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AASRI Procedia 1 (2012) 344 – 352

AASRI Procedia

www.elsevier.com/locate/procedia

2012 AASRI Conference on Computational Intelligence and Bioinformatics

Classification of Chinese Medicinal Tree Peony Cultivars Based on Chloroplast DNA Sequences

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Abstract

Tree peonies are deciduous subshrubs of the *Paeonia* sect. *Moutan* of the Paeoniaceae. They have been used as medicine for more than 2000 years in China. In contrast with the traditional knowledge of one medicinal lineage, cpDNA sequences revealed that the cultivars of Chinese medicinal tree peonies were classified into four lineages. *P*. 'Xiang Dan' (from Hunan production area) and *P*. *ostii* (from Anhui production area) belong to two different lineages, and *P*. 'Hu Lan' (from Hubei production area) belongs to another lineage. *P*. 'Jinpao Hong', 'Jianshi Fen' and 'Taiping Hong', with a sympatric distribution in Hubei Province and Chongqing municipality, together formed a different lineage by sharing a single cpDNA genotype, although they have certain phenotypic differences. A taxonomic key to the Chinese medicinal tree peony cultivars was compiled for identification. The highest nucleotide difference (0.004) occurred between *P*. *ostii* and the rest cultivars (such as *P*. 'Jinpao Hong', 'Jianshi Fen', 'Taiping Hong' and *P*. 'Xiang Dan'). The significant genetic differentiation among the medicinal cultivars suggested that it is necessary to conduct an intensive evaluation on the core germplasm resources in order to improve conservation, rational utilization, quality control, breeding and development of medicinal tree peony industry.

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doi:10.1016/j.aasri.2012.06.053

Keywords. Medicinal tree peony cultivar, Chloroplast DNA, SNP, lineage, classification, conservation

1. Introduction

The root bark of tree peonies has been used as medicine for more than 2000 years in China. It has been included in over 1000 Chinese prescriptions as one of the main raw materials. The effective chemical components have been used extensively in cosmetics and healthy food. There is a growing demand for high quality root bark of tree peonies in the market [1-4].

Tree peonies are deciduous subshrubs in *Paeonia* sect. *Moutan* of the Paeoniaceae [5, 6]. At present, nine species have been recognized in *P*. sect. *Moutan* based on multiple DNA fragments and morphological data [5, 6]. *P.* sect. *Moutan* includes two subsections: subsect. *Vaginatae* and subsect. *Delavayanae*. The medicinal cultivars of tree peonies belong to *P*. subsect. *Vaginatae*.

Compared to the large amount of literature involving the ornamental cultivars of tree peonies, studies on the medicinal tree peony cultivars are fewer, mostly focusing on effective component isolation, chemical structure determination, improvement of cultivation methods, storage and processing techniques of the root bark [1, 9].

Medicinal tree peony cultivars have been described in detail by Li (1999) [2] and Xiao and Yang (2001) [1] in morphology, biogeography, cultivation and utilization. Shen (2001) revised a documentation of medicinal plants in *P.* sect. *Moutan* [10]. Isoenzyme analysis indicated that there exist genetic differentiations between *P. ostii* (Tongling Peony) and *P*. 'Taiping Hong' (Dianjiang Peony) [11].

The Chinese medicinal tree peony cultivars which were bred through long-term artificial selections are planted on large scale in the production areas. In China, there are four important areas (Anhui, Hunan and Shandong provinces, and Chongqing manicipality) producing tree peony root bark. There are particular medicinal cultivars of tree peonies in each production area.

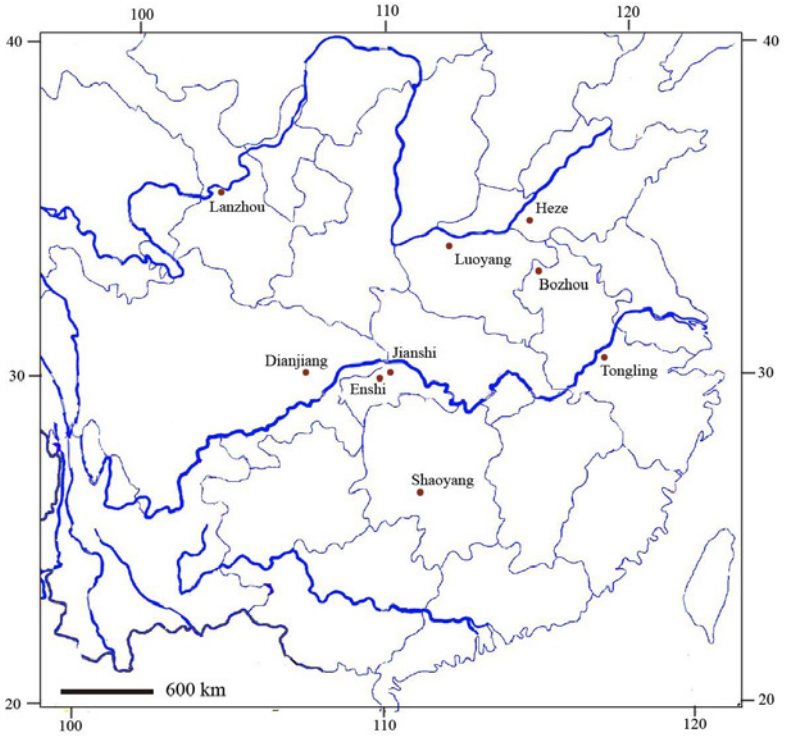
Nomenclature

RAPD	Random amplified polymorphic DNA
ISSR	Intersimple sequence repeat
AFLP	Amplified fragment length polymorphism
SRAP	Sequence-related amplified polymorphism
SSR	Microsatellites or polymorphic simple sequence repeats
SNP	single nucleotide polymorphism
cpDNA	Chloroplast DNA
PCR	Polymerase Chain Reaction
BGI	Beijing Genome Institute
NJ tree	Neighbor-joining tree

As a matter of fact, there exist morphological differences among the medicinal cultivars although the products have the same commercial name of "Danpi" (i.e., root bark of tree peony in Chinese) in the market [2]. However, no comparative study has been reported on the medicinal cultivars at DNA level. Meanwhile, affected by the traditional modes of thought that the root bark of wild tree peonies has a better medicinal efficacy, digging for root bark by local people is severely destroying the wild resources of tree peonies [1-4].

The single copy chloroplast DNA has been widely used for genetic characterization of plants at genus, species and population levels [12, 13]. The development of DNA sequencing technology and the growing

number of published chloroplast genome sequences of flowering plants have provided an opportunity for genetic evaluation of medicinal tree peony cultivars with higher accuracy. In this study, we will report new insights into the Chinese medicinal tree peony cultivars based on cpDNA sequences and discuss the strategy for the germplasm resources conservation.



Map 1. The sites where the samples were from in China.

2. Materials and Methods

2.1 Plant materials

Paeonia 'Xiang Dan' was the local medicinal cultivar particularly grown in the production area (e.g., Shaoyang, Shaodong, Qining and Changning) in southwest Hunan Province, China (Table 1) [4]. *P. ostii* was the medicinal tree peony traditionally grown in the production areas (Tongling and Bozhou) of Anhui Province, China [1]. *P.* 'Hu Lan', 'Jinpao Hong' and 'Jianshi Fen' were the medicinal cultivars particularly grown in the production area (Enshi and Jianshi) of Hubei Province [2]. *P.* 'Taiping Hong' was the local medicinal cultivar grown in Dianjiang production area of Chongqing municipality, China [1, 2]. The herbaceous *P. anomala* subsp. *veitchii* of *P.* sect. *Paeonia* subsect. *Albiflorae* (Salm-Dyck) D. Y. Hong was used as an outgroup. Four artificial crosses of tree peonies including the F_1 hybrid (s) and both of the male and female parents (i.e., *P. decomposita* x *P. qiui*, *P. qiui* x *P. decomposita*, *P. ostii* x *P. delavayi* and *P. delavayi* x *P.* 'Yufei Yanzhuang') from the resource nursery of General Station for Extension of Forestry Science and Technology, Gansu Forestry Department, Lanzhou, Gansu Province, China were used to confirm the maternal inheritance mode of the cpDNA regions in *Paeonia* sect. *Moutan*[14].

Fresh leaves of the samples for DNA extraction were collected in spring and dried immediately using

silica gel. Thirty-five sequences from five chloroplast DNA regions of the 7 accessions were deposited in GenBank under accession numbers shown in Table 1.

Table 1. Materials used in this study

No.	Name of sample	Accession No.
1	*P.* 'Jinpao Hong'	JQ396320, JQ396332, JQ396344, JQ396356, JQ396368
2	*P.* 'Taiping Hong'	JQ627019, JQ627021, JQ627023, JQ627025, JQ627027
3	*P.* 'Jianshi Fen'	JQ396325, JQ396337, JQ396349, JQ396361, JQ396373
4	*P.* 'Hulan'	JQ396319, JQ396331, JQ396343, JQ396355, JQ396367
5	*P. ostii*	JQ228279, JQ228301, JQ228323, JQ228345, JQ228367
6	*P.* 'Xiang Dan'	JQ396318, JQ396330, JQ396342, JQ396354, JQ396366
7 *	*P. anomala* subsp. *veitchii*	JQ627020,JQ627022, JQ627024, JQ627026, JQ627028

Notes: * Sect. *Paeonia*. Other accessions belong to *P.* sect. *Moutan*.

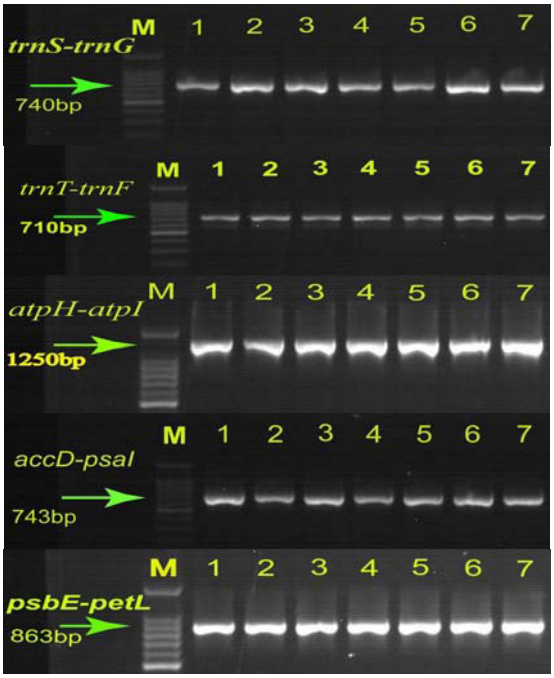


Fig. 1. PCR products of each of the five chloroplast regions of the seven samples of Paeonia. 1 to 7 are sample numbers corresponding to those in Table 1. M is the 100-bp Ladder DNA size marker.

As shown in Table 1 and Map 1, samples 1, 3, 4 were collected in Yutian Tree Peony Nursery, Heze, Shandong Province where they were introduced from the production areas (Enshi and Jianshi) of Hubei Province, China (Map 1). Sample 2 was collected in Chongqing Institute of Medicinal Plants Cultivation, Nanchuan, Chongqing, China. Sample 5 was collected in General Station for Extension of Forestry Science and Technology, Gansu Forestry Department, Lanzhou, Gansu Province, it was originally introduced from Tongling, Anhhui Province, China. Sample 6 was collected at Shaoyang, Hunan Province, China. Sample 7 was collected in Peace Tree Peony Garden, Lanzhou, Gensu Province, China.

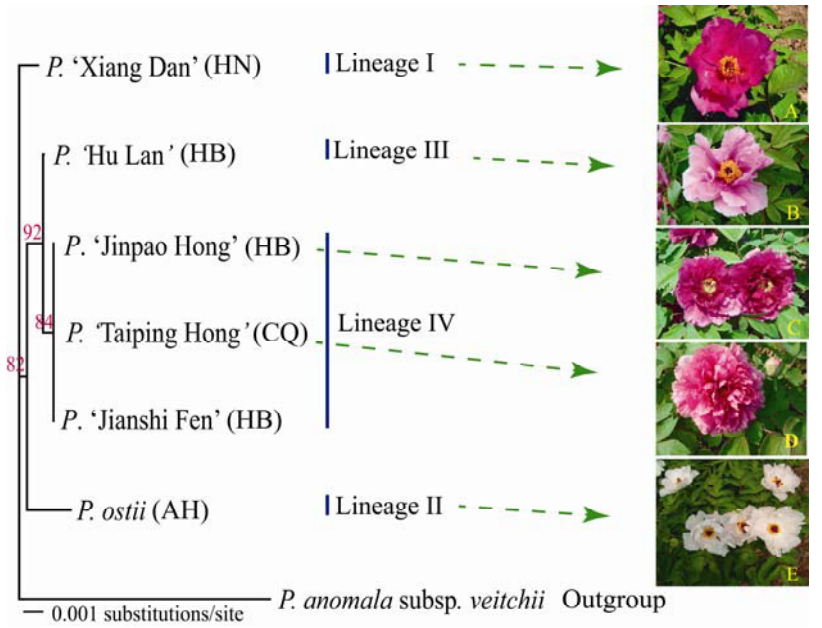


Fig. 2. The Neighbor-joining (NJ) tree generated with PAUP 4.0b10 based on sequences from the five chloroplast DNA regions of the six accessions showing the major lineages of medicinal cultivars of tree peonies in China. Tree length = 65, Consistency index (CI) = 0.9846, Homoplasy index (HI) = 0.0154, Retention index (RI) = 0.9167. The numbers above the branch are bootstrap values (%) of 1000 replicates. The letters in the brackets following the cultivar name indicate the production area: HN= Hunan Province, HB= Hubei Province, CQ= Chongqing municipality and AH= Anhui Province. The photos show the flowers of the medicinal tree peony cultivars in each of the four lineages. **A:** *P.* 'Xiang Dan', **B:** *P.* 'Hu Lan', **C:** *P.* 'Jinpao Hong', **D:** *P.* 'Taiping Hong', **E:** *P. ostii*. See Li (1999) [2] for the photo of flower of *P.* 'Jianshi Fen'.

2.2 DNA extraction and primers

Total genomic DNA was extracted following the procedure of Plant Genomic DNA Kit (DP305) from Tiangen Biotech (Beijing) Co., Ltd., China.

Five chloroplast DNA regions were sequenced. The *trnT-trnF* intergenic spacer region was amplified using primers *trn_a* and *trn_b* (equal to *trnT* and *trnF*) reported by Taberlet et al. (1991) [15]. Internal primers *trn_a*_289R (5'—GGCAGACCAGACCTTGGA—3') and *trn_a*_438F (5'—ATGGGAAAGCGAGAGGAAGAA—3') were newly designed as sequencing primers to overcome the poly $(T)_{13\text{-}17}$ structure located in the mid-part (ranging from positions 254 to 272) of the *trnT-trnF* region. The *trnS-trnG* intergenic spacer region was amplified using primers *trnS* and *trnG* reported by Zhao et al. (2008) [7] and Zhang et al. (2009) [16]. The *atpH-atpI* intergenic spacer region was amplified using newly designed primers *atpH-atpI*-83F (5'—GGCTGTCTCGCAATACCTTCTA—3') and *atpH-atpI*-1340R (5'—CGTGGCTCAAGATAATCTACTTAGG—3'). The *accD-psaI* intergenic spacer region was amplified using newly designed primers *accD-psaI*-21F (5'—AACATTGAATAAGACAGTACCTGAG—3') and *accD-psaI*-747R (5'—GTAAGTTAAGAGTTGTCATAGGATGG—3'). The *psbE-petL* intergenic spacer region was amplified using newly designed primers *psbE-petL*-356F (5'—CCTTCTTCTGACACAGCAATG—3') and *psbE-petL*-1219R (5'—TTACCATTATAGACAGCACTAACAA—3').

2.3 PCR amplification and DNA sequencing

Taq DNA polymerase and PCR buffer (TaKaRa Code: DR100B) were from TaKaRa Biotechnology (Dalian) Co., Ltd. PCR amplification was conducted following the protocol of TaKaRa Code: DR100B. PCR program is as follows: 94°C for 4 min; 35cycles of 94°C for 1 min, 56°C (annealing temperature) for 50 s and 72°C for 1.5 min; 8 min at 72°C for final extension. PCR amplification was performed in an Applied Biosystems Veriti™ 96-Well Thermal Cycler (Model#: 9902, made in Singapore). The amplicons were resolved simultaneously on 2% agarose gels (Promega) run in 1 x TAE buffer at 3 V cm^{-1} for 2.5 h and were stained with ethidium bromide. Band patterns were documented and photographed with the Gel Documentation System of Transilluminator BINTA2020D (Liaoning Langke Business and Trade Co. Ltd., China). The 100-bp Ladder DNA size marker (100 to 1500bp) was from Tiangen Biotech (Beijing) Co., Ltd., China. The fragments were sent to BGI for PCR direct sequencing using a 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA).

2.4 Data analysis

Nucleotide sequences were edited and manually corrected by eye using Sequencher (v. 4.6). Alignment was conducted using Clustalx [17]. The poly $(T)_{13\text{-}17}$ structure was deleted from the mid-part (ranging from positions 254 to 272) of the *trnT-trnF* region before computation. The final dataset included 35 sequences from five cpDNA regions of the seven accessions in an alignment of 3343bp. Neighbor-joining tree was created with PAUP 4.0b10 [18] using nucleotide evolutionary model of Jukes-Cantor model.

Table 2. Pairwise base differences among the six medicinal cultivars based on the cpDNA sequences

	P. 'Jinpao Hong'	*P*. 'Taiping Hong'	*P*. 'Jianshi Fen'	*P*. 'Hu Lan'	*P. ostii*
P. 'Jinpao Hong'					
P. 'Taiping Hong'	0.0000				
P. 'Jianshi Fen'	0.0000	0.0000			
P. 'Hu Lan'	0.0006	0.0006	0.0006		
P. ostii	0.0040	0.0040	0.0040	0.0033	
P. 'Xiang Dan'	0.0030	0.0030	0.0030	0.0024	0.0039

3. Results

3.1 Genetic analysis of Chinese medicinal tree peony cultivars

The maternal inheritance of the five cpDNA regions was confirmed by sequencing of the four artificial crosses of tree peonies. Three or more individual plants were sampled and sequenced per cultivar/species, and identical results were obtained among individual plants within each cultivar/species. Therefore, one sample of each cultivar/species was included in the dataset for formal computation.

In total, seven parsimony-informative characters were obtained in the cpDNA sequence alignment of the six medicinal cultivars. In the Neighbor-joining (NJ) tree (Fig. 2), four lineages were recognized among the Chinese medicinal tree peony cultivars with high bootstrap support values (≥82%).

P. 'Xiang Dan', *P. ostii* and *P*. 'Hu Lan' belong to three different lineages (lineage I, II and III), respectively. *P*. 'Jinpao Hong', 'Jianhi Fen' and 'Taiping Hong', with sympatric distribution in Hubei Province and Chongqing municipality, together formed a single lineage (IV). The largest differentiation occurred between lineage II (*P. ostii*) and lineage IV. In general, lineages I and II are quite diverged from the other medicinal lineages (Table 2 and Fig. 2).

3.2 Key to the six Chinese medicinal tree peony cultivars

1a. Lineage I: Chloroplast *psbE-petL*_region_aln_728bp_**$T_3T_{249}C_{301}G_{416}G_{712}$**
Petal purplish red and suffused with slight blue, with a red blotch at the base of the upper surface of the petal and a white streak on the lower surface of the petal. Disc deep purplish red, stigma purplish red. Filament purple. Single flower form. …… *P.* 'Xiang Dan'

1b. Lineage II: Chloroplast *psbE-petL*_region_aln_728bp_**$G_3C_{249}C_{301}G_{416}G_{712}$**
Both upper and lower surfaces of the petal white, without blotches. Disc deep purplish red, stigma purplish red. Filament purple. Single flower form. …… *P. ostii*

1c. Lineage III: Chloroplast *psbE-petL*_region_aln_728bp_**$T_3C_{249}A_{301}G_{416}T_{712}$**
Petal slight pink tinted with blue, with a deep red blotch at the base of the upper surface of the petal. Disc deep purplish red, stigma purplish red. Filament purple. Lotus flower form. …… *P.* 'Hu Lan'

1d. Lineage IV: Chloroplast *psbE-petL*_region_aln_728bp_**$T_3C_{249}A_{301}A_{416}T_{712}$**

2a. With a deep red blotch at the base of the upper surface of the petal

3. Petal slightly purplish red tinted with blue …… *P.* 'Taiping Hong'

4. Petal slightly pink tinted with red. …… *P.* 'Jianshi Fen'

2b. With no obvious blotches at the base of the upper surface of the petal
Petal reddish purple, with a white streak on the lower surface of the petal. Disc purplish red, stigma purplish red. Filament purplish red. Rose flower form. …… *P.* 'Jinpao Hong'

Note: The nucleotide molecular formula, e.g., chloroplast *psbE-petL*_728bp_**$T_3T_{249}C_{301}G_{416}G_{712}$**, was used to indicate the lineage of Chinese medicinal tree peony cultivars, where "chloroplast *psbE-petL*_region" refers to the name of the chloroplast *psbE-petL* intergenic spacer region used, "aln_728bp" refers to the length of the sequence alignment (728 base pairs), "**$T_3T_{249}C_{301}G_{416}G_{712}$**" refers to the nucleotide molecular formula containing five SNP sites extracted from the aligned sequence of the cpDNA region, the figure following the nucleotide character (**T, C,** G or **A**) indicates the position of the corresponding SNP site from the 5' end of the sequence alignment. The nucleotide molecular formulas are used to first divide the six medicinal tree peony cultivars into four lineages, and then morphological characters are used within the lineages to make classification to cultivars.

4. Discussion and conclusion

4.1 Cultivation of medicinal tree peony cultivars in China

Bozhou and Tongling of Anhui Province have the highest production of "Danpi" of China by the cultivated *P. ostii* (about 1.5 x 10^6 kg per year). Because of the damp and hot tolerance as well as resistance to disease and insect pest, *P. ostii* has been playing an important role as parental plants in breeding of Chinese ornamental cultivars of tree peonies [2-4, 19].

Both *P.* 'Xiang Dan' and *P. ostii* are cultivated for root bark in the production areas of southwest Hunan Province. According to local farmers and the annals of the local counties, *P.* 'Xiang Dan' has been transplanted to and cultivated in the current production area for at least 600 years since Ming Dynasty from an unkown place probably in the south of Hunan Province, China. In the other production areas, such as Heze in Shandong Province and Luoyang in Henan Province, the tree peony industry mainly focuses on production of ornamental cultivars. Their medicinal tree peony plants of *P. ostii* are introduced from Anhui Province. *P.* 'Jinpao Hong' (Hubei Province), 'Jianshi Fen' (Hubei Province) and 'Taiping Hong' (Chongqing manicipality) which share a single cpDNA-genotype, have a sympatric distribution. Some of the medicinal tree peony cultivars, such as 'Jinpao Hong' and *P. ostii*, are also planted for ornamental use in gardens in China. Many plants of *P. ostii* have been introduced to other countries for ornamental use as well. *P. ostii* is

propagated commonly by seed, while the other medicinal cultivars are propagated by division.

4.2 Traditional use of the wild tree peonies as medicine in China

There are documentations about medicinal use of the wild tree peonies [1, 2]. *P. jishanensis* is frequently dug in its natural distribution areas (southern Shanxi Province and Northern Shaanxi Province) for root bark. The root bark of *P. rockii* is dug commonly for medicinal use by the local people of its distribution areas in southeast Gansu Province, southwest Shaanxi province, northwest Sichuan Province and western Hubei Province (e.g., Shengnongjia Nature Reserve). *P. ostii*, as a distinct species with both wild and cultivated plants, has a wide distribution but only a minor morphological and genetic variations [7, 8, 16, 20]. The root bark of *P. decomposita* in northwest Sichuan Province is commonly used in prescriptions by the local people. The root bark of *P. delavayi* is called "Xichang danpi" or "Yunnan chi danpi" by the local people and used as medicine [1, 2].

4.3 Strategy for resources conservation and rational utilization

Chloroplast DNA markers unambiguously revealed the genetic lineages of tree peony cultivars that were unknown previously. This made an integrated management possible concerning medicinal tree peonies. The wild plants of tree peony species are third-class conservation plants in China. A comparative study showed that the content of main medicinal components of the wild species was not higher than those of the cultivated tree peony varieties for ornamental or medicinal uses. However, the component content was significantly different in the root bark among tree peony cultivars. Under the same soil conditions (Changping district, Beijing, China), the paeoniflorin content of *P*. 'Jianshi Fen' (2.05%) was lower than those of *P*. 'Jinpao Hong' (2.24%) and *P. ostii* (3.96%), but the paeonol content of 'Jianshi Fen' (0.75%) was higher than those of *P*. 'Jinpao Hong' (0.51%) and *P. ostii* (0.47%) [9]. The medicinal component content of *P. ostii* varied among different cultivation areas (Dianjiang, Tongling and Heze) [9]. Selection of cultivars and soil conditions is important in tree peony cortex production [9]. Paeonol content is higher in the root bark of medicinal cultivars (0.33% to 1.43%) than in that of wild species (0.10% to 0.61%) [9]. Unfortunately, *P*. 'Xiang Dan' was not be sampled and compared in the previous studies. Thus, a study should be conducted among the lineages detected in this study at both phytochemical and DNA levels to construct a fundamental database for the germplasm resources conservation and quality control [21, 22]. Publicity and implementation of rational use of the cultivated medicinal varieties instead of the wild plants will efficiently eliminate destruction of the wild tree peony resources.

5. Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (grant 30972412), by the Program of "Guidelines for the Techniques of Molecular Tests of New Plant Varieties" (No 2007011) of the Science and Technology Development Centre of the State Forestry Administration of China, by the State Forestry Administration of China (grant 2008-09) and by the Chongqing Bureau of Health (grant 2011-1-12). The authors acknowledge the help of the following experts: Yuping Zou and Shiliang Zhou for advice in molecular experiments, Prof. Yongqi Zheng, Prof. Zhiming Zhang, Huijin Zhang, Wenhai Sun, Mr. Taiping Hong, Dezhong Chen, Fuhui Chen, Fufei Chen and Xikun Yang for instructions in material collections.

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