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Evidence for the Use of Karst Tiankengs as Shelters: The Effect of Karst Tiankengs on Genetic Diversity and Population Differentiation in *Manglietia aromatica*

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Abstract: Karst tiankengs in China are globally significant locations for studying ecological environments and plant diversity. However, there are few reports on how the unique geographical environment of tiankengs affects plant genetic diversity and genetic structure. This study used Hyper-seq gene sequencing technology to develop large-scale genomic SNPs of *Manglietia aromatica*, both within and outside the tiankengs. Its aim was to investigate the impact of tiankengs on the genetic diversity and genetic structure of the *M. aromatica* population. The analysis results indicate that the genetic diversity of the populations within the tiankeng ($\pi = 0.2044$) is higher than that of the populations outside of it ($\pi = 0.1671$), indicating that the tiankengs have a positive impact on species diversity. The genetic differentiation coefficient (F_{ST}) between the populations inside and outside the tiankeng was 0.0534 and the F_{ST} values of populations within the tiankeng were 0.077, 0.082, and 0.141, meaning that the genetic variation in the tiankengs is very high. The genetic similarity outside the tiankengs is also very high, indicating that the tiankengs are effectively preserving the genetic diversity of *M. aromatica*. Furthermore, the gene introgression analysis results gave no proof of gene flow between the three tiankeng populations. This suggests that the tiankengs not only protect species diversity, but also hinder gene flow between populations to some extent. However, this hindrance may gradually subside with the evolution of the tiankengs. The genetic structure analysis revealed that the *M. aromatica* population in Guangxi, China, can be classified into three subpopulations. The first is the tiankeng subpopulation, including all the populations in tiankengs. The second subpopulation consists of populations surrounding the tiankengs. These two subpopulations are distributed in Leye County in northwestern Guangxi, China, and are very close to each other. The third is the Huanjiang subpopulation, which is located far away from the tiankengs. Considering the direction of gene flow and genetic structure, it is speculated that the populations in the tiankengs evolved from the populations near the pit mouth. This study confirms that the tiankengs are shelters and provide a suitable habitat for the endangered plant *M. aromatica*, because its genetic diversity is well conserved and the species is well adapted to the habitat within the tiankengs.

Keywords: endangered tree; SNP; Hyper-seq; genetic characters; gene integration; evolution stages

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1. Introduction

China has the most extensive distribution of karst terrain globally, covering an area of 344 km^2. The unique development process of karst landforms has resulted in the formation of distinctive landscapes, including peak clusters, peak forests, caves, and karst sinkholes [1,2]. One of the recent discoveries in karst topography is the karst tiankengs, which are large funnels formed due to karstification. This particular funnel has a volume of

over 1,000,000 m^3 [3]. Currently, there are approximately 300 known tiankengs worldwide, with over half of them located in China, earning it the title of the 'world karst tiankeng kingdom' [4]. The Dashiwei tiankeng group in Leye, Guangxi, China is the largest tiankeng group in the world, with the largest number, scale, and densest distribution [5]. Due to the surrounding cliffs, tiankengs have unique hydrothermal conditions, which are different from the conditions in the surrounding areas, giving rise to rich and unique flora and fauna [6]. They can be a natural conservation pool for germplasm resources and a refuge for surface-native species [3]. At present, research on the ecosystem of tiankeng has mainly focused on the distribution and adaptive evolution of their vegetation [7,8].

Manglietia aromatica is a rare and endangered tree of the genus *Manglietia*, family Magnoliaceae. It is a native to the tiankeng region of China. *M. aromatica* is found mainly in the karst rocky mountain areas of Guizhou, as well as in the southwestern part of Guangxi and the southeastern part of Yunnan. It is a nationally protected plant with a limited range. *M. aromatica* is a tree species with an attractive shape, large flowers, and a pleasant fragrance. It is commonly used as an ornamental tree in landscaping. Additionally, due to the plant's aroma, it can be used as a raw material for spice preparation, making it a valuable and sought-after commodity. Its economic value and broad market appeal has garnered significant attention. Therefore, the depletion of natural resources is a threat to germplasm resources. Furthermore, *M. aromatica* has a low ability to germinate pollen and to pollinate in poor conditions. At the same time, the natural regeneration of the population has been hampered by the degeneration and abortion of megaspores and female gametes [9]. Therefore, in habitats other than tiankeng, the wild populations of *M. aromatica* are mostly scattered, and some populations consist of only a single plant. Nevertheless, the wild populations of *M. aromatica* in the tiankeng habitats are relatively concentrated. The most concentrated and largest community of *M. aromatica* is currently the Dashiwei tiankeng in Leye, Guangxi. This karst tiankeng is steep and mostly distributed in mountainous areas with limited transportation, which has helped to protect the resources of *M. aromatica*. Bátori et al. [10] studied the vegetation patterns inside and outside sinkholes in Hungary and found that the population able to grow in the natural environment outside the sinkhole tended to develop inside the sinkhole. With the current threat of global warming to biodiversity, there have been few reports on the effectiveness of tiankengs in conserving species. There is an overall absence of exploration on the genetic diversity of *M. aromatica*, with current research focusing on growth characteristics [11], seedling breeding [12] and transcriptome analysis [13].

Assessing genetic diversity is essential for developing conservation and management strategies. However, genetic structure and diversity can vary significantly between species [14,15]. Therefore, it is vital to establish security measures and methodologies to defend species. Due to its advantages of high polymorphism, high genetic stability and easy automated detection, the third-generation molecular marker technology represented by single-nucleotide polymorphisms (SNPs) has become the best choice for population genetic analysis and genomics research [16,17]. In this study, we utilized a novel, viable and adaptable marker-assisted determination and genotyping strategy: Hyper-seq. This method has the effect of gene region enrichment. By using a special PCR method, a large number of sample data can be efficiently processed and a large number of genotype data can be generated, providing an accurate scientific basis for studying the genetic diversity of species [18]. In summary, we speculated that the populations of *M. aromatica* in the tiankengs has gradually evolved from the populations near the pit mouth in the past and relatively isolated karst tiankengs may play a certain protective role in the genetic diversity of *M. aromatica* populations. However, the diversity of the tiankeng environment and the restriction of gene exchange may lead to greater genetic differentiation between populations inside and outside tiankeng, as well as within tiankeng populations. Therefore, we used Hyper-seq technology to investigate the genetic diversity, gene flow differences and genetic differentiation between *M. aromatica* populations inside and outside tiankengs. The aims were to: (1) clarify the influence of the karst tiankeng on the genetic characteristics of the

M. aromatica, (2) explore the influence of the evolution of the tiankengs on the evolution of plants in this area, (3) put forward corresponding protection measures and suggestions for the genetic characteristics of *M. aromatica* and the evolution of tiankengs.

2. Materials and Methods

2.1. Plant Materials

Leaf samples were collected from 35 *Manglietia aromatica* individuals from 10 populations (Figure 1 and Table 1). The DC, LJ and SM populations were distributed inside the karst tiankengs. The CW-MBL, FD, FY, XB and CY-LH populations were distributed outside the karst tiankengs. The phenomenon of single individual *M. aromatica* plants outside the tiankeng populations was significant. The majority of individuals in the populations were mature plants with a short tree shape, and there was a scarcity of saplings and seedlings. The number of individuals of *M. aromatica* in tiankengs was large and the age composition was reasonable, comprising both adult plants and young trees. Additionally, the adult individuals were tall and had mature fruits. Compared to the ecological environment of populations outside the tiankeng, the temperature inside the three tiankengs studied in this paper is lower, and the soil is moist with rich humus. The 10 populations of *M. aromatica* are distributed in Guangxi, China. These populations can be divided into 3 categories according to geographical location: the Napo County populations, Leye County populations and Huanjiang County populations. Among these, Leye County has the largest number of *M. aromatica* populations of 7. There are 2 populations in Napo County, but the number of individuals available for research is scarce. Huanjiang County has 1 population.

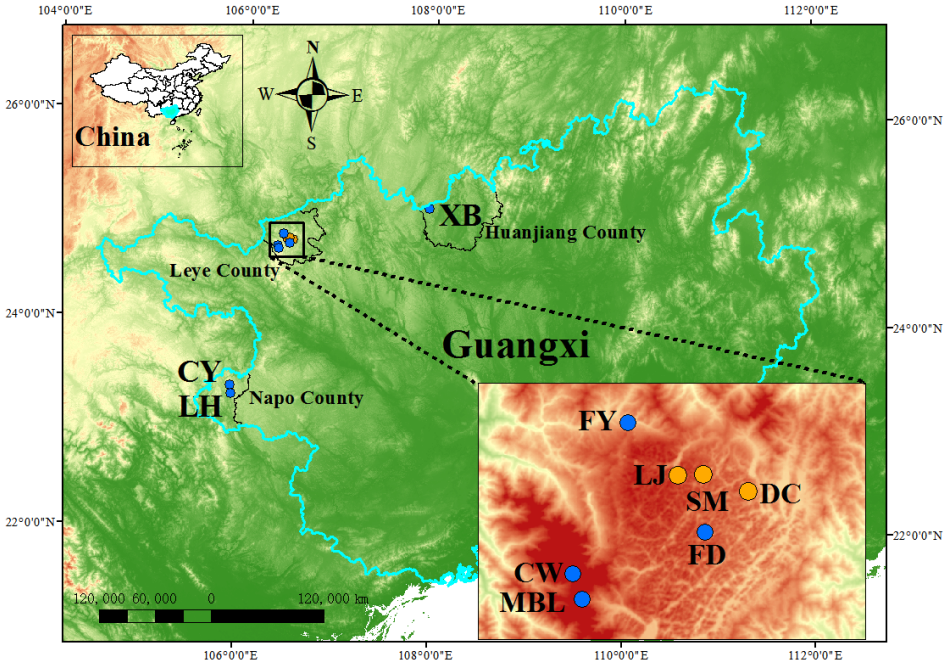

Figure 1. The distribution map of 10 populations of *Manglietia aromatica*. Note: The orange points represent the populations of *M. aromatica* inside the karst tiankeng and the blue points represent the populations of *M. aromatica* outside the karst tiankeng. CW-MBL, FD, FY, XB, CY-LH, DC, LJ and SM are the abbreviation of sampling site, the full names are shown in Table 1.

Because the total number of samples for some *M. aromatica* populations is small, we balanced the total number of samples with the sampling distance in order to collect as many samples as possible. In the end, the distance between all the samples taken is more than 5 m. Three to five healthy, pest-free leaves were collected from each individual and immediately placed in stained silica gel for dehydration and preservation. The number of samples that were collected for each of the populations is shown in Table 1.

Table 1. Sampling information of *Manglietia aromatica*.

Population	Location	Type	Latitude	Longitude	No. of Samples
CW-MBL	Caowang Mountain and Maobiliang Mountain, Leye County, Guangxi, China	Outside the tiankeng	24°43′46″ N	106°21′21″ E	7
FD	Fengdong village, Leye County, Guangxi, China	Outside the tiankeng	24°45′42″ N	106°28′20″ E	2
FY	Fengyandong Mountain, Leye County, Guangxi, China	Outside the tiankeng	24°51′01″ N	106°24′21″ E	3
XB	Xiabai Mountain, Huanjiang County, Guangxi, China	Outside the tiankeng	25°8′34″ N	107°56′21″ E	5
CY-LH	Chengyang Town and Liuhua village, Napo County, Guangxi, China	Outside the tiankeng	23°22′48″ N	105°53′35″ E	2
DC	Dacao Tiankeng, Leye County, Guangxi, China	Inside the tiankeng	24°47′39″ N	106°30′38″ E	4
LJ	Luojia Tiankeng, Leye County, Guangxi, China	Inside the tiankeng	24°48′26″ N	106°26′58″ E	10
SM	Shenmu Tiankeng, Leye County, Guangxi, China	Inside the tiankeng	24°48′28″ N	106°28′19″ E	2

Note: Due to the rare number of individuals in CY, LH and MBL, these populations were merged with nearby populations to calculate the overall genetic diversity parameters. The same below.

2.2. Hyper-seq Library Construction and Sequencing

DNA was extracted from 35 *M. aromatica* leaf samples using the Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the instructions of the manufacturer. In total, 1% agarose gel electrophoresis and a Nanodrop 2000 spectrophotometer (Thermo Fisher, Walsham, MA, USA) were used to assess DNA quality. Extracted DNA was measured using a Qubit 3.0 (Thermo Fisher, Walsham, MA, USA) to ensure each sample met the following criteria: total mass > 3 μg, concentration > 30 ng/μL, and OD_{260}/OD_{280} ratio between 1.80 and 1.20. After, barcode adaptor ligation, fragment size selection and PCR amplification were performed to obtain a hyper-seq library [18]. The library was submitted for 150 bp paired-end sequencing on the Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA).

2.3. SNP Calling

The initial data quality was evaluated utilizing FastQC [19]. A Trimmomatic v0.36 (default parameters) [20] was used to extract adapter sequences, cleavage sites (first 6 base pairs of reads) and low-quality reads (Q20 < 20, length < 36 bp) from raw sequence data [20]. The clean reads were then aligned with the *Magnolia hypoleuca* (Magnoliaceae) reference genome [21] using BWA v0.7.12 [22]. The Stacks v.2.59 software's rm-pcr-duplicates parameter was utilized to eliminate PCR repeats and conduct SNP calling [23]. The SNPs were filtered using Vcftools v0.1.11 [24]. Sites with a population loss rate of over 40%, maf greater than 0.05, and a sequencing depth of less than 3 were removed.

2.4. Population Genetics, Population Structure and Genetic Relationship Analysis

Using the selected high-quality SNP data, we computed the genetic diversity parameters of the 10 populations of *M. aromatica* separately, including observed heterozygosity (H_O), expected heterozygosity (H_E), nucleotide diversity (π) and inbreeding coefficient (F_{IS}), using Stacks v2.59 [23]. The π, Tajima' D value, and the genetic differentiation coefficient (F_{ST}) and absolute differentiation coefficients (D_{XY}) between populations outside and inside

the tiankeng were computed using PIXY software v1.2.7 [25]. The sliding window size of the operation was set to 100 kb.

A maximum likelihood (ML) phylogenetic tree was constructed in IQtree v2.0 for 35 *M. aromatica* individuals [26], with *Manglietia insignis* set as the outgroup. Admixture v1.3.0 [27] was used to perform Bayesian clustering on all individuals, with clustering values (K) set from 1 to 9. The minimum cross-validation (CV) error was used to determine the optimal K value.

PCA and phylogenetic analyses were carried out using MingPCACluster v1.38, a simple and efficient piece of software for working with population VCF files [28].

2.5. Gene Introgression Analysis

The D-statistics of 120 combinations between *M. aromatica* populations were calculated using Dsuite software v0.3 [29] with *Manglietia insignis* as outgroup O, and significance tests were conducted using Z-scores. The D-statistic, also known as the ABBA-BABA test, is a method for the detection of gene exchange or gene infiltration between populations or related species on the basis of differences in DNA sequence. This method usually uses four populations ((P1, P2) P3) O) as the object of analysis, where O is the outgroup, P1 and P2 are sister groups, and P3 is the group of possible sources of gene exchange. The D-statistics was used as the reference index. If $D > 0$, it indicates that P3 has an introgression effect on P2, that is, gene flow occurs from P3 to P2, and the genetic relationship between P3 and P2 is closer. If $D < 0$, it shows that P3 has an introgression effect on P1, that is, gene flow occurs from P3 to P1, and the genetic relationship between P3 and P1 is closer; if $D = 0$, the number of ABBA and BABA in the population is the same, and there is no obvious infiltration between the groups. According to the D value, the Z-score was calculated (Z-score = D/std_err (D)), where $Z > 3$ and <-3 represent positive and negative significance, respectively.

3. Results

3.1. SNP Calling Based on the Reference Genome

A total of 290,361 raw reads were generated from 35 *M. aromatica* samples. After filtering, a total of 4884 high quality SNP loci were identified and were found to be evenly distributed across 19 chromosomes (Figure 2).

Figure 2. The distribution of 6596 SNPs on 19 chromosomes.

3.2. Analysis of Genetic Diversity and Genetic Differentiation among Populations of Manglietia aromatica inside and outside Tiankeng

At the population level, the genetic diversity level of the population inside the tiankengs (π = 0.2044) was higher than that of the population outside the tiankengs (π = 0.1671) (Figure 3a). Neutral test results (Figure 3b) showed that *M. aromatica* populations outside the tiankengs had more low-frequency alleles (Tajima' D = −0.0086). However, the tiankeng population had more intermediate frequency alleles (Tajima' D = 0.0678). The genetic differentiation coefficient (F_{ST}) between the individuals outside and inside the tiankeng was 0.0534 (Figure 3c), and the nucleotide ambiguity (D_{XY}) was 0.2189 (Figure 3d).

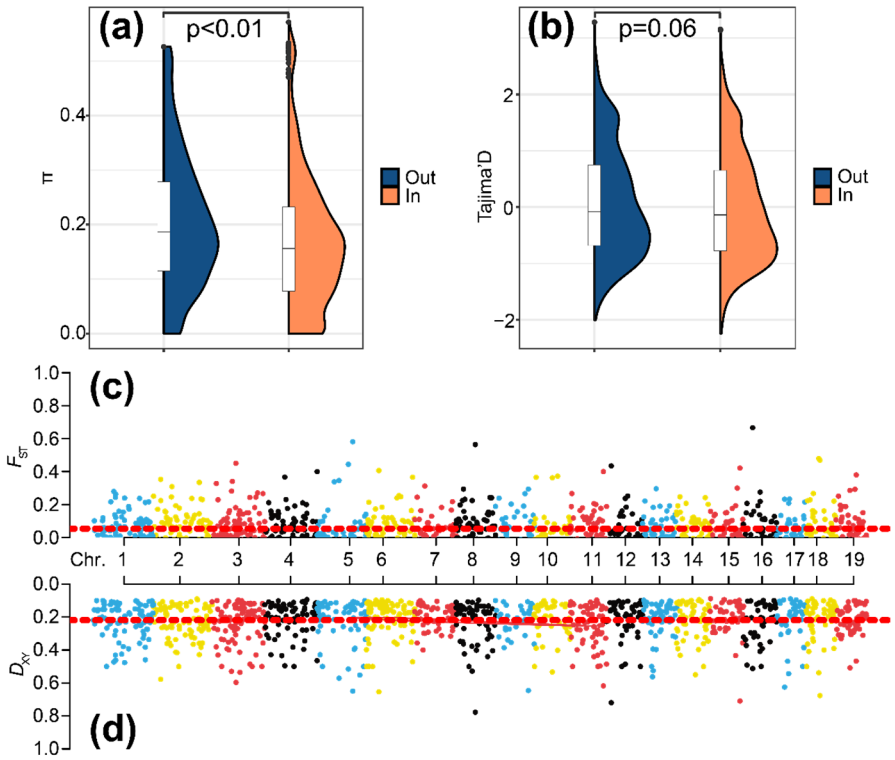

Figure 3. Genetic diversity and genetic differentiation of populations of *Manglietia aromatica* in tiankeng and outside the tiankengs. (**a**) Nucleotide diversity (π) of *M. aromatica* populations inside and outside the tiankengs; (**b**) the Tajima' D of *M. aromatica* populations inside and outside the tiankengs; (**c**) the genetic differentiation coefficient (F_{ST}) of *M. aromatica* populations inside and outside the tiankengs; (**d**) the nucleotide ambiguity (D_{XY}) of *M. aromatica* populations inside and outside the tiankengs. Note: DC, LJ, SM populations were distributed in the karst tiankengs. The CW-MBL, FD, FY, XB, CY-LH populations were distributed outside the karst tiankengs. Different color points represent selected 19 chromosomes in (**c**,**d**). The red dotted lines represent the mean values of F_{ST} and D_{XY} in (**c**,**d**).

3.3. Analysis of Genetic Diversity and Genetic Differentiation among 10 Populations of Manglietia aromatica

At the population level, the genetic diversity (π) of the 10 populations increased from 0.144 to 0.205 (Table 2). The FY population had the highest genetic diversity (π = 0.205), and the CY-LH population had the lowest (π = 0.144) (Table 2). A comparison of genetic diversity among the populations in the tiankengs showed that LJ > SM > DC. According to the geographical distribution, the genetic diversity of *M. aromatica* populations (CW-MBL, FD, FY) distributed in Leye County, was higher than that of populations from Huanjiang County (XB, π = 0.149) and Napo County (CY-LH, π = 0.144) (Table 2). In the 10 populations, the inbreeding coefficient (F_{IS}) increased from −0.026 to 0.1 (Table 2), of which 6 (67%) populations had an $F_{IS} > 0$, indicating that individuals in these populations were inbreeding, and 3 (33%) populations had an $F_{IS} < 0$, indicating that there was no

inbreeding between individuals. Besides the DC population (F_{IS} = 0.011), no evidence of inbreeding was found in the populations in the two tiankengs (F_{IS} = −0.026, −0.015) (Table 2), whereas the populations outside the tiankengs were basically inbred. The range of observed heterozygosity (H_O) in the 10 populations was 0.130–0.205, and the range of observed heterozygosity (H_E) was 0.085–0.176 (Table 2). Among the 10 populations, there were only 2 (20%) populations in which H_E was greater than H_O (Table 2). Both of these populations were populations outside the tiankeng, while the populations inside the tiankeng showed that the H_O was greater than the H_E.

Table 2. Genetic diversity information of 10 populations of *Manglietia aromatica*.

Pop ID	Private SNP	H_O	H_E	π	F_{IS}
CW-MBL	101.000	0.155	0.173	0.199	0.100
FD	0.000	0.173	0.106	0.167	−0.008
FY	1.000	0.153	0.158	0.205	0.093
XB	329.000	0.143	0.126	0.149	0.016
CY-LH	4.000	0.130	0.085	0.144	0.020
DC *	21.000	0.175	0.146	0.179	0.011
LJ *	195.000	0.205	0.176	0.190	−0.026
SM *	1.000	0.195	0.120	0.185	−0.015

Note: H_O, observed heterozygosity; H_E, expected heterozygosity; π, nucleotide diversity; F_{IS}, inbreeding coefficient. * represents the population in the karst tiankeng.

The genetic differentiation coefficients (F_{ST}) between the 10 populations were 0.077 (LJ-SM) and 0.325 (FD-XB) (Table 3). Of these, XB had the highest level of distinction from the other populations (F_{ST} = 0.204 to 0.325), and the LJ population had the lowest level of distinction from the other populations (F_{ST} = 0.077 to 0.204). The F_{ST} values between populations outside the tiankengs were 0.101 (CY-LH-LJ) and 0.310 (XB-FD), while the degree of differentiation between the three tiankeng populations remained at a low level (F_{ST} = 0.077, 0.082, 0.141).

Table 3. Coefficients of genetic differentiation among 10 populations of *Manglietia aromatica*.

	CW-MBL	FD	FY	XB	CY-LH	DC *	LJ *	SM *
CW-MBL		0.204	0.109	0.206	0.149	0.174	0.147	0.184
FD			0.218	0.325	0.31	0.154	0.085	0.231
FY				0.246	0.194	0.165	0.11	0.191
XB					0.268	0.263	0.204	0.297
CY-LH						0.188	0.101	0.264
DC *							0.082	0.141
LJ *								0.077

Note: * represents the population in the karst tiankeng.

3.4. Gene Flow among Manglietia aromatica Populations

Not all the 120 combinations showed gene exchange events (Figure 4). With $D \neq 0$ and $|Z| > 3$ as the reference standard, a total of 26 groups of combinations with significant gene flow were screened out, removing the combination of P3 and P2 repeats and retaining the combination with the largest D statistic, leaving 16 combinations (Table 4). From the table, in comparison with gene flow between other combinations, when the topological structure is (((CY, FY) MBL) O) the gene flow is the most significant (D-statistic = 0.32, Z-score = 5.81). XB outside the tiankeng had the most gene exchange with (LJ and SM) inside the tiankengs, followed by FY and CW, but both were unidirectional.

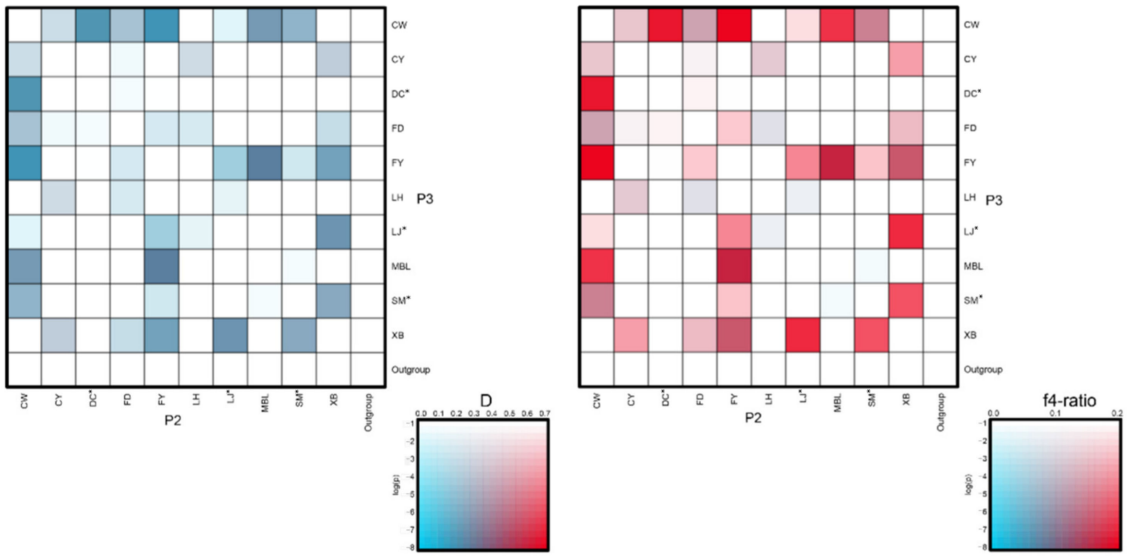

Figure 4. D-statistic and f4-ratio among 10 populations of *Manglietia aromatica*. Note: P2 is the horizontal axis, P3 is the vertical axis. The redder the color of the heat map is, the larger the D value and f4-ratio are, the deeper the color is, the smaller the *p*-value is, and the higher the degree of infiltration between the two is. * represents the population in the karst tiankeng.

Table 4. The results of D-statistics analysis among populations of *Manglietia aromatica*.

P1	P2	P3	D-Statistic	Z-Score	*p*-Value	f4-Ratio
CY	FY	MBL	0.32	5.81	0.0000	0.18
XB	DC *	CW	0.21	5.55	0.0000	0.66
CW	LJ *	XB	0.25	5.31	0.0000	0.21
MBL	CW	DC *	0.25	5.26	0.0000	0.18
LJ *	CW	MBL	0.25	5.19	0.0000	0.21
MBL	FY	XB	0.23	5.13	0.0000	0.16
CW	SM *	XB	0.25	4.81	0.0000	0.2
CY	FY	CW	0.2	4.67	0.0000	1.78
MBL	CW	SM *	0.23	4.62	0.0000	0.15
MBL	CW	FD	0.24	4.18	0.0000	0.13
CY	LJ *	FY	0.17	4.09	0.0000	0.7
CW	CY	XB	0.27	3.74	0.0002	0.2
CW	FD	XB	0.2	3.4	0.0007	0.17
MBL	CW	CY	0.22	3.3	0.0010	0.15
SM *	CY	LH	0.26	3.29	0.0010	0.14
CY	SM *	FY	0.15	3.06	0.0022	0.94

Note: * represents the population in the karst tiankeng.

The populations in the three tiankengs had different gene flow characteristics. LJ accepted the gene flow of the two populations outside the tiankengs at the same time, but its own genes were not found to flow to the other populations. SM only accepted gene flow from XB outside the tiankeng, but at the same time there was gene flow to CW outside tiankeng. Unlike the other two populations inside the tiankeng, DC and CW outside the tiankeng had significant gene flow at the same time, representing the only two-way gene flow that occurred in the selected combination. No gene flow was found between the populations in the three tiankengs. Overall, the gene flow of the *M. aromatica* population originated from XB in Huanjiang County. It moved in a divergent manner from northeast to southwest. In addition, it was basically a one-way flow, with most of the gene flow moving from the population outside the tiankengs to the population inside. As demonstrated via the screening results, there were five gene flows that occurred among the populations

in the tiankeng, including one two-way flow, which accounted for only 31% of the total screening results.

3.5. Analysis of Genetic Structure of Manglietia aromatica Population

The genetic structure of the 10 populations was analyzed using mixed analysis, and the CV error value was smallest when K = 2, which was an indication that the populations can be divided into two groups (Figure 5). But when we combined this analysis with the genetic structure analysis (Figure 6), it can be seen that the ten populations divided into two groups (Figure 5) can be roughly divided into three subpopulations. The first subpopulation was the tiankeng subpopulation in southwest Guangxi, which included the DC, SM and LJ populations; the second subpopulation was the subpopulation around the tiankengs in southwest Guangxi, which included the CW, MBL, FD, FY, LH and CY populations; the third subpopulation was the Huanjiang subpopulation in the north-eastern part of Guangxi, which contained only the XB population and was far away from the first and second subpopulations. In the first and third subpopulation (Figure 6c), the genetic components were single, but the genetic components of the second subpopulation were more complex, and some individuals in the FD, CW, LH and CY populations were mixed with the genetic components of another subpopulation.

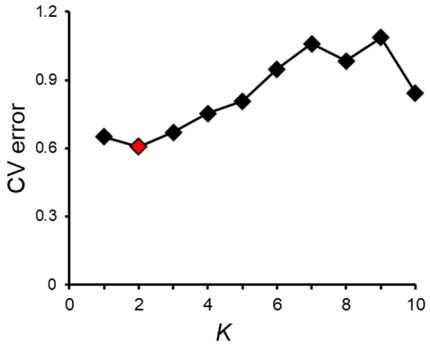

Figure 5. Cross-validation (CV) error distribution for K from 1 to 10, with K of the smallest. CV value marked in red.

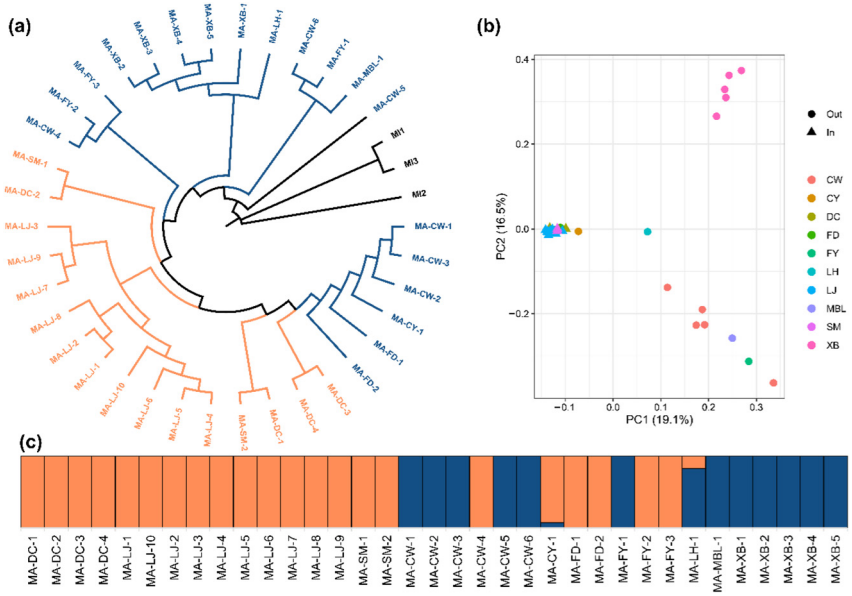

Figure 6. Genetic structure of 10 populations of *Manglietia aromatica*. (**a**) Phylogenetic relationship; (**b**) principal component analysis; (**c**) population genetic structure. Note: Different colors represent different genetic information in (**c**).

The positional distance on the two-dimensional map can intuitively show the genetic relationships between the various populations of *M. aromatica*. According to the two-dimensional diagram obtained through the principal component analysis (Figure 6b), the contribution rates of PCA1 and PCA2 were 16.5% and 19.1%, respectively, and the cumulative contribution rate was 35.6%, indicating that these two principal components can best reflect the genetic relationship between populations. The individuals of the first subpopulation (DC, SM, LJ) were mainly distributed in the third quadrants, and they were gathered together. The individuals in the second subpopulation around the tiankeng (CW, MBL, FD, FY, LH, CY) were mainly located in the fourth quadrant, but some individuals in this subpopulation exhibited a tendency to converge with the first subpopulation, indicating that there was a close genetic relationship between these two subpopulations. The XB population in the third subpopulation was distributed in the first quadrant and far away from other populations, indicating that the genetic relationship between this subpopulation and the other two subpopulations was not close and there was large genetic differentiation.

4. Discussion

4.1. Karst Tiankengs Can Play a Protective Role in the Genetic Diversity of Manglietia aromatica Populations

Population genetics suggest that the greater the genetic diversity of a species, the greater its ability to adapt to the environment and the wider its range [30,31]. Compared with the changing and complex environment outside tiankengs, the environment inside tiankeng is more stable and single. The species distributed in tiankeng will inevitably lose their genetic diversity under the influence of single natural selection. Interestingly, this study found that the overall level of genetic diversity ($\pi = 0.2044$) in the tiankeng population was higher than that outside the tiankeng population ($\pi = 0.1671$). We suggest two possible reasons for this phenomenon. Firstly, *M. aromatica* has experienced population expansion events throughout its history (Tajima' $D = -0.0086 < 0$) [32]. As part of its long-term evolutionary process, it has accumulated much more genetic variation than is currently present. However, due to climatic and ecological changes in recent years, combined with human interference, the population size and the number of individuals are gradually decreasing. Habitat fragmentation is increasing and gene exchange between populations is decreasing, which ultimately leads to a reduction in the genetic diversity of the population. However, the populations forced to migrate to the tiankengs under natural selection may retain a large amount of genetic information, even if they have experienced a sudden contraction in population size [33,34] or a bottleneck effect (Tajima's $D = 0.0678 > 0$) and have lost part of their genetic information. On the other hand, we also found evidence of selfing or inbreeding ($F_{IS} = 0.100, 0.093, 0.016, 0.020 > 0$) in four (80%) populations outside tiankengs [35]. Although this mating strategy may maintain and increase population size in the presence of limited pollinators, it will result in a loss of genetic diversity in the population. Of the three populations in tiankengs, only one engaged in selfing or inbreeding, while the other two bred through outcrossing. This outcrossing-based mating strategy is beneficial for maintaining and increasing genetic diversity.

The genetic diversity of the three populations (SM, DJ, LJ) in the tiankengs was compared and analyzed. The results showed that, despite their close geographical locations and similarity to the populations in the tiankengs, there were differences in their genetic diversity. Their specific performance was as follows: LJ (0.190) > SM (0.185) > DC (0.175). Karst tiankengs typically develop in three stages: the underground river stage, the underground-hall-collapse stage, and the tiankeng stage. The first two stages occur entirely underground without the participation of higher plants. The tiankeng stage starts with the collapse of the ceiling of the underground hall. From that point on, external vegetation begins to grow in the tiankeng. According to the origin and evolution of the flora in the tiankeng, it can be divided into three stages of evolution: the early stage, middle stage and late stage. Tiankengs exhibit varying ecological microenvironments due to differences in their evolutionary periods [36]. During the early stages of tiankeng evolution, the interior is

devoid of soil, light intensity is low, and the ecological environment is poor. This stage is known as the 'bare land' stage of primary succession in the plant community. Primitive lichen plants, which require only weak light, became the pioneer plant in the primary succession of tiankeng plant communities. During the middle stage of evolution, the area of the pit mouth increases compared to the previous period, resulting in more light at the bottom of the pit, thicker soil and humid air. The plant propagules around the tiankeng enter the tiankeng through the pit mouth, and the original bryophyte lichens are gradually replaced by species that thrive in damp and shady conditions. The shaded tree species gradually gain ecological dominance and form a forest. In later stages of evolution, the crater mouth continues to widen, increasing light at the bottom of the crater, and falling boulders block the underground river channel, resulting in a dry crater floor. Additionally, the ability to resist external cold air increases in the later stages of evolution. The original shade-wet trees gradually disappear and are replaced by tall, neutral and sunny tree species, forming a composite forest community [37]. Among the three tiankengs in this study, the Luojia tiankeng (LJ) and Shenmu tiankeng (SM) are tiankengs in the middle stage of evolution, while the Dacao tiankeng (DC) is in the late stage of evolution [38]. It has been found that *M. aromatica* thrives in shady areas and has shade-tolerant characteristics, but has a weak resistance to drought, high temperatures and bright light [39]. This investigation revealed that *M. aromatica* is primarily found in mid-term tiankengs with strong tropical attributes, such as the Luojia tiankeng and Shenmu tiankeng. The shaded and unsaturated light conditions in these tiankengs promote seedling growth and population formation. During the late stages of tiankeng evolution, environmental factors such as increased light and intensified drought can impact the growth of *M. aromatica*, hindering individual development and ultimately affecting population reproduction. Simultaneously, greater accessibility to tiankengs in the later stage also exposes *M. aromatica* populations to significant risk from human interference, ultimately resulting in a decline in the number of *M. aromatica* individuals.

The tiankengs' evolution and population changes indirectly suggest that they serve as a refuge for *M. aromatica*, and also preserve the more primitive community characteristics. Therefore, we believe that one of the important factors affecting the population size and genetic diversity of *M. aromatica* is the difference in natural conditions caused by the different periods of tiankengs' evolution. Although tiankengs have the ability to resist external environmental factors, their development can lead to loss of species and genetic diversity. In the three tiankengs communities of *M. aromatica*, it is important to strengthen the protection of the Dacao (DC) population. Furthermore, the underground rivers at the bottom of tiankengs have significant carrying capacity, which limits the available evidence inferring the tiankeng's establishment time and evolution mechanism [40,41]. Studying the genetic evolutionary history of species in tiankengs can provide a scientific basis for establishing tiankeng theory.

4.2. Geographical Isolation Hinders Gene Exchange between Populations of Manglietia aromatica

In the protection and management of endangered plants, ex situ conservation is used as an artificial means to manage and protect species whose native habitats have been destroyed [42,43]. However, because of the distance from their native environment, gene exchange between the ex situ population and the native population is greatly reduced, resulting in greater genetic differentiation [44,45]. As a relatively isolated environment, tiankengs may experience this phenomenon. For this reason, it is essential to assess the gene flow of *M. aromatica* populations both inside and outside of the tiankengs. The results of the ABBA-BABA analysis indicate effective gene exchange is mostly concentrated outside of the tiankeng, but we still found significant evidence of gene exchange between the populations that are distributed in the tiankeng and some of the populations outside of the tiankeng. In addition, there was a moderate level of genetic differentiation between the populations within and outside the tiankengs (F_{ST} = 0.0534) [46]. Surprisingly, no evidence of gene exchange was found among the three tiankengs populations, and there was moderate

genetic differentiation among the populations. Put simply, due to the geographic isolation between the tiankeng populations, there was no effective gene exchange between them, resulting in moderate genetic differentiation; however, there was significant gene exchange between the populations outside the tiankengs and those far away from the geographical location, but there was still a medium–high level of genetic differentiation.

The reasons for this are unlikely to be revealed by contemporary gene flow. Gene flow plays a crucial role in the differentiation of populations. It can limit evolution and reduce genetic differentiation among populations by preventing them from adapting to new conditions. However, it can also promote evolution and increase differentiation among populations by spreading new genes throughout the species [47]. Therefore, it is believed that the population size of the ancestors of *M. aromatica* was much larger in ancient times than it is currently. During the initial stages of species formation, there was extensive gene exchange, resulting in the accumulation of rich genetic variation. Prior to the formation of tiankengs, there were numerous populations of *M. aromatica* in the surrounding area. Subsequently, individuals who were compelled to migrate to the tiankengs engaged in continuous gene exchange with those already present, acquiring new genetic information to adapt to the new environment. Populations have adapted to the different tiankeng environments through natural selection. However, significant gene flows are mostly unidirectional. They flow from XB and FY outside the tiankengs into the tiankengs. It should be noted that only the gene flows between CW and DC populations are bidirectional in these gene flows, with one being outside the tiankeng and the other inside. As a tiankeng in the later stage of evolution, Dacao tiankeng's natural environment has gradually become similar to that outside the tiankeng due to geographical factors such as the uplift of the crust. Consequently, its biodiversity has also become similar to that of the surrounding area. We believe that in order to better adapt to its future environment, *M. aromatica* must gradually relinquish control of the natural environment and begin undergoing gene exchange with other *M. aromatica* populations. Based on the above data, it can be seen that tiankengs will hinder gene exchange between the populations within and outside them, but this hindering ability may gradually decrease with the natural evolution of the sinkholes.

4.3. Genetic Structure and Genetic Relationship of Manglietia aromatica Populations

The investigation of genetic structure is significant in investigating the hereditary connections and developmental history among species. The degree of genetic relationship or differentiation (F_{ST}) between populations is commonly quantified using a genetic differentiation coefficient. Past examinations have shown that assuming the F_{ST} between populations is in the scope of 0 to 0.05, there is no separation between populations. Assuming the F_{ST} is somewhere in the range of 0.05 to 0.15, there is moderate separation, and in the event that the F_{ST} is somewhere in the range of 0.15 to 0.25, there is high separation [46]. Based on this standard, the results of this study showed that the degree of genetic differentiation between populations outside tiankeng is basically highly differentiated and higher than that of populations inside tiankeng. However, the three tiankeng populations were found to be closely related to each other, but still moderately genetically differentiated from each other, as supported by the results of the phylogenetic tree and principal component analysis (Table 3) (F_{ST} = 0.082, 0.141, 0.077). The results of the genetic differentiation analysis showed that the genetic differentiation coefficient between the populations of the first and second subpopulations was smaller than that of the third subpopulation, which is far from the tiankeng (Table 3). The genetic relationship analysis revealed that the populations in the first tiankeng subpopulation had the same genetic information as the second subpopulation around the Tiankeng (Figure 6c), and the individuals in the two subpopulations were relatively close in the principal component analysis (Figure 6b), indicating that there was a close genetic relationship between the populations distributed in tiankengs and the populations around tiankengs. These results, on the other hand, comprehensively confirm our previous theory on the evolutionary history of the tiankeng populations, that is, the populations in the tiankeng gradually evolve from the populations

near the pit mouth; furthermore, different genetic differentiations are produced to adjust to various habitats. It is worth noting that in the phylogenetic tree, we also found that in the phylogenetic branch of the tiankeng DC population, some CW population individuals were also included. Our blended investigation of hereditary construction additionally found that the two populations had similar hereditary parts. This coincides with the two-way gene flow we found above.

In the analysis of genetic structure, it was found that the XB population distributed in Huanjiang was significantly separated from other populations, indicating clear genetic differentiation. This was in addition to the identification of the population in the tiankeng. The F_{ST} between this population and other populations reached a high level of differentiation ($F_{ST} > 0.25$) (Table 3). At the same time, it also shows that the genetic relationship between the Huanjiang subpopulation, which is composed of the XB population, and the first and second subpopulations, is distant and there is a great deal of genetic differentiation. This can be attributed to long-term geographical isolation and unique geographical conditions, such as karst and danxia landforms [48,49], which hinder gene exchange between populations. As a result, natural selection and genetic drift are strengthened, leading to increased genetic differentiation between populations. Therefore, we believe that as a plant that relies on insects for pollination, the transmission of genetic information over long distances may be challenging. Moreover, this species has few populations, and the absence of effective gene exchange among them has brought about a serious level of hereditary separation. Furthermore, the results of gene introgression indicate that the gene flow direction of the *M. aromatica* population as a whole diverges from the XB population towards the tiankeng and the populations surrounding it. Combined with the analysis of the phylogenetic tree, it is believed that the *M. aromatica* found in Guangxi is derived from the XB population in Huanjiang County. The developmental direction of the population is from the temperate zone in the northeast to the tropical zone in the southwest, which is consistent with *M. aromatica*'s preference for heat and humidity.

4.4. Protection Suggestions and Measures

In recent years, human activities in karst tiankengs have increased due to their growing economic and research value. This has put significant pressure on the survival of species such as *M. aromatica*. It is important to consider the impact of these activities on the environment and take measures to protect this species. In addition, compared with other endangered plants, *M. aromatica* is only a national second-class protected plant, but as a species with high economic value, it faces threats from humans and the evolution of sinkholes. Based on our research results, we propose the following protection measures for *M. aromatica*. (1) Screening high-quality germplasm resources is crucial for conserving species. The genetic diversity of the *M. aromatica* populations in the tiankengs was higher than that outside the tiankengs. Therefore, the populations of *M. aromatica* in the tiankengs can serve as an excellent germplasm gene pool, and its population size can be expanded through asexual reproduction, such as tissue culture and cutting. (2) The gradual evolution and disappearance of karst tiankengs may lead to the disappearance or loss of adaptability to the special microenvironment of tiankengs. Therefore, it is necessary to strengthen the protection of the *M. aromatica* population in the Dacao tiankeng, Leye County, China, during the later stages of evolution and implement necessary ex situ protection. (3) Due to the tiankengs' unique geographical isolation, the populations inside and outside of them have undergone moderate genetic differentiation. To improve genetic diversity at the species level, it is necessary to facilitate gene exchange between the populations through artificial pollination. (4) The relevant government departments should enhance the investigation of plant resources and improve the overall planning of these resources. Additionally, they should increase public awareness and protection of the native environment and establish natural protection communities in areas with a high population density, such as the Luojia tiankeng in Leye County, China.

5. Conclusions

Karst tiankengs, which have a relatively isolated natural environment, are refuges for the endangered plant *M. aromatica* that can effectively protect the species' genetic diversity, but at the same time limit gene exchange between populations, resulting in greater genetic differentiation between populations. However, this protection and limitation of gene flow gradually weakens as the tiankengs develop. Therefore, in the conservation of biodiversity, we should pay more attention to the protection of species in tiankengs that are in the later stage of evolution, and at the same time, strengthen the exchange of genes among different groups, so as to improve the level of genetic diversity of the population and prevent the gradual disappearance of species due to changes in the natural environment. Combined with the direction of gene flow and genetic structure, it is speculated that the distribution of *M. aromatica* in Guangxi originated from the Huanjiang County. The population development direction is from the temperate zone in northeast China to the tropical zone in the southwest China. The populations in the tiankengs evolved from the populations near past craters. In conclusion, this study determines the influence of karst tiankengs on the genetic diversity of *M. aromatica* populations and confirms that tiankeng serve as shelters and provide a suitable habitat for the endangered plant *M. aromatica*, because its genetic diversity is well conserved in the Tiankeng and the species is well adapted to the habitat within the tiankengs. Consequently, this study provides a scientific basis for the study of the evolution and origin of species in the tiankengs.

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