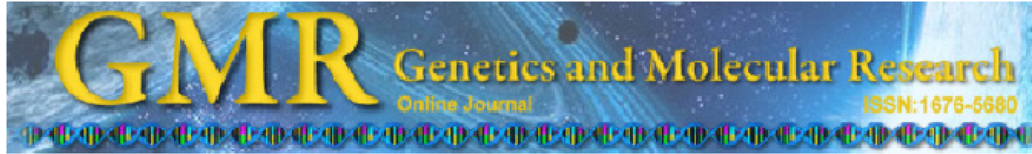

Genetic diversity of *Broussonetia papyrifera* populations in southwest China

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ABSTRACT. *Broussonetia papyrifera* is an important native tree species with high economic value in southwest China. Its resources are drastically reduced because of over-harvesting and habitat fragmentation. In this study, 17 natural populations of *B. papyrifera* were analyzed using inter-simple sequence repeat (ISSR) markers to assess the genetic diversity and population structure. In total, 100 bands were obtained from 16 ISSR primers. The *B. papyrifera* populations showed relatively high genetic diversity at the species level [percentage of polymorphic bands (PPB): 96%; Nei's genetic diversity (H_E): 0.3074; Shannon's information index (I): 0.4617], while the genetic diversity at the population level was relatively low (PPB: 53.2%; H_E: 0.1826; I: 0.2735). Relatively high level of genetic differentiation among populations (41%) was disclosed by analysis of molecular variance, which agrees with the Nei's genetic diversity statistics (40.59%) and Shannon's information measure (40.76%). Gene flow among populations (N_M) was only 0.7318. A significant correlation was observed between genetic and geographic distance among the studied

populations (r = 0.2948). We conjectured that the genetic diversity of *B. papyrifera* resulted from human disturbance, habitat fragmentation, small effective population size, and geographic barrier. Given the high genetic differentiation among populations, some utilization and conservation strategies were proposed. This study provides a reference for the sustainable use of the species in southwest China.

Key words: *Broussonetia papyrifera*; Population differentiation; Inter-simple sequence repeat (ISSR) marker; Genetic diversity

INTRODUCTION

Broussonetia papyrifera L. Vent. belongs to Moraceae and is a broad-leaved woody species that is a deciduous, dioecious, and dichogamous plant. These characteristics agree with those of anemophilous pollination (Liu et al., 2009a). It is native to southern China and Japan, and it is now distributed in China, Malay Peninsula, Japan, and Pacific islands (Zerega et al., 2005; Liu et al., 2009b). Although native to temperate climates, it has adapted to diverse latitudes and grows in many types of habitat. *B. papyrifera* has strong stress resistance, and it can grow normally at arid hillside, valley, and roadside. Its leaf, bark, fruit, and latex can act as raw material for medicine, and its leaf is a perfect feed. Additionally, its bark can be used to make textiles and paper, from which the common name "paper mulberry" is derived (Andrews, 1940).

B. papyrifera is a native tree in southwest China that has rich germplasm resources. Its bark is an important raw material for local papermaking because of the excellent performance of the fiber (Liao et al., 2006). Especially, it is well known that its bark can make high-grade paper for special purposes, such as banknote paper and battery separator paper.

Under the push of economic benefits, denudation is very severe, and the natural resources are drastically reduced. In addition, southwest China has an extremely complex terrain, and different populations are separated by mountains and rivers; this provides an advantage for population diffusion. Therefore, the pattern of genetic variation of *B. papyrifera* in southwest China is very anfractuous. However, studies related to this have not been reported. In the effort to conserve and sustainably exploit *B. papyrifera* resources, genetic diversity and structure studies are urgently needed.

The use of molecular markers to evaluate neutral genetic variation is an important tool to study population genetics (Rodrigues et al., 2013). Inter-simple sequence repeat (ISSR) markers permit the detection of polymorphic loci without knowing the DNA sequences of species (Barth et al., 2002). At the same time, the cost of the analyses is relatively lower than that of other markers such as amplified fragment length polymorphisms (AFLPs) and microsatellites (Fang and Roose, 1997). ISSRs have been extensively used in population genetic studies, especially in genetic diversity studies (Xiao et al., 2004; Xia et al., 2007; Lin et al., 2013).

In this study, ISSR markers were used to evaluate the genetic variation and differentiation among 17 natural populations of *B. papyrifera* in southwest China. This study will identify the degree of genetic differentiation and also provide a reference for the utilization of its genetic resources.

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MATERIAL AND METHODS

Plant material

In a typical distribution area, 510 individuals, corresponding to 17 natural populations of *B. papyrifera* from four provinces in southwest China, were collected in 2009 and 2010. Specific information is listed in Table 1 and Figure 1. Fresh, healthy leaf tissue was randomly sampled and dried with silica gel and stored at 4°C until DNA extraction.

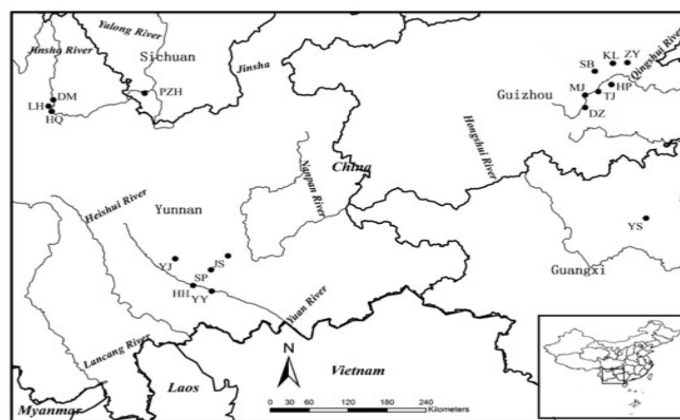

Figure 1. Distribution of 17 populations of *Broussonetia papyrifera* in southwest China.

Table 1. Location of the *Broussonetia papyrifera* populations sampled.

Population code	Location in China	Geographic coordinates	Sample size	Elevation (m asl)
DM	Yunnan	26°15'22"N 100°24'50"E	30	1210
LH	Yunnan	26°27'01"N 100°26'12"E	30	1241
HQ	Yunnan	26°20'32"N 100°22'20"E	30	2367
YS	Guangxi	24°29'59"N 108°38'42"E	30	178
KL	Guizhou	26°35'00"N 107°58'59"E	30	697
TJ	Guizhou	26°41'55"N 108°09'50"E	30	708
ZY	Guizhou	27°03'31"N 108°23'10"E	30	573
SB	Guizhou	27°02'57"N 108°11'06"E	30	637
HP	Guizhou	26°55'08"N 107°56'02"E	30	769
MJ	Guizhou	26°31'32"N 107°48'01"E	30	617
DZ	Guizhou	26°19'18"N 107°47'59"E	30	814
PZH	Sichuan	26°33'18"N 101°42'02"E	30	1046
JS	Yunnan	23°52'56"N 102°51'20"E	30	1516
SP	Yunnan	23°39'11"N 102°37'15"E	30	1415
YJ	Yunnan	23°50'03"N 102°07'29"E	30	865
HH	Yunnan	23°23'36"N 102°22'19"E	30	502
YY	Yunnan	23°18'01"N 102°37'50"E	30	457

DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted using the cetyltrimethylammonium bromide method described by Doyle (1991). Purified genomic DNA was quantified using a DU-800 spectrophotometer (Beckman, USA). Sixteen primers that showed good repetition and distinct poly-

morphism were chosen from 70 pre-screened primers that were obtained from the Biotechnology Laboratory, University of British Columbia, Canada. Amplifications were performed in a C1000 thermal cycler (Biorad, USA).

The 20-μL reaction mixture contained 10 ng DNA template, 2.0 μL 10X PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTPs (TIANGEN BIOTECH, China), 200 nM ISSR primer, 1.5 units Taq DNA polymerase (TIANGEN BIOTECH, China), and double-distilled water. According to the procedure of Xiao et al. (2004), amplification conditions were as follows: initial denaturation at 94°C for 5 min; 40 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 2 min; and a final extension at 72°C for 7 min. PCR products were electrophoretically separated on 2.0% agarose gels buffered with 0.5X Tris, boric acid, ethylenediaminetetraacetic acid buffer. A 100-bp DNA ladder (Fermentas, Vilnius, Lithuania) was used as a size marker. The DNA fragments were identified by image analysis software for gel documentation (Lab Works Software, Version 3.0; UVP, USA) after staining with ethidium bromide. The bands that showed consistent amplification were analyzed, and smeared and weak bands were excluded.

Data analysis

Each distinct and reproducible band was scored as present (1) or absent (0) for each marker and entered into a binary matrix. Assuming Hardy-Weinberg equilibrium, the POPGENE 3.2 software (Yeh et al., 1999) was used to process these data. The percentage of polymorphic bands (PPB), number of alleles (N_A), effective number of alleles (N_E), Nei's genetic diversity (H_E), and Shannon's information index (I) were analyzed. Nei's (1973) gene diversity statistics, total genetic diversity (H_T), genetic diversity within populations (H_S), genetic diversity among populations (D_{ST}), and the coefficient of genetic differentiation (G_{ST}) were measured at the species level. In addition, the genetic distance (D) between populations was also calculated according to the model presented by Nei (1972). Gene flow estimates (N_M) were calculated as $N_M = (1\text{-}G_{ST})/4G_{ST}$ (Slatkin and Barton, 1989). The total diversity of species (H_{SP}) and the mean intra-population diversity (H_{POP}) were measured using Shannon's index of phenotypic diversity. The proportion of diversity between populations was then calculated as $(H_{SP}\text{-}H_{POP})/H_{SP}$. The NTSYS-pc software (Rohlf, 2000) was used to test the correlations between genetic and geographical distances among populations. A dendrogram based on the genetic distance was constructed, implementing an unweighted pair-group method of cluster analysis that used arithmetic averages (UPGMA) with NTSYS-pc 2.1 (Rohlf, 2000). To determine the genetic structure, analysis molecular variance (AMOVA) was performed by GENALEX 6.4 (with 999 permutations). Mantel test (Mantel, 1967) between Nei's pairwise genetic distances and pairwise geographical distances was implemented by GENALEX 6.4 (Peakall and Smouse, 2006).

RESULTS

Amplification results of ISSR markers

Thirty primers were screened to detect polymorphic bands (putative loci). Of these, 16 primers generated interpretable polymorphic amplifications. Thus, 510 individual genomes from 17 populations were amplified by 16 ISSR primers. One hundred clear bands were am-

plified, and an average of 6.25 bands was produced per primer. The average number of polymorphic bands was 6, and the PPB for this species was 96% (Table 2).

Table 2. Sequences and numbers of bands for 16 primers.

Primer	Sequence 5'→3'[a]	No. of amplified bands	No. of polymorphic bands	% polymorphic bands
IS04	$(GTGC)_4$	8	8	100
IS05	$(GT)_6CC$	7	7	100
IS10	$(CA)_6GG$	7	7	100
IS11	$(CTC)_4GG$	10	10	100
IS13	$(CT)_8TG$	4	4	100
IS14	$(AG)_8TA$	5	4	80
IS18	$(CT)_8GC$	6	5	83.3
IS19	$(AG)_8TC$	7	7	100
IS20	$(CT)_8AC$	7	6	85.7
IS21	$(GTC)_6$	6	5	83.3
IS25	$(AG)_8YC$	4	4	100
IS26	$(CT)_6RC$	4	4	100
IS27	$(AC)_8YC$	7	7	100
IS30	$(AC)_8YT$	7	7	100
IS32	$(AG)_8YC$	6	6	100
IS33	$(AC)_6T$	5	5	83.3
Total		100	96	96
Average		6.25	6	

[a]R = (A, G); Y = (C, T)

Genetic diversity of *B. papyrifera*

At the population level, the average PPB was 53.2% and ranged from 31 (DM) to 68% (PZH). The Nei's genetic diversity (H_E) ranged from 0.1244 (KL) to 0.2258 (PZH), and it was estimated to be 0.1826 at the population level and 0.3074 at the species level. Shannon's diversity indices (*I*) were 0.2735 at the population level and 0.4617 at the species level. The results of H_E and *I* displayed a trend that was similar to that of PPB. According to these indices, the most diverse populations are PZH (PPB: 68%, H_E: 0.2258, *I*: 0.3394) and HP (PPB: 66%, H_E: 0.2257, *I*: 0.3384). The least diverse populations are DM (PPB: 31%, H_E: 0.1375, *I*: 0.1965) and HQ (PPB: 40%, H_E: 0.1446, *I*: 0.2142) (Table 3).

Genetic differentiation of *B. papyrifera*

According to the Nei's genetic diversity, the total genetic diversity (H_T) was 0.3074, and the genetic diversity within populations (H_S) was 0.1826 while the genetic diversity among populations was 0.1248 (H_{ST}). The Nei's coefficient of gene differentiation (G_{ST}) was 0.4059, which indicated that 40.59% of the genetic variation existed among populations and that 59.41% of it existed within populations. The gene flow between populations was estimated to be 0.7318, which is a relatively small value (Table 4).

Shannon's coefficient of gene differentiation [$(H_{SP}$-$H_{POP})/H_{SP}$] was 0.4076, indicating that 40.76% of the genetic variation was distributed among populations and 59.24% of it distributed within populations. In addition, the AMOVA (Table 5) showed that 41% of the genetic variation existed among populations and 59% of it existed within populations. The trends of genetic differentiation based on these analysis methods were consistent. These methods indi-

cated that the genetic variation within populations of *B. papyrifera* was higher than that among populations.

Table 3. Genetic diversity of 17 populations of *Broussonetia papyrifera* based on inter-simple sequence repeat (ISSR) markers.

Code[a]	PPB (%)	N_A (SD)	N_E (SD)	H_E (SD)	I (SD)
DM	31	1.3100 (0.4648)	1.2528 (0.3909)	0.1375 (0.2089)	0.1965 (0.2970)
LH	48	1.4800 (0.5021)	1.3469 (0.4008)	0.1948 (0.2149)	0.2835 (0.3070)
HQ	40	1.4000 (0.4924)	1.2517 (0.3632)	0.1446 (0.1981)	0.2142 (0.2845)
YS	45	1.4500 (0.5000)	1.2504 (0.3561)	0.1463 (0.1931)	0.2205 (0.2770)
KL	42	1.4200 (0.4960)	1.2027 (0.3136)	0.1244 (0.1735)	0.1928 (0.2538)
TJ	43	1.4300 (0.4976)	1.2054 (0.3151)	0.1256 (0.1751)	0.1944 (0.2553)
ZY	59	1.5900 (0.4943)	1.3324 (0.3765)	0.1940 (0.2006)	0.2920 (0.2843)
SB	63	1.6300 (0.4852)	1.3658 (0.3630)	0.2170 (0.1958)	0.3264 (0.2801)
HP	66	1.6600 (0.4761)	1.3862 (0.3758)	0.2257 (0.1994)	0.3384 (0.2817)
MJ	59	1.5900 (0.4943)	1.3459 (0.3579)	0.2057 (0.1970)	0.3089 (0.2834)
DZ	50	1.5000 (0.5025)	1.2940 (0.3794)	0.1694 (0.2013)	0.2536 (0.2863)
PZH	68	1.6800 (0.4688)	1.3891 (0.3856)	0.2258 (0.2003)	0.3394 (0.2802)
JS	54	1.5400 (0.5009)	1.3391 (0.3848)	0.1950 (0.2067)	0.2891 (0.2947)
SP	60	1.6000 (0.4924)	1.3574 (0.3800)	0.2079 (0.2024)	0.3109 (0.2875)
YJ	61	1.6100 (0.4902)	1.3273 (0.3569)	0.1961 (0.1924)	0.2983 (0.2750)
HH	55	1.5500 (0.5000)	1.3168 (0.3729)	0.1854 (0.1994)	0.2788 (0.2846)
YY	61	1.6100 (0.4902)	1.3620 (0.3886)	0.2089 (0.2038)	0.3126 (0.2875)
Average	53.2	1.5324 (0.1037)	1.3133 (0.0598)	0.1826 (0.0345)	0.2735 (0.0514)
Species	96	1.96 (0.1969)	1.5321 (0.3604)	0.3074 (0.1738)	0.4617 (0.2250)

[a]Population codes are explained in Table 1. N_A = number of alleles; N_E = effective number of alleles; H_E = Nei's genetic diversity; I = Shannon's information index.

Table 4. Nei's analysis of genetic diversity in *Broussonetia papyrifera* populations.

	H_T	H_S	D_{ST}	G_{ST}	N_M
Mean	0.3074	0.1826	0.1248	0.4059	0.7318
SD	0.0302	0.0128			

H_T = total genetic diversity; H_S = genetic diversity within populations; D_{ST} = genetic diversity among populations; G_{ST} = coefficient of genetic differentiation; N_M = gene flow.

Table 5. Analysis of molecular variance (AMOVA) for *Broussonetia papyrifera* based on inter-simple sequence repeat (ISSR) data.

Source of variance	SSD[a]	MSD[b]	Variance component	Total variance (%)	P value[c]
Variance among populations	1518.471	94.904	7.072	41%	<0.0001
Variance within populations	1877.333	10.039	10.039	59%	<0.0001

[a]Sum of squared deviation; [b]Mean of squared deviation; [c]P values are the probabilities of having high and extreme variance component than the observed values alone. Probabilities were calculated by 1000 random permutations of individuals across populations.

Genetic relationships of *B. papyrifera*

To represent the relationship among populations, a UPGMA tree was generated based on Nei's genetic distance between the 17 populations that were studied (Figure 2). It showed that all populations could be separated into three clusters if 0.155 (genetic similarity coef-

ficient) was set as a boundary. Cluster A included three populations (DM, LH, and HQ) from northwest Yunnan Province. Seven populations (KL, TJ, ZY, SB, HP, MJ, and DZ) from southeast Guizhou Province and the YS population from northern Guangxi Province were grouped in cluster B. The populations that were located in southeast Yunnan Province (JS, SP, YJ, HH, and YY) were grouped in cluster C. Also, the PZH population that is geographically close to northwest Yunnan Province was grouped in cluster C. Mantel tests showed that the genetic distance was significantly correlated with the geographic distance (r = 0.2948, P = 0.0001; Figure 3), which suggests that geographic isolation influenced the genetic differentiation among populations to some extent.

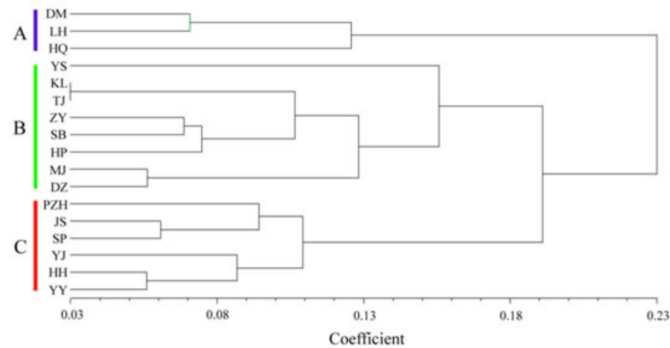

Figure 2. Dendrogram after unweighted pair-group method with arithmetic mean of *Broussonetia papyrifera* populations based on Nei's genetic distance. Population codes are explained in Table 1.

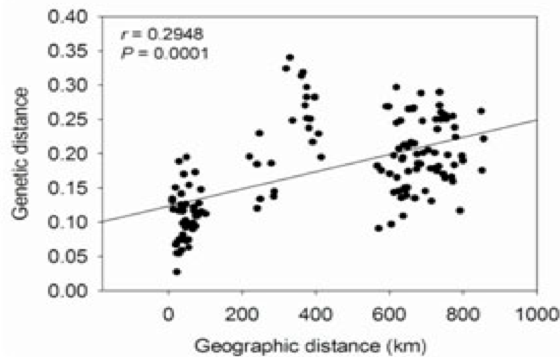

Figure 3. Correlation between the geographic distance and genetic distance of populations.

DISCUSSION

Genetic diversity of *B. papyrifera*

Generally, widespread species have a higher genetic diversity than species with a narrow distribution (Hamrick and Godt, 1996). *B. papyrifera* has a wide distribution, but it is scattered in southwest China. Based on ISSR analysis, the overall genetic diversity is relative-

ly high at the species level (PPB: 96%) compared with other Moraceae species, such as *Ficus carica* (PPB: 72.23%) (Salhi-Hannachi et al., 2006), *Morus serrata* (PPB: 67%) (Vijayan et al., 2004), *Artocarpus heterophyllus* (PPB: 22%) (Shyamalamma et al., 2008), and *Artocarpus altilis* (PPB: 85%) (Sreekumar et al., 2007). However, the genetic diversity is relatively low at the population level (PPB: 53.2%, H_E: 0.1826, I: 0.2735). The H_E value is lower than the average value of widespread species (H: 0.22) and is close to the average value of endemic species (H: 0.20) according to the Nybom statistic, which calculated the genetic diversity of many plants at the population level [based on random amplified polymorphic DNA (RAPD), AFLP, and ISSR dominant markers] (Nybom, 2004). This suggested that the high overall genetic diversity that was presented by the species itself could be explained mostly by differences among populations that originated from the high genetic structure (Rodrigues et al., 2013).

Population size was reported to be associated with genetic variation in wildlife, both within and among species (Francisco-Ortega et al., 2000). Because *B. papyrifera* in southwest China had considerable economic benefits, and the supervision by the local government was ineffective, the excess deforestation and denudation was extremely serious, and its resources were drastically reduced. Actually, we observed that the population size became smaller and the habitat fragmentation was aggravated. This resulted in *B. papyrifera* being vulnerable to fluctuations in climate and habitat disturbance (Travis et al., 1996). For example, the DM, LH, and HQ populations belong to the Jinsha River Basin, and the JS, SP, YJ, HH, and YY populations belong to the Yujiang River Basin. Compared with the Yujiang River Basin, the Jinsha River Basin has more severe human disturbance and a smaller population size. Interestingly, the DM, LH, and HQ populations displayed lower genetic diversity (PPB = 31, 48, and 40%, respectively), while the JS, SP, YJ, HH, and YY populations showed higher genetic diversity (PPB = 54, 60, 61, 55, and 61%, respectively). This indicated that populations in fragmented habitats and with a small effective size are sensitive to stochastic events, genetic drift, and inbreeding, leading to a low genetic diversity and the high genetic structure pattern (Hartl and Clark, 1997; Rodrigues et al., 2013).

Genetic structure of *B. papyrifera*

The genetic structure of plant populations depends on the genetic basis, breeding system, and the historical process of its origin and evolution, and it is also influenced by natural selection, genetic drift, recolonization, and gene flow (Ishihama et al., 2005). At the species level, the taxonomic status and distribution range are main factors for genetic variation. However, at the population level, the breeding system is the most important factor for genetic variation (Hamrick and Godt, 1990). *B. papyrifera* is characterized by perennial, dioecious, dichogamous, and anemophilous features. Therefore, it is a typical outbreeding plant. Bussell (1999) summarized the RAPD results of 35 species and found that the genetic variation among populations of 29 outbreeding species accounted for 0.9-41.3% (G_{ST}) of the total genetic variation, while that of the other 6 inbreeding species accounted for 44.8-66.9% of the total genetic variation. We found that the case of *B. papyrifera* agreed with this law because its genetic variation among populations was 41%.

In this study, the G_{ST} of *B. papyrifera* reached 0.41, which far exceeds the average G_{ST} of long-lived perennial species (0.19) and anemophilous species (0.17) according to the Nybom statistic (Nybom, 2004). According to the Mantel test (r = 0.29), the diversity pat-

tern could be partially explained by the geographic distance. In this study, many sampling sites were located at Yungui Plateau, where a mountain is closely interlaced with river and dense alpine gorges. The mountain and river are believed to be major geographical barriers that might largely hinder gene flow via seed and pollen dispersal among populations (Pfeifer and Jetschke, 2006). The moderate gene flow not only helps to maintain the stability of the populations but also enhances the ability to adapt to the changing environment (Irwin et al., 2005). It is generally believed that populations can prevent genetic differentiation that results from genetic drift when their gene flow is ≥ 1 (Slatkin, 1987). The gene flow of *B. papyrifera* populations was only 0.7318, which is possibly due to the geographical barriers. In addition, the seriously fragmented habitat led to a lack of effective flow. This limited gene flow caused strong genetic differentiation among populations.

Conservation implication

Current conservation plans mainly focus on maintaining species diversity in detriment of the intraspecific genetic diversity (Margules and Pressey, 2000). A loss of genetic diversity would decrease a species' ability to survive environmental changes and demographic fluctuations in the short and long term (Ellstrand and Elam, 1993; Reisch et al., 2003). Therefore, the elucidation of the genetic diversity and genetic structure of a species can provide important information to assess the protective value and to design adequate protection strategies. In this study, *B. papyrifera* in southwest China showed a low genetic diversity at the population level and a high population differentiation. Based on these results, some protection approaches must be proposed. First, the local forestry department should strengthen supervision to reduce the human disturbance and excess deforestation. Given its high economic value, the tissue culture seedling and plantation should be put into market. In this way, the fragmented habitat can be effectively relieved and the natural population size can be maintained. Second, *in situ* conservation should be implemented because the majority of the genetic variation existed within populations. Some forestry tending measures can be performed to promote natural regeneration. The ecological environment of the Jinsha River Basin is very fragile, and corresponding populations should be paid attention. The DM population had the lowest genetic diversity of the populations. In addition, as observed in our field survey, the habitat of the DM population was seriously destructed by the building of highway. Therefore, it is necessary to construct nature reserves to protect the DM population. Third, the manual transfer of mature seeds and artificial pollination among populations can enhance the gene flow. Taking into account the observation that *B. papyrifera* populations are highly structured genetically, outbreeding depression might be a potential genetic threat for already weakened populations (Sagvik et al., 2005). Therefore, it may be wise to protect a network of populations that exchange genetic material and are able to reinforce each other (Rodrigues et al., 2013). For these reasons, we propose that individuals within a cluster can be exchanged. Fourth, *ex situ* conservation should be adopted to preserve the core germplasm. Thus, the populations with the highest genetic diversity can be good representatives of the species. The sample size should be maximized to collect as much genetic information as possible.

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