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Genetic diversity in *Callicarpa kwangtungensis* Chun. based on morphological, biochemical and ISSR markers

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ABSTRACT

An evaluation of genetic variation and genetic structure of 17 wild populations of *Callicarpa kwangtungensis* in China were studied using morphological traits, biochemical and ISSR markers. The 17 accessions of *C. kwangtungensis* genotypes exhibited considerable variation for morphological characters, which plant height, ground diameter, leaf length, leaf width and leaf area were the main traits according to the principal coordinate analysis (PCA). High performance liquid chromatography (HPLC) results show that poliumoside, forsythiaside B are higher than the criterion standard for Callicarpae caulis et folium in Chinese Pharmacopoeia. ISSR results revealed that the 20 selected primers generated a total of 217 clear and distinguishable bands fragments, 187 bands (85.69%) of which were polymorphic. There was a high level of inter-population genetic variability, relatively low genetic diversity within populations, which indicated the same trend by G_{st} (0.438). This was also confirmed by the limited gene flow among populations (N_m 0.642), which indicated the difficulty in genetic exchange among populations. Mantel test revealed a low association between the ISSR data and geographic position (r = 0.390), ISSR and morphological traits (0.283), and a weak correlation between ISSR and content of phytochemicals (0.078). Finally, a preliminary proposal for conservation and breeding of this germplasm was put forward based on these findings.

1. Introduction

Callicarpa kwangtungensis Chun., a perennial deciduous shrub belonging to the family Verbenaceae, is mainly distributed in the Jiangxi, Hunan, Hubei, Guizhou, Guangdong and Guangxi provinces of China. It grows on sunny forested slopes, bushy and grassy places. The wild populations exhibit extensive morphological variability, especially on the leaves (Editorial Committee of the Flora of China, 1982). As a special medicinal material cultivated primarily in the Jiangxi province, *C. kwangtungensis* has been embodied in the Pharmacopoeia of the People's Republic of China (2010, 2015 editions), and its twigs and leaves constitute the primary raw materials of the Chinese patent medicine "Kang-gong-yan Tablet", which is used to treat gynecological diseases. Chemical constituents reported from this species include terpenoids, phenylpropanoids, flavonoids and essential oils, which exhibit various biological effects, such as antioxidant, analgesic, anti-inflammatory, hemostatic, antiplatelet aggregation and NO inhibitory activities (Xie et al., 2009; Jia et al., 2012; Cai et al., 2014; Hu et al., 2014; Zhou et al., 2015; Yuan et al., 2015; Ding et al., 2015; Xu et al., 2016; Li et al., 2016). Poliumoside and forsythiaside B are phenylpropanoids and comprise the principal medicinally effective components in *C. kwangtungensis*. Their relative contents have thus been used as the primary quality indicator for different *C. kwangtungensis* plants. In recent years, the domestic and international market demand for herbal medicines has led to the increased harvesting of *C. kwangtungensis*, resulting in the wild populations becoming endangered as a result of the lack of protective measures and excessive commercial collections. Additionally, as it has grown as a wild plant for a long time, it has adapted to a variety of ecological habitats, resulting in inconsistencies in the constituents of the crude drug, seriously affecting its efficacy for clinical use. Thus, a resource assessment of *C. kwangtungensis* is necessary.

As one of the most important medicinal herbs of China, most studies to date have focused on cultivation technology (Zou and Liu, 2009; Hu et al., 2016), the effective ingredients (Xie et al., 2009; Hu et al., 2014; Zhou et al., 2015; Yuan et al., 2015; Ding et al., 2015; Xu et al., 2016; Li et al., 2016) and pharmacology of *C. kwangtungensis* (Jia et al., 2012; Cai et al., 2014). However, no study has been reported on its genetic diversity and relationships, although such information is necessary for

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the conservation of this species.

Morphological, biochemical and molecular markers can be used to assess genetic diversity (Fang et al., 2012a,b; Kremer et al., 2015). Of these methods, morphological and biochemical markers are likely to be affected by the environment and cultivation conditions (Liu et al., 2013a,b), resulting in the underrepresentation of the total genetic diversity (Falconer, 1964). Conversely, molecular DNA methods successfully eliminate the influence of environmental factors. Inter simple sequence repeat (ISSR) markers, developed by Zietkiewicz et al. (1994), are based on simple sequence repeats (SSR) and have the advantages of high reproducibility, efficiency, high levels of polymorphism and high information capacity. Hence, they have been widely used in assessments of genetic diversity (Heidari et al., 2016; Tiwari et al., 2017), cultivar identification (Zheng et al., 2011), taxonomy (Zvyagina et al., 2016), phylogeny (Hundsdoerfer et al., 2005) and molecular mapping (Tomar et al., 2017).

In this study, we used a combination of morphological, biochemical and ISSR markers to elucidate the genetic diversity and genetic relationships among 17 natural populations of *C. kwangtungensis* distributed primarily in China. Our objectives were: (1) to assess the genetic diversity and relationships of natural populations of *C. kwangtungensis*; (2) to examine the genetic variation and differentiation within and among populations; and (3) to propose an appropriate conservation strategy for *C. kwangtungensis*.

2. Materials and methods

2.1. Plant materials

According to field surveys, a total of 170 individuals of *C. kwangtungensis* were collected from seventeen natural populations in China, including Jiangxi, Hunan, Guangdong, Guangxi and Guizhou Provinces (Table 1, Fig.1). In order to avoid sampling from the same rhizomes, each individual sample from the same population was collected from different locations at least 20 m apart. The vouchers were deposited in the Botanical Museum of the Jiangxi Academy of Forestry. Young leaf tissues were collected randomly from 10 individuals in each population, and dried in silica gel for transportation, then been frozen in −80 °C freezer for DNA extraction. An amount of the collected sample was dried at 45 °C for chemical analysis. In addition, all the materials were transplanted in the Medicinal Plant Germplasms Nursery of the Jiangxi Academy of Forestry for germplasm conservation.

2.2. Morphological study

Ten individuals were selected and tagged in order to collect data based on the 9 quantitative morphological traits that were assessed across the populations. From these measurements, the maximum, minimum, standard deviation (S.D.) and coefficient of variation (C.V.) were calculated (Table 2).

2.3. Chemical composition study

The detection method for poliumoside and forsythiaside B content was carried out following the Pharmacopoeia of the People's Republic of China (2015 editions) with few modifications. The collected samples were dried at 45 °C for 24 h and ground into a fine powder (< 0.5 mm). An accurately weighed sample of 1 g powder was first extracted with 50 mL 50% aqueous methyl alcohol (MeOH) for 40 min by ultrasound. Followed by filtration using filter paper, the samples were extracted twice with the extraction solvent. The extracts were combined and diluted with extraction solvent to 100 mL, and then filtered through a syringe filter (0.45 μm). The filtrate was directly injected (20 μL) into the HPLC system. Each extract was injected in triplicate. HPLC was performed on a Waters HPLC system (Binary Pump 600 controller), a Waters 2487 UV detector (Waters, USA), auto sampler (2707) and C_{18} column (4.6 mm × 250 mm, 5 μm). Empower software was used for the data collection. The mobile phase consisted of acetonitrile: 0.1% phosphoric acid solution (82:18, V:V). The flow rate was 1 mL/min and the detection wavelength was set at 332 nm.

The detection method for ursolic acid and oleanolic acid content was performed according to Fang et al. (2015).

2.4. ISSR study

Total genomic DNA was extracted from fresh leaves using the method of Doyle and Doyle (1990). The quality and quantity of DNA were determined using 0.8% agarose gels. The DNA samples were diluted to 20 ng/μL with 1 X TE buffer and stored at −20 °C.

PCR conditions were optimized using different concentrations of template DNA, primers, *d*NTPs, Mg^{2+} and *Taq* polymerase as well as different annealing temperatures. Based on the optimization experiments, the PCR was performed in 25 μL final volumes as follows: 1 × PCR buffer, 100 ng of DNA, 0.25 mM *d*NTPs, 2.0 mM Mg^{2+}, 1.0 U *Taq* DNA polymerase and 0.75 μM primer. Amplification reactions were carried out in a PTC-100 thermal cycler (MJ Research Thermocycler) according to the following temperature profile: 5 min initial denaturation at 94 °C; 34 cycles of 94 °C for 30 s of denaturation, 50–60 °C for

Table 1
Sampling locality, abbreviations, geographical coordinates and the contents of chemical composition of the *C. kwangtungensis*.

No.	Locality	Abbreviations	Latitude/°	Longitude/°	Voucher No.	Poliumoside/%	Forsythiaside B/%	Ursolic acid/%	Oleanolic acid/%
1	Nanchang, Jiangxi	NC	28.68	115.89	Jxfang2013-12	0.718 ± 0.021	1.304 ± 0.025	0.190 ± 0.013	0.075 ± 0.011
2	Luxi, Jiangxi	LX	27.63	114.03	Jxfang2013-18	3.546 ± 0.014	4.138 ± 0.022	0.329 ± 0.027	0.205 ± 0.034
3	Yujiang, Jiangxi	YJ	28.22	116.82	Jxfang2013-22	0.438 ± 0.023	0.281 ± 0.011	0.239 ± 0.021	0.148 ± 0.025
4	Tonggu, Jiangxi	TG	28.53	114.37	Jxfang2013-31	1.048 ± 0.014	1.054 ± 0.032	0.136 ± 0.015	0.154 ± 0.031
5	Wuning, Jiangxi	WN	29.26	115.09	Jxfang2013-14	3.669 ± 0.024	4.236 ± 0.029	0.350 ± 0.053	0.196 ± 0.028
6	Anfu, Jiangxi	AF	27.39	114.62	Jxfang2013-9	1.528 ± 0.027	1.918 ± 0.081	0.181 ± 0.034	0.111 ± 0.017
7	Anyuan, Jiangxi	AY	25.15	115.41	Jxfang2013-38	0.502 ± 0.015	0.712 ± 0.034	0.257 ± 0.024	0.103 ± 0.033
8	Ganzhou, Jiangxi	GZ	25.85	114.92	Jxfang2013-26	0.401 ± 0.023	1.248 ± 0.034	0.232 ± 0.042	0.104 ± 0.026
9	Yichun, Jiangxi	YC	27.81	114.38	Jxfang2013-16	2.949 ± 0.021	2.523 ± 0.024	0.270 ± 0.022	0.119 ± 0.035
10	Jinggangshan, Jiangxi	JGS	26.57	114.17	Jxfang2013-21	0.703 ± 0.017	1.559 ± 0.028	0.171 ± 0.051	0.104 ± 0.019
11	Xunwu, Jiangxi	XW	24.96	115.64	Jxfang2013-36	0.531 ± 0.011	1.161 ± 0.033	0.277 ± 0.027	0.183 ± 0.030
12	Yanshan, Jiangxi	YS	28.32	117.71	Jxfang2013-4	0.689 ± 0.013	0.945 ± 0.047	0.163 ± 0.018	0.089 ± 0.021
13	Pingxiang, Jiangxi	PX	27.65	113.73	Jxfang2013-11	2.098 ± 0.026	2.415 ± 0.017	0.229 ± 0.044	0.099 ± 0.028
14	Huitong, Hunan	HT	26.86	109.71	Jxfang2013-25	0.831 ± 0.015	1.204 ± 0.042	0.146 ± 0.035	0.081 ± 0.022
15	Nanxiong, Guangdong	NX	25.24	114.33	Jxfang2013-23	0.421 ± 0.019	0.605 ± 0.032	0.291 ± 0.029	0.094 ± 0.031
16	Guilin, Guangxi	GL	25.29	110.28	Jxfang2013-30	3.318 ± 0.033	2.446 ± 0.031	0.255 ± 0.048	0.119 ± 0.036
17	Rongjiang, Guizhou	RJ	25.94	108.50	Jxfang2013-33	0.641 ± 0.021	0.823 ± 0.026	0.128 ± 0.031	0.071 ± 0.024