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GENETIC DIVERSITY OF *SYMPLOCOS PANICULATA* OF HUNAN PROVINCE REVEALED BY INTER-SIMPLE SEQUENCE REPEAT (ISSR)

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Abstract

The natural populations' genetic diversity of *Symplocos paniculata* in Hunan province was evaluated by using Inter-simple sequence repeat (ISSR) markers. In all 122 loci were identified in 149 individual plants of six populations using nine selected ISSR primers (UBC815, UBC817, UBC824, UBC835, UBC845, UBC853, UBC862, UBC873 and UBC879) and 92.2% loci were polymorphic. Nei's gene diversity (*H*) and shannon information index (*I)* were 0.3264 and 0.4873, respectively. The coefficient of gene differentiation (*Gst*) was 0.5397. Gene flow among populations (*Nm*) was 0.4763. Analysis of molecular variance (AMOVA) demonstrated that the genetic differentiation among *S. paniculata* populations was relatively high ($p<0.001$). The among-population component accounted for 51.1% of the total variation, while the within-population component accounted for 49.8%. Six populations were clustered into three groups by the unweighted pair-group method arithmetic average (UPGMA). The results showed that populations of Da Wei Shan A (DWSA), Da Wei Shan B (DWSB) and Xiang Xi Long Shan (XXLS) were in the first group, populations of Yue Yang Jun Shan (YYJS) and Yong Zhou Dao Xian (YZDX) were in another group, and Heng Yang Hen Shan (HYHS) population separated from all the populations in the dendrogram. The cluster results were mutually related to the geographical elevation, but not to geographical distribution.

Key words: *Symplocos paniculata*, Inter-simple sequence repeat (ISSR), Natural populations, Genetic diversity.

Introduction

Inter-simple sequence repeat (ISSR) is a molecular marker technique that has been recently developed to explore polymorphism in microsatellite regions (Zietkiewicz, 1994). This technique is robust under different environmental conditions and plant development stage (Esselman *et al.,* 1999). In addition, the technique of ISSR needs only a few markers and does not require DNA prior sequence information. It is also fast and cost-efficient to obtain molecular intraspecific polymorphism to achieve the correct fingerprinting pattern and abundant polymorphism (Kuras *et al.,* 2004). Previous studies indicated that the ISSR technique had been widely used to investigate the biogeographic patterns (Sica *et al.,* 2005), genetic diversity (Kumar *et al.,* 2009; Lucas *et al.,* 2011; Grativol *et al.,* 2011) and gene mapping (Sankar & Moore, 2001) during the germplasm identification of plant species.

Symplocos paniculata belonging to the family of *Symplocaceae*, is deciduous stunned shrub or small tree endemic to China (Liu *et al.,* 2012). The fruits enriched unsaturated fatty acid in oil containing (Liu *et al*., 2016). Due to high fruit yield and oil content, *S. paniculata* serves as an ideal bio-diesel feedstock (Liu *et al.,* 2011) and edible oil plant (Guan *et al.,* 1991) like other whole fruit oil containing woody oil plant (Chen *et al*., 2018). In addition, *S. paniculata* has a high adaptability to different temperature zones and varying soil conditions, which grows well in barren, salty, and severe drought soil like marginal land and hyper arid areas. Because of its developed root system with large active absorption root surface and high tolerance of disease and insect, this species plays a very important role in maintaining ecosystem function and eliminating desertification and erosion (Guan, 1991). The phytochemicals from stem bark of *S. paniculata* has antimicrobial, analgesic, and anti-inflammatory activities and been used in India as traditional medicine for checking abortion (Ruchi *et al.,* 2011). Protein Tyrosine Phosphatase-1B (PTP1B) was also extracted from leaves and stems of this species and was suggested as a therapy for the type 2 diabetes and obesity treatment (Na *et al.,* 2006). The purpose of current study was to investigate the genetic diversity of six natural populations in Hunan province from five different regions.

Materials and Methods

Plant materials: Fresh young leaves of *Symplocos paniculata* were collected from six natural populations in five different regions of Hunan province, China (Table 1 and Fig. 1). The samples were immediately stored in liquid nitrogen and then transferred to -80ºC freezer in Botany Research Center of Central South University of Forestry & Technology, Changsha, China.

DNA extraction: According to the modified method of the Cetyl Trimethyl Ammonium Bromide (CTAB) (Nunes *et al.,* 2011), the total genomic DNA was extracted, and the gel electrophoresis and spectrophotometry were used for DNA quality and quantity assessment. DNA samples were diluted to 3% for PCRs, stored at -20ºC for PCR analysis.

Table 1. The geographic data of different *Symplocos paniculata* populations.

Population ID	Origins	Sample size	Elevatio (M)	Longitude (E)	Latitude (N)
DWSA	Da Wei Shan A , Liu Yang	21	1550	114°09′	28°25′
DWSB	Da Wei Shan B, Liu Yang	25	1263	114°08′	28°26′
XXLS	Long Shan, Xiang Xi	28	1402	109°10′	28°44′
HYHS	Heng Shan, Heng Yang,	26	650	112°42′	27°15′
YYJS	Jun Shan, Yue Yang,	30	80	113°06′	29°22′
YZDX	Dao Xian, Yong Zhou,	19	100	111°46′	25°24′

Table 2. Sequence and loci of selected ISSR markers.

Primer	Sequence (5′to 3′)	Optimum temperature (°C)	No. of bands	No. of polymorphic bands	Percentage of polymorphic bands (%)
UBC815	$(CT)_8G$	52	15	15	100
UBC817	$(CA)_8A$	51	14	12	85.7
UBC824	$(TC)_8G$	52	14	13	92.9
UBC835	$(AG)_8YC$	51	10	8	80
UBC845	$(CA)_8RC$	54	15	15	100
UBC853	$(TC)_8RG$	52	11	10	90.9
UBC862	$(AGC)_6$	50	13	12	92.3
UBC873	$(GACA)_4$	52	17	15	88.2
UBC879	$CTTCA)_3$	48	13	13	100
Mean			13.5	12.5	92.2

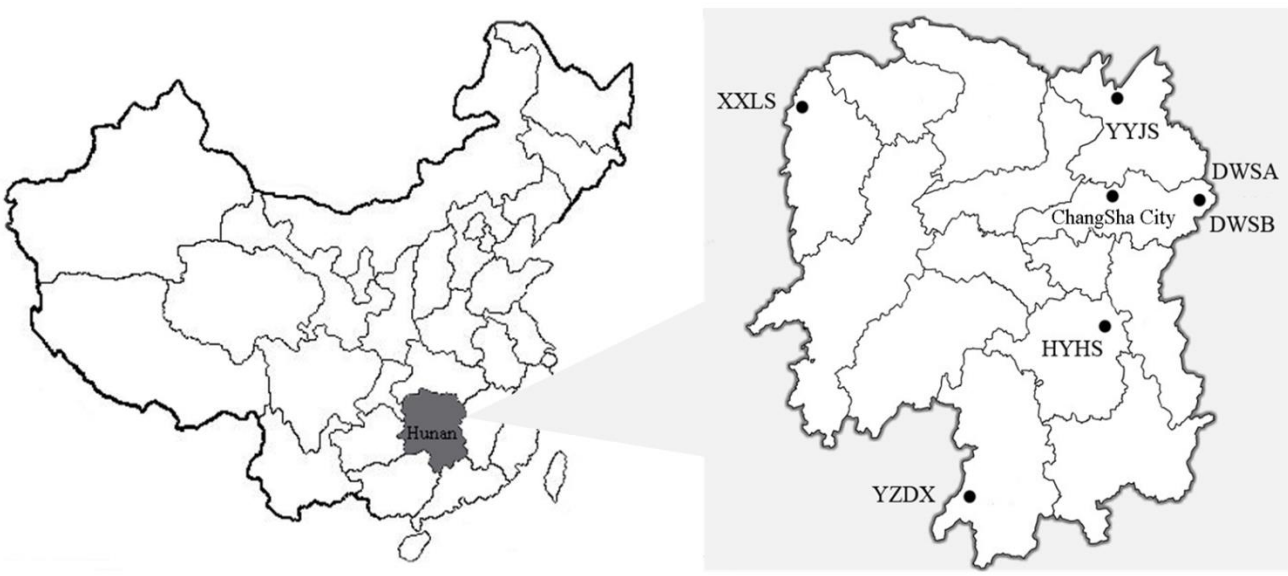

Fig. 1. The geographic distributions of six natural populations of *Symplocos paniculata* in Hunan, China.

Primers and ISSR amplification: Of 100 primers tested, 9 primers revealed polymorphism and exhibited good resolution (Table 2). The PCR mix (20 µL) for ISSR analysis contained 80 ng of *Symplocos paniculata* fruit genomic DNA, 10× Buffer (Mg^{2+} free) 2 µL, ddH_2O 10.9 µL, 3 mmol/L $MgCl_2$, 1.6µL (0.2 mmol/L) each dNTPs, 1 µL (0.5 umol/L) each primer, and 0.5 µL (1.25 U) Taq DNA polymerase (Takara Bio Group Co Ltd, Japan). Amplification reactions were performed in an AB2720 PCR amplification (Applied Biosystems Co Ltd, America). The PCR program was initial denaturation at 94°C for 4 min; and then followed by 30 cycles of 94°C for 30 s, annealing at 48°C~54°C for 40 s, and 72°C for 90 s, with a final extension at 72°C for 7 min (Spagnuolo *et al.,* 2009). After the PCR reaction, the samples were stored at 4°C.

Data analysis: The bands obtained from PCR reaction were standardized as a binary data matrix with number of 1 or 0 (1 = present, 0 = absent). The polymorphic markers were used to analyze Nei's genetic diversity using software PopGenes 32 (Yeh *et al.,* 1999) which based on the Simple Matching coefficient of similarity (Nei, 1973).

The genetic structure of the 149 individuals was analyzed using the application of "Structure 2.3.4" with Bayesian method. Markov Chain Monte Carlo (MCMC) algorithm was applied in the admixture model with

independent allele frequencies, and 10,000 steps were set in length of burn-in period at the beginning run, followed by 100,000 of MCMC replicates after burn-in.

A number of groups (k) were set as the most liable adjust table parameter, for each value of k (ranging from 2 to 10), twenty simulations were performed. The Δk is obtained from the standard deviation of L (K) (the log probability of the data [lnP(D)]) divided by mean of |L″(K)|,L'(K)=L(K)–L(K–1), |L″(K)|=|L'(K+1)–L'(K)|. The optimum Δk value was selected and estimated from the greatest Δk value. A graph for each replicate run was obtained, and different color in the graph for each replicate run represented a group of structured individuals (Evanno *et al.,* 2005; Claudinéia *et al.,* 2013).

Gene diversity analysis was performed using Analysis of Molecular Variance (AMOVA) at the different spatial scales in Arlequin software (version3.1) (Peakall *et al*., 2006). The total gene diversity (Ht), within-population (Hs) of polymorphic loci and the gene diversity of among populations (Dst) were calculated by the equation of Ht=Hs+Dst. Differentiation among populations (Gst) were obtained from the equation of Gst=Dst/Ht. Effective gene flow among populations was estimated using the equation Nm=0.5(1-Gst)/Gst (McDermott *et al.,* 1993).

The Nei's genetic distance was figured out and used for cluster analysis (Nei, 1978). Based on Unweighted Pair Group Method with Arithmetic means (UPGMA), dendrogram was generated using software of NTSYS pc (Version 2.02).

Results

The nine selected ISSR primers have been used to test the 149 individual plants from six populations and a total of 122 clear bands were generated, of which 113 were polymorphic. The total percentage of polymorphic bands was high to 92.2% (Table 2; Fig. 2). The number of bands per primer ranged from 10 to 17 with an average of 13.5. The size of DNA fragments produced were between 200 and 2500 bp. Primer UBC845, trinucleotide repeat $(CA)_8$ RC, produced the highest number of bands of 15 with the highest polymorphism rate of 100 %. PrimerUBC835, the dinucleotide repeat $(AG)_8$ YC, showed the lowest number of 10 and polymorphism rate of 80%.

The percentage of polymorphic bands (PPB) ranged from 31.0% (XXLS) to 64.6% (DWSA) at the population level, The total Shannon's information index was 0.4873 and ranged from 0.1633 (XXLS) to 0.3635 (DWSB) with an average of 0.2664 at the population level. Population of DWSB and DWSA had the highest percentage of polymorphic bands of 0.3635 and 0.3348, respectively, which indicated the greatest adaption to environment. The population of XXLS had the lowest genetic diversity of 0.1633 (Table 3). All gene frequency of 149 individuals were assumed independent. The highest ΔK value was generated by K=5 (Fig. 3A and B) with the most stable module value of LnP(D) indicating that all 149 genotypes could likely be divided into five structured groups. The genetic structure of all the individuals was presented in Figure 3C. The genetic exchanging among *S. paniculata* populations with the exception of the populations of DWSA and XXLS was difficult. The populations of DWSA and XXLS were not geographically close but performed abundant genetic exchanging.

As shown in the Table 4, Analysis of among population differentiation at the ISSR level was conducted. The coefficient of gene differentiation (Gst) was 0.5397 and Nm equaled to 0.4763<1. There were poor gene flows among populations. The percentage of genetic variability among the six groups (DWSA, DWSB, YZDX, HYHS, YYJS and XXLS) calculated by AMOVA showed that the among-population component accounted for 51.1% of the total variation, while the within-population component accounted for 48.9% (Table 5). The levels of genetic differentiation within-population were much lower than those among-populations, which indicated that the populations have diverged.

In our study, Nei's genetic distance among six populations ranged from 0.08811 to 0.65637. The highest genetic similarity was found between two populations of HYHS and YYJS, with a genetic distance of 0.65637 (Table 6). A UPGMA dendrogram was obtained using Nei's genetic coefficients of similarity. All populations were clustered into three groups (Fig. 4), the result showed that populations of DWSA, DWSB, and XXLS were in Group I, populations of YYJS and YZDX were in Group II, population of HYHS was in Group III which was separated from all the populations in the dendrogram. XXLS population was genetically closer to the DWSA population, but was not geographically close to DWSA population (Fig. 1). The results of cluster analysis were similar to those of genetic structure. Although populations of DWSA and DWSB were geographically close, they were not clustered. This suggested that the geographical distribution was not related to the cluster results.

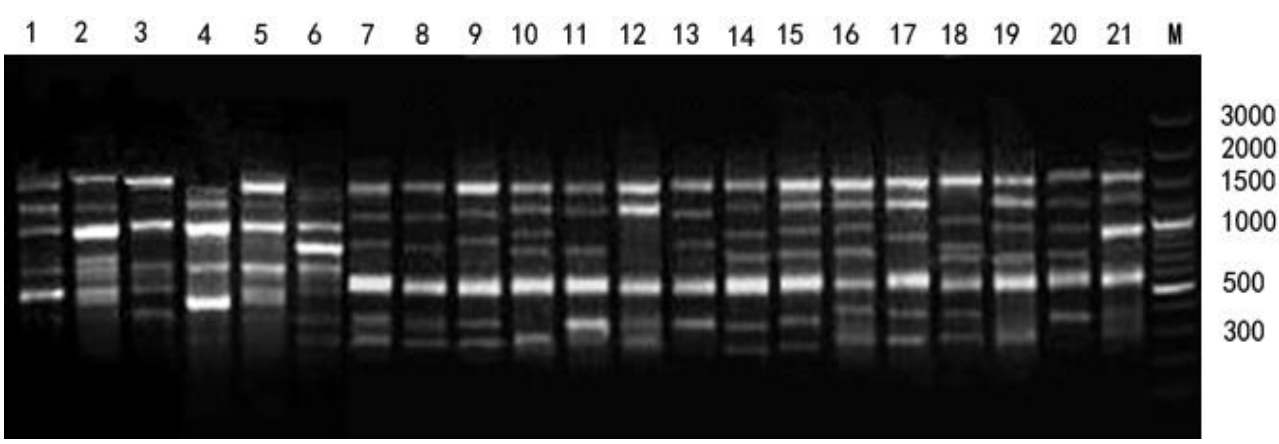

Fig. 2. The ISSR amplification map of the population of Dai Wei Shan A using primer UBC845.

Table 3. Genetic diversity within populations of *Symplocos paniculata*.

Population	No. of polymorphic bands	% of polymorphic bands	*Ae*	*Nei's* genetic diversity index (*He*)	*Shannon* information index *(I)*
DWSA	73	64.6	1.3841	0.2238	0.3348
DWSB	72	63.7	1.4400	0.2482	0.3635
XXLS	35	31.0	1.1960	0.1106	0.1633
HYHS	39	34.5	1.2665	0.1445	0.2079
YYJS	53	46.9	1.3327	0.1879	0.2745
YZDX	55	48.7	1.3040	0.1717	0.2544
Mean	54.5	48.2	1.3206	0.1809	0.2664
At population level	113	92.2	1.5685	0.3264	0.4873

Table 4. Coefficient of genetic differentiation for geographic population of *Symplocos paniculata*.

Primer	*Ht*	*Hs*	*Dst*	*Gst*	*Nm*
UBC815	0.3295	0.1899	0.1396	0.4237	0.6800
UBC817	0.3125	0.1502	0.1623	0.5194	0.4627
UBC824	0.3291	0.1298	0.1993	0.6055	0.3257
UBC835	0.3518	0.1964	0.1554	0.4417	0.6320
UBC845	0.3893	0.2304	0.1589	0.4081	0.7252
UBC853	0.3289	0.1814	0.1475	0.4484	0.6151
UBC862	0.2128	0.0463	0.1665	0.7824	0.1390
UBC873	0.2557	0.1193	0.1364	0.5334	0.4874
UBC879	0.2480	0.0757	0.1723	0.6947	0.2197
Mean	0.3064	0.1466	0.1598	0.5397	0.4763

Nm=estimate of gene flow from *Gst* or *Gcs;* E.g., *Nm*=0.5(1-*Gst*)/*Gst; Ht.* Gene diversity of species; *Hs.* Gene diversity within populations; *Gst*. Coefficient of gene differentiation; *Dst*. Gene diversity among populations

Table 5. Analysis of molecular variance (AMOVA) in *Symplocos paniculata* populations.

Source of variance	df	SS	Variance component	Total variation (%)	*P*-value
Among population	5	1291.236	11.1238	51.07	<0.001
Within population	143	1344.333	10.6576	48.93	<0.001
Total	148	2635.569	21.78141		

Table 6. Genetic distance among populations of *Symplocos paniculata*.

Population	DWSA	XXLS	HYHS	DWSB	YYJS	YZDX
DWSA	0.00000					
XXLS	0.08811	0.00000				
HYHS	0.56749	0.56457	0.00000			
DWAB	0.32892	0.45723	0.63191	0.00000		
YYJS	0.42669	0.41673	0.65637	0.56349	0.00000	
YZDX	0.46364	0.45022	0.61467	0.46529	0.44437	0.00000

Discussion

The abundance of polymorphic loci is one of the vital parameters to evaluate the genetic diversity (Jan *et al.,* 2017; Jan *et al.,* 2016; Khan *et al.,* 2016; Lin *et al.,* 2012; Rehman *et al.,* 2015; Shinwari *et al.,* 2013). Our study revealed a relatively high polymorphism in *S. paniculata*. The total proportion of polymorphic bands was as high as 92.2%, while the proportion of polymorphic bands ranged between 30.97% (XXLS) and 64.64% (DWSA) at the population level. The total Shannon's information index was high to 0.4873 and ranged between 0.1633 (XXLS) and 0.3635 (DWSB) at the population level, with an average value of 0.2664. Populations of DWSA and DWSB had a highest percentage of polymorphic bands, which indicated the greatest adaption to environment. *S. paniculata* is a widely distributed species extending from Russia to India. The high level of polymorphism observed in current study was consistent with previous comparable researches (Bi *et al.,* 2010; Xu *et al.,* 2013). Population of XXLS was in the Chinese-fir plantation, the habitat destruction and disturbance from human overexploitation might result in the decline of the population. Small populations are more vulnerable to stochastic events such as genetic drift and it further reduces genetic variability (Stefano *et al.,* 2012).

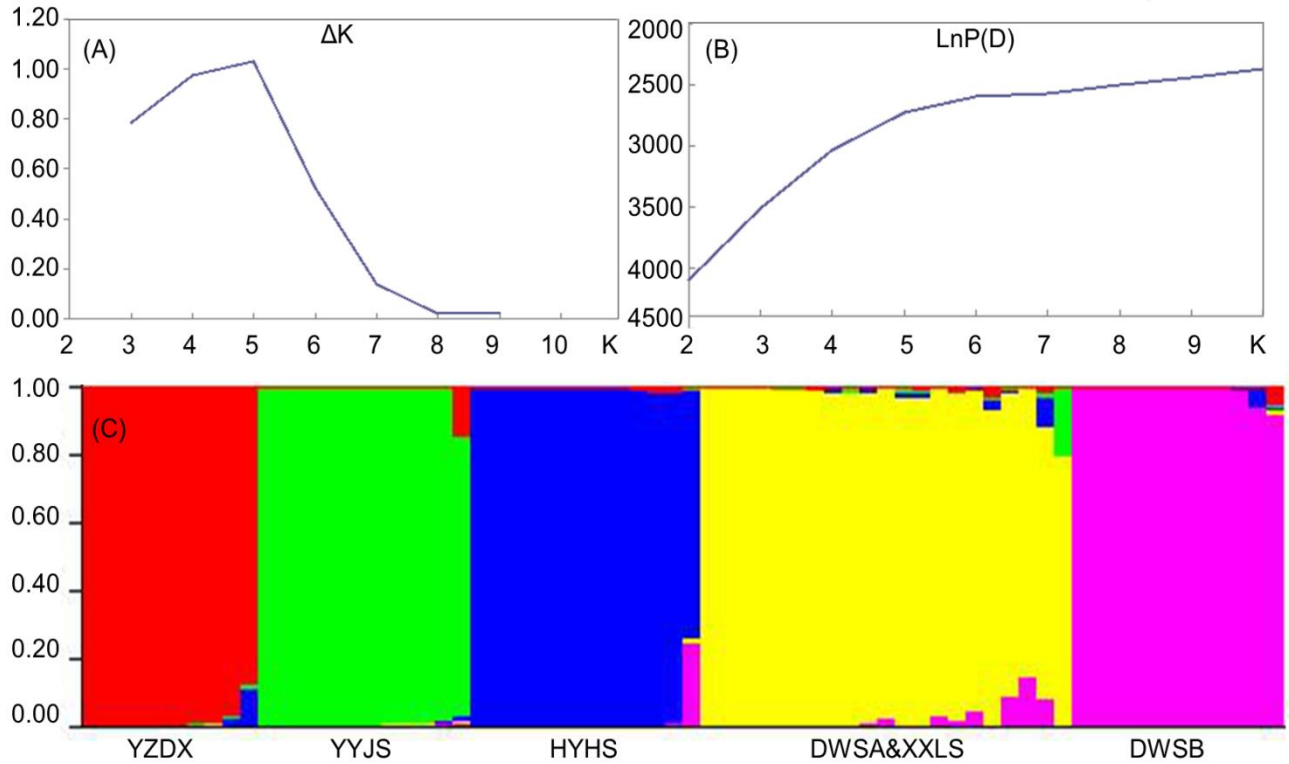

Fig. 3. The genetic structure of *Symplocos paniculata* populations. A: Plot of the Δ*K* on the number of population genetic clusters; B: Plot of mean posterior probability *lnP(D)* values; C: the genetic structure of 149 *Symplocos paniculata* individuals.

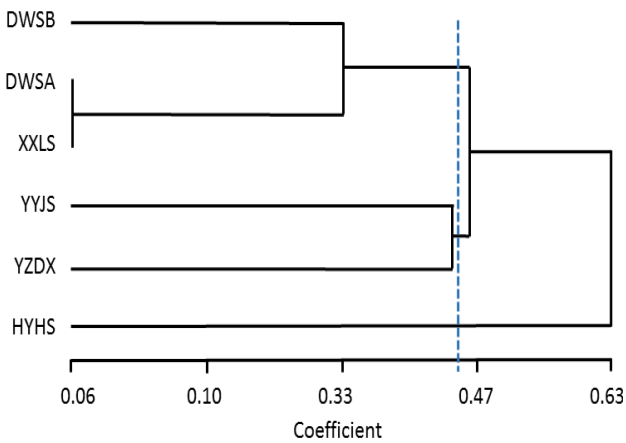

Fig. 4. UPGMA tree based on the genetic similarity coefficients of six natural *Symplocos paniculata* populations.

The gene flow among populations is one of vital evolutionary factors greatly affected the genetic diversity in a species (Nybom *et al.,* 2000; Rabbani *et al.,* 2010; Shah *et al.,* 2015; Turi *et al.,* 2012). A high gene flow among populations counteract genetic drift and genetic differentiation and plays an important role in maintaining a random and panmictic population structure. When Nm<1, the effects of genetic drift is greater than gene flow ultimately results in genetic differentiation in present population, and moderate value of gene flow could not make a great contribution to stability, but also adaptability of natural populations to environment (Irwin *et al.,* 2005).

Analysis of molecular variance (AMOVA) demonstrated that the genetic differentiation among populations was relatively high. Nm=0.4763<1, as there were poor gene flows among populations. Similar results were reported in other comparable studies (Juliana *et al.,* 2012, Li *et al.,* 2011). The Geographical isolation due to the separate distribution of collected site, heterogenous habitats and the elevation differences made habitat fragmentation, high level of inbreeding, random genetic drift and limited gene flow, cause the distinct genetic variation among-population (Cozzolino *et al.,* 2003).

A dendrogram was obtained by the UPGMA cluster analysis according to Nei's genetic coefficients of similarity, all natural populations were clustered into 3 groups, the results showed that populations of DWSA, DWSB and XXLS were in the first group (Group I), the first subgroup included DWSA and XXLS populations, YYJS and YZDX populations were in another group (Group II), HYHS population separated from all the populations in the dendrogram (Group III), These results were similar to the analysis of genetic structure.

The genetic diversity mostly was not correlated to geographical distance or climate type, but was mutually related to the geographical elevation, which has been reported by many previous studies (Fan *et al*., 2014; Hamick *et al*., 1989; Michalski *et al*., 2010). Therefore, selection pressure of the altitudinal gradient became the main factors of genetic differentiation, which changed physical characteristics of *S. paniculata* species, temperature difference caused florescence difference, which limited pollen spread and random mating among populations. Effects of bird seed dispersal of seeds and human activity causes less genetic variation in large genetic distance at the similar altitudinal gradient that could explain the long-distance dispersal between populations of XXLS and DWSA (Yang *et al.,* 2011).

Conclusion

The results of genetic diversity indicate that the germplasm resources of *S. paniculata* species may be suffering gene erosion in Hunan province, population of XXLS is most likely endangered due to the human activities.

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