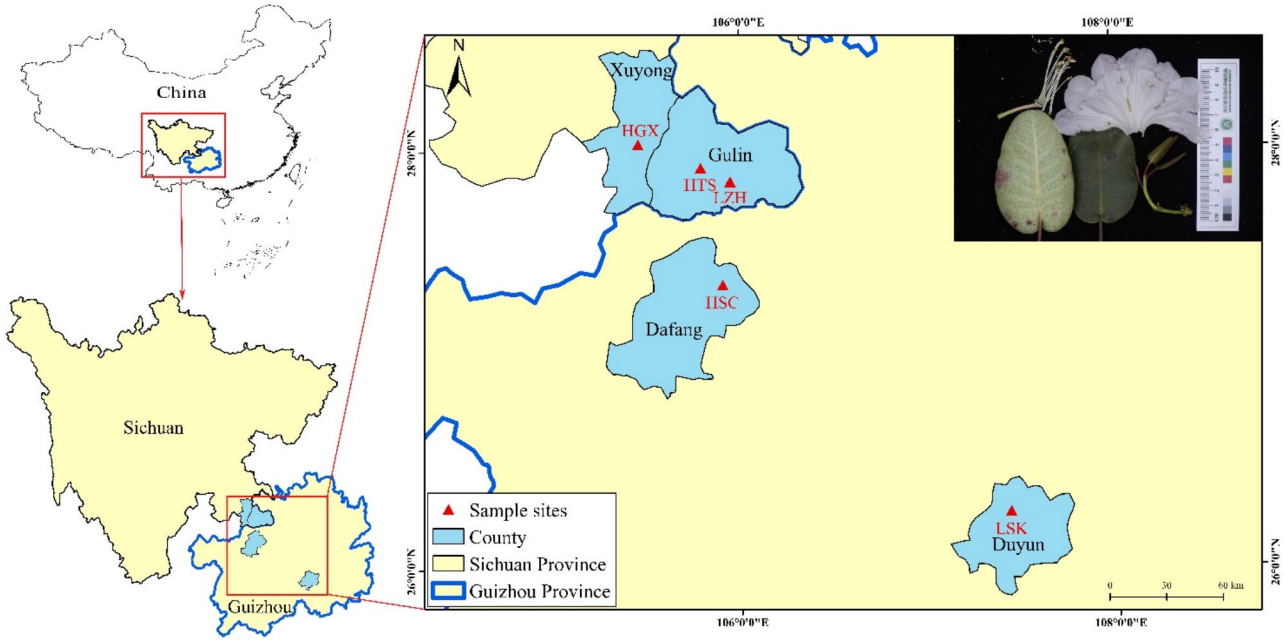

Fig. 6 Geographic locations of *R. nymphaeoides* populations sampled in this study. LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

exhibits a high level of polymorphism [27, 28, 29, 30, 31]. This study utilizes microsatellite markers for the first time to reveal the genetic diversity and population structure of *R. nymphaeoides*, providing a basis for scientifically formulating conservation and management plans for endangered plants. The natural populations of *R. nymphaeoides* maintain a high level of genetic diversity, significant historical gene flow, and reduced recent gene flow, with no genetic bottlenecks experienced under the Two-Phase Model (TPM) assumption. There is substantial genetic differentiation among the populations, and based on STRUCTURE analysis, principal coordinate

analysis (PCoA), and UPGMA analysis, the five natural populations are divided into three genetic clusters. These populations provide valuable genetic resources for the conservation of endangered plants, significantly impacting the scientific formulation of conservation and utilization strategies for *R. nymphaeoides*.

Genetic diversity of populations in *R. nymphaeoides*

Rich genetic diversity is the foundation for species to cope with environmental changes, influenced by factors such as life history or geographical characteristics. The higher the level of genetic diversity, the richer the alleles contained within the species, the stronger its ability to adapt to the environment, and the greater its evolutionary potential [32]. This study utilized 15 pairs of microsatellite markers to evaluate the genetic diversity of five populations of *R. nymphaeoides*. From the level of loci, the number of observed alleles (N_a) average was 14.2667, and the effective number of alleles (N_e) average was 7.0324, with no null alleles (N_{null}) present. This could be due to certain low-frequency alleles becoming randomly fixed due to genetic drift, but the high mutation rate of microsatellites may continuously introduce new rare alleles, maintaining a dynamic equilibrium [33]. The shannon information index (I) had an average value of 2.0692, the polymorphic information index (PIC) averaged 0.7832, and the mean expected heterozygosity (H_e) was 0.8102, indicating a high level of polymorphism at the microsatellite loci and that the *R. nymphaeoides* populations hold a rich store of genetic information. This may be related to the species having a relatively high historical migration rate (average value = 1.0850) and a lower recent migration rate (average value = 0.2849). Genetic bottleneck tests indicated that the populations did not experience a genetic bottleneck under the the Two-Phase Model (TPM) assumption. The isolated status of these populations may be a recent event, and their genetic diversity may not have been significantly eroded by genetic drift yet, as previous gene flow with other groups may have left a rich allele pool [34].

From the population level analysis, the observed heterozygosity (H_o = 0.6375) for all five populations was lower than the expected heterozygosity (H_e = 0.6663), and the fixation index (F) was greater than zero. This indicates that the *R. nymphaeoides* populations have an excess of homozygotes and a deficiency of heterozygotes, with a certain degree of inbreeding present. This not only increases the rate of unfavorable genes becoming homozygous but also causes random fluctuations in gene frequencies, leading to genetic drift, reducing the species' genetic diversity, and decreasing its adaptability to changing environments, ultimately raising the risk of extinction [26]. Therefore, when conserving *R. nymphaeoides*, the issue of inbreeding within the population should also be considered. Additionally, the HTS population exhibits relatively higher shannon information index (I), observed heterozygosity (H_o), and expected heterozygosity (H_e) compared to other populations, which is associated with its larger population size and lower level of human disturbance. Field surveys revealed that the HTS population is located within the Huangjinglaolin Nature Reserve in Gulin County, Sichuan Province, and has the highest number of individuals among the five populations. The local government has designated *R. nymphaeoides* for protected status, significantly reducing human interference, thereby maintaining the highest genetic diversity in the HTS population. Conversely, the HSC population has the lowest shannon information index (I), observed heterozygosity (H_o), and expected heterozygosity (H_e), which correlates with its smaller population size and poorer habitat conditions. Field surveys found that the mature plant count in the HSC population is just over ten, the fewest among the five populations, and is located on steep cliffs at an altitude close to 1700 m. The absence of birds and animals that could aid in seed dispersal severely limits genetic exchange with the external environment, imposing significant selective pressure on the HSC population. Additionally, the high humidity in the mountainous air impedes pollen distribution by wind to distant locations, increasing the difficulty of reproduction and further restricting genetic exchange, leading to the lowest genetic diversity in the HSC population.

Generally, rare and endangered species with narrow geographic distributions have lower genetic diversity than species with wide distributions [34, 35]. However, this study indicates that although *R. nymphaeoides* is a rare and endangered species, it exhibits a relatively high level of genetic diversity (H_o = 0.6375, H_e = 0.6663). This is consistent with findings in some endangered woody plants such as *Parotia subuequalis* (H_o = 0.5200, H_e = 0.5580) [36], *Changiostyrax dolichocarpus* (H_o = 0.594, H_e = 0.6430) [2], and *Phoebe chekiangensis* (H_o = 0.621, H_e = 0.478) [37]. Compared to other species in the Rhododendron genus, such as *Rhododendron dauricum* (H_o = 0.4507, H_e = 0.5710) [38], *Rhododendron protistum* var. *giganteum* (H_e = 0.6020) [39], *Rhododendron cyanocarpum* (H_o = 0.0442, H_e = 0.0675) [40], and *Rhododendron longipedicellatum* (H_e = 0.5590) [41], *R. nymphaeoides* also shows a relatively higher level of genetic variation among endangered species. This outcome may be associated with its breeding system, life form, and natural selection. Firstly, plants with different breeding system types exhibit significant differences in levels of genetic diversity, with outcrossing species generally having higher diversity than self-pollinating species [42]. Previous studies have shown that most plants in the Rhododendron genus are predominantly outcrossing and rely on pollinating insects [43]. Our field observations also revealed

that pollination in *R. nymphaeoides* is carried out by insects and birds, and its seeds are small and winged, dispersed by wind and gravity, indicating an outcrossing breeding system. Additionally, as a small tree with a long lifespan and evident generational overlap, *R. nymphaeoides*'s stands include several age classes. Each age class, when established, encounters the selective pressures of its time, allowing suitable genes to be retained and accumulate, forming a broad genetic base. Moreover, studies have noted that high genetic diversity can also be preserved in species that were previously continuous or widely dispersed but have recently experienced rapid population reduction and fragmentation. Such high levels of genetic diversity could potentially be derived from ancestral populations [39, 44, 45, 46]. It is hypothesized that *R. nymphaeoides* might have historically been one continuous population that fragmented into the current five smaller populations due to geological and historical events.

Genetic differentiation and gene flow among populations in *R. nymphaeoides*

The genetic differentiation coefficient (F_{ST}) and gene flow (N_m) are commonly used indicators to describe the degree of differentiation between natural populations. There is a negative correlation between gene flow and the genetic differentiation coefficient [47, 48]. If gene flow is low, the genetic exchange between two populations is limited, leading to a higher genetic differentiation coefficient [49, 50].

The genetic relationships between populations indicate that significant genetic divergence exists within natural populations when the genetic differentiation coefficient (F_{ST}) exceeds 0.25, reflecting significant genetic differences [51]. In this study, the average genetic differentiation coefficient (F_{ST}) for *R. nymphaeoides* is 0.2685, suggesting a high degree of genetic differentiation between populations. The analysis of molecular variance (AMOVA) results also support this differentiation, revealing molecular differences within and between populations, with the majority of genetic variation (73%) originating within populations. This could be due to the breeding system of *R. nymphaeoides* and the geographical environments of the populations. In outcrossing and long-lived plants, most genetic variation exists within populations, whereas in self-fertilizing plants, most genetic variation is maintained between populations [52, 53, 54]. *R. nymphaeoides*, being an outcrossing plant, emits fragrances that attract insect pollinators, and its pollen can be dispersed long distances by wind and gravity, facilitating gene flow between different populations. However, the populations of *R. nymphaeoides* are located in Gulin County and Xuyong County in Sichuan Province, as well as Dafang County and Duyun City in Guizhou Province, encompassing major mountain ranges such as Miaoling, Wumengshan, and Daloushan, and major water systems like the Chishui River, Wujiang River, and Yuanjiang River, which create geographical barriers between populations, resulting in significant genetic differentiation. This finding is consistent with the results of Mantel tests.

By calculating the genetic distances between the five populations of *R. nymphaeoides*, it was found that the populations HGX and LSK are the most distantly related, while HTS and LZH are the closest genetically. This corresponds with the calculated geographical distances between the populations, where the distance between HGX and LSK is the greatest (286.958 km), and the distance between HTS and LZH is the smallest (4.464 km). Through Mantel test analysis, a significant positive correlation was found between genetic distance and geographical distance among the populations ($r = 0.8456$, $P = 0.0021$), which conforms to the isolation-by-distance (IBD) model. This further confirms that the high level of genetic differentiation among the *R. nymphaeoides* populations is due to geographical barriers separating different gene pools. Therefore, geological changes and varying habitat conditions play a crucial role in the genetic differentiation of populations [55]. provided a more detailed exposition on the effects of climate change and plant movement, noting that the severe impact of Pleistocene glaciations significantly affected plant survival. During this period, a large number of plants died because they could not adapt to the extreme harsh climates, but some plants survived owing to the existence of refugia. Furthermore, according to the substitutional evolution hypothesis proposed by [56, 57], which suggests that the emergence of barriers led to the fragmentation of ancestral distribution areas, this can explain the discontinuous distribution patterns. It is inferred that the current genetic differentiation and distribution patterns of the *R. nymphaeoides* populations may be related to geological and climatic changes.

Gene flow is crucial for the stability of population genetic structure, and alterations in gene flow reflect changes in population numbers, environmental factors, and population structure on genetic structure. Particularly, the recent or contemporary gene flow determines the future genetic structure of populations. Hence, for the conservation of endangered plants, recent gene flow may be more critical than historical gene flow [58, 59]. In this study, historical gene flow (N_m), derived through the genetic differentiation coefficient (F_{ST}), had an average value of 1.2607, greater than 1. Utilizing migration version 3.6.4 software, the average historical migration rates estimated among five populations was 1.0850, also exceeding 1. Meanwhile, recent migration rates between these populations, estimated using BayesAss version

3.0.0 software, had an average value of 0.2849, less than 1. This indicates that *R. nymphaeoides* has a higher historical gene flow and lower recent gene flow. The current five isolated populations have affected the species dispersion, potentially leading to substantial genetic differentiation among these populations in the future. The lower recent gene flow also suggests that current seed and pollen flow are obstructed [60], reconfirming the geographic barriers between populations. Given the small size of these five populations, this might further lead to genetic drift in small populations, thereby reducing the species' genetic diversity levels, and possibly leading to inbreeding depression. Additionally, genetic bottleneck tests suggest that under the Two-Phase Model (TPM) assumption assumption, the five populations conform to the mutation-migration model, indicating that they have not experienced genetic bottlenecks and genetic drift has not yet become a major factor affecting the genetic diversity of *R. nymphaeoides*. Under the Stepwise Mutation Model (SMM) assumption, only the HTS population deviated from mutation-migration equilibrium, suggesting a historical genetic bottleneck, yet this population has the highest genetic diversity, indicating that the bottleneck event might not have been long enough to significantly impact the population's genetic diversity. However, *R. nymphaeoides* populations are now affected by fragmentation and vandalism, with lower contemporary gene flow, making future descendant populations likely to experience gene flow bottlenecks reducing their genetic diversity levels [61].

Population structure and genetic relationships

In genetic diversity analysis, it is common to use a combination of methods to achieve more comprehensive results and interpretations [59, 62]. STRUCTURE analysis software can infer the genetic structure of populations using genetic markers, while principal coordinate analysis (PCoA) and UPGMA analyses can cluster populations based on genetic distances or dissimilarities, constructing hierarchical clustering dendrograms that visually represent the differences between samples. Therefore, we believe that combining principal coordinate analysis (PCoA), STRUCTURE analysis, and UPGMA analysis can better reveal the genetic structure of the populations.

In this study, although the STRUCTURE analysis indicated that the maximum value of delta K was at K = 2, combining this with the genetic differentiation coefficient (F_{ST}) suggests that it is more reasonable to classify the five populations of *R. nymphaeoides* into three genetic clusters. This conclusion was further validated by principal coordinate analysis (PCoA). Based on Nei's genetic distance, principal coordinate analysis (PCoA) was conducted on five populations and 79 plant samples. It was found that the genetic distance between the HSC and LSK populations was relatively large (0.8351), with a relatively high genetic differentiation coefficient (0.3901), indicating they should be classified into two different genetic clusters. The cumulative percentage variance of the first three axes in the principal coordinate analysis (PCoA) was 96.63%, suggesting that these axes captured most of the genetic variation. The remaining 3.37% of the variance can be attributed partly to genetic drift within small populations, which may accelerate the random loss or fixation of rare alleles, leading to subtle genetic differences among individuals. These differences might be distributed along subsequent principal coordinate analysis (PCoA) axes but are overlooked by the first three axes due to their low contribution. Additionally, these populations are distributed across different habitats; local adaptation might drive variations in functional genes near microsatellites. These adaptive variations might be weakly distributed along subsequent principal coordinate analysis (PCoA) axes but are not sufficiently captured by the first three axes due to the resolution limitations of the neutral markers (microsatellites). The UPGMA clustering tree also supports the principal coordinate analysis (PCoA) results, showing that there are three independent evolutionary units within the *R. nymphaeoides* populations, corresponding to the analysis results of geographic distance, genetic distance, gene flow, and Mantel test among the five populations. The HTS, LZH, and HGX populations, all located in the southeastern part of Sichuan Province (Gulin and Xuyong counties), are geographically close with consistent environmental conditions and frequent gene flow, and are classified into one genetic cluster. Meanwhile, the LSK population is positioned in Duyun city, Guizhou Province, at the greatest geographic distance from the HGX population, with the HSC population positioned in between these two in Dafang County, Guizhou Province, experiencing lower gene flow, hence classified into two distinct genetic clusters. This further confirms the presence of geographic barriers within the *R. nymphaeoides* populations.

Conservation of populations

Preserving the genetic diversity of species is crucial to preventing the loss of unique germplasm, particularly for rare and endangered plants like *R. nymphaeoides*, where maintaining species diversity and long-term survival holds significant importance. It is evident that the evolutionary potential, resistance to adverse environments, ecosystem resilience, and stability of a species are all contingent upon the magnitude of genetic diversity [63]. Thus, genetic variation information within and between populations of endangered and rare plants plays a vital role in formulating conservation and management strategies [64]. Although *R. nymphaeoides* populations possess high genetic diversity, their distribution range

Table 10 The sampling information of 5 populations of *R. nymphaeoides*

Population	Latitude(°*N*)	Longitude(°E)	locality	Altitude(m)	Gradient(°)	Sample size
LSK	26.250237°N	107.396605°E	Duyn, Guizhou	1643	35	17
HSC	27.421144°N	105.893159°E	Dafang, Guizhou	1696	50	10
LZH	28.112746°N	105.802008°E	Gulin, Sichuan	1559	40	17
HTS	28.124544°N	105.758501°E	Gulin, Sichuan	1708	47	25
HGX	28.220467°N	105.521633°E	Xuyong, Sichuan	1533	30	10

LSK: Luosike population; HSC: Heshancun population; LZH: Longzhaohe population; HTS: Hutoushan population; HGX: Huagaoxi population

is limited, population sizes are small, and recent gene flow is low. To effectively maintain the genetic diversity of *R. nymphaeoides* and to achieve population restoration, we propose the following recommendations: First, based on analysis of molecular variance (AMOVA) results, genetic variation principally originates within populations, with each population potentially containing unique allelic combinations. Genetic clustering has also classified the five populations into three evolutionary units. Therefore, we recommend establishing three conservation management units corresponding to these evolutionary units. Given that the HTS, LZH, and HGX populations are already within nature reserves, conservation management units for the LSK and HSC populations should also be established promptly to strengthen the protection of this endangered species. Second, the HTS and LZH populations, which exhibit the richest genetic diversity, should be prioritized during germplasm collection and reintroduction efforts. By designing seed collection strategies and establishing a seed germplasm bank, samples from each population across the entire natural geographical distribution of the different genetic clusters should be collected. Additionally, plant tissue culture techniques should be utilized to preserve germplasm. Third, a strategy combining ex-situ conservation with in situ reintroduction should be implemented. By assessing the ecological factors of the current habitats of *R. nymphaeoides* and identifying suitable habitable areas, near-site translocation conservation of seedlings can be conducted to expand the distribution of *R. nymphaeoides* populations. Further research on the asexual reproduction techniques of *R. nymphaeoides*, such as tissue culture and cutting, should be pursued to achieve rapid propagation. After transplanting seeds collected from the field, seedlings should be introduced back into their original locations to revitalize the populations and facilitate the natural return of artificially established populations, ultimately preventing the extinction of this species.

Conclusions

This study is the first to reveal the genetic diversity and population structure of *R. nymphaeoides* using microsatellite markers, providing a basis for the scientific formulation of conservation and management strategies for endangered plants. The natural populations of *R. nymphaeoides* maintain a high level of genetic diversity, yet due to geographical barriers, have also maintained a high level of genetic differentiation, with the majority of genetic variation arising within populations. *R. nymphaeoides* exhibits higher historical gene flow and lower contemporary gene flow; seed and pollen flow are impeded, and there is a certain degree of inbreeding within populations. Under the Two-Phase Model (TPM) assumption, the species has not experienced genetic bottlenecks. Future generations may face a decline in genetic diversity due to genetic drift. The five natural populations have been divided into three genetic clusters, for which corresponding conservation management units should be established to strengthen the protection of this species.

The primers used in this study are suitable for the majority of *Rhododendron* species and can be applied to research on the population structure, genetic diversity, germplasm collection, and conservation strategies of *Rhododendron* plants. They provide important information about the genetic structure, which will reduce the cost of primer development and enable faster research through microsatellite markers. This will provide scientific basis for the conservation, development, and utilization of *Rhododendron* resources.

Methods

Plant materials

After years of investigation, we have identified five wild populations of *R. nymphaeoides*, including the Longzhaohe population (LZH) and the Hutoushan population (HTS) located in Gulin County, Sichuan Province; the Huagaoxi population (HGX) in Xuyong County, Sichuan Province; the Luosike population (LSK) in Duyun City, Guizhou Province; and the Heshancun population (HSC) in Dafang County, Guizhou Province. These are currently the only five known wild populations of *R. nymphaeoides*. To better study the genetic diversity among these populations, we collected 79 individuals from these five populations in the spring of 2024. The sample collection followed the principles and methods of population genetics, adhering to the principles of representativeness and comparability (with a minimum spacing of 5 m between samples). Details of the sampling are listed in Table 10; Fig. 6. Fresh leaves were collected, placed in test tubes, and stored in a liquid nitrogen tank. They were

then transported to the laboratory and stored in a -80 °C ultra-low temperature freezer for DNA extraction. The collection of plant materials complied with institutional, national, or international guidelines. The formal identification of the samples used in this study was performed by Xiao-Yong Dai of taxonomist of the genus Rhododendron spp. Voucher specimens were deposited in the Herbarium of Guizhou Provincial Academy of Forestry (GF), the deposition number was DY20210411 and DF20220428 and GL20240922-1 and GL20240922-2 and XY20240924.

DNA extraction and PCR amplification

First, total genomic DNA was extracted from *R. nymphaeoides* leaf material using a plant genomic DNA extraction kit (200) (Qingke Biotechnology Co., Ltd.; TSP101-200) for microsatellite marker analysis. The DNA concentration and quality were determined using 1% agarose gel electrophoresis and spectrophotometry methods, respectively. The extracted DNA was diluted to 50 ng/μl and then stored at -20℃. From the *R. nymphaeoides* samples, ten individuals were randomly selected for screening with developed primers of the evergreen *Rhododendron* subgenus. The data for accurate locus analysis were processed using the GeneMapper 4.1 software. A total of 15 pairs of highly polymorphic microsatellite primers [19, 20, 21, 22] were selected for further analysis. Next, based on these 15 polymorphic primers, forward primers labeled with FAM, HEX, ROX, and TAMRA fluorescent dyes were synthesized at the 5′ end. PCR amplification and capillary electrophoresis analysis were then conducted for 79 samples. The amplification system was as follows: The PCR reaction system consists of 17 μl of Golden Mix (Green) (Qingke Biotech Co., Ltd.; TSE101), 1 μl of 10 μM Primer F, 1 μl of 10 μM Primer R, and 1 μl of Template (gDNA), making a total volume of 20 μl. The PCR reaction program is as follows: 98 ℃ for 2 min, one cycle; 98 ℃ for 10 s, annealing temperature (Tm) for 10 s, 72 ℃ for 10 s, 35 cycles; 72 ℃ for 5 min, one cycle. The amplified PCR products are subjected to agarose gel electrophoresis at 300 V for 12 min to obtain the gel image. The gel image is used to determine the template concentration, and then it is diluted with water to the required concentration for capillary electrophoresis. Finally, specific and polymorphic loci with high specificity and good polymorphism are selected for statistical analysis.

Data analyses

To facilitate better data analysis, data correction and organizing are required. The specific process is as follows: the specific bands of each individual are counted based on band size (bp). Utilizing the peak charts analyzed by GeneMapper 4.1 [65], bands were selected based on the criterion that peaks at the same locus were similar in shape and free from interference by additional non-specific peaks. The usable data were then used to construct an original data matrix.

Using Popgen32 and GenAlEx version 6.5 software [66, 67], various genetic diversity indices at both the locus and population levels were calculated, including the number of observed alleles (N_a), effective number of alleles (N_e), null alleles (N_{null}), shannon information index (I), polymorphic information index (PIC), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{is}), genetic differentiation coefficients (F_{ST}) and genetic distance (GD). The Hardy-Weinberg equilibrium (HXE) and linkage disequilibrium tests were conducted using the online software Genepop v.4.7 (https://genepop.curtin.edu.au/). Based on the genotype data of each individual at multiple microsatellite loci, the total number of unique genotype combinations across all individuals was counted. The 'vegan' package in RStudio was utilized to construct a Genotype Accumulation Curve, which was used to analyze whether the current sample size covers the majority of genotypes. This evaluation helped determine whether the 15 pairs of primers selected are adequate for analyzing the genetic diversity of the *R. nymphaeoides* populations [68].

The population genetic structure of this experiment was analyzed using the Bayesian model-based clustering method in STRUCTURE version 2.3.3 software [69]. The Markov Chain Monte Carlo (MCMC) method was employed, which allows for pre-setting the number of population groups (K) and simultaneously calculates, samples, and assigns individuals based on allele frequencies. The parameters were set with K values ranging from 1 to 10, and 10 independent runs were performed for each K value, with 100,000 iterations per run. Finally, the most appropriate K value was determined using Structure Selector (https://lmme.ac.cn/StructureSelector/) [70].

The genetic distances were calculated using the unweighted pair-group method with arithmetic means (UPGMA) to construct a clustering tree for the individuals [71]. Specifically, the UPGMA tree was constructed in the Populations-1.2.30 software with 1000 bootstrap replicates. The beautification and editing of the clustering tree were performed in the FigTree version 1.4.2 software. Additionally, the principal coordinate analysis (PCoA) was performed using GenAlEx version 6.5 to further analyze the phylogenetic relationships between populations [71].

Based on the latitude and longitude coordinates of each population, geographic distance (GGD) between populations were calculated using the online geographic distance calculator (https://www.hhlink.com/). The geographic distances were then combined with the genetic distance (GD) between populations to perform a Mantel test and construct an isolation-by-distance (IBD) model.

According to the results of the analysis of population genetic structure, variation and differentiation between populations were calculated using GenAlEx version 6.5 software. Significance tests were conducted [69].

The historical gene flow (N_m) was calculated using formula: N_m = (1 - F_{ST})/4F_{ST} [72]. The historical migration rates among populations were estimated using the Maximum Likelihood method in the Migrate-n v3.6.4 software, to assess the long-term gene flow among the five populations of *R. nymphaeoides*. Within the software, we selected the Brownian motion model. The settings involved running a long chain for three repetitions, recording every 10,000 generations, and short chains were set to run ten times, recording every 1,000 generations, with other settings at default [73]. Recent migration rates among populations were estimated using the BayesAss v3.0.0 software, to evaluate the recent gene flow among the five populations of *R. nymphaeoides*. This software employs a Bayesian Markov chain Monte Carlo approach (MCMC), based on the assumption that populations are in linkage equilibrium and have not experienced genetic drift in the past 2–3 generations at the time of sampling, with all parameters set to default [74].

The software BOTTLENECK v.1.2.02 was used to analyze whether the five populations of *R. nymphaeoides* have recently experienced a genetic bottleneck effect. The settings included a variance of 30, a probability of 90.00%, and 1000 repetitions. The sign test and Wilcoxon test were applied to determine whether there was a significant excess or deficiency of heterozygotes. The analysis assessed compliance with the mutation-migration model under the Two-Phase Model (TPM) and Stepwise Mutation Model (SMM) assumptions. Additionally, the mode-shift graphical method was used to analyze whether the five populations conform to a normal L-shaped distribution [75].

Abbreviations

AFLP	Amplified fragment length polymorphism
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
ISSR	Inter simple sequence repeat
SSR	Simple sequence repeat
SNP	Single nucleotide polymorphism
AMOVA	Analysis of molecular variance
PCoA	Principal coordinate analysis

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

JL and XYD designed the experiments.JL, XYD, JC, SH, CJY and DLL carried out the experiments.JLanalyzed the data and wrote the main manuscript. All authors have read and approved the final manuscript.

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Data availability

The sequencing data of the 15 polymorphic microsatellite primers were listed in the manuscript, and no other DNA sequences were applied to this study.

Declarations

Ethics approval and consent to participate

Because this study was carried out on an endangered species, I confirmed that I complied with all relevant institutional, national and international guidelines. This study was supported by Guizhou Provincial Natural Science Foundation, including handling these plants and collecting samples.

Consent for publication

Not applicable.

Clinical trial number

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Guizhou Academy of Forestry, Guiyang, Guizhou 550005, China
[2]Guizhou Libo Karst Forest Ecosystem National Observation and Research Station, Libo, Guizhou 558400, China
[3]Key Laboratory of National Forestry and Grassland Administration on Bioaffiliationersity Conservation in Karst Mountainous Areas of Southwestern China, Guiyang, Guizhou 55005, China
[4]College of forestry, Guizhou University, Guiyang, Guizhou 550025, China

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