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Full Length Research Paper

Genetic characterization of the nine medicinal *Dendrobium* species using RAPD

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The nine species of *Dendrobium* were distinguished from each other by the banding pattern generated by the sixteen 10-mer oligonucleotide primers in the random amplified polymorphic DNA (RAPD) reaction. Distinctive bands generated from the nine species were observed. RAPD analysis was also applied to estimate the genetic relationship among the nine species. A dendrogram was constructed based on a data matrix of 323 polymorphic bands originated by the sixteen random primers. Four groups were identified, one consisting of *Dendrobium huoshanense* and *Dendrobium loddigesii*, the second consisting of *Dendrobium bellatulum* and *Dendrobium fimbriatum*. The third cluster contained *Dendrobium candidum*, *Dendrobium densiflorum* and *Dendrobium exile*. The rest were grouped in the fourth cluster. The principal coordinate analysis (PCA), that is, the plot drawn on the basis of the RAPD data, clearly distinguished the nine species into four groups, which also support the notion in the dendrogram described above.

Key words: DNA fingerprinting, genetic relationship, *Dendrobium*, RAPD, RAPD marker, species identification.

INTRODUCTION

Dendrobium, a perennial plant belonging to the orchid family, is mainly distributed in China, Japan, Thailand and other Southeast Asian countries. Among them, there are more than thirty species not only used for traditional patent medicine production such as *Mailuoning Injection*, but also used for the health care foods production such as *Fengdou* and *Fengdoujing* (Chinese). According to the pharmacopoeia of People's Republic of China, it is a herb for shortness of cataract, peripheral obstruction and chronic superficial gastrists (The State Pharmacopoeia Commission of People's Republic of China, 2000). Recent pharmacological studies have also shown that it can enhance general body immunity, scavenge active-oxygen and inhibit platelet aggregation (Chen and Guo, 2000; Zha et al., 2007a,b). As is well known, significant differences, both in quality and quantitative, in the chemical composition of different species and its corresponding health care activity exist in different species. Nevertheless, due to the similar distinctive morphology and the subsequent morphology of dry materials, that is, fengdou (chinese), prone to alternation by various processing method, their replacement is always seen in market. Therefore, to improve their use and enhance the reproducibility and the efficacy of commercial products, it is urgent to establish a better identification method.

Traditional morphological inspection and histological method provide little help, as some species from different localities are similar in their morphologies and anatomical features. Although physico-chemical methods have been applied to some medicinal plants (Sloley et al., 2001; Bauer et al., 1988), it is difficult to distinguish *Dendrobium* species, due to the little research on components in *Dendrobium*. Recently, genetic polymorphism in many plant genera has been documented by use of various fingerprinting systems including the analysis of isozymes, restriction fragment length polymorphism (RFLP), AFLP, SSR and random amplified polymorphic DNA (RAPD), etc. Results showed that analysis of isozymes and RFLP revealed relatively little polymorphism (Keizo et al., 2000). The disadvantages of complex procedures and expensive costs strongly restrict the application of AFLP

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Abbreviations: PCA, Principal coordinate analysis; **RAPD**, Random amplified polymorphic DNA.

Table 1. Source of nine *Dendrobium* species in China.

Sample	Common name	Locality
D_1	*D. huoshanense*	Anhui
D_2 [a]	*D. nobile*	Yunnan Sichuan Hainan
D_3 [a]	*D. candidum*	Yunnan Anhui Zhejiang
D_4	*D. densiflorum*	Yunnan Hainan Guangxi
D_5	*D.exile*	Yunnan
D_6 [a]	*D. fimbriatum*	Yunnan Guizhou Guangxi
D_7 [a]	*D. loddigesii*	Guizhou Guangxi Xizhang
D_8	*D. lohohense*	Chongqing Hubei Guangdong
D_9	*D. bellatulum*	Guizhou

[a]Species of *Dendrobium* listed in the Pharmacopoeia of People's Republic of China

and SSR. By contrast, RAPD developed by Williams et al. (1990) not only can reveal high levels of polymorphism, but also has advantages of speed, low cost and requirement for only minute amounts of plant materials. This method has been successfully used in identification of many medicinal plants (Zhang et al., 2001; Cheng et al., 1998; Zhang et al., 1999), and can be applied for differentiation of herbal medicine. Nine species of *Dendrobium* as the popular materials in China listed in Table 1 were identified by RAPD in the present work. Furthermore, the genetic relationships were also analyzed by the resultant DNA fragments using the software of Phylip 3.5C and MVSP.

MATERIALS AND METHODS

Plant materials and preparation of template DNA

The nine species of *Dendrobium* used and the sources of specimens are shown in Table 1. Among these, four species were noted in the Pharmacopoeia of Peoples' Republic of China (Table 1) and the rest were all the traditional medicinal herbs in China. Young leaves were harvested from wild samples and stored at -20°C until use.

Total genomic DNA was isolated from the stored leaves with the modified CTAB procedure (Tel-Zur et al., 1999). The isolated DNA was dissolved in 50 µl TE buffer. The quality and quantity of DNA extracted were estimated by eletrophoresis on agarose gel (1.2%) and by UV spectrophotometry, respectively. The concentration of extracted DNA was adjusted to 10 - 20 ng/µl and samples were stored at -20°C.

DNA amplification

RAPD assays were carried out in 50 µl reaction mixture containing template 1 µl DNA (10 - 20 ng), 25 µl of Taq PCR Master Mix (Takara Biotechnology, Dalian, China) (1.25 U of Takara Ex Taq, 2×Ex Taq buffer and 0.4 mM dNTP Mixture), 1 µl primer mixture (Kit J and I: primers OPJ1-20 and OPI1-20, 5 pM, Operon) and distilled water up to 50 µl. The sequences of the primers applied are given in Table 2. Amplification was performed in a GeneAmp PCR system 2400 (P.E., USA). DNA amplification was obtained as follows: 94°C for 10 min, 45 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, followed by one cycle of 72°C for 10 min. The amplified DNA products were resolved on 1.2% agarose gel, visualized by ethidium bromide staining, and photographed under UV light.

Data collection and analysis

RAPD bands were scored from photographs into a binary data matrix as 1 (present) or 0 (absent). The genetic relationship among the species was estimated using the similarity index (S) proposed by Nei and Li (1979):

$$S = 2 N_{AB} (N_A+N_B)^{-1}$$

Where N_{AB} is the number of common bands shared by species A and B, N_A the number of bands in species A and N_B the number of bands in species B.

The similarity matrix was processed by the NEIGHBOR program on PHYLIP 3.5C (Felsenstein, 1993). Genetic distance estimates based on pair-wise comparisons served as elements in the proximity matrix in a cluster analysis by UPGMA and plotted with the use the DRAWGRAM program on PHYLIP 3.5C. The resulting clusters were represented as a dendrogram. To evaluate the strength of the resulting branches, bootstrap probabilities were calculated using 1000 bootstrap resampling data with the program free-tree. The RAPD data were also evaluated by principal coordinate analysis (PCA) as an average distance, using the MVSP package (Kovach, 1999).

RESULTS AND DISCUSSION

RAPD analysis was used to discriminate the nine *Dendrobium* species and their genetic relationships were further analyzed according to the resultant RAPD markers. The PCR reactions were repeated three times for each of the nine samples and the resultant DNA bands were highly reproducible (data not shown). Results showed that each species collected from different localities seemed similarity in RAPD profiles by using different primers. Therefore, one sample of each species was selected to do the following experiments. Sixteen random primers that we used generated a total of 340 RAPD fragments containing 17 species-specific fragments and 323 variable bands from the nine species (Table 2). Each primer gave 14 ~ 29 bands and the average was 21.3. One (1700 bp), one (1900 bp) and one (3000 bp) species-specific fragments from the nine *Dendrobium* species were generated by Primer OPI-12.

Table 2. The sequences of oligonucleotide primers used for RAPD analysis and banding patterns obtained from the nine species of *Dendrobium*.

Primers	Sequences	T	V	P (%)	Distinctive markers								
					D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
OPI01	ACCTGGACAC	15	14	93.3	0	0	0	0	1	1	0	2	1
OPI02	GGAGGAGAGG	28	27	96.4	1	0	1	0	3	2	0	0	1
OPI03	CAGAAGCCCA	23	22	95.7	0	1	1	0	1	0	0	0	0
OPI04	CCGCCTAGTC	24	23	95.8	1	1	1	0	0	1	0	1	0
OPI06	AAGGCGGCAG	25	24	96.0	1	1	2	2	0	1	0	1	0
OPI07	CAGCGACAAG	21	20	95.2	1	1	1	1	1	0	2	1	1
OPI09	TGGAGAGCAG	17	16	94.1	1	0	0	0	1	2	1	2	1
OPI10	ACAACGCGAG	14	13	92.9	0	0	2	0	1	1	1	1	0
OPI11	ACATGCCGTG	21	19	90.5	0	0	1	0	2	0	0	2	1
OPI12	AGAGGGCACA	29	26	89.7	0	0	0	0	0	0	1	0	2
OPI13	CTGGGGCTGA	23	22	95.7	0	0	0	1	3	1	1	0	0
OPI14	TGACGGCGGT	19	18	94.7	0	0	0	0	1	2	0	0	3
OPI16	TCTCCGCCCT	18	18	100.0	2	0	0	2	1	0	0	1	0
OPI17	GGTGGTGATG	26	26	100.0	2	0	3	3	1	2	1	1	2
OPI20	AAAGTGCGGG	14	13	92.9	3	0	1	2	0	0	0	1	1
OPJ20	AAGCGGCCTC	23	22	95.7	2	2	1	0	0	0	0	0	0
Total		340	323	95.0	14	6	14	11	16	13	7	13	13

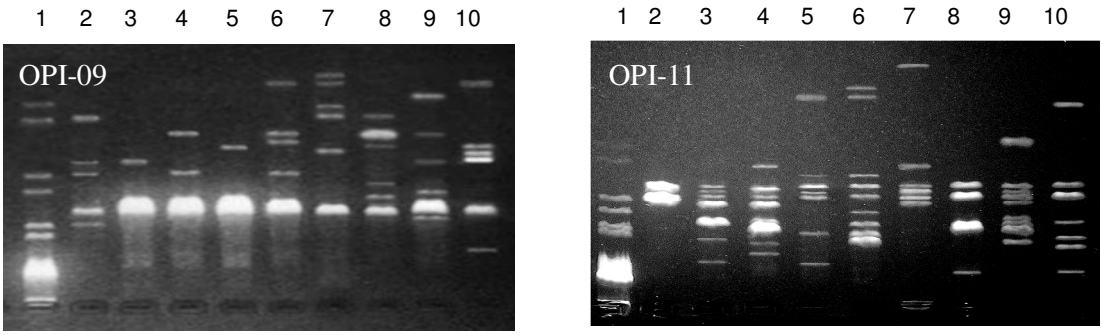

Figure 1. RAPD fingerprint of the nine *Dendrobium* species obtained with OPI-09 (left) and OPI-11 (right). Lane 1: λDNA/EcoRI + HindШ marker; Lane 2: *D. huoshanense*; Lane 3: *D. nobile*; Lane 4: *D. candidum*; Lane 5: *D. densiflorum*; Lane 6: *D. exile*; Lane 7: *D. fimbriatum*; Lane 8: *D. loddigesii*; Lane 9: *D. lohohense*; and Lane 10: *D. bellatulum*

Among the total DNA bands, some distinctive bands generated by the 16 primers were found in every species where 14 bands were for *Dendrobium huoshanense*, 6 for *Dendrobium nobile*, 14 for *Dendrobium candidum*, 11 for *Dendrobium densiflorum*, 16 for *Dendrobium moniliforme*, 13 for *Dendrobium fimbricatum*, 7 for *Dendrobium loddigedii*, 13 for *Dendrobium lohohense* and 13 for *Dendrobium bellatulum*, respectively. These RAPD markers had high diagnostic value since they were highly distinctive for each species and absent from other species, with no exceptions. Representative fingerprints generated from OPI-09 and OPI-11 is shown in Figure 1.

The banding patterns generated by the sixteen primers were scored in the form of a binomial matrix and were used to calculate the similarity index values of the DNA fingerprints according to Nei and Li (1979). The similarity index is shown in Table 3. Among the nine *Dendrobium* species, the range of genetic similarity was from 0.5614 to 0.7047. Although the two species of *D. nobile* and *D. lohohense* were more closely related with a genetic similarity of 0.7047, the difference existing between them. *D. densiflorum* and *D. lohohense* were more divergent and had a genetic similarity of 0.5614. A dendrogram was constructed according to UPGMA cluster analysis by using the Neighbor-joining method (NJ) (Figure 2). Based on the dendrogram, the nine species could be clearly grouped in four clusters. *D. huoshanense* and *D. loddigesii* were grouped with *D. candidum* in cluster I. *D.*

Table 3. Pair-wise similarity matrix (Nei and Li, 1979) of the nine species of *Dendrobium* (see Table 1) studied.

Sample	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
D_1	****								
D_2	0.6550	****							
D_3	0.6404	0.6813	****						
D_4	0.6287	0.6170	0.6491	****					
D_5	0.5819	0.6404	0.6433	0.6667	****				
D_6	0.6404	0.5819	0.6082	0.6550	0.5965	****			
D_7	0.6637	0.6520	0.6491	0.6433	0.6257	0.6491	****		
D_8	0.5877	0.7047	0.5965	0.5614	0.5906	0.5673	0.6550	****	
D_9	0.6257	0.6082	0.6520	0.6754	0.6228	0.6813	0.6637	0.6111	****

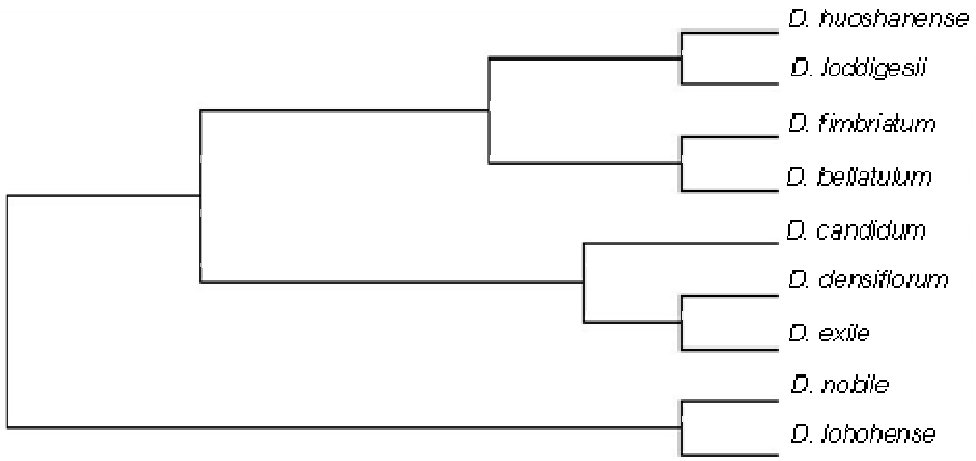

Figure 2. Dendrogram constructed according to UPGMA cluster analysis, based on the similarity index of Nei and Li (1979), showing the genetic relationships among the nine species of *Dendrobium*.

bellatulum and *D. fimbriatum* were grouped in cluster. The third cluster contained *D. candidum*, *D. densiflorum* and *D. exile*. The rest were grouped in the fourth cluster. In line with the dendrogram, four clear groups were also distinguishable by PCA analysis, with the two species in cluster I, two in cluster II, three in cluster III and two in the third cluster, respectively.

Quality control is one of the key issues in the modernization of Chinese medicine. However, the existing traditional methods of authentication by morphological and histological approaches are not effective for differentiating dry materials of different species in the phytopharmaceutical market, as they have similarity morphologies by different processing method or distinctive similarities. The chemical fingerprint is also not always reliable as the difference in the chemical profiles can be due to many non-genetic factors such as age and processing methods. The present work indicates that RAPD technology is an effective method to differentiate *Dendrobium* species. The genetic variation and dendrogram constructed by this method in this report may also demonstrate the genetic relationships among the nine *Dendrobium* species. The RAPD technique has been applied to some research aspects, such as plant species identification (Zhang et al., 2001; Zhang et al., 1999), diversity assessment (Jarret and Austin, 1994), detection of genetic variations in N fertilizer-treated plants (Lo et al., 2004), detection of intraclonal variations (Villordon and LaBonte, 1995), and estimation of genetic diversity (Sagredo et al., 1998). The RAPD approach described in this paper is highly sensitive and reproducible, and as little as 10 - 20 ng DNA template is sufficient for analysis. Currently, the more other herbal Chinese medicinal materials belong to orchid are being

defined by this approach.

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