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Full Length Research Paper

Genetic diversity and population structure in *Meconopsis quintuplinervia* (Papaveraceae)

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Meconopsis quintuplinervia **is regarded as a valuable medicinal plant in Tibetan medicinal system. This species is distributed in Qinghai, Xizang, Sichuan, Shanxi ,Gansu and Hubei provinces of the People's Republic of China. Genetic variation of 16 *M. quintuplinervia* populations sampled from Qinghai and Gansu of China was examined by random amplified polymorphic DNA markers (RAPDs). In total, 225 scored DNA bands were amplified from the 17 primers used. Of the 225 loci, 192(85.33%) were polymorphic, and total genetic diversity (Ht) was 0.2954 and Shannon's information index (I) was0.4371, suggesting a relatively high rate of genetic variation at the species level. The average within-population diversity also appeared to be high, with PPB, He and I values of 70.50%, 0.2408 and 0.3347, respectively. Analysis of molecular variance (AMOVA) revealed 78.3% of variation within populations and only 21.7% between populations. Nei's coefficient of differentiation (G_{ST}) was found to be high (0.2320), also confirming the relatively high level of genetic differentiation within populations. By UPGMA cluster analysis, based on Nei's standard genetic distance, the populations were divided into three groups including the populations distributed in same location together in every group. The results exhibit a strong genetic differentiation which is obviously due to genetic drift in the isolated populations. The genetic structure of *M. quintuplinervia* has probably been shaped by its breeding modes, biogeographic history and human impact (both grazing and collection for medicinal purposes). This research might be an efficient way to conserve genetic resources of the medicinal plant, in addition to its effective uses.**

Key words: *Meconopsis quintuplinervia* Regel, genetic diversity, random amplified polymorphic DNA markers, the Qinghai-Xizang plateau, conservation.

INTRODUCTION

Meconopsis quintuplinervia Regel, a plant belonging to the Papaveraceae family and distributed in the northwest of China, is one of the most important Tibetan medicinal materials in Meconopsis (Luo et al., 1984). Based on early collections and sightings, *M. quintuplinervia* is mostly distributed on Qinghai-Xizang plateau (S Gansu, W Sichuan, SE Qinghai and E Xizang) of the northwest of China in altitudes from 1800 to 4600 m, growing commonly in low scrub (dominated by *Potentilla fruticosa, Spiraea alpine* Pall., *Salix oritrepha*, *Sibiraea angustata*, *Caragana* spp. and *Rhododendron* spp) and mountain meadows and the edge of forests (dominated by *Picea* spp. and *Sabina przewalskii Kom*) (Luo et al., 1984). This species is used as a traditional Tibetan medicine for treatments of various diseases, such as inflammation, pain, hepatitis and tuberculosis (Shang et al., 2006). There are 14% prescriptions that use *M. quintuplinervia* in 200 traditional Tibetan medicine prescriptions which are formally approved by the Chinese government (Peng, 2007). Because of its medicinal importance (associated

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Abbreviations: PPB, Percentage of polymorphic loci; **Na,** number of alleles; **Ne,** effective number of alleles; **He,** expected heterozygosity; **Ht,** total genetic diversity; **Hs,** genetic diversity within populations; **I,** Shannon' s information indices; **Nm,** gene flow; **POP,** population.

Table 1. Locations of the sampled populations' information.

Populations	Collection site	Latitude	Longitude	Altitude (m)	Symble
Pop1	Banma county , Qinghai	32°47.823′	100°33.458′	4574	Banma-1
Pop2	Banma county , Qinghai	32°48.977′	100°34.640′	4130	Banma-2
Pop3	Banma county , Qinghai	32°49.512′	100°36.198′	3976	Banma-3
Pop4	Banma county, Qinghai	32°50.583′	100°37.827′	3940	Banma-4
Pop5	Dari county in Qinghai	33°16.688′	100°24.261′	4225	Dari
Pop6	Gande county, Qinghai	33°48.488′	99°48.986′	4014	Gande
Pop7	Maqin county, Qinghai	34°29.977′	100°24.535′	3997	Maqin
Pop8	Guide county, Qinghai	35°0.120′	100°50.381′	3945	Guide
Pop9	Xunhua county, Qinghai	35°34.368′	102°44.458′	3587	Xunhua
Pop10	Pingan county, Qinghai	36°16.714′	101°58.114′	3224	Ping'an
Pop11	Huangzhong, county, Qinghai	36°21.66′	101°26.744′	3804	Huangzhong
Pop12	Huzhu county, Qinghai	37°00.427′	102°8.378′	3126	Huzhu
Pop13	Menyuan county, Qinghai	37°17.026′	101°48.278′	2915	Menyuan-1
Pop14	Menyuan county, Qinghai	37°23.449′	101°24.690′	3229	Menyuan-2
Pop15	Minle county , Gansu	38°3.827′	100°53.706′	3325	Minle
Pop16	Qilian county, Qinghai	38°04.147′	100°13.035′	3440	Qilian

with high biologically active alkaloid contents) and wide distribution, the species has been subjected to extensive collection and is used increasingly every year. Destruction of the species habitat resulting from overgrazing, over-exploitation and clearing has led to the fragmentation of populations and a decrease of their size and number gradually (Peng, 2007). The maintenance of populations for sustainable use is determined by the level of their genetic diversity. Therefore, the analysis of the genetic variation within and among populations of the species is crucial for understanding of their future maintenance and developing improvement and conservation programs.

Except for its chemical components (Wang et al., 1991, 1995; Shang et al., 2002, 2003, 2006; Wu et al., 2006), this species has been poorly studied up to now. No data had been reported prior to this study about its population genetic structure or intra-specific differentiation across its distribution in the Qinghai-Xizang plateau. The present study aims at assessing the genetic diversity and population structure of *M. quintuplinervia* using random amplified polymorphic DNA markers (RAPDs).

MATERIALS AND METHODS

Plant material

The study was carried out from 16 wild populations of *M. quintuplinervia* sampled from Qinghai and Gansu in china. For each population, 15 individuals were sampled randomly with a minimum distance of 10 m between individuals to avoid multiple sampling from the same parent. In total, 240 individuals of *M. quintuplinervia* were sampled. The location and main ecological characteristics of sample sites are shown in Table 1 and Figure 1. Young leaves were collected from each sampled individual and dried in silica gel and stored at 4℃ for subsequent DNA extraction.

DNA extraction and RAPD analysis

The DNA was extracted from the silica gel-dried leaves using the modified 2× cetyltrimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). About 0.2 g dried leaf was put in a mortar with liquid nitrogen and incubated for 60 min at 65℃ with shaking every 10 min, in 1000 uL of CTAB extraction buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl,); 10 uL of mercaptoethanol was added for the previous soluting and subsequently mixed. Proteins were extracted twice with 500 uL chloroform–isoamyl alcohol (24:1) for 10 min and then centrifuged at 10,000 rpm for 10 min. The supernatant was reserved and mixed with 500 uL ice-cold isopropanol. The tubes were placed at –20℃ for 1 h to allow precipitation of DNA. DNA was collected as a pellet by centrifugation at 10,000 rpm at 4℃, washed with 500 uL 70% ethanol thrice, dried, and dissolved in 100 uL of 1× TE buffer. DNA quality and quantity were determined in 1.0% agarose gels.

DNA samples from three individuals representing three populations located at least 100 km away from each other were screened with100 RAPD primers (from Sangon Biotechnology, Shanghai), 17 of which were selected for analysis of all individuals base on their ability to amplify DNA, band intensity, number of loci amplified, and reproducibility of the products (Table 2). To increase the reproducibility and consistency in amplification efficiencies among samples, the reaction conditions were kept constant by using the same polymerase chain reaction (PCR) reaction kits, PCR machine and electrophoresis instrument to minimize systematic errors. The PCR reaction mixture was 25 uL containing DNA template (about 20 ng), 1.5 unit Taq polymerase enzyme, 0.2mMdNTP, 0.2 uM primer and 10×PCR buffer. Amplification was carried out in a thermocycler (PTC-100, Bio-RAD Corporation). The PCR amplification was performed using a program of 94℃ for 5 min followed by 40 cycles of denaturation at 94℃ for 50 s, annealing at 38℃ for 60 s, DNA elongation at 72℃ for 80 s, and a final extension at 72℃ for 7 min. Amplified products were separated by electrophoresis on 1.5% agarose gels in 1×TBE (Sambrook et al.,

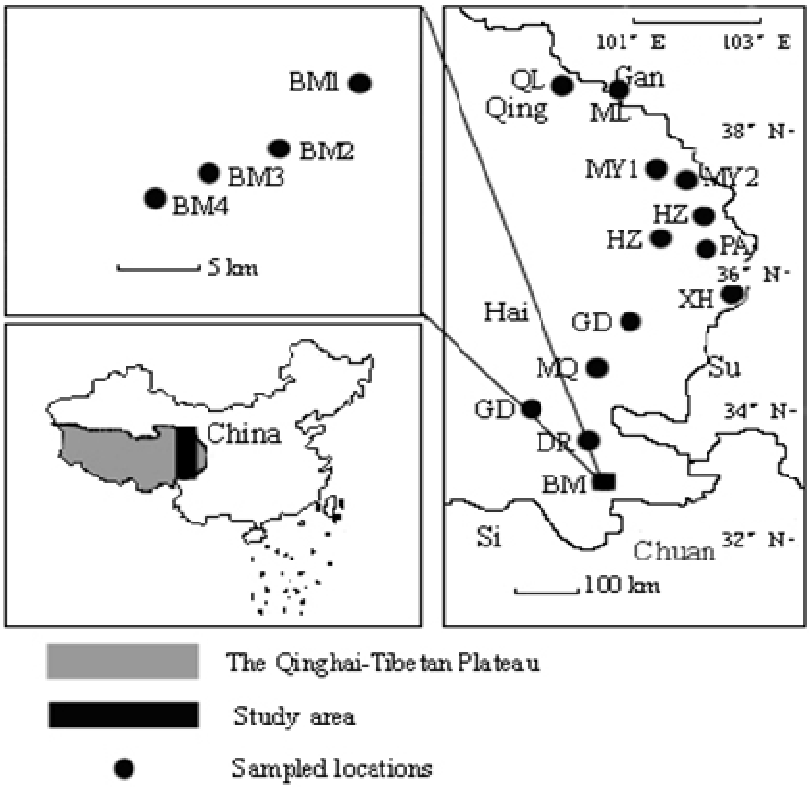

Figure 1. Locations of sixteen study populations of *M. quintuplinervia* from the Qinghai-Xizang Plateau, China.

Table 2. RAPD primers used for PCR amplification of *M. quintuplinervia* Regel and bands of amplification products in all sampled individuals.

Primer	Sequence of the primer	Total number of bands	Primer	Sequence of the primer	Total number of bands
S209	5'-CACCCCTGAG-3'	14	S274	5'-CTGCTGAGCA-3'	16
S214	5'-AATGCCGCAG-3'	9	S275	5'-ACACCGGAAC-3'	15
S224	5'-CCCCTCACGA-3'	13	S276	5'-CAGCCTACCA-3'	13
S226	5'-ACGCCCAGGT-3'	14	S285	5'-GGCTGCGACA-3'	14
S227	5'-GAAGCCAGCC-3'	13	S287	5'-AGAGCCGTCA-3'	12
S228	5'-GGACGGCGTT-3'	11	S289	5'-AGCAGCGCAC-3'	14
S252	5'-TCACCAGCCA-3'	13	S290	5'-CAAACGTGGG-3'	13
S262	5'-ACCCCGCCAA-3'	15	S294	5'-GGTCGATCTG-3'	12
S268	5'-GACTGCCTCT-3'	14			

1989) and stained with ethidium bromide for visualization on a UV transilluminator.

Data analysis

The presence/absence of each RAPD band of a locus was coded as 1/0, and a binary data matrix was constructed. Bands showing the same gel mobility were assumed to be homologous and the same molecular weight was considered to belong to the same locus. The binary data matrix was treated with Popgene 1.31 (Yeh et al., 1999) as computer parameters depicting genetic diversity. These parameters which include the percentage of polymorphic loci (PPB), number of alleles(Na), effective number of alleles(Ne) expected heterozygosity (Nei's (1978) gene diversity, He), genetic diversity within populations (Hs), Shannon' s information indices (I) and gene flow(Nm) were calculated to represent the gene diversity and distribution of the variation. We used AMOVA software (Analysis of Molecular Variance, ver. 1.55; Excoffier et al., 1992) to calculate the variance components and their significance levels (by evaluation Euclidean distance matrix) within and between populations for all 192 individuals. The variance components were tested by nonparametric randomization tests using 1000 permutations. To represent the correlation between geographic distance and level of genetic

Table 3. Genetic variations in populations of *M. quintuplinervia* detected in the RAPD analysis.

Population	PPB (%)	Na(±SD)	Ne(±SD)	He(±SD)	I(±SD)	Nm
1	65.78	1.6578±0.4624	1.2892±0.3387	0.2043±0.1756	0.2848±0.1982	
2	70.67	1.7067±0.4524	1.3752±0.3482	0.2406±0.1843	0.3310±0.2154	
3	70.22	1.7022±0.4583	1.3706±0.3475	0.2368±0.1571	0.3295±0.2216	
4	71.55	1.7155±0.4602	1.3708±0.3381	0.2536±0.1647	0.3221±0.2198	
5	71.11	1.7111±0.4597	1.3698±0.3547	0.2470±0.1632	0.3516±0.2307	
6	72.89	1.7289±0.4569	1.4017±0.3605	0.2601±0.1527	0.3633±0.2194	
7	73.33	1.7333±0.4578	1.3951±0.3024	0.2581±0.1604	0.3659±0.2133	
8	68.44	1.6844±0.4576	1.3605±0.3445	0.2334±0.1592	0.3250±0.2206	
9	69.33	1.6933±0.4426	1.3817±0.3501	0.2522±0.1587	0.3563±0.2245	
10	69.78	1.6978±0.4530	1.3501±0.3375	0.2346±0.1636	0.3243±0.2186	
11	67.11	1.6711±0.4601	1.3106±0.3215	0.2133±0.1658	0.2960±0.2013	
12	67.56	1.6756±0.4615	1.3182±0.3469	0.2071±0.1524	0.2903±0.2239	
13	72.00	1.7200±0.4557	1.3825±0.3475	0.2423±0.1539	0.3425±0.2565	
14	75.11	1.7511±0.4368	1.4018±0.3517	0.2634±0.1573	0.3727±0.2258	
15	72.44	1.7244±0.4507	1.4218±0.3329	0.2610±0.1608	0.3561±0.2351	
16	70.67	1.7067±0.4527	1.3678±0.3357	0.2458±0.1592	0.3436±0.2302	
Average	70.50	1.7050	1.3728	0.2408	0.3347	
Species	85.33	1.8428±0.3529	1.5326±0.3196	0.2860±0.1798	0.4371±0.2253	0.9025

differentiation among the populations, a cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) method was performed using the NTSYS program (Rohlf, 1994). To estimate the magnitude of differences between the dendrograms constructed based on Nei's (1978) genetic distances, cophenetic value matrices were computed for each dendrogram, and these cophenetic matrices were compared by the Mantel matrix-correspondence test using the NTSYS program (Rohlf, 1994).

RESULTS

Genetic diversity of *M. quintuplinervia* was detected. The calculated results are as shown in Table 3.

Genetic diversity of *M. quintuplinervia*

Seventeen primers generated a total of 225 reliable bands, the amplified products of different primer was from 9 to 16, 13.2 on average, and ranging from 200 bp to 2.0 kb. Among the 225 bands, 85.3% (191 in total) were polymorphic loci (Table 3). At the population level, the percentage of polymorphic loci (PPB) ranged from 65.78% to75.11%, with an average of 70.50%. The Menyuan population had the highest value of polymorphism while the Pingan population had the smallest**.**

At the population level, the observed number of alleles (Na) in the sixteen populations of the *M. quintuplinervia* varied from 1.6578 to 1.7511, with an average of 1.7050 (Table 3). The effective number of alleles (Ne) ranged from 1.2892 to 1.4218, with an average of 1.3728 (Table 3). At species level, the observed number of alleles (Na) was 1.8428 and the effective number of alleles was 1.5326 (Table 3).

The average Nei's unbiased genetic diversity (He) was estimated to be 0.2408 at the population level (from 0.2034 to 0.2643), and 0.2860 at the species level (Ht). The Shannon's indices (I) was estimated to be 0.3347 (from 0.2848 to 0.3727) at the population level, and 0.4371 at the species level**.**

The genetic structure of populations

To further evaluate the relationships among the populations, individual RAPD haplotypes were used to estimate population differentiation. The result shown that the genetic distances between populations of *M. quintuplinervia* ranged from 0.0276 to 0.1962, with an average value 0.1053. The maximum value appeared between the samples from Pingan and Banma, while the minimum value appeared between the samples Huangzhong and Pingan. Likewise, the average Nei's genetic similarity coefficient between populations was 0.8947(ranged from 0.8038 to 0.9724).

The MOVA analysis of RAPD data of *M. quintuplinervia* in Qinghai-Xizang Plateau indicated that the major proportion (78.3%) of the total variation was found within populations and 21.7% ($\Phi_{ST,\ 21.7\%}$) of the variation comes from among populations (Table 4).

An unweighted pair-group cluster analysis method (UPGMA) was used, using arithmetic average based on Nei-Li genetic distance, and the result is shown in Figure 2. The UPGMA dendrogram separated the populations of M. quintuplinervia into three main groups: five populations (POPs 1, 2, 3, 4, 5) from the southern of QH comprised a

Table 4. Analysis of molecular variance among and within sixteen populations of *M. quintuplinervia* detected by RAPD.

Source of variation	d.f.	Sum of squares	Mean squares	Variance component	Total variance (%)	p-value
Among populations	15	934.8052	324.251	7.09	21.8	< 0.001
Within populations	239	1785.4320	25.432	25.43	78.2	< 0.001

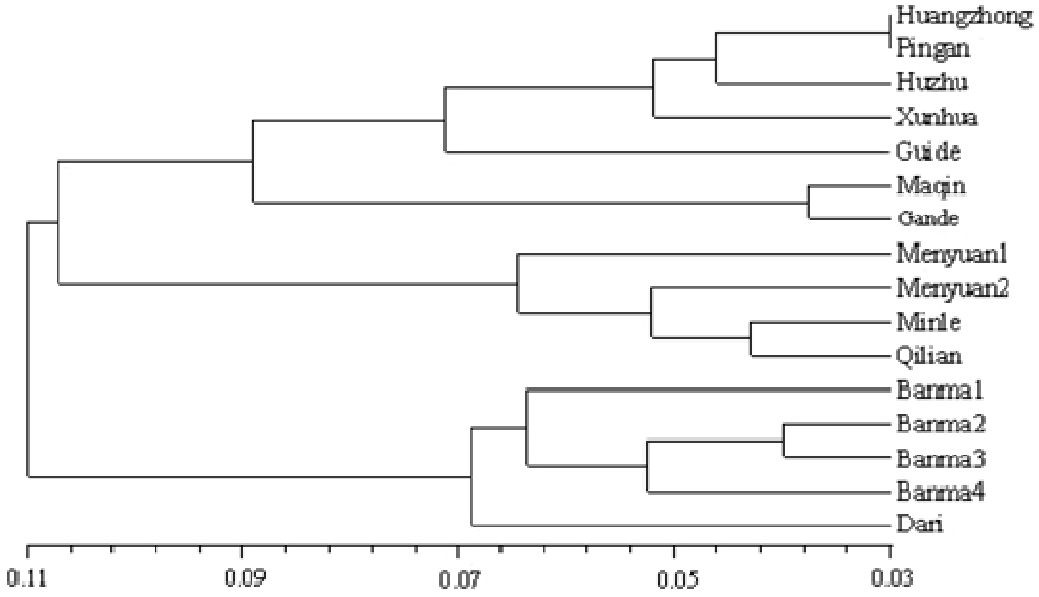

Figure 2. UPGMA dendrogram of *Meconopsis quintuplinervia* based on Nei's (1978) genetic distances, indicating the clustering relationships of sampled populations.

distinct group, seven populations (POPs 6, 7, 8, 9, 10, 11, 12) from the middle of Qinghai comprised another distinct group, while the remaining four, including those located on the Northern of Qinghai and eastern of Gansu, clustered as the third group.

DISCUSSION

The results of this work demonstrated that the species of *M. quintuplinervia* from Qinghai and Gansu Province, E Qinghai-Xizang plateau maintain a high level of genetic diversity, with 85.33% of polymorphic bands and 0.2860 Ht value in all 16 populations. The percentage of polymorphic bands (PPB) in each population ranged from 65.78 % to 75.11%. The genetic variation detected was a little lower than other species by the same method in the same genus. By using 38 RAPD primers, Sulaiman et al. (1996) estimated the genetic diversity of *Meconopsis paniculata* for seven populations and *Meconopsis simplicifolia* for four populations from Sikkim (Eastern Himalaya) and found that the average PPB values of the two species was 86.4%. However, using enzymes, Sulaiman et al. (1996) revealed low genetic diversity for three endangered species of Meconopsis from Sikkim. Allelic frequencies for loci acid phosphatases, esterases and glutamate dehydrogenase in *M. paniculata*, *M. simplicifolia* and *Meconopsis sinuata* were more or less similar at most loci, and in all these loci suggesting a low level of polymorphism. The level of genetic variability of *M. quintuplinervia* obtained in this study are relatively higher than the average values for long-lived herbaceous perennials (PPB= 0.413, He = 0.116) and widespread species (PPB = 0.589, He = 0.202) (Hamrick and Godt 1989). Hamrick and Godt, (1996) and Nybom et al. (2000) concluded that the level of genetic variability strongly depended on plant life form, geographic range, pollen dispersal mechanism, and natural selection. *M. quintuplinervia* is a perennial plant species with very broad ecological amplitude, growing commonly in low scrub and mountain meadows and the edge of forests. These biological characters may contribute to create and maintain the observed high level of genetic variability, and also suggest that this is wind-pollinated and allogamous plant species in the present study.

From the genetic parameters (PPB, He and I) in *M. quintuplinervia*, it was found that the values of the Menyuan population located in Qilian Mountain were the highest of all the sixteen populations. The high genetic diversity imbedded in the Menyuan populations may reflect the general genetic diversity pattern of this species in its original center, and Banma population located in

Bayankala Mountain is another original center.

In this study, we found the high values of variability within populations and the low levels of genetic variation among populations to *M. quintuplinervia*. This result suggests the existence of a reproduction model without inbreeding. The genetic variability within populations is a very important measure of species adaptation to environmental changes and of species survival (Sofia, 2006). When the gene pool of a population narrows and loses genetic plasticity, it becomes increasingly susceptible to changes in the environmental conditions and hence more prone to extinction (Louis, 1980). High heterozygosity could result from high amounts of self-pollination within the populations by our field surveys.

M. quintuplinervia is an important medicinal plant with high content of alkaloids. These chemicals are used against inflammation, pain, hepatitis and tuberculosis as anticholinergic. Over-collection to use may be one of the major reasons that this species is decreased. Therefore, collection in the wild should be restricted, and medicinal supplies of this species also should be restricted to commercial utilization. At present, the importance of *M. quintuplinervia* and the genetic diversity conservation in alpine ecosystem has not been well recognized. It is necessary to preserve as many populations as possible in the wild in any *ex situ* conservation or artificial cultivation programs, introductions should be performed to include representatives of as many populations as possible. The high genetic diversity of the available material that could be used in artificial propagation programs should reduce inbreeding depression and facilitate quality control of drug production from the plants. According to the results of this study, RAPDs would provide a valuable tool for the molecular characterization of gene bank accessions.

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