Pak. J. Bot., 52(6): 2087-2093, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-6(26)

GENETIC DIVERSITY AND STRUCTURE OF *SINOPODOPHYLLUM HEXANDRUM* POPULATIONS IN THE TIBETAN REGION OF QINGHAI-TIBET PLATEAU, CHINA

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Abstract

Sinopodophyllum hexandrum is a perennial herb with medicinal value for local Tibetans living in the Qinghai-Tibet Plateau (QTP), China. The species has been classified as endangered in the Chinese Plant Red Book because of the decline of wild resources and lack of artificial cultivation. For the conservation of resources in the Tibetan region of QTP, the genetic diversity and structure of *S. hexandrum* distributed in this area were investigated based on nuclear microsatellite molecular markers. All populations showed low genetic diversity. Genetic differentiation occurred mainly among populations JCX, MTS, and 10 others on Tibetan region of the QTP. Such genetic differentiation pattern might have been due to geographic isolation and lack of pollination in QTP. The strategies for conservation based on these results are suggested.

Key words: *Sinopodophyllum hexandrum*, Himalayan mayapple, Genetic diversity, Genetic structure.

Introduction

Sinopodophyllum hexandrum (Royle) Ying, also known as *S. emodi* (Wall) Ying, *Podophyflum hexandrum* (Royle) Ying and *P. emodi* (Wall) Ying. It is commonly called Himalayan mayapple, and is a perennial medicinal herb. It belongs to the family Berberidaceae, and bears beautiful pink flowers and red fruits (Figs. 1A and 1B), and is mainly distributed in the plateau region of the Himalayas, which has an elevation of 2,700-4,500 meters above sea level (masl) in China, and neighboring regions of Bhutan, Nepal, India and Pakistan. In China, this wild resource is mainly distributed in the Tibet region of Qinghai-Tibet Plateau (QTP) (Li & Guo, 2016). It is also found in the Qinling Mountains which possesses a lower elevation of 1300-2800 masl to the east of Northwest China (Liu *et al*., 2016).

The roots of *S. hexandrum* contain lignans with anticancer activity, among which podophyllotoxin has the highest anticancer activity (Li *et al*., 2018a; Liu *et al*., 2015). The unique natural environment of the QTP, i.e., with scarce oxygen and abundant ultraviolet, the accumulation of podophyllotoxin is increased at higher altitude compared with the other regions (Li *et al*., 2018b). As a traditional Tibetan medicine, the roots and fruits are used in the treatment of diseases for a long time by local people. At present, the medicinal value of *S. hexandrum* is widely valued, but it has not been artificially cultivated on a large-scale. Medicinal materials are mainly obtained from the wild plants, especially from the roots and fruits. The fresh of the fruit is delicious, thereby, attracting people and animals (Fig. 1). Thus, its natural ability to reproduce is weakened.

Great disturbance affects *S. hexandrum* population reproduction, with no effective protection measures, thereby resulting in serious decline in resources and risking species extinction. It has been classified as an endangered species (grade 3) in the Chinese Plant Red Book (Fu, 1992) and is listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Lata *et al*., 2010).

The great value of the plant and current resource situation in the world has attracted research attention. The evaluation of genetic diversity and structure among natural population will provide scientific basis for the conservation of wild resources, especially for endangered species. Therefore, the genetic diversity of *S. hexandrum* populations in Northern India and in the Qinling mountains of Shaanxi Province, Tibetan region of Sichuan Province, and the Himalaya-Hengduan Mountains of China was evaluated based on different molecular markers such as RAPD, ISSR, AFLP, and chloroplast DNA sequences (Alam *et al*., 2008; Li *et al*., 2011; Liu *et al*., 2014; Liu *et al*., 2016; Naik *et al*., 2010; Xiao *et al*., 2006a; Xiao *et al*., 2006b). However, research that may provide a theoretical basis of the wild resource conservation, i.e., research on the distribution, genetic diversity and structure of *S. hexandrum*, is still lacking in the Tibetan region of QTP, where species distribution is the highest.

Nuclear SSR markers are widely used in population genetic analysis because of they cover the whole genome (Wang *et al*., 2020), and possess abundant quantity, high polymorphism and co-dominance (Govindaraj *et al*., 2015; Xue *et al*., 2018). To provide a theoretical reference for the effective conservation and rational utilization of *S. hexandrum* population in this region of China, investigated the genetic diversity and structure in different *S. hexandrum* populations by using nuclear SSR markers.

Materials and Methods

Plant materials: Leaf samples were obtained from 12 natural populations in the Tibetan region of QTP. Sampled individuals were placed more than 50 m apart, and leaf samples were collected and immediately placed in labeled bags with dried silicon. The geographic distribution of the populations is shown in Fig. 1C.

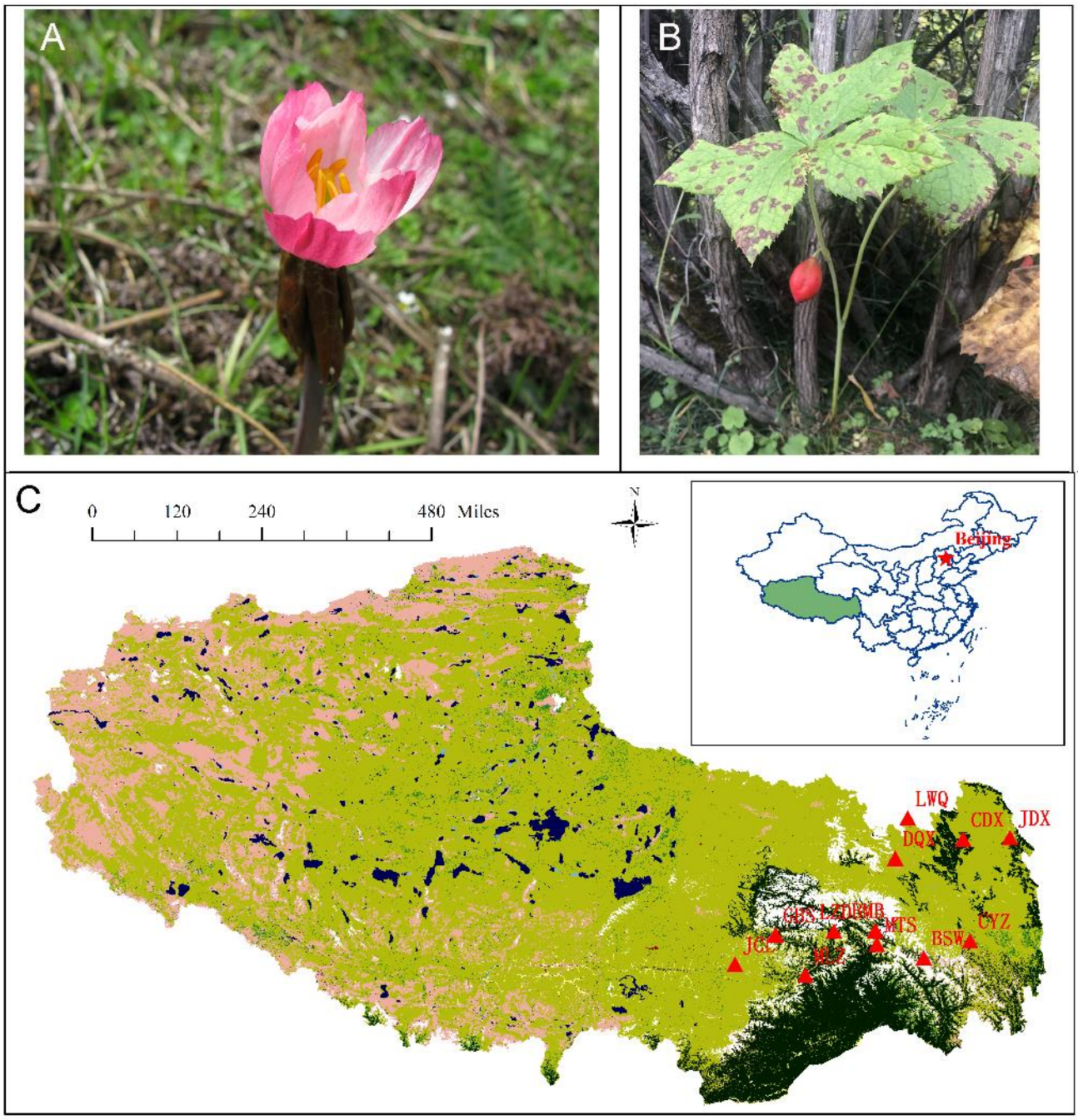

Fig. 1. Plant population in the habitats and geographical distribution of *S. hexandrum* in QTP. A. flowering plant; B. fruiting plant; C. geographic distribution of 12 sampling populations in the Tibetan region of QTP.

DNA isolation and the development of polymorphic SSR markers: DNA was isolated from leaves by using modified CTAB method. Each DNA sample was verified by running agarose gel, and the concentration and purity was detected using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Twenty-five individuals from 12 locations were randomly sampled for developing polymorphic SSR markers. Part of the screened SSR markers were obtained from published makers of *S. hexandrum* and *Dysosma* species from family Berberidaceae (Guan *et al*., 2008; Nag *et al*., 2013; Guo *et al*., 2014; Mao *et al*., 2016). The other markers were newly designed from 160 SSR loci from the partial genomic sequences of *S. hexandrum* (Li *et al*., 2014). For the developed markers, PCR was performed in 20 µL mixtures, including 2×Taq buffer, 0.1 µM forward and reverse primers (Shenggong, China), 1 Unit HotsrarTaq polymerase (Qiagen, China) and 1 µL of template DNA. The PCR products were separated using polyacrylamide gel and silver staining, and the alleles were analyzed manually.

SSR genotyping: PCR was performed using SSR primers labeled with FAM-fluorescence. The PCR products were analyzed on Analyzer ABI 3730xl (Applied Biosystems, USA), and the alleles were identified using GeneMapper 4.0 (Applied Biosystems, UK).

Data analysis: PowerMarker v3.0 (Liu & Muse, 2005) was used to evaluate the parameters of each locus: the number of alleles (N_a), the number of effective alleles (N_e), the observed heterozygosities (H_o), expected heterozygosities (H_e), deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium, and p value of last two parameters was further controlled by false discovery rate procedure of Moran (Moran, 2003).

PopGen3.2 (Yeh *et al*., 1997) was used to calculate the population genetic diversity parameters including N_a, N_e, H_o, H_e, Shannon's information index (*I*), *Nei*'s gene diversity (*H*) and fixation indices (F_{st}). GenAlEx v6.5 (Peakall & Smouse 2006; Peakall & Smouse 2012) was used to estimate the population genetic differentiation parameters including: genetic distance and identity, molecular variance (AMOVA), principal coordinates analysis (PCoA, 999 permutations for calculating) and Mantel test (999 permutations for calculating). Based on the calculated genetic distance, the PCoA plot was generated using Sigmaplot 10.0 (Systat, USA), and the UPGMA tree was generated based on the population *Nei*'s genetic distance by using MEGA X (Kumar *et al*., 2018). STRUCTURE v2.3.4 (Pritchard *et al*., 2000) was used to cluster the population. The *K* values were set from 1-13 and repeat 20 times for each, and the running bum-in and Markov Chain Monte Carlo were set to 50,000 and 100,000 times, respectively. Delta*K* corresponding to each *K* value was calculated, and the *K* value for the optimal group number was determined according to the method of Evanno (Evanno *et al*., 2005).

Results and Discussion

Development of polymorphism SSR markers: After the strict control of the analysis, 10 polymorphic unlinked loci in HWE were selected for further genotyping (Table 1). Among them, seven markers (marker 1-7) were selected from 74 published SSR markersof *Dysosma* species (Guan *et al*., 2008; Nag *et al*., 2013; Guo *et al*., 2014; Mao *et al*., 2016), whereas three markers (marker 8-10) were newly developed from 160 SSR loci from the partial genomic sequences of *S. hexandrum*. However, the 20 reported polymorphic SSR markers developed from *S. hexandrum* distributed in Indian (Nag *et al*., 2013) did not show polymorphism in the samples distributed in the Tibet regions of QTP, probably due to genetic differentiation in different areas or other unknown reasons. Interestingly, some of the reported polymorphic SSR loci developed from *Dysosma* species of family Berberidaceae (Guan *et al*., 2008; Guo *et al*., 2014; Mao *et al*., 2016) showed polymorphism in *S. hexandrum*, thereby indicating their good transferability across the genera.

Overall, 48 alleles were detected based on 25 individuals by using 10 markers, and the number of alleles varied between 3 (DVA5802, DVA3866 and DVA3931) and 9 (EDV-40) with an average of 4.8 alleles per locus. H_o and H_e ranged from 0.366 (DVA5802) to 0.736 (STR18) and from 0.2 (DVA5802, DVA3866 and DVA3931) to 0.92 (STR18), respectively. Marker STR18 had the highest value. This finding suggested that the markers could be used in further genotyping.

Genetic diversity among populations: In total, 53 alleles were obtained in 267 samples by using 10 selected polymorphic SSR markers, at an average of 5.3 for each. The average N_a of each population varied from 2.1 (CDX and LWQ) to 3.2 (MLZ) (Table 2). The average N_e varied from 1.333 (BMB) to 1.653 (MLZ). The average *I* of each population varied from 0.330 (BMB) to 0.572 (MLZ). The mean H_o and H_e for all populations were 0.257 and 0.483, respectively. The H_o values of each population ranged from 0.180 (JCL) to 0.355 (MTS), whereas the H_e values ranged from 0.180 (BMB) to 0.333(JDX and LWQ). The average *H* was 0.482 and ranged from 0.177 (BMB) to 0.326 (JDX), thereby suggesting a low level of genetic diversity for all populations ($p<0.5$), especially in population BMB (Table 2). The low genetic diversity may be due to the reasons of lack of insect pollination and geographic isolation in QTP regions.

Although the H_e values of *S. hexandrum* in Tibet are low, these values are still much higher than those in the populations distributed in the Qinling Mountain (0.0226-0.1229) (Liu *et al*., 2014) and other areas in China (0.0141-0.0963) (Liu *et al*., 2016) as determined by AFLP and ISSR markers, respectively. These findings probably resulted from the calculation of H_e values, which was based on different molecular maker methods and the different *S. hexandrum* distributions.

The results of *Nei*'s genetic distance showed that population JDX and CDX had the lowest genetic distance of 0.004) (Table 3), population MTS and CYZ had the highest at 2.138. Moreover, the analysis of *Nei*'s genetic identity showed that populations MTS and CYZ had the lowest genetic identity at 0.118, whereas populations JDX and BSW had the highest at 0.993. Thus the genetic divergence between populations MTS and CYZ was higher than that between any other two populations. Moreover, populations JDX and BSW were closely related (Table 3) and possibly have a recent common ancestor.

Population genetic differentiation and structure: UPGMA clustering analysis was performed based on *Nei's* genetic distance (Table 3). Twelve populations were clearly clustered into 3 groups (Fig. 2A). Ten populations, except JCX and MTS, were clustered into a big group. JCX and MTS were clustered into separate groups. Similarly, the PCoA analysis showed that the samples were scattered into three groups, one big group and two small groups (Fig. 2B). The two small groups were mainly composed of samples from populations JCX and MTS. The big group was mainly composed of samples from the other 10 populations. This result was consistent with that of UPGMA. Furthermore, the genetic structure analysis showed that the peak point of the estimated Ln probability of data [Ln *P* (*D*)] was obtained when $K = 3$, thereby suggesting an optimal group number of three. Among the three groups, the big group consisted of 10 populations, whereas the two small groups consisted of populations JCX and MTS (Fig. 3). The results of UPGMA clustering, PCoA analysis, and genetic structure consistently and clearly supported the genetic differentiation among populations JCX, MTS and the other 10 populations on the Tibetan region of QTP.

Table 1. Characterization of 10 polymorphic microsatellite loci in *Sinopodophyllum hexandrum* (*n*=25).

Locus	Primer sequences (5'-3')	Repeat motif	T_A(°C)	Size range (bp)	N_A	H_o	H_e	*P* (Seq-Bon)	Source
EDV-30	F: CTGGATTCTTCACAGACCAAGAC R: GTGACCGTCTTTCCATTCTATCA	$(GAA)_7$	60	132-152	4	0.430	0.280	0.039(0.017)	Guo *et al*., 2014
EDV-40	F: GTCGTAAGATAAGCGATTTCTGC R: TTGCAGCTGTATTCATCATCAAC	$(GGAT)_5$	60	121-149	9	0.632	0.440	0.036(0.013)	Guo *et al*., 2014
DVA5806	F: AGGACTTGAGGAGAGATGCTGAT R: GAGGAGATGAAGAATGTGTGAGG	$(TC)_6/(TC)_6$	60.45	115-145	5	0.405	0.280	0.017(0.007)	Mao *et al*., 2016
DVA5802	F: GAGAGGACGGTTAGGTTTTGATT R: AAACCCCACTGCTCTCTTTTATT	$(AG)_6/(AG)_8$	59.7	121-129	3	0.366	0.200	0.017(0.006)	Mao *et al*., 2016
DVA3866	F: ACTTCCCAATGAAGAAGAAAACC R: ACTTTGGATGATTCTTCAAACCA	$(GAA)_5/(GAA)_5$	59.8	124-136	3	0.367	0.200	0.011(0.005)	Mao *et al*., 2016
DVA3931	F: AGTTGAATCCGGAAACCAGTATT R: CATACAACTAACCGCAAACCTCT	$(AGG)_7/(AGG)_7$	59.4	157-183	3	0.367	0.200	0.015(0.006)	Mao *et al*., 2016
DVA4242	F: TAGGGCTGACAGAGTGAGAGAAG R: CAATTGATTTTTCACTTCCGTTC	$(AGC)_5/(AGC)_5$	60	129-137	4	0.666	0.520	0.02(0.009)	Mao *et al*., 2016
Sh72	F: CCACAAACATAACATGCAAAC R: CGATACCATCCTTATGGTAG	$(CTG)_5$	61	139-187	7	0.632	0.680	0.574(0.050)	KR094979*
STR13	F: TCAGTGCGCCTTCTTTTAGTTG R:TGGACTATATGATGAAAATCCCACTC	$(CT)_{16}$	61	141-149	4	0.631	0.440	0.031(0.010)	MH998518*
STR18	F: GGCGCTTGAAGATGCACTAGA R: AAAATGCAACCACCCTCACAC	$(GA)_{12}$	61	102-136	6	0.736	0.920	0.048(0.025)	MH998519*

T_A annealing temperature (^{O}C); N_A number of alleles within the examined population; H_o observed heterozygosity; H_e expected heterozygosity; *P*exact *P*-value for Hardy -Weinberg equilibrium (HWE) test; *SB*, threshold from the false discovery rate (FDR) controlling procedure of Moran (2003); *n* number of individuals assayed. "*" indicate the Genbank accession number

Table 2. Details of genetic diversity varied among *Sinopodophyllum hexandrum* populations in the Tibetan region of QTP.

Population Abbreviation	Population location	Sample size	Latitude	Longitude	Altitude (m)	N_a	N_e	*I*	H_o	H_e	*H*
LZD	Lizhi county	25	29°57′37″	94°46′45″	3673	2.900	1.522	0.498	0.224	0.289	0.283
MLZ	Milin county	25	29°11'08″	94°11′46″	2953	3.200	1.635	0.572	0.236	0.328	0.322
GBS	Gongbujiangda county	25	29°53′48″	93°34′57″	3306	3.100	1.425	0.457	0.220	0.250	0.237
JCL	Jiacha county	25	29°22′39″	92°45′13″	3854	2.800	1.490	0.516	0.180	0.296	0.290
BMB	Bomi county	25	29°58′28″	95°37′35″	2715	2.600	1.333	0.330	0.236	0.180	0.177
MTS	Motuo county	20	29°43′44″	95°39′23″	3295	2.800	1.581	0.548	0.355	0.318	0.310
CYZ	Chayu county	25	28°47′31″	97°33′47″	3308	3.000	1.456	0.451	0.280	0.240	0.235
CDX	Changdu county	25	31°33′36″	97°25′25″	3725	2.100	1.512	0.480	0.260	0.308	0.301
BSW	Basu county	25	29°29′31″	96°37′03″	3815	2.200	1.481	0.467	0.252	0.290	0.284
JDX	Jiangda county	25	31°35′17″	98°22′38″	3261	2.300	1.551	0.528	0.324	0.333	0.326
LWQ	Leiwuqi county	9	31°55'30"	96°17'05"	3979	2.100	1.515	0.499	0.311	0.333	0.315
DQX	Dingqing county	13	31°13'49"	96°01'58"	3932	2.200	1.469	0.443	0.269	0.277	0.266

H_O observed heterozygosity; H_e expected heterozygosity; N_a the number of alleles, N_e the number of effective alleles, H_o the observed heterozygosities, expected (H_e) heterozygosities, *I* Shannon's information index, *H Nei*'s *gene diversity*.

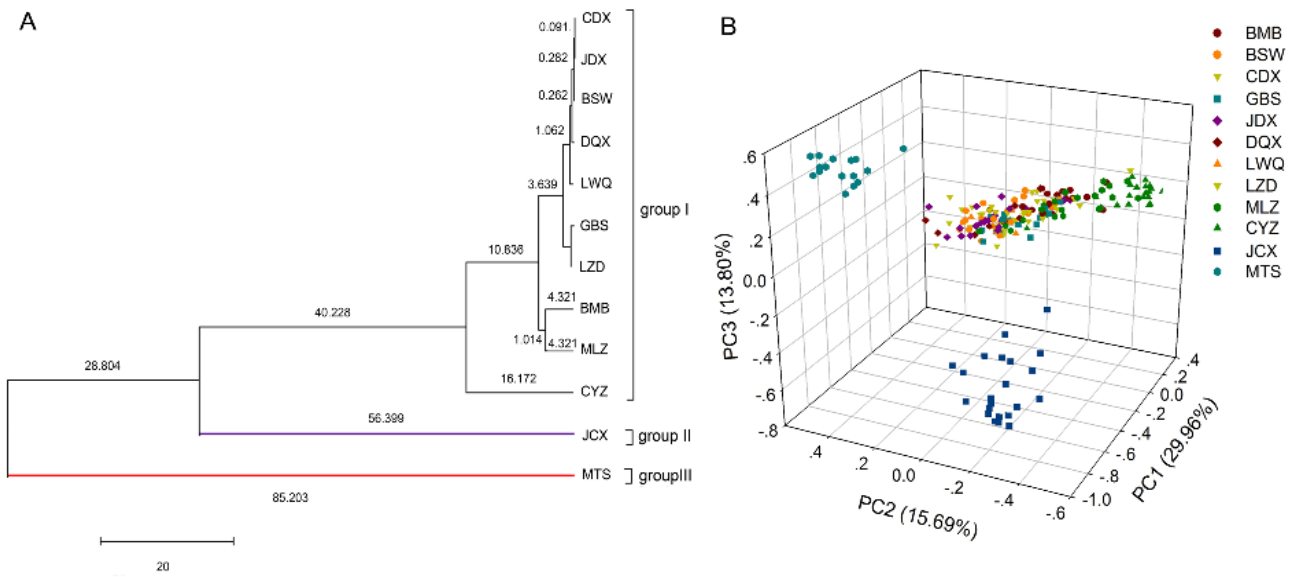

Fig. 2. UPGMA clustering illustrates the phylogenetic relationship and the scatter plot obtained from the principal coordinate analysis of a genetic distance matrix derived from 267 individuals of 12 *S. hexandrum* populations from the Tibetan region of QTP.

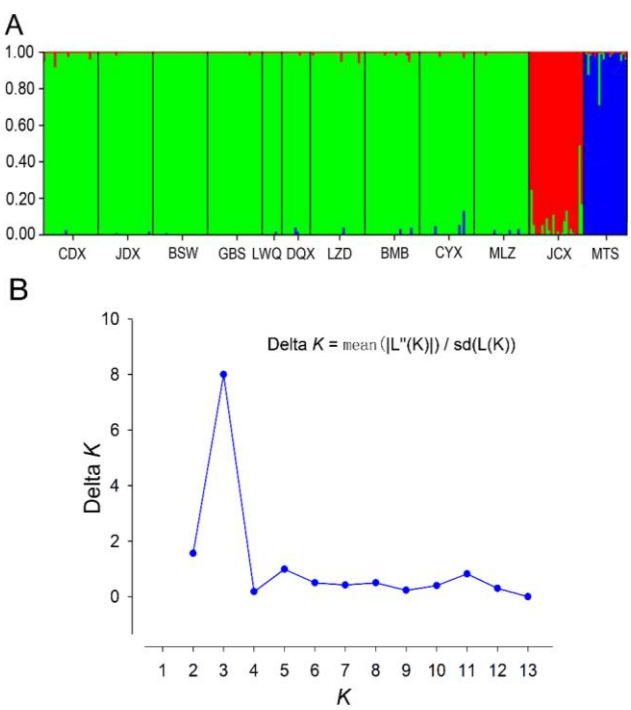

Fig. 3. Genetic structure of the 12 *S. hexandrum* populations from the Tibetan region of QTP.

AMOVA analysis showed the genetic variation mainly existed within populations (74%) and was higher than the genetic variation among populations (26%) (R_{st}=0.336, p<0.001), thereby suggesting that molecular variation mainly existed between populations. The average number of migrants per generation (gene flow, N_m) among populations was 0.494. A low N_m (<1) value suggested that limited gene flow increased the genetic differentiation among populations, thereby increasing the genetic variation.

The QTP is generally between 3,000 and 5,000 m above sea level, with an average elevation of more than 4,000 m. Many mountains and peaks exist in the QTP, and some of them are more than 6000 m high. *S. hexandrum* populations in this region are separately distributed. The low genetic diversity and gene flow, together with the high genetic population differentiation may due to the geographic isolation and lack of pollination. Furthermore, population MTS was located in the southern slopes of the eastern Himalayan and Gangzhigabo mountains. Population JCX is located in the valley of Kailas Range-Nyenchen Tanglha and the Himalayan Mountains. These two populations were isolated severely by mountains that are over 6000 m from each other and from other populations. As a natural barrier, the Himalayan Mountains separate the different populations of *S. hexandrum*. The climate and water supply of the southern and northern sides of the mountain range greatly differ. Barrier isolation limits the genetic exchange among populations, and different ecological environments may eventually result in the genetic differentiation of *S. hexandrum* in the Tibetan region of QTP. Geographic differentiation also occurs among populations of other species distributed in the QTP, such as *Incarvillea sinensisas* (Chen *et al*., 2012), *Allium przewalskianum* (Liang *et al*., 2015), 'Zangli' pear (Xue *et al*., 2017) and *Cupressus chengiana* (Xu *et al*., 2017).

Geographic and genetic correlations: No significant correlation was detected between the pairwise *F*st values and geographic distances among the 12 populations (r^2=0.0513, P=0.119), similarly, no significant correlation was detected between the pairwise genetic distance and geographic distances among the populations of *S. hexandrum* in QTP (r^2=0.0487, P=0.170) (detailed data not shown). This finding may be due to the topography and climate changes caused by the uplift of the QTP. Many irregular valleys and natural barriers are formed. Ina relatively small-scale region, these barriers eventually lead to the lack of relationship between the genetic parameters of the *S. hexandrum* populations and their geographic distances from one another.

Table 3. The pairwise Nei's genetic distance (up) and Nei's genetic identity (bottom) among the *Sinopodophyllum hexandrum* populations in the Tibetan region of QTP.

Population	BMB	BSW	CDX	CYZ	GBS	JCX	JDX	JQX	LWQ	LZD	MLZ	MTS
BMB		0.132	0.125	0.237	0.102	1.205	0.123	0.141	0.147	0.054	0.090	1.784
BSW	0.876		0.006	0.376	0.029	1.195	0.007	0.013	0.018	0.040	0.123	1.688
CDX	0.883	0.994		0.377	0.038	1.205	0.004	0.014	0.027	0.046	0.127	1.594
CYZ	0.789	0.687	0.686		0.345	1.158	0.376	0.413	0.395	0.262	0.167	2.138
GBS	0.903	0.972	0.963	0.708		1.039	0.027	0.035	0.039	0.015	0.072	1.679
JCX	0.300	0.303	0.300	0.314	0.354		1.132	1.121	1.128	1.070	1.072	1.753
JDX	0.884	0.993	0.996	0.686	0.973	0.322		0.013	0.016	0.036	0.112	1.556
JQX	0.869	0.987	0.986	0.662	0.966	0.326	0.987		0.025	0.052	0.131	1.544
LWQ	0.863	0.982	0.973	0.674	0.962	0.324	0.984	0.975		0.049	0.123	1.668
LZD	0.948	0.961	0.955	0.770	0.985	0.343	0.965	0.949	0.952		0.048	1.728
MLZ	0.914	0.884	0.881	0.846	0.931	0.342	0.894	0.877	0.884	0.953		1.672
MTS	0.168	0.185	0.203	0.118	0.187	0.173	0.211	0.214	0.189	0.178	0.188	

Conservation

Proper management will effectively conserve the resources, and secure the future with rational development and utilization. Due to the effective medicinal value of *S. hexandrum*, the wild resource quantity decreases daily. To conserve this valuable resource in the Tibet region of QTP, the following strategies are suggested: (1) Populations JDX and MLZ should be prioritized for conservation because of their relatively high genetic diversity, and the storage library should be established accordingly. (2) A nature reserve should be established in the relatively concentrated distribution area such as the sampled locations, to protect the wild resources from exploitation.

Acknowledgements

The authors would like to thank Dr. Longshan Zhao (College of Forestry, Guizhou University, China) for generating the distribution map. This research was supported by the National Natural Science Foundation of China (31460079) , Technological Projects of Guizhou Province, China (20163022-06), and the construction project for first-class ecology discipline in Guizhou Province, China (GNYL [2017] 007).

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(Received for publication 19 December 2018)