Journal of Medicinal Plants Research Vol. 5(18), pp. 4549-4559, 16 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Comparison of genetic diversity in *Pogostemon cablin* from China revealed by RAPD, morphological and chemical analyses

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Accepted 29 July, 2011

The genetic diversity of 16 patchouli populations by random amplified polymorphic DNA (RAPD), morphology and chemical composition was presented in this paper. Polymorphism of RAPD primers and the percentage of polymorphic bands revealed with RAPD were 79.21 and 78.40%, respectively, which indicated a high level of genetic diversity existed among *Pogostemon cablin* (Blanco) populations. A dendrogram based on RAPD analysis showed most populations from the same or adjacent regions were classified together. High polymorphism in morphological parameters was found among populations, but did not reflect completely the differences by geographic location. According to the relative contents of nine compounds in *P. cablin*, the tested samples were divided into two main clusters, pogostone-type and patchouliol-type. A correlation between genetic and chemical polymorphism (r^2 = 0.816) was higher than that between genetic and morphological variations (r^2 = 0.629). High morphological and chemical variability as well as genotypic polymorphism provided ecological advantages that might explain the extensive distribution of *P. cablin*.

Key words: *Pogostemon cablin* (Blanco) Benth, genetic diversity, random amplified polymorphic DNA (RAPD), morphology, essential oil.

INTRODUCTION

Pogostemon cablin (Patchouli), a plant of the Lamiaceae family, is extensively cultivated in Indonesia, the Philippines, Malaysia, China, India and Brazil (Miyazawa et al., 2000; Singh et al., 2002; Wu et al., 2009). The aerial part of *P. cablin* has been used for the treatment of the common cold, headache, fever, vomiting, indigestion and diarrhea in China and its surrounding region ("Pharmacopoeia of the People's Republic of China", 2010). Patchouli from southeast Asia was introduced into China for perfume and medicinal purposes as early as the Liang dynasty or potentially earlier (Wu et al., 2007). Currently, Patchouli is widespread in southern China, including Guangdong (Guangzhou, Zhaoqing, Zhanjiang, etc.) and Hainan (Wanning and Haikou) Province. Due to its vast cultivation in different localities in China under varying environmental conditions, *P. cablin* populations have evolved diverse morphological characteristics that is, growth habit, maturity and surface texture (Luo and Zeng, 2002; Li et al., 2003). Due to this high diversity, commercially available seeds of *P. cablin* are divided into Paixiang (cultivated in Guangzhou), Zhaoxiang (cultivated in Zhaoqing), Zhanxiang (cultivated in Zhanjiang) and Nanxiang (cultivated in Hainan) (Wu et al., 2008).

Knowledge of genetic diversity has been successfully used for effective germplasm management and utilization, genetic fingerprinting and genotype selection (Fu et al., 2005). Various methods have been employed to estimate the genetic diversity. Fingerprinting with

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molecular markers based on genomic DNA nucleotide variations allows precise, objective and rapid population identification. Conducting molecular level investigation is important not only for understanding ecological and genetic consequences of populations, but also for the design and implementation of management strategies. The random amplified polymorphic DNA (RAPD) marker technique is a simple and cost effective way to detect nucleotide sequence polymorphisms using a single primer of arbitrary nucleotide sequence requiring no prior sequence information (Williams et al., 1990) and RAPD has been used extensively for classification of populations, identification of cultivars and diversity estimation (Archak et al., 2003; Ravishankar et al., 2004; Kafkas et al., 2006). Currently, by using RAPD marker, two studies have been reported on the genetic diversity in *P. cablin* species: One is that only five populations cultivated in different localities in China were analyzed by RAPD (Pan et al., 2006). The other is that some samples obtained in Guangdong province were studied utilizing RAPD labeling technique (Zhang, 2007). However, these samples or populations used in the previous two studies are relatively limited and incomplete.

The measurement of morphological trait is commonly used since it provides a simple technique of quantifying genetic variation while simultaneously assessing genotype performance under normal growing environments (Fu et al., 2008). Furthermore, morphological trait is a technologically and financially low demand system of evaluating populations. In previous studies, patchouli germplasm resources were identified with different methods such as the flower observations of *P. cablin* from three populations (Li et al., 2003) and qualitative description of ramification angle, old stem shape, growth habit, maturity, epidermis texture and leaf color of Paixiang and Nanxiang (Luo and Zeng, 2002). However, there were few reports that germplasm resources of *P. cablin* were systematically evaluated with quantitative agronomic parameters such as plant height, ratio of leaf length to leaf width, internodal length and petiole length.

Essential oil, commonly used to give a base and lasting character to a fragrance in the perfume industry, was obtained from the aerial part of *P. cablin*. Patchouli oil was very appreciated for its pleasant and long lasting odor (Deguerry et al., 2006; Betts, 1994). It also mainly contributed to the pharmacological activities (Hu et al., 2006) and the therapeutic properties of the essential oils were directly correlated with their qualitative and quantitative compositions which were obviously different and derived from various cultivation regions of Patchouli (Reverchon and Marrone, 1997). The main components of the essential oils extracted from *P. cablin* collected from Wanning City were patchouli alcohol (37.74%, relative content) and pogostone (0.85%) (Luo et al., 2002), While essential oil was mainly composed of patchouli alcohol (30.87%) and pogostone (18.90%) in the population from Guangzhou city (Li and Tang, 2007).

The present study was conducted to understand the genetic diversity of the various populations sampled from 16 representative populations by the analysis of 5 morphological traits, 9 main compounds of patchouli oil and RAPD marker. Our objectives were to: (i) compare RAPD, morphological, and chemical analyses for the suitability of the data sets for assessing genetic diversity in *P. cablin* populations and for the compatibility of the results, (ii) analyse the correlation among cluster results based on 5 morphological traits, UPGMA clustering results using RAPD marker, and cluster results based on the contents of 9 main compounds of patchouli oil, and (iii) explain the reasons of the extensive distribution of patchouli germplasm.

MATERIALS AND METHODS

Plant materials

From 2006 to 2008, we investigated the distribution pattern of *P. cablin* in southern China. According to the field survey information, a total of 16 populations (Wu et al., 2010) representing a wide geographic distribution, were sampled for DNA analysis. In each of the 16 populations, 12 individuals were collected randomly with about 5 g of young and clean leaves per plant. Since *P. cablin* is a clonal plant, in order to minimize collecting the ramet of the same clone, each individual sample from the same population was collected from locations at least 500 m apart. In total, 192 samples were collected and immediately placed in sealed plastic bags containing about 50 g of silica gel which sped up the drying process and samples were transferred to the laboratory for genomic DNA extraction.

DNA extraction

Total genomic DNA of every sample was extracted according to the CTAB method (Liu et al., 2008). DNA sample concentration was determined using a fluorometer employing a Hoechst dye (Hoefer Inc., San Francisco, CA, USA) and the DNA samples were diluted to a final concentration of 10 ng/µl with 1×TE buffer and stored at -20°C prior to polymerase chain reaction (PCR) amplification.

RAPD amplification and selection of optimum primers

One hundred and fifty primers [Invitrogen Biotech (Shanghai) Co., China] were initially screened in four representative samples from the 16 populations. Under the optimized condition, 48 primers generated strong amplified products. Then 21 primers which produced clear and reproducible bands were selected for further analyses (Tables 1 and 2). PCR amplification was performed in a total volume of 20 µl containing 1 × Taq buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 µM primer, 0.75 units of Taq DNA polymerase and 40 ng of template DNA. RAPD programmes involved an initial denaturation step of 5 min at 94°C, cycle denaturation at 94°C for 45 s, annealing at 36°C for 45 s, initial extension at 72°C for 90 s for 45 cycles with final extension at 72°C for 7 min. The PCR reactions were carried out in a PTC 200^{TM} programmable thermal controller (MJ Research, USA).The amplified products were resolved electrophoretically on 2.0% agarose gels run at 100 V in 1.0 × TBE buffer, visualized by staining with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light by bio-imaging system (Syngene, Genegenuis). The amplifications were repeated twice and only clear repetitive bands were used in data analysis

Table 1. Sequence and polymorphism of RAPD primers.

RAPD primer	Sequence (5′ to 3′)	Rb	Polymorphism		RAPD primer	Sequence (5′ to 3′)	Rb	Polymorphism	
			Pb	%				Pb	%
B-1	GTTTCGCTCC	9	8	88.89	AT-3	GACTGGGAGG	9	7	77.78
B-4	GGACTGGAGT	10	8	80.00	AW-5	CTGCTTCGAG	12	9	75.00
U-13	GGCTGGTTCC	11	10	90.91	AN-1	ACTCCACGTC	8	6	75.00
P-9	GTGGTCCGCA	8	7	87.50	AA-5	GGCTTTAGCC	10	8	80.00
G-4	AGCGTGTCTG	10	8	80.00	AK-16	CTGCGTGCTC	9	7	77.78
X-17	GACACGGACC	14	10	71.43	AN-3	AGCCAGGCTG	10	8	80.00
V-1	TGACGCATGG	11	9	81.82	AU-17	TTGGCATCCC	9	6	66.67
V-14	AGATCCCGCC	15	12	80.00	Z-18	AGGGTCTGTG	10	8	80.00
AI-5	GTCGTAGCGG	9	7	77.78	B-8	GTCCACACGG	9	8	88.89
AK-6	TCACGTCCCT	10	7	70.00	B-2	TGATCCCTGG	10	8	80.00
AH-3	GGTTACTGCC	10	8	80.00					
Total of all primers		213	169						
Mean of all primers		10.1	8.0	79.21					

Note: Rb represents No. of recorded bands; Pb represents No. of polymorphism bands.

Table 2. Genetic diversity of *P. cablin* among and between populations.

Population no.	RAPD					Population no.	RAPD				
	PPB (%)	Ao	Ae	He	Ho		PPB (%)	Ao	Ae	He	Ho
DM	27.70	1.28	1.20	0.11	0.16	ZW	49.30	1.49	1.39	0.21	0.30
CF	26.76	1.27	1.21	0.12	0.17	CQ	33.31	1.33	1.25	0.14	0.20
LJ	27.71	1.28	1.20	0.11	0.16	ZP	50.21	1.50	1.37	0.20	0.29
DA	26.77	1.27	1.18	0.10	0.15	LT	24.88	1.25	1.17	0.10	0.15
HD	20.19	1.20	1.12	0.07	0.11	GH	21.13	1.21	1.15	0.09	0.13
TS	33.33	1.33	1.25	0.14	0.20	HT	14.55	1.15	1.11	0.06	0.09
CY	50.23	1.50	1.39	0.21	0.30	LD	25.82	1.26	1.18	0.10	0.15
YL	33.33	1.33	1.25	0.14	0.20	LG	17.37	1.17	1.11	0.06	0.10
Population level	30.16	1.30	1.22	0.12	0.18						
Species level	78.40	1.78	1.32	0.21	0.33						

Note: PPB represents percentage of polymorphic band; Ao represents observed number of alleles; Ae represents effective number of alleles; He represents Nei's (1973) gene diversity; Ho represents Shannon's Information index.

and molecular weights were estimated using a DNA marker DL2000 (Takara Biotech Co., Ltd).

Morphological measurements

For each population, 12 branches (15 cm in length, including more than 3 nodes and 2 young leaves) were collected from the same 12 individuals as the ones for DNA extraction and propagated by cuttings in medicinal plant garden (Altitude = 5.8 m, East Longitude=110°19′28″, North Latitude = 20°3′29″), located in Hainan University, Haikou, China. Plants are grown under field conditions in a 1 × 1 m area in row and between rows with normal cultivation and management and a live germplasm collection of *P. cablin* was established. Populations of *P. cablin* collected from different locations were maintained together under similar conditions. Therefore, the germplasm offered the possibility to assess the genetic basis for the phenotypic variation. After being cut and cultivated for one year, the morphological traits of these ripe plants were measured and evaluated (Table 3).

Extraction of patchouli essential oil

For every population, the ripe aerial parts of *P. cablin* were collected from the same 12 individuals as the ones for morphological measurements. According to preliminary experiments, raw materials were dried at 35°C for 48 h and were ground into powder of mesh 40 in order to standardize the size of the particles. Patchouli essential oil was extracted from powder of 20.0 g with supercritical carbon dioxide (SFE-CO_2, Model Speed SFE-2, Applied Separations Inc., America) under the optimized conditions: pressure, 18.0 Mpa; temperature 40°C; extraction time 2.5 h. Extract was transferred to a 25 ml volumetric flask which was

Table 3. The morphological traits of samples.

Population no.	Plant height (cm)	*L.L.* / *L.W.*	Internodal length (cm)	Petiole length (cm)	Ramification angle (°)
DM	103.60±8.52	1.78±0.10	4.89±0.71	3.31±0.59	63.95±3.65
CF	101.45±7.74	1.70±0.11	5.93±0.84	3.44±0.60	60.55±5.61
LJ	87.65±2.60	1.68±0.07	5.61±0.75	3.33±0.55	61.10±4.71
DA	102.95±7.90	1.78±0.08	5.22±0.79	3.26±0.51	62.75±4.08
HD	90.30±10.31	1.72±0.06	5.63±0.68	3.44±0.42	62.25±4.74
TS	96.05±5.43	1.73±0.07	5.62±0.85	3.43±0.55	60.60±3.86
CY	109.30±4.03	1.81±0.09	5.49±0.55	3.40±0.59	64.75±6.04
YL	105.60±9.51	1.78±0.08	5.40±0.80	3.29±0.59	62.85±3.27
ZW	86.95±5.61	1.75±0.08	5.60±0.85	3.29±0.55	61.90±4.93
CQ	93.95±2.50	1.72±0.07	5.99±0.71	3.31±0.55	61.45±4.89
ZP	98.20±7.49	1.80±0.07	4.99±0.67	3.23±0.49	63.70±5.19
LT	85.15±4.00	1.38±0.09	5.45±0.72	3.24±0.55	55.40±5.27
GH	87.55±3.15	1.41±0.09	5.71±0.81	3.19±0.55	54.55±5.84
HT	91.40±4.03	1.40±0.07	5.79±0.80	3.41±0.52	53.60±3.27
LD	78.45±7.35	1.28±0.09	4.74±0.60	3.51±0.60	55.35±5.76
LG	77.05±3.25	1.24±0.07	4.39±0.64	3.36±0.55	55.05±6.21

Note: The data is presented as average of the ripe 12 individuals and represents mean ± standard deviation. L.L. / L.W: represents ratio of leaf length to leaf width.

brought up to its volume with extraction solvent and filtered through a 0.45 µm Econofilter (Agilent Technologies) prior to injection into the GC–MS system.

Gas chromatography- mass spectrometry (GC-MS)

GC-MS was performed with an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and an Agilent ChemStation software (Agilent Technologies, Palo Alto, CA, USA). Compounds were separated on a 30 m × 0.25 mm i.d. capillary column coated with 0.25 µm film. The column temperature was at 60°C for injection and held for 8 min temperature program began at 4°C min^{-1} to 120°C, then at 8°C min^{-1} to 250 °C and held for 5 min. The carrier gas was helium at a flow rate of 1.0 ml/min and the sample volume injected was 0.6 µl of the pure oil with a split rate of 1:100. The spectrometers were operated in electron-impact (EI) mode, the ionization energy was 70 eV, the scan range was 35 to 550 amu and the scan rate was 0.34 s per scan. The injector temperature was 250°C and the ionization, inlet source temperature was 230 and 270°C, respectively. The 9 components (β-patchoulene, caryophyllene, α-guaiene, seychellene, β-guaiene, δ-guaiene, α-patchoulene, patchouli alcohol and pogostone) in common established the characterisitic fingerprint of patchouli volatile oil (Guo et al., 2004). Thus, the relative contents were determined by using the calibrated GC-MS that the experimental procedures (Table 4), that is, the calibration curves, the stability and the recovery for the analytes, were validated.

Data analysis

The amplified fragments were scored for band presence (1) or absence (0) with all the populations studied. Jaccard similarity coefficients were performed by using the NTSYS-pc version 2.1 computer program package to construct a dendrogram using the unweighted pair group method (UPGMA) (Rohlf, 2000). The observed number of alleles (Ao), the percentage of polymorphic bands (PPB), Shannon's Information index (Ho), Nei's (1973) gene diversity (He) and effective number of alleles (Ae) were calculated using POPGENE 32 software and genetic diversity within and among populations was measured by PPB. The morphological data were standardized, prior to use in the calculation of genetic similarity and the Euclidean distance between the different populations. The statistical software package to construct a dendrogram using chemical or morphological data was the procedure cluster of SAS 9.1 for windows (SAS Institute Inc., USA). Correlation between each similarity matrix was estimated using Mantel's matrix correspondence test which yields a product moment correlation (r) that defines the relatedness between the two matrices (Mantel, 1967).

RESULTS

Polymorphism analysis by RAPD marker

Using 21 selected RAPD primers, a total of 213 bands were generated of which 169 bands were polymorphic. The number of recorded bands varied from 8 (primer AN-1 and P-9) to 15 (primer V-14) with an average of 10.1 per primer, the number of polymorphic bands varied from 6 (primer AN-1 and AU-17) to 12 (primer V-14) with an average of 8.0 per primer and the percentage of polymorphic bands tested ranged from 66.67% for primer AU-17 to 90.91% for primer U-13 with an average of 79.21% (Table 1). The size of the amplified fragments varied from 100 to 1000 bp.

A summary of the genetic data for 16 populations of *P. cablin* was given in Table 2. First, we examined the

Table 4. Relative contents (%) of investigated compounds in samples collected from the same 12 individuals as the ones for morphological traits.

Population no.	Patchouli alcohol	Pogostone	α-Patchoulene	β-Patchoulene	Caryophyllene	α-Guaiene	Seychellene	β-Guaiene	δ-Guaiene
DM	64.34±2.25	10.27±0.45	1.36±0.06	0.70±0.04	1.30±0.02	7.77±0.11	4.29±0.09	2.69±0.06	11.64±0.56
CF	61.10±2.28	12.27±0.39	0.77±0.04	0.54±0.02	0.75±0.03	5.88±0.13	3.36±0.06	2.24±0.07	10.15±0.24
LJ	61.77±1.59	13.87±0.62	0.89±0.03	0.60±0.04	0.87±0.02	5.04±0.08	2.76±0.09	1.89±0.06	7.95±0.11
DA	63.19±1.94	13.92±0.55	1.02±0.04	0.72±0.04	0.93±0.04	6.27±0.07	3.55±0.09	1.95±0.07	8.94±0.19
HD	61.98±2.36	13.83±0.63	1.22±0.06	0.59±0.04	1.14±0.02	6.54±0.11	3.05±0.08	1.83±0.05	9.46±0.22
TS	57.80±2.00	15.77±0.66	0.99±0.05	0.48±0.03	1.30±0.04	4.58±0.14	3.59±0.07	1.80±0.04	8.49±0.18
CY	63.35±2.49	13.97±0.62	0.97±0.04	0.75±0.04	1.27±0.01	6.91±0.12	2.81±0.05	1.87±0.05	10.60±0.54
YL	62.52±2.46	13.84±0.57	1.08±0.04	0.58±0.03	1.43±0.02	7.80±0.13	3.61±0.04	2.46±0.06	12.55±0.21
ZW	60.07±2.72	14.57±0.69	1.24±0.04	0.53±0.01	1.19±0.04	7.04±0.08	3.69±0.05	2.05±0.07	6.85±0.12
CQ	60.59±2.49	15.74±0.65	1.13±0.05	0.68±0.03	1.06±0.04	7.37±0.09	3.28±0.06	1.99±0.07	7.19±0.27
ZP	59.75±2.87	15.90±0.62	1.07±0.05	0.76±0.04	1.12±0.04	6.98±0.14	3.41±0.04	2.04±0.04	7.09±0.24
LT	47.49±2.03	27.29±1.27	0.26±0.01	0.29±0.02	1.50±0.06	0.67±0.03	0.60±0.02	0.78±0.02	0.32±0.01
GH	48.18±2.50	26.27±1.30	0.25±0.01	0.30±0.02	1.38±0.06	0.41±0.02	0.51±0.02	0.74±0.04	0.41±0.02
HT	50.64±2.20	25.58±1.32	0.25±0.01	0.28±0.01	1.44±0.02	0.52±0.03	0.41±0.02	0.72±0.03	0.34±0.01
LD	42.88±2.19	27.74±1.25	0.23±0.01	0.27±0.02	1.64±0.06	0.00±0.00	0.13±0.01	0.17±0.01	0.23±0.01
LG	43.83±3.04	27.31±1.25	0.19±0.01	0.16±0.01	1.52±0.06	0.12±0.01	0.00±0.00	0.14±0.01	0.00±0.00

genetic variation within each of 16 populations, respectively (12 individuals for each population) by RAPD markers. For calculating genetic data from the species level, the percentage of polymorphic bands (PPB) of *P. cablin* was 78.40%, the observed average number of alleles (Ao) was 1.78, the effective mean number of alleles (Ae) was 1.32, the mean Nei's gene diversity (He) was 0.21 and the mean Shannon's Information index (Ho) was 0.33. Genetic diversity varied among 16 populations with PPB values ranging from 14.55% in Hetai (HT) population to 50.23% in Chengyue (CY) population with an average of 30.16%. Four indexes of Ao, Ae, He and Ho also indicated that the CY population had the greatest variation (1.50, 1.39, 0.21 and 0.30, respectively), while the HT population showed less variation (1.15, 1.11, 0.06 and 0.09, respectively). The genetic variations of other populations were between HT and CY population. The genetic relationships among 16 populations of Patchouli were analyzed by an UPGMA method. According to the RAPD data, the dendrogram (Figure 1A) indicated that the 16 populations were distinctly separated into three major groups (Group I to III) at the Jaccard similarity coefficient level of 0.857. The Jaccard similarity coefficient between all the populations ranged from 0.580 to 0.976. Group I was composed of 11 *P. cablin* populations, which could be further separated into two sub-groups. All the 5 populations in the I sub-group came from Hainan province, including Damao (DM), Haidian (HD), Changfeng (CF), Dongao (DA) and Liji (LJ) population, and the II sub-group was composed of 6 populations of Tanshui (TS), Yingli (YL), Zhengwen (ZW), Zhangpu (ZP), Changqi (CQ) and CY. Group II consisted of 3 populations [namely, Liantang (LT), HT and GH population] which were geographically from Zhaoqing City. Group III includes the rest of these two populations.

Morphological characteristics

Plant height ranged from 77.05 cm [Luogang (LG) population] to 109.30 cm (CY) and the ratio of leaf length to leaf width ranged from 1.81 (LG) to 1.24 (CY) for the same populations as the ones for plant height (Table 3). Population CQ had the longest internodal length (5.99 cm), while LG was shortest (4.39 cm). Petiole length ranged from 3.19 (GH) to 3.51 cm [Longdong (LD) population],

and ramification angle was from 53.60 (HT) to 64. 75° (CY) with an average of 59.99° (Table 3). Cluster analysis of the morphological data grouped the 16 populations into three main cluster groups (r^2 = 0.621, Figure 1B) at 0.4. Two populations, LG and LD, coming from the suburb of Guangzhou City were found in the Group I.

Group II was composed of three populations of HT, GH and LT, of which LT and GH were closely clustered together coming from the suburb of Zhaoqing City. Group III was composed of 11 populations, which could be further separated into two sub-groups. The first sub-group included branch 1 (namely, ZP population) and branch 2(CQ, TS, HD, ZW and LJ population). The second sub-group was consisted of other five populations (CY, CF, YL, DA, DM population).

Chemical composition of essential oils

According to GC-MS results of the essential oils by SFE-CO_2 from the ripe aerial parts of *P. cablin*, patchoulene, caryophyllene, guaiene, seychellene, patchouli alcohol and pogostone were the major compounds and the relative contents were seen Table 4. Population DM had the maximum in the patchouli alcohol, α-patchoulene, seychellene and β-guaiene and their value was 64.34, 1.36, 4.29 and 2.69%, respectively. The maximum of pogostone, β-patchoulene, caryophyllene, α-guaiene and δ-guaiene was presented to population LD (27.74%), ZP (0.76%), LD (1.64%), YL (7.80%) and YL (7.80%), respectively. The minimum value of the 9 compounds had not presented regularity (Table 4). Cluster analysis (Figure 1C) divided the 16 populations into two main groups according to their contents of 9 compounds (r^2 = 0.949). The first Group (I) was characterized by high contents of caryophyllene (1.38 to 1.64%), pogostone (25.58 to 27.74%) and low contents of the rest compounds.

Group I was further divided into two subgroup I (including LG and LD population) and II (HT, GH and LT population). The second main group (II), characterized by low contents of caryophyllene (0.75 to 1.43%), pogostone (10.27 to 17.91%) and high contents of the rest compounds, was composed of 11 *P. cablin* populations divided into two subgroups. The first subgroup was YL and DM populations clustered together and the rest 9 populations were turned into the second subgroup. In order to detect reliably and completely phenotypic variation among 16 populations, the combinations of morphological traits and investigated compounds were employed in this study.

It also indicated that 2 populations (LG and LD) from the suburb of Guangzhou City were stapled together, HT, GH and LT populations coming from Gaoyao City were found in the same group, and the rest populations were clustered together at 0.4 from Figure 1D. This conclusion was the same as the ones gained by using the RAPD marker.

DISCUSSION

Genetic diversity among and within populations

By using RAPD marker, this study showed that there was a high level of genetic diversity among populations and low level of genetic variations within populations of *P. cablin* from China. Similar conclusions were obtained when ISSR and SRAP markers were applied to DNA fingerprinting and genetic diversity analysis of *P. cablin* (Wu et al., 2010). Between 16 populations, the percentage of polymorphic bands detected by RAPDs was 78.40%, but within 16 populations, the mean of PPB was only 30.16% (Table 2). These analyses showed that most of the genetic variations were partitioned between populations rather than within populations. This result was consistent with previous studies on *Oryza granulata* by Wu et al. (2004). This might have been due the possibility that the chosen populations lived in the different regions for rather a long time and were seldom transplanted. In general, long introduced populations had higher genetic diversity than those introduced only a few times. Patchouli had been introduced into China and planted in the different regions for rather a long time (Wu et al., 2007). It adapted differently to selection pressures imposed by the distinctive environmental conditions, which included phenotypic plasticity responding to new habitats, competitive ability increased by rapid clonal growth and a strong tolerance to environmental stresses, in accordance with the idea of Singh et al. (1998). Each well-adapted ecotype, which was resulted from a long process of selection, represented a conservation entity of different genes. This might contribute to its high genetic diversity.

The insights gained from exploring the genetic diversity of patchouli populations were important in developing a sound program for its conservation and utilization. The results from this study indicated that there was a high level of genetic diversity between populations but a low level genetic variation within populations (Table 2). Therefore, we suggest that *P. cablin in situ* conservation areas for core populations of low genetic diversity is immediately established. According to our field survey, some populations' genetic diversity was degraded due to consecutive asexual propagation by cuttings leading to serious influences on the stability of medicinal materials. So, it is imperative to protect these populations of *P. cablin*, particularly in populations of low polymorphism.

Cluster analysis and geographic distribution

All populations in this study were collected from Hainan and Guangdong province of the southern China. According to their geographic distribution, populations

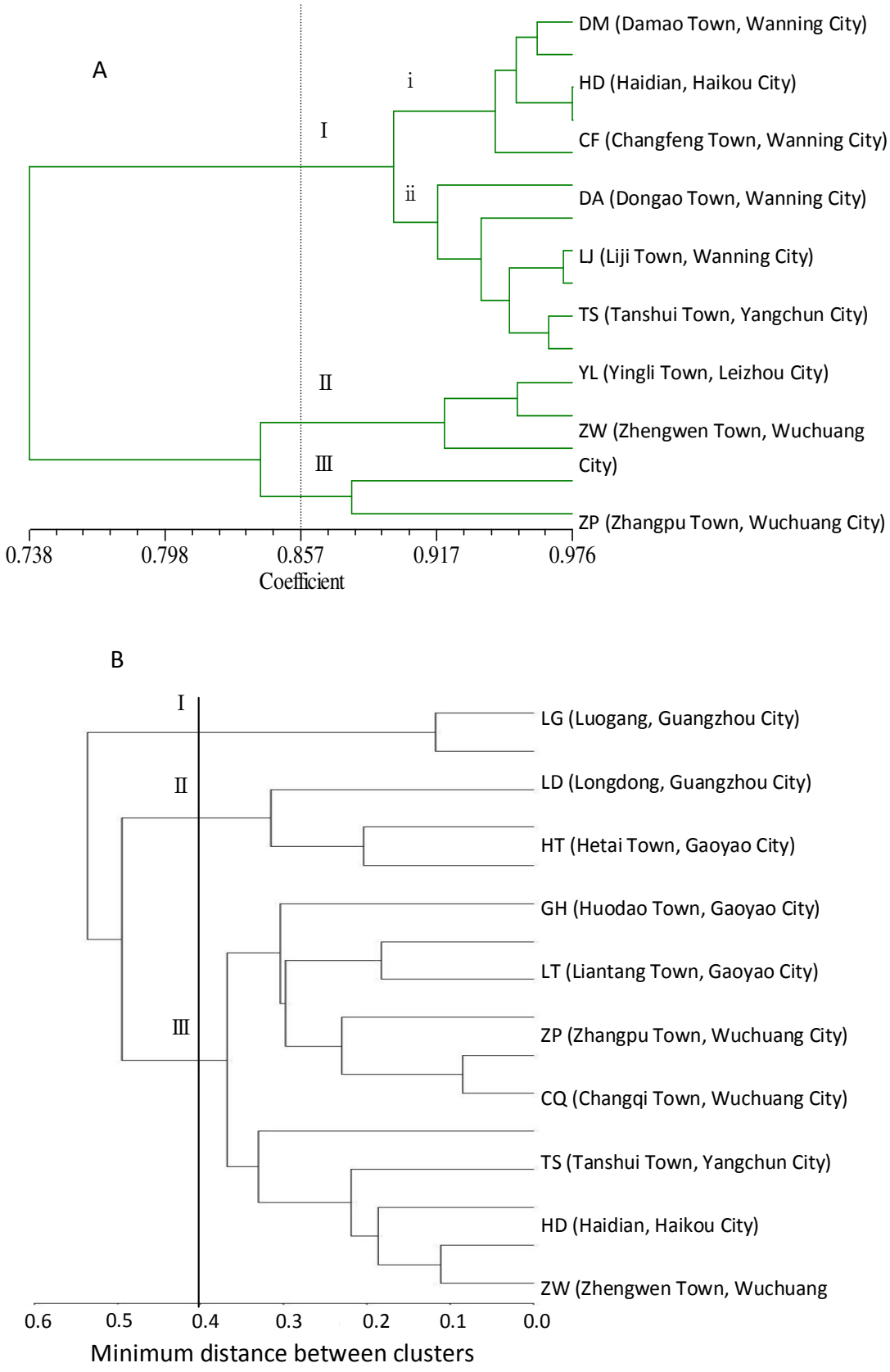
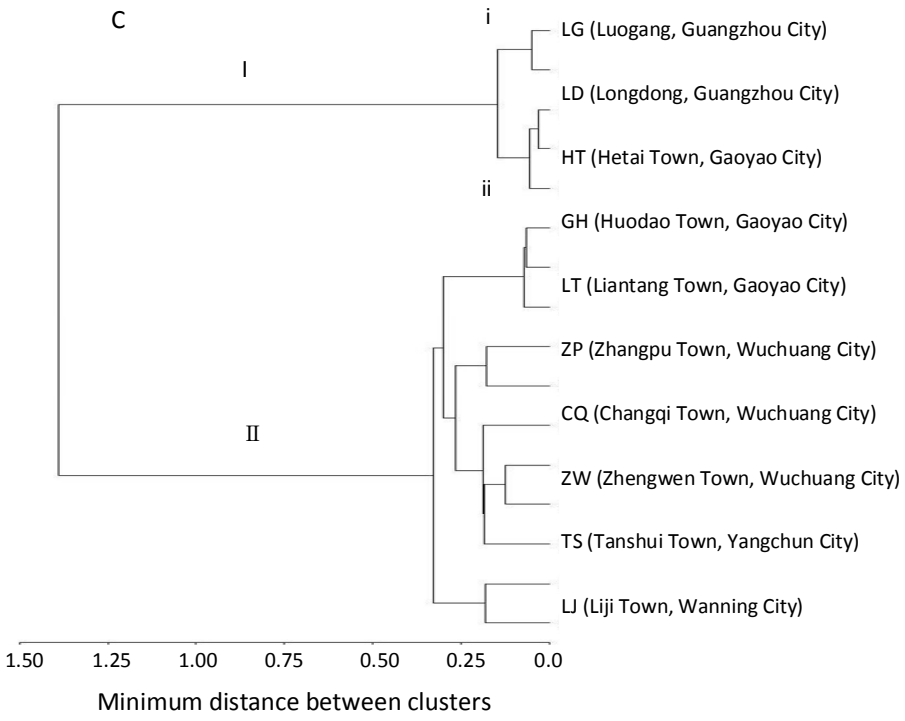


Figure 1. UPGMA dendrogram of *P. cablin* based on RAPD markers (A), morphological traits (B), investigated compounds (C) and combination of morphological traits and investigated compounds (D).



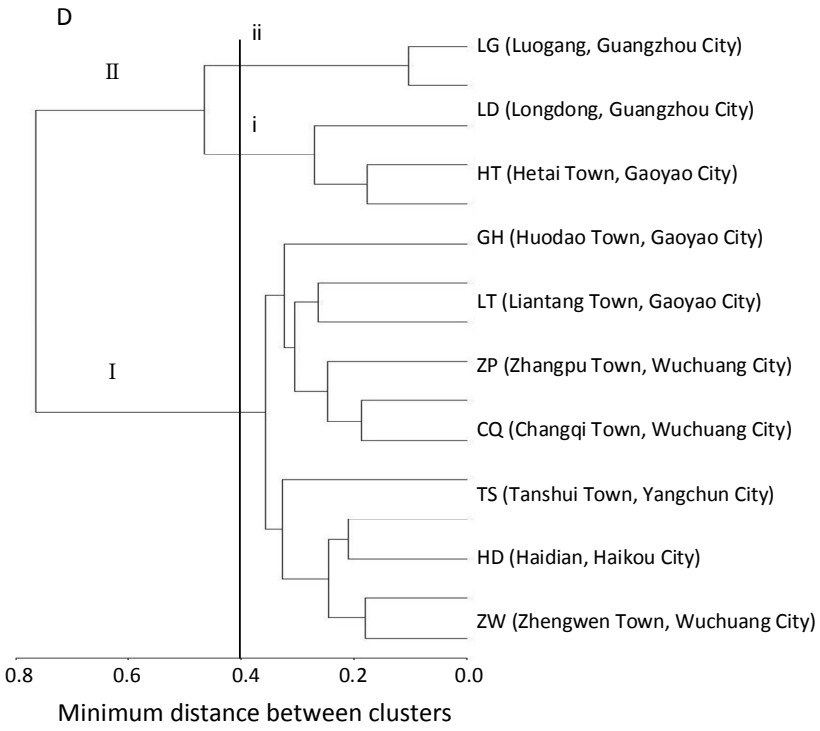


Figure 1. Contd.

were divided into four traditional types, that is, Nanxiang, Zhanxiang, Zhaoxiang and Paixiang in China. It was not difficult to find that most populations from the same or adjacent regions were classified together from the UPGMA clustering results using RAPD marker. There was an indication that the clustering results were consistent with the geographic distributions of the populations and the cluster analysis clearly separated

four types of *P. cablin* from each other (Figure 1A). This result showed that RAPD marker was suitable for assessing genetic diversity in *P. cablin* populations.

Morphological and chemical analyses

Sometimes, utilizing only one method for evaluating the genetic relationships among and between populations leaves a lot of questions. Investigating morphological traits and determining the relative contents of essential oil was labor-intensive and the phenotypic plasticity of plants made environmental variation a major problem (Van Beuningen and Busch, 1997), but it could result in more comprehensive and reliable analysis of genetic diversities.

Phytochemical and morphological characters showed high phenotypic variability existed among studied populations, but this did not entirely reflect the differences by geographic location (Figure 1D). On the basis of the relative contents of 9 compounds, cluster analysis divided the tested 16 samples into two main groups according to what appears to be chemotypic groups rather than to their geographic origin (Figure 1C). Our study showed that herbs from Guangzhou and Gaoyao City of Guangdong Province had high content of pogostone and low content of patchouli alcohol, while high content of patchouli alcohol and low content of pogostone were included in the *P. cablin* material from Leizhou, Zhanjiang, Wuchuan and Yangchun regions of Guangdong Province and Haikou and Wanning region of Hainan Province. From the results obtained by Principal Component Analysis, Group I had high contents of pogostone (pogostone-type), while Group Ⅱ was characterized by high contents of patchouli alcohol (patchouliol-type). This was similar to the viewpoint of Luo et al. (2003) and Guo et al. (2004).

By comparison, the compounds and their relative contents from the plants in the present study were slightly different from the previous reported results (Huang et al., 2001; Wang and Fu, 2000; Luo et al., 2002; Li et al., 2007). Different extraction and determination methods of patchouli oil might be the cause for the observed differences. However, the effect of environmental conditions on phenotypic plasticity was also likely. The effect of environmental stress on chemical composition of essential oil had been reported in several studies. Llusia and Penuelas (1998) reported that under drought conditions total terpene concentration in *P. lentiscus*, grown under irrigated conditions, was increased by 21.3% compared to control. Nutrient deficiency (phosphorus and/or nitrogen) enhanced allocation of fixed carbon into the biosynthesis of essential oils in *Rosmarinus officinalis* and *Lavandula latifolia* (Ross and Sombrero, 1991). Therefore, it was possible that the category and contents of some compounds had taken place various changes under the environmental conditions, different from their origins, in our germplasm garden of Hainan University, Haikou. This assumption was consistent with the conclusions of Li et al. (2004).

Genetic, morphological and chemical variations in *P. cablin*

Cluster results between *P. cablin* populations provided by three methods were partially different in our study (Figure 1A, B and C). This difference might be related to phenotypic plasticity of the plants in response to changes in the habitat environment. This relevant aspect of this study showed that genomic similarity did not necessarily reflect similarity or difference in output traits, such as contents of oil composition, morphological characters or agronomic traits. For example, populations DM and HD were a little different in their morphological traits but genetically very similar (Table 3 and Figure 1A and B). Based on the findings of this study, RAPD dendrogram might be used for taxonomic studies, while dendrograms based on quantitative agro-morphological traits and chemical characteristics, might be of practical interest, but did not necessarily correlate with taxonomy. DNA genotyping offered the unique capacity to classify populations regardless of environmental condition and plant growth stage. Morphological characters, which were the easiest to determine, might only provide a primary classification. Morphological traits were found to be the least effective genetic markers and the relative effectiveness of the three systems as markers for genetic diversity was RAPD marker > chemical characteristics > morphological traits. In our study, a higher correlation was obtained between genetic and chemical polymorphism (r^2 = 0.816) rather than between genetic and morphological variations (r^2 = 0.629).

The present study was conducted with the aim of assessing levels of diversity of the patchouli populations by the analysis of 5 morphological traits, 9 main compounds of patchouli oil and RAPD marker. A high level of genetic diversity existed among *P. cablin* populations by using RAPD marker (Tables 1 and 2). This finding was in good agreement with earlier reports by Pan et al. (2006) and Zhang (2007). Wu et al. (2010) reported that high diversity of the patchouli germplasm was found among populations by using ISSR and SRAP markers. This further demonstrated the conclusions in this paper were correct. High polymorphism was also revealed among studied populations by analyzing morphological parameters or main compounds of patchouli oil (Tables 3 and 4). The results demonstrated that these three analysis systems could be useful for identification and diversity analysis of *P. cablin*. In addition, high morphological and chemical variability as well as genotypic polymorphism might contribute to the wide distribution of *P. cablin* around the southeast Asia, which was consistent with the current situation that patchouli had been extensively cultivated in Indonesia, the Philippines, Malaysia, China, India and so on. In

conclusion, our report was the first time to use RAPD, morphological and chemical analyses for evaluating genetic diversity in *P. cablin*. There was a high level of genetic diversity among populations and low level of genetic variations within populations of *P. cablin* from China. The clustering results using RAPD marker were consistent with the geographic distributions of the populations. High morphological polymorphism and chemical variability were found among populations. On one hand, these natural biodiversity could be exploited for breeding programs of *P. cablin* such as the identification and selection of populations (chemotypes) with active compounds (patchouli alcohol and pogostone). On the other hand, high morphological and chemical variability as well as genotypic polymorphism provided ecological advantages that might explain the extensive distribution of *P. cablin* around the southeast Asia.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Natural Science Foundation of China (30960533), the Natural Science Foundation of Hainan Province (310022 and 30814), the College Science and Research Programme of Education Department of Hainan Province in 2009 (Hjkj2009-25), the Science and Research Programme of Hainan University in 2009 (hd09xm58), the Science and Research Programme of Hainan University (kyqd1002).

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