

•Research articles•

## Rapid identification of *Dendrobium officinale* using Loop-Mediated Isothermal Amplification (LAMP) method

YANG Lu<sup>1</sup>, WU Wen-Ru<sup>1\*</sup>, ZHOU Hua<sup>2,3</sup>, LAI Hui-Li<sup>4</sup>, FU Fei<sup>1</sup>

<sup>1</sup> School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou 510006, China;

<sup>2</sup> Faculty of Chinese Medicine, Macau University of Science and Technology, Macau, China;

<sup>3</sup> State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Macau, China;

<sup>4</sup> School of Nursing, Guangdong Food and Drug Vocational College, Guangzhou 510520, China

Available online 20 May, 2019

**[ABSTRACT]** *Dendrobium officinale* is not only an ornamental plant, but also a valuable medicinal herb that is widely used in traditional Chinese medicine. However, distinguishing *D. officinale* from other *Dendrobium* species is usually a difficult task. In this study, we developed a rapid identification protocol for *D. officinale* using the loop-mediated isothermal amplification (LAMP) method. A set of primers were specifically designed to detect a modified internal transcribed spacer region of *D. officinale* at 65 °C within 40 min after adding SYBR<sup>®</sup> Green I, which was used for the detection of *D. officinale*. Unlike commonly used adulterants, reaction mixtures containing *D. officinale* DNA changed from orange to green, and this color change was easily observed with the naked eye. Thus, this methodology can be used to accurately differentiate *D. officinale* from other *Dendrobium* species, is quick as all *D. officinale* samples were amplified within 40 min, and specific as samples of the adulterants were not amplified. The specificity of this LAMP-based method was confirmed by testing 17 samples of *D. officinale* and 32 adulterant samples from other *Dendrobium* species. This LAMP-based rapid identification method does not require expensive equipment or specialized techniques and can be used in field surveys for accurate and fast on-site identification.

**[KEY WORDS]** *Dendrobium officinale*; ITS; Loop-mediated isothermal amplification; Identification

**[CLC Number]** Q949    **[Document code]** A    **[Article ID]** 2095-6975(2019)05-0337-09

### Introduction

*Dendrobium officinale* Kimura et Migo, an Orchidaceae, is a popular and valuable traditional Chinese herbal medicine. Because it nourishes the stomach and enhances the production of body fluids, this herbal medicine has been commercialized as a herbal tonic and health food in China and many other Asian countries for hundreds of years. In Chinese medicine, *D. officinale* is considered to have the best medicinal properties among *Dendrobium Sw.* species due to the rich bioactive

polysaccharides present in its stems and leaves<sup>[1]</sup>. Modern pharmacological studies have shown that *D. officinale* has several benefits including functioning as an anti-oxidant and immunomodulator, reducing fatigue, and ameliorating pulmonary function<sup>[2–5]</sup>. In recent years, many other effects, such as preventing cancer, functioning as an anti-angiogenic, and treating colitis, have also been reported<sup>[6–8]</sup>. Due to its economic value and great market demand, many varieties of *D. officinale* commodities are available. However, this has led to the production and distribution of several adulterated products in the market<sup>[9]</sup>.

According to Chinese Pharmacopoeia<sup>[10]</sup>, Fengdou is a relatively well-known commodity of *Dendrobium* species, often considered an invaluable product; *D. officinale* is the only botanical source of processed Fengdou. *Dendrobium officinale* was distinguished from other medicinal *Dendrobium* species in the latest edition of the Chinese Pharmacopoeia<sup>[10]</sup>. However, a variety of wild *Dendrobium* species are being used for Fengdou processing and marketed<sup>[11]</sup>. Currently, more than 30 species belonging to *Dendrobium*,

**[Received on]** 17-Feb.-2019

**[Research funding]** This work was supported by the Opening Project of State Key Laboratory of Quality Research in Chinese Medicine (Macau University of Science and Technology) (No. MUST-SKL-2016-07) and the National Training Program of Innovation and Entrepreneurship for Undergraduates (No. 201710572024).

**[\*Corresponding author]** Tel: 86-20-39358296, E-mail: wuwenu@gzucm.edu.cn

These authors have no conflict of interest to declare.

Published by Elsevier B.V. All rights reserved

and to other plant genera, such as *Pholidota*, *Ephemerantha*, and *Bulbophyllum*, have been used and marketed as *D. officinale* and other medicinal *Dendrobium* dried products [12], which seriously affects the quality and efficacy of *D. officinale* commodities. These dried products are generally twisted into a spiral or spring form and have very similar morphological characteristics to those of *D. officinale*, therefore making them difficult to distinguish from *Dendrobium* species using conventional methods. Furthermore, common chemical composition analysis methods are time-consuming, involve complicated operations and exhibit poor specificity. Thus, it is important to adopt effective and scientific identification methods for *D. officinale*.

In recent years, molecular diagnostic techniques have been established and used as fast alternatives to traditional detection techniques. A wide variety of molecular technologies, especially polymerase chain reaction (PCR)-based methods such as random amplified polymorphic DNA, simple sequence repeats, bidirectional PCR, and DNA barcoding [13–16], have been developed for the identification of *D. officinale* and are considered robust nucleic acid amplification techniques for molecular analysis platforms. However, these methods can only be performed in the laboratory due to long processing times and need for thermocycling equipment and technical expertise [17]. Thus, researchers were encouraged to develop DNA amplification techniques that are not subject to these constraints.

The introduction of isothermal nucleic acid amplification technology, represented by loop-mediated isothermal amplification (LAMP) [18], has solved many problems previously associated with the identification of nucleic acid molecular markers by allowing their rapid identification. LAMP is a new nucleic acid amplification technology that amplifies DNA/RNA under isothermal conditions (60–65 °C) for 30–60 min using a set of four specifically designed primers and a high strand-displacement activity *Bacillus stearothermophilus* (*Bst*) DNA polymerase. Compared with traditional PCR technology, LAMP is more efficient and can be applied in resource-limited laboratories that do not have a PCR thermal cycler. Furthermore, LAMP products are more readily detectable than PCR products due to the large amount of pyrophosphate precipitate that is produced in the reaction mixture [19], therefore it is suitable for rapid identification of the samples. The LAMP method is characterized by its high specificity and high sensitivity as well as simplicity, speed, and low cost, and has been successfully used in many areas, such as pathogen detection, rapid molecular diagnosis of disease, and food testing [20–22]. Due to its growing popularity, LAMP has been gradually applied to the molecular identification of plants used in traditional Chinese medicine and has been used as an alternative technology for herbal medicine identification [23]. Since Sasaki *et al.* successfully distinguished *Curcuma longa*, *C. aromatica*, and *Panax ginseng* using LAMP [24–25], *Hedyotis diffusa*, *Taraxacum formosanum*, and toxic *Aristolochia*

*manshuriensis* [26–28] have been identified using this method. In their study comprising the identification of the valuable herb *Crocus sativus* using the LAMP method, Zhao *et al.* presented a practical standard operating procedure for herbal authentication by LAMP, aiming to provide an immediate testing method for many other herbs [29].

In the current study, the LAMP method was used to design a set of primers for *D. officinale* internal transcribed spacer (ITS) sequences. At the 5' end of the inner primer, B1c, a mismatch in the second-to-last base was artificially introduced to allow the amplification of *D. officinale* with fluorescent signals, while closely related species were not amplified. Contrary to traditional molecular identification methods that require complicated processes like electrophoresis, detection of *D. officinale* using the LAMP method was directly performed, and observed with the naked eye after 40 min at constant temperature during ITS amplification. The highly reproducible results demonstrated that this method is easy, highly efficient, and reliable for gene diagnosis and rapid identification of *D. officinale*.

## Materials and Methods

### Plant samples

The 17 samples of *D. officinale* and 146 samples of 32 other *Dendrobium* species used in this study were collected from Zhejiang, Anhui, and Yunnan Provinces, China, and were identified based on morphological characters by WU Wen-Ru at the School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine. The number of samples per species and the origin of all samples used in the present study are summarized in Table 1. All materials were stored at the Guangzhou University of Chinese Medicine.

### Primer design

Based on the bioinformatics analysis, primers for LAMP analysis of *D. officinale* were designed to target the ITS gene. The GenBank sequence HQ114245.1 was used for primer design on Primer Explorer (<http://primerexplorer.jp/lampv5e/index.html>), a LAMP primer designing software. After screening several primers, we chose the subset for which the mismatch site was located in the 5' end of the B1c primer (PCR start site). In the second-to-last position on the 5' end, the C base was artificially changed to A. Thus, the difference between *D. officinale* and other species of *Dendrobium* was expanded to increase the specificity of LAMP primers. Fig. 1 and Table 2 show the design process and primer sequences, respectively.

### DNA preparation

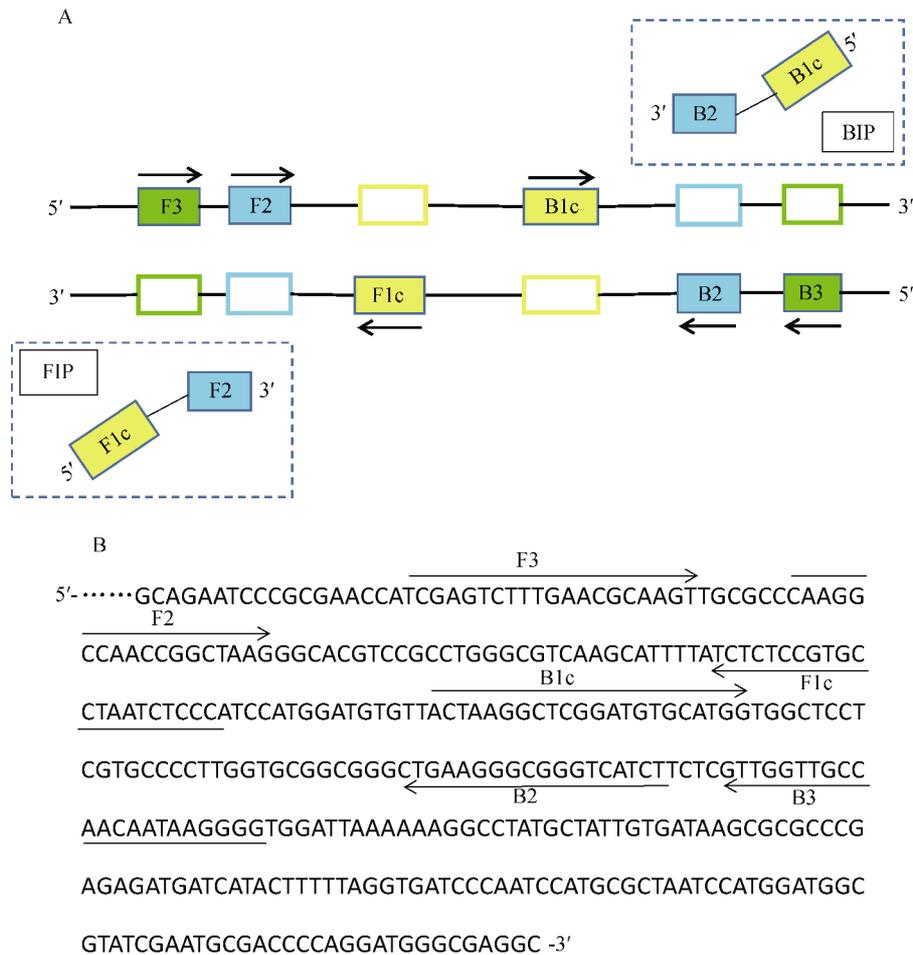
Leaf and stem tissues (100-mg samples) were obtained from each accession and either immediately used to extract DNA or stored at –80 °C for isolating DNA later. To extract genomic DNA, samples were shaken at high speed in 2-mL microcentrifuge tubes with stainless steel beads in a Qiagen TissueLyser LT (QIAGEN, Germany). After pulverization, 750 µL of cetyltrimethylammonium bromide (CTAB) precipitation solution [2% CTAB, 10 mmol·L<sup>-1</sup> EDTA, 100 mmol·L<sup>-1</sup>

**Table 1 Plant materials used in this study**

No.	Species	Sample Number	Origin
1	<i>Dendrobium officinale</i>	2	Bozhou, Anhui Province, China
2	<i>Dendrobium officinale</i>	3	Huoshan, Anhui Province, China
3	<i>Dendrobium officinale</i>	10	Kunming, Yunnan Province, China
4	<i>Dendrobium officinale</i>	1	Pu'er, Yunnan Province, China
5	<i>Dendrobium officinale</i>	1	Zhejiang Province, China
6	<i>Dendrobium polyanthum</i>	3	Yunnan Province, China
7	<i>Dendrobium gratiosissimum</i>	3	Pu'er, Yunnan Province, China
8	<i>Dendrobium stuposum</i>	3	Pu'er, Yunnan Province, China
9	<i>Dendrobium trigonopus</i>	3	Pu'er, Yunnan Province, China
10	<i>Dendrobium falconeri</i>	3	Pu'er, Yunnan Province, China
11	<i>Dendrobium wardianum</i>	3	Yunnan Province, China
12	<i>Dendrobium denneanum</i>	3	Pu'er, Yunnan Province, China
13	<i>Dendrobium cucullatum</i>	3	Yunnan Province, China
14	<i>Dendrobium capillipes</i>	3	Pu'er, Yunnan Province, China
15	<i>Dendrobium ellipsophyllum</i>	3	Yunnan Province, China
16	<i>Dendrobium findlayanum</i>	9	Pu'er, Yunnan Province, China
17	<i>Dendrobium chrysotoxum</i>	21	Pu'er, Yunnan Province, China
18	<i>Dendrobium williamsonii</i>	3	Pu'er, Yunnan Province, China
19	<i>Flickingeria calocephala</i>	3	Pu'er, Yunnan Province, China
20	<i>Dendrobium dixanthum</i>	3	Pu'er, Yunnan Province, China
21	<i>Dendrobium tosaense</i>	3	Pu'er, Yunnan Province, China
22	<i>Dendrobium heterocarpum</i>	3	Pu'er, Yunnan Province, China
23	<i>Dendrobium acinaciforme</i>	3	Pu'er, Yunnan Province, China
24	<i>Dendrobium nobile</i>	9	Pu'er, Yunnan Province, China
25	<i>Dendrobium crystallinum</i>	3	Yunnan Province, China
26	<i>Dendrobium fimbriatum.</i>	3	Pu'er, Yunnan Province, China
27	<i>Dendrobium crepidatum</i>	3	Yunnan Province, China
28	<i>Dendrobium loddigesii</i> Rolfe	3	Pu'er, Yunnan Province, China
29	<i>Dendrobium thyrsiflorum</i> Rchb.	3	Yunnan Province, China
30	<i>Dendrobium gibsonii</i> Lindl.	3	Pu'er, Yunnan Province, China
31	<i>Dendrobium chrysanthum</i>	3	Pu'er, Yunnan Province, China
32	<i>Dendrobium furcatopedicellatum</i>	3	Pu'er, Yunnan Province, China
33	<i>Dendrobium harveyanum</i>	3	Pu'er, Yunnan Province, China
34	<i>Dendrobium moniliforme</i>	3	Yunnan Province, China
35	<i>Dendrobium hancockii</i>	3	Pu'er, Yunnan Province, China
36	<i>Dendrobium exile</i> Schltr.	3	Yunnan Province, China
37	<i>Dendrobium falconeri</i>	3	Pu'er, Yunnan Province, China

Tris-HCl (pH 8.0)] was added to the extraction mixture and placed in a water bath at 65 °C for 40 min. The mixture was centrifuged at 5000 r·min<sup>-1</sup> for 5 min, and the supernatant was discarded. This step was repeated twice and DNA from the material remaining in the tube was then extracted using the modified CTAB method as described by Cui *et al.* [30]. The

above steps aimed to increase the amount of polysaccharides removed from *Dendrobium* sp. samples, thereby improving the success rate of the extraction. Subsequently, extracted templates were examined in the Ultramicro UV spectrophotometer (Thermo Fisher Scientific, USA) at A260/A280 and A260/A230.



**Fig. 1 (A) Schematic diagram of two inner primers (FIP, BIP), and two outer primers (F3, B3), for LAMP. (B) ITS target DNA fragment. This sequence was used to design two pairs of primers, which are shown in different colors, with the arrows showing the orientation of the primers.**

**Table 2 LAMP species-specific primers**

Name	Sequence (5'-3')
FIP	TGGGAGATTAGGCACGGAGAGACAAGGCCAACCGGCTAAG
BIP	A <u>A</u> TAAGGCTCGGATGTGCATGGAGATGACCCGCCCTTCAG
F3	TCGAGTCTTTGAACGCAAGT
B3	CCCCTTATTGTTGGCAACCA

**LAMP reaction**

LAMP reactions were performed in a total volume of 25  $\mu$ L, and the reaction mix consisted of primers FIP and BIP (1.6  $\text{mmol}\cdot\text{L}^{-1}$  each), primers F3 and B3 (0.2  $\text{mmol}\cdot\text{L}^{-1}$  each), 1.4  $\text{mmol}\cdot\text{L}^{-1}$  dNTPs (TaKaRa, Japan), 10  $\times$  Isothermal Amplification Buffer (New England Biolabs, USA), 6  $\text{mmol}\cdot\text{L}^{-1}$   $\text{MgSO}_4$  (New England Biolabs), 8 U of *Bst* 2.0 WarmStart<sup>®</sup> DNA Polymerase (New England Biolabs), and 2  $\mu$ L of template DNA. Sterile deionized water was used as the template for the negative control. These reactions were carried out in 0.2-mL microtubes, and 1  $\mu$ L of 1/10 diluted original SYBR<sup>®</sup> Green I (Invitrogen, USA) was added to the inner lid of every microtube before incubating the reactions in the Arktik<sup>™</sup>

Thermal Cycler (Thermo Scientific, USA). Since color change depends on the amount of double-stranded DNA in a sample, solutions with LAMP amplicons present would turn green after brief centrifugation.

**Optimal reaction in real time**

Time of positivity ( $T_p$ , expressed in min) and melting temperature ( $T_m$ , expressed in  $^{\circ}\text{C}$ ) are two basic parameters for determining positive fluorescence signals and correspond to the time at which the second derivative of fluorescent amplification reaches its peak above the baseline value, and to the temperature at which amplification products melt into two single-stranded DNA molecules, respectively. To determine the optimal  $T_p$  and  $T_m$ , the fluorescence of five samples was

measured in real time at temperatures ranging from 63 to 67 °C. For real-time LAMP analysis, ROX reference dye II (Thermo, USA) (0.05 µL) and 1/500 diluted original SYBR Green I (Thermo, USA) (1.0 µL) were added to the LAMP reaction. The real-time thermal cycler ABI 7500 (Applied Biosystems, USA) operating version 2.3 was used to amplify and measure fluorescence at different reaction temperatures and different cycles. The program was set to the following conditions: 1 s to react and 40 s to collect data for either 60 or 90 cycles (1 cycle is 40 s).

**Specificity and sensitivity of LAMP reaction**

To determine the specificity of the primers, eight representative samples, including *D. officinale* and closely related *Dendrobium* species, were analyzed by real-time LAMP. To assess the sensitivity of the LAMP method, a series of decimal dilutions ranging from 100 ng·µL<sup>-1</sup> to 10 fg·µL<sup>-1</sup> were prepared. Genomic DNA was extracted as mentioned above and subjected to a 10 × dilution gradient. After that, genomic DNA (2 µL) from each dilution series was added to the LAMP reaction mixture as template.

**Visual detection of the LAMP reaction**

After adding 1 µL of 1/10 diluted SYBR Green I to the inner lid of the PCR tube containing DNA samples and reaction system, the tubes were instantaneously centrifuged after LAMP reaction for thorough mixing; Color changes in LAMP products were then observed in natural light, with naked eye.

To further investigate primer specificity, 40 representative samples, including *D. officinale* and closely related *Dendrobium* species, were amplified by the LAMP method. Sterile deionized water (2 µL) was used as template for the negative control in the LAMP assay.

**Results**

**Bioinformatics analyses**

Bioinformatics analysis were conducted to find a suitable target gene for LAMP detection. In this study, we selected the ITS (nuclear gene), maturase K (*matK*), ribulose-bisphosphate carboxylase (*rbcL*), intergenic spacers *trnH-psbA* and *trnL-trnF* (chloroplast regions), and NADH dehydrogenase subunit 1 (*nad1*, mitochondrial gene) of *D. officinale* and its adulterants, *D. fimbriatum*, *D. nobile*, *D. chrysotoxum*. All sequences were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and aligned (Table 3). The bioinformatics analyses, such as genetic diversity of the *Dendrobium* species were performed in DnaSP ver 4.5 software<sup>[31]</sup>.

The bioinformatics analyses of the *Dendrobium* species showed that all sequences contained some variation. However, the number of variable sites (Vs), Parsimony informative sites (Ps), insertions/deletions (Indels; I), and DNA polymorphisms (Pis) of four *Dendrobium* species (Table 4) revealed that DNA polymorphism was highest in ITS sequences and, therefore, this gene was the most suitable for LAMP targeting.

**Table 3** *Dendrobium* sequence accession number of GenBank

Species	Accession Number of GenBank					
	ITS	<i>rbcL</i>	<i>matK</i>	<i>trnH-psbA</i>	<i>trnL-trnF</i>	<i>nad1</i>
<i>D. officinale</i>	HQ114245.1	FJ216567.1	FJ794048.1	GQ153537.1	EF397937.1	JQ362951.1
<i>D. fimbriatum</i>	JN388588.1	AB519784.1	AB847758.1	KF177500.1	KF143567.1	AY974223.1
<i>D. nobile</i>	JN388579.1	AB519785.1	AB847821.1	EU887942.1	KP749341.1	JQ362952.1
<i>D. chrysotoxum</i>	JN388585.1	KT778725.1	KF143654.1	EU672792.1	EF397915.1	JQ350916.1

**Table 4** DNA polymorphism of *Dendrobium*

Sequence	L/bp	Vs	Ps	I	Pi
ITS	668	110	50	7	0.142 13
<i>rbcL</i>	630	5	0	0	0.003 97
<i>matK</i>	1413	15	7	3	0.008 63
<i>trnH-psbA</i>	850	9	3	54	0.008 17
<i>trnL-trnF</i>	704	14	32	49	0.045 90
<i>nad1</i>	711	41	1	5	0.030 68

L: Length. Vs: Variable sites. Ps: Parsimony informative sites. I: InDel sites. Pi: DNA Polymorphism

**Analysis of ITS sequence alignment and primer design**

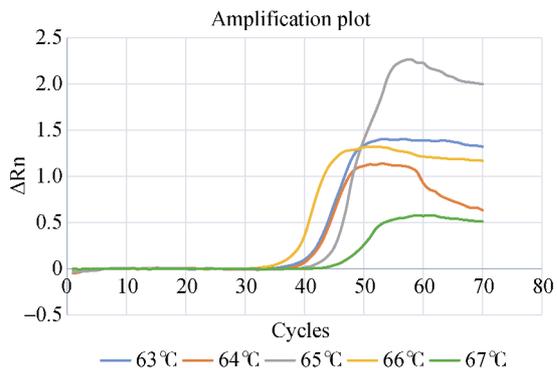
To design the LAMP primers for the authentication of *D. officinale*, the ITS regions of *D. officinale* was finally chosen as the target DNA region. Multiple sequences were aligned using DNAMAN ver 6 software<sup>[32]</sup>. Based on the interspecies and intraspecies sequence variation of ITS, four specific LAMP primers, F3, B3, FIP, and BIP, were designed to specifically amplify the *D. officinale* target DNA under isothermal conditions. In order to enhance the specificity of this set

of LAMP primers, we added a mismatch site that was located in the 5' end of the B1c primer (PCR start site). In the second-to-last position on the 5' end, the C base was artificially changed to A. Fig. 1 and Table 1 show the design process and primer sequences of LAMP species-specific primers, respectively.

**Optimal reaction temperature and time for real-time LAMP curve analysis**

To analyze LAMP reactions in real time, genomic DNA

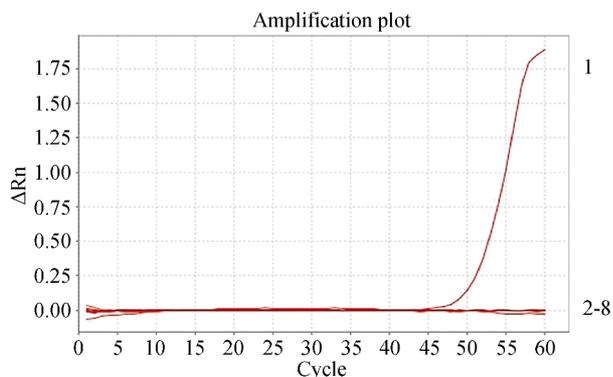
was used as template to amplify the target sequence in the real-time thermal cycler ABI 7500. The real-time kinetics of LAMP reactions were studied by monitoring fluorescence as described in the Materials and Methods. These reactions were performed at temperatures ranging from 63 to 67 °C, and it was found the reaction at 65 °C was the most productive as well as the fastest (Fig. 2). Thus, 65 °C for 40 min were the most appropriate conditions for the LAMP reaction.



**Fig. 2** Reactions were carried out at different temperatures from 63 to 67 °C. When tested at 65 °C, the LAMP assay had the highest amplification efficiency, compared with the reactions at other temperatures (1 cycle = 40 seconds).

*Specificity of LAMP*

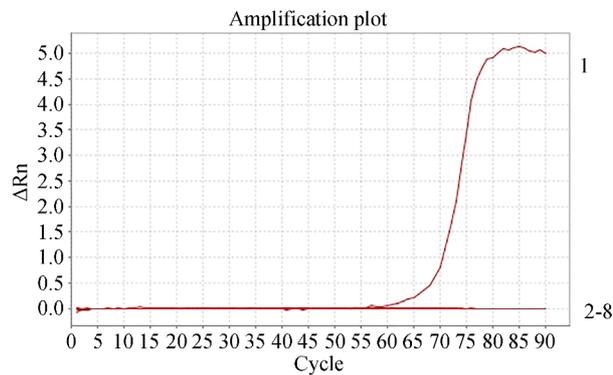
To detect specificity of the primers, eight representative samples of *D. officinale* and closely related *Dendrobium* species were tested at 65 °C for 40 min. First, the real-time PCR method was used to identify *D. officinale*. Positive reactions reached a plateau after a few minutes, while negative ones remained acyclic. The amplification rate of only *D. officinale* plateaued within 40 cycles, while other species were not successfully amplified (Fig. 3). Thus, the primers selected for this experiment were specific and suitable for rapid identification of *D. officinale*.



**Fig. 3** Specificity of LAMP by Real-time PCR. 1: *Dendrobium officinale*; 2: *D. fimbriatum*; 3: *D. nobile*; 4: *D. chrysotoxum*; 5: *D. stuposum*; 6: *D. ellipsophyllum*; 7: *Flickingeria calocephala*; 8: Negative control

*Sensitivity of LAMP*

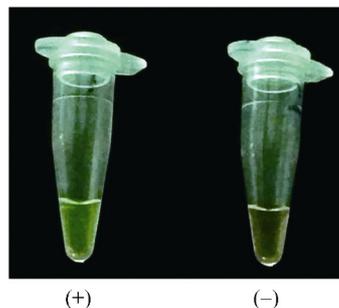
The initial concentration of genomic *D. officinale* DNA was 100 ng·μL<sup>-1</sup> and this was diluted to 10 fg·μL<sup>-1</sup> via 10-fold dilution; 2 μL of each diluted DNA solution was amplified via LAMP for 60 min at 65 °C to evaluate reaction sensitivity. The result shows that the detection-limit of template DNA was 100 ng·μL<sup>-1</sup> using the LAMP primers with base mismatch introduced (Fig. 4).



**Fig. 4** Sensitivity of LAMP. 1: 100 ng·μL<sup>-1</sup>; 2: 10 ng·μL<sup>-1</sup>; 3: 1 ng·μL<sup>-1</sup>; 4: 100 pg·μL<sup>-1</sup>; 5: 10 pg·μL<sup>-1</sup>; 6: 1 pg·μL<sup>-1</sup>; 7: 100 fg·μL<sup>-1</sup>; 8: 10 fg·μL<sup>-1</sup>

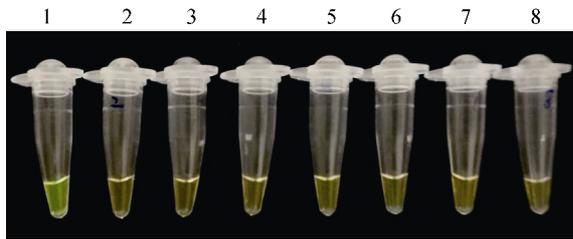
*Visual inspection of LAMP-initiated fluorescence*

One of the characteristics of LAMP is its ability to produce results visible with naked eye due to the color change of the LAMP product when it binds to the fluorescent SYBR Green I dye. As expected, DNA samples of *D. officinale* turned green, while the negative control remained orange (Fig. 5).



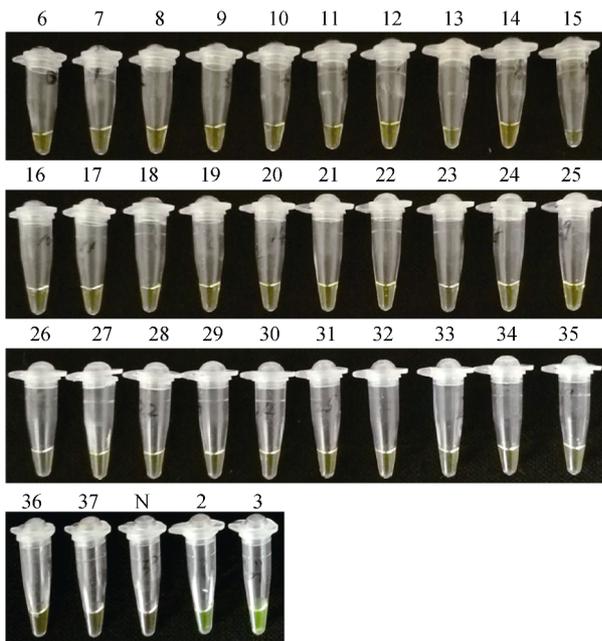
**Fig. 5** Visual assessment of DNA amplification by LAMP. The solution turned green (+) in the presence of a LAMP amplification, while it remained orange (-) with no amplification.

The LAMP reaction mixture containing amplified fragments turned green immediately after it was mixed with the internal tube SYBR Green I, whereas solutions without amplicons retained the original orange color of SYBR Green I. The samples used in the fluorescence curve analysis were also used to investigate the feasibility of the fluorescent visual inspection method, and the results were identical to that of real-time LAMP analysis (Fig. 3); only *D. officinale* samples changed color, while the other *Dendrobium* species and the negative control retained the original orange color (Fig. 6).



**Fig. 6 Application of fluorescent visual method. 1: *Dendrobium officinale*; 2: *D. fimbriatum*; 3: *D. nobile*; 4: *D. chrysotoxum*; 5: *D. stuposum*; 6: *D. ellipsophyllum*; 7: *Flickingeria calcephala*; 8: Negative control**

To confirm that this method can be used to distinguish *D. officinale* from its congeners, DNA samples from other 32 *Dendrobium* species were tested using this method (Fig. 7). Our results indicated that the LAMP method using the specific primers designed in the present study were able to rapidly discriminate *D. officinale* from its common adulterants under isothermal conditions within 40 min after the addition of SYBR Green I.



**Fig. 7 Specificity of LAMP detection of fluorescent visual method. Tubes 2–37 correspond to 2–37 listed in Table 4. Tube N: negative control**

## Discussion

Among the 1000 *Dendrobium* species identified globally, 74 (and two varieties) are known from China<sup>[9]</sup>. Due to its popularity and effectiveness as an herbal medicine, a rapid and precise diagnostic method of *D. officinale* is urgently required. At present, the most commonly used *D. officinale* detection and identification methods involve amplification of a specific DNA fragment via PCR, and the most common

technique to demonstrate the presence of amplified DNA sequences is gel electrophoresis, which also allows estimating the quantity and size of the DNA molecule. The identity of the amplified DNA can be determined by sequencing or by digesting it with restriction enzymes followed by fragment analysis to determine the sizes of the resulting fragments. However, all these methods have some limitations, such as involving relatively long and complex operations. In contrast to conventional PCR, LAMP is suitable for on-site detection in the field because of its speed and robustness, and it can also be applied by laypeople without the need for elaborate laboratory equipment<sup>[33]</sup>. To date, LAMP has been used to detect various bacteria and viruses<sup>[20, 34]</sup>, as well as to identify several valuable and toxic Chinese medicines<sup>[25, 28-29]</sup>. However, no LAMP-based method for the detection of *Dendrobium* species had been developed so far.

In the present study, we developed a highly practical and valid LAMP-based method for detecting *D. officinale*. We achieved this by designing a four-primer set consisting of F3, B3, FIP, and BIP, which recognized six distinct sequences on the target DNA. The reactions can be completed in a water bath, as opposed to requiring expensive instruments, and the amplified products can be detected visually by the naked eye within 1 hour.

In previous studies, DNA extraction was performed using the Kit<sup>[35]</sup>, CTAB<sup>[36]</sup>, and modified CTAB methods<sup>[37]</sup> to obtain high quality DNA. However, because preliminary experiments showed that the high content of polysaccharides in *D. officinale* negatively affected its DNA extraction efficiency<sup>[11]</sup>, in the present study we applied the modified CTAB method for DNA extraction as it uses the CTAB precipitation solution to separate polysaccharides, resulting in a higher concentration and purity of the DNA template compared to other methods.

The core of the LAMP process is primer design, which is also the key problem that hinders the development of LAMP technology. *Dendrobium officinale* and related species are very similar genetically thus making it difficult to design specific primers. In this study, LAMP technology was first used to identify *D. officinale* through the analysis of several DNA sequences and then to design LAMP primers. The design of specific LAMP primers for *D. officinale* was a major difficulty in the present study. Although we tried using several target DNA regions, including ITS, *matK*, *rbcL*, *trnH-psbA*, *trnL-trnF*, and *nad1*, we ultimately selected the most distinct ITS sequence as the target because it presented the highest number of DNA polymorphisms among screened sequences. Moreover, Dong *et al.* have used ITS sequences to successfully identify *D. officinale*<sup>[15]</sup>. The multicopy ITS sequence has good amplification efficiency, so even processed *D. officinale* products with a high degree of DNA degradation can be effectively identified<sup>[30]</sup>. While designing primers, we artificially introduced a mismatch (C modified to A) at the second to last base position at the 5' end of the inner primer,

B1c. Thus, amplified ITS fragments of the several *Dendrobium* species differed by more than five nucleotides at this location. The experiment showed that the introduction of this mismatch might have considerably increased the specificity of LAMP primers allowing us to easily distinguish *D. officinale* from related species. In the present study, the detection-limit of template DNA was 100 ng·μL<sup>-1</sup> using the LAMP primers targeting the introduced mismatch, and LAMP sensitivity was not as high as that observed in other studies<sup>[34]</sup>. This difference might be due to the increase in specificity caused by the introduced mismatch, i.e., some sensitivity was offset. However, this method was as sensitive as PCR. The method of primer mismatch applied here provides reference for future LAMP experiments.

Another advantage of LAMP for molecular identification is that there are several simple methods available for detecting the LAMP product. It can be directly determined whether the reaction has occurred or not. Because LAMP reactions produce many bands of different sizes forming a ladder of DNA fragments of 100 bp or more, it is common to verify the amplification results with gel electrophoresis. However, this step increases the risk of amplicon contamination. Because the concentration of LAMP amplification products is extremely high, once we open the tube, the reaction products can easily create aerosol pollution, which would result in higher false positive rates<sup>[23]</sup>. In addition, the time and energy required for gel electrophoresis reduce the applicability of the LAMP method in the field. Although amplification processes are visible by gel electrophoresis, its detection costs are greatly increased. A relatively simple approach to detect LAMP amplicons is to observe the change in turbidity due to the formation of a white magnesium pyrophosphate precipitate. However, this result is not obvious and is easily influenced by subjective observation. Thus, we used the low cost and easy to operate visual fluorescence method.

In the present study, 1 μL of 1/10 diluted original SYBR Green I was added to the inner lid of every centrifuge tube before reactions were initiated. Because color change depends on the amount of DNA in the tube, solutions containing LAMP amplicons turned green after brief centrifugation and mixture. Using this closed-tube method, the aerosol pollution caused by opening the lids was avoided, visual LAMP detection was improved, and the time and cost of the analysis were reduced. The established LAMP-based method performs well in a thermocycler, a water bath, on a heating block, and even in an insulated mug<sup>[34]</sup>, thus allowing fast on site detection.

An important improvement in point-of-care DNA extraction methods<sup>[38]</sup>, and the advances in commercial rapid molecular detection, have greatly promoted the rapid identification of Chinese medicinal materials. We have applied the cellulose-based DNA extraction method<sup>[39]</sup> to this experiment, and it greatly simplified the whole experiment process, but to ensure the stability and accuracy of the result, the method is still in the process of being perfected. We will do further research, such as combining the LAMP method with lateral

flow dipstick, gene chips, and other technologies<sup>[39–40]</sup>. It is expected to provide a practical standard operating procedure (SOP) for the point-of-care herbal authentication.

This method will be of great value for the identification of *D. officinale* products in the herbal medicine market, also providing a technical guarantee for the quality, stability, and safety of *D. officinale* products.

## Conclusion

The LAMP-based method developed in the present study can be used for the rapid and accurate identification of *D. officinale*. Coupled with visual inspection of LAMP-initiated fluorescence, this method is particularly suitable for rapid detection of TCM in the field and provides a reference for the detection of other medicinal materials.

## References

- [1] Teixeira da Silva JA, Ng TB. The medicinal and pharmaceutical importance of *Dendrobium* species [J]. *Appl Microbiol Biotechnol*, 2017, **101**(6): 2227-2239.
- [2] Zhang JY, Guo Y, Si JP, et al. A polysaccharide of *Dendrobium officinale* ameliorates H<sub>2</sub>O<sub>2</sub>-induced apoptosis in H9c2 cardiomyocytes via PI3K/AKT and MAPK Pathways [J]. *Int J Biol Macromol*, 2017, **104**: 1-10.
- [3] He TB, Huang YP, Yang L, et al. Structural characterization and immunomodulating activity of polysaccharide from *Dendrobium officinale* [J]. *Int J Biol Macromol*, 2016, **83**: 34-41.
- [4] Wei W, Li ZP, Zhu T, et al. Anti-fatigue effects of the unique polysaccharide marker of *Dendrobium officinale* on BALB/c mice [J]. *Molecules*, 2017, **22** (1): 155.
- [5] Song TH, Chen XX, Tang SCW, et al. *Dendrobium officinale* polysaccharides ameliorated pulmonary function while inhibiting mucin-5AC and stimulating aquaporin-5 expression [J]. *J Funct Foods*, 2016, **21**: 359-371.
- [6] Zheng QP, Qiu DS, Liu XJ, et al. Antiproliferative effect of *Dendrobium catenatum* Lindley polypeptides against human liver, gastric and breast cancer cell lines [J]. *Food Funct*, 2015, **6**(5): 1489-1495.
- [7] Yue H, Liu YQ, Qu HH, et al. Structure analysis of a novel heteroxylan from the stem of *Dendrobium officinale* and anti-angiogenesis activities of its sulfated derivative [J]. *Int J Biol Macromol*, 2017, **103**: 533-542.
- [8] Liang J, Chen SX, Chen JH, et al. Therapeutic roles of polysaccharides from *Dendrobium officinale* on colitis and its underlying mechanisms [J]. *Carbohydr Polym*, 2018, **185**: 159-168.
- [9] Chu C, Yin HM, Xia L, et al. Discrimination of *Dendrobium officinale* and its common adulterants by combination of normal light and fluorescence microscopy [J]. *Molecules*, 2014, **19**(3): 3718-3730.
- [10] *Pharmacopoeia of the People's Republic of China* [S]. Vol 1. 2015: 282-283.
- [11] Xu HJ, Hou BW, Zhang JZ, et al. Detecting adulteration of *Dendrobium officinale* by real-time PCR coupled with ARMS [J]. *Int J Food Sci Tech*, 2012, **47**(8): 1695-1700.
- [12] Zhang Q, Duan CL. Studies on the authentication of *Den-*

- drobium officinale* [J]. *Biotechnol Bull*, 2008, (6): 69-72.
- [13] Jin B, Jiang FS, Yu J, et al. Study on sequence characterized amplified region (SCAR) markers in *Dendrobium candidum* [J]. *Chin Med Mat*, 2010, 33(3): 343-346.
- [14] Lu JJ, Suo NN, Hu X, et al. Development and characterization of 110 novel EST-SSR markers for *Dendrobium officinale* (Orchidaceae) [J]. *Am J Bot*, 2012, 99(10): 415-420.
- [15] Dong XM, Jiang C, Yuan Y, et al. Study on identification of *Dendrobium officinale* and related species by bidirectional PCR amplification of mismatched and specific alleles [J]. *Chin J Chin Mater Med*, 2017, 42(5): 896-901.
- [16] Feng SG, Jiang Y, Wang S, et al. Molecular identification of *Dendrobium* Species (Orchidaceae) based on the DNA barcode ITS2 region and its application for phylogenetic study [J]. *Int J Mol Sci*, 2015, 16(9): 21975-21988.
- [17] Law JWF, Ab Mutalib NS, Chan KG, et al. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations [J]. *Front Microbiol*, 2015, 5: 770.
- [18] Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA [J]. *Nucleic Acids Res*, 2000, 28(12): 63.
- [19] Zhang XZ, Lowe SB, Gooding JJ. Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP) [J]. *Biosens Bioelectron*, 2014, 61: 491-499.
- [20] Zhang JH, Feng YJ, Hu D, et al. Rapid and sensitive detection of H7N9 avian influenza virus by use of reverse transcription-loop-mediated isothermal amplification [J]. *J Clin Microbiol*, 2013, 51(11): 3760-3764.
- [21] Besuschio SA, Murcia ML, Benatar AF, et al. Analytical sensitivity and specificity of a loop-mediated isothermal amplification (LAMP) kit prototype for detection of *Trypanosoma cruzi* DNA in human blood samples [J]. *PLoS Neglect Trop D*, 2017, 11(7): e0005779.
- [22] Kim MJ, Kim HY. Direct duplex real-time loop mediated isothermal amplification assay for the simultaneous detection of cow and goat species origin of milk and yogurt products for field use [J]. *Food Chem*, 2018, 246: 26-31.
- [23] Li JJ, Xiong C, Liu Y, et al. Loop-mediated isothermal amplification (LAMP): Emergence as an alternative technology for herbal medicine identification [J]. *Front Plant Sci*, 2016, 7: 1956.
- [24] Sasaki Y, Nagumo S. Rapid identification of *Curcuma longa* and *C. aromatica* by LAMP [J]. *Biol Pharm Bull*, 2007, 30(11): 2229-2230.
- [25] Sasaki Y, Komatsu K, Nagumo S. Rapid detection of *Panax ginseng* by loop-mediated isothermal amplification and its application to authentication of ginseng [J]. *Biol Pharm Bull*, 2008, 31(9): 1806-1808.
- [26] Li M, Wong YL, Jiang LL, et al. Application of novel loop-mediated isothermal amplification (LAMP) for rapid authentication of the herbal tea ingredient *Hedyotis diffusa* Willd. [J]. *Food Chem*, 2013, 141(3): 2522-2525.
- [27] Lai GH, Chao J, Lin MK, et al. Rapid and sensitive identification of the herbal tea ingredient *Taraxacum formosanum* using loop-mediated isothermal amplification [J]. *Int J Mol Sci*, 2015, 16(1): 1562-1575.
- [28] Wu L, Wang B, Zhao MM, et al. Rapid identification of officinal *Akebiae Caulis* and its toxic adulterant *Aristolochiae Manshuriensis Caulis* (*Aristolochia manshuriensis*) by loop-mediated isothermal amplification [J]. *Front Plant Sci*, 2016, 7: 887.
- [29] Zhao MM, Shi YH, Wu L, et al. Rapid authentication of the precious herb saffron by loop-mediated isothermal amplification (LAMP) based on internal transcribed spacer 2 (ITS2) sequence [J]. *Sci Rep*, 2016, 6: 25370.
- [30] Li XW, Yang Y, Henry RJ, et al. Plant DNA barcoding: from gene to genome [J]. *Biol Rev*, 2015, 90(1): 157-166.
- [31] Rozas J, Sanchez-DelBarrio JC, Messeguer X, et al. DNA polymorphism analyses by the coalescent and other methods [J]. *Bioinformatics*, 2003, 19(18): 2496-2497.
- [32] Woffelman C. DNAMAN for Windows, Version 5.2.10. Lynon Biosoft, Institute of Molecular Plant Sciences, Leiden University: Leiden, The Netherlands, 2004.
- [33] Kong XJ, Qin WT, Huang XQ, et al. Development and application of loop-mediated isothermal amplification (LAMP) for detection of *Plasmopara viticola* [J]. *Sci Rep*, 2016, 6: 28935.
- [34] Huang W, Zhang H, Xu JS, et al. Loop-mediated isothermal amplification method for the rapid detection of *Ralstonia solanacearum* phylotype I mulberry strains in China [J]. *Front Plant Sci*, 2017, 8(76): 560.
- [35] Wang X, Liao HH, Huang HM, et al. Comparative test for DNA extraction methods of Citrus Huanglongbing pathogen [J]. *J South Arg*, 2013, 44(2): 225-229.
- [36] Doyle JJ, Doyle JL. A rapid total DNA preparation procedure for fresh plant tissue [J]. *Focus*, 1990, 12: 13-15.
- [37] Cui GH, Tang XJ, Huang LQ. Study on the extraction method of DNA in Chinese medicinal materials containing starch and polysaccharide [J]. *Chin J Chin Mater Med*, 2006, 31(16): 1365-1367.
- [38] Zou YP, Mason MG, Wang YL, et al. Nucleic acid purification from plants, animals and microbes in under 30 seconds [J]. *PLoS Biol*, 2017, 15(11): e2003916.
- [39] Foo PC, Chan YY, Mohamed M, et al. Development of a thermostabilised triplex LAMP assay with dry-reagent four target lateral flow dipstick for detection of *Entamoeba histolytica* and non-pathogenic *Entamoeba* spp. [J]. *Anal Chem Acta*, 2017, 966: 71-80.
- [40] Fang XE, Liu YY, Kong JL, et al. Loop-mediated isothermal amplification integrated on microfluidic chips for point-of-care quantitative detection of pathogens [J]. *Anal Chem*, 2010, 82(7): 3002-3006.

**Cite this article as:** YANG Lu, WU Wen-Ru, ZHOU Hua, LAI Hui-Li, FU Fei. Rapid identification of *Dendrobium officinale* using Loop-Mediated Isothermal Amplification (LAMP) method [J]. *Chin J Nat Med*, 2019, 17(5): 337-345.