Acta Pharmaceutica Sinica B ▮▮▮▮;▮(▮):▮▮▮–▮▮▮

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Acta Pharmaceutica Sinica B

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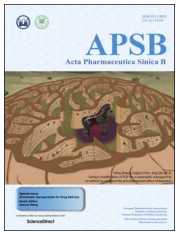

ORIGINAL ARTICLE

# Plastome-wide comparison reveals new SNV resources for the authentication of *Dendrobium huoshanense* and its corresponding medicinal slice (Huoshan Fengdou)

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Received 13 September 2017; revised 24 October 2017; accepted 8 November 2017

**KEY WORDS**

*Dendrobium huoshanense*;
Plastome;
Slices;
Single nucleotide variants;
RT-ARMS;
Authentication

**Abstract** *Dendrobium* species and their corresponding medicinal slices have been extensively used as traditional Chinese medicine (TCM) in many Asian countries. However, it is extremely difficult to identify *Dendrobium* species based on their morphological and chemical features. In this study, the plastomes of *D. huoshanense* were used as a model system to investigate the hypothesis that plastomic mutational hotspot regions could provide a useful single nucleotide variants (SNVs) resource for authentication studies. We surveyed the plastomes of 17 *Dendrobium* species, including the newly sequenced plastome of *D. huoshanense*. A total of 19 SNVs that could be used for the authentication of *D. huoshanense* were detected. On the basis of this comprehensive comparison, we identified the four most informative hotspot regions in the *Dendrobium* plastome that encompass *ccsA* to *ndhF*, *matK* to *3′trnG*, *rpoB* to *psbD,* and *trnT* to *rbcL*. Furthermore, to established a simple and accurate method for the authentication of *D. huoshanense* and its medicinal slices, a total of 127 samples from 20 *Dendrobium* species including their corresponding medicinal slices (Fengdous) were used in this study. Our results suggest that *D. huoshanense* and its medicinal slices can be rapidly and unequivocally identified using this method that combines real-time PCR with the amplification refractory mutation system (ARMS).



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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

https://doi.org/10.1016/j.apsb.2017.12.004


Please cite this article as: Niu Zhitao, et al. Plastome-wide comparison reveals new SNV resources for the authentication of *Dendrobium huoshanense* and its corresponding medicinal slice (Huoshan Fengdou). *Acta Pharmaceutica Sinica B* (2018), https://doi.org/10.1016/j.apsb.2017.12.004

## 1. Introduction

*Dendrobium*, one of the largest genera in Orchidaceae, includes approximately 1500 species. They are distributed mainly in tropical Asia, Australasia, and Australia, with a few species extending into the temperate Asian regions and New Zealand[1,2]. In China, there are about 80 species of this genus[3]. Because of their excellent medicinal merits, such as nourishing Yin, benefiting the stomach, enhancing the body's immunity, resisting cancer, and prolonging life, *Dendrobium* species have been extensively used as traditional Chinese medicine (TCM) in many Asian countries[4]. The price varies among different kinds of TCM products of *Dendrobium* due to their medical values. For example, a famous medicinal slice called "Huoshan Fengdou", made from the stem of *Dendrobium huoshanense*, is sold at $8000–10,000 per kg, which is much higher than other TCM products of *Dendrobium*. However, *Dendrobium* species and their corresponding medicinal slices are similar in appearance and tissue structure, which make them notoriously difficult to identify[5,6]. Therefore, a simple and accurate method for the authentication of *Dendrobium* species and their corresponding medicinal slices (Fengdous) is urgently needed.

The phytochemical approaches, *i.e.*, multiple fingerprint techniques, such as capillary electrophoresis (CE)[7] and high-pressure liquid chromatography (HPLC)[8], have been adopted for the authentication of *Dendrobium* species and their corresponding medicinal slices by determining their different chemical constituents and percentage compositions. Although these methods have played an important role in the identification of *Dendrobium* species, they are inadequate because they are unstable, complicated in operation or time-consuming. With the development of molecular biotechnology, a variety of molecular fingerprinting markers, including microsatellite (SSR) markers, inter-simple sequence repeat (ISSR) markers and amplified fragment length polymorphism (AFLP) markers has been developed for the identification of *Dendrobium* species[9–11]. Recently, the DNA barcodes have also showed a better specificity for *Dendrobium* species[12], involving different loci or their combinations, *e.g.*, *rbcL*+*matK*[13], ITS2[14] and ITS+*matK*[15]. However, the DNA contained in TCM products of *Dendrobium* is always wrapped with high percentage of polysaccharides, which result in a low DNA extraction and PCR amplification efficiency. Therefore, these methods can only be used to distinguish fresh materials, but are helpless for the authentication of their medicinal slices[5,16,17].

Single nucleotide variants (SNVs), widely present in DNA sequences, have been successfully applied in studies of phylogeny, population genetics and species identification[5,18,19]. The amplification refractory mutation system (ARMS), which has been developed for detecting SNVs, is well suited to authenticate the TCM products of *Dendrobium*, *i.e.*, *D. fimbriatum*[20] and *D. loddigesii*[21]. Recently, real-time fluorescent quantitative polymerase chain reaction (real-time PCR), a highly sensitive method of detecting DNA, has been widely adopted to identify food species, such as wheat, soybean and grapevine[22–24]. Therefore, the RT-ARMS method that combines real-time PCR with ARMS was established for the authentication of *D. officinale* and its medicinal slice (Tiepi Fengdou)[5]. However, this method has not been tested on other *Dendrobium* species due to the lack of SNV data.

Plastomic mutational hotspot regions are the most commonly used tools for identification studies of plants. A number of hotspot loci, including *rbcL*, *matK*, and *psbA-trnH*, have been selected as mutational hotspots in various lineages[25–27]. These hotspots might provide new resources of genetic information of SNVs that could be used for species identification. However, mutational hotspots for orchid species are known to be genus-specific[28]. Recently, more than 30 complete plastome sequences of *Dendrobium* species were sequenced and analyzed[28–30]. However, detailed analyses of SNVs remain very limited in this genus. *D. huoshanense*, also known as "Huoshan shihu", is an endangered herb endemic to China and only distributed in Anhui, Jiangxi and Henan provinces[31]. The unparalleled health effects of *D. huoshanense* make its price much higher than that of other TCM products of *Dendrobium*. Consequently, commercially available slices of "Huoshan shihu" are often adulterated with other kinds of *Dendrobium* slices. Therefore, in this study, the plastome of *D. huoshanense* were used as a model system to address three questions, as follows: (1) Could the plastomic mutational hotspot regions provide a useful SNVs resource for the authentication of *D. huoshanense*? (2) If so, which are the most informative hotspot regions for *Dendrobium* species? (3) Is the RT-ARMS method a simple and accurate method for the authentication of *D. huoshanense* and its corresponding medicinal slices (Huoshan Fengdou)?

To address these questions, we sequenced the complete plastome of *D. huoshanense* and compared it with other 16 *Dendrobium* species which were most likely to be mixed up with *D. huoshanense* according to documented information[4,31]. The sequences of 117 syntenic non-coding regions were retrieved from these plastomes and compared to detected *D. huoshanense*-specific nucleotides. Furthermore, the RT-ARMS method was used to distinguish *D. huoshanense* and its medicinal slices (Huoshan Fengdou) from other adulterants.

## 2. Material and methods

### 2.1. Plant materials and DNA extraction

A total of 127 samples from 20 *Dendrobium* species, including 107 fresh samples and 20 medicinal slices (Fengdous) were used in this study (Table 1). In addition to *D. huoshanense*, the other 19 *Dendrobium* species are often used as adulterants of *D. huoshanense*. These plant samples were collected from 2012 to 2016 in southern provinces of China, *i.e.*, the Anhui, Guangxi, Guizhou, Henan, Sichuan and Yunnan provinces (Table 1). Their corresponding medicinal slices were purchased from different markets. All the samples were identified by Prof. Dr. Xiaoyu Ding and stored in College of Life Sciences, Nanjing Normal University, Nanjing, China.

For the extraction of DNA from fresh plant material, two grams of fresh leaves were harvested from an individual plant of each tested *Dendrobium* species. The total genomic DNA was extracted by using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The genomic DNA of medicinal slices was isolated from about 0.2 g of small flakes per sample using the method provided in Xu et al.[5]. All extracted DNA samples were subjected to 1% agarose gel electrophoresis to evaluate the quality.

### 2.2. Plastome sequencing, assembly and annotation

Extracted DNA sample of *D. huoshanense* that met the quality standards of concentration > 300 ng/μL, A260/A280 between 1.8 and 2.0, and A260/A230 > 1.7 were used for plastome

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