GENETIC DIVERSITY AND GENETIC STRUCTURE OF THE RARE AND ENDANGERED RELICT PLANT *CYCAS SHIWANDASHANICA*

TANG, J. M.[1,2,3#] – ZOU, R.[3#] – WEI, X.[3] – LI, D. P.[3] – ISHIMARU, K.[1,2*]

[1]*The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan*

[2]*Faculty of Agriculture, Saga University, Saga 840-8502, Japan*

[3]*Guangxi Key Laboratory of Plant Functional Substances and Resources Sustainable Utilization, Guangxi Institute of Botany, Guilin 541006, China*

[#]*Jianmin Tang and Rong Zou contributed equally to this work*

[*]*Corresponding author*
e-mail: tangjianmin889@hotmail.com

(Received 28th Feb 2023; accepted 18th May 2023)

Abstract. Understanding the genetic diversity and structure of the rare and endangered relict plant *Cycas shiwandashanica* is the basis and prerequisite for formulating effective conservation and management strategies. In this study, the genetic diversity and genetic structure of 103 individuals from five populations of *C. shiwandashanica* were analyzed based on 6 pairs of SSR primers with good polymorphism and stable amplification. The results showed that at the population level, the Shannon information diversity index (I) was 0.709. The mean value of Ho was 0.453. The results indicated that *C. shiwandashanica* had high genetic diversity. There were significant differences among all populations. The total mean value of Wright fixed index F of the polymorphic loci of each population was -0.147, indicating that there was an excess of heterozygotes in each population. The analysis of molecular variance showed that 21% of the genetic variation existed in the population level and 79% in the individual level. The individual variation was the main source of the total variation of *C. shiwandashanica*. The results indicated that *C. shiwandashanica* had abundant genetic variation and high evolutionary potential. The maximum genetic distance between the five populations was 0.268423 (SY1/ZWS1) and the minimum was 0.049418 (SY1/MZT1). MZT1 and SY1 get together, then PFA1, and finally WWL1; This is consistent with the distribution location and distance of the wild population of *C. shiwandashanica*. Considering the current situation of the wild population of this species, it is suggested to establish a protection plot, carry out in situ protection, and strengthen the ex-situ protection measures such as introduction and artificial breeding. This study can provide theoretical support for the conservation of plant resources of *C. shiwandashanica* and has important theoretical and practical significance.
Keywords: *hereditary, conservation strategies, artificial breeding, individual variation, level*

Introduction

Cycads, as the oldest plants, originated in the Paleozoic period and reached its peak distribution in the Jurassic period, serving as the main food of dinosaurs (Clugston and Kenicer, 2022; Gomankov, 2022). The fossil record shows that cycads were once widely distributed (Wang et al., 2005). However, the existing cycads are mostly distributed in tropical, subtropical, or temperate regions, their species and number sharply reduced. Climate change, habitat change, and human activities have posed a great threat to the survival of cycas. Nearly 63% of the world's cycas have been included in the Red List of Endangered Plants of the International Union of Nature (IUCU) (Zheng et al., 2017). There are six species of Cycas, of which there are 23 species in four categories in China, of which

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

16 (69.57%) are listed as threatened species by the International Union for Conservation of Nature, and 6 (26.09%) are listed as endangered species (Zheng et al., 2017). All the genera of Cycas are listed as the primary protected plants of China (Ren et al., 2012), mainly distributed in Taiwan, Yunnan, Fujian, Hainan, Sichuan, Guangdong, and Guangxi, etc. Most species of cycas in Yunnan and Guangxi are listed as minimal population wild plants (Ma et al., 2013). It accounts for 9.17% of the total number of wild plants in China.

Cycas shiwandashanica is a rare wild plant of the genus Cycad unique to Guangxi, which is only distributed in the monsoon rain forests of the low mountains and hills of Shiwandanshan, Fangchenggang City, Guangxi Province, with an altitude of 100~750 m (Zhang, 1997), with very few wild resources. Less than 500 strains exist (Liu, 2018). At present, only morphological description, taxonomic studies and karyotype analysis have been studied (Yang, 2013), etc., but there is a lack of studies on the genetic diversity and genetic differentiation level of *C. shiwandashanica*. Therefore, in this study, SSR molecular marker fluorescence technology (Wang et al., 2008; Yang et al., 2008) was used to design specific primers and add fluorescent groups according to microsatellite conservative sequences for fluorescent PCR amplification, and to detect the genetic diversity and genetic structure of *C. shiwandashanica*. The research results can provide scientific data support for the protection of plant resources of *C. shiwandashanica*, which has important theoretical and practical significance.

Materials and methods

Experimental materials

The experimental materials were collected from four populations of *Cycas shiwandashanica* (MZT1, WWL1, SY1, PFA1) in Fangchenggang City, Guangxi, and one introduced species population of Guilin Botanical Garden (ZWS1). A total of 103 young leaves of 65 wild plants were collected, stored in a -20°C refrigerator for safekeeping and dried in silica gel (*Figure 1*, *Table 1*).

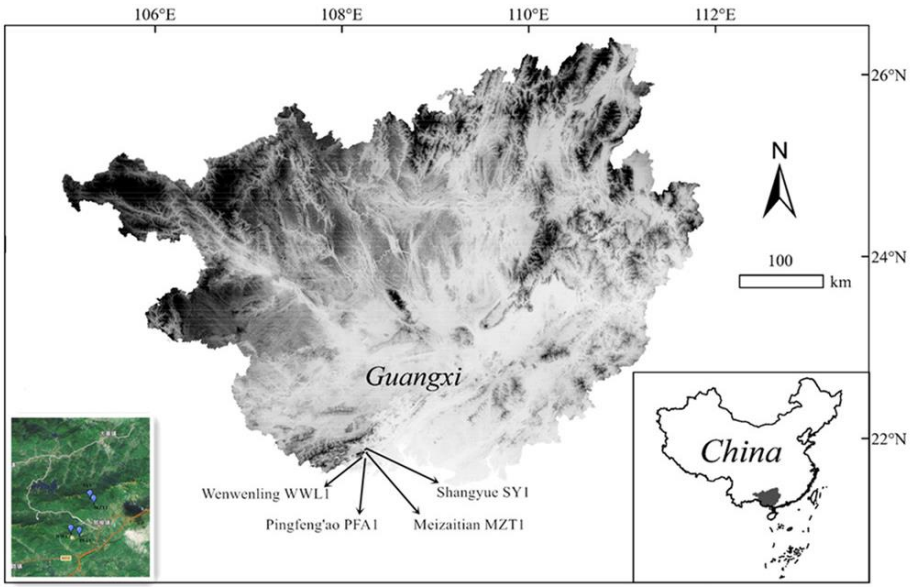

Figure 1. *Wild distribution and collection sites of C. shiwandashanica in Shiwandashan Mountains. The map can be downloaded from the Geospatial Data Cloud (https://www.gscloud.cn)*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

Table 1. *Sample collection of Cycas shiwandashanica*

Population	Location	Habitat	Latitude (N)	Longitude (E)	Altitude (*m*)
PFA1	Pingfeng'ao	slope side	21º41′59″	108º04′21″	261
WWL1	Wenwenling	slope side	21º42′19″	108º03′38″	270
SY1	Shangyue	slope side	21º45′41″	108º05′36″	152
MZT1	Meizaitian	slope side	21º45′10″	108º06′01″	230
ZWS1	Guilinzhiwuyuan	flat ground	25º4′14.88″	110º17′57″	180

DNA extraction and detection

The magnetic bead plant genome extraction kit and an automated workstation (USA Bio-Rad Laboratories Inc.) were used to extract nucleic acid from the samples of *C. shiwandashanica*, and the DNA quality was detected by gel electrophoresis: concentration (ng/μL) ≥30 ng/μL, too low concentration is not good for subsequent PCR (USA Bio-Rad Laboratories Inc.) experiments.

Primer synthesis and fluorescent PCR

Amplification SSR primers were designed according to the whole genome sequence analysis, and 96 pairs of primers were obtained for screening. Primers were synthesized by joint method, that is, 21bp joint sequence was added to upstream primers during synthesis. When using the splitter method for PCR amplification, the upstream primers with splitter in the first step and the downstream primers with splitter in the first step are combined with the template to obtain PCR products with splitter sequences; the joint primers with fluorescent groups in the second step are combined with the downstream primers with PCR products in the first step to obtain PCR products with fluorescent groups (Shershov et al., 2017) and 21bp splitter sequences.

A total of 16 screened samples were selected from each population, 96 pairs of primers were amplified, and the reaction was performed on Veriti 384 PCR apparatus (25 μL reaction system with approximately 30 ng DNA, 2.5 μL 10 × buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.6 μM primer, 0.75 U Taq enzyme). The PCR amplification program was set as: 95℃ pre-denaturation for 5 min; Denaturation at 95℃ for 30 s, gradient annealing at 62~52℃ for 30 s, extension at 72℃ for 30 s, 10 cycles were run. 25 cycles were run with the following settings: denaturation at 95℃ for 30 s, annealing at 52℃ for 30 s, extension at 72℃ for 30 s; It was extended at 72℃ for 20 min and stored at the last 4℃. After PCR reaction, the amplified products were detected by fluorescence capillary electrophoresis. GeneMarker software was used to analyze the results, and 6 pairs of primers with stable amplification and good polymorphism were obtained. The fluorescence PCR products diluted to a unified concentration were added to the upper board, and the detection file corresponding to the name of the board to be detected was selected according to the on-board operation process of ABI 3730xl, and the SSR sample analysis and detection program was run.

Analysis of original data

The original data in fsa format were exported from the ABI 3730xl instrument, classified, and archived according to the detection sites, and imported into the GeneMarker analysis software for reading the genotype data, and exported the original

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

Excel genotype data and PDF genotype peak map file respectively according to the name of the site.

Analysis of genetic diversity

In GenAlEx version 6.501, various genetic diversity indices of SSR loci and populations were calculated. They included observed alleles (Na), effective alleles (Ne), Shannon index (I), polymorphism information index (PIC), observed heterozygosity (Ho), expected heterozygosity (He) and inbreeding coefficient (Fis). The genetic distance between populations was calculated by powermarker software. The UPGMA method was used for cluster analysis and ring cluster graph was drawn. According to the results of population genetic structure analysis, the inter-population and intra-population variation, differentiation and significance test were calculated in GenAlEx version 6.501 software. Genetic differentiation coefficient (Fst) and gene flow (Nm) were calculated according to Wright's (1931) formula: Nm= 0.25(1-Fst)/Fst. The genetic distance between populations was calculated in powermarker (Nei, 1983).

Result

Primers' screening results

96 pairs of primers were obtained from the whole genome sequence, and a total of 6 pairs were selected and amplified successfully with good peak type (Qin et al., 2022) (*Table 2*).

Table 2. *Screened out information of 6 pairs of SSR primers*

Name of site	Repeat unit	Forward primer	Reverse primer	Allelic interval
GZST002	(GA)6	TGTGGAACGTGGAATGGTAA	AGGAATCCCGAAGGAAGAAA	158-160
GZST019	(ATAA)5	GATGAGGAAGCCTACGCAGT	GAAAGACCTCACCATCCGAG	212-221
GZST055	(AT)6	TCATGAAGATGGCAACCAAC	TCCCTTCCAAGCAAATGTCT	161-184
GZST013	(GAG)5	ACCGGTCGACTAGATGGATG	AGGTCCGAAGCTTTCCTCTC	252-265
GZST088	(AG)7	TGGCTTTCGATTTCCACACT	GAACGCTCGCTCTCTCTCTC	136-159
GZST065	(CGA)5	GCTTGGCTGTACCGTTCTTT	CGCCATTGACAACAACAGAC	157-174

Primer polymorphism

A total of 26 alleles (Na) were detected by 6 pairs of primers in 103 samples, among which the minimum number of alleles was 2, the maximum number of alleles was 9, and the average number of alleles per locus was 4.33 (*Table 3*). The total number of effective alleles (Ne, the more evenly distributed the alleles in the population, the closer the Ne was to the number of actually detected alleles) was 14.528, with the range of 1.062 (GZST013) to 5.083 (GZST065), and the average number of effective alleles per locus was 2.4213. The Shannon index (I) ranges from 0.135 (GZST013) to 1.835 (GZST065), with an average value of 0.9073. The observed heterozygosity (Ho) ranged from 0 (GZST002) to 0.794 (GZST055), with an average value of 0.443. The expected heterozygosity (He) ranged from 0.058 (GZST013) to 0.803 (GZST065), with an average value of 0.471. The polymorphic information content (PIC) ranges from 0.057 (GZST013) to 0.78 (GZST065), with an average value of 0.4267. The mean inbreeding coefficient was -0.002 and the value was -0.405 (GZST055) -1.000 (GZST002) (*Table 4*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

Table 3. *Polymorphisms of 6 pairs of SSR primers*

Locus	Na	Ne	I	Ho	He	F	PIC	Prob	Signif
GZST002	2	1.587	0.557	0	0.37	1	0.302	0.000	***
GZST013	2	1.062	0.135	0.059	0.058	-0.021	0.057	0.758	ns
GZST019	2	1.561	0.545	0.471	0.36	-0.309	0.295	0.002	**
GZST055	4	2.552	1.032	0.794	0.608	-0.306	0.53	0.000	***
GZST065	9	5.083	1.835	0.763	0.803	0.05	0.78	0.522	ns
GZST088	7	2.683	1.34	0.571	0.627	0.089	0.596	0.023	*
Mean	4.333	2.421	0.907	0.443	0.471	0.084	0.427		
St Dev	3.011	1.446	0.619	0.342	0.263	0.481			

Note: Na: alleles observed; Ne: effective allele; I: Shannon index; Ho: observed heterozygosity; He: expected heterozygosity; F: Fixed index, an index to evaluate the degree of deviation between actual observed values and theoretical values, PIC: polymorphic information index; Prob: P value; Signif: significant (ns means not significant, i.e., the population conforms to HWE; * indicates significant difference. 0.05, ** indicates significant difference. 0.01, *** indicates significant difference. 0.001)

Table 4. *Inbreeding coefficients and gene flow of 6 pairs of primers*

Locus	Fis	Fit	Fst	Nm
GZST002	1.000	1.000	0.930	0.019
GZST013	-0.059	-0.031	0.027	8.926
GZST019	-0.391	-0.261	0.093	2.430
GZST055	-0.405	-0.298	0.077	3.014
GZST065	-0.088	-0.028	0.056	4.234
GZST088	-0.067	0.041	0.101	2.234
Mean	-0.002	0.071	0.214	3.476
SE	0.211	0.194	0.144	1.226

Note: Fis: inbreeding coefficient within a population, Fit: overall inbreeding coefficient, Fst: genetic differentiation coefficient, Nm: gene flow (Nm = 0.25(1-Fst)/Fst)

Population genetic diversity

At the population level, the Shannon information diversity index (I) of each population in the wild was between 0.6789 and 0.735, with an average value of 0.709 (*Table 5*). The heterozygosity Ho was 0.349-0.533, with an average value of 0.453. The expected heterozygosity (He) of Nei's diversity index was between 0.348-0.406, with an average value of 0.384. The order of size is MZT1>WWL1>ZWS1>SY1>PFA1, there were significant differences among all populations; The Wright fixation index F of the polymorphic loci of each population ranged from -0.0234 to -0.3932, and the total mean value was -0.147, indicating that there was excessive heterozygote in each population of *C. shiwandashanica*. The results of Nybom's study on genetic diversity of 307 species showed that the mean values of I at the population level of perennials, widespread species and outcrosses were 0.25, 0.22 and 0.27, respectively. According to statistics, the average H of dicotyledonous plants, perennials, short-lived plants, narrow distribution species, outcrossing plants and gravity seed dispersing species at population level was 0.191, 0.20, 0.28, 0.27, 0.19. The values of I and Ho were 0.709 and 0.453, respectively, higher than the above average plant genetic diversity, indicating that the genetic diversity of *C. shiwandashanica* was at a high level (*Table 6*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

Table 5. *Genetic diversity among the five populations*

Pop	Na	Ne	I	Ho	He	F
MZT1	2.833333	2.17305	0.734946	0.484127	0.406274	-0.1746
PFA1	2.833333	2.340647	0.678826	0.349129	0.348239	-0.02341
SY1	3.833333	1.928616	0.711659	0.417488	0.368689	-0.1329
WWL1	2.833333	2.219296	0.731671	0.47963	0.404923	-0.03164
ZWS1	2.666667	2.207403	0.68945	0.533333	0.39	-0.39326
Mean	3	2.173802	0.70931	0.452741	0.383625	-0.14655

Na: observed alleles; Ne: effective allele; I: Shannon Information Index; Ho: observed heterozygosity; He: expected heterozygosity; F: Fixed index (The same of follows)

Table 6. *Genetic diversity of population loci*

Pop	Locus	Na	Ne	I	Ho	He	F
MZT1	GZST002	1.000	1.000	0.000	0.000	0.000	-
	GZST013	2.000	1.153	0.257	0.143	0.133	-0.077
	GZST019	2.000	1.630	0.575	0.524	0.387	-0.355
	GZST055	3.000	2.443	0.968	0.762	0.591	-0.290
	GZST065	5.000	4.618	1.566	1.000	0.783	-0.276
	GZST088	4.000	2.194	1.044	0.476	0.544	0.125
PFA1	GZST002	1.000	1.000	0.000	0.000	0.000	-
	GZST013	1.000	1.000	0.000	0.000	0.000	-
	GZST019	1.000	1.000	0.000	0.000	0.000	-
	GZST055	3.000	2.227	0.938	0.667	0.551	-0.210
	GZST065	6.000	4.983	1.680	0.706	0.799	0.117
	GZST088	5.000	3.834	1.455	0.722	0.739	0.023
SY1	GZST002	1.000	1.000	0.000	0.000	0.000	-
	GZST013	2.000	1.056	0.124	0.054	0.053	-0.028
	GZST019	2.000	1.810	0.640	0.676	0.447	-0.510
	GZST055	4.000	2.785	1.129	0.919	0.641	-0.434
	GZST065	8.000	3.330	1.538	0.485	0.700	0.307
	GZST088	6.000	1.591	0.839	0.371	0.371	0.000
WWL1	GZST002	2.000	1.198	0.305	0.000	0.165	1.000
	GZST013	2.000	1.105	0.199	0.100	0.095	-0.053
	GZST019	2.000	1.308	0.398	0.273	0.236	-0.158
	GZST055	2.000	1.862	0.655	0.727	0.463	-0.571
	GZST065	5.000	4.667	1.574	1.000	0.786	-0.273
	GZST088	4.000	3.176	1.259	0.778	0.685	-0.135
ZWS1	GZST002	1.000	1.000	0.000	0.000	0.000	-
	GZST013	1.000	1.000	0.000	0.000	0.000	-
	GZST019	2.000	1.724	0.611	0.600	0.420	-0.429
	GZST055	2.000	1.867	0.657	0.733	0.464	-0.579
	GZST065	6.000	4.592	1.632	1.000	0.782	-0.278
	GZST088	4.000	3.061	1.237	0.867	0.673	-0.287

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

Genetic variation

Molecular analysis of variance (ANOVA) is a method to measure and calculate genetic variation between haplotypes (or genotypes) by evolutionary distance. The analysis of molecular variance showed that 21% of the genetic variation existed in the population and 79% in the individual (*Table 7*). The individual variation was the main source of the total variation of *Cycas dahuaensis* (*Table 8*).

Table 7. *Molecular variance analysis (AMOVA)*

Source	df	SS	MS	Est. Var.	%
Among Pops	4	57.456	14.364	0.335	21%
Among Indiv	98	115.762	1.181	0.000	0%
Within Indiv	103	131.000	1.272	1.272	79%
Total	205	304.218		1.607	100%

Source: source of variation; df: Freedom of freedom; SS: total variance; MS: mean square error; Est. Var.: estimated variance; %: variation percentage; Among Pops; Among Indiv. Within Indiv refers to the genetic difference caused by a heterozygous allele, the size of which is related to the number of heterozygous sites in an individual, i.e., the genetic diversity of an individual

Table 8. *Inter-population gene flow (upper triangle) and genetic differentiation coefficient (lower triangle)*

	MZT1	PFA1	SY1	WWL1	ZWS1
MZT1	-	3.031027	11.61966	1.300784	1.058489
PFA1	0.076	-	2.509679	1.237132	0.666417
SY1	0.021	0.091	-	1.185457	0.966217
WWL1	0.161	0.168	0.174	-	5.870171
ZWS1	0.191	0.273	0.206	0.041	-

Principal coordinate analysis

Principal coordinate analysis (PCoA) is a non-binding method of data dimensionality reduction, which can present the visual coordinates of the similarity or difference of the research data (*Figure 2*). It can also be used to study the similarity or difference of the composition of the sample population. PcoA analysis can reflect the difference between 2 samples or 2 groups of samples by intuitively comparing the linear distance between the samples in the coordinate axes. If the linear distance between 2 samples or 2 groups of samples is close, it means that the difference between the 2 samples or 2 groups of samples is small. On the contrary, if the straight-line distance between two samples or two groups of samples is far, it indicates that they are different from each other. PCoA analysis was performed by GenAIex software.

Population genetic structure

Structure analysis

Six molecular markers were used to evaluate the population structure of 103 samples of *Cycas shiwandashan*. According to the principle of maximum likelihood value, the optimal K value was judged to be equal to 2, and 103 samples of *Cycas shiwandashan* could be divided into two subgroups (*Figures 3, 4*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

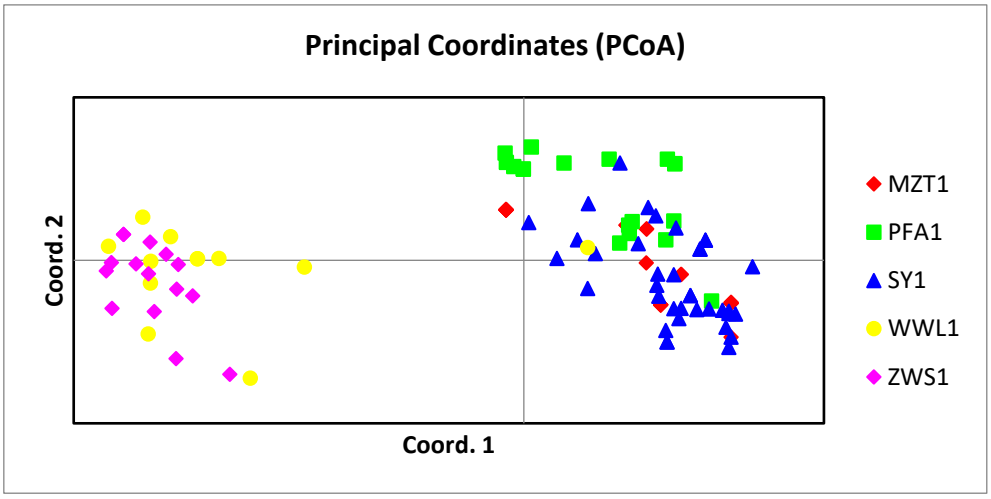

Figure 2. *Principal coordinate analysis of samples*

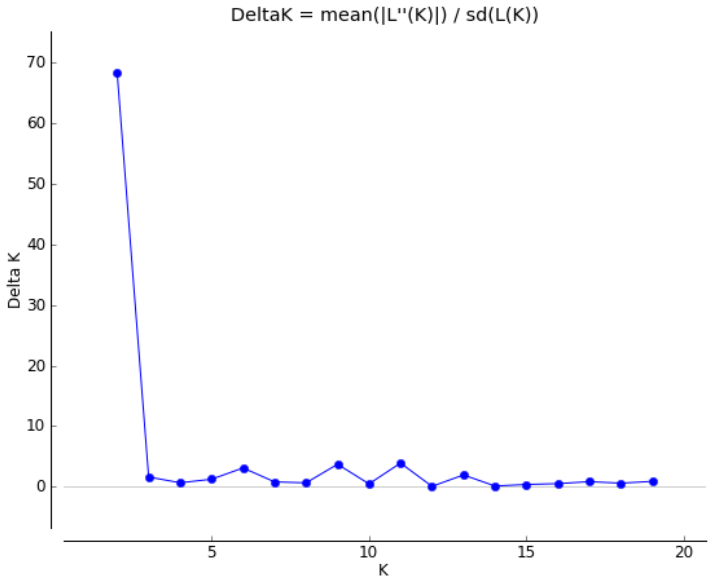

Figure 3. *K value change graph drawn by ΔK method of structure analysis*

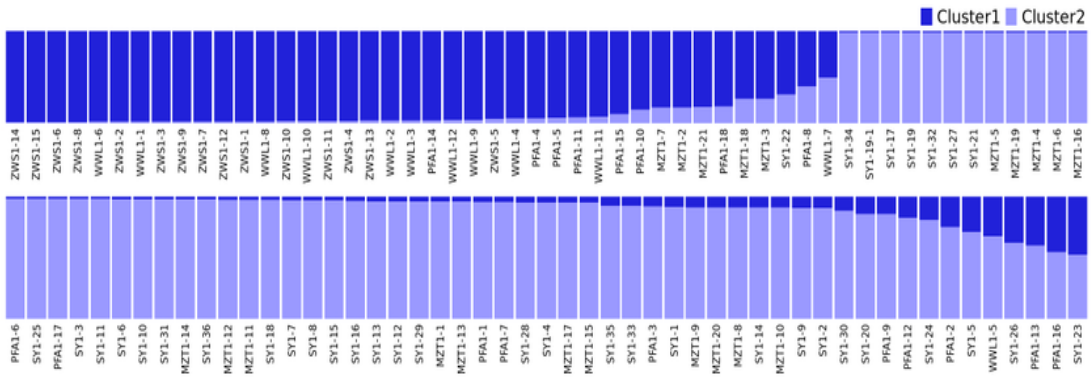

Figure 4. *structure results of samples at K=2*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

Genetic distance and cluster analysis

The genetic distance between populations was calculated in powermarker (Nei, 1983). The maximum genetic distance between the five populations was 0.268423 (SY1/ZWS1) and the minimum was 0.049418 (SY1/MZT1) (*Table 9*). Unweighted group mean method (UPGMA) based on Nei genetic distance was used for cluster analysis (*Figures 5*, *6*).

Table 9. *Genetic distance between populations*

	MZT1	PFA1	SY1	WWL1	ZWS1
MZT1	-	0.119875	0.049418	0.20147	0.265438
PFA1	0.119875	-	0.11575	0.19229	0.265168
SY1	0.049418	0.11575	-	0.202089	0.268423
WWL1	0.20147	0.19229	0.202089	-	0.091688
ZWS1	0.265438	0.265168	0.268423	0.091688	-

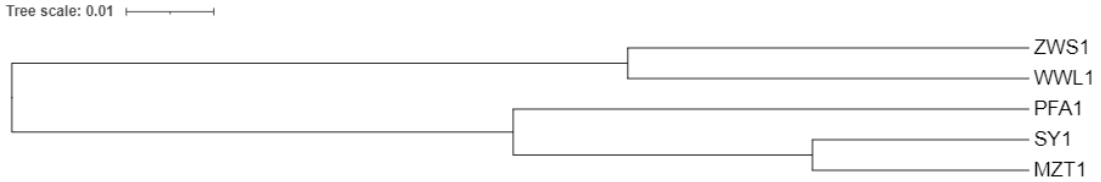

Figure 5. *UPGMA clustering results between groups*

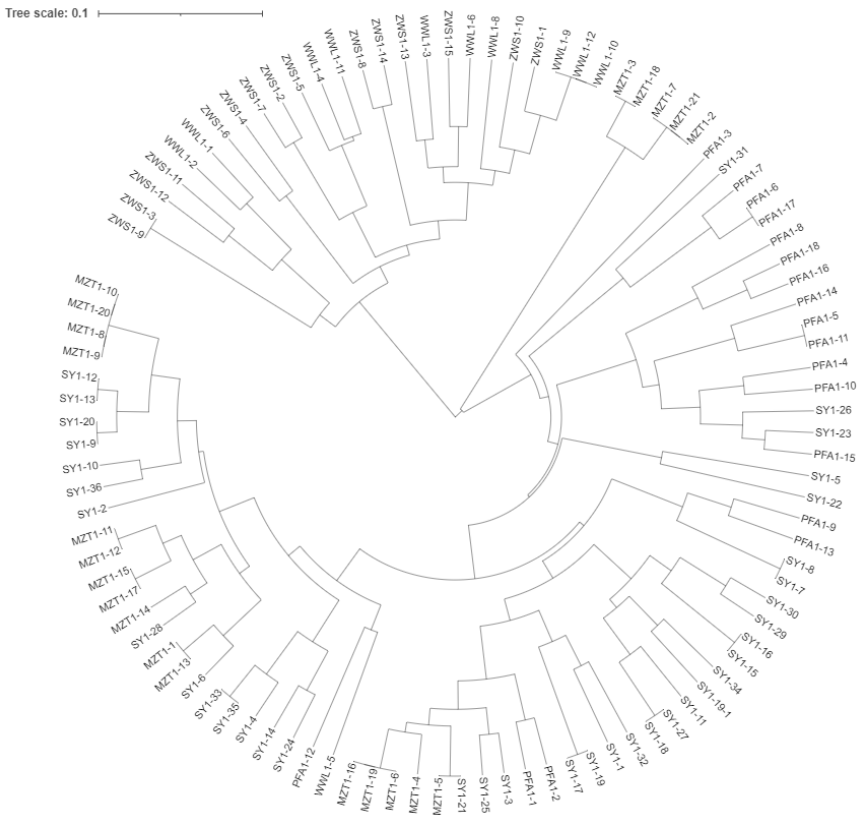

Figure 6. *UPGMA clustering results between individuals*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

Conclusion and discussion

A total of 26 alleles (Na) were detected by 6 pairs of primers in 103 samples, among which the minimum number of alleles was 2, the maximum number of alleles was 9, and the average number of alleles per locus was 4.33. The total number of effective alleles (Ne, the more evenly distributed the alleles in the population, the closer the Ne was to the number of actually detected alleles) was 14.528, with the range of 1.062 (GZST013) to 5.083 (GZST065), and the average number of effective alleles per locus was 2.4213. The Shannon index (I) ranges from 0.135 (GZST013) to 1.835 (GZST065), with an average value of 0.9073. The observed heterozygosity (Ho) ranged from 0 (GZST002) to 0.794 (GZST055), with an average value of 0.443. The expected heterozygosity (He) ranged from 0.058 (GZST013) to 0.803 (GZST065), with an average value of 0.471. The polymorphic information content (PIC) ranges from 0.057 (GZST013) to 0.78 (GZST065), with an average value of 0.4267. The mean inbreeding coefficient was -0.002 and the value was -0.405 (GZST055) -1.000 (GZST002).

At the population level, the Shannon information diversity index (I) of each population in the wild was between 0.6789 and 0.735, with an average value of 0.709. The heterozygosity Ho was 0.349-0.533, with an average value of 0.453. The expected heterozygosity (He) of Nei's diversity index was between 0.348-0.406, with an average value of 0.384. The order of size is MZT1>WWL1>ZWS1>SY1>PFA1, there were significant differences among all populations; The Wright fixation index F of the polymorphic loci of each population ranged from -0.0234 to -0.3932, and the total mean value was -0.147, indicating that there was excessive heterozygote in each population of *C. shiwandashanica*. The results of Nybom's study on genetic diversity of 307 species showed that the mean values of I at the population level of perennials, widespread species and outcrosses were 0.25, 0.22 and 0.27, respectively. According to statistics, the average H of dicotyledonous plants, perennials, short-lived plants, narrow distribution species, outcrossing plants and gravity seed dispersing species at population level was 0.191, 0.20, 0.28, 0.27, 0.19 (Xiong, 2012). The values of I and Ho were 0.709 and 0.453, respectively, higher than the above average plant genetic diversity, indicating that the genetic diversity of *C. shiwandashanica* was at a high level. Among them, MZT1 and WWL1 populations have high genetic diversity, which is suggested to be the priority populations for conservation and utilization.

C. shiwandashanica had high genetic diversity. And its seed germination is higher and easier (Tang et al., 2021). Therefore, the main reasons for the endangered species are: (1) The natural regeneration of the wild *C. shiwandashanica* completely depends on seeds, with few flowering and fruiting plants and low reproductive rate. As a result, the population of *C. shiwandashanica* lacks seedling reserve resources, and the population size gradually decreases and tends to be endangered. (2) Habitat fragmentation will lead to the reduction of the area of a original habitat, the formation of a large number of boundary habitat areas and the distance from the distribution area to the boundary is greatly reduced, and often leave habitat fragments like patches. The impact of habitat fragmentation on the population growing in the habitat is very obvious, because after the habitat fragmentation of *C. shiwandashanica*, the population was divided and isolated, resulting in heterogeneous populations. (3) Due to narrow distribution area, lack of gene exchange and long-term degradation, its ecological adaptability is weak. From the perspective of reproductive strategy, the seed generation cycle is long, the reproductive ability is low, and the survival performance is K strategy. (4) According to the investigation, the threat to *C. shiwandashanica* was mainly caused by human interference,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531

especially for *C. shiwandashanica* growing outside the protected area, such as plants growing in the cinnamon plantation. Since the people had to weed and care every year, *C. shiwandashanica* was removed as a weed. As well as the destruction of large areas of forests, the environment on which *Cycas* live has deteriorated dramatically, making this ancient child plant endangered. Therefore, it is suggested that populations with high genetic diversity should be prioritized for conservation and utilization.

Acknowledgments. The research is supported by National Natural Science Foundation of China (No. 32160091), Guangxi Natural Science Foundation of China (No. 2020GXNSFAA259029), and Guangxi Key Laboratory of Plant Functional Phytochemicals Research and Sustainable Utilization Independent project (ZRJJ2022-2).

REFERENCES

[1] Clugston, J. A. R., Kenicer, G. J. (2022): Sexing cycads - a potential saviour. – Nature Plants 8(4): 326-327.
[2] Gomankov, A. V. (2022): Cycads in the Permian of the Subangara Region. – Paleontological Journal 56(3): 317-326.
[3] Liu, B., Liu, Y. J. (2018): A new community of cycad was discovered in Shiwandashan Protected Area. – Guangxi Forestry 2018(9).
[4] Ma, Y. P., Chen, G., Grumbine, R. E., Dao, Z. L., Sun, W. B., Guo, H. J. (2013): Conserving plant species with extremely small populations (PSESP) in China. – Biodivers. Conserv. 22(3): 803-809.
[5] Qin, H. Z., Yang, X. D., Tang, J. M. (2022): Establishment of SSR and ISSR reaction system and screening of primers for Cycas Shiwandaashan, a wild plant with minimal population in 2022. – Molecular Plant Breeding 20(8).
[6] Shershov, V. E., Lapa, S. A., Kuznetsova, V. E., Spitsyn, M. A., Guseinov, T. O., Polyakov, S. A., Stomahin, A. A., Zasedatelev, A. S., Chudinov, A. V. (2017): Comparative Study of Novel Fluorescent Cyanine Nucleotides: Hybridization Analysis of Labeled PCR Products Using a Biochip. – Journal of Fluorescence 27(6): 2001-2006.
[7] Tang, J. M., Qin, H. Z., Zou, R., Zhu, C., Wei, X., Jiang, Y., Xiong, Z. (2021): Photosynthetic physiological characteristics of Cycas Dawandashan seedlings from a small population of wild plant. – Molecular Plant Breeding 19(11).
[8] Wang, Y. D., Zhang, W., Zheng, S. L., Sanki, K. C., Li, N. (2005): New discovery of fossil cycad-like plants from the Middle Jurassic of West Liaoning, China. – Chinese Sci. Bull. 50(16): 1805- 1808.
[9] Wang, Z. F., Ye, W. H., Cao, H. L., Li, Z. C., Peng, S. L. (2008): Identification and characterization of EST-SSRs and cpSSRs in endangered *Cycas Hainanensis*. – Conserv. Genet. 9(4): 1079- 1081.
[10] Xiong, Z. C., Shi, Y. C., Qi, X. X., Wei, J. Q. (2012): ISSR analysis of genetic diversity of germplasm resources of Zhuangyaozhanzi. – Chinese Journal of Traditional Chinese Medicine 43(10): 2040-2044. (In Chinese).
[11] Yang, Y., Li, Y., Li, L. F., Ge, X. J., Gong, X. (2008): Isolation and characterization of microsatellite markers for *Cycas debaoensis* Y. C. Zhong et C. J. Chen (Cycadaceae). – Mol. Ecol. Resour. 8(4): 913-915.
[12] Yang, Z. X., Gong, X. (2013): Karyotype analysis of five Cycas from China. – Plant Taxa and Resources 35(5): 601-604.
[13] Zhang, H. D., Zhong, Y. C. (1997): New species of Cycas from Guangxi. – Journal of Sun Yatsen University (Natural Science Edition) 36(3): 69-71.
[14] Zheng, Y., Liu, J., Feng, X. Y., Gong, X. (2017): The distribution, diversity, and conservation status of Cycas in China. – Ecol. Evol. 7(9): 3212-3224.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2104_35213531