Article

Loop-Mediated Isothermal Amplification (LAMP) Method for the Rapid Identification of *Dendrobium officinale*

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Abstract: Background: *Dendrobium officinale* is not only an ornamental plant, but also a valuable medicinal herb that is both effective and widely used in traditional Chinese medicine. However, distinguishing *D. officinale* from other *Dendrobium* species is usually a difficult task that need much time and complex technologies due to their very similar external morphologies. The aim of this study is to develop a fast, even on-spot approach to identify *D. officinale*.

Methods: We used DNA barcode-based loop-mediated isothermal amplification (LAMP) method with species-specific LAMP primers targeting the internal transcribed spacer (ITS) region of the rDNA of *D. officinale*. LAMP reaction time and temperature were optimized and the specificity and sensitivity of LAMP species-specific primers were assessed. Results: This technique showed a high specificity and sensitivity to amplify the genomic DNA of *D. officinale* and allowed for rapid amplification (within 40 min) of the ITS region under a constant and mild temperature range of 65 °C without using thermocyclers. Besides, by using SYBR® Green I dye as the color developing agent, the color change was easily observed with naked eye. Reaction mixture containing DNA of *D. officinale* changed from orange to green, while the other *Dendrobium* species and the negative control retained original orange color. The specificity of this LAMP-based method was confirmed by testing 17 samples of *D. officinale* and 32 adulterant samples from other *Dendrobium* species. Conclusions: 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.

Keywords: *Dendrobium officinale*; ITS; loop-mediated isothermal amplification; identification; rapid
1. 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 an 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 [1]. 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 [2–5]. In recent years, many other effects, such as preventing cancer, functioning as an anti-angiogenic, and treating colitis, have also been reported [6–8]. 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 [9].

According to Chinese Pharmacopoeia [10], 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 [10]. However, a variety of wild *Dendrobium* species are being used for Fengdou processing and marketed [11]. Currently, more than 30 species belonging to *Dendrobium*, 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 being difficult to identify those 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 being 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. aromatic, 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. (2016) 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 close 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 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.

2. Results

2.1. 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), 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 1). The bioinformatics analyses such as genetic diversity of the Dendrobium species were analysed by DnaSP ver 4.5 software [30].
The bioinformatics analyses of the *Dendrobium* species showed that all sequences contained some variation. But the number of variable sites (Vs), degenerate loci (Ps), insertions/deletions (Indels; I), and DNA polymorphisms (Pis) of four *Dendrobium* species (Table 2) revealed that DNA polymorphism was highest in ITS sequences and, therefore, this gene was the most suitable for LAMP targeting.

### Table 1. *Dendrobium* sequence Accession Number of GenBank

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

### Table 2. DNA Polymorphism of *Dendrobium*

<table>
<thead>
<tr>
<th>Sequence</th>
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<th>Vs</th>
<th>Ps</th>
<th>I</th>
<th>Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>668</td>
<td>110</td>
<td>50</td>
<td>7</td>
<td>0.14213</td>
</tr>
<tr>
<td>rbcL</td>
<td>630</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0.00397</td>
</tr>
<tr>
<td>matK</td>
<td>1413</td>
<td>15</td>
<td>7</td>
<td>3</td>
<td>0.00863</td>
</tr>
<tr>
<td>trnH-psbA</td>
<td>850</td>
<td>9</td>
<td>3</td>
<td>54</td>
<td>0.00817</td>
</tr>
<tr>
<td>trnL-trnF</td>
<td>704</td>
<td>14</td>
<td>32</td>
<td>49</td>
<td>0.04590</td>
</tr>
<tr>
<td>nad1</td>
<td>711</td>
<td>41</td>
<td>1</td>
<td>5</td>
<td>0.03068</td>
</tr>
</tbody>
</table>


### 2.2. Analysis of ITS2 Sequence Alignment and Primer Design

To design the LAMP primers for the authentication of *D. officinale*, the ITS2 regions of *D. officinale* was finally chosen as a target DNA region. Multiple sequences were aligned using DNAMAN ver 6 software [31]. Based on the interspecies and intraspecies sequence variation of ITS2, 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. Figure 1 and Table 3 showed the design process and primer sequences of LAMP species-specific primers, respectively.
Figure 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 3. LAMP species-specific Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-TP-FIP</td>
<td>TGGGAGATTAGGCACGGGACAGAAGCCACCCGCTAAG</td>
</tr>
<tr>
<td>3-TP-BIP</td>
<td>AATAAGGCCTCGGATGTCCATGGAGATGACCCGCCCTTCAG</td>
</tr>
<tr>
<td>3-TP-F3</td>
<td>TCGAGTCTTTGAACGCAAGT</td>
</tr>
<tr>
<td>3-TP-B3</td>
<td>CCCCTTATGGGCGAGGCCAACCAG</td>
</tr>
</tbody>
</table>
2.3. 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 (Figure 2). Thus, 65 °C for 40 min were the most appropriate conditions for the LAMP reaction.

![Amplification Plot](image)

Figure 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).

2.4. 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 acclinic. Only *D. officinale* plateaued within 40 cycles, while other species were not successfully amplified (Figure 3). Thus, the primers selected for this experiment were specific and suitable for rapid identification of *D. officinale*.
Figure 3. Specificity of LAMP by Real-time PCR. 1, Dendrobium officinale; 2, Dendrobium fimbriatum; 3, Dendrobium nobile; 4, Dendrobium chrysotoxum; 5, Dendrobium stiposum; 6, Dendrobium ellipsophyllum; 7, Flickingeria calocephala; 8, Negative control.

2.5. Sensitivity of LAMP

The initial concentration of genomic D. officinale DNA was 100 ng/μL and this was diluted to 10 fg/μL 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 using the LAMP primers with base mismatch introduced (Figure 4).
Figure 4. Sensitivity of LAMP. 1, 100ng/μl; 2, 10ng/μl; 3, 1ng/μl; 4, 100pg/μl; 5, 10pg/μl; 6, 1pg/μl; 7, 100fg/μl; 8, 10fg/μl.

2.6. 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 (Figure 5).

The LAMP reaction mixture containing amplified fragments turned green immediately after the addition of 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 (Figure 3); only *D. officinale* samples changed color, while the other *Dendrobium* species and the negative control retained the original orange color (Figure 6).

To confirm that this method can be used to distinguish *D. officinale* from its congener,
DNA samples from other 32 Dendrobium species were tested using this method (Figure 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.

Figure 7. Specificity of LAMP detection of fluorescent visual method. Tubes 2-37 correspond to 2-37 listed in Table 4. Tube N, negative control.

3. Discussion

Among the 1000 Dendrobium species identified globally, 74, plus two varieties, are known from China [9]. 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 including relatively long and complex operations. In contrast to conventional PCR, LAMP is suitable for onsite detection in the field because of its speed and robustness, and it can also be applied by
laymen without the need for elaborate laboratory equipment [32]. To date, LAMP has been used to detect various bacteria and viruses [33, 20], as well as to identify several valuable and toxic Chinese medicines [25, 29, 28]. 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 [34], CTAB [35], and modified CTAB methods [36] 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 [11], 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. (2017) have used ITS sequences to successfully identify *D. officinale* [15]. 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 [37]. 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 easily distinguishing *D. officinale* from related species. In the present study, the detection-limit of template DNA was 100 ng/μL using the LAMP primers targeting the introduced mismatch, and LAMP sensitivity was not as high as that observed in other studies [33]. 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 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 [23]. 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 turn green after brief centrifugation. Using this method, the aerosol pollution caused by opening the lids is avoided, visual LAMP detection is improved, and the time and cost of the analysis are reduced. The established LAMP-based method performs well in a thermocycler, in a water bath, on a heating block, and even in an insulated mug [33], thus allowing on site fast detection.

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.

4. Materials and methods

4.1. 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 Wenru Wu at the School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong Province, China. The number of samples per species and the origin of all samples used in the present study are summarized in Table 4. All materials were stored at the Guangzhou University of Chinese Medicine.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Sample Number</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dendrobium officinale</td>
<td>2</td>
<td>Bozhou, Anhui province, China</td>
</tr>
<tr>
<td>2</td>
<td>Dendrobium officinale</td>
<td>3</td>
<td>Huoshan, Anhui province, China</td>
</tr>
<tr>
<td>3</td>
<td>Dendrobium officinale</td>
<td>10</td>
<td>Kunming, Yunnan province, China</td>
</tr>
<tr>
<td>4</td>
<td>Dendrobium officinale</td>
<td>1</td>
<td>Pu’er, Yunnan province, China</td>
</tr>
<tr>
<td>5</td>
<td>Dendrobium officinale</td>
<td>1</td>
<td>Zhejiang province, China</td>
</tr>
<tr>
<td>6</td>
<td>Dendrobium polyanthum</td>
<td>3</td>
<td>Yunnan province, China</td>
</tr>
<tr>
<td>7</td>
<td>Dendrobium gratiosissimum</td>
<td>3</td>
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</tr>
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<td>8</td>
<td>Dendrobium stiposum</td>
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<td>Dendrobium trigonopus</td>
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<td>13</td>
<td>Dendrobium cucullatum</td>
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<td>Yunnan province, China</td>
</tr>
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</table>
4.2. Methods

4.2.1. 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. Figure 1 and Table 3 show the design process and primer sequences, respectively.

4.2.2. 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 later DNA isolation. To extract genomic DNA, samples were high-speed shaken in 2-mL microcentrifuge tubes with stainless steel beads in a Qiagen TissueLyser LT (QIAGEN, Germany). After pulverization, 750 μL of 65 °C cetyltrimethylammonium bromide (CTAB) precipitation solution [2% CTAB, 10 mM EDTA, 100 mM Tris-HCl (pH 8.0)] was added to the extraction mixture and placed in a 65 °C water bath for 40 min. The mixture was centrifuged at 5000 rpm for 5 min, and the supernatant was
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. (2006) [36]. 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 $A_{260}/A_{280}$ and $A_{260}/A_{230}$.

### 4.2.3. LAMP reaction

LAMP reactions were performed in a total volume of 25 μL, and the reaction mix consisted of primers FIP and BIP (1.6 mM each), primers F3 and B3 (0.2 mM each), 1.4 mM dNTPs (TaKaRa, Japan), 10 × Isothermal Amplification Buffer (New England Biolabs, USA), 6 mM MgSO$_4$ (New England Biolabs), 8 U of *Bst* 2.0 WarmStart® DNA Polymerase (New England Biolabs), and 2 μL 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 μL of 1/10 diluted original SYBR® Green I (Invitrogen, USA) was added to the inner lid of every microtube before incubating the reactions in the Arktik™ 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.

### 4.2.4. Optimal reaction in real time

Time of positivity ($T_p$, expressed in min) and melting temperature ($T_m$, expressed in °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).

### 4.2.5. Specificity and sensitivity of LAMP reaction

To determine the specificity of the primers, eight representative samples, including *D. officinale* and close *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 to 10 fg/μL 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.

### 4.2.6. 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.

5. Conclusion

The LAMP-based method developed in the present study can be used for the rapid and accurate identification of *D. officinale*. Due to its ease of operation and lack of need for sophisticated equipment, this method might be used in the field to accurately and efficiently identify medicinal herbs. Moreover, the continuous development of the LAMP method, especially the advances in commercial rapid molecular detection, has greatly promoted the rapid identification of Chinese medicinal materials. The LAMP method has been combined with lateral flow dipstick, gene chips and other technologies. It is expected to allow the rapid identification of traditional Chinese medicine related products on site. We will further optimize the DNA extraction method and try to improve the sensitivity of the LAMP method, and to combine monitoring methods to improve the detection step.

**Author Contributions:** WW and LY designed this experiment and helped to collecting the plant samples. LY performed this experiment and wrote this manuscript. HZ and HL provided assistance during the experiment. WW, LY, HZ, HL, and FF revised this manuscript.

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