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

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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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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

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*[a,b]	Huoshan Fengdou	Anhui, Henan, Jiangxi	15	Niu16009	LC269310
2	*D. moniliforme*[a,b]	Tongpi Fengdou	Yunnan, Anhui, Sichuang, Guangxi, Guizhou	12	Niu12007	NC_035154
3	*D. flexicaule*[b]	Jing Huodou	Henan, Sichuang	7	Niu13017	
4	*D. aduncum*[b]	Shuicao	Guangxi, Guizhou	6	Niu14002	
5	*D. primulinum*[a,b]	Shuidabang	Guangxi, Yunnan	6	Niu15021	LC192810
6	*D. aphyllum*[a,b]	Shuicao	Sichuang	3	Niu12008	LC192953
7	*D. loddigesii*[a,b]	–	Yunnan, Guangxi, Guizhou	8	Niu14007	LC192960
8	*D. crepidatum*[a,b]	Zipilan	Guizhou	3	Niu14009	LC193509
9	*D. hercoglossum*[a,b]	Jizhualan	Yunnan	3	Niu15011	LC192959
10	*D. wardianum*[a,b]	Tiepibianlan	Yunan	3	Niu15012	LC192961
11	*D. devonianum*[a,b]	Zipi Fengdou	Guizhou, Yunnan	6	Niu15008	LC192956
12	*D. denneanum*[a,b]	Tieshuijie	Yunan	3	Niu15015	LC192955
13	*D. chrysanthum*[a,b]	Shuicao	Guizhou	3	Niu14010	LC193514
14	*D. officinale*[a,b]	Tiepi Fengdou	Anhui, Jiangxi, Guizhou, Yunnan	11	Niu16008	NC_024019
15	*D. pendulum*[a,b]	Gangjiecao	Yunan	3	Niu15003	NC_029705
16	*D. falconeri*[a,b]	Zhumilan	Guangxi	3	Niu12006	LC192957
17	*D. lohohense*[a,b]	–	Yunnan	3	Niu15002	LC193516
18	*D. crystaclinum*[a,b]	Shuicao	Guizhou	3	Niu14005	NC_028549
19	*D. lituiflorum*[b]	Shuicao	Guizhou	3	Niu14018	
20	*D. gratiosissimum*[a,b]	Gangjiecao	Yunnan	3	Niu15016	LC192958

[a]The species that used in plastome comparison analysis.
[b]The species that used in the authentication studies.

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 $< 50\times$ 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 with PCR amplification. Genes were annotated using DOGMA[32] and tRNAscan-SE 1.21[33]. The boundaries of each annotated gene were manually confirmed by comparison with orthologous genes from other *Dendrobium* plastomes.

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 3.8.31[34] with the "refining" option implemented in Mega 5.2[35]. The gaps located at the 5′- and 3′-ends of alignments were excluded. The SNVs were pairwise-analyzed using DnaSP v5[36]. 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 mis-matching 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
	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	2	2	5	8	7	7	1	0	1	3	3	4	6	1	4	4	4	5	6
	0	5	1	5	0	7	3	3	5	6	6	8	8	9	0	5	7	8	2
	2	3	8	3	5	2	3	9	6	8	9	0	8	3	8	1	9	2	4
D. huoshanense	C	T	A	C	T	G	A	T	T	T	G	G	G	G	G	C	C	C	T
D. moniliform	C	C	A	A	C	G	G	G	C	A	T	G	G	A	G	A	C	A	T
D. flexicaule	A	–	A	G	C	G	G	–	A	A	T	G	T	A	–	A	C	A	G
D. primulinum	T	–	G	A	C	T	G	G	C	A	T	C	–	A	–	A	A	A	–
D. aphyllum	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	A
D. crepidatum	T	–	G	A	T	T	G	G	C	A	T	C	G	A	–	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
D. denneanum	T	–	G	A	C	T	G	G	C	A	T	A	–	A	T	A	A	A	G
D. chrysanthum	T	–	G	A	T	T	G	G	C	A	T	C	–	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. C_t values of all the *Dendrobium* slices were analyzed and each sample was tested in triplicate by both PCR assays. The average C_t 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. huoshanase* 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.
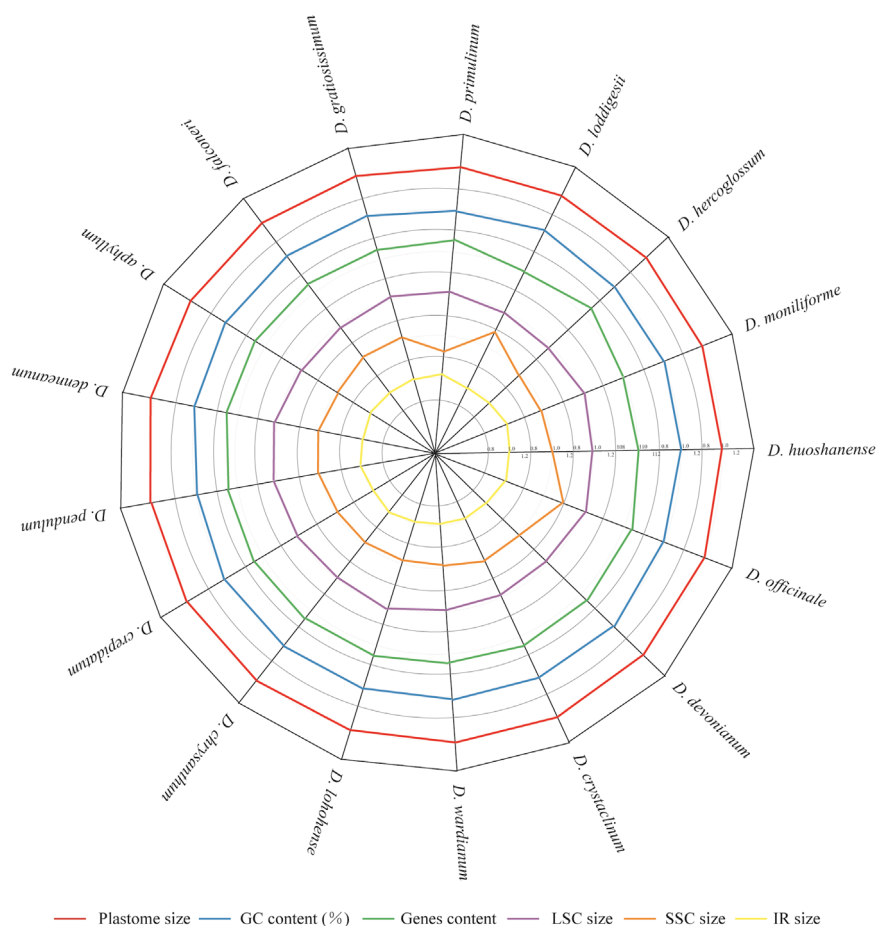

Figure 1 A radar-plot comparing features of 17 plastomes of *Dendrobium* species. The circles from the outermost to the innermost show the comparison results (%) of plastome size, GC content, genes content and the size of LSC, SSC and IR. The plastome features of *D. huoshanense* was used as reference.
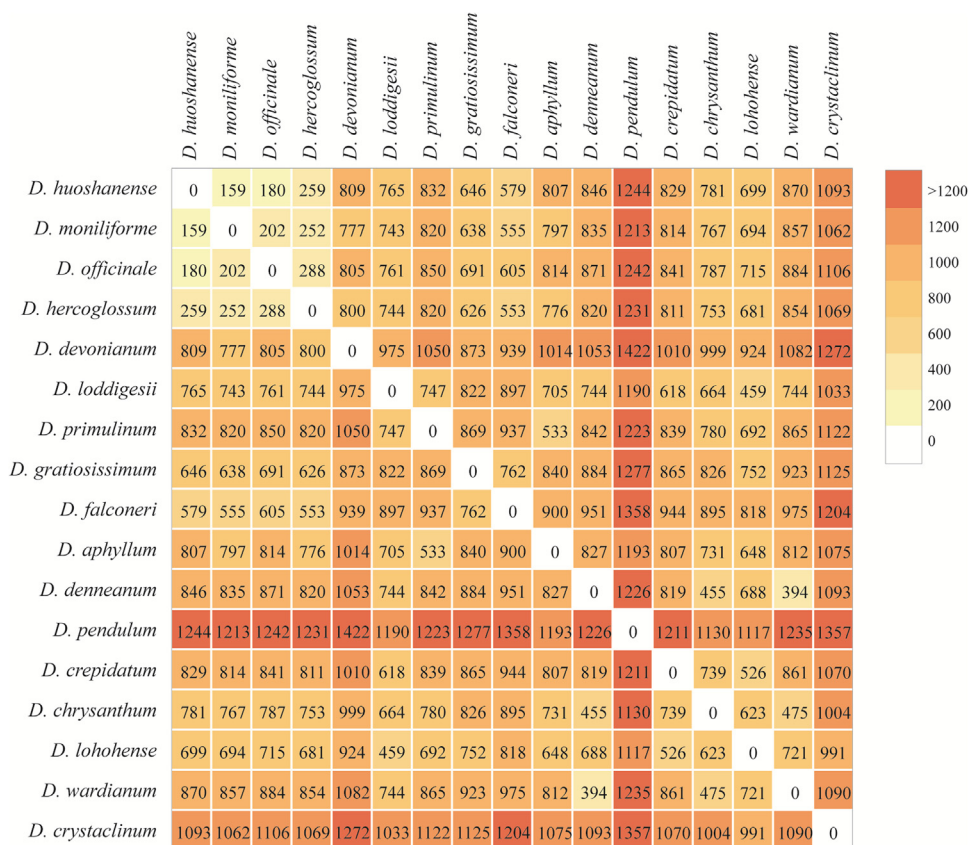
	D. huoshanense	*D. moniliforme*	*D. officinale*	*D. hercoglossum*	*D. devonianum*	*D. loddigesii*	*D. primulinum*	*D. gratiosissimum*	*D. falconeri*	*D. aphyllum*	*D. denneanum*	*D. pendulum*	*D. crepidatum*	*D. chrysanthum*	*D. lohohense*	*D. wardianum*	*D. crystaclinum*
D. huoshanense	0	159	180	259	809	765	832	646	579	807	846	1244	829	781	699	870	1093
D. moniliforme	159	0	202	252	777	743	820	638	555	797	835	1213	814	767	694	857	1062
D. officinale	180	202	0	288	805	761	850	691	605	814	871	1242	841	787	715	884	1106
D. hercoglossum	259	252	288	0	800	744	820	626	553	776	820	1231	811	753	681	854	1069
D. devonianum	809	777	805	800	0	975	1050	873	939	1014	1053	1422	1010	999	924	1082	1272
D. loddigesii	765	743	761	744	975	0	747	822	897	705	744	1190	618	664	459	744	1033
D. primulinum	832	820	850	820	1050	747	0	869	937	533	842	1223	839	780	692	865	1122
D. gratiosissimum	646	638	691	626	873	822	869	0	762	840	884	1277	865	826	752	923	1125
D. falconeri	579	555	605	553	939	897	937	762	0	900	951	1358	944	895	818	975	1204
D. aphyllum	807	797	814	776	1014	705	533	840	900	0	827	1193	807	731	648	812	1075
D. denneanum	846	835	871	820	1053	744	842	884	951	827	0	1226	819	455	688	394	1093
D. pendulum	1244	1213	1242	1231	1422	1190	1223	1277	1358	1193	1226	0	1211	1130	1117	1235	1357
D. crepidatum	829	814	841	811	1010	618	839	865	944	807	819	1211	0	739	526	861	1070
D. chrysanthum	781	767	787	753	999	664	780	826	895	731	455	1130	739	0	623	475	1004
D. lohohense	699	694	715	681	924	459	692	752	818	648	688	1117	526	623	0	721	991
D. wardianum	870	857	884	854	1082	744	865	923	975	812	394	1235	861	475	721	0	1090
D. crystaclinum	1093	1062	1106	1069	1272	1033	1122	1125	1204	1075	1093	1357	1070	1004	991	1090	0

Legend: >1200, 1200, 1000, 800, 600, 400, 200, 0

Figure 2 Number of SNVs among 17 *Dendrobium* species based on pair-wised comparison.

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. officinale* 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.
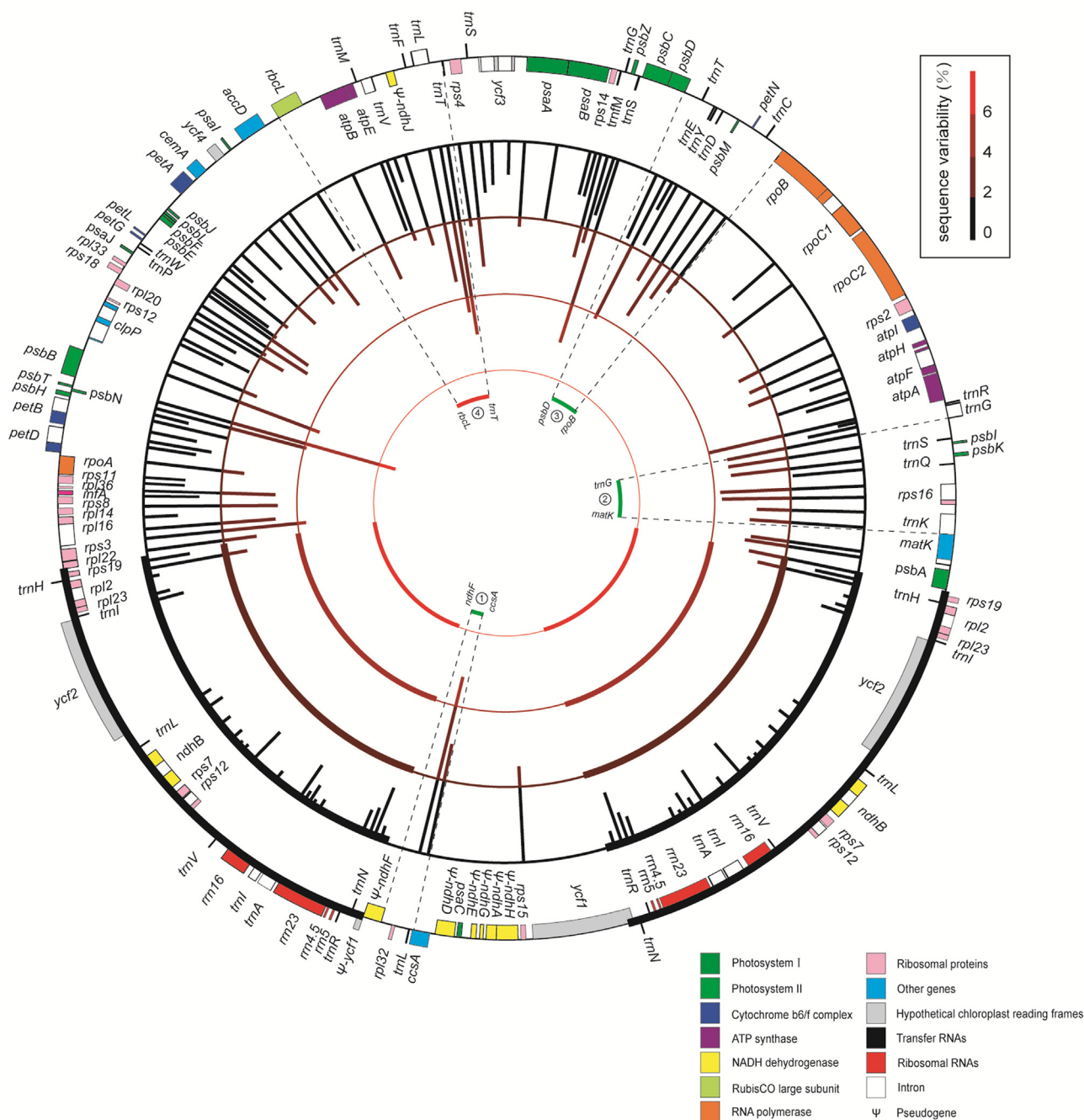
Figure 3 Four most informative hotspot regions in *Dendrobium* plastome. The genome map was drawn on the base of the complete plastome sequence of *D. huoshanense*. The inner bars show the average percentage of pair-wised SV values for each non-coding region. The colors from black to red indicate increasing 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.[40]. The region ④ is proposed as the mutational hotspot in this study.

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
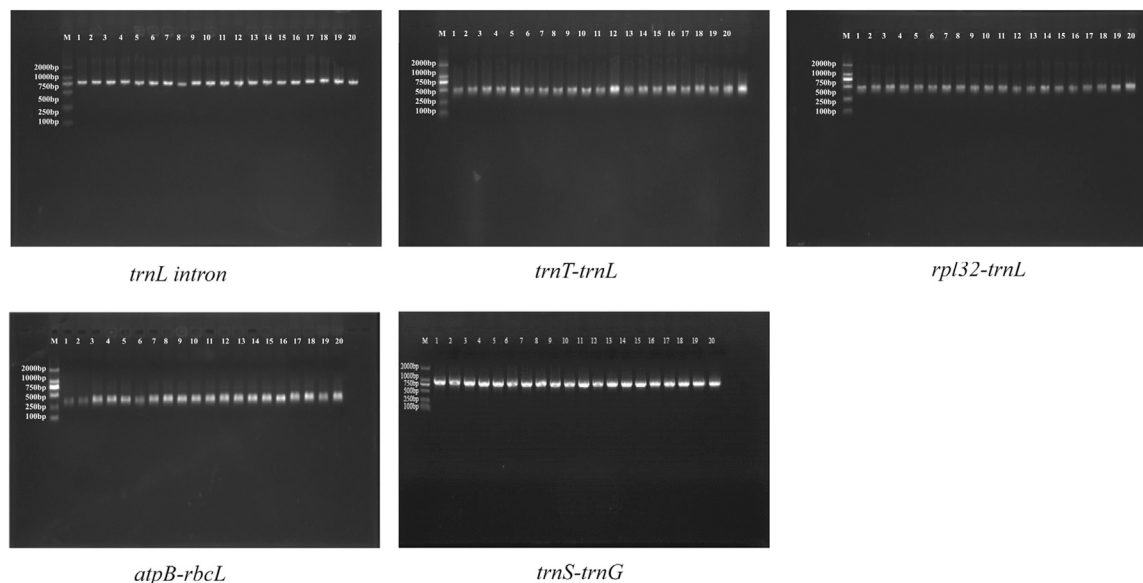
trnL intron *trnT-trnL* *rpl32-trnL*

atpB-rbcL *trnS-trnG*

Figure 4 Pre-amplification of the five non-coding regions.

Table 3 Average C_t values for *D. huoshanense* and nineteen reference *Dendrobium* species and their corresponding medicinal slices resulting from the real-time PCR.

Species names	NL1-NL3		NL1-NL4		NL1-NL5		NL4-NL6		NT-NT2		TP1-TP2	
	C_t	±SD	C_t	±SD	C_t	±SD	C_t	±SD	C_t	±SD	C_t	±SD
D. huoshanense	17.58	0.03	20.22	0.10	30.06	0.33	24.73	1.12	30.91	0.86	23.42	0.46
D. moniliform	26.46	0.09	26.74	0.22	32.98	0.19	31.25	1.31	–	–	27.18	0.11
D. flexicaule	39.97	0.34	34.57	0.45	42.07	1.78	41.07	0.18	–	–	32.45	0.50
D. aduncum	39.94	1.17	35.05	0.56	41.82	0.76	41.57	0.04	40.73	0.38	31.96	0.22
D. primulinum	38.56	1.14	35.05	0.44	41.10	0.35	42.16	0.47	–	–	31.34	0.30
D. aphyllum	37.96	0.85	34.59	0.31	39.84	0.15	–	–	39.47	0.38	31.52	0.27
D. loddigesii	39.79	1.01	34.10	1.00	42.03	2.38	–	–	38.15	0.10	31.83	0.06
D. crepidatum	38.61	0.18	34.27	0.25	41.29	0.84	–	–	37.13	0.08	31.57	0.35
D. hercoglossum	38.44	0.59	28.13	0.20	36.85	0.68	–	–	39.17	0.11	31.58	0.24
D. wardianum	30.43	0.19	33.37	0.09	41.52	1.12	–	–	–	–	30.49	0.24
D. devonianum	36.91	0.93	34.08	0.70	39.53	0.59	–	–	–	–	30.75	0.21
D. denneanum	33.93	0.29	33.48	0.47	41.88	0.23	–	–	–	–	31.37	0.44
D. chrysanthum	30.61	0.15	32.31	0.05	40.53	0.48	38.73	0.30	–	–	31.03	0.48
D. officinale	26.58	0.04	28.49	0.04	39.25	0.31	33.54	1.12	–	–	31.26	0.28
D. pendulum	30.14	0.06	31.80	0.36	40.69	1.26	37.02	0.19	–	–	31.35	0.51
D. falconeri	32.44	0.17	33.03	0.11	40.03	0.41	40.50	0.82	–	–	31.52	0.32
D. lohohense	37.79	0.44	35.32	0.42	42.34	0.62	–	–	38.82	0.32	30.96	0.18
D. crystaclinum	35.52	0.38	33.07	0.13	40.55	0.28	–	–	36.32	0.74	30.39	0.45
D. lituiflorum	29.30	0.47	29.67	0.06	39.16	0.76	39.46	0.68	38.27	0.31	30.43	0.21
D. gratiotissimum	26.10	0.09	32.3	0.10	40.55	0.96	37.23	0.65	–	–	30.38	0.23

– No amplification in the species.
C_t: average C_t value.

(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
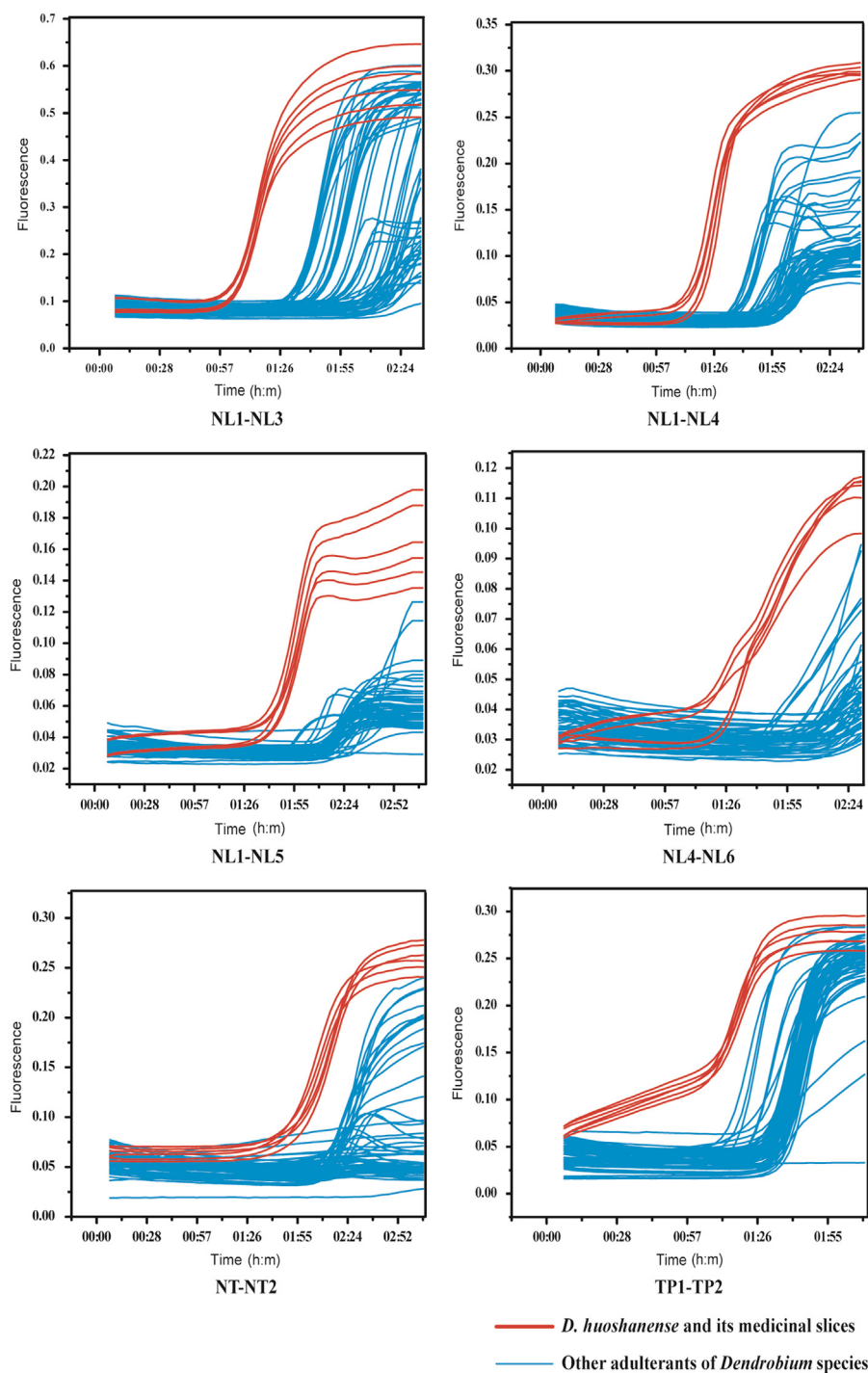
Figure 5 The identification of *D. huoshanense* and its medicinal slices based on the real-time PCR assay. The red lines indicate the *D. huoshanense* and its medicinal slices. The blue lines indicate other adulterants of *Dendrobium* species.

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*[5] and *D. loddigesii*[21] 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.[3] 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. moniliform*, Geng et al.[37] 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[38,39]; thus, mutational hotspots of

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.[20] successfully distinguished *D. fimbriatum* from other *Dendrobium* species based on the sequence of *psbA–trnH* intergenic spacers.

Recently, Niu et al.[30] assessed 30 complete plastome sequences of *Dendrobium* species and proposed that the top-ten mutational hotspots *psbB-psbT*, *ndhF-rpl32*, *trnT-trnL*, *rpl32-trnL*, *clpP-psbB*, *trnL* intron, *rpl16-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 mutational hotspots reported in Niu et al.[30]. 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.[40]. 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 mutational hotspots, newly put forth by Niu et al.[30]. 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.[40] 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[41–43].

Furthermore, after intensive processing, the medicinal slices of *Dendrobium* species become more difficult to distinguish[44]. Recently, well-developed molecular marker techniques, *i.e.*, SSR, ISSR, AFLP and DNA barcodes became available for the identification of medicinal plants[9–11,45–49]. 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[50–52]. After intensive processing, the concentrations of polysaccharides and other secondary metabolites 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[5,17]. 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 (Fengdous).

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*[5,17]. Moreover, real-time PCR, a highly sensitive method of detecting DNA, would be effective with "Fengdou" DNAs[17]. 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[16]. 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[53–55]. It has been successfully applied to the identification of species and commercial products[56,57]. In *Dendrobium*, the species of *D. fimbriatum*[20], *D. loddigesii*[21] and *D. officinale*[5] can be identified by ARMS-primers. 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[58,59]. Our results show that all the 6 pairs of ARMS-primer amplified in *D. huoshanense* have significantly lower C_t 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[5]. 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 can not be used for identifying several important *Dendrobium* species, *i.e.*,

D. huoshanense and its closely related species *D. moniliforme* because of their similar intra-specific nucleotide variation[3,37]. Secondly, the authentication study based on the SNVs in only one DNA locus may not be reliable. Thirdly, whether the RT-ARMS method is suitable for the authentication of other *Dendrobium* species still needs to be testified. To overcome those disadvantages, the plastome sequence 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.

Acknowledgments

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 Dendrobiinae (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 (bibenzyl, phenanthrene, and fluorenone) 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, Dobránszki 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. Asahina 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 41: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 fimbriatum* 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, Zhu S, Sun J, Liu W, Ding X. The complete plastome sequences of four orchid species: insights into the evolution of the Orchidaceae and the 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 chloroplast genomes of photosynthetic orchids: insights into evolution of the Orchidaceae and development of molecular markers for phylogenetic applications. *PLoS One* 2014;**9**:e99016.

30. Niu Z, Zhu S, Pan J, Li L, Sun J, Ding X. Comparative analysis of *Dendrobium* plastomes and utility of plastomic mutational hotspots. *Sci Rep* 2017;**7**:2073.

31. Tsi ZH. *Flora of China*. Science Press; 1999.

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

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

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

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

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

37. Geng LX, Zheng R, Ren J, Niu ZT, Sun YL, Xue QY, et al. Applylication of new type combined fragments: nr DNA ITS+ nad 1-intron 2 for identification of *Dendrobium* species of Fengdous. *Acta Pharm Sin* 2015;**50**:1060.

38. 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.

39. Drouin G, Daoud H, Xia J. Relative rates of synonymous substitutions in the mitochondrial, chloroplast and nuclear genomes of seed plants. *Mol Phylogenet Evol* 2008;**49**:827–31.

40. 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.

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

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

43. 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.

44. 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.

45. 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.

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

47. 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.

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

49. 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>.

50. 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.

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

52. 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.

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

54. 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.

55. 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 1model studies. *Nucleic Acids Res* 1990;**18**:999–1005.

56. Li XX, Ding XY, Chu BH, Ding G, Gu S, Qian L, et al. Molecular authentication of *Alisma orientale* by PCR-RFLP and ARMS. *Planta Med* 2006;**72**:1–4.

57. Park MJ, Kim MK, In JG, Yang DC. Molecular identification of Korean ginseng by amplification refractory mutation system-PCR. *Food Res Intern* 2006;**39**:568–74.

58. 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.

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