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Analysis of the Genetic Diversity and Mating System of the Endangered Plant *Keteleeria davidiana* var. *calcarea*

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Abstract: *Keteleeria davidiana* var. *calcarea* is an endangered plant endemic to China. It is mainly distributed in the karst areas of the Guangxi and Guizhou Provinces. It is characterized by small populations and intermittent distribution. This study aimed to explore the genetic diversity and mating system of wild populations of *Keteleeria davidiana* var. *calcarea* in fragmented habitats. To achieve this, we genotyped 46 maternal trees and 214 progenies from four fragmented populations of *Keteleeria davidiana* var. *calcarea* using nine pairs of microsatellite primers. The genetic diversity of *Keteleeria davidiana* var. calcarea (Ho = 0.68, He = 0.63) was lower than that of the species overall but higher than that of other *Keteleeria* plants. The incidence of unbiased expected heterozygosity (uHe) and allelic richness (Ar) was higher in the maternal generation than in the progeny. This suggests that the genetic diversity of the progeny was lower than that of the maternal generation. *Keteleeria davidiana* var. *calcarea* is divided into four populations, but there is significant genetic exchange between the populations according to STRUCTURE and gene flow analyses. The multilocus mating system analysis (MLTR) results indicate that the multilocus outcrossing rate (t_m) was 0.902, the single-locus outcrossing rate (t_s) was 0.606, the bimaternal inbreeding coefficient (t_m-t_s) was 0.295, and the coefficient of inbreeding depression(δ) was 0.904. These results suggest a certain degree of selfing and inbreeding in *Keteleeria davidiana* var. *calcarea*. To prevent problems associated with inbreeding and conserve the genetic diversity of *Keteleeria davidiana* var. *calcarea*, we recommend establishing seed gardens, using artificial pollination, and employing asexual propagation techniques for conservation intervention.

Keywords: SSR; mating system; *Keteleeria davidiana* var. *calcarea*; genetic diversity; endangered plants

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1. Introduction

Keteleeria davidiana var. *calcarea* is a rare and endangered species belonging to the genus *Keteleeria* in the Pinaceae family. It is a valuable plant resource in China, mainly distributed in the karst and rocky mountains at the junction of Guangxi and Guizhou Provinces [1]. This species is drought-tolerant and excellent for afforestation in limestone mountains. Its hard wood can also be used as timber for building [2]. *Keteleeria davidiana* var. *calcarea* has been listed as a second-level key protected wild plant in China due to its weak natural regeneration ability and excessive logging by human beings, which has gradually reduced the habitat area and drastically decreased the population such that it is on the verge of extinction [3,4].

To expedite the effective conservation of wild populations of *Keteleeria davidiana* var. *calcarea*, Jiang et al. conducted a study on the effects of various treatments on the seedlings of endangered *Keteleeria davidiana* var. *calcarea* cuttings [2]. Jiang et al. examined the seed germination characteristics of *Keteleeria davidiana* var. *calcarea* and discovered that the

low seed germination rate and the significant influence of environmental factors on seed germination may be two primary reasons for the population's endangerment [5]. Shi et al. successfully deciphered the complete chloroplast genome sequence of *Keteleeria davidiana* var. *calcarea*, filling a gap in the evolutionary and phylogenetic studies at the molecular level [6]. However, there are few studies on genetic diversity and mating systems, and these are crucial for developing conservation management strategies for *Keteleeria davidiana* var. *calcarean* [7].

There is a positive correlation between genetic diversity and a species' ability to adapt to its environment. The more variable the genetic structure, the better the species can adapt to mutations and harsh environments. Furthermore, greater genetic diversity allows for a wider ecological niche and increases the chances of survival when the environment stabilizes. However, some studies show no significant correlation between the ecological niche of a species and its genetic diversity [8]. Studying the genetic diversity of species can provide insights into their evolutionary events and population dynamics and serve as a basis for molecular classification and phylogeny. However, most studies have focused on changes in genetic diversity between populations, with little emphasis on changes across generations [8–10]. The genetic monitoring of different generations within populations is crucial for characterizing the developmental status of populations, as changes in the genetic composition of populations depend on generation turnover [11]. The use of simple sequence repeats (SSRs) in studying mating systems in plants provides insight into genotype distribution and population dynamics, including the effective population size, sex ratio, and degree of stochastic mating. This correlation enhances our understanding of genetic diversity within species [12]. *Keteleeria davidiana* var. *calcarea* is currently facing population survival problems due to habitat fragmentation. It is difficult for us to assess the degree to which habitat fragmentation has occurred [13]. Fragmentation impacts plant adaptations and mating systems [14]. Therefore, mating results reflect evidence of habitat fragmentation.

Microsatellites, also known as SSRs, are tandem repeat sequences composed of 1–6 base pair repeat units [15,16]. They have been extensively utilized as markers of genetic differences due to their abundance and ease of detection [17]. Several mating systems based on microsatellite technology have been investigated [18]. For example, Sun et al. studied *Vitex negundo* var. *heterophylla* using SSR markers and found a mixed mating system that predominantly comprised outcrossing and inbreeding depression [19]. Zhang et al., using 11 pairs of SSR primers, showed that *Camellia oleifera* "Ruan Zhi" has a high degree of outcrossing [20]. Fuchs et al. found that *Chamaedorea tepejilote* populations have moderate to high nuclear simple sequence repeat (SSR) genetic diversity, possibly due to their large population size and the use of an outcrossing mating system [21].

SSR markers were utilized to investigate the mating system of the natural population of *Keteleeria davidiana* var. *calcarea*. The study aimed to reveal the mating mechanism of the species' natural population, explore differences in genetic diversity between the maternal tree and progeny, and assist the long-term conservation of the natural population of *Keteleeria davidiana* var. *calcarea*.

2. Materials and Methods

2.1. Plant Materials

This study is based on a detailed survey of the natural range of *Keteleeria davidiana* var. *calcarea* in 2020 [22]. Samples were collected from four natural populations: Rong'an, Fuchuan, Lingui, and Gongcheng (Table 1, Figure 1). The landscape of the four natural habitats is karst and stony mountains, and all have a subtropical monsoon climate (average temperature 18–19 °C). Soils in the *Keteleeria davidiana* var. *calcarea* range are black calcareous, brown calcareous, or loess, which are acidic, neutral, or weakly alkaline. The community is mainly in the well-preserved geomantic forests by the village and roadside, with fragmented distribution and an obvious vertical structure divided into three layers: tree, shrub, and herb. *Keteleeria davidiana* var. *calcarea* is the dominant species in the tree

layer (Figure S3). There are differences in terms of the number of individuals and population structure in different populations, with both GC and FC populations having over 400 individuals, mainly middle-aged or elderly, and a higher number of fruiting plants. The number of individuals in both the RA and GL populations is under 150. The RA population is mainly composed of middle-aged individuals, while the GL population is mainly composed of small and medium-sized individuals. Both populations have fewer fruiting plants.

Table 1. The survey of the stands of *Keteleeria davidiana* var. *calcarea* community.

Population	Elevation (m)	Aspect	Slope (°)	Latitude	Longitude	Maternal Trees	Progeny
GC	670	WS	45	25°50′26″	110°05′47″	20	98
LG	160	WS	40	25°12′47″	110°11′51″	5	22
FC	334	ES	35	25°02′24″	111°18′47″	15	73
RA	480	WS	45	25°01′46″	109°34′50″	6	21

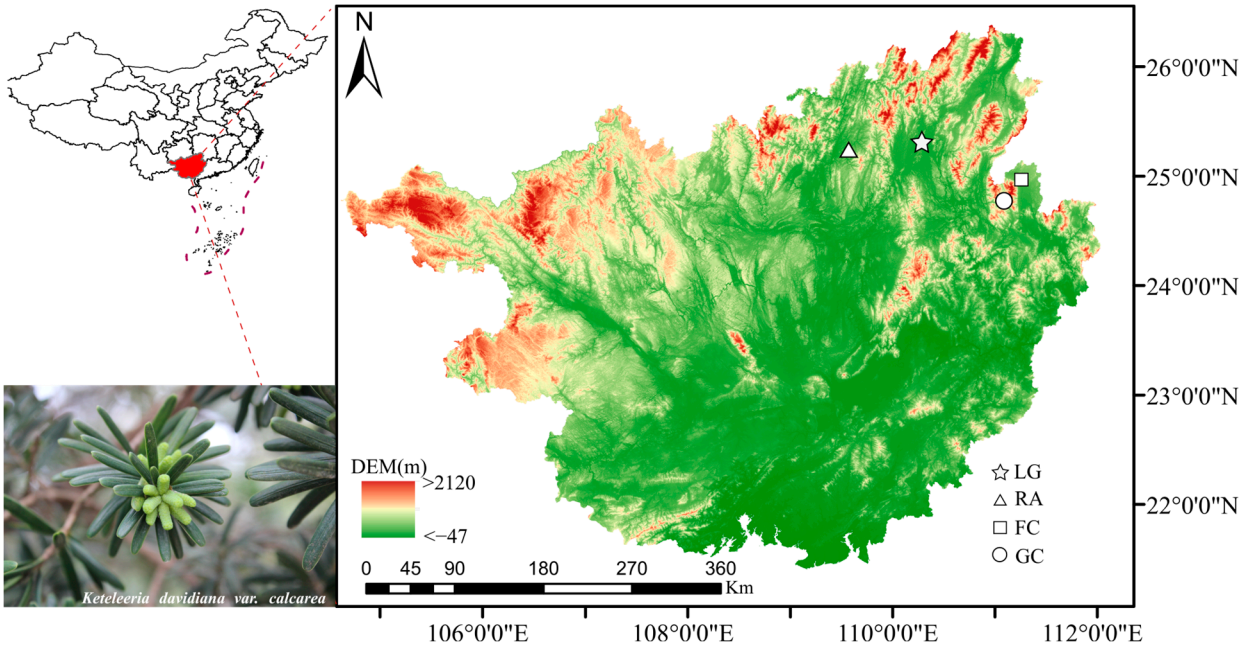

Figure 1. Sampling locations of *Keteleeria davidiana* var. *calcarea*. Different shapes represent populations in different locations: pentagram refers to LG; triangle refers to RA; square refers to FC; round refers to RA.

The number of fruiting plants of *Keteleeria davidiana* var. *calcarea* in the four populations varied greatly. We collected samples of all fruiting plants from the LG and RA populations and balanced the total number of samples through sampling spacing to obtain as many representative samples as possible from the GC and FC populations. We used random sampling and spaced them more than 20 m apart. Five to twenty plants were selected as maternal tree plants in each population according to the actual situation, and seeds were obtained as progeny for planting. One year later, 2–5 progeny plants were selected from each of the 46 maternal trees from the progeny forests, resulting in a total of 214 plants in the progeny population. The blade material was stored at −20 °C for future use.

2.2. Data Analysis

2.2.1. DNA Extraction and SSR-PCR Amplification

The total genomic DNA of the maternal trees and progeny populations was extracted using the magnetic bead method genomic DNA extraction kit from Tiangen Biochemicals,

and the concentration and quality were detected using 1% agarose gel electrophoresis. The DNA was then stored at −20 °C for spare use.

Based on the findings of Shi et al. [23] and according to the SSR loci information obtained from the transcriptome sequencing data, 325 pairs of primers were selected for amplification and validation, and 9 pairs of SSR primers with clear bands and high stability were selected. Nine pairs of polymorphic primer results were selected for PCR amplification (Table 2). A PCR amplification reaction was carried out using the TP-M13-SSR technique [24]. A 15 μL system was used for PCR, in which 2 × Taq PCR Master Mix 7.5 μL, mix primer 2.0 μL, DNA template 1 μL (50–200 ng), and ddH20 4.5 μL were used. The PCR amplification procedure was as follows: 96 °C for 3 min (96 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min) for 30 cycles and 72 °C for 10 min. The amplified products were detected for length polymorphism on ABI 3730 XL DNA sequences.

Table 2. Primer sequence information.

Primer No.	Repeat Motif	Primer Sequence (5′-3′)	Expected Size (bp)	Tm (°C)
P7	(AGA)8	F: CCAACATTGCAGTTGACGAC R: CATCATCCTCACCACATTGC	132	54
P49	(TC)10	F: TTGGCCAGGTCAGAAATAGG R: CCCATTGCCTCAAGAGAGAG	171	60
P56	(TA)11	F: GTCAAAGACAACAGACGCCA R: CTTGGGGATACAACCAAGGA	212	59.8
P60	(AT)11	F: GAACCCCCACCTGTACCTCT R: CGAGTATGCATCCACGTCAA	238	60.4
P64	(TA)11	F: GCTGCGAAGCTGCTAAAACT R: CGGCCTCTCACTTCTGGTAG	255	60
P130	(TA)11	F: CAAATTCTCCCAGAGGAAGC R: GGTCAGTGTCCTTCCTCCAA	234	55
P150	(TG)12	F: ATCTCCTTGCTGATTGGGTG R: CCTCCTGCAACGGTTATGTT	194	55
P153	(GT)10	F: CCTTTCACACGCACTAGCAA R: CGCACCTCTTATCCACCACT	267	56.5
P156	(AT)11	F: ATCCGTATCCGTTTCCGTTT R: TTTTTGCTGGAGTGTGTTGC	110	53.5

2.2.2. Genetic Diversity Analysis

Using LIZ500 as an internal standard, the fluorescence signal detection results of PCR products were analyzed for peak patterns and allele reading using GeneMarker software version 3.0.1 (available at https://softgenetics.com/information/request-trial/, accessed on 17 May 2019). Subsequently, the genetic diversity was analyzed using FSTAT version 2.9.3.2 [25]. Diversity parameters included the number of alleles (*N*), average number of alleles (*Na*), effective number of alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), unbiased expected heterozygosity (*uHe*), and percentage of polymorphism loci (*PPL*). Allele richness (*Ar*) was assessed by ADZE. Micro-checker version 2.2.3 software was used to determine the presence of null alleles and to screen for genotyping errors before subjecting the data to statistical analysis. Fixation indices (*f*) were calculated by GENEPOP to determine each population's Hardy–Weinberg equilibrium [26]. Additionally, 1000 simulations were performed with FSTAT 2.9.3.2 to detect differences in the aforementioned genetic diversity parameters between mature plants and seeds within each patch, as well as across each life history stage.

2.2.3. Genetic Structure and Gene Flow Analysis

The genetic relationships between populations were analyzed using GenAlEx version 6.5 software to perform the analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA) based on the genetic distance matrix [27]. The simulations were performed using an "admixture" model for mixed individuals, assuming correlated allelic frequencies. The preliminary analysis involved 20 independent runs of the Markov Monte Carlo chain (MCMC), with 10,000 and 30,000 iterations each, testing for possible numbers

of clusters (K) between 1 and 20. The optimal K value was determined using the highest ΔK method in STRUCTURE HARVESTER [28].

GenAlEx version 6.5 was also used to generate values for the genetic differentiation (F_{st}), gene flow (N_m), Nei's genetic distance (D), and genetic identity (I). Nm, determined based on G_{st}, was calculated using the formula $N_m = (1 - G_{st})/2\ G_{st}$. Visualization of the results for F_{st}, N_m, D, and I used R version 4.3.3. In order to ascertain whether there is a correlation between genetic distance and the geographic distance between pairs of populations, we employed Mantel's generalized regression procedure [29]. The geographic distance between populations was calculated using the latitude and longitude coordinates.

2.2.4. Mating System Analysis

The mating system parameters were calculated using MLTR [30] version 3.4 software, employing the maximum likelihood estimation method with 1000 bootstrap resamples within a 95% confidence interval. Parameters included the multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), bimaternal inbreeding coefficient (t_m-t_s), single-locus inbreeding coefficient (F), single-locus paternity correlation ($r_{p(s)}$), and multilocus paternity correlation ($r_{p(m)}$). The t_m-t_s value estimates the level of bimaternal inbreeding, with higher values indicating closer kinship between maternal individuals; r_p estimates the paternal correlation, representing the proportion of full-sibs (i.e., outcrossed progeny sharing the same father) within a family. The average effective number of pollen donors $N_{ep} = 1/r_{p(m)}$ and the coefficient of inbreeding depression (δ) was estimated using t_m and F values, $\delta = 1 - 2Ft_m/(1 - t_m)(1 - F)$. Descriptive statistical values are expressed as the mean (standard deviation) [31]. F is the single-locus inbreeding coefficient, and t_m is the multilocus outcrossing rate.

3. Results

3.1. Genetic Diversity

Nine pairs of microsatellite primers amplified 48 alleles (N) ranging from 2 to 11 in each of the four wild populations of *Keteleeria davidiana* var. *calcarea*, with an average of 5.33 alleles per locus (Table 3). The maternal trees and progeny in the four wild populations had moderate genetic diversity, with the total number of alleles ranging from 22 to 40 in the maternal trees and 24 to 48 in the progeny. The null allele frequencies estimated by Micro-checker (Table S1) were relatively high (over 0.10) in the P56 loci, but null allele frequencies at the P56 locus did not show differences at the population level.

Table 3. Number of alleles amplified by nine pairs of microsatellite primers in wild populations of *Keteleeria davidiana* var. *calcarea*.

Primer	Maternal Trees				Progeny			
	LG	RA	FC	GC	LG	RA	FC	GC
P130	2	2	2	2	2	2	2	2
P150	2	1	2	2	2	1	2	2
P153	3	5	3	5	3	6	7	7
P49	3	1	2	3	3	2	3	3
P56	2	2	5	8	2	3	7	11
P60	2	2	3	4	2	3	3	5
P64	6	4	7	10	7	5	6	11
P156	1	3	3	3	2	3	5	4
P7	1	1	2	3	1	2	10	3
total	22	21	29	40	24	27	45	48

Nine microsatellite loci (Table 3) showed that a total of 112 individual alleles (Na) were detected in the maternal population, an average of 12.44 per locus; 144 Na were detected in the progeny population, an average of 16 per locus. The mean values of the effective number of alleles (Ne) and the fixation index (f) mean values were 3.11 and 0.05, respectively; the Ne and f mean values of the progeny of the four habitats were 4.00 and

0.17, respectively; and the percent polymorphic loci (*PPL*) of the maternal trees and progeny were 86.11% and 94.44%. In addition, the mean values of observed heterozygosity (*Ho*) and expected heterozygosity (*He*) of the maternal trees were 0.38 and 0.41, respectively, and the mean values of Ho and He of the progeny were 0.35 and 0.41, respectively (Table 4). Due to the large difference in the number of samples in the population, there was unbiased expected heterozygosity (*uHe*) with allelic richness (*Ar*), and the mean values of *uHe* and *Ar* were 0.435 and 2.099, respectively, while the mean values of *uHe* and *Ar* of the progeny were 0.427 and 2.049, respectively. The *f* of the progeny and the maternal trees were both greater than 0, and the value for the progeny was higher than for the maternal trees. This indicates an excess of homozygous in both the progeny and the maternal trees and the fact that inbreeding is occurring to stabilize the populations. The *uHe* and *Ar* values of the progeny of the four wild populations of *Keteleeria davidiana* var. *calcarea* were lower than those of the maternal trees, which shows that the genetic diversity of the progeny was lower.

Table 4. Genetic diversity of maternal and progeny in wild populations of the *Keteleeria davidiana* var. *calcarea*.

Stage	Population	*Ne* (SE)	Ho (SE)	He (SE)	uHe (SE)	Ar (SE)	*F* (SE)	PPL (%)
Maternal trees	LG	2.444(0.322)	0.333(0.042)	0.387(0.088)	0.430(0.075)	2.058(0.032)	0.106(0.008)	77.78
	RA	2.333(0.182)	0.361(0.003)	0.341(0.046)	0.383(0.003)	1.964(0.091)	−0.007(0.000)	66.67
	FC	3.222(0.029)	0.375(0.039)	0.402(0.037)	0.418(0.022)	2.059(0.003)	0.010(0.001)	100.00
	GC	4.444(0.090)	0.450(0.036)	0.496(0.098)	0.509(0.093)	2.315(0.005)	0.085(0.002)	100.00
	average	3.111(0.355)	0.380(0.65)	0.406(0.033)	0.435(0.033)	2.099(0.048)	0.048(0.014)	86.11
Progeny	LG	2.670(0.035)	0.350(0.091)	0.390(0.066)	0.398(0.025)	1.901(0.009)	0.120(0.002)	88.89
	RA	3.000(0.165)	0.330(0.082)	0.360(0.014)	0.365(0.071)	1.907(0.071)	0.070(0.000)	88.89
	FC	5.000(0.175)	0.310(0.034)	0.450(0.039)	0.456(0.033)	2.164(0.038)	0.270(0.000)	100.00
	GC	5.330(0.092)	0.380(0.033)	0.480(0.064)	0.488(0.019)	2.224(0.048)	0.220(0.007)	100.00
	average	4.000(0.316)	0.340(0.020)	0.420(0.040)	0.427(0.067)	2.049(0.048)	0.170(0.001)	94.44

Ne, the number of effective alleles; *Ho*, the observed heterozygosity; *He*, the expected heterozygosity; *uHe*, unbiased expected heterozygosity; *Ar*, allelic richness; *f*, fixation index; *PPL*, percent polymorphic loci.

3.2. Genetic Structure

Principal coordinate analysis (PCoA) of the genetic distances of 260 samples from four populations of *Keteleeria davidiana* var. *calcarea* was performed (Figure 2A). PC1 and PC2 explained 13.87% and 23.68% of the variation, respectively. The GC population is independent of the LG, RA, and FC populations. The UPGMA were similar to the PCoA results (Figures 2A and S1). Using the online tool STRUCTURE HARVESTER, we calculated the optimal Delta K value (i.e., the optimal population stratification scenario). Extreme values occurred at K = 2 (Figure 2B). At K = 2, all samples were categorized into two subgroups, with orange representing the first subgroup and blue representing the second subgroup. The orange subgroup included LG, RA, and FC, and the blue subgroup only included GC (Figure 2C). Meanwhile, an admixture of different populations in the genetic structure suggested the presence of interspecific gene flow (Figure 3).

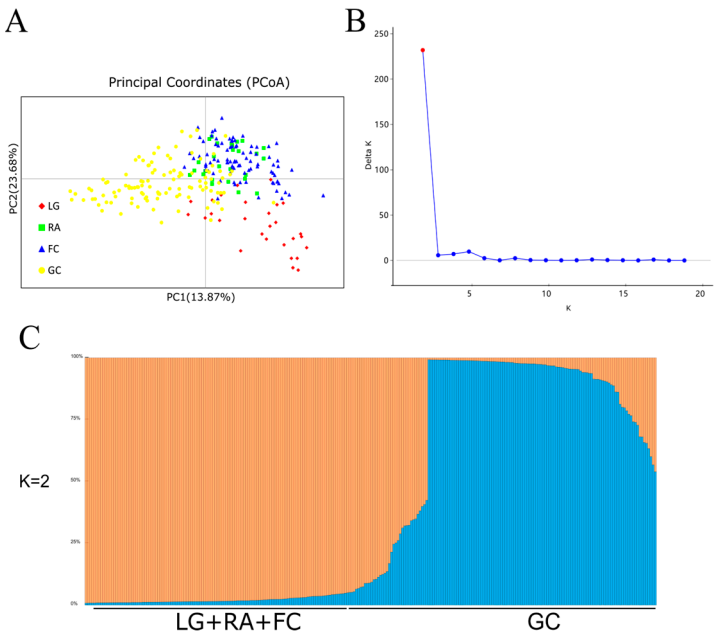

Figure 2. Genetic structures of *Keteleeria davidiana* var. *calcarea*. (**A**) Principal coordinate analysis (PCoA) plot generated for the SSR data. (**B**) Delta K for K = 1–20. (**C**) Genetic structure bar plots at K = 2.

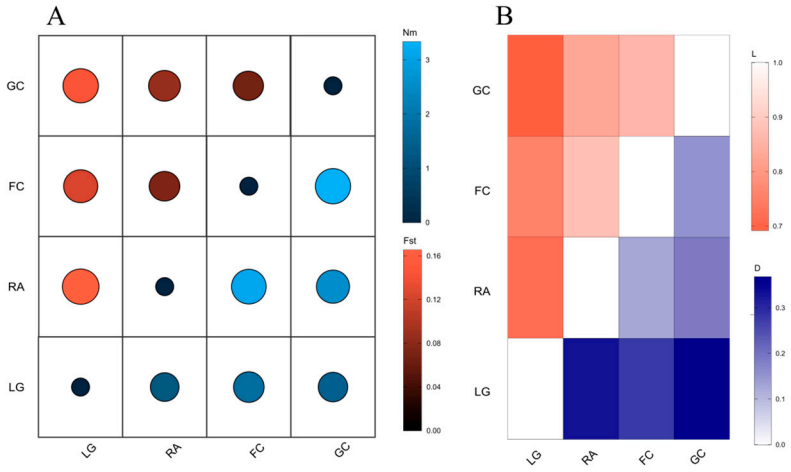

Figure 3. Genetic differentiation (F_{st}), gene flow (N_m), Nei's genetic distance (D), and genetic identity (I). (**A**) The top right indicates F_{st}, and the bottom left indicates N_m between populations. (**B**) The top right indicates Nei's genetic distance, and the bottom left indicates genetic identity between populations.

3.3. Genetic Differentiation and Gene Flow

The population genetic differentiation coefficient was greatest between RA and LG (F_{st} = 0.16), and it is noteworthy that the genetic differentiation coefficients between LG and the other two populations were all greater than 0.1 (LG vs. FC, F_{st} = 0.12; LG vs. GC, F_{st} = 0.14), with the remaining three populations less than 0.1 from each other (Figure 3A). Correspondingly, the three populations, RA, FC, and GC, had gene flow greater than 2.5 with each other, with FC having the largest gene flow value with GC (N_m = 3.33). Gene flow between LG and the other three was less than 2 (Figure 3A). In terms of Nei's genetic distance, LG was greater than 0.25 with the other three populations, with less than 0.2 between the other three populations (Figure 3B). In terms of genetic identity, LG was less than 0.76 with the other three populations and greater than 0.8 between the other

three populations (Figure 3B). Based on Mantel analyses, there was no linear relationship between geographic and genetic distance ($p > 0.05$) (Figure S2).

The analysis of molecular variance (AMOVA) of *Keteleeria davidiana* var. *calcarea* populations shows that 59.310% of the genetic variation occurred within the individual, 25.120% between individuals, and 15.570% between populations (Table S2). The AMOVA results indicate that the genetic variation of *Keteleeria davidiana* var. *calcarea* populations was mainly at the within-individual level.

3.4. Mating System

Table 5 shows that, at the species level, the mating system parameters of *Keteleeria davidiana* var. *calcarea* were (multilocus outcrossing rate (t_m)) 0.902 (0.025) and (single-locus outcrossing rate (t_s)) 0.606 (0.035). Therefore, the percentage of selfing (1-t_m) was 0.098. The bimaternal inbreeding coefficient was 0.295 (0.028). The multilocus correlation of paternity ($r_{p(m)}$) was 0.490 (0.057). The above indicators suggest that *Keteleeria davidiana* var. *calcarea* has some degree of inbreeding and self-crossing at the species level. The coefficient of inbreeding depression (δ) and effective number of pollen donors (N_{ep}) were 2.04 and 0.967, respectively, indicating the presence of inbreeding decline in *Keteleeria davidiana* var. *calcarea*.

Table 5. Mating system parameters for *Keteleeria davidiana* var. *calcarea*.

	t_m (SE)	t_s (SE)	t_m-t_s (SE)	r_t (SE)	$r_{p(m)}$ (SE)	$r_{p(s)}$ (SE)	$r_{p(s)}$-$r_{p(m)}$ (SE)	N_{ep}	δ
LG	1.000 (0.000)	0.918 (0.037)	0.082 (0.037)	0.103 (0.000)	0.064 (0.028)	0.073 (0.019)	0.009 (0.015)	15.384	0
RA	1.134 (0.079)	0.977 (0.090)	0.158 (0.090)	−0.443 (0.512)	0.043 (0.395)	0.021 (0.293)	0.001 (0.268)	23.255	−0.057
FC	0.859 (0.046)	0.603 (0.079)	0.257 (0.049)	−0.215 (0.318)	0.584 (0.137)	0.808 (0.248)	0.224 (0.165)	1.712	1.029
GC	0.810 (0.060)	0.671 (0.061)	0.140 (0.024)	0.325 (0.179)	0.192 (0.080)	0.095 (0.084)	−0.097 (0.070)	5.208	1.048
Species level	0.902 (0.025)	0.606 (0.035)	0.295 (0.028)	0.145 (0.114)	0.490 (0.057)	0.461 (0.099)	−0.028 (0.069)	2.040	0.967

t_m: The multilocus outcrossing rate; t_s: the single-locus outcrossing rate; t_m-t_s: bimaternal inbreeding coefficient; r_t: outcrossing rate; $r_{p(m)}$: multilocus correlation of paternity; $r_{p(s)}$: single-locus correlation of paternity; δ: coefficient of inbreeding depression. N_{ep}: effective number of pollen donors.

At the population level, the tm values of LG and RA were greater than or equal to 1 and higher than FC and GC, indicating that self-crossing was only found in FC and GC. The t_m-t_s values varied considerably between populations, ranging from 0.082 to 0.275. The t_m-t_s values of LG were smaller than in the other three populations, suggesting that mating among relatives is occurring in the RA, FC, and GC populations. LG (15.38) and RA (23.25) were higher than FC (1.71) and GC (5.20) in N_{ep}. According to δ, FC (1.02) and GC (1.04) showed inbreeding depression except for RA (−0.057) and LG (0).

4. Discussion

4.1. Genetic Diversity

Keteleeria davidiana var. *calcarea* exhibits lower genetic diversity compared to more widespread plants (*Ho* = 0.68, *He* = 0.63) and similar levels of diversity to endemic plants (*Ho* = 0.42, *He* = 0.32) [32]. The distribution range of *Keteleeria* is narrow, and the genetic diversity is closely related to its distribution range [33]. *Keteleeria davidiana* var. *calcarea* has a high level of genetic diversity (*Ho* = 0.340, *He* = 0.420) compared to other *Keteleeria plants* (*Keteleeria davidiana* var. *formosana* (*Ho* = 0.100, *He* = 0.075) [33] and *Keteleeria evelyniana* (*He* = 0.418) [34]), which indicates that *Keteleeria davidiana* var. *calcarea* is more capable of adapting to its environment and has a higher evolutionary potential. Therefore, its endangerment is not caused by internal factors of low genetic diversity. The presence of 32 distinct alleles in the progeny may be attributed to unsampled adults in the population, new allele forms being introduced from neighboring populations, or gene flow from other

sources. Similar observations have been reported in studies of *Acrocomia aculeata* [35] and *Butia eriospatha* [36].

The detailed analysis and comparison of the genetic diversity of the maternal trees and progeny reveal that, while both He and Ne were lower than in the progeny, *Ho*, *Ar*, and *uHe* were higher than in the progeny. This suggests that, even in the presence of gene flow and unsampled maternal trees [19,37], there is still a downward trend in the genetic diversity of *Keteleeria davidiana* var. *calcarea*. The primary reason for this may be that many of the naturally regenerating progeny come from the same maternal trees, resulting in inbreeding. This phenomenon has also been observed in other endangered plants. For example, Lu et al. found higher genetic diversity in adult trees than in seedlings of both the mangrove species, *Rhizophora apiculata* and *Avicennia marina* [38]. Compared with the decline of 14.59% Ho in *Vatica guangxiensis,* the decline in genetic diversity of *Keteleeria davidiana* var. *calcarea* is not serious but still worthy of concern [39].

The genetic diversity of the progeny was lower than that of the maternal trees. Furthermore, the f of the progeny was higher than that of the maternal trees, and both the progeny and maternal trees had an f value greater than 0. These results clearly indicate that the progeny were more severely self-crossing or inbreeding compared to the maternal trees. Related research suggests that self-crossing and inbreeding are effective strategies to increase the plant population in the context of habitat fragmentation and limited wind pollination [40,41]. An f value greater than 0 also indicates that heterozygote deficiency is prevalent in *Keteleeria davidiana* var. *calcarea*. This is due to some degree of inbreeding decline and genetic drift, which, in turn, leads to reduced genetic diversity [42,43]. In order to further investigate the differences in genetic diversity between genetic progeny and maternal trees, the mating system should be analyzed.

4.2. Gene Flow and Gene Structure

Wind pollination has been demonstrated in *Castanopsis sclerophylla* and *Corylus mandshurica* to maintain high genetic diversity and gene flow and to prevent population differentiation in plants subjected to habitat fragmentation [44,45]. A thorough analysis of genetic variation and genetic structure is crucial for uncovering genetic traits and comprehending evolutionary history. Additionally, it serves as a valuable resource for developing conservation policies for *Keteleeria davidiana* var. *calcarea*. The sample variation between populations in this study was small, and the AMOVA results detected 15.570% genetic variation between populations (Table S2). *Keteleeria davidiana* var. *calcarea*, as a wind pollinator, has a longer pollination distance relative to animal pollinators, which may be related to its lower genetic variance. Shih et al. also reported low genetic variation variability in *K. davidiana* var. *formosana* from Taiwan [33]. The gene structure was not completely differentiated according to population, and results matching the gene structure appeared in the cluster analysis of the evolutionary tree. This could mean that there is a more frequent exchange of genes between populations or that populations have experienced similar selective pressures and environmental influences, resulting in similar genetic traits. It is also likely that the species has historically had a large distribution area, with more extensive gene flow between populations [46,47]. The progeny were closely related, which would ultimately lead to a decrease in genetic diversity. Similar results were also found in a study of *Vatica guangxiensis* [39].

In this study, LG was found to have larger F_{st} and Nei's genetic distance values and smaller N_m and genetic identity than those corresponding to the other three populations. This may be due to the fact that there is less gene exchange between this population and the other three populations, resulting in a higher degree of genetic differentiation. This may be caused by factors such as geographical isolation, selection pressure, and differences in biological behavior that limit gene flow [48,49]. These factors may prevent gene exchange between different populations, thus increasing genetic differentiation. After excluding the effect of geographical distance (Figure S2), LG was found to differ from other populations in the height of elevation. It is hypothesized that the lower elevation of *Keteleeria davidiana* var.

calcarea, a wind-pollinated species, may have formed a natural barrier to LG, limiting gene flow and leading to greater genetic differentiation from the other three populations. The genetic diversity of LG populations and their corresponding conservation are important.

4.3. Mating System

Unfortunately, there have been no systematic studies on the reproductive strategies of *Keteleeria davidiana* var. *calcarea*. This study found that *Keteleeria davidiana* var. *calcarea* had a high multilocus outcrossing rate at the population level (t_m = 0.902), but it was lower than other species in the Pinaceae family, such as *Cedrus libani* A. Rich (t_m = 0.940) [50] and *Pinus sylvestris* L. (t_m = 0.998) [51]. This suggests that *Keteleeria davidiana* var. *calcarea* is a mixed mating system dominated by outcrossing, but 1-t_m = 0.098 and t_m-t_s = 0.295 > 0, suggesting that there is a degree of selfing and inbreeding. The degree of self-crossing (or inbreeding) is influenced by population size and habitat fragmentation. The spatial genetic structure (SGS) is an important reason for the generation of mating systems [52]. The SGS is caused by seed dispersal around fruiting maternal trees, which results in the relatedness of nearby individuals. This phenomenon has been characterized in *Acrocomia aculeata* [53,54]. FC and GC had higher t_m-t_s values and smaller t_m and had more plants in the habitat than RA and LG. Therefore, it was hypothesized that the SGS may have some relationship with the population size of *Keteleeria davidiana* var. *calcarea*. There are similar findings in *Dipteryx alata vogel*: low population density resulted in lower levels of outcrossing and mating among relatives but longer pollen dispersal distances. In contrast, higher-density populations showed higher levels of outcrossing, more mating among relatives, and shorter pollen dispersal distances [55].

The diversity of pollen found in progeny is described by the multilocus correlation of paternity ($r_{p(m)}$) [30]. This correlation measures the probability that two individuals taken at random from the same matrix are full siblings. *Keteleeria davidiana* var. *calcarea* had a higher value of multilocus correlation, which suggests that the progeny derived from crosses with several closely related pollen donors. Among the four populations, FC and GC had higher $r_{p(m)}$ values, and N_{ep} values were also the highest among the populations. This result is most likely related to fewer N_{ep}. In the study of the habitat and population structure of *Keteleeria davidiana* var. *calcarea* forests, both populations of FC and GC were characterized by a high number of middle-aged and old-aged individuals, a low number of seedlings and young trees, and a declining population trend, which matches the results of our mating system. Similar results were found in *Euterpe precatoria* and *Ormosia hosiei* [56,57]. Therefore, for the conservation of FC and GC populations, captive breeding may be necessary.

This study detected inbreeding depression at the species level in *Keteleeria davidiana* var. *calcarea* (δ = 0.96 > 0). Many rare and endangered species use inbreeding to increase the number of individuals in their progeny in order to prevent extinction under harsh environmental conditions [58–60]. For plants, inbreeding can improve the adaptability of populations to fragmented habitats and mitigate the effects of insufficient numbers of pollination patterns. However, inbreeding leads to a loss of genetic diversity and, ultimately, worsens survival and development [61,62]. Prolonged inbreeding is capable of causing inbreeding depression, thereby making progeny vulnerable to extinction in the face of environmental fluctuations. The presence of a certain degree of inbreeding depression in the mating system may, therefore, be an important factor in the endangerment of *Keteleeria davidiana* var. *Calcarea*.

5. Conclusions

The results of the SSR-based genetic diversity detection show that *Keteleeria davidiana* var. *calcarea* had high genetic diversity in Keteleeria plants. However, the progeny exhibited a decreasing trend in genetic diversity when compared to the maternal trees. This suggests that, while wind-pollinated species can tolerate some environmental and anthropogenic influences, excessive habitat fragmentation still has a negative impact on genetic diversity. Analyzing the reasons for the decline in genetic diversity, we found that self-crossing and

inbreeding depression existed at the species level and in the mating system of *Keteleeria davidiana* var. *calcarea*. Therefore, it is possible that excessive habitat fragmentation has caused self-crossing and inbreeding depression in *Keteleeria davidiana* var. *calcarea*, resulting in a decrease in genetic diversity.

To conserve the genetic diversity of *Keteleeria davidiana* var. *calcarea*, it is important to take measures, such as manually selecting different maternal trees to avoid inbreeding during pollination. This task involves establishing seed gardens and collecting seeds from different populations for artificial propagation. Additionally, asexual propagation technology for *Keteleeria davidiana* var. *calcarea* should be developed to rescue the population.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15050793/s1, Figure S1: Deprogram of UPGMA cluster analysis base on Nei's genetic distance between four populations; Figure S2: Linear regression analysis of genetic distance versus geographic distance. R^2, coefficient of determination; Figure S3: Plant and habitat conditions of *Keteleeria davidiana* var. *Calcarea*. (A) Photographs of *Keteleeria davidiana* var. *calcarea* plants. (B) Photographs of *Keteleeria davidiana* var. *calcarea* habitat. Table S1: The null allele frequencies estimated by Micro-checker; Table S2: Analysis of coefficients of variation for individuals and populations; degree of freedom; SS, sum of squares; MS, mean square; Est. Var, estimate of variance; %, percentage of total variation based on 999 permutations.

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