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Genetic Structure and Differentiation of Endangered *Cycas* Species Indicate a Southward Migration Associated with Historical Cooling Events

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Abstract: Understanding the genetic structure and differentiation in endangered species is of significance in detecting their phylogenetic relationships and prioritizing conservation. Here we sampled five endangered *Cycas* species endemic to southwest China and genotyped genetic structure and differentiation among them using the genotyping-by-sequencing (GBS) method. *C. hongheensis* showed high genetic diversity, but the other four species showed low genetic diversity. The genetic diversity between wild and cultivated populations was similar for *C. debaoensis* and *C. guizhouensis*, respectively. Low genetic differentiation and high gene flow were found among *C. debaoensis*, *C. guizhouensis*, and *C. fairylakea*, and *C. hongheensis* differentiated from them at ~1.74 Mya. TreeMix results showed historic migration events from *C. guizhouensis* to *C. hongheensis*, showing southward migration pathways. *C. hongheensis* showed increased effective population size with time, while the other four species underwent bottleneck events at ~1–5 Mya when continuous cooling events occurred. Our results indicate that the migration, differentiation, and speciation of *Cycas* species are associated with historical cooling events.

Keywords: *Cycas*; endangered species; species differentiation; genotyping-by-sequencing; genetic diversity

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1. Introduction

The genetic diversity of species determines their adaptation and survival to local environments, particularly within the context of global climatic change [1]. Historical climate change and habitat fragmentation caused by human activities pose threats to small and isolated populations of plants [2]. Low genetic diversity likely increases the extinction risk of species [3]. Gene flow and genetic differentiation between populations are generally influenced by habitat fragmentation, overexploitation, and reproductive behavior [4]. Stresses from extreme environmental conditions can exacerbate inbreeding, accumulated genetic load, and other latent genetic issues [5], which likely decrease low effective population size and genetic diversity [6]. Genetic variations, or polymorphisms, reflect the viability and evolutionary potential of natural populations [7], which is crucial for understanding the evolutionary history of extant populations, particularly for endangered species that need effective conservation and management strategies.

It is necessary to study the genetic diversity of endangered plants to scientifically guide protection because the extinction of endangered plants may lead to the destruction of the entire ecosystem [8]. To study genetic diversity, gene marker techniques have been used in plant protection, such as single nucleotide polymorphisms (SNPs), microsatellites (SSRs) [9,10], and random amplified polymorphisms (RAPDs) [11]. SNPs are the most diverse at the DNA level and can reflect the genetic variation of endangered species [12],

particularly in small populations [13]. *Qian* et al. [12] evaluated the genetic structure and differentiation of endangered *Pinus bungeana* using SNPs and proposed potential historical migration events between populations. In a previous study, we used SNPs to evaluate the differentiation history of short-leaved yellow cedar (*Pseudotsuga brevifolia*) populations and proposed that climate change led to their southward migration [14]. *Cai* et al. [15] studied the genetic variation of *Horsfieldia tetratea*, one plant species with extremely small populations (PSESP), supporting the development of effective conservation strategies for species.

As one of the most primitive gymnosperm species in the world, cycad species are key objectives in the evolutionary history of seed plants [16,17]. Cycads comprise two families, Cycadaceae and Zamiaceae, with 10 genera and 344 accepted species [18]. Around ~40% of the species are threatened based on the International Union for Conservation of Nature (IUCN) Red List [17,19]. East Asia is the ancestral area of Cycadaceae, and the extant Cycadaceae originated before the Eocene period (~43 Mya) [20]. *Cycas* is the oldest genus in the monotypic Cycadaceae family, with ~118 species [21–23], and the *Cycas* genus is divided into six sections, i.e., *Stangerioides*, *Asiorientales*, *Indosinenses*, *Cycas*, *Panzhihuaenses,* and *Wadeae* [23]. The section *Indosinenses* is regarded as a sister section to the other sections. We here sampled five endangered *Cycas* species with small population sizes, i.e., *Cycas debaoensis*, *C. guizhouensis*, *C. fairylakea* (*C. szechuanensis* W.C.Cheng & L.K.Fu in POWO and *C. szechuanensis* subsp. fairylakea (D.Yue Wang) in WFO), *C. diannanensis*, and *C. hongheensis*. The first four species belong to the section *Stangerioides*, and *C. hongheensis* belongs to the *Indosinenses* section [17]. The *C. debaoensis* and *C. hongheensis* are mainly distributed in Yunnan and Guangxi provinces, China [17,24,25]; the *C. guizhouensis* is an endangered plant endemic to southwest China [26,27]; wild *C. fairylakea* species live in Guangdong and Guangxi provinces [28]; and the *C. diannanensis* is endangered and endemic to the Red River region in Yunnan province [29]. Furthermore, *C. guizhouensis* and the other four species are plant species with extremely small populations (PSESP) [30]. *Cycas* species have been facing potential endangerment challenges due to the overexploitation of ornamental plants in nature.

Previous studies mainly focused on cycad phylogeography, population genetics, and conservation strategies [20,25,31–37], and found low genetic diversity and high genetic differentiation among *Cycas* species [20,29,34,38,39]. However, most of these studies were based on a few molecular markers, and little is known about the genetic evolution and demographic history of *Cycas* species [40,41]. Thus, it is urgent to detect the genetic diversity, differentiation, and historical population dynamics of *Cycas* species [38,41]. We investigated genetic diversity and differentiation for five *Cycas* species and their historical dynamics with climate change using genotyping-by-sequencing (GBS). Understanding the genetic background of *Cycas* species provides the basis for developing in situ and ex situ conservation strategies.

2. Material and Methods

2.1. Sample Collection

We sampled 133 individuals from five *Cycas* species, *C. debaoensis*, *C. diannanensis*, *C. fairylakea*, *C. guizhouensis*, and *C. hongheensis,* in Yunnan, Guizhou, Guangxi, and Guangdong provinces, China (Figure 1 and Table 1). Among these samples, there were 29 cultivated *C. debaoensis* and 31 cultivated *C. guizhouensis* individuals that had been transplanted from nature (Table 1). Around ~50 g of fresh leaves per plant were sampled and dried in allochronic silica gel for DNA extraction.

The genomic DNA of young leaves from these 133 individuals was extracted using a plant genomic DNA extraction kit (TIANGEN BIOTECH, Beijing, China). The purity of the extracted DNA was detected using a Nanodrop spectrophotometer (ND-1000, Thermos Fisher Scientific, Wilmington, NC, USA), and DNA electrophoresis was simultaneously performed in a 1% agarose gel to ensure DNA integrity. A Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) was used to accurately measure the DNA concentration. High-quality DNA was used for subsequent GBS library construction and sequencing.

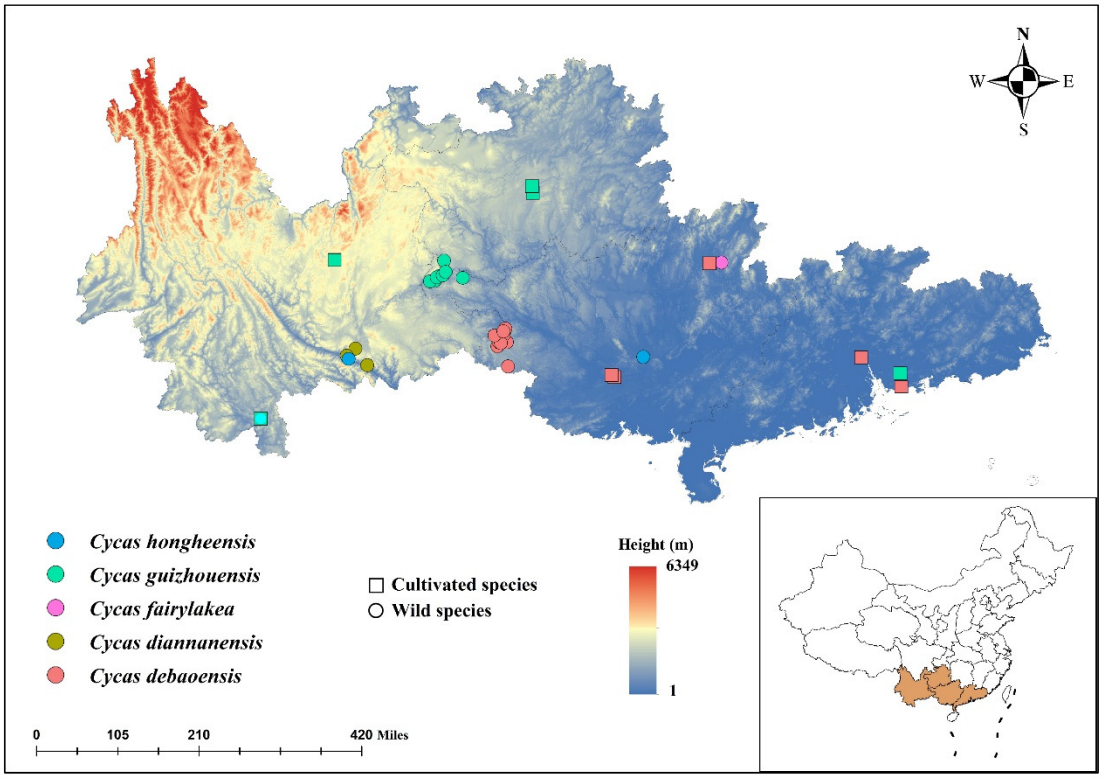

Figure 1. Sampling sites and geographic distributions of the five *Cycas* species in China.

Table 1. Genetic diversity of *Cycas* species based on all sits (variant and fixed).

Species	*N*	Populations	Variant Sites	Private	Poly sites	Poly (%)	*p*	*Ho*	*He*	π
C. debaoensis	53	CS 29 WS 24	1121	21	1011	90.187	0.877	0.242 0.268	0.201 0.220	0.200 0.198
C. diannanensis	17		1121	1	862	76.896	0.894	0.203	0.184	0.234
C. fairylakea	21		1121	9	854	76.182	0.899	0.190	0.171	0.222
C. guizhouensis	36	CS 31 WS 5	1121	10	1026	91.525	0.890	0.186 0.400	0.166 0.336	0.200 0.198
C. hongheensis	6		1121	0	311	27.743	0.928	0.428	0.360	0.447

Abbreviation: *N*, population sample size; Variant sites, variant nucleotide sites; Private, the number of variable sites unique to each population; Poly, a percentage of polymorphic loci; *Ho*, the average observed heterozygosity per locus; *He*, the average excepted heterozygosity per locus; π, the average nucleotide diversity; *FIS*, the average Wright's inbreeding coefficient; CS, cultivated population; WS, wild population.

A total of 0.1–1 ug genomic DNA per sample was digested with two restriction enzymes, EcoRI and PstI (New England Biolabs, Beverly, MA, USA), at 37 °C for 8 h. The ligation products of all samples were equally pooled and size-selected into 300–500 bp fragments using agarose gel electrophoresis. After manipulating gel purification, derived fragments were used as templates for PCR amplification via 25 cycles with EcoRI and PstI adapter universal primers using Prime Star Max DNA Polymerase (Takara, Dalian, China). Finally, the amplicons were size-selected once more into 350–500 bp fragments. The resulting ddRAD library was sequenced on the Illumina HiSeq X ten platform with the paired-end 150 (PE 150) sequencing strategy (Novogene Bioinformatics Technology Co., Ltd., Beijing, China). We matched the clean reads individually to the barcodes and remnant restriction sites at both ends [42].

2.2. SNP Calling

Quality control of the FASTQ-format raw data was performed with the software FastQC [43], while adapter sequences and abnormal nucleotide bases at the 5′terminus were removed. Preprocessed sequence reads were subjected to *Stacks* v2.0's, "*process_radtags*" module to confirm the demultiplexed reads and to check the restriction enzyme sites using default parameters. The quality control for the per-base quality of reads and removal of potential adaptor sequences was performed using FastQC and Cutadapt. Reads were then mapped to *C. hongheensis* as a reference genome using Bowtie2 [44]. The bash command cat was used to combine the two sequences of each sample generated by paired-end sequencing into one sequence. SNP calling for each sample was performed using the *Stacks* pipeline to build loci (*ustacks*), create a catalog of loci (*cstacks*), match samples back to the catalog (*sstacks*), transpose the data (tsv2bam), add paired-end reads to the analysis, call genotypes, and perform population genomics analysis [45]. For the *stacks* parameter, m = 5 was set to the minimum coverage depth, and m = 12 was set to the maximum distance between stacks within an individual. The *cstacks* module-built directories for all samples have n = 12, set as the maximum number of mismatches allowed between individuals. In the population module, we set p = 8 and r = 0.6 to call consensus SNPs. The remaining parameters were defaults.

2.3. Genetic Diversity and Structure Analysis

We calculated the number of private alleles, expected heterozygosity (*He*), observed heterozygosity (*Ho*), nucleotide diversity (π), and inbreeding coefficient (*FIS*) using the "*populations*" module in Stacks [45].

Population structure was performed from a Bayesian-based analysis using the software Admixture v 1.3.0 [46], and results were visualized in Plink v 1.90 [47]. A population structure analysis of 1–6 clusters was set up (K = 1–6), and the cross-validation error (CV error) was calculated by Admixture v 1.3.0 with the sum of the values of 10 permutations. Principal component analysis (PCA) was performed using the R package *adegenet* to identify the genetic variation of populations [48].

2.4. Gene Flow and Genetic Differentiation

We used a composite-likelihood approach implemented in TREEMIX (v1.13) to test gene flow among the five *Cycas* species [49]. The TREEMIX algorithm was run from 0 to 6 migration events using the –m parameter. Residuals were used to select the best-fit model.

The coefficient of genetic differentiation (*FST*) among populations was calculated in the program vcftools [50]. The values of *Nm* were estimated from *FST*, as $Nm = (1 - FST)/4\,FST$ for indirectly estimating gene flow [51]. Analysis of molecular variance (AMOVA) was conducted to assess genetic differentiation within populations in Arlequin 3.5.2.1 [52], and the significant level of the variance components was computed using 1000 permutations.

2.5. Population Demographic History

The maximum likelihood (ML) phylogenetic tree of the populations was constructed using the IQ-tree with the recode-INFO-all model [53].

Effective population size was inferred by Stairway Plot v2, a model-flexible method for inferring historical changes in population size based on site frequency spectrum (SFS) [54]. We set the mutation rate at 1.0×10^{-8} per site and the generation time at 40 years (International Union for Conservation of Nature, 2020). A folded SFS-formatted file was generated by the Python script "*easySFS*".

Fastsimcoal 2 (v 2.5) was used to detect bottleneck events based on *Ne* [55]. The mutation rate was set to 1.0×10^{-8} because the common mutation rate of the Cycas family is 1.0×10^{-8} [56]. Statistical models were estimated 50 times, each with 10,000 simulations and 40 executed loops (ECM cycles) for each estimation [57]. The optimal model was selected with the highest parameters.

3. Results

3.1. SNP Characteristics of Cycas Populations

The *cstacks* module processing generated 3,649,319,605 ± 832,280,494.5 reads, and the average depth per site was 7.5×. We identified 538,982 raw SNPs and then obtained 18,597 loci with 5605 variant sites after SNP calling and filtering with the *population* module.

3.2. Genetic Diversity of Cycas Species

The populations of five *Cycas* species showed similar observed heterozygosity (*Ho* = 0.145–0.428), expected heterozygosity (*He* = 0.128–0.360), and nucleotide diversity (π = 0.151–0.447) (Table 1). Among the five *Cycas* species, *C. hongheensis* (*Ho* = 0.428, *He* = 0.360, π = 0.447) (Table 1) showed the highest genetic diversity, while *C. debaoensis* exhibited the lowest genetic diversity (*Ho* = 0.145, *He* = 0.128, π = 0.151) (Table 1). The genetic diversity (*Ho*, *He*, and π) between cultivated populations and wild populations was similar for *C. debaoensis* and *C. guizhouensis*, respectively (Table 1).

3.3. Genetic Phylogenetic Relationship of the Five Cycas Species

The population structure analysis showed that the *C. debaoensis* species differed from the other four species (*K* = 2) (Figure 2 and Table S1). When *K* = 3 (best delta *K*, Figure 2), *C. debaoensis*, *C. fairylakea*, and *C. guizhouensis* separated, while *C. hongheensis* and *C. diannanensis* showed introgression from *C. fairylakea* and *C. guizhouensis* (Figure 2). When *K* = 4, *C. hongheensis* and *C. diannanensis* still clustered together, corresponding to their closest geographic distribution (Figures 1 and 2). The cultivated and wild populations were not separated for *C. debaoensis* and *C. guizhouensis*, respectively (Figure 2).

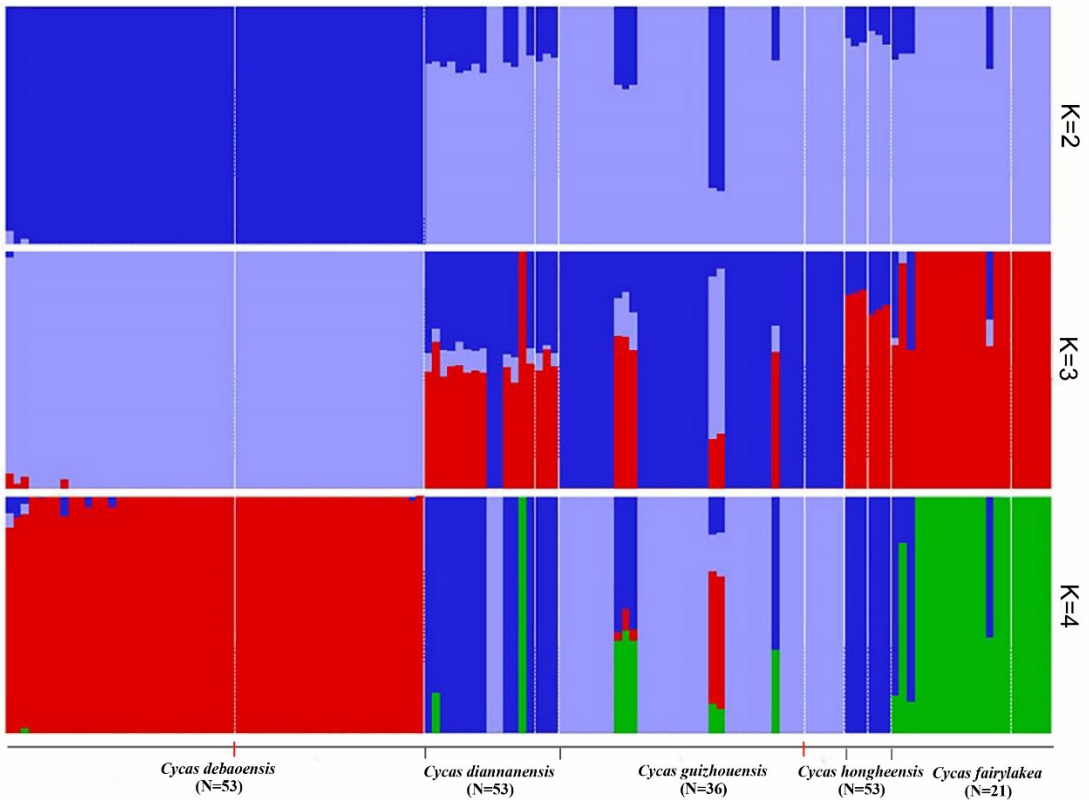

Figure 2. Genetic structures of populations of five *Cycas* species. Population structure bar plots show the clustering of samples from K = 2 to 4. The best K = 3. Each vertical bar indicates a single individual, and the height of each colored bar represents the proportion of assignments to a given cluster. The red line segment distinguishes between wild species (**left**) and cultivated species (**right**) within the species.

The principal component analysis (PCA) analysis confirmed the structure results (K = 3). *C. debaoensis*, *C. fairylakea*, and *C. guizhouensis* separated, while *C. hongheensis* and *C. diannanensis* clustered in the center (Figure 3).

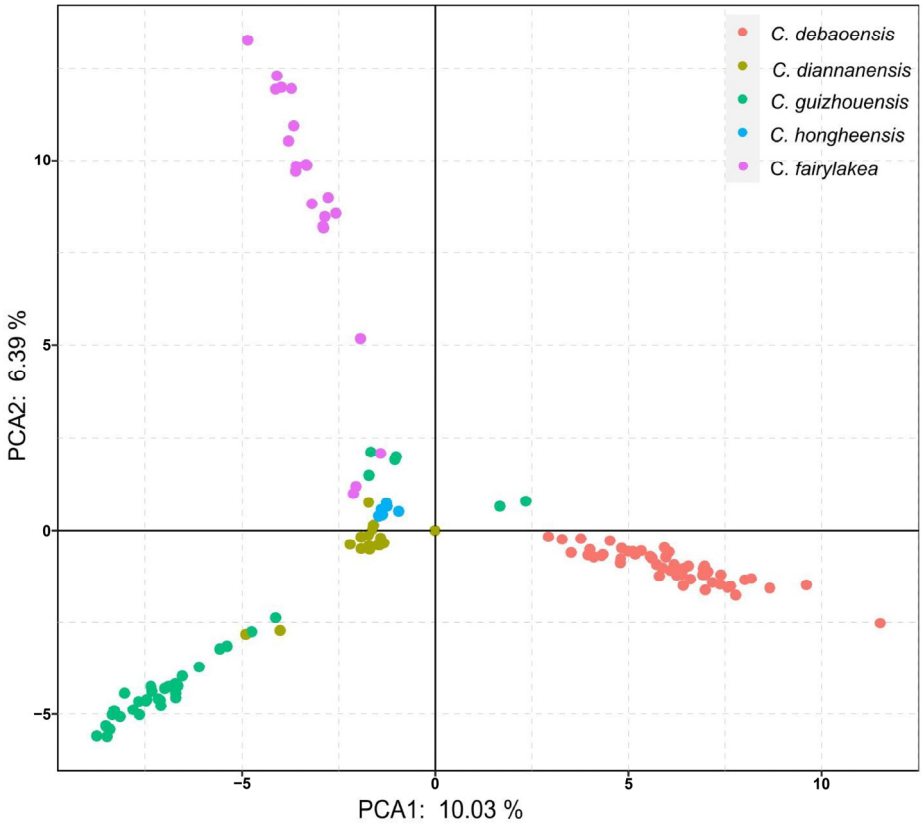

Figure 3. Plots of the first two dimensions of a principal component analysis (PCA) for all individuals of the five *Cycas* species.

3.4. Genetic Differentiation and Gene Flow among the Five Cycas Species

The genetic differentiation coefficients (*FST*) ranged from 0.005 to 0.591 among the five *Cycas* species (Table 2). We detected low levels of genetic differentiation (*FST* = 0.005–0.012) and high levels of gene flow (*Nm* = 20.58–49.75) among *C. debaoensis*–*C. fairylakea*–*C. guizhouensis* (Table 2). *C. hongheensis* showed relatively high genetic differentiation and low gene flow compared to other *Cycas* species (Table 2). Analysis of molecular variance (AMOVA) showed that 18.66% of the genetic variation of the five *Cycas* species was attributed to populations and 81.34% to individuals (Table 3).

Table 2. Matrix of pairwise *FST* and *Nm* coefficient of *Cycas* species.

	C. debaoensis	*C. diannanensis*	*C. fairylakea*	*C. guizhouensis*	*C. hongheensis*
C. debaoensis		1.688	20.583	41.417	0.239
C. diannanensis	0.129		1.353	1.439	0.285
C. fairylakea	0.012	0.156		49.750	0.173
C. guizhouensis	0.006	0.148	0.005		0.188
C. hongheensis	0.511	0.467	0.591	0.571	

Top-right matrix refers to the pairwise gene flow coefficient. Lower-left matrix refers to the pairwise genetic differentiation coefficient.

Table 3. Analysis of molecular variance (AMOVA) in five *Cycas* species.

Source of Variation	df	SS	σ	%
Among species	4	9.489	0.052	18.66
Among individuals within species	112	24.383	−0.009	−3.12
Within species	117	27.5	0.235	84.45
Total	223	61.372	0.278	

Note: df: degree of freedom; SS: sum of squares; MS: mean of squares; σ: each species and the percent of the total variance explained by each source of variance; %: percentage of variance.

Among 1–3 migration events in TREEMIX, *C. Guizhouensis* has a strong gene flow pointed to *C. hongheensis*, which revealed historic migrations from *C. guizhouensis* to *C. hongheensis*, indicating a southward migration of *Cycas* species (Figures 1, 4 and S2).

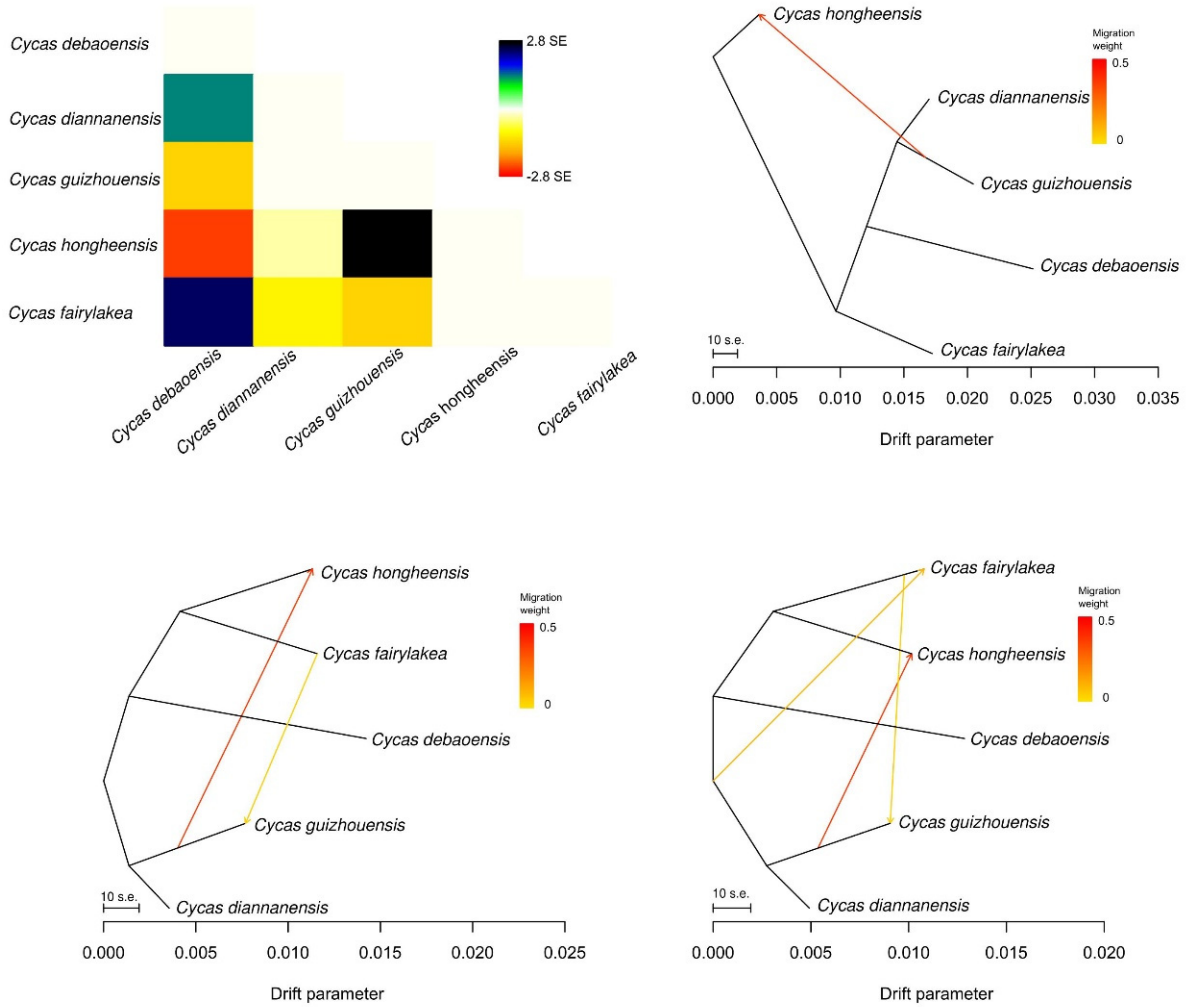

Figure 4. TREEMIX results showing historical mitigation events among the five *Cycas* species. Upper left corner shows residual fit plots in the TREEMIX analysis. We divided the residual covariance between each pair of populations by the average standard error across all pairs. We then plot in each cell this scaled residual. Colors are described in the palette on the right. Residuals above zero represent populations that are more closely related to each other in the data than in the best-fit tree and thus are candidates for admixture events.

3.5. Demographic History of the Five Cycas Species

The optimal result model was confirmed based on the minimum Δ Likelihood (Table S1). Fastsimcoal results showed that *C. hongheensis* differentiated from the other four *Cycas* species at ~1.74 Mya (Figure 5). The differentiation time between *C. guizhouensis* and the other three *Cycas* species was at ~0.40 Mya. The recent differentiation event occurred at ~0.16 Mya between *C. debaoensis* and *C. fairylakea* (Figure 5).

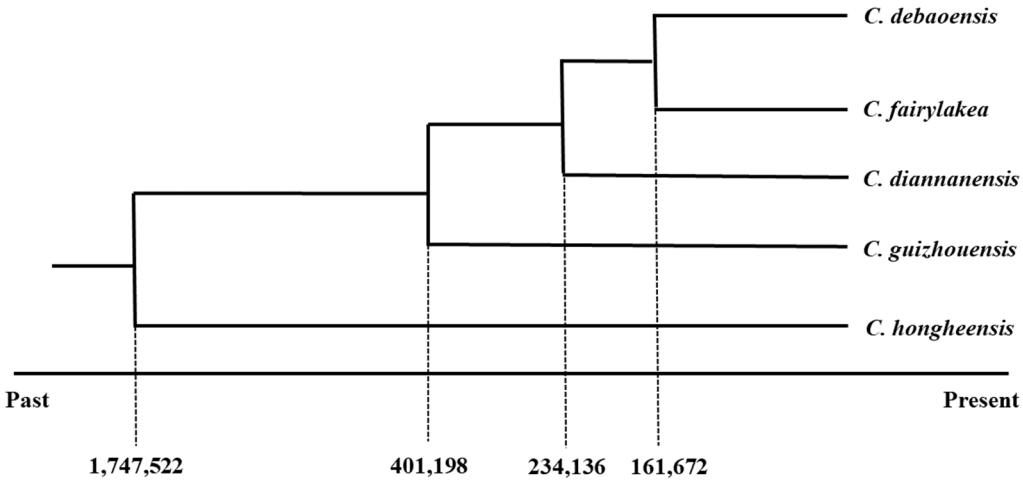

Figure 5. Best-fitting model inferring demographic histories and differentiation for the five *Cycas* species implemented by the Fastsimcoal 2.5. Number unit: years ago.

The effective population size of *C. hongheensis* increased at ~5 Mya, while the other four species underwent bottleneck events at ~1–5 Mya. The effective population size of *C. fairylakea* and *C. guizhouensis* started to decrease at ~ 5–10 Mya, and *C. debaoensis* and that of *C. diannanensis* started to decrease at ~4–5 Mya (Figure 6).

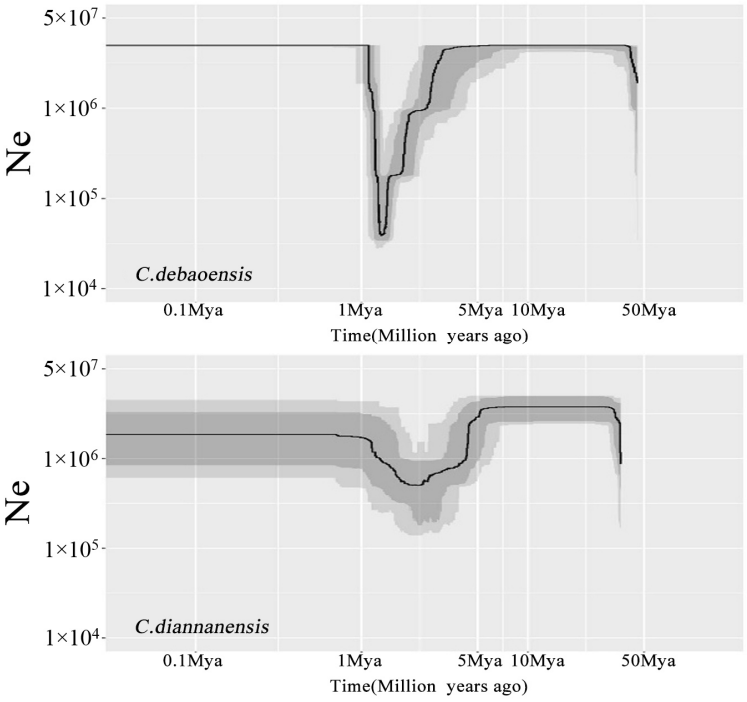

Figure 6. *Cont.*

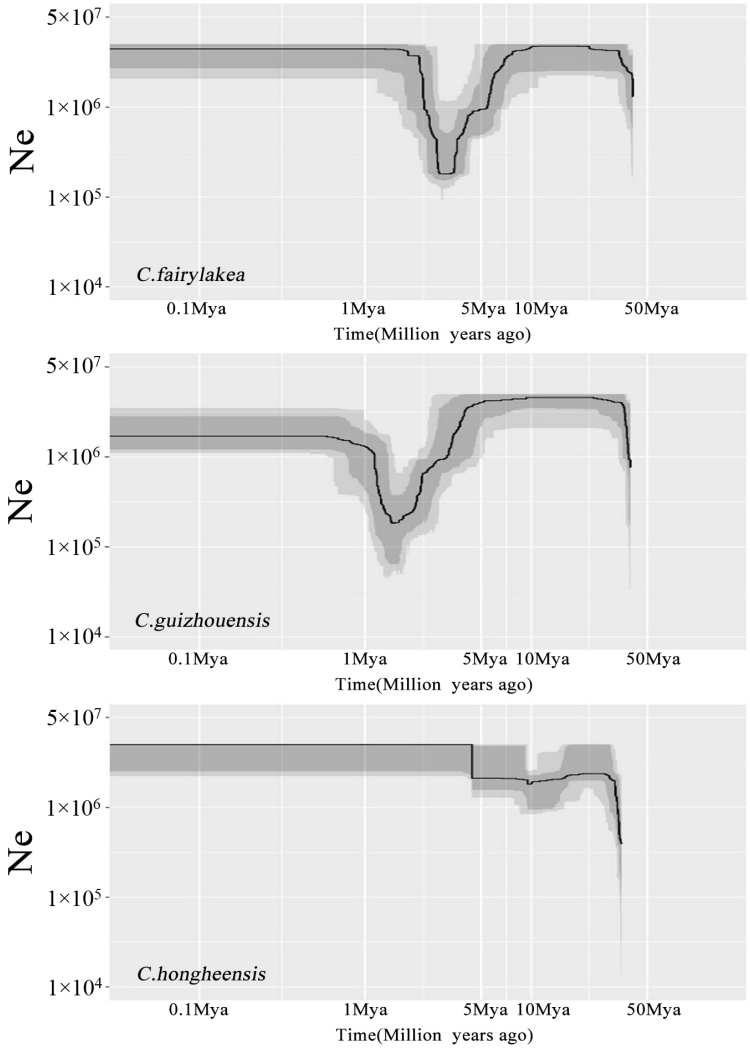

Figure 6. Historical effective population sizes of five *Cycas* species. Black lines and gray shadows represent the medians and the 2.5 and 97.5 percentiles, respectively.

4. Discussion

Climate change and human activities likely result in habitat fragmentation, limit the geographic ranges of plants and even lead to their extinction [58–60]. Maintaining the genetic diversity of natural populations is key to the survival and evolutionary potential of species [61,62]. Cycads, as one of the extant gymnosperm groups, are important for conserving genetic diversity and understanding the origin and early evolution of seed plants [20]. Most *Cycas* species are narrowly distributed [63], but they have experienced a long evolution process and likely possess high genetic diversity [64]. Here, using genotyping-by-sequencing (GBS), we found that the migration, differentiation, and speciation of *Cycas* species are associated with historical cooling events.

As an ancient gymnosperm species, cycads are considered to possess high genetic diversity and low genetic differentiation among populations [65,66]. Genetic structure in plant populations is shaped by mating systems, population density, and the continuity of geographical distribution [67–69]. Due to the dioicous characteristics and long life cycle of *Cycas* species, they are considered to have high genetic diversity [20]. However, a lack of pollinators or seed dispersal limits gene flow between populations [70,71]. Liu et al. [20] found that the distribution and phylogeography of *Cycas* species are shaped by the long-distance seed dispersal driven by ocean current systems. In this study, *C. hongheensis* has a longer evolutionary history than the other four *Cycas* species [65,66], which likely explains its high levels of genetic diversity. Maintaining the genetic diversity of *C. hongheensis*

is important to ensure its continued survival and evolutionary potential [61]. However, the other four Cycas species showed low genetic diversity. Previous studies showed relatively high genetic diversity among the four species, e.g., *C. debaoensis* (*Ho* = 0.389, *He* = 0.484 for cpDNA) [72], *C. guizhouensis* (*Ho* = 0.311, *He* = 0.419 for cpDNA and SSR) [27], *C. diannanensis* (*HT* = 0.627 for cpDNA) [29], and *C. fairylakea* (mean *Ho* = 0.550 and *He* = 0.420 for SSR) [73]. The difference in genetic diversity is likely due to different methods, i.e., several loci in the SSR and cpDNA analyses but 18,597 loci in this study with GBS.

One reason to explain the relatively low genetic diversity of the four Cycas species that experienced bottleneck events at ~1–5 Mya [74,75] is that the loss of heterozygosity is positively correlated to bottlenecks [76]. In addition, an alternative reason is that four *Cycas* species have a limited geographical range with small and isolated populations, which likely results in high levels of genetic drift and inbreeding [61,62]. It is consistent with other narrow-ranged species, for example, the threatened species *Thuja sutchuenensis* (*FST* = 0.011–0.191 for cpSSR) [77] and three endangered *Rhododendron* species (*FST* = 0.128–0.387 for RAD-seq) [78]. Thus, rare species with small populations generally have low genetic diversity compared to those species with large and widespread geographical populations [79,80], such as *Paeonia decomposita* [81], *Mentha cervine* [82], and *Omphalogramma souliei* [83]. The endangered status likely results from intensive human activities [84], e.g., deforestation [85], grazing [86], and road construction [87], which lead to habitat fragmentation and low population size [88].

Understanding long-term demographic history is important not only to elucidate the genetic characteristics of species [89,90] but also to detect the effects of climate change and habitat fragmentation on historical population dynamics [91]. Cycads originated before the mid-Permian and showed the greatest species diversity during the Jurassic-Cretaceous [92,93]. However, the extant cycads have undergone a synchronous global re-diversification at ~12 Mya [16]. We here found that *C. hongheensis* increased population size, but the other four species underwent bottleneck events at 1~5 Mya, which is consistent with previous studies [16,27,29,72,94]. *C. hongheensis* differentiated from the other four *Cycas* species at ~1.74 Mya, which supports the hypothesis that *Cycas* L. originated in the Quaternary in south China [31]. The divergence time (~1.74 Mya) among *Cycas* species and bottleneck times (1~5 Mya) of *Cycas* species correspond to the Pliocene epoch (2.6~5.3 Mya), which was a period of global cooling and drying. Climate change is generally considered an important factor in threatening the survival of plants, particularly those rare and endangered plants with narrow distributions and small population sizes [95]. For example, during the Quaternary (~2.58 Mya), climatic oscillations exerted significant impacts on the genetic diversity of plants in the northern hemisphere [96]. Most cycad plants prefer to live in warm and moist habitats such as valleys or slopes of ridges and cliffs [21]. This likely explains the southward migration of cycad species from *C. guizhouensis* to *C. hongheensis* found here. Thus, the glacial-interglacial fluctuation restricted the dispersal of Cycad plants and thus gene flow between populations [25,29,34,39].

Overexploitation not only directly threatens the survival of Cycas species but also destroys their habitats. Thus, it is urgent to take effective measures for the conservation of Cycas species. In situ and ex situ protection are effective for the protection of cycads [97,98]. Ex situ conservation and reintroduction measures can improve the population size and genetic diversity of endemic and endangered species, but it is likely that introgression occurred from cultivated to wild *Cycas* species [97,99]. This is common in *Populus*, as many varieties are intentionally introduced, providing conditions for artificial hybridization and introgression [100]. Compared to wild populations, cultivated ones generally have low genetic diversity because founder effects and genetic drift occurred during the process of demonstration and cultivation [77,101], such as *Spondias purpurea* [102], *Morus* species [103], and *Zanthoxylum* [104]. In this study, cultivated *C. debaoensis* and *C. guizhouensis* species transplanted from nature to be reintroduced after breeding did not differ from wild populations. That is because wild seedlings are currently transferred to parks [72], and individuals cultivated in the same place might have come from multiple wild source populations.

However, limited pollinators and seed dispersal may negatively affect the reproductive systems of *Cycas* species [105]. For example, the pollination limitation of the alpine shrub *Rhododendron aureum* weakens its reproductive ability [106]. Thus, it is key to protect the species in its natural habitat (in situ) through setting up nature reserves and protection stations, strengthening artificial pollination, raising local farmer conservation awareness, limiting human activities, conducting research on fast reproduction, and strengthening government management [107,108].

5. Conclusions

In summary, we utilized genotyping by sequencing (GBS) to analyze the genetic structure and differentiation of five endangered cycad species in southwestern China. Results indicate that *C. hongheensis* showed high genetic diversity, but the other four species showed low genetic diversity that likely resulted from bottleneck events at ~1–5 Mya. The genetic diversity between wild and cultivated populations was similar for *C. debaoensis* and *C. guizhouensis*, which is consistent with the results of genetic structure, PCA, and *Fst*. The population differentiation history and gene flow analysis showed that *Cycas* species had a southward migration pathway. Moreover, the migration, differentiation, and speciation of *Cycas* species are associated with historical cooling events. Thus, we proposed strategies for protecting cycad germplasm resources in their natural habitats (in situ) through the construction of nature reserves and other protection stations to strengthen field monitoring.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15050643/s1, Table S1: Parameters of seven demographic models with the Fastsimcoal 2.5.

Author Contributions: Y.L.(Yongbo Liu) and Y.L.(Youzhi Li) designed the study. Z.H., Z.Y., K.W. and Y.L.(Youzhi Li) collected the data. Y.L.(Yongbo Liu), Z.H. and Z.Y. analyzed the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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