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

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**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 establish 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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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. In China, there are about 80 species of this genus. 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. 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. 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) and high-pressure liquid chromatography (HPLC), 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 technology, 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. Recently, the DNA barcodes have also showed a better specificity for *Dendrobium* species, involving different loci or their combinations, e.g., *rbcL*-mat*K*, *ITS2*, and *ITS*+*mat*K*. 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.

Single nucleotide variants (SNVs), widely present in DNA sequences, have been successfully applied in studies of phylogeny, population genetics and species identification. 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* and *D. loddigesii*. 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. 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). 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. 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. Recently, more than 30 complete plastome sequences of *Dendrobium* species were sequenced and analyzed. 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. 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. 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. 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/mL, A260/A280 between 1.8 and 2.0, and A260/A230 > 1.7 were used for plastome
sequencing. A total of 7.3 Gb of pair-end reads were sequenced on an Illumina Hiseq. 4000 sequencer. After the adaptors were removed, the raw reads were trimmed under the threshold with an error probability <0.05 and then de novo assembled on the CLC Genomics Workbench 6.0.1 (CLC Bio, Aarhus, Denmark). Contigs <50× sequencing depths were discarded. The remaining contigs were searched against the plastomic sequences of *D. moniliforme* (NC_035154) using blastn. Matched contigs with E-values of <10⁻¹⁰ were used for plastome assembly. Gaps between contigs were closed by PCR amplification and Sanger sequencing. The four junctions between LSC/SSC and IRs were validated by PCR amplification and real-time PCR analysis.

### Table 1  Plant samples of *D. huoshanense* and 19 adulterant *Dendrobium* species and the corresponding medicinal slices.

| No. | Species name                  | Product name          | Sampling site                             | Simple size | Voucher No. | Accession No. |
|-----|-------------------------------|-----------------------|-------------------------------------------|-------------|-------------|---------------|
| 1   | *D. huoshanense*<sup>a</sup> | Huoshan Fengdou       | Anhui, Henan, Jiangxi                     | 15          | Nia16009    | LC269310      |
| 2   | *D. moniliforme*<sup>b</sup> | Tongpi Fengdou        | Yunnan, Anhui, Sichuang, Guangxi, Guizhou | 12          | Nia12007    | NC_035154     |
| 3   | *D. flexicaule*<sup>b</sup>  | Jing Huodou           | Henan, Sichuang                           | 7           | Nia13017    |               |
| 4   | *D. adoncun*<sup>b</sup>     | Shuicao               | Guangxi, Guizhou                          | 6           | Nia14002    |               |
| 5   | *D. primitivum*<sup>b</sup>  | Shuidiabang           | Guangxi, Yunnan                           | 6           | Nia15021    | LC192810      |
| 6   | *D. aphyllum*<sup>b</sup>    | Shuicao               | Sichuang                                  | 3           | Nia12008    | LC192953      |
| 7   | *D. lodgesis*<sup>b</sup>    | –                     | Yunnan, Guangxi, Guizhou                  | 8           | Nia14007    | LC192960      |
| 8   | *D. crepidatum*<sup>b</sup>  | Zipilan               | Guizhou                                   | 3           | Nia14009    | LC193509      |
| 9   | *D. hercoglossum*<sup>b</sup>| Jizhuilan             | Yunnan                                    | 3           | Nia15011    | LC192959      |
| 10  | *D. wardianum*<sup>b</sup>   | Tiepibianlan          | Yunnan                                    | 3           | Nia15012    | LC192961      |
| 11  | *D. devonianum*<sup>b</sup>  | Zipi Fengdou          | Guizhou, Yunnan                           | 6           | Nia15008    | LC192956      |
| 12  | *D. demeanum*<sup>b</sup>    | Tieshuijie            | Yunnan                                    | 3           | Nia15015    | LC192955      |
| 13  | *D. chrysanthum*<sup>b</sup> | Shuicao               | Guizhou                                   | 3           | Nia14010    | LC193514      |
| 14  | *D. officinal*<sup>b</sup>   | Tiepi Fengdou         | Anhui, Jiangxi, Guizhou, Yunnan           | 11          | Nia16008    | NC_024019     |
| 15  | *D. pendulum*<sup>b</sup>    | Gangjiecao            | Yunnan                                    | 3           | Nia15003    | NC_029705     |
| 16  | *D. falconeri*<sup>b</sup>   | Zhumilin              | Guangxi                                   | 3           | Nia12006    | LC192957      |
| 17  | *D. lohoense*<sup>b</sup>    | –                     | Yunnan                                    | 3           | Nia15002    | LC193516      |
| 18  | *D. crysclinum*<sup>b</sup>  | Shuicao               | Guizhou                                   | 3           | Nia14005    | NC_028549     |
| 19  | *D. litaflorum*<sup>b</sup>  | Shuicao               | Guizhou                                   | 3           | Nia14018    |               |
| 20  | *D. gracioissimum*<sup>b</sup>| Gangjiecao            | Yunnan                                    | 3           | Nia15016    | LC192958      |

<sup>a</sup>The species that used in plastome comparison analysis.

<sup>b</sup>The species that used in the authentication studies.

2.3. Single nucleotide variants (SNVs) and sequence variability analysis

The sequences of non-coding loci, including intergenic and intronic regions, were retrieved from the 17 sequenced *Dendrobium* plastomes. The loci flanked by the same genes/exons were identified as syntenic. Loci that smaller than 150 bp were discarded. We obtained 117 syntenic non-coding loci and performed sequence alignments using MUSCLE 3.8.318 with the “refining” option implemented in Mega 5.2.25. The gaps located at the 5′- and 3′-ends of alignments were excluded. The SNVs were pairwise-analyzed using DnaSP v5.7. Sequence variability was estimated according to the method of Niu et al. 28,30.

2.4. Universal and ARMS primers design

A total of 9 *D. huoshanense* specific SNVs were identified in five non-coding regions (rpl32-trnL, trnS-trnG, atpB-rbcL, trnT-trnL, and trnL intron). Therefore, the universal primers were designed based on the conserved regions of their flanked genes/exons by using the Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA, Supplementary Information Table S1). From the 5 non-coding regions, the other 10 SNVs, which could be incorporated into the specific primers, were also identified and consequently 14 pairs of specific primers were designed for use on *D. huoshanense* (Table 2 and Supplementary Information Table S2). Based on the specific primer pairs, 14 pairs of ARMS primer were designed by mismatching the third and fourth nucleotide from the 3′ end of the primer, respectively (Supplementary Information Table S2). The underlined boldface bases are the bases that were deliberately destabilized via the substitution of A for C or the substitution of G for T (T/A pair replaced by a T/C pair, C/G pair replaced by a C/T pair) in the *D. huoshanense* specific primers, in order to ensure absolute specificity.

2.5. PCR pre-amplification and real-time PCR analysis

According to Xu et al. 5 and Yan et al. 17, pre-amplification of target DNA regions by conventional PCR with universal primers is an effective method to increase the DNA content of the processed samples. Therefore, pre-amplification of the five non-coding regions was carried out in this study. The universal primer pairs are listed in Supplementary Information Table S1. A total volume of 30 μL of the PCR mixture was prepared, including 10 ng of genomic DNA, 3 μL of 10× PCR buffer, 2 μL of 25 mmol/L MgCl₂, 2 μL of 2.5 mmol/L dNTP, 0.2 μL of 5 U/μL *Taq* DNA polymerase (TaKaRa, Otsu, Japan) and 1 μL of each universal primer at 10 μmol/L. The PCR amplification process was performed according to the following programme: an initial denaturation at 95 °C for 4 min; thirty cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 30 s and extension at 72 °C for 2 min; and then a final
Table 2  The single nucleotide variants in the five non-coding regions.

| Species name         | rpl32-trnL | trnS-trnG | atpB-rbcL | trnT-trnL | trnL intron |
|----------------------|------------|-----------|-----------|-----------|------------|
|                      | PL1 | PL2* | PL3 | PL4* | NS1 | NS2 | NS3* | TP1* | TP2* | NT1* | NT2* | NT3 | NT4 | NL1* | NL2 | NL3* | NL4 | NL5 | NL6 |
| D. huoshanense       | C   | T    | A   | C    | T    | G    | A    | T    | G    | G    | G    | G   | C   | C    | C   | C   | T   |
| D. moniliform        | C   | C    | A   | A    | C    | G    | G    | G    | C    | A    | T    | G    | G    | A    | G   | A   | C    | A   | G   |
| D. flexicaule        | A   | −    | A   | G    | C    | G    | G    | −    | A    | A    | T    | G    | T    | A    | −    | A   | C    | A   | G   |
| D. primulinum        | T   | −    | G   | A    | G    | C    | T    | G    | G    | C    | A    | T    | C    | −    | A    | −    | A   | A    | A   | −    |
| D. aphylatum         | T   | C    | G   | A    | C    | T    | G    | G    | C    | A    | T    | C    | −    | A    | −    | A   | A    | A   | −    |
| D. loddigesii        | A   | −    | A   | G    | C    | T    | G    | −    | A    | A    | T    | C    | −    | A    | −    | A   | A    | A   | −    |
| D. crepidatum        | T   | −    | G   | A    | T    | G    | T    | G    | C    | A    | T    | C    | G    | A    | −    | A   | A    | A   | −    |
| D. hercoglossum      | C   | C    | A   | A    | C    | G    | G    | G    | C    | A    | T    | G    | −    | A    | T    | A   | C    | A   | G   |
| D. wardianum         | T   | −    | G   | A    | C    | T    | G    | G    | C    | A    | T    | C    | T    | A    | −    | −   | −    | A   | G   |
| D. devonianum        | T   | −    | G   | A    | C    | T    | G    | G    | C    | A    | T    | C    | −    | A    | A    | A   | A    | A   | A   |
| D. denneanum         | T   | −    | G   | A    | C    | T    | G    | C    | A    | T    | −    | A    | T    | −    | A    | T   | A    | A   | G   |
| D. chrysanthum       | T   | −    | G   | A    | T    | T    | G    | G    | C    | A    | T    | C    | −    | A    | A    | A   | A    | A   | A   |
| D. officinale        | T   | C    | G   | A    | C    | G    | G    | G    | C    | A    | T    | G    | T    | A    | T   | A    | A    | A   | G   |
| D. pendulum          | A   | −    | A   | G    | C    | T    | G    | −    | A    | A    | T    | G    | G    | A    | T    | A    | A    | A   | G   |
| D. falconeri         | T   | C    | G   | A    | C    | T    | G    | G    | C    | A    | T    | G    | G    | A    | T    | A    | A    | A   | G   |
| D. lohohense         | T   | −    | G   | A    | T    | T    | G    | G    | C    | A    | T    | C    | −    | A    | −    | A   | A    | C   | A   |
| D. gratiotissimm     | T   | −    | G   | A    | C    | T    | G    | G    | C    | A    | T    | C    | T    | A    | −    | −   | −    | A   | G   |

− indicates gaps in the aligned sequences.

* indicates the D. huoshanense specific nucleotides.
extension at 72 °C for 7 min. The pre-amplified DNA products were diluted to 10 ng/μL for the real-time PCR analysis.

The real-time PCR analysis was performed in a fast optical 96-well reaction plate (Eppendorf Mastercycler ep Realplex 2; Hamburg, Germany). Six pairs of primers were employed in the real-time PCR analysis (Supplementary Information Table S3). A total volume of 20 μL of the reaction mixture which contained 1 μL of 10 ng/μL DNA, 0.5 μL of primer and 8 μL of SYBR Green Mix Taq II (TaKaRa) was prepared. The SYBR reactions were run under the following thermal cycling protocol: 95 °C for 1 min, followed by forty cycles of 95 °C for 30 s and 60 °C for 30 s. A melting curve (95 °C for 15 s, 60 °C for 15 s and 95 °C for 20 s) was also added to detect nonspecific amplification products or primer dimers were being formed. Ct values of all the Dendrobium slices were analyzed and each sample was tested in triplicate by both PCR assays. The average Ct values were compared to distinguish D. huoshanense and its medicinal slices from the adulterants.

2.6. Statistical analyses

Statistical analyses of Mann-Whitney 2-sides test were performed using SPSS Statistics 20.0.

3. Results

3.1. Plastome features of D. huoshanense

The newly sequenced plastome of D. huoshanense (LC269310) was circular with 148,590 bp in size. The plastome contains a pair of inverted regions (IRs), which separated the single copy (SC) region into two subsets: large SC (LSC) and small SC (SSC) regions. The size of the LSC, SSC and IR regions were 84,694, 11,964 and 25,966 bp, respectively. The overall GC content was 37.54%, with 35.32% in LSC, 30.57% in SSC and 43.53% in IRs. Except for the independent loss of ndh genes, the plastome of D. huoshanense possesses 68 unique protein-coding genes, 30 unique tRNA genes and four unique rRNA genes. Comparison of plastomes between D. huoshanense and 16 other Dendrobium species showed no dramatic differences in compared features (Fig. 1). The most prominent difference was observed in the size of SSC region, with the largest in D. officinale and the smallest in D. primulinum. Plastome size, GC content and the size of the LSC and IRs did not vary greatly, while the number of genes was slightly less for D. huoshanense and D. loddigesii. The variation in size of the SSC regions and the gene content was caused by the selective loss/retention of ndh genes.

Figure 1 A radar-plot comparing features of 17 plastomes of Dendrobium species. The circles from the outermost to the innermost show the comparison results (%) of plastome size, GC content, genes content and the size of LSC, SSC and IR. The plastome features of D. huoshanense was used as reference.
3.2. Exploration of single nucleotide variants (SNVs)

Sequences of 117 syntenic non-coding loci were retrieved from the plastomes of 17 Dendrobium species (Table 1). The SNVs between D. huoshanense and other Dendrobium species were compared (Fig. 2). Our results showed that the base composition of non-coding loci in the D. huoshanense plastome was similar to D. moniliforme, D. officinarum and D. hercoglossum. Fig. 3 illustrates the sequence variability estimated from each syntenic non-coding locus. Notably, the mutational hotspots of Dendrobium species are mainly distributed in the LSC and SSC regions, which reinforces the view that substitution rates are significantly slower in the IR than in the SC regions. Therefore, only non-coding loci in SC regions were used for exploring D. huoshanense specific SNVs. Although only few SNVs were detected between D. huoshanense and these species, a total of 19 SNVs which could be incorporated into the specific primers were detected, including 9 D. huoshanense specific SNVs and 10 other SNVs. The 19 SNVs were distributed in 5 non-coding loci (rpl32-trnL, trnS-trnG, atpB-rbcL, trnT-trnL and trnL intron) that have higher SV values than most other non-coding loci. Additionally, 2 belonged to the hotspot regions: from ccsA to ndhF and matK to 3′trnG. These results suggest that the plastomic mutational hotspot regions could be new resources for SNV selection.

3.3. ARMS primers selection

The 19 SNVs are shown in Table 2. The trnL intron has six nucleotide mutations at positions 193, 408, 451, 479, 582 and 624. The sequence of rpl32-trnL and trnT-trnL contain four single-nucleotide mutations (at positions 202, 253, 518 and 853 of rpl32-trnL and at positions of 368, 369, 480 and 688 in trnT-trnL). The remaining two sequences of trnS-trnG and atpB-rbcL contained three mutations (at positions of 705, 772, and 1133) and two single nucleotide mutations (at positions of 39 and 156). These SNVs were verified to be conserved among different species with the PCR amplification of all the sampled species, which indicates that these SNVs of cpDNA could be used for the authentication of D. huoshanense.

Using the ARMS technology, 14 pairs of primers were designed based on these specific nucleotides (two pairs within rpl32-trnL, two pairs within trnS-trnG, one pair within atpB-rbcL, two pairs within trnT-trnL and seven pairs within trnL intron, Supplementary Information Table S2). Among them, 9 pairs of ARMS-primer were proven to have good specificity and amplification efficiency for D. huoshanense by testing fresh Dendrobium species. Considering the requirements of real-time PCR for primers and the length of the amplified DNA fragment, 6 pairs of primers were employed in the real-time PCR analysis, which were used for identifying D. huoshanense and its medicinal slices (Supplementary Information Table S3).

3.4. Authentication of D. huoshanense and its medicinal slices

The total genomic DNA samples were extracted from both fresh Dendrobium species and their corresponding medicinal slices. However, the DNA samples extracted from Dendrobium slices showed such low concentration that DNA bands were not visible.
Therefore, in order to improve the specificity and amplification efficiency of real-time PCR, the non-coding regions of *atpB-rbcL*, *trnT-trnL* and *trnL* intron were pre-amplified before real-time PCR analysis (Fig. 4 and Supplementary Information Table S1). After that, the DNA samples from fresh *Dendrobium* species and pre-amplified DNA products were diluted to 10 ng/μL and used as template DNA for the real-time PCR analysis.

In the real-time PCR analysis, the DNA fragments of *D. huoshanense* were successfully amplified from all 6 pairs of ARMS-primers, with the average *C*\(_t\) values of 17.58 for NL1/NL3, 20.22 for NL1/NL4, 30.06 for NL1/NL5, 24.73 for NL4/NL6, 30.91 for NT1/NT2 and 23.42 for TP1/TP2 (Table 3, Fig. 5). The average *C*\(_t\) values of *D. huoshanense* were significantly lower than those of other *Dendrobium* species.

**Figure 3** Four most informative hotspot regions in *Dendrobium* plastome. The genome map was drawn on the base of the complete plastome sequence of *D. huoshanense*. The inner bars show the average percentage of pair-wised SV values for each non-coding region. The colors from black to red indicate increasing of SV values for each locus, as demonstrated by the scale bar. The four regions showed innermost are the predicted most informative hotspot regions in *Dendrobium* plastome. The regions ①–③ are also documented as the mutational hotspots in Shaw et al. 472. The region ④ is proposed as the mutational hotspot in this study.
(Mann-Whitney 2-sided test, \( P < 0.01 \)), which indicated that the \( D. \) huoshanense could be amplified from the 6 pairs of ARMS-primer. Moreover, the data from melting temperatures suggested that non-specific amplification did not occur in the real-time PCR analysis. Therefore, we recommend that the 6 pairs of ARMS-primer listed in the Supplementary Information Table S3 can be used to distinguish \( D. \) huoshanense and its medicinal slices from other adulterants based on real-time PCR analysis.

4. Discussion

4.1. Plastomic mutational hotspot regions provide useful SNV resource for the authentication of \( D. \) huoshanense and its corresponding medicinal slices (Huoshan Fengdou)

Sequences of the nrDNA ITS region are highly variable in angiosperms and have been proposed for DNA barcoding\(^{25}\). The genetic information of this region provides a useful resource for
the authentication of *Dendrobium* species. Therefore, based on the SNVs from the sequence of ITS, several *Dendrobium* species and their medicinal slices, such as *D. officinale* and *D. loddigesii* have been distinguished from other kinds of *Dendrobium* slices. However, recent molecular studies showed that the sequences of ITS regions could not be used easily for identifying many important *Dendrobium* species. For example, Xiang et al. showed that the species of *D. officinale*, *D. tosaense*, *D. shixingense*, *D. flexicaule*, *D. scoriarum* and *D. aduncum* in *D. officinale* complex and the species of the *D. fanjingshanense*, *D. henanense*, *D. huoshanense*, *D. moniliforme*, *D. okinawense* and *D. xichouense* in *D. moniliforme* complex were closely related to each other. After having carefully examined nine ribotypes of ITS sequences of *D. huoshanense* and *D. moniliforme*, Geng et al. proposed that the ITS sequences could not be easily used for the identification of the species in *D. moniliforme* complex. Compared to nuclear and mitochondrial genomes, plastome sequences have slower evolutionary rates; thus, mutational hotspots of

**Figure 5** The identification of *D. huoshanense* and its medicinal slices based on the real-time PCR assay. The red lines indicate the *D. huoshanense* and its medicinal slices. The blue lines indicate other adulterants of *Dendrobium* species.
plastome have been demonstrated to be conserved among different populations of each species, but polymorphic between various species. Therefore, the mutational hotspots of the plastome are more suitable for the authentication of Dendrobium species than those of the ITS region. For example, Lu et al. successfully distinguished D. fimbriatum from other Dendrobium species based on the sequence of psbA–trnH intergenic spacers.

Recently, Niu et al. assessed 30 complete plastome sequences of Dendrobium species and proposed that the top-ten mutual plastome hotspots psbB-psbT, ndhF-rip32, rnl-trnL, rpl32-trnL, clpP-psbB, trnL intron, rip16-rps3, trnE-trnT, trnR-atpA and rps16-trnQ, which contain highest degree of sequence variability, can be used for the identification of Dendrobium species. In this study, 117 syntenic non-coding regions were compared between D. huoshanense and 16 other Dendrobium species. After having carefully examined the data, 19 SNVs, which could be used to authenticate D. huoshanense, were detected in five non-coding regions. Among them, three (rpl32-trnL, trnT-trnL and trnL intron) are within the top-10 mutual hotspots reported in Niu et al. The other two (trnS-trnG and atpB-rbcL) are also listed in the top-20 in this study. These results indicate that the mutational hotspots of plastome sequences could provide new resources of genetic information of single nucleotide polymorphisms that can be used for identifying D. huoshanense and its corresponding medicinal slices.

4.2. The most informative hotspot regions for the identification of Dendrobium species

Three regions (ccsA to ndhF, matK to 3’trnG and rpoB to psbD) of the plastome that contain high levels of sequence variability have been proposed as the most informative hotspots by Shaw et al. In this study, the non-coding regions of rpl32-trnL and atpB-rbcL belonged to the regions of ccsA to ndhF, matK to 3’trnG, which suggested that the three hotspots could provide useful SNV resources for the identification of Dendrobium species. Furthermore, the remaining three loci were distributed in the area of the plastome from trnT to rbcL. This region also contains several variable loci; for example, the protein-coding gene of rbcL, one of the most valuable DNA barcode candidates; the non-coding loci of trnT-trnL and trnL intron, within the top-10 mutual hotspots, newly put forth by Niu et al. These mutational hotspot loci led to the selection of this region for SNVs identification. Therefore, based on the extensive plastome survey included in Shaw et al. and the present study, we propose that the 4 regions, ccsA to ndhF, matK to 3’trnG, rpoB to psbD and trnT to rbcL, would be the most informative hotspot regions in Dendrobium species, and which are most likely to contain the greatest number of SNVs. The results of our work could guide researchers who want to identify Dendrobium species by using the most variable portions of the Dendrobium plastome, whether they are exploring specific SNVs or amplified independently.

4.3. The RT-ARMS method could be used for the authentication of D. huoshanense and its corresponding medicinal slices (Huoshan Fengdou)

Traditionally, the authentication of Dendrobium species is based on their morphological or chemical characteristics. However, many species have overlapping characteristics due to environmental and development factors during plant growth. Furthermore, after intensive processing, the medicinal slices of Dendrobium species become more difficult to distinguish. Recently, well-developed molecular marker techniques, i.e., SSR, ISSR, AFLP and DNA barcodes became available for the identification of medicinal plants. However, these methods can only be used to distinguish fresh Dendrobium materials, but are useless for their medicinal slices due to two reasons. Firstly, the Dendrobium species are rich in polysaccharides and after intensive processing, the concentrations of polysaccharides in these slices was greatly increased, which makes it difficult to purify and detect their genomic DNA, resulting in the low yield and purity. Secondly, the intensive processing also causes the degradation of their genomic DNA. For example, there are a series of steps of processing fresh Dendrobium plants into the “Fengdou” products. The stems of D. huoshanense plants are cut into 4–6 cm segments and then baked to dehydrate. The dried stems of D. huoshanense also need to twist into a curl for Fengdou. These steps badly damage the genomic DNA of Dendrobium plants. Consequently, the genomic DNA extracted from these medicinal slices always failed to meet the requirements of conventional PCR. Therefore, it is necessary to develop a simple and accurate method for the authentication of Dendrobium species and their corresponding medicinal slices (Fengdou).

In this study, 19 Dendrobium species, which are most likely to be mixed up with D. huoshanense, were collected as the reference adulterants. Nineteen SNVs were detected in 5 non-coding regions. Recent studies revealed that the pre-amplification of target sequences could greatly improve the quantity of DNA TCM products of Dendrobium. Moreover, real-time PCR, a highly sensitive method of detecting DNA, would be effective with “Fengdou” DNAs. Therefore, the pre-amplification of these regions by conventional PCR with universal primers was firstly used to improve the quantity of the DNA samples. Then, the RT-ARMS method was employed to authenticate D. huoshanense and its medicinal slices. Allele-specific polymerase chain reaction (AS-PCR) has been adopted in the identification of medicinal plants. However, in some cases, this method may not be effective for species-level identification. The ARMS, which introduces a mismatched nucleotide into the 3’ end of the primer, yields more repeatable and accurate results than the AS-PCR method. It has been successfully applied to the identification of species and commercial products. In Dendrobium, the species of D. fimbriatum, D. loddigesii and D. officinale can be identified by ARMS-primer. In line with this observation, the 6 pairs of ARMS-primer developed in this study also showed high specificity for D. huoshanense. Moreover, combined with real-time PCR technology, PCR products can be detected quantitatively and directly through the intensity of fluorescent signals. Our results show that all the 6 pairs of ARMS-primer amplified in D. huoshanense have significantly lower C values than those of other Dendrobium species. All the experiments were repeated at least 3 times with similar results, indicating the reproducibility of this method. Hence, the method used in this study will rapidly and unequivocally distinguish D. huoshanense and its medicinal slices from other adulterants.

The RT-ARMS method has been used for the authentication of D. officinale and its medicinal slices based on the SNVs in the sequences of the ITS regions. However, this method has not been tested on other Dendrobium species due to three reasons. Firstly, as mentioned above, the sequence of the ITS region cannot be used for identifying several important Dendrobium species, i.e.,
indicate that the RT-ARMS method, based on the plastomic SNVs for the authentication of D. huoshanense was sequenced and compared with other Dendrobium species. Our plastome-wide comparison revealed a total of 19 SNVs that could be used for the authentication of D. huoshanense. Moreover, D. huoshanense and its medicinal slices could be quickly identified through all the six pairs of ARMS-primer. These results indicate that the RT-ARMS method, based on the plastomic SNVs could be used for the authentication of D. huoshanense and its medicinal slices.

5. Conclusions

In conclusion, this study is the first to uncover a new resource of SNVs in plastomic mutational hotspot regions that could be used for the authentication of D. huoshanense and its medicinal slice (Huoshan Fengdou). On the basis of the explored SNVs, D. huoshanense and its medicinal slices were quickly distinguished from its adulterants. Moreover, we also proposed that the RT-ARMS method could be used for the authentication of D. huoshanense and its corresponding medicinal slices. In comparison to other species-identification methods, our method has great advantages: (1) based on the ARMS-primer, D. huoshanense could be easily distinguished from other similar species; (2) the real-time PCR has good sensitivity in tracing DNA and is effective in the authentication of D. huoshanense and its corresponding medicinal slices; (3) the method was a repeatable and convenient process.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2017.12.004.

References

1. Wood HP. The Dendrobiums. Timber Press; 2006.
2. Adams P. Systematics of Dendrobium (Orchidaceae), with special reference to Australian taxa. Bot J Linn Soc 2011;166:105–26.
3. Xiang XG, Schuiteman A, Li DZ, Huang WC, Chung SW, Li JW, et al. Molecular systematics of Dendrobium (Orchidaceae, Dendrobieae) from mainland Asia based on plastid and nuclear sequences. Mol Phylogenet Evol 2013;69:950–60.
4. Bao XS, Shun QS, Chen LZ. The medicinal plants of Dendrobium (Shi-hu) in China. Shanghai Medicinal University Press and Fudan University Press; 2001.
5. Xu H, Hou B, Zhang J, Min T, Yuan Y, Niu Z, et al. Detecting adulteration of Dendrobium officinale by real-time PCR coupled with ARMS. Int J Food Sci Tech 2012;47:1695–700.
6. Paul P, Wang ZT, Shaw PC. Current approaches for the authentication of medicinal Dendrobium species and its products. Plant Genet Resour 2005;3:144–8.
7. Zha XQ, Luo JP, Wei P. Identification and classification of Dendrobium candidum species by fingerprint technology with capillary electrophoresis. S Afr J Bot 2009;75:276–82.
8. Yang L, Wang Z, Xu L. Simultaneous determination of phenols (bienzyl, phenanthrene, and fluorone) in Dendrobium species by high-performance liquid chromatography with diode array detection. J Chromatogr A 2006;1104:230.
9. Yue GH, Lam-Chan LT, Hong Y. Development of simple sequence repeat (SSR) markers and their use in identification of Dendrobium varieties. Mol Ecol Notes 2006;6:832–4.
10. Shen J, Ding X, Liu D, Ding G, He J, Li X, Tang F, Chu B. Inter simple sequence repeats (ISSR) molecular fingerprinting markers for authenticating populations of Dendrobium officinale Kimura et Migo. Biol Pharm Bull 2006;29:420–2.
11. Li XX, Ding XY, Chu BH, Zhou Q, Ding G, Gu S. Genetic diversity analysis and conservation of the endangered Chinese endemic herb Dendrobium officinale Kimuraet Migo (Orchidaceae) based on AFLP. Genetica 2008;133:159–66.
12. Teixeira da Silva JA, Jin X, Dobranszki J, Lu J, Wang H, Zotz G, Cardoso JC, Zeng S. Advances in Dendrobium molecular research: applications in genetic variation, identification and breeding. Mol Phylogenet Evol 2016;95:196.
13. Ashahina H, Shinozaki J, Masuda K, Morimitsu Y, Satake M. Identification of medicinal Dendrobium species by phylogenetic analyses using matK and rbcL sequences. J Nat Med 2010;64:133–8.
14. Feng S, Jiang Y, Wang S, Jiang M, Chen Z, Ying Q, et al. Molecular identification of Dendrobium species (Orchidaceae) based on the DNA barcode ITS2 region and its application for phylogenetic study. Int J Mol Sci 2015;16:21975–88.
15. Xu S, Li D, Li J, Xiang X, Jin W, Huang W, et al. Evaluation of the DNA barcodes in Dendrobium (Orchidaceae) from mainland Asia. PLoS One 2015;10:e0115168.
16. Zhang WC, Ding XY, Xie ML, Feng ZY, Lu S, Li XX, et al. Authentication of three valuable Dendrobium species by adapter ligation-mediated allele-specific amplification. Eur Food Res Technol 2009;229:1–7.
17. Yan WJ, Zhang JZ, Zheng R, Sun YL, Ren J, Ding XY. Combination of SYBR Green II and TaqMan Probe in the adulteration detection of Dendrobium devonianum by fluorescent quantitative PCR. Int J Food Sci Tech 2016;50:2572–8.
18. Paun O, Turner B, Trucchi E, Munzinger J, Chase MW, Samuel R. Processes driving the adaptive radiation of a tropical tree (Diospyros, Ebenaceae) in New Caledonia, a biodiversity hotspot. Syst Biol 2016;61:syv076.
19. Wang XQ, Zhao L, Eaton DA, Li DZ, Guo ZH. Identification of SNP markers for inferring phylogeny in temperate bamboos (Poaceae: bambusoideae) using RAD sequencing. Mol Ecol Resour 2013;13:938–45.
20. Lu S, Ding X, Ma Y, Han L, Zhang W, Qian L, et al. Confirming the genetic identity of Dendrobium lamianum using an amplification refractory mutation system (arms). Plant Mol Biol Rep 2010;28:712–6.
21. Qian L, Ding G, Zhou Q, Feng Z, Ding X, Gu S, et al. Molecular authentication of Dendrobium loddigesii rolfe by amplification refractory mutation system (arms). Planta Med 2008;74:470–3.
22. Sonnante G, Montemurro C, Morgese A, Sabetta W, Blanco A, Pasqualone A. DNA microsatellite region for a reliable quantification of soft wheat adulteration in durum wheat-based foodstuffs by real-time PCR. J Agr Food Chem 2009;57:10199–204.
23. Brod FCA, Arisi ACM. Quantification of roundup Ready (TM) soybean in Brazilian soy-derived foods by real-time PCR. Int J Food Sci Tech 2009;43:1027–32.
24. Gambino G. Multiplex RT-PCR method for the simultaneous detection of nine grapevine viruses. Methods Mol Biol 2015;1236:39–47.

25. CBOL. Plant Working Group. A DNA barcode for land plants. Proc Natl Acad Sci U S A 2009;106:12794–7.

26. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci U S A 2005;102:8369–74.

27. Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. PLoS One 2007;2:e508.

28. Niu Z, Xue Q, Zha S, Sun J, Liu W, Ding X. Comparative chloroplast genomes of photosynthetic orchids: insights into evolution of the Orchidaceae and utility of plastomic mutational hotspots. Front Plant Sci 2017;8:715.

29. Luo J, Hou BW, Niu ZT, Liu W, Xue QY, Ding XY. Comparative analysis of Dendrobium plastomes and utility of plastomic mutational hotspots. Sci Rep 2017;7:2073.

30. Tsi ZH. Flora of China. Science Press; 1999.

31. Wyman SK, Jansen RK, Boore JL. Automatic annotation of organellar genomes with DOGMA. Bioinformatics 2004;20:3252–5.

32. Schattner P, Brooks AN, Lowe TM. The tRNAscan-SE, snocan and snoGPS web servers for the detection of tRNAs and snoRNAs. Nucleic Acids Res 2005;33:W686–9.

33. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792–7.

34. Tamura K. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9.

35. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 2009;25:1451–2.

36. Geng LX, Zheng R, Ren J, Niu ZT, Sun YL, Xue QY, et al. Application of new type combined fragments: nr DNA ITS+ nad1-intron 2 for identification of Dendrobium species of Fengdou. Acta Pharm Sin 2015;50:1060.

37. Wolfe KH, Li WH, Sharp PM. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci U S A 1987;84:9054–8.

38. Drouin G, Daoud H, Xia J. Relative rates of synonymous substitutions in the mitochondrial, chloroplast and nuclear genomes of seed plants. Mol Phylogenet Evol 2006;49:827–31.

39. Shaw J, Shafer HL, Leonard OR, Kovach MJ, Schorr M, Morris AB. Chloroplast DNA sequence utility for the lowest phylogenetic and phylogeographic inferences in angiosperms: the tortoise and the hare IV. Am J Bot 2014;101:1987.

40. Morris MW, Steen WL, Judd WS. Vegetative anatomy and systematics of subtribe Dendrobiinae (Orchidaceae). Bot J Linn Soc 1996;120:89–144.

41. Yukawa T, Uchihara K. Vegetative diversification and radiation in subtribe Dendrobiinae (Orchidaceae): evidence from chloroplast DNA phylogeny and anatomical characters. Plant Syst Evol 1996;201:1–14.

42. Li TX, Wang JK, Lu ZH. Accurate identification of closely related Dendrobium species with multiple species-specific gDNA probes. J Biochem Biophys Methods 2005;62:111–23.

43. Ding XY, Wang ZT, Xu H, Xu LS, Zhou KY. Database establishment of the whole rDNA ITS region of Dendrobium species of “Fengdou” and authentication by analysis of their sequences. Acta Pharm Sin 2002;37:567–73.

44. Sharma S, Shrivastava N. Internal transcribed spacer guided multiplex PCR for species identification of Convolvulus prostratus and Evolvulus alsinoides. Acta Pharm Sin B 2016;3:253–8.

45. Niu Z, Pan J, Zhu S, Li L, Xue Q, Liu W, Ding X. Comparative analysis of the complete plastomes of Apostasia wallchii and Neuwiedia singapureana (Apostasioidae) reveals different evolutionary dynamics of IR/SSC boundary among photosynthetic orchids. Front Plant Sci 2017;8:1713.

46. Wang L, Kong W, Yang M, Han J, Chen S. Safety issues and new rapid detection methods in traditional Chinese medicinal materials. Acta Pharm Sin B 2015;1:38–46.

47. Zhang Y, Xu Z, Ji A, Luo H, Song J. Genomic survey of bZIP transcription factor genes related to tanshinone biosynthesis in Salvia miltiorrhiza. Acta Pharm Sin B 2018. Available from: <http://dx.doi.org/10.1016/j.apsb.2017.09.002>.

48. Xin T, Xu Z, Jia J, Leon C, Hu S, Lin Y, et al. Biomonitoring for traditional herbal medicinal products using DNA metabarcoding and single molecule, real-times equencing. Acta Pharm Sin B 2018. Available from: <http://dx.doi.org/10.1016/j.apsb.2017.10.001>.

49. Li J, Li S, Huang D, Zhao X, Cai G. Advances in the of resources, constituents and pharmacological effects of Dendrobium officinale. Sci Technol Rev 2011;29:74–9.

50. Weng D. FAAS determination of trace elements in Dendrobium candidum using suspension sampling with ultrasonic agitation. Chin Pharm J 2003;38:704–6.

51. Zhang J, He C, Wu K, JATD Silva, Zeng S, Zhang X, et al. Transcriptome analysis of Dendrobium officinale and its application to the identification of genes associated with polysaccharide synthesis. Front Plant Sci 2016;7:5.

52. Newton CR, Graham A, Hepkinsall LE, Powell SJ, Summers C, Kulasheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989;17:2503–17.

53. Hayashi K, Hashimoto N, Daigen M, Ashikawa I. Development of PCR based SNP markers for rice blast resistance genes at the Piz locus. Theor Appl Genet 2004;108:1212–20.

54. Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, et al. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res 1990;18:999–1005.

55. Li XX, Ding XY, Chu BH, Ding G, Gu S, Qian L, et al. Molecular authentication of Salvia miltiorrhiza. Acta Pharm Sin B 2015;1:38–46.

56. Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, et al. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res 1990;18:999–1005.

57. D’Andrea M, Coisson JD, Travaglia F, Garino C, Arlorio M. Development and validation of a SYBR-Green I real-time PCR protocol to detect hazelnut (Corylus avellana L.) in foods through calibration via plasmid reference standard. J Agr Food Chem 2009;57:11201–8.

58. Gambino G. Multiplex RT-PCR method for the simultaneous detection of nine grapevine viruses. Methods Mol Biol 2015;1236:39–47.