Industrial Crops & Products 112 (2018) 569–576

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Conventional octaplex PCR for the simultaneous identification of eight mainstream closely related *Dendrobium* species

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ARTICLE INFO

Keywords:
Multiplex PCR
Species-specific
Dendrobii caulis
Molecular identification
Authenticity

ABSTRACT

As one of the most valuable medicinal genus in folk medicine and industrial crop, *Dendrobium* is cultivated extensively in south of China with distinct species. Pharmacological functions vary greatly among different *Dendrobium* species but their identification is somewhat difficult since they do not have unsophisticated method to distinguish the given species in an admixture. In the present study, an octaplex PCR assay was developed for rapid and reliable identification of eight mainstream, closely related *Dendrobium* species or their admixture consisting of *D. devonianum*, *D. aphyllum*, *D. strongylanthum*, *D. officinale*, *D. nobile*, *D. chrysotoxum*, *D. huoshanense,* and *D. fimbriatum*. The optimized multiplex PCR in this study utilized one specific primer derived from chloroplast *trnL-F* region and seven specific primer pairs from internal transcribed spacer sequences. Multiplex PCR yielded products of 148 bp, 210 bp, 265 bp, 340 bp, 397 bp, 448 bp, 491 bp, and 584 bp amplicons in the presence of *D. fimbriatum*, *D. huoshanense*, *D. chrysotoxum*, *D. nobile*, *D. officinale*, *D. strongylanthum*, *D. aphyllum*, and *D. devonianum*, respectively. The multiplex PCR approach was validated in 242 *Dendrobium* specimens from different production areas. As the results were species specific, the botanical origin of 20 samples of commercial Dendrobii caulis could be identified. These results show that the developed multiplex PCR assay provides a rapid and reliable approach for routine terminal market control of Dendrobii caulis.

1. Introduction

The substitutes of closely related species in herbal medicinal or herbal dietary supplements are difficult to discriminate because they share similar morphological appearances, textures, chemical or microscopic characteristics (Jiang et al., 2016). Dendrobii caulis (Shihu), is a common herb derived from the stem of any among several closely related species of the genus *Dendrobium* sw., traditionally used in folk medicine. As one of the most valuable medicinal genus in folk medicine and industrial crop, *Dendrob*ium is cultivated extensively in south of China with distinct species, particular in tissue culture industrials. According to the Pharmacopoeia of the People's Republic of China (2015 ed.), three cultivated species, *D. nobile*, *D. chrysotoxum*, and *D. fimbriatum*, are used as the authorized sources of Dendrobii caulis, while another *Dendrobium* species, *D. officinale,* is used to obtain Dendrobii officinalis caulis. A few other species, particularly *D. devonianum*, *D. aphyllum*, *D. strongylanthum,* and *D. Huoshanense,* have also been commonly employed as sources of Dendrobii caulis in various herbal commodity markets in China (Wu et al., 2009; Ma et al., 1991). Based on the processing method and morphological characters, Dendrobii caulis are classified as "Fengdou" or "Huangcao"; four twisted stems, namely "Tiepi Fengdou" (*D. officinale*), "Huoshan Fengdou" (*D. huoshanense*), "Zipi Fengdou" (*D. devonianum*) and "Shuicao Fengdou" (*D. aphyllum*), belong in the first category, whereas other yellow processed stems are called "Huangcao Shihu" (*D. nobile*, *D. strongylanthum, D. chrysotoxum*, and other related species), belong in the second one. The biological activities and pharmacological actions of Dendrobii caulis vary greatly between the two different categories. As an example, *D. huoshanense* and *D. chrysotoxum* exhibit stronger antioxidant and free radical scavenging activities, whereas *D. fimbriatum* considerably relieves the symptoms of Sjögren's syndrome (Lam et al., 2015). Precise identification of Dendrobii caulis at the species level is critical to guarantee therapeutic efficacy and medicinal safety. However, almost all commercial Dendrobii caulis originate from closely related *Dendrobium* species. Commercial samples often share similar textures, chemical and microscopic characteristics after processing and preparation as decoction pieces, which makes each, hard to distinguish from the rest by traditional analytical methods.

Molecular techniques have been used to differentiate among closely related species (Xu et al., 2006; Xu et al., 2012), thus improving the

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https://doi.org/10.1016/j.indcrop.2017.12.048
Received 4 September 2017; Received in revised form 14 December 2017; Accepted 19 December 2017
Available online 04 January 2018

accuracy of identification of *Dendrobium* species. Several DNA identification techniques, including DNA sequencing (Lau et al., 2001), allele-specific PCR (Ding et al., 2003), real-time PCR (Lam et al., 2015) and DNA barcoding (Feng et al., 2015) have been used for authentication of botanical *Dendrobium* and Dendrobii caulis. However, the mainstream substitute approaches used to obtain commercial products involve simultaneous admix of the authentic *Dendrobium* and other closely related counterfeit species. Most single target assays can identify only a few *Dendrobium* species, but are of limited value in discriminating within a mixture of Dendrobii caulis samples.

To resolve this problem and improve throughput and efficiency, a multiplex PCR assay for the determination of botanical identity of the components in a mixture of products has been proposed (Babaei et al., 2014; Giusti et al., 2016; Safdar and Junejo, 2015; Zha et al., 2011). Multiplex PCR methods show great potential in herbal authentication and drug safety control because of their ability for simultaneous detection of multiple biological components in the same reaction. Herein, the practicality of an octaplex PCR assay for rapid and reliable was demonstrated to identification of the species present in Dendrobii caulis products containing eight mainstream closely related *Dendrobium* species. Since PCR amplification yields distinct length amplicon products in the presence of different *Dendrobium* species, the octaplex PCR method was also used in identifying mixture samples. The application of this method to determine botanical origin of commercial Dendrobii caulis revealed the occurrence of several substitutes in herbal markets. Therefore, the present multiplex PCR assay provides a rapid and reliable approach for routine terminal market control of Dendrobii caulis.

2. Materials and methods

2.1. Samples

Two hundred and forty-two samples were collected from different production areas in China (Table 1), including 115 fresh leaves of *Dendrobium chrysotoxum*, *D. nobile*, *D. aphyllum*, *D. strongylanthum*, *D. fimbriatum*, *D. devonianum*, *D. officinale* or *D. huoshanense,* obtained from the germplasm bank of the botanical garden of Sipsongpanna South Medicine, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (IMPLAD, Sipsongpanna, China), and 13 batch samples kindly provided by Jin XH, associate Professor at the Institute of Botany, Chinese Academy of Sciences. “Xiantiao” samples were identified by Professor Yu Nianjun and “Fengdou” samples were identified by Director Zhang Yazhong based on morphological characteristics. Fresh plant samples were morphologically identified by orchid experts, associate Professor Li Ge and Jin Xiaohua, before collection and used as certified reference materials. To prevent DNA degradation, samples were kept at −20 °C until ready to use.

Twenty commercial Dendrobii caulis samples were purchased from traditional herbal markets in different provinces of China, including Kuming Juhuayuan herbal market (5 batches, Yunnan, China), Bozhou Kangmei herbal market (10 batches, Anhui, China), and Anguo herbal market (5 batches, Hebei, China), (Table 2). All samples were dried with silica gel and kept at room temperature until ready to use (Fig. 1).

2.2. Genomic DNA extraction

Materials were frozen in liquid nitrogen and ground to a fine powder with a MM 400 Mixer Mill (Retsch Technology GmbH, Haan, Germany). At least 2 g of commercial Dendrobii caulis sample were powdered and homogenized thoroughly. Homogeneous samples were split into quadruplicates, from which one sample was randomly selected and again split to obtain approximately 50 mg of powder for DNA extraction.

Genomic DNA was extracted from each certified reference material using a DNeasy Plant Mini Kit (QIAGEN, CA, USA) as per the instructions of the manufacturer. DNA extraction from twenty commercial Dendrobii caulis samples was performed using the two-step cetyltrimethylammonium bromide (CTAB) method (Dong et al., 2017a). Concentration of isolated DNA, OD_{260}/OD_{280} ratio, and OD_{260}/OD_{230} ratio were measured with a NanoDrop ND-1000 spectrophotometer (Gene, Hong Kong, China).

Table 1
Dendrobium samples used in the present study.

NO.	Species	Type	Location	Sample Size	Voucher number
1	*D. huoshanense*	Xiantiao	Heishuidu Town, Anhui Province	15	HS001-HS015
2	*D. huoshanense*	Xiantiao	Taipingfan Town, Anhui Province	15	HS016-HS030
3	*D. huoshanense*	Fengdou	Heishuidu Town, Anhui Province	4	20151003001 – 20151003004
4	*D. huoshanense*	Fengdou	Taipingfan Town, Anhui Province	4	20151003005 – 20151003008
5	*D. huoshanense*	Fengdou	Jinzhai County, Anhui Province	4	20151003009 – 20151003012
6	*D. huoshanense*	plant	Huoshan County, Anhui Province	1	72
7	*D. officinale*	Xiantiao	Huoshan County, Anhui Province	20	TP001-TP020
8	*D. officinale*	Xiantiao	Jing County, Anhui Province	20	TP021-TP040
9	*D. officinale*	Xiantiao	Yunnan Province	20	TP041-TP060
10	*D. officinale*	Fengdou	Heishuidu Town, Anhui Province	6	20151003013 – 20151003018
11	*D. officinale*	Fengdou	Taipingfan Town, Anhui Province	6	20151003019 – 20151003024
12	*D. officinale*	Plant	Bose, Guangxi, Province	1	77
13	*D. officinale*	Plant	Jinxiu, Guangxi, Province	1	20170718001
14	*D. officinale*	Plant	Xiuning, Anhui Province	1	20170718002
15	*D. chrysotoxum*	Plant	Jinhong, Yunnan Province	15	01001 – 01015
16	*D. nobile*	Plant	Jinhong, Yunnan Province	20	03001 – 03020
17	*D. nobile*	Plant	Simao, Yunnan Province	1	282
18	*D. aphyllum*	Plant	Jinhong, Yunnan Province	20	05001 – 05020
19	*D. strongylanthum*	Plant	Jinhong, Yunnan Province	20	28001–28020
20	*D. strongylanthum*	Plant	Jinhong, Yunnan Province	1	185
21	*D. strongylanthum*	Plant	Honghe, Yunnan Province	1	183
22	*D. strongylanthum*	Plant	Nujiang, Yunnan Province	1	146
23	*D. crystallinum*	Plant	Jinhong, Yunnan Province	20	10001–10020
24	*D. crystallinum*	Plant	Simao, Yunnan Province	1	286
25	*D. crystallinum*	Plant	Jinhong, Yunnan Province	1	107
26	*D. devonianum*	Plant	Jinhong, Yunnan Province	20	02001 – 02020
27	*D. devonianum*	Plant	Simao, Yunnan Province	1	285
28	*D. devonianum*	Plant	Nyingchi, Tibet, Province	1	144
29	*D. devonianum*	Plant	Jinhong, Yunnan Province	1	91