GENETIC DIVERSITY ANALYSIS OF *PAPHIOPEDILUM MALIPOENSE* AND *PAPHIOPEDILUM MICRANTHUM* BASED ON SSR MOLECULAR MARKERS

LU, L.[1,2] – ZOU, R.[2] – LI, X.[1,2] – HE, G. H.[2,3] – TANG, F. L.[2] – LI, J. W.[1] – TANG, J. M.[2*] – WEI, X.[1,2,3*]

[1]*Key Laboratory of the Ministry of Education for Ecology of Rare and Endangered Species and Environmental Protection, Guangxi Normal University, Guilin 541004, China*

[2]*Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization, Guangxi Institute of Botany, Guilin 541006, China*

[3]*College of Tourism and Landscape Architecture, Guilin University of Technology, Guilin 541006, China*

**Corresponding authors*
e-mail: 18877384841@163.com; weixiao@gxib.cn

(Received 13th Nov 2024; accepted 5th Feb 2025)

Abstract. *Paphiopedilum* plants are over - collected due to their attractive features and habitat destruction threatens wild populations. This study focused on the first - class protected *Paphiopedilum malipoense* and second - class protected *Paphiopedilum micranthum*, using SSR technology to study genetic diversity and structure for germplasm protection. Results showed *Paphiopedilum malipoense* had low genetic diversity, while *Paphiopedilum micranthum* had high genetic diversity with most variation within individuals. Clustering was not strictly by location. Based on these, in - situ, ex - situ protection, regression conservation and artificial breeding are recommended to protect resources and raise public awareness.
Keywords: *Paphiopedilum malipoense, Paphiopedilum micranthum, wild populations, SSR makers, genetic differentiation*

Introduction

Paphiopedilum is a perennial herb in the *Paphiopedilum* family (Cypripedaceae). There are about 80 species in *Paphiopedilum*, and about 27 species in China, mainly distributed in Yunnan, Guizhou, Guangxi and other places (Liu et al., 2009; Yin et al., 2022). *Paphiopedilum* plants have unique appearance, large and colorful flowers, and long flowering period, which contribute to high ornamental value and are sought after by enthusiasts at home and abroad (Zhang et al., 2024) In recent years, the wild resources of *Paphiopedilum* have been seriously threatened due to excessive excavation and habitat destruction (Long and Long, 2006). At present, the plants of this genus are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and their trading is prohibited (Zhang et al., 2024; Long and Long, 2006). *Paphiopedilum malipoense* is the most primitive type of *Paphiopedilum*, representing a transitional group between *Cypripedium* to *Paphiopedilum* (Liu et al., 2002). It is characterized by short rhizomes with a few fleshy hairy fibrous roots; basal leaves are lingual, dorsal purple or purple spots, with hairy edges; flowers yellow or pale green, with purple stripes (Zhou, 2017). *Paphiopedilum malipoense* is distributed in the southeast of Yunnan, the west of Guangxi and the northwest of Guizhou in China. It was born in the limestone slope of 800-1600 m above sea level (Wang, 2015). *Paphiopedilum micranthum* is one of the most widely distributed species in the *Paphiopedilum Subgen*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

(Zhang et al., 2021). It is distributed in the shady areas of limestone mountainous areas with shallow soil layers in southwest Guizhou, southeast Yunnan and north Guangxi (Zhang et al., 2022; Li and Li, 2016). *Paphiopedilum malipoense* and *Paphiopedilum micranthum* are distributed in karst areas with fragile ecological environment and are characterized by cluster distribution. They are vulnerable to local extinction and thus lead to a decline in genetic diversity (Luo et al., 2003a).

Simple Sequence Repeat (SSR) molecular markers, also known as microsatellite markers, have the characteristics of rich polymorphism, high repeatability, co-dominant inheritance, mature technology and simple analysis (Luo et al., 2010). It is widely used in genetic diversity research, variety identification, genetic pedigree construction, molecular marker assisted selection breeding and other fields (Wang et al., 2017; ITOO et al., 2023; Li et al., 2002b; Antanynienė et al., 2023). At present, the research on *Paphiopedilum malipoense* and *Paphiopedilum micranthum* mainly focuses on organelle genome sequence (Li et al., 2019; Yang et al., 2023), DNA extraction (Shao et al., 2020), photosynthetic physiology (Zhang et al., 2014), rhizosphere microorganisms (Li et al., 2023), germplasm resources protection (Yang et al., 2021), etc. However, the research on genetic diversity and genetic structure is still insufficient. It is urgent to carry out the evaluation of germplasm genetic diversity and promote the sustainable development of its wild resources. In this study, 100 samples of 5 wild populations of *Paphiopedilum malipoense* and 129 samples of 6 wild populations of *Paphiopedilum micranthum* were used as research materials. SSR molecular marker technology was used to analyze the genetic diversity and genetic structure of *Paphiopedilum malipoense* and *Paphiopedilum micranthum*. The utilization and protection of wild resources provide theoretical support and scientific basis.

Materials and methods

Materials

A total of 100 samples of Paphiopedilum malipoense were collected from Yizhou Liusanjie Township (MLPDL-YZLSJX), Xiazhai Houshan (MLPDL-HJHS), Xiazhai Haodong (MLPDL-HJHD), Xiabaishan (MLPDL-HJXBS) and Tianlin (MLPDL-TL) in Huanjiang Mulun Nature Reserve. A total of 129 samples of Paphiopedilum micranthum were collected from Leye Lanjiawan (YY-LYLJW), Liusanjie Township (YY-YZLSJX), Tiane Longtan Grand Canyon Forest Park (YY-TELT), Liaowangta (YY-HJLWT) in Huanjiang Mulun Nature Reserve, Yongfu Xiadong Village (YY-YFDXC) and Tianlin Station (YY-TLZ). The sample collection location was plotted using GIS (*Fig. 1*). A total of 3-10 healthy and disease-free leaves were collected from each sample. The fresh leaves were stored in a sealed bag containing silica gel, and the specific information of each sample was recorded. They were identified as Paphiopedilum malipoense (Orchidaceae) and Paphiopedilum micranthum (Orchidaceae) by Wei Xiao, a researcher of Guangxi Institute of Botany, Chinese Academy of Sciences, Guangxi Zhuang Autonomous Region.

Methods

DNA extraction

From the primers developed by Xu et al. (2018) in *P. henryanum*, 10 pairs of primers with successful amplification and good peak shape were used to analyze the genetic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

diversity of *P. malipoense* and *P. micranthum* (*Table 1*). PCR amplification system: 2×Taq PCR Master Mix 5 uL, DNA 1 uL (-20 ng), The upstream and downstream primers were 0.5 uL each (10 pmol/uL), ddH_2O 3 uL. PCR amplification procedure: Pre-denaturation at 95℃ for 5 min, denaturation at 95℃ for 30 s, annealing at 62℃-52℃ for 30 s, extension at 72℃ for 30 s, reaction cycle 10 times, each cycle decreased by 1℃; denaturation at 95℃ for 30 s, annealing at 52℃ for 30 s, extension at 72℃ for 30 s, repeated 25 cycles; 72℃ terminal extension for 20 min; heat preservation at 4℃. The SSR genotypes were read on the ABI 3730 XL genetic analyzer using GeneMapper v4.1 software.

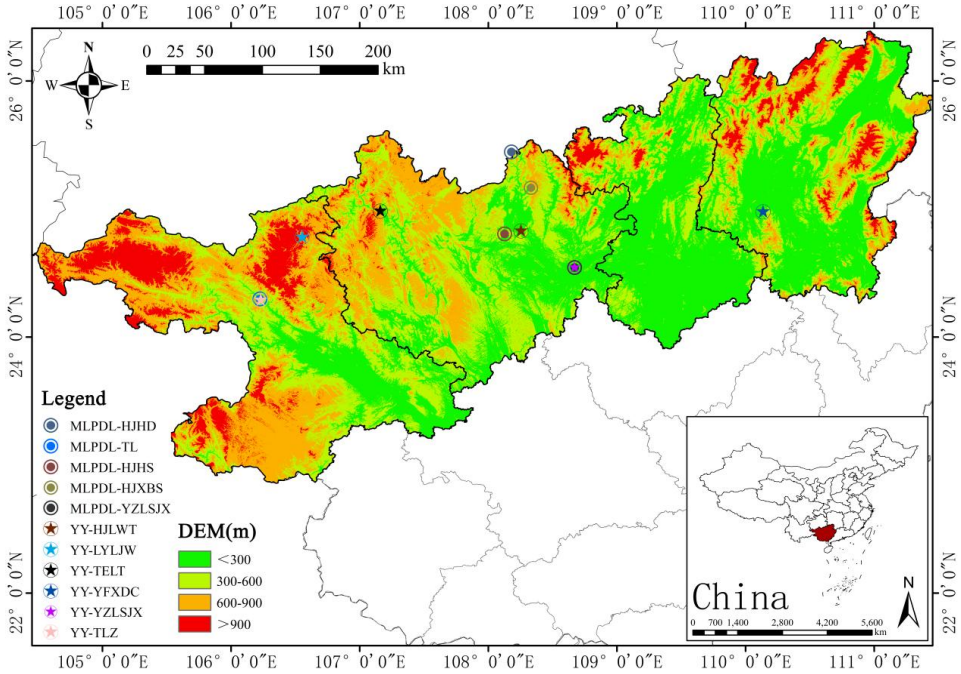

Figure 1. *Sample collection location*

Table 1. *10 pairs of SSR primer information*

Locus	Repeat unit	Upstream primer	Downstream primer	Allelic interval
DL014	(CTC)6	TTCCTTCCCTACCCTTTCCA	CAGCGGTGTCGTTGATGTT	259-267
DL020	(GCC)6	GGCCAAGTACATGCACCCAT	TTCCCACCTCGGTTATGCAC	290-314
DL021	(CAG)6	GCAAATCCATTCAGCCCTGC	CGACATGGTCTGAGAGGAGC	95-219
DL023	(AGA)6	CTTGGGACTCTTTCCTCGGC	CCAGGAGGCTCTCAGCTTTC	235-270
DL030	(CCG)6	CAGGTTGACAGCAATGTCGC	GCCGCAGCTTTTCGGATAAG	202-208
DL032	(AAAC)5	AGCGTGTTTGGACTAGAGCA	TCGGGGATGCACATGGAAAA	249-284
DL034	(CGG)6	GGGTGGGGAGAGTAGGAGTT	GCCACAACTTGTTTTCCCGG	239-251
DL036	(CGT)6	CCACGTGTGACAGAATCCCA	GGCTCCCGACGAGGAATTAC	246-255
DL039	(ATC)6	CCACCAGCTTTCATATCCTCCA	GCCCATGCTGTGCAAAAAGA	223-259
DL040	(TCT)6	AAGAAGTGGCTTCCATGGCA	GCAAAACCAAGGTGTCGTCC	227-242

Data analysis

Genetic diversity analysis

Gene AIEx version 6.501 software was used to calculate the genetic diversity indexes of SSR loci and populations, including observed alleles (N_a), effective alleles

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

(N_e), Shannon index (I), polymorphism information index (PIC), observed heterozygosity (H_o), expected heterozygosity (H_e) and fixed index (F) (Peakall and Smouse, 2012).

Analysis of molecular variance (AMOVA) and gene flow estimation

According to the results of population genetic structure analysis, Gene AIEx version 6.501 software was used to calculate the changes and differentiation among and within populations, and the genetic differentiation coefficient (F_{st}) and gene flow (N_m) were calculated. According to the formula $N_m = 0.25 (1-F_{st}) / F_{st}$ (Wright, 1931), Origin 2024 software was used to plot.

Analysis of population genetic structure

STRUCTURE 2.3.4 software was used to analyze the population structure of 100 samples of *P. malipoense* and 129 samples of *P. micranthum*. K = 1~20, Burn-in period was 1000, MCMC (Markov Chain Monte Carlo) was set to 100000, and each K value was run for 20 times. The online tool STRUCTURE HARVESTER was used to calculate the optimal △K value to determine the optimal population stratification and plot according to the △K value. Principal coordinate analysis (PCoA) was performed using GenAIex software to represent the genetic distance between individuals (Zhang et al., 2023).

Phylogenetic analysis

The Nei's genetic distance between two samples was calculated by powermaker v 3.25 software. Based on the Nei's genetic distance matrix, the genetic differentiation coefficient and geographical distance were tested by Mantel test using R software, and the Spearman correlation coefficient was calculated. Finally, the ggplot package of R software draws a linear relationship between geographic distance and genetic distance.

Results and analysis

Polymorphism analysis of SSR primers

SSR polymorphism analysis was carried out on 100 *P. malipoense* samples and 129 *P. micranthum* samples using 10 pairs of primers (*Table 2*). The results showed that a total of 32 observed allele (N_a) were detected in *P. malipoense* samples and the distribution of observed allele number was 1 (DL014) ~ 8 (DL032); The effective allele number (N_e) was 12.023, with an average of 1.2 023; The average value of Shannon's information index (I) was 0.2 692; The observed heterozygosity (H_o) values were between 0 (DL014, DL040) ~ 0.28 (DL020), and the average value of 0.0 701 indicated that the population heterozygosity was low; The expected heterozygosity (H_e) ranged from 0 (DL014, DL040) ~ 0.394 (DL020), and the average value was 0.1 303, indicating that the genetic diversity was low; The fixation index (F) ranged from-0.037 (DL021) to 0.786 (DL032). The polymorphism information content (PIC) of SSR loci ranged from 0 (DL014) to 0.408 (DL032), with an average of 0.1 197. A total of 64 observed alleles (N_a) were detected in *P. micranthum* samples, and the number of observed alleles ranged from 2 (DL040) to 13 (DL032); The effective allele number (N_e) was 20.308, with an average of 2.0 308; The average value of Shannon's

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

information index (*I*) was 0.7 355; The observed heterozygosity (H_o) ranged from 0.031 (DL040) to 0.696 (DL032), with an average of 0.224; The expected heterozygosity (H_e) ranged from 0.031 (DL040) to 0.807 (DL032), with an average of 0.3 263; The fixation index (*F*) ranged from-0.03 (DL036) to 0.587 (DL032); The polymorphism information content (PIC) ranged from 0.031 (DL040) to 0.783 (DL032), with an average of 0.3097. The results showed that most of the SSR primers used in this study had good polymorphism and could be used to analyze the genetic diversity of *P. malipoense* and *P. micranthum*. Among them, the locus DL032 performed best in various genetic indicators, and the polymorphism of DL040 was significantly lower than that of other primers.

Table 2. *Polymorphism of SSR primers*

Species	Locus	N_a	N_e	*I*	H_o	H_e	*F*	PIC
P. malipoense	DL014	1	1	0	0	0	0	0
	DL020	4	1.649	0.734	0.28	0.394	0.289	0.36
	DL021	3	1.084	0.183	0.08	0.077	-0.037	0.075
	DL023	2	1.01	0.031	0.01	0.01	-0.005	0.01
	DL030	2	1.01	0.031	0.01	0.01	-0.005	0.01
	DL032	8	1.877	0.886	0.1	0.467	0.786	0.408
	DL034	3	1.084	0.196	0.02	0.078	0.742	0.076
	DL036	4	1.144	0.304	0.051	0.126	0.594	0.122
	DL039	4	1.165	0.327	0.15	0.141	-0.06	0.136
	DL040	1	1	0	0	0	0	0
	Mean	3.2	1.202	0.2 692	0.0 701	0.1 303	0.2 304	0.1 197
P. micranthum	DL014	5	1.243	0.471	0.085	0.196	0.564	0.19
	DL020	7	2.348	1.082	0.357	0.574	0.379	0.502
	DL021	3	1.297	0.453	0.178	0.229	0.222	0.213
	DL023	10	1.182	0.443	0.085	0.154	0.447	0.152
	DL030	3	1.134	0.275	0.047	0.118	0.607	0.115
	DL032	13	5.185	1.901	0.696	0.807	0.137	0.783
	DL034	9	1.516	0.799	0.141	0.34	0.587	0.325
	DL036	3	1.047	0.128	0.047	0.045	-0.03	0.045
	DL039	9	4.323	1.721	0.573	0.769	0.255	0.741
	DL040	2	1.033	0.082	0.031	0.031	0.015	0.031
	Mean	6.4	2.0 308	0.7 355	0.224	0.3 263	0.3 183	0.3 097

Genetic diversity analysis

The genetic diversity index among populations showed (*Table 3*). The observed allele number (N_a), effective allele number (N_e), Shannon's information index (*I*), observed heterozygosity (H_o) and expected heterozygosity (H_e) of *P. malipoense* were 1.74, 1.216, 0.204, 0.080 and 0.113, respectively. The observed allele number (N_a), effective allele number (N_e), Shannon's information index (*I*), observed heterozygosity (H_o) and expected heterozygosity (H_e) of *P. micranthum* were 2.77, 1.693, 0.502, 0.224 and 0.262. At the population level, the observed allele number (N_a) of the 11 populations ranged from 1.1 (MLPDL-HJHD) to 3.7 (YYDL-HJLWT), with an average of 2.3, and the effective allele

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

numbe (N_e) ranged from 1.018 (MLPDL-HJHD) to 1.888 (YYDL-TELT), with an average of 1.476. The Shannon's information index (I) ranged from 0.0287 (MLPDL-HJHD) to 0.6230 (YYDL-TELT), with an average of 0.3 667. The observed heterozygosity (H_o) ranged from 0.000 (MLPDL-HJHD) to 0.3 077 (YYDL-TELT), with an average of 0.1 585. The expected heterozygosity (H_e) was between 0.0 153 (MLPDL-HJHD) ~ 0.3 327 (YYDL-TELT), with an average of 0.1 945. The above data indicate that the average value of the fixation index (F) of the 11 populations is low, indicating that there is a certain degree of gene exchange and gene flow between these populations. The genetic diversity of *P. malipoense* is lower than that of *P. micranthum*. Among them, the genetic diversity of MLPDL-HJHD was the lowest, and the genetic diversity of MLPDL-YZLSJX was the highest. The genetic diversity of YYDL-LYLJW was the lowest, and the genetic diversity of YYDL-TELT was the highest.

Table 3. *Genetic diversity between populations*

Species	Pop	N_a	N_e	I	H_o	H_e	F
P. malipoense	MLPDL-HJHD	1.1	1.018	0.029	0.000	0.015	1.000
	MLPDL-HJHS	1.7	1.158	0.174	0.043	0.099	0.504
	MLPDL-HJXBS	2.1	1.241	0.274	0.086	0.152	0.318
	MLPDL-TL	1.2	1.147	0.123	0.078	0.085	0.112
	MLPDL-YZLSJX	2.6	1.515	0.421	0.195	0.216	0.073
	Mean	1.74	1.216	0.204	0.080	0.113	0.295
P. micranthum	YYDL-HJLWT	3.7	1.773	0.586	0.252	0.281	0.115
	YYDL-LYLJW	1.9	1.362	0.282	0.171	0.160	0.057
	YYDL-TELT	3.1	1.888	0.630	0.308	0.333	0.063
	YYDL-TLZ	2.6	1.506	0.404	0.064	0.204	0.640
	YYDL-YFXDC	2.7	1.790	0.541	0.262	0.285	0.124
	YYDL-YZLSJX	2.6	1.838	0.570	0.286	0.311	0.074
	Mean	2.767	1.693	0.502	0.224	0.262	0.169

Genetic structure and differentiation

The results of molecular variance analysis (AMOVA) (*Table 4*) showed that the genetic variation of *P. malipoense* and *P. micranthum* mainly came from individuals, which were 82% and 79%, respectively. There was little variation between the two species populations, only 18% of *P. malipoense* and 21% of *P. micranthum*. The results of genetic differentiation coefficient (F_{st}) and gene flow (N_m) among the populations of *P. malipoense* were shown in *Figure 2*. The gene flow between MLPDL-HJHS and MLPDL-HJXBS populations was the largest (11.081), and the genetic differentiation coefficient was the smallest (0.022). The minimum gene flow between MLPDL-TL and MLPDL-HJHD populations was 0.677, while the maximum genetic differentiation coefficient was 0.270. The average F_{st} value was 0.0 961, and the average N_m value was 4.3 791. The results of genetic differentiation coefficient and gene flow among populations are shown in *Figure 3*. The maximum gene flow between populations YYDL-HJLWT and YYDL-YFXDC was 4.020, and the genetic differentiation coefficient was 0.059, which was the minimum value among populations. The gene flow between YYDL-TLZ and YYDL-YZLSJX populations was the smallest (1.167) and the genetic differentiation coefficient was the largest (0.176). The mean F_{st} was 0.1 048, and the mean N_m was 2.356. The average F_{st} of the two species ranged from 0.05 to 0.15, and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

the degree of genetic differentiation among populations was not high. The frequency of gene exchange in the population of *P. micranthum* was higher. Structure genetic structure analysis was carried out on the samples of *P. malipoense* and *P. micranthum* (*Figs. 4* and *5*). The genotypes of 5 populations of *P. malipoense* could be divided into 3 subgroups, and the genotypes of 6 populations of *P. micranthum* could be divided into 6 subgroups. The different degrees of mixed color blocks between samples indicated that the genotypes were unevenly distributed in the gene pool and could not be distributed according to the genotypes, and there were different degrees of gene exchange.

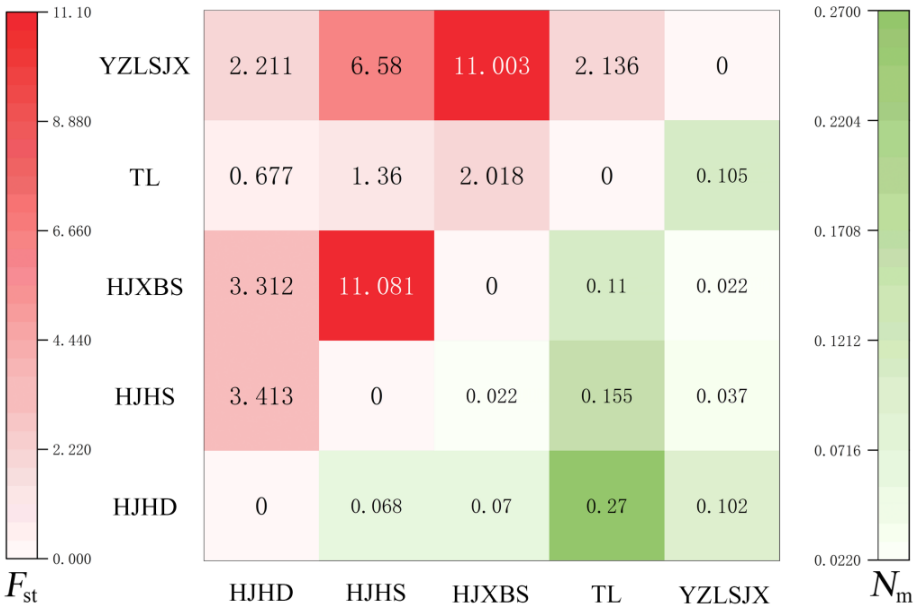

Figure 2. *Gene flow between populations and coefficient of genetic differentiation of P. malipoense*

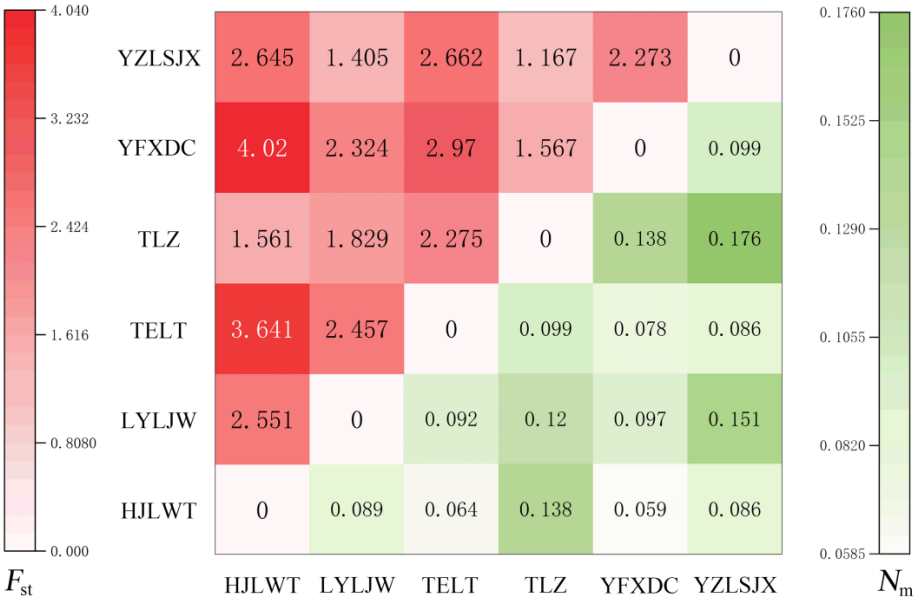

Figure 3. *Gene flow between populations and coefficient of genetic differentiation of P. micranthum*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

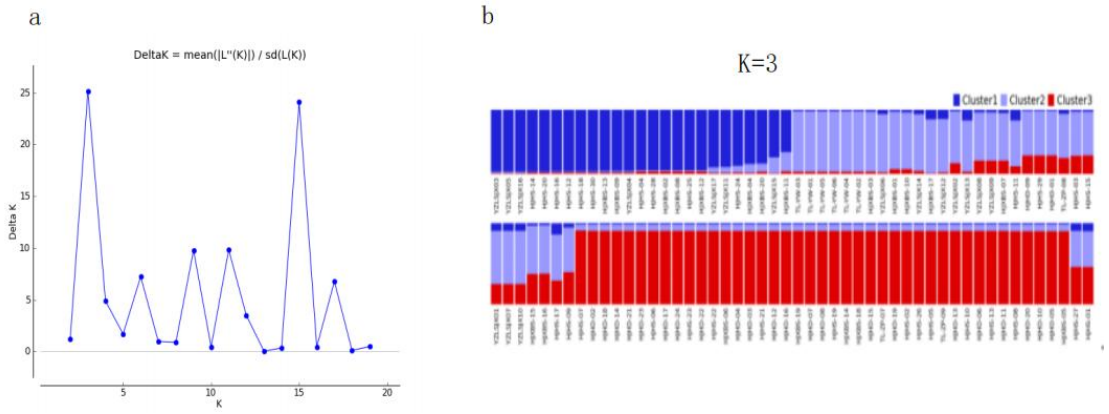

Figure 4. *Structure analysis results of P. malipoense. (a) Trend of the rational groups number K and estimated value Delta K.(b) Structure analysis results of 100 samples (K = 3)*

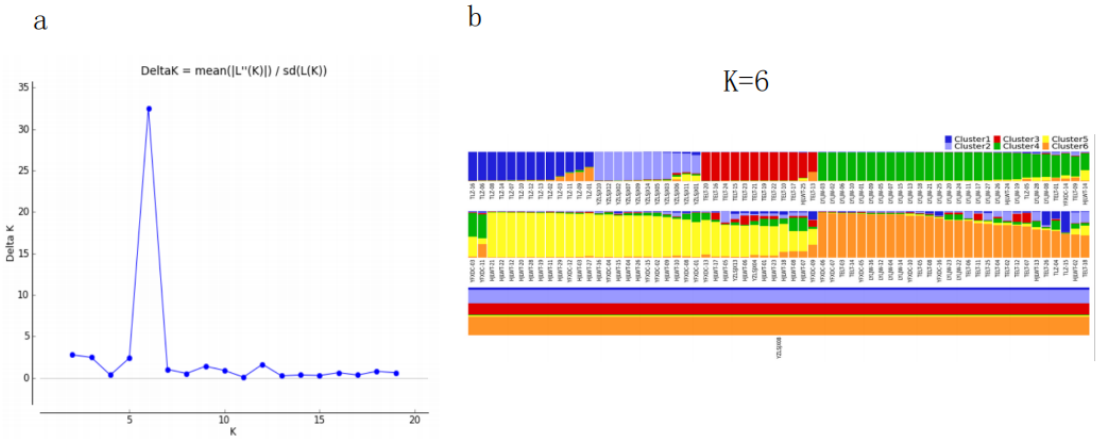

Figure 5. *Structure analysis results of P. micranthum. (a) Trend of the rational groups number K and estimated value Delta K. (b) Structure analysis results of 129 samples (K = 6)*

Table 4. *Molecular variance analysis of populations*

Species	Source of variation	df	SS	MS	Est. Var.	Percentage of variation (%)
P. malipoense	Among Pops	4	22.704	5.676	0.126	18%
	Among Indiv	95	76.051	0.801	0.225	32%
	Within Indiv	100	35.000	0.350	0.350	50%
P. micranthum	Among Pops	5	85.731	17.146	0.365	21%
	Among Indiv	123	209.630	1.704	0.329	19%
	Within Indiv	129	135.000	1.047	1.047	60%

Genetic distance and principal coordinate analysis

The genetic distance among populations was analyzed based on Nei's genetic distance method. The results showed that the genetic distance among the five populations of *P. malipoense* was between 0.03 394 (MLPDL-HJHD / MLPDL-HJHS) and 0.10 746 (MLPDL-TL / MLPDL-YZLSJX), and the genetic distance among the six populations of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

P. micranthum was between 0.10 655 (YYDL-HJLWT / YYDL-YFXDC) and 0.23 972 (YYDL-TLZ / YYDL-YZLSJX). There was no correlation between genetic distance and geographical distance of two species of *Paphiopedilum* (*Figs. 6* and *7*). The results of principal coordinate analysis (*Fig. 8*) showed that the first principal coordinate and the second principal coordinate explained 39.13% and 19.3% of the total variables, respectively, indicating that there was germplasm confusion in the population of *P. malipoense*. The punctate dispersion between MLPDL-TL and MLPDL-YZLSJX populations indicated that the genetic similarity was low, which was similar to the UPGMA clustering results. The results of principal coordinate analysis (*Fig. 9*) were basically consistent with the results of cluster analysis. The first principal coordinate accounted for 16.40% of the total variation, and the second principal coordinate accounted for 11.63% of the total variation. The sample points were evenly distributed in the two-dimensional map of the principal coordinate, indicating that *P. micranthum* was divided into 6 branches, but it was not clearly divided by geographical location.

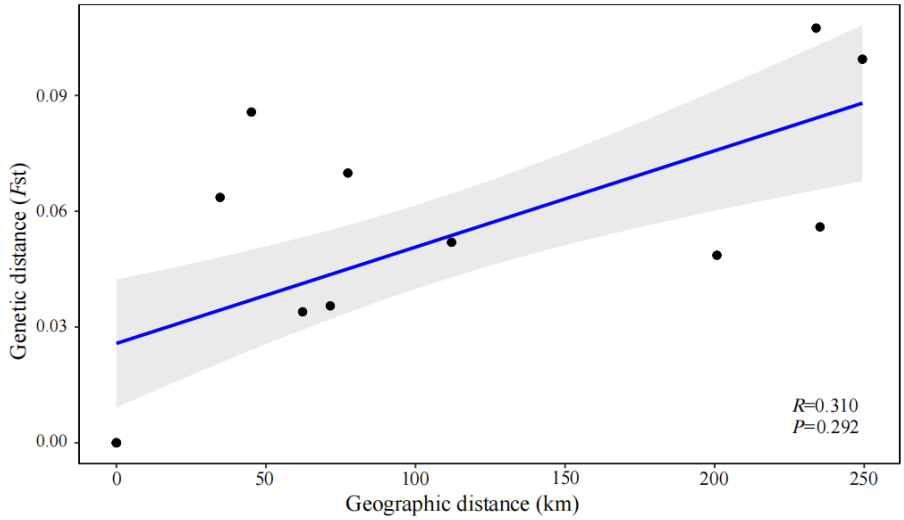

Figure 6. *Genetic distance and geographical distance of P. malipoense*

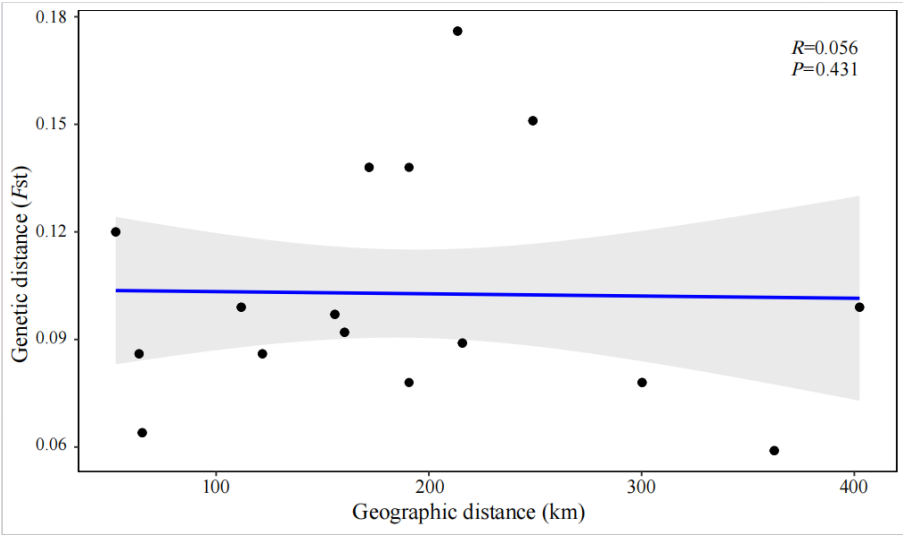

Figure 7. *Genetic distance and geographical distance of P. micranthum*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

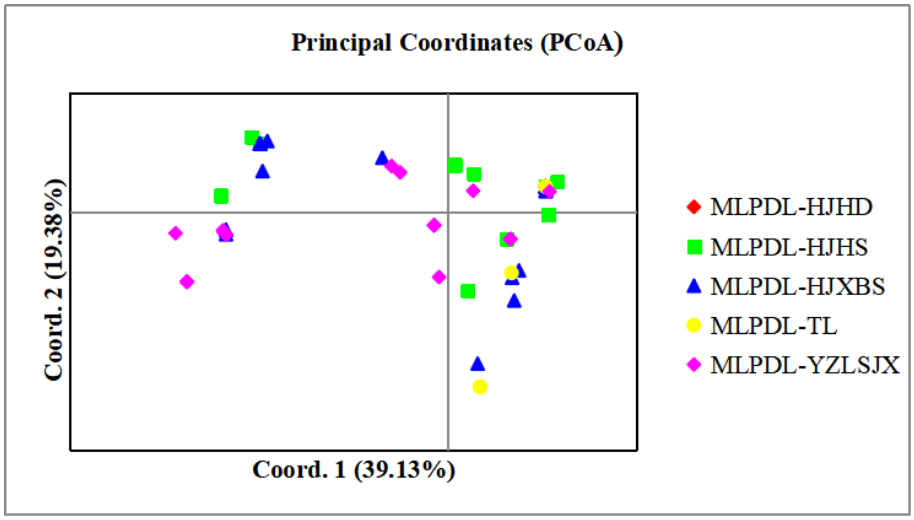

Figure 8. *Principal coordinate analysis of P. malipoense populations*

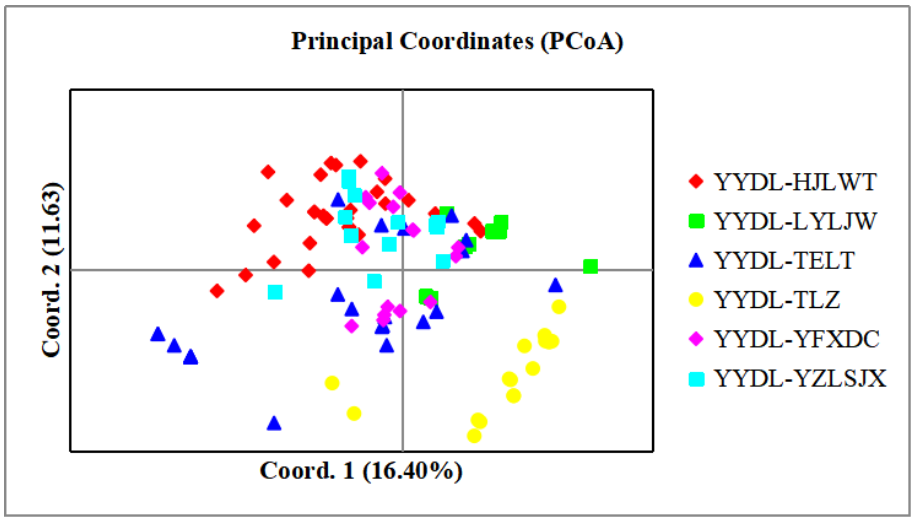

Figure 9. *Principal coordinate analysis of P. micranthum populations*

Discussion

Genetic diversity

Genetic diversity is an important part of biodiversity, which reflects the ability of species to adapt to the environment. The study on the genetic diversity of *P. malipoense* and *P. micranthum* is of great significance for estimating the distribution of gene resources, developing and utilizing germplasm resources, determining the core germplasm and preserving them (Feng et al., 2006). In this study, 10 pairs of SSR primers were selected for genetic diversity analysis. The mean observed allele number (N_a), effective allele number (N_e), Shannon's information index (I), observed heterozygosity (H_o) and expected heterozygosity (H_e) of *P. malipoense* were 1.74, 1.216, 0.204, 0.080 and 0.113, respectively. The mean values of observed allele number (N_a), effective allele number (N_e), Shannon's information index (I), observed heterozygosity (H_o) and expected heterozygosity (H_e) of *P. micranthum* were as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

follows: 2.77, 1.693, 0.502, 0.224, 0.262. The results showed that the genetic diversity of *P. malipoense* was low, and the genetic diversity of *P. micranthum* was higher than that of *P. malipoense*. Compared with the same genus and other orchids, the genetic diversity of *P. micranthum* was higher than that of six wild *Paphiopedilum* species in Beipanjiang River basin of Guizhou province ($N_a = 1.845$, $N_e = 1.449$, $I = 0.403$, $H_e = 0.265$) (Tian et al., 2023a); Wild *Paphiopedilum* plants in southern Guizhou ($N_a = 1.763$, $N_e = 1.506$, $I = 0.424$, $H_e = 0.288$) (Zhu et al., 2017); Wild *Paphiopedilum* plants in southeast Yunnan ($N_a = 1.813$, $N_e = 1.402$, $I = 0.371$, $H_e = 0.242$) (Li et al., 2013); A very small population of *Cypripedium palangshanense* ($N_a = 2$, $N_e = 1.5\ 026$, $I = 0.4\ 856$, $H_e = 0.2\ 205$) (Yang et al., 2022); Wild *Dendrobium officinale* populations in Taining, Fujian ($N_a = 2.00$, $N_e = 1.6069$, $I = 0.5228$, $H_e = 0.3507$) (Jiang et al., 2016). But lower than *Cymbidium Sw.* in Chongqing-Guizhou-Sichuan region ($N_a = 3.9\ 17$, $N_e = 2.7\ 48$, $I = 1.0\ 490$, $H_e = 0.600$) (Kou et al., 2020); *Renanthera coccinea* in Hainan island ($N_a = 3.18$, $N_e = 2.37$, $I = 0.86$, $H_e = 0.49$) (Zhao et al., 2023) so it has high genetic diversity. Polymorphic information content (PIC) can describe the degree of variation of SSR loci. The higher the PIC value, the more information the marker can provide. The average PIC value of *P. malipoense* samples was 0.1197, no highly polymorphic loci ($PIC > 0.5$), two (DL020, DL032) moderate polymorphic loci ($0.50 \geq PIC > 0.25$), and the remaining eight loci were low polymorphic loci ($PIC \geq 0.25$). The average PIC value of *P. micranthum* was 0.3097, with three highly polymorphic loci (DL020, DL032, DL039), one moderate polymorphic locus (DL034) and six low polymorphic loci. Relatively lower than Guangxi *P. helenae* (PIC = 0.444) (Zhu et al., 2023) and wild *P. hirsutissimum* populations in southwest China (PIC = 0.3 996) (Xu et al., 2023).

Genetic structure, genetic differentiation and phylogeny

From the perspective of genetic structure, the genetic variation between the populations of *P. malipoense* and *P. micranthum* was less, accounting for 18% and 21%, respectively, and the main genetic variation occurred in individuals. In this study, the genetic differentiation coefficients of *P. malipoense* and *P. micranthum* were analyzed, and the average genetic differentiation coefficients (F_{st}) were 0.096 and 0.105, respectively, indicating that the degree of genetic differentiation among populations was moderate (Dong et al., 2019). The study also used AMOVA, Structure and PCoA methods for analysis. Through AMOVA molecular variance analysis, it was found that most of the genetic variation occurred in individuals, and the genetic variation of individuals was the main source of the total variation. This may be due to the gene flow of *P. malipoense* ($N_m = 4.379$) and *P. micranthum* ($N_m = 2.356$) were greater than 1. Strong gene flow helps to reduce genetic differences between populations, maintain genetic diversity of populations, have a positive impact on population adaptability and long-term survival of species, and can offset genetic drift. The genetic differentiation between populations caused by genetic drift promotes individual variation (SLATKIN, 1981). The mode of pollen transmission and the distribution of seeds are the determinants of gene flow in natural populations (Li et al., 2008). The pollination method of *Paphiopedilum* plants is mainly insect-mediated outcrossing, and its seeds are difficult to germinate under natural conditions (Chen et al., 2004). *P. malipoense* mainly reproduces by asexual tillering (Xie et al., 2011). Compared with other *Paphiopedilum* plants, the seeds of *P. micranthum* have a shorter seed dispersal distance (Huang et al., 2014). Therefore, the genetic background between

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

the populations of *P. malipoense* and *P. micranthum* is relatively simple. The results of Structure, UPGMA clustering and PCoA analysis were consistent. The geographical division of *P. malipoense* was divided into three branches, and *P. micranthum* was divided into six branches, but it was not strictly divided according to the geographical location. The results were similar to those of *P. helenae* (Zhu et al., 2023) and *P. emersonii* (Qin et al., 2022), indicating that the genetic differentiation among populations was small and the genetic relationship was close. It may be due to the habitat fragmentation caused by habitat destruction, and the overexploitation and harvesting of *Paphiopedilum* plants, resulting in a sharp decline in the number of wild populations of *P. malipoense* and *P. micranthum*, thus forming the current genetic model (Xie et al., 2011; Li et al., 2002a).

Protection recommendations

Paphiopedilum plants are national first-class protected plants except *P. micranthum* and *P. hirsutissimum*. They are extremely dispersed in the natural environment, and the number of wild individuals is very small. In addition to their own biological reasons, habitat destruction and degradation, illegal collection and wild plant trade also pose a serious threat to the natural growth of *Paphiopedilum* in the wild (Zhang et al., 2022; Li and Li, 2016; Luo et al., 2003b). *P. malipoense* has a very small number of populations in China and the number of individuals in a single population is less than 50, which is extremely endangered (Tan and Liu, 2007; Tan, 2009). *P. micranthum* is the most widely distributed species in *Paphiopedilum*. Cluster growth makes it easier to be found by pirates and pirated (Zhang et al., 2021; Tian et al., 2023b). Based on the results of this study, we can propose the following protection measures for *P. malipoense* and *P. micranthum*: Most of the *Paphiopedilum* plants belonging to *P. malipoense* and *P. micranthum* need to rely on specific insects for pollination, and the germination of seeds and the growth of plants need to be symbiotic with specific fungi. We should focus on in-situ protection and monitor at the population level. When implementing in-situ protection measures, we should evaluate and determine the natural distribution areas of these plants to ensure that these areas are effectively protected and managed (Ye et al., 2022; Tian et al., 2017; Yao et al., 2023). Due to the low genetic diversity and few individuals in the field of *P. malipoense*, it is necessary to carry out ex-situ conservation on the basis of in-situ conservation to prevent the population composed of a small number of genotypes from being extinct in response to disasters (Xu and Zang, 2022). However, *P. malipoense* has poor adaptability to ex-situ conservation (Li et al., 2015). Therefore, it can be used for regression conservation by re-introduction of Orchidaceae plant into appropriate habitats in many countries (Liang et al., 2017). *P. micranthum* has rich genetic diversity, and should protect as many populations as possible in situ and maintain a sufficient number of individuals in a population to ensure the integrity of the population (Cheng et al., 2020). It is recommended to focus on the protection of YYDL-HJLWT and YYDL-TELT populations with high genetic diversity. Actively carry out research on the breeding mechanism of *Paphiopedilum* plants and develop artificial breeding techniques to achieve the return of *Paphiopedilum* and other rare orchids to nature; the establishment of protected areas combined with species basic research and popular science propaganda to protect the resources of *Paphiopedilum.* These measures will help to protect the population and habitat of wild *P. malipoense* and *P. micranthum*, ensure its important role in the ecosystem, and improve the public's awareness and participation in the protection of the species.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

Conclusion

The results showed that the genetic diversity of *P. micranthum* was low (N_a = 1.74, N_e = 1.216, I = 0.204, H_o = 0.080, H_e = 0.113); The genetic diversity of *P. micranthum* was high (N_a = 2.77, N_e = 1.693, I = 0.502, H_o = 0.224, H_e = 0.262). The genetic differentiation coefficient between the two *Paphiopedilum* populations was small, indicating that the degree of genetic differentiation between the populations was low; the gene flow between populations was large, which was helpful to maintain the genetic diversity of *P. malipoense* and *P. micranthum*. Genetic variation mainly comes from within the individual. The genetic diversity of MLPDL-YZLSJX in *P. malipoense* populations and YYDL-HJLWT and YYDL-TELT in *P. micranthum* populations were high, which should be protected as key populations. Most of the genetic variation of the two species occurs in individuals, and the genetic variation of individuals is the main source of total variation. In view of the slow growth rate of *Paphiopedilum* plants, strict environmental requirements, and scattered distribution in the wild state, they have been stolen and excavated due to their extremely high ornamental value. It is suggested to protect germplasm resources by means of in-situ protection, ex-situ protection, return conservation, artificial breeding and seed bank establishment, and to raise public awareness of the importance of Paphiopedilum protection through education and publicity activities.

REFERENCES

[1] Antanynienė, R., Šikšnianienė, J. B., Stanys, V., Frercks, B. (2023): Fingerprinting of Plum (*Prunus domestica*) genotypes in Lithuania using SSR markers. – Plants (Basel, Switzerland) 12(7): 1538.

[2] Chen, Z. L., Ye, X. L., Liang, C. Y., Duan, J. (2004): Seed germination in vitro of *Paphiopedilum armeniacum* and *P. micranthum.* – Acta Horticulturae Sinica 31(4): 540-542.

[3] Cheng, H., Xu, Y. F., Wang, W. X., Zhu, J., Jia, R. D., Yang, S. H., Zhao, X., Ge, H. (2020): Phenotypic diversity of *Paphiopedilum micranthum* populations from main distribution areas in China. – Acta Horticulturae Sinica 47(6): 1098-1110.

[4] Dong, L. M., Dai, L. F., Bai, L. W. D., Liu, M. L., Xie, J. K., Luo, X. D. (2019): Genetic diversity of wild seedlings of endangered *Rhododendron molle* G. Don by SSR. – Acta Botanica Boreali-Occidentalia Sinica 39(4): 613-619.

[5] Feng, X. L., He, C. Z., Zhang, Z. Y., An, X. M., Wang, D. M. (2006): Summarization on research methods of plant genetic diversity. – Journal of Southwest Forestry College 26(1): 69-74 + 79.

[6] Huang, J. L., Li, S. Y., Hu, H. (2014): ISSR and SRAP marker reveal genetic diversity and population structure of an endangered slipper orchid, *Paphiopedilum micranthum* (Orchidaceae). – Plant Diversity and Resources 36(2): 209-218.

[7] Itoo, H., Shah, R. A., Qurat, S., Jeelani, A., Khursheed, S., Bhat, Z. A., Mir, M. A., Rather, G. H., Zargar, S. M., Shah, M. D., Padder, B. A. (2023): Genome-wide characterization and development of SSR markers for genetic diversity analysis in northwestern Himalayas Walnut (*Juglans regia* L.). – 3 Biotech 13(5): 136.

[8] Jiang, J. L., Ye, W., Li, Y. Q., Fan, Z. M., Lei, F. G., Zhang, R., Zhou, J. J. (2016): Genetic Relationships among Wild *Dendrobium officinale* Populations in Taining, Fujian by ISSR. – Journal of Tropical and Subtropical Botany 24(3): 259-266.

[9] Kou, S., Li, Z., Li, X. Y., Sui, S. Z., Li, M. Y., Li, Z. N. (2020): Development of SSR primers in Cymbidium faberi Rolfe and study on genetic diversity of *Cymbidium* Sw. in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

Chongqing-Guizhou-Sichuan region. – Journal of Plant Genetic Resources 22(2): 338-348.

[10] Li, A., Luo, Y. B., Xiong, Z. T., Ge, S. (2002a): A preliminary study on conservation genetics of three endangered orchid species. – Acta Botanica Sinica 44(2): 250-252.

[11] Li, L. Q., Huang, J., Chen, L. J., Zhang, Q. Y., Chen, J. B. (2019): The complete chloroplast genome of *Paphiopedilum malipoense* (Orchidaceae). – Mitochondrial DNA Part B 4(2): 2617-2618.

[12] Li, X. L., Wang, X. G., Li, C. N., Zhou, J. Y., Deng, J. L., Zeng, S. J., Bu, C. Y., Lu, J. S. (2015): Adaptability evaluation of ex situ conservation of thirteen wild *Paphiopedilum* species by gray-correlation analysis. – Plant Science Journal 33(3): 326-335.

[13] Li, X. X., Ding, X. Y., Chu, B. H., Zhou, Q., Ding, G., Gu, S. (2008): Genetic diversity analysis and conservation of the endangered Chinese endemic herb *Dendrobium officinale* Kimura & Migo (Orchidaceae) based on AFLP. – Genetica 133(2): 159-166.

[14] Li, Y. C., Korol, A. B., Fahima, T., Beiles, A., Nevo, E. (2002b): Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. – Molecular Ecology 11(12): 2453-65.

[15] Li, Y. X., Fang, L., Chen, H., Li, L., Wu, K. L., Zeng, S. J. (2023): Research advance in mycorrhiza of *Paphiopedilum.* – Chinese Journal of Tropical Crops 44(11): 2157-2166.

[16] Li, Z. Y., Li, M. Y. (2016): Accompanying seed plant of *Paphiopedilum micranthum* community in Southeast of Yunnan. – Journal of Southwest University (Natural Science Edition) 38(7): 94-100.

[17] Li, Z. Y., Li, J., Zeng, W. B., Li, M. Y. (2013): Genetic structure of *Paphiopedilum micranthum* population from core distribution in Southeast Yunnan. – Journal of Plant Genetic Resources 14(3): 407-413.

[18] Liang, Y., Wei, K. H., Zhang, Z. J., Xiao, D., Qiao, Z., Li, M. J., Zhang, C. Y., Liao, J. H. (2017): Strategies for conservation of medicinal plants in South China karst. – Modern Chinese Medicine 19(2): 226-231.

[19] Liu, Z. J., Chen, X. Q., Zhang, J. Y., Lei, S. P. (2002): A taxonomical note of *Paphiopedilum malipoense* and its allies. – Acta Botanica Yunnanica 24(2): 193-198.

[20] Liu, Z. J., Chen, X. Q., Chen, L. J., Lei, S. P. (2009): The Genus *Paphiopedilum* in China. – Science Press, Beijing.

[21] Long, B., Long, C. L. (2006): Amazing *Paphiopedilum* and its research status. – Chinese Journal of Nature 28(6): 341-344.

[22] Luo, R., Wu, W. L., Zhang, Y., Li, Y. H. (2010): SSR marker and its application to crop genetics and breeding. – Genomics and Applied Biology 29(1): 137-143.

[23] Luo, Y. B., Jia, J. S., Wang, C. L. (2003a): A general review of the conservation status of Chinese orchids. – Chinese Biodiversity 11(1): 70-77.

[24] Luo, Y. B., Jia, J. S., Wang, C. L. (2003b): Conservation strategy and potential advantages of the Chinese *Paphiopedilum.* – Chinese Biodiversity 11(6): 491-498.

[25] Peakall, R., Smouse, P. E. (2018): 2GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. – Bioinformatics (Oxford, England) 28(19): 2537-2539.

[26] Qin, H. Z., Pan, B., Zhao, J., Zou, R., Wei, X., Tang, F. L. (2022): Genetic diversity analysis by ISSR of *Paphiopedilum emersonii*, a plant species with extremely small populations. – Guangxi Sciences 29(6): 1134-1140.

[27] Shao, Y. L., Si, J. B., Chang, W., Shi, J. L., Ding, Y. (2020): Comparison of genomic DNA extraction methods from five species of *Paphiopedilum.* – Molecular Plant Breeding 18(15): 4965-4974.

[28] SLATKIN (1981): Estimating levels of gene flow in natural populations. – Genetics 99(2): 323-335.

[29] Tan, W. N. (2009): The present situation and protection countermeasures of *Paphiopedilum* (Orchidaceae) plant resources in Guangxi Mulun National Nature Reserve. – Guangxi Forestry Science 38(3): 187-189 + 196.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

[30] Tan, W. N., Liu, Y. (2006): A rapidly declining species *Paphiopedilum malipoense*. – Newton 2007(6): 44.
[31] Tian, F., Liao, X. F., Wang, P. H., Zhu, G. S., Gui, Y., Bai, X. X. (2017): Classification and identification of mycorrhizal fungi of *Paphiopedilum micranthum.* – Northern Horticulture (24): 116-122.
[32] Tian, L., An, M. T., Shi, J. Z. (2023a): Genetic diversity analysis of wild *Paphiopedilum* in Beipan River. – Guizhou Province Journal of West China Forestry Science 52(2): 88-97.
[33] Tian, L., An, M. T., Zhang, Y., Wu, Y. X. (2023b): Characteristics of soil fungi community in wild populations of three *Paphiopedilum Subgen Brachypetalum* species. – China Environmental Science 43(4): 1995-2006.
[34] Wang, L. L., Chen, D. L., Huang, C. L., Xing, Z. (2017): Application of SSR molecule marker technique in plant research. – Journal of Anhui Agricultural Sciences 45(36): 123-126, 130.
[35] Wang, X. J. (2015): 'Yutuo' *Paphiopedilum malipoense.* – China Flowers & Horticulture (6): 38.
[36] Wright, S. (1931): Evolution in mendelian populations. – Genetics 16: 97-159.
[37] Xie, D. Z., Qin, W. G., Tang, X. P., Wei, L., Qin, W. Q., Fu, Y. J. (2011): Preliminary research on characteristics of *Paphiopedilum malipoense* community in Mulun Nature Reserve, Guangxi. – Northern Horticulture (13): 111-114.
[38] Xu, Y., Zang, R. G. (2022): Theoretical and practical research on conservation of Wild Plants with Extremely Small Populations in China. – Biodiversity Science 30(10): 84-105.
[39] Xu, Y., Chen, Z. G., Xu, Y. F., Ge, H., Yang, S. H., Zhao, X., Kou, Y. P., Yu, X. N., Jia, R. D. (2023): Genetic diversity of wild *Paphiopedilum hirsutissimum* populations in Southwest China with SSR markers. – Chinese Journal of Tropical Crops 44(11): 2208-2218.
[40] Xu, Y. F., Jia, R. D., Zhou, Y. H., Cheng, H., Zhao, X., Ge, H. (2018): Development and characterization of polymorphic EST-SSR markers for *Paphiopedilum henryanum* (Orchidaceae). – Applications in Plant Sciences 6(5): e01152.
[41] Yang, J. X., Dierckxsens, N., Bai, M. Z., Guo, Y. Y. (2023): Multichromosomal Mitochondrial genome of *Paphiopedilum micranthum*: compact and fragmented genome, and rampant intracellular gene transfer. – International Journal of Molecular Sciences 24 (4): 3976-3976.
[42] Yang, S. T., Ma, X. N., Bai, X. L., Tan, K. F., Zhang, A. L., Luo, J., Bai, J., Tan, Y. C., He, T. M. (2022): CDDP genetic diversity analysis of a very small population of wild plant *Cypripedium palangshanense.* – Journal of Sichuan University (Natural Science Edition) 59(6): 155-163.
[43] Yang, Y. J., Huang, J. L., Hu, H., Zhang, S. B. (2021): Progress on conservation and utilization of *Paphiopedilum* species in China. – Journal of West China Forestry Science 50(5): 108-112 + 119.
[44] Yao, N., Zheng, B. Q., Wang, T., Cao, X. L. (2023): Isolation of *Tulasnella spp.* from cultivated *Paphiopedilum* orchids and screening of germination-enhancing fungi. – Journal of fungi (Basel, Switzerland) 9(6): 597.
[45] Ye, C., Liu, F., An, M. T., Yang, Y. B. (2022): Analysis on in situ conservation status and conservation vacancy of wild Orchidaceae in Guizhou Province. – Guihaia 42(2): 240-246.
[46] Yin, Y. Y., Fang, L., Li, L., Chen, Y., Fu, W. Q., Wu, K. L., Zeng, S. J. (2022): Advances in flowering regulation of *Paphiopedilum.* – Chinese Journal of Tropical 43(4): 769-778.
[47] Zhang, H. R., Li, X., Chen, Z. X., Hu, W. B., Li, W., Huang, M., Yu, F. L., Yang, L. L. (2023): Genetic diversity analysis and DNA fingerprints construction of *Callicarpa nudiflora* germplasm resources based on SSR markers. – Chinese Traditional and Herbal Drugs 54(12): 3971-3982.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688

[48] Zhang, J. R., Fang, L., Zeng, J. J., Li, L., Wu, K. L., Zeng, S. J. (2024): Species and geographical distribution of *Paphiopedilum.* – Chinese Journal of Tropical 45(8): 1572-1584.
[49] Zhang, Y., Wu, J. Y., An, M. T., Xu, J., Ye, C., Shi, J. Z. (2021): Geographical distribution pattern and prediction of the potential distribution of *Paphiopedilum micranthum* in China. – Acta Botanica Boreali-Occidentalia Sinica 41(11): 1932-1939.
[50] Zhang, Y., An, M. T., Wu, J. Y., Yang, Y. B., Li, Z., Ye, C. (2022): Survival status and protection effectiveness of *Paphiopedilum Subgenus. Brachypetalum* in China. – China Environmental Science 42(7): 3461-3472.
[51] Zhang, Y. Y., Fang, Z. M., Huang, W. T., Zeng, S. J., Wu, K. L., Zhang, J. X., Duan, J. (2014): Study on change of leaf morphology and photosynthetic characters of three *Paphiopedilum* species introduced in south China. – Guangdong Agricultural Sciences 41(5): 92-95 + 9.
[52] Zhao, H. Q., Li, D. L., Zhao, H. L., Wu, F. F., Huang, S., Zhang, Y. H., Zhang, L., Yang, J., Ren, J. F. (2023): SSR market development, genetic diversity and population structure analyses of *Renanthera coccinea* in Hainan Island. – Molecular Plant Breeding 21(7): 2300-2310.
[53] Zhou, Q. (2017): Guizhou Yesheng Lanke Zhiwu. – Guizhou Science and Technology Publishing House, Guiyang.
[54] Zhu, S. J., Zou, R., Chai, S. F., Tang, J. M., Tang, F. L., Chen, T. G., Wei, X. (2023): Genetic Diversity of *Paphiopedilum helenae* in Guangxi based on EST-SSR Markers. – Guangxi Sciences 30(6): 1060-1067.
[55] Zhu, Y. Y., Wang, G., Hou, N., Wang, L. H. (2017): Genetic diversity analysis of wild *Paphiopedilum* species in the South of Guizhou. – Journal of Southwest Forestry University 37(1): 10-14.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2304_66736688