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RESEARCH ARTICLE

# Genetic diversity and population structure of *Garcinia paucinervis*, an endangered species using microsatellite markers

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## Abstract

Genetic diversity influences the fitness of species and provides variation for adaptation. *Garcinia paucinervis* Chun et How (Clusiaceae) is an endangered species with important ecological, medicinal and ornamental values endemic to Southwest China and Northern Vietnam, whose populations were severely fragmented in island habitats and population sizes were influenced by human. The assessment of genetic variation of *G. paucinervis* is anticipated to provide essential information for efficient conservation strategies. In this study, a suite of population genetics tests and analyses were used to investigate genetic diversity and structure of the 11 natural populations (a total of 360 individuals) of *G. paucinervis* in Guangxi and Yunnan Provinces, China, based on genotypes at 14 loci. Our results revealed a low to moderate genetic diversity in *G. paucinervis* remnants ($H_E = 0.487$, $I = 0.924$, $A_R = 3.420$). The global inbreeding coefficient ($F_{IS} = 0.004$) showed significant deviation from Hardy–Weinberg equilibrium, implying that the risk of inbreeding depression accompanied by heterozygote deficiency was probably due to severe habitat fragmentation and decreasing population sizes. Significant bottlenecks were detected in two populations. There has been little recent exchange of genes between most of the population pairs. Mantel test revealed that the genetic distance was not related to the geographical distance, suggesting a limitation of gene flow. A population from Yunnan Province could be classified as an independent cluster separated from the other populations, which should be considered as a prior conservation unit.

**Keywords** *Garcinia paucinervis* · Microsatellite · Genetic diversity · Genetic differentiation · Population structure · Conservation strategies

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## Introduction

Studies on conservative genetics of endangered plants are of fundamental importance for establishing management plans to protect biodiversity (Ávila-Díaz and Oyama 2007). Genetic diversity influences the current fitness of species and provides variation for adaptation (Neel and Commings 2003). Loss of genetic diversity could lead to a decrease in survival ability of species to adapt to environmental changes (Reisch et al. 2003). Understanding the level of genetic diversity within and among natural populations is therefore considered to have a high priority for proposing successful conservation strategies for imperilled plant species (Francisco-Ortega et al. 2000; Honjo et al. 2004), such as choosing conservation units and giving priority because of high ecological and genetic distinctiveness (Funk et al. 2012).

Accompanying with current over exploitation and the environmental deterioration in karst areas in Southwest

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China, the habitats of some rare or endangered karst plants have been seriously destroyed and their resources have decreased drastically, leading to their highly fragmented and relatively narrow distributions in China. These species have been forced into small and isolated populations, which will experience an erosion of random genetic drift and non-random mating (Karron 1997; Lande 1999). Earlier studies have suspected that numerous endangered species were generally prone to lose genetic variation with low genetic diversity (Fagen and Xia 2005), and some of them have even experienced bottleneck effect (Chan et al. 2011). Their fitness often reduced and the ability to adapt to changes in the environment might be diminished (Yu et al. 2006).

*Garcinia paucinervis* Chun et How (Clusiaceae) is an evergreen tree and one of the most well-known hardwoods with a distribution restricted to forests on dry karst limestone mountains at 194–830 m above sea level in Western Guangxi and Southeastern Yunnan, China and Northern Vietnam (Liang 2015; Zhang et al. 2017). The wood is heavy, hard, mothproof and especially water-tolerant and can be used in furniture, ship, and building manufacture (Toure et al. 2010). This karst endemic tree species is a valuable timber species but vulnerable to extirpation, because of its limited and dispersive distribution, excessive deforestation, weak seed germination, poor natural regeneration capacity (Fu, 1991), and rare adult trees (Zhang et al. 2017). Thus *G. paucinervis* was categorized as an endangered species by the International Union for Conservation of Nature (IUCN, http://www.iucnredlist.org), and was classified under State Protection Category II in China, as well as was listed as a vulnerable plant species in the *China Plant Red Data Book* (Fu 1991). In Zhuang medicine (a traditional medicine of the Chinese minorities), its bark, branches and leaves are used externally for treatment of scalding and burns (Zhang et al. 2015). The tree is tall and straight with lustrous evergreen foliage and bright red young leaves. Accordingly, it is a beautiful ornamental colour-leaved tree species.

Unfortunately, up to now, little information exists regarding the genetic status of the rare and endangered plants *G. paucinervis*, with the exception of some mapped microsatellite loci published for it (Hu et al. 2017). Microsatellites are one of the most commonly employed markers for studying genetic variation because of the high level of polymorphism and codominant transmission (Zhang and Hewitt 2003; Tautz 1989). In the present study, a set of new microsatellites were developed using restriction site-associated DNA sequencing (RAD-seq) method and some previously developed microsatellites with high polymorphism were screened to construct genotypes for 360 individuals from 11 populations of *G. paucinervis* in Guangxi and Yunnan Provinces, China. A suite of population genetics analyses and tests were used to investigate genetic diversity and structure based on genotypes at 14 loci. Our main aims were to provide baseline genetic information and establish more efficient strategies for appropriate conservation and management of *G. paucinervis*. Because of habitat fragmentation and decreasing population sizes, we hypothesized that the species had lost genetic variation with low genetic diversity, and genetic differentiation had occurred between populations far away from each other. There may be significant correlation between genetic distance and geographic distance.

## Materials and methods

### Plant material and DNA extraction

A total of 360 individuals were sampled from 11 natural populations of *G. paucinervis*, covering Western Guangxi (HJ, TE, BM, DA, XC, LA, JX, CZ and LZ populations) and Southeastern Yunnan (MLP and HK populations), China, between June to August 2016 (Table 1; Fig. 1). The minimum geographical distance between populations was 46 km. Individual numbers within populations (population sizes) were roughly estimated in the field by counting, ranging from approximately 30–200 individuals. Leaf samples were randomly collected with a minimum distance of 5 m apart from each other, covering the whole area in each population. The sample sizes for each population ranged from 21 to 58, based on population sizes and procurability. Leaf samples collected were desiccated by silica gel in sealed polyethylene bags until DNA extraction. Genomic DNA of all individuals was extracted from one leaf of each sample using the CTAB method (Doyle 1987). After passing DNA quality testing, each DNA sample was diluted a final concentration of approximately 50 ng $\mu l^{-1}$.

### Development of microsatellite markers and primer selection

To obtain the whole genome DNA sequences of *G. paucinervis*, RAD-seq libraries for one DNA sample were constructed. Following the methods of Wang et al. (2017), sequencing and assembling sequences as well as screening microsatellites from assembled sequences were carried out. Consequently, a total of 13,102,370 raw reads was obtained. After removing low-quality reads and PCR duplicates, we finally got 12,767,030 clean reads. Then, 13,750 microsatellites were screened from assembled sequences, but only 133 of these microsatellites containing appropriate flanking regions to design primers. Twenty-seven microsatellites having the dinucleotide and trinucleotide motifs with a minimum of seven repeats were selected for designing primers, and then the primer pairs were synthesized and screened.

The reaction mixture and PCR procedures were carried out as described by Wang et al. (2017). After checking PCR

**Table 1** Description of all studied populations for *G. paucinervis* including geographical locations, sample sizes and population sizes

| Population code | Location | Latitude (N) | Longitude (E) | Altitude (m) | Sample size | Population size |
|---|---|---|---|---|---|---|
| HJ | She Village, Chuanshan Town, Huanjiang County | 25°9′10″ | 108°3′34″ | 360 | 33 | 40–50 |
| TE | Guangxi Longtan Grand Canyon National Forest Park in Tian'e County | 24°58′57″ | 107°9′29″ | 681 | 25 | About 30 |
| BM | Changgan Village, Xishan Town, Bama County | 24°17′2″ | 107°13′46″ | 590 | 21 | 180–200 |
| DA | La'ren Village, La'ren Town, Du'an County | 24°22′59″ | 108°15′57″ | 194 | 33 | 150–180 |
| XC | Cuipingshan Park in Xincheng County | 24°4′8″ | 108°40′3″ | 220 | 36 | 80–100 |
| LA | Long'an Longhushan Nature Reserve | 22°57′39″ | 107°37′27″ | 280 | 26 | 40–50 |
| JX | Gupang Village, Anning Town, Jingxi | 22°54′13″ | 106°16′11″ | 830 | 43 | 150–180 |
| CZ | Chongzuo White-headed Langur National Nature Reserve | 22°33′33″ | 107°23′54″ | 376 | 22 | 30–40 |
| LZ | Nonggang National Nature Reserve in Longzhou County | 22°27′58″ | 106°57′18″ | 280 | 34 | 180–200 |
| MLP | Tianbao Farm, Tianbao Town, Malipo County | 22°57′50″ | 104°48′37″ | 330 | 58 | About 75 |
| HK | AnjiaheVillage, Nanxi Town, Hekou County | 22°40′42″ | 103°55′58″ | 410 | 29 | 50–60 |

Sample size was estimated based on the individuals with their height larger than 1 m

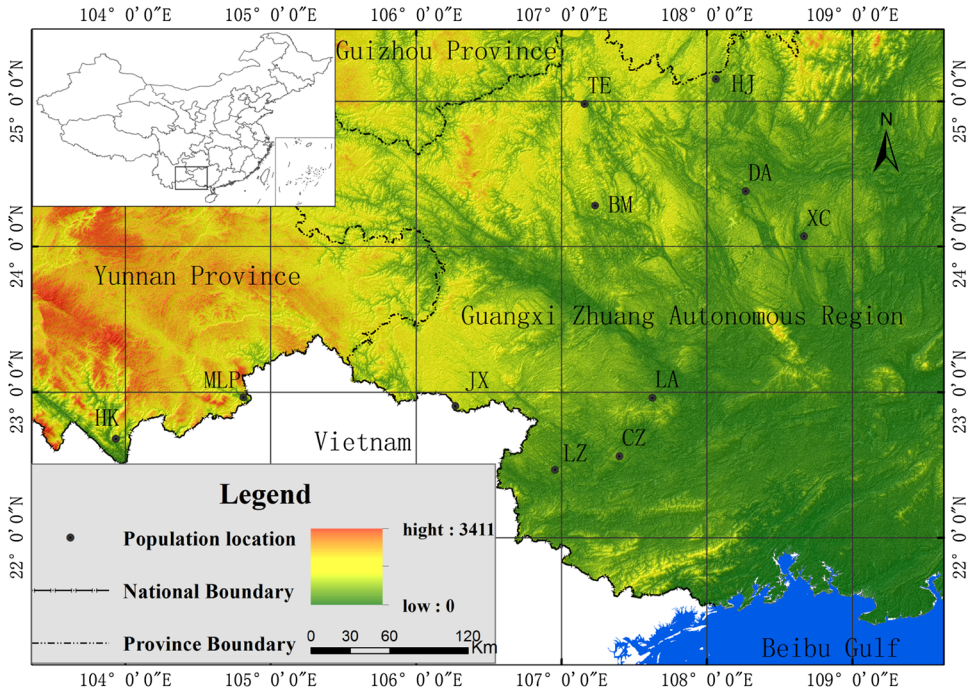


**Fig. 1** Locations of the 11 natural *G. paucinervis* populations sampled. See Table 1 for population codes and detailed information of populations. The same below. (Color figure online)

products on 2% agarose gels, six individuals were selected randomly from different populations to perform PCR and examine the effectiveness of primers for further marker screen. The fluorescently labeled PCR products were electrophoretically run on an ABI 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA), and their product sizes were visualized and recorded using the GeneMarker 2.2.0 with GS-500 (LIZ) as a size standard, subsequently. Consequently, five of 27 primer pairs amplified the target regions and showed unambiguously and polymorphic banding patterns with a maximum of two alleles for each locus per individual. The characteristics of five microsatellite markers were listed in Table 2. The newly-developed microsatellites may be valuable for investigating the genetic variation in *G. paucinervis* and other *Garcinia* species. Furthermore, twenty-two microsatellite loci previously published by Hu et al. (2017) were tested. Fourteen microsatellites of them showed stable and clear polymorphism. We used a combination of our five markers and previously developed 14 markers to construct genotypes for 360 individuals in 11

**Table 2** Characteristics of five microsatellite markers developed in *G. paucinervis*

| Locus | (5′–3′) Primer sequence (5′–3′) | Repeat motif | $T_a$ (°C) | GenBank accession No. |
|---|---|---|---|---|
| GP13 | F:GTGAAGGAGATGACTGAGAGAGA<br>R:TGGTATGGAATGTACAGATACCGT | $(AT)_7$ | 54 | MH269376 |
| GP17 | F:GGAAGACGATTGGATGGGTAGAG<br>R:CCAAGCATATGGTCCTTACGGTA | $(CT)_{11}$ | 55 | MH269377 |
| GP18 | F:AATTCTGAAAGTTTGCACACCCT<br>R:ATGTTGGGATTTGGTGAAATCGT | $(CA)_{13}$ | 57 | MH269378 |
| GP23 | F:TTCTCGACTCAAGTCCTAACCCT<br>R:GCCGAAACTACATTGGAAGCTAG | $(TC)_8$ | 57 | MH269379 |
| GP24 | F:CCTCTGTAGCAAACCCACAATTG<br>R:GGCAAAACTAGCTTGGAACATGT | $(TC)_8$ | 54 | MH269380 |

*F* forward primer, *R* reverse primer, $T_a$ annealing temperature

*G. paucinervis* populations. When we find loci with high frequency of null alleles (larger than 0.05) and deviate from Hardy–Weinberg equilibrium, they should be removed from further analyses.

## PCR amplification and product detection

Forward primers for all loci were labeled with fluorescent dyes (FAM, HEX or TAM) at the 5′end. PCR was performed in a 20-μl reaction consisting of 3 μl of template DNA, 10 μl of 2 × *Taq* PCR MasterMix (Novoprotein Scientific Inc, Shanghai, China), 5 μl of sterilized dd$H_2O$, and 1 μl of each primer (5 μlM). The PCR cycling condition included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51–57 °C (Table 2) for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. PCR products for 3–4 microsatellite loci were pseudo-multiplexed (Guichoux et al. 2011) and separated via capillary electrophoresis on an ABI 3730XL DNA Analyzer using GS-500 (LIZ) as a size standard, then allele sizes were performed in GeneMarker 2.2.0.

## Microsatellite and genetic diversity analysis

A set of genetic statistics for each microsatellite locus and each population were calculated using following methods. The GenAlEx 6.5 software (Peakall and Smouse 2006) was run to estimate the number of different alleles per locus ($N_A$), mean number of alleles per locus within populations ($A$), effective number of alleles ($N_E$), observed and expected heterozygosity ($Ho$, $H_E$), Shannon's diversity index ($I$), number of private alleles ($N_p$, i.e., alleles restricted to a single population). Number of rare alleles ($N_R$, i.e., the frequency of allele < 0.05) and polymorphism information content ($PIC$) were calculated in CERVUS 3.0.7 (Kalinowski et al. 2007). The frequency of null alleles ($A_n$) per locus was examined with Brookfield method (Brookfield 1996) using the program MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). Take the minimum individual number among populations as the standard, allelic richness ($A_R$) for each population was calculated using ADZE 1.0 software (Szpiech et al. 2008). In addition, inbreeding coefficient ($F_{IS}$, i.e., an estimation of deviation from random mating), departure from Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium ($LD$) between all pairs of loci were assessed using GENEPOP 4.1.1 (Rousset 2008). The significance level was adjusted with the Bonferroni correction of SGoF method (Carvajal-Rodríguez et al. 2009) using Myriads 1.1 (Carvajal-Rodriguez 2018). The criteria of 95% confidence intervals (CI) for the parameters above were generated by permutation using SPSS 21.0. Since the two-phased model of mutation (TPM) has been reported to be more conservative and powerful (Spencer et al. 2000), Wilcoxon test for mutation-drift equilibrium under TPM run based on 10,000 coalescent simulations were performed to exam population bottleneck using INEST 2.2 (Carvalho et al. 2014).

## Genetic differentiation and population structure analysis

The software GenAlEx 6.5 was used to calculate pairwise Nei's unbiased genetic distances (Nei 1978) and estimate pairwise allelic differentiation ($D_{est}$) based on 999 permutations between all pairs of populations (Jost 2008). For further study on the genetic structure of *G. paucinervis* populations, the principal coordinate analysis (PCoA) was performed with GenAlEx 6.5 based on the standard-convariance matrix. Mantel test (Mantel 1967) was used to assess the correlation between Nei's unbiased genetic distance matrix and geographical distance matrix of all population pairs employing the program GenAlEx 6.5. A Neighbor-joining

(NJ) tree based on allele frequency between population pairs was computed using POPTREE v.2, bootstrapping at 10,000.

In addition, a Bayesian approach was applied to estimate recent migration rates (i.e. over the last several generations) implemented in BAYESASS ed. 3 (Wilson and Rannala 2003). The program was run with Markov chain Monte Carlo (MCMC) 50,000,000 iterations, discarding 5,000,000 iterations as burn-in, and provides estimates of migration rates. Analysis of molecular variance (AMOVA) was also performed with GenAlEx 6.5.

A Bayesian cluster analysis was further implemented to study the genetic structure of the 11 populations using STRUCTURE 2.3.4 (Pritchard et al. 2000). Using an admixture ancestry model with correlated allele frequencies and assuming no prior concerning of individuals' origin populations, the number of tested populations (ln Pr(X|$K$) refers to the mean log likelihood estimate, and $K$ ranged from 1 to 11) was estimated to which population each individual belonged with 20 independent runs. The simulations were conducted with $10^6$ iterations and $10^5$ burn-in steps on total multiloci genotypes. The choice of the preferred $K$ value and the highest $\Delta K$ value was tested, and the genetic components were determined according to Evanno et al. (2005). The $K$ value with the maximal $\Delta K$ is usually the most appropriate.

## Results

### Characteristics of microsatellite markers

The characterization of the 19 polymorphic loci used in this study was presented in Table 3, including ten dinucleotide and nine trinucleotide repeats. The number of different alleles ranged from 3 to 17 with an average of 8.3 per locus, with a total of 158 alleles across the 360 analyzed individuals. The $H_O$ and $H_E$ for each locus ranged from 0.039 to 0.792 and 0.063 to 0.735, with an average of 0.451 and 0.468, respectively. The number of rare alleles ($N_R$) ranged from 1 to 11. Across 19 microsatellites, mean *PIC* was 0.538, varying from 0.148 to 0.816, indicating 10 out of 19 loci with high polymorphism ($PIC > 0.5$) (Botstein 1980). Shannon's index ($I$) varied from 0.116 to 1.527, with a mean value of 0.886. Significant deviation from HWE after Bonferroni correction were observed in nine (GP18, GP23, JSL5, JSL17, JSL19, JSL22, JSL27, JSL29 and JSL43) out of 19 Loci ($P < 0.05$). Chapuis and Estoup (2007) considered a negligible frequency with $A_n < 0.05$. Five loci (GP13, GP18, JSL3, JSL27 and JSL29) have high frequency of null alleles with $A_n > 0.05$, and three of them also show deviation from HWE. Accordingly, the five loci should be excluded from further analyses otherwise the interpretation of results can be misleading. No locus pairs showed consistently significant *LD* across populations after Bonferroni correction. Accordingly, the 14 (GP17, GP23, GP24, JSL2, JSL5, JSL17, JSL19, JSL22, JSL26, JSL32, JSL39, JSL42, JSL43 and JSL45) out of 19 microsatellite markers would be valuable for assessments in genetic diversity and population structure of *G. paucinervis*.

### Genetic diversity

Genetic diversity among the 14 microsatellite loci and among the 11 populations of *G. paucinervis* showed that the $A$ value varied from 2.9 (CZ) to 5.7 (MLP). Across all populations, the mean $N_E$ was 2.5, and the mean $I$ was 0.924, varying from 0.727 (XC) to 1.064 (JX, HK). The lowest $A_R$ was 2.702 (XC) and the highest $A_R$ was 3.886 (MLP). The private alleles were observed in seven populations, indicating the most considerable variation in MLP population ($N_p = 16$). A low to moderate genetic diversity level was detected across all studied populations, with the global mean $H_O$ of 0.492 and the global mean $H_E$ of 0.487, ranging from 0.409 (MLP) to 0.582 (JX) and 0.402 (XC) to 0.549 (JX), respectively (Table 4). These genetic diversity indexes revealed BM, LA, JX, MLP and HK populations with moderate genetic diversity, nevertheless HJ and XC populations with relatively low genetic diversity.

The $F_{IS}$ for each population ranged from $-0.155$ to 0.215, with a mean $F_{IS}$ over all populations of 0.004 and significant deviation from HWE ($P < 0.05$) after Bonferroni correction, implying a general significant deficiency of heterozygote. Three populations significantly deviated from HWE after Bonferroni correction. The $F_{IS}$ of DA and MLP populations showed positive values, while that of XC population exhibited a negative value, indicating significant excess of heterozygote. Wilcoxon test for the 11 populations revealed significant bottleneck in CZ and HK populations ($P < 0.05$) under TPM.

### Population differentiation and gene flow

The Nei's unbiased genetic distances between all population pairs were distinctly different, ranging from 0.026 (between XC and TE) to 0.677 (between MLP and BM). The genetic distance between MLP population and other populations (larger than 0.401) was higher than that between other population pairs (Table 5). Mantel test showed that no significant correlation ($r^2 = 0.0271$, $P = 0.19 > 0.05$) was detected between Nei's unbiased genetic distances and geographic distances. We observed that the pairwise allelic differentiation ($D_{est}$) values ranged from 0.028 (between XC and TE) to 0.486 (between MLP and BM) between population pairs. In HJ, DA, XC, JX and MLP populations, the proportion of native individuals was high (larger than 0.9). By contrast, the recent migration rates between some populations occupied a considerable proportion (larger than 0.1), such as from XC

**Table 3** Diversity detected of investigated microsatellite loci in 360 *G. paucinervis* individuals

| Locus | $T_a$ (°C) | $N_A$ | $N_E$ | $N_R$ | *PIC* | *I* | *Ho* | $H_E$ | $F_{IS}$ | $P_{HWE}$ | $A_n$ | Size range (bp) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GP13 | 54 | 9 | 1.5 | 6 | 0.374 | 0.445 | 0.266 | 0.240 | −0.137 | 1.000 | 0.072 | 160–174 |
| GP17 | 55 | 4 | 2.1 | 1 | 0.475 | 0.836 | 0.470 | 0.492 | 0.055 | 0.120 | 0.003 | 186–192 |
| GP18 | 57 | 14 | 2.9 | 10 | 0.694 | 1.233 | 0.582 | 0.605 | 0.073 | 0.003* | 0.138 | 101–125 |
| GP23 | 57 | 3 | 1.1 | 1 | 0.210 | 0.116 | 0.039 | 0.063 | 0.512 | 0.003* | 0 | 151–155 |
| GP24 | 54 | 3 | 1.4 | 1 | 0.325 | 0.383 | 0.262 | 0.242 | 0.005 | 0.060 | −0.031 | 184–188 |
| JSL2 | 53 | 9 | 1.9 | 7 | 0.418 | 0.786 | 0.416 | 0.417 | 0.015 | 0.082 | −0.088 | 305–344 |
| JSL3 | 55 | 4 | 1.2 | 2 | 0.148 | 0.258 | 0.112 | 0.138 | 0.205 | 0.085 | 0.126 | 237–243 |
| JSL5 | 53 | 7 | 2.4 | 3 | 0.626 | 1.007 | 0.539 | 0.556 | 0.102 | 0.000* | 0.041 | 138–156 |
| JSL17 | 55 | 13 | 2.1 | 11 | 0.485 | 0.847 | 0.332 | 0.430 | 0.223 | 0.000* | 0.036 | 254–284 |
| JSL19 | 54 | 5 | 1.8 | 2 | 0.509 | 0.686 | 0.311 | 0.417 | 0.274 | 0.000* | −0.043 | 192–202 |
| JSL22 | 54 | 9 | 3.2 | 4 | 0.709 | 1.277 | 0.685 | 0.662 | 0.021 | 0.002* | 0.018 | 153–177 |
| JSL26 | 53 | 14 | 3.6 | 8 | 0.806 | 1.412 | 0.792 | 0.704 | −0.110 | 1.000 | −0.037 | 245–290 |
| JSL27 | 56 | 10 | 2.7 | 4 | 0.782 | 1.154 | 0.388 | 0.608 | 0.364 | 0.000* | 0.215 | 154–175 |
| JSL29 | 54 | 7 | 2.0 | 4 | 0.467 | 0.799 | 0.323 | 0.473 | 0.307 | 0.000* | 0.093 | 161–187 |
| JSL32 | 51 | 10 | 3.6 | 5 | 0.779 | 1.354 | 0.762 | 0.702 | −0.068 | 0.148 | −0.094 | 164–189 |
| JSL39 | 55 | 10 | 3.2 | 5 | 0.756 | 1.331 | 0.750 | 0.670 | −0.086 | 0.442 | 0.044 | 178–205 |
| JSL42 | 52 | 3 | 1.1 | 1 | 0.175 | 0.128 | 0.071 | 0.078 | 0.137 | 0.212 | −0.001 | 292–337 |
| JSL43 | 51 | 7 | 3.1 | 2 | 0.671 | 1.251 | 0.705 | 0.651 | −0.088 | 0.014* | 0.024 | 118–139 |
| JSL45 | 51 | 17 | 3.9 | 11 | 0.816 | 1.527 | 0.759 | 0.735 | 0.013 | 0.060 | −0.044 | 196–248 |
| Total | | 158 | 44.8 | 88.0 | | | | | | | | |
| 95%CI | | (6.3, 10.3) | (1.9, 2.8) | (3.0, 6.3) | (0.431, 0.645) | (0.668, 1.103) | (0.332, 0.570) | (0.361, 0.574) | (0.009, 0.181) | | (−0.131, 0.063) | |
| Mean | | 8.3 | 2.4 | 4.6 | 0.538 | 0.886 | 0.451 | 0.468 | 0.096 | | | |

Loci start with "JSL" published by Hu et al. (2017)

$T_a$ optimal annealing temperature. $N_A$ number of different alleles, $N_E$ effective number of alleles, $N_R$ number of rare alleles (< 5%), *I* Shannon's information index, *Ho* observed heterozygosity, $H_E$ expected heterozygosity, *PIC* polymorphism information content, $F_{IS}$ inbreeding coefficient, $P_{HWE}$ Hardy–Weinberg *P* value. $A_n$ frequency of null allele, *95% CI* confidence interval

*Significant deviation from the Hardy–Weinberg epuilibrium after correction of SGoF(0.05) in Bonferroni correlation ($P < 0.05$)

**Table 4** Genetic diversity parameters of *G. paucinervis* within populations

| Population code | *N* | *A* | $N_E$ | *I* | $A_R$ | $N_p$ | *Ho* | $H_E$ | $F_{IS}$ | $P_{HWE}$ | TPM |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HJ | 33 | 3.6 | 2.1 | 0.751 | 2.908 | 0 | 0.476 | 0.405 | −0.107 | 0.112 | 0.6508 |
| TE | 25 | 3.7 | 2.3 | 0.832 | 3.206 | 0 | 0.455 | 0.437 | −0.013 | 0.174 | 0.8673 |
| BM | 21 | 4.1 | 2.7 | 1.020 | 3.709 | 1 | 0.525 | 0.536 | 0.048 | 0.205 | 0.3920 |
| DA | 33 | 4.1 | 2.4 | 0.895 | 3.433 | 1 | 0.451 | 0.466 | 0.073 | 0.000* | 0.5945 |
| XC | 36 | 3.4 | 2.0 | 0.727 | 2.702 | 1 | 0.451 | 0.402 | −0.155 | 0.009* | 0.1913 |
| LA | 26 | 4.7 | 2.6 | 1.032 | 3.817 | 0 | 0.526 | 0.535 | 0.037 | 0.080 | 0.3567 |
| JX | 43 | 4.6 | 2.8 | 1.064 | 3.770 | 2 | 0.582 | 0.549 | −0.049 | 0.258 | 0.3634 |
| CZ | 22 | 2.9 | 2.1 | 0.769 | 2.816 | 0 | 0.485 | 0.461 | −0.028 | 0.553 | **0.0042** |
| LZ | 34 | 4.3 | 2.7 | 0.972 | 3.554 | 6 | 0.491 | 0.503 | 0.043 | 0.428 | 0.3972 |
| MLP | 58 | 5.7 | 2.6 | 1.043 | 3.886 | 16 | 0.409 | 0.519 | 0.215 | 0.000* | 0.8731 |
| HK | 29 | 4.2 | 2.9 | 1.064 | 3.818 | 3 | 0.565 | 0.546 | −0.017 | 0.797 | **0.0083** |
| 95%CI | (25.6, 39.9) | (3.6, 4.6) | (2.3, 2.7) | (0.835, 1.014) | (3.124, 3.716) | (−0.47, 5.92) | (0.457, 0.528) | (0.450, 0.525) | (−0.062, 0.070) | | (0.227, 0.628) |
| Mean | 32.727 | 4.1 | 2.5 | 0.924 | 3.420 | 2.727 | 0.492 | 0.487 | 0.004 | 0.000* | |

The bold values in TPM column indicate that the populations with these values experienced genetic bottleneck. Codes for populations correspond to those in Table 1; *95% CI* confidence interval. The same below

*N* number of individual per population, *A* mean number of alleles per locus, $N_E$ effective number of alleles, *I* Shannon's diversity index, $A_R$ allelic richness standardized by the minimum population, $N_p$ number of private alleles, *Ho* observed heterozygosity, $H_E$ expected heterozygosity, $F_{IS}$ inbreeding coefficient calculated over all loci, $P_{HWE}$ Hardy–Weinberg *P* value. *TPM* the model of bottleneck test

*Significant deviation from HWE after correction of SGoF (0.05) in Bonferroni correlation ($P < 0.05$)

**Table 5** Pairwise Nei's unbiased genetic distances (below the diagonal) and allelic differentiation ($D_{est}$) (above the diagonal) between population pairs

| Population | HJ | TE | BM | DA | XC | LA | JX | CZ | LZ | MLP | HK |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HJ | – | 0.057 | 0.203 | 0.230 | 0.079 | 0.098 | 0.187 | 0.128 | 0.108 | 0.368 | 0.189 |
| TE | 0.055 | – | 0.113 | 0.106 | **0.028** | 0.093 | 0.095 | 0.125 | 0.072 | 0.354 | 0.125 |
| BM | 0.215 | 0.117 | – | 0.106 | 0.156 | 0.084 | 0.035 | 0.163 | 0.100 | **0.486** | 0.078 |
| DA | 0.243 | 0.109 | 0.111 | – | 0.146 | 0.129 | 0.100 | 0.214 | 0.120 | 0.454 | 0.153 |
| XC | 0.081 | **0.026** | 0.162 | 0.151 | – | 0.134 | 0.152 | 0.114 | 0.093 | 0.331 | 0.165 |
| LA | 0.092 | 0.091 | 0.089 | 0.136 | 0.134 | – | 0.092 | 0.123 | 0.084 | 0.402 | 0.098 |
| JX | 0.194 | 0.095 | 0.036 | 0.104 | 0.155 | 0.097 | – | 0.189 | 0.094 | 0.476 | 0.105 |
| CZ | 0.136 | 0.135 | 0.175 | 0.235 | 0.119 | 0.127 | 0.205 | – | 0.137 | 0.430 | 0.160 |
| LZ | 0.110 | 0.077 | 0.109 | 0.124 | 0.097 | 0.087 | 0.100 | 0.151 | – | 0.435 | 0.121 |
| MLP | 0.454 | 0.435 | **0.677** | 0.612 | 0.401 | 0.521 | 0.652 | 0.564 | 0.572 | – | 0.478 |
| HK | 0.203 | 0.129 | 0.081 | 0.157 | 0.169 | 0.104 | 0.109 | 0.172 | 0.133 | 0.658 | – |

Significant difference among all Pairwise $D_{est}$ estimators ($P<0.001$). The maximum and minimum values are in bold

**Table 6** Pairwise recent migration rates between populations based on Bayesian estimates using individual multilocus genotypes

| Immigrants | From | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Into | HJ | TE | BM | DA | XC | LA | JX | CZ | LZ | MLP | HK |
| HJ | *0.9021* | 0.0076 | 0.0076 | 0.009 | 0.0179 | 0.0084 | 0.0114 | 0.009 | 0.0108 | 0.0076 | 0.0086 |
| TE | 0.0502 | *0.6760* | 0.0092 | 0.0169 | **0.1888** | 0.0093 | 0.0101 | 0.0095 | 0.0110 | 0.0094 | 0.0094 |
| BM | 0.0105 | 0.0105 | *0.6771* | 0.0200 | 0.0104 | 0.0106 | **0.2183** | 0.0105 | 0.0111 | 0.0105 | 0.0106 |
| DA | 0.0086 | 0.0076 | 0.0076 | *0.9013* | 0.0172 | 0.0088 | 0.0154 | 0.0079 | 0.0096 | 0.0075 | 0.0085 |
| XC | 0.0107 | 0.0071 | 0.0071 | 0.0088 | *0.9171* | 0.0073 | 0.0088 | 0.0099 | 0.0086 | 0.0071 | 0.0075 |
| LA | **0.1000** | 0.0091 | 0.0091 | 0.0266 | 0.0403 | *0.7101* | 0.0487 | 0.0135 | 0.0210 | 0.0091 | 0.0126 |
| JX | 0.0066 | 0.0061 | 0.0062 | 0.0166 | 0.0074 | 0.0067 | *0.9203* | 0.0068 | 0.0095 | 0.0063 | 0.0075 |
| CZ | 0.0185 | 0.0101 | 0.0102 | 0.0110 | 0.0144 | 0.0105 | 0.0164 | *0.8773* | 0.0106 | 0.0101 | 0.0109 |
| LZ | 0.0175 | 0.0074 | 0.0074 | 0.0109 | 0.0245 | 0.0092 | 0.0223 | 0.0108 | *0.8736* | 0.0074 | 0.009 |
| MLP | 0.0057 | 0.0049 | 0.0048 | 0.0049 | 0.0055 | 0.0054 | 0.0144 | 0.0048 | 0.010 | *0.9228* | 0.0168 |
| HK | 0.0091 | 0.0083 | 0.0083 | 0.0097 | 0.0151 | 0.0141 | 0.0274 | 0.0101 | 0.0131 | 0.0084 | *0.8765* |

The migration rates from populations listed across the top into populations listed along the side of the table. The proportions of individuals derived from the source population are in italics; the values of migration rates above 0.100 are highlighted in bold

to TE, JX to BM, and HJ to LA population pairs (Table 6), implying a recent dispersal trend between these population pairs. Note that these pairwise populations were located more than 181.56 km away from another pairwise population at least. AMOVA analysis assigned 28.190% ($P<0.01$) of the total genetic variance among populations, indicating low differentiation among populations, while AMOVA showed 71.810% ($P<0.01$) of that was distributed within populations.

## Population structure

NJ tree revealed the patterns of genetic relationships among the studied populations and the tree was clearly classified into two major genetic clusters (Fig. 2). Cluster I contained ten populations most from Guangxi Province. BM and JX populations clustered most closely together first, then they

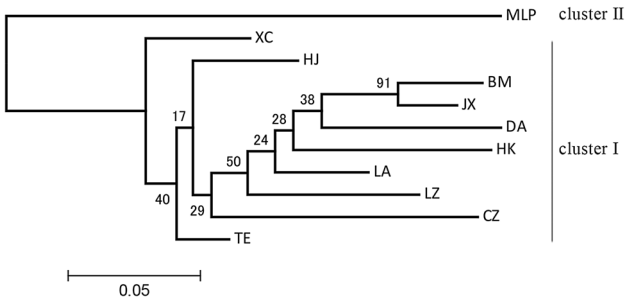


**Fig. 2** NJ tree of the 11 populations of *G. paucinervis* based on allele frequency between population pairs. See Table 1 for population codes. Bootstrap values are indicated on each node of the NJ tree

clustered with DA, HK, LA, LZ, CZ, HJ, TE and XC populations successively. MLP population from Yunnan Province was independently assigned into cluster II. MLP population

was the most genetically distinct from the other populations thereby.

PCoA also showed a genetic relationship among the 11 studied populations. The first two axes (two principal coordinates) accounted for 39.15% and 21.27% of the total variation, respectively, and mainly split the 11 populations into two distinct groups. BM, JX, DA and HK populations clustered closely together, combined with LA, LZ, TE, CZ, XC and HJ populations to correspond to Group 1. Of them, DA and BM populations are physically closer. However, MLP population was clearly differentiated from the other ten populations, forming Group 2 independently (Fig. 3). The clustering pattern of populations in bootstrap NJ tree was very similar to that in PCoA.

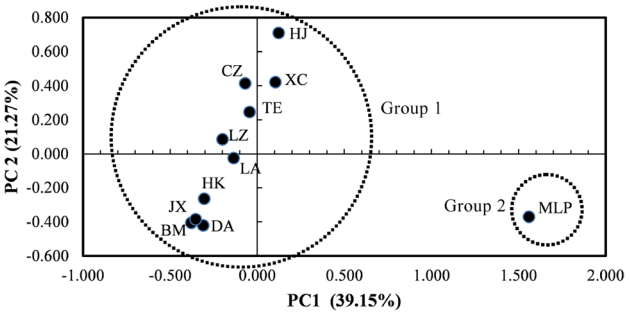


**Fig. 3** Principal coordinates analysis (PCoA) among the 11 populations of *G. paucinervis* based on their genotypic genetic distances. A, B and C refers to populations are divided into three groups, respectively. See Table 1 for population codes

The mean log-likelihood [Ln Pr(X\K)] increased progressively from $K=1$ to $K=11$. The STRUCTURE analysis showed a peak in $\Delta K$ at $K=2$ (Fig. 4a). Thus, we consider that the optimal value of genetic clusters was two, suggesting these genotypes was partitioned into two clusters. The nine populations from Guangxi Province and HK population from Yunnan Province constituted one cluster mainly in green, while MLP population from Yunnan Province corresponded to the other cluster commonly in red (Fig. 4b). In addition, the clustering of the second best $K$ ($K=3$) assignment was also shown in Fig. 4a. Here, the 360 individuals were assigned into three genetic clusters. MLP population also corresponded to one cluster in blue separately, and BM, DA, JX and HK populations were assigned to the cluster basically

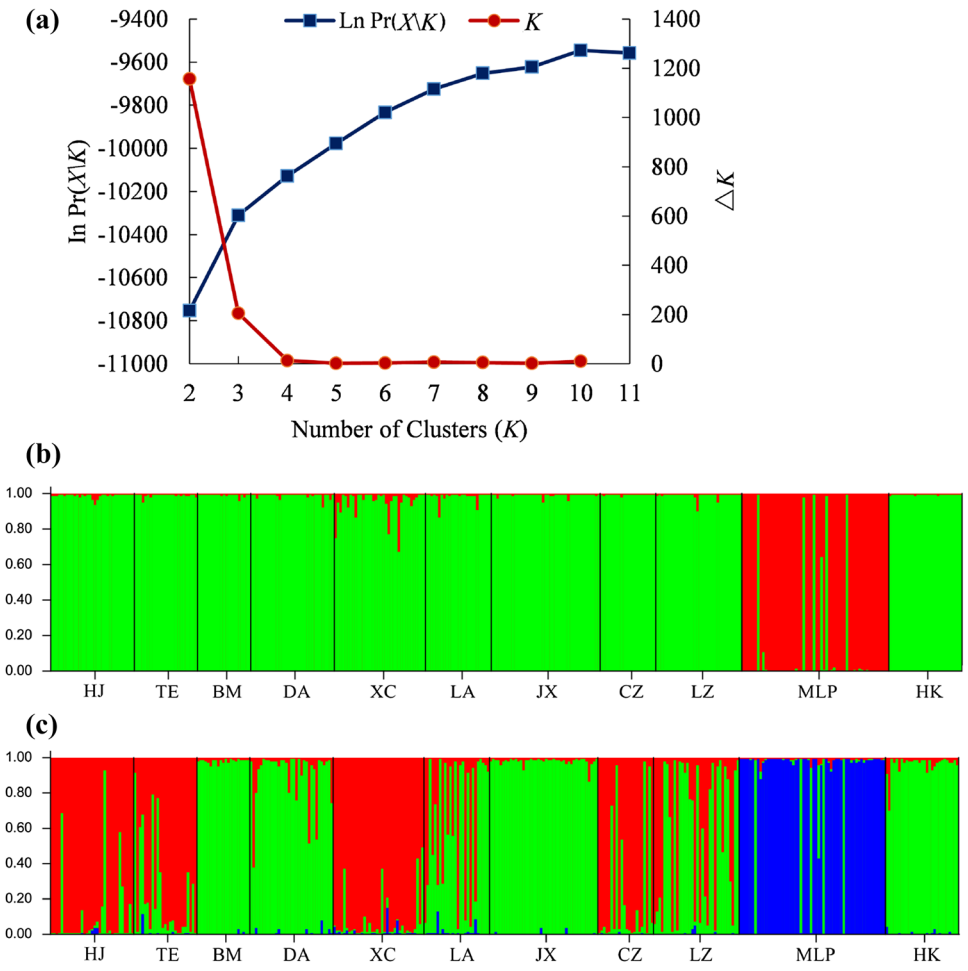


**Fig. 4** Genetic component clusters of the 11 *G. paucinervis* populations based on STRU CTURE analysis. **a** Plotted the mean likelihood Ln Pr(X*K*) and $\Delta K$ value; **b**, **c** Assignments proportion of each individual from all populations when $K=2$ and $K=3$, respectively. Each individual is represented by a thin vertical bar, which is partitioned into *K* colored segments. The colour represents the proportion of its genotype assigned to each cluster. Black lines separate different populations. Population codes according to Table 1. (Color figure online)

in green. HJ, TE, XC and CZ populations corresponded to another cluster in red. The frequent gene introgression was observed among LZ and LA proportions with a very high degree of admixture (Fig. 4c). NJ tree had the similar topology with the STRUCTURE of $K=2$. Likewise, the result of PCoA tallied with $K=2$.

## Discussion

### Genetic variation of *G. paucinervis*

*G. paucinervis* is an imperilled plant species with restricted geographic ranges and a low to moderate level of genetic diversity ($H_E=0.487$). When compared genetic diversity data for microsatellites of *G. paucinervis* compiled from the literature with those of other species with similar distribution, life history and seed dispersal forms (Nybom 2004), its $H_E$ across all the loci was lower than the average values reported for narrowly distributed species ($H_E=0.56$) and long-lived perennials ($H_E=0.68$), and comparable to species with gravity seed-dispersal ($H_E=0.47$). Nevertheless, its average values for $H_E$ were higher than that of endemic species ($H$E$=0.42$).

Among all studied populations, XC population displayed the lowest genetic diversity ($H_E=0.402$). Our field survey indicated that the XC population is located in the Cuipingshan Park in Xincheng County, and some *G. paucinervis* plants even live beside hiking trails. Under the circumstances, their survival and reproduction (e.g. insect pollination and seed dispersal) were seriously affected by frequent human activities, leading to potential inbreeding and a low genetic diversity. The demographic bottleneck was detected in CZ and HK populations, suggesting the two populations are not under mutation-drift equilibrium and further genetic erosion is very likely to be expected.

Our field observations found that many populations of *G. paucinervis* have been severely fragmented in island habitats, except for the BM population with a relatively widespread distribution. The global $F_{IS}$ was positive ($F_{IS}=0.004$) and significantly deviated from HWE, implying existence of the risk of inbreeding (Cullingham et al. 2012). The habitats in natural populations of *G. paucinervis* have suffered from frequent fragmentation and the rapid demographic decline associated with anthropogenic activities since the second half of the 20th century. From a genetic standpoint, habitat fragmentation and decreasing population sizes could lead to reduction of gene flow among populations and increase of inbreeding and genetic drift within populations (Jump and Peñuelas 2006). Such demographical process might consequently result in genetic losses such as a reduction of allelic richness and loss of alleles including rare alleles (Kang et al. 2005). The small remnant populations are vulnerable to stochastic environment (Frankham 2005), which will eventually influence the long-term persistence of *G. paucinervis* species. It is likely the main reasons for significant departure from HWE in DA, XC and MLP populations.

As a long-lived perennial and successional climax species, *G. paucinervis* plants grow slowly in early 30–40 years and an estimate of generation time exist for *G. paucinervis* is approximately 25 years (Fu 1991), suggesting that obvious changes in genetic diversity caused by habitat fragmentation may take several generations to emerge (Lowe et al. 2005; Kramer et al. 2008). It was only 40–60 years from the large-scale destruction period of the last century, the genetic variation caused by fragmentation may have not yet fully therefore emerged. Reduction of genetic diversity may take longer. If the habitats of *G. paucinervis* are not protected, the level of genetic diversity is likely to decrease, and the species probably raise inbreeding level and face extinction in a few generations. There were similar conclusions on previous studies of *Changiostyrax dolichocarpa* (Yao et al. 2007), *Cymbidium goeringii* (Chung et al. 2014) and *Theobroma speciosum* (Dardengo et al. 2018).

Additionally, the breeding system (such as the mating system and the patterns of pollination) of *G. paucinervis* have not been reported yet, which may be related to the inconvenience of investigation due to the height of adult trees (approximately 22 m) (Fu 1991), small and unattractive flowers (with approximately 10.90 mm in diameter and yellowish white, translucent petals, observed by authors). In field, highly scattered distributions of populations are not conducive to pollination and propagation. It is imperative to carry out the research on the breeding system of *G. paucinervis*, especially the important factors affecting the genetic characteristics such as pollinators and self-compatibility.

### Genetic differentiation and population structure of *G. paucinervis*

Gene flow is an essential microevolutionary force affecting genetic differentiation among populations (Slatkin 1994). In general, geographical isolation plays a non-negligible role in genetic isolation, such as the Pyrenees in Spain, which contributed greatly to the genetic differentiation among the *Borderea chouardii* populations (Segarra-Moragues et al. 2005). The fragmented island distributions restricted the long-distance genetic dispersal and the exchanges via pollen and seeds among populations.

Noticeably, the cluster analysis by using NJ tree, PCoA and STRUCTURE method suggested that populations of the same regions or close distances (i.e., four populations in Northwest Guangxi, five populations in Southeast Guangxi, and two populations in Yunnan) did not clustered strictly together. Mantel test also revealed that the genetic distance was not closely related to the geographical distance,

indicating no pattern of isolation by distance (IBD). The lack of IBD perhaps may be due to low levels of inter-population gene flow (Deacon and Cavender-Bares 2015; Zhang et al. 2018b). In addition, migration rates unequivocally indicated that there has been little recent exchange of genes between most of the population pairs. Also significant recent migration rates were detected between three pairwise populations located relatively far away from each other, which would be due to some long-distance dispersal factors likely to play a role in gene flow, such as human activities or bird migration. Berries and seeds of *G. paucinervis* are big (Zhang et al. 2018a) and dispersed primarily by gravity and animals, such as monkeys (Fu 1991). The destruction of habitats resulted in the reduction of frugivorous animals, which may lead to seed dispersal mainly by gravity and the decrease of recent gene flow. The increased habitat fragmentation has contributed to shrinking population sizes and restricted gene flow, which may cause the random genetic drift under low rate of natural regeneration observed (Cao et al. 2006).

Genetic diversity analysis showed there were the most private alleles in MLP population. The highest unbiased genetic distance was found between MLP population and other populations. In accordance with the results of NJ tree, PCoA, STRUCTURE, pairwise Nei's unbiased genetic distances and pairwise allelic differentiation showed strong genetic differentiation between MLP population and the other populations. Hence MLP population could be classified as a unique clade separated from the other populations. Interestingly, the location of MLP population is 330 m above sea level, while average elevation of Malipo County is 1,053 m. Some (Laoshan, Laojunshan, Daheishan, and Daping) mountains screen MLP population in the valley, serving as natural barriers to gene flow, hindering genetic exchanges and enhancing differentiation (Yang et al. 2015). In contrast, the terrain in Guangxi Province consists of plains and hills, without high mountains and big rivers (Fig. 1). Therefore, MLP population was differentiated from the other populations. Frequent economic and trade activities in Hekou County may be the reasons why HK population clustered closely together with the populations in Guangxi. Henceforth, an integrated analysis of nuclear and chloroplast DNA sequences should be conducted in a more detailed study focusing on phylogeographic and habitat differences, which will be likely to provide more insights into the underlying genetic differentiation (Zhai et al. 2018).

## Establishment of conservation management strategies

Habitat destruction and excessive collection for timber, fuel and herb are major threats to *G. paucinervis*, faced with a risk of genetic erosion. Currently, LZ, LA and CZ populations have been relatively well protected and preserved since the foundation of relevant national nature reserves in 1980, 1987 and 2012, respectively. But other populations located outside nature reserves may owing to lack of habitat protection and management, might be risking genetic erosion and our tasks will be particularly urgent to remedy the situation. For example, XC and TE populations are located in parks, with high human disturbance. HJ, DA, BM and JX populations are located on the limestone mountains beside roads, and the habitats have been fragmented with the risk of destruction and illegal deforestation. Furthermore, banana trees are planted at the foot of the mountains where the HK and MLP populations lies, and the two populations are therefore seriously affected by farming. All these factors above contribute to the extinction risk of this endangered species and could jeopardize its survival. These human behaviors have impeded gene flow among *G. paucinervis* populations and increased the probability of genetic drift and inbreeding. Given the severe fragmentation of habitats and relatively small population sizes, and in order to avoid the decrease of heterozygosity, the occurrence of inbreeding depression and the loss of private alleles (probably containing some specific genetic information), as well as to minimize further loss of genetic variation, it is necessary to develop the sustainable management strategies to protect and recover this species resources. Consequently, protecting the ecological habitats of the remaining populations and preventing illegal logging are particularly urgent.

The ultimate goals of conservation are to ensure sustainable survival of populations and to preserve their evolutionary potential (Cao et al. 2006). Given the current endangered level of *G. paucinervis*, surely the critical measures should be attentively taken into account to monitor and conserve all remaining populations of *G. paucinervis* at their original sites. The in situ conservation plans for all extant populations should be executed to decrease the impact from human activities, ensure their viability in field, promote natural regeneration, and maintain the genetic diversity of populations. However, because measures for all populations are likely extremely expensive, realistic measures may have to be focused on some populations. For HJ and TE populations with smaller population sizes and lower genetic diversity, vulnerable to stochastic environmental factors, it is suggested that artificial breeding, transplantation and reintroduction should be implemented to expand the existing population sizes on the basis of in situ conservation measures.

Moreover, we should select candidate populations covering genotypes as much as possible to be given priority for ex situ conservation. Firstly, the population with high level of genetic variation should be selected as its prior conservation unit. In the studied population, MLP population should also be a special target and deserves special prior conservation consideration, because it may contain some specific genes and excellent properties from isolation and local adaptation

(in view of the most private alleles and allelic richness), as well as it was highly differentiated with other populations.

Secondly, in order to maximize the protection of its genetic integrity with the smallest conservation units (Templeton 2010) and achieve genetic representation of *G. paucinervis,* the genetic backgrounds of the plants should be paid attention to, avoiding the homogeneity of germplasm sources or genetic composition. Conservation activities should take into account the two genetically differentiated clusters. Every cluster of heterogeneous genetic components should be included, and the population of relatively pure genetic components without low genetic diversity should be chosen, such as MLP and JX population. Furthermore, to meet the timber demand and commercial demand for this species, effective cultivation facilities could be established as alternative sources of raw materials. And a seed bank for ex situ collection urgently needs to be established.

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