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.

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

80 polymerase. Compared with traditional PCR technology, LAMP is more efficient and can be
81 applied in resource-limited laboratories that do not have a PCR thermal cycler. Furthermore,
82 LAMP products are more readily detectable than PCR products due to the large amount of
83 pyrophosphate precipitate that is produced in the reaction mixture [19], therefore being
84 suitable for rapid identification of the samples. The LAMP method is characterized by its high
85 specificity and high sensitivity as well as simplicity, speed, and low cost, and has been
86 successfully used in many areas, such as pathogen detection, rapid molecular diagnosis of
87 disease, and food testing [20–22]. Due to its growing popularity, LAMP has been gradually
88 applied to the molecular identification of plants used in traditional Chinese medicine and has
89 been used as an alternative technology for herbal medicine identification [23]. Since Sasaki et
90 al. successfully distinguished *Curcuma longa*, *C. aromatic*a, and *Panax ginseng* using LAMP [24–
91 25], *Hedyotis diffus*a, *Taraxacum formosanum*, and toxic *Aristolochia manshuriensis* [26–28] have
92 been identified using this method. In their study comprising the identification of the valuable
93 herb *Crocus sativus* using the LAMP method, Zhao et al. (2016) presented a practical standard
94 operating procedure for herbal authentication by LAMP, aiming to provide an immediate
95 testing method for many other herbs [29].

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

105 **2. Results**

106 *2.1. Bioinformatics analyses*

107   Bioinformatics analysis were conducted to find a suitable target gene for LAMP detection.
108 In this study, we selected the ITS (nuclear gene), maturase K (*mat*K), ribulose-bisphosphate
109 carboxylase (*rbc*L), intergenic spacers *trn*H-*psb*A and *trn*L-*trn*F (chloroplast regions), NADH
110 dehydrogenase subunit 1 (*nad*1, mitochondrial gene) of *D. officinale* and its adulterants, *D.*
111 *fimbriatum, D. nobile, D. chrysotoxum.* All sequences were obtained from GenBank
112 (https://www.ncbi.nlm.nih.gov/genbank/) and aligned (Table 1). The bioinformatics analyses
113 such as genetic diversity of the *Dendrobium* species were analysed by DnaSP ver 4.5 software
114 [30].

115

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

Species	Accession Number of GenBank					
	ITS	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***trn*L-*trn*F**	***nad*1**
D. officinale	HQ114245.1	FJ216567.1	FJ794048.1	GQ153537.1	EF397937.1	JQ362951.1
D. fimbriatum	JN388588.1	AB519784.1	AB847758.1	KF177500.1	KF143567.1	AY974223.1
D. nobile	JN388579.1	AB519785.1	AB847821.1	EU887942.1	KP749341.1	JQ362952.1
D. chrysotoxum	JN388585.1	KT778725.1	KF143654.1	EU672792.1	EF397915.1	JQ350916.1

117   The bioinformatics analyses of the *Dendrobium* species showed that all sequences contained
118   some variation. But the number of variable sites (Vs), degenerate loci (Ps), insertions/deletions
119   (Indels; I), and DNA polymorphisms (Pis) of four *Dendrobium* species (Table 2) revealed that
120   DNA polymorphism was highest in ITS sequences and, therefore, this gene was the most
121   suitable for LAMP targeting.

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

Sequence	L/bp	Vs	Ps	I	Pi
ITS	668	110	50	7	0.14213
rbcL	630	5	0	0	0.00397
matK	1413	15	7	3	0.00863
trnH-psbA	850	9	3	54	0.00817
trnL-trnF	704	14	32	49	0.04590
*nad*1	711	41	1	5	0.03068

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

124   *2.2. Analysis of ITS2 Sequence Alignment and Primer Design*

125   To design the LAMP primers for the authentication of *D. officinale*, the ITS2 regions of *D.*
126   *officinale* was finally chosen as a target DNA region. Multiple sequences were aligned using
127   DNAMAN ver 6 software [31]. Based on the interspecies and intraspecies sequence variation
128   of ITS2, four specific LAMP primers, F3, B3, FIP and BIP, were designed to specifically
129   amplify the *D. officinale* target DNA under isothermal conditions. In order to enhance the
130   specificity of this set of LAMP primers, we added a mismatch site that was located in the 5'
131   end of the B1c primer (PCR start site). In the second-to-last position on the 5' end, the C base
132   was artificially changed to A. Figure 1 and Table 3 showed the design process and primer
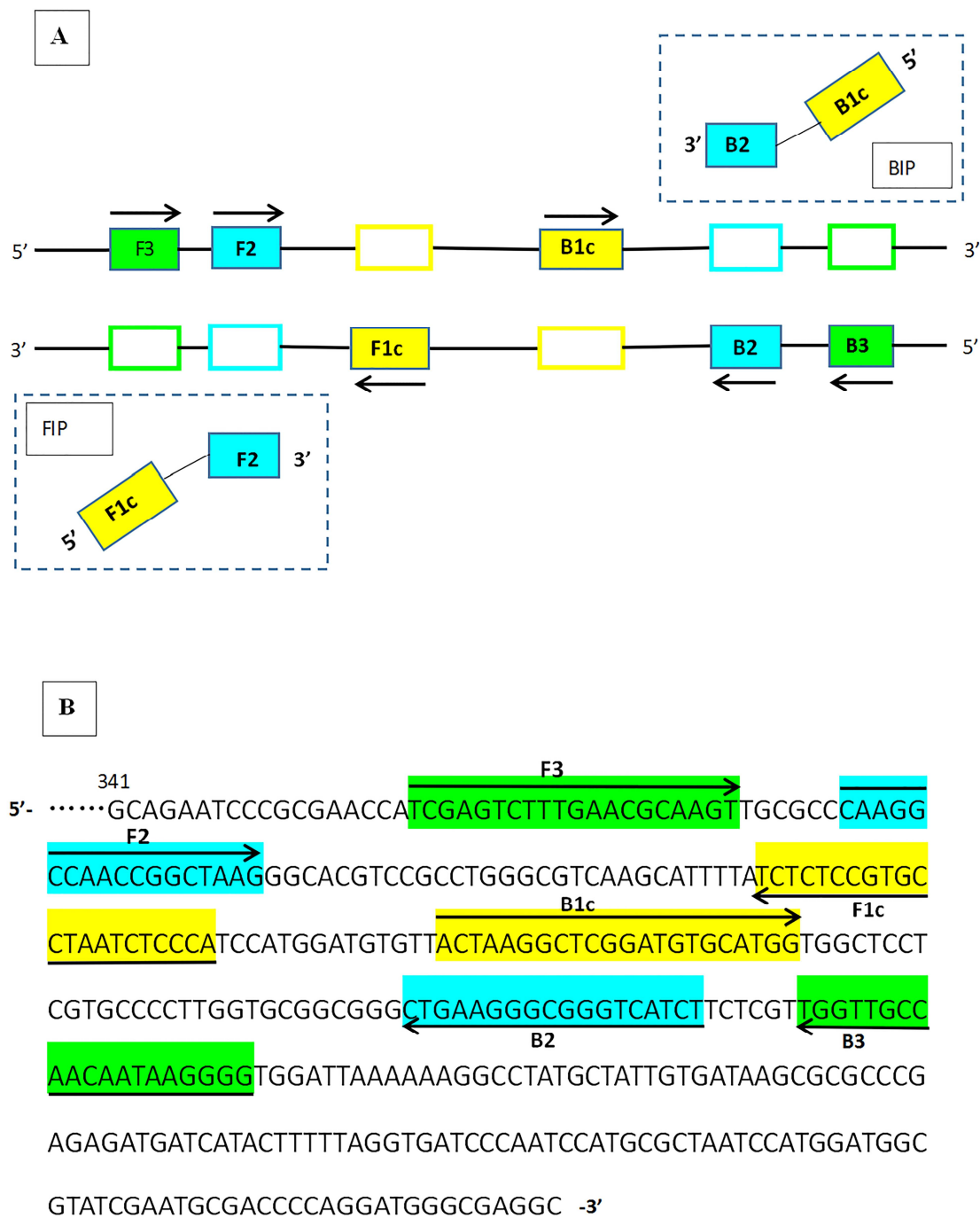133   sequences of LAMP species-specific primers, respectively.

A

BIP

5'
B1c
3' B2

F3   F2   B1c   3'
5'

3'   F1c   B2   B3   5'

FIP

F1c
5'   F2 3'

B

341
5'- ······GCAGAATCCCGCGAACCA**TCGAGTCTTTGAACGCAAGT**TGCGCC**CAAGG**

F3

F2
CCAACCGGCTAAGGGCACGTCCGCCTGGGCGTCAAGCATTTTA**TCTCTCCGTGC**

F1c

B1c
CTAATCTCCCATCCATGGATGTGTT**ACTAAGGCTCGGATGTGCATGG**TGGCTCCT

CGTGCCCCTTGGTGCGGCGGG**CTGAAGGGCGGGTCATCT**TCTCGT**TGGTTGCC**

B2   B3

AACAATAAGGGGTGGATTAAAAAAGGCCTATGCTATTGTGATAAGCGCGCCCG

AGAGATGATCATACTTTTTAGGTGATCCCAATCCATGCGCTAATCCATGGATGGC

GTATCGAATGCGACCCCAGGATGGGCGAGGC **-3'**

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

Name	Sequence(5'-3')
3-TP-FIP	TGGGAGATTAGGCACGGAGAGACAAGGCCAACCGGCTAAG
3-TP-BIP	AA**A**TAAGGCTCGGATGTGCATGGAGATGACCCGCCCTTCAG
3-TP-F3	TCGAGTCTTTGAACGCAAGT
3-TP-B3	CCCCTTATTGTTGGCAACCA

139   *2.3. Optimal Reaction Temperature and Time for Real-time LAMP curve analysis*

140   To analyze LAMP reactions in real time, genomic DNA was used as template to amplify

141   the target sequence in the real-time thermal cycler ABI 7500. The real-time kinetics of LAMP

142   reactions were studied by monitoring fluorescence as described in the Materials and Methods.

143   These reactions were performed at temperatures ranging from 63 to 67 °C, and it was found

144   the reaction at 65 °C was the most productive as well as the fastest (Figure 2). Thus, 65 °C for

145   40 min were the most appropriate conditions for the LAMP reaction.
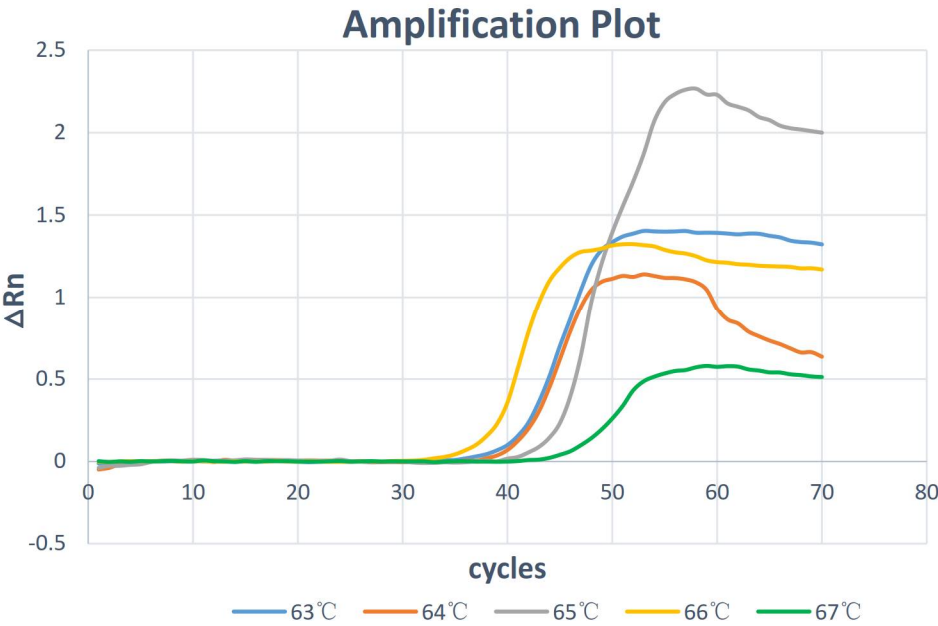
146

147   **Figure 2. Reactions were carried out at different temperatures from 63 to 67 °C.** When tested at 65 °C,

148   the LAMP assay had the highest amplification efficiency, compared with the reactions at other

149   temperatures (1 cycle = 40 seconds).

150   *2.4. Specificity of LAMP*

151   To detect specificity of the primers, eight representative samples of *D. officinale* and closely

152   related *Dendrobium* species were tested at 65 °C for 40 min. First, the real-time PCR method was

153   used to identify *D. officinale.* Positive reactions reached a plateau after a few minutes, while

154   negative ones remained aclinic. Only *D. officinale* plateaued within 40 cycles, while other

155   species were not successfully amplified (Figure 3). Thus, the primers selected for this

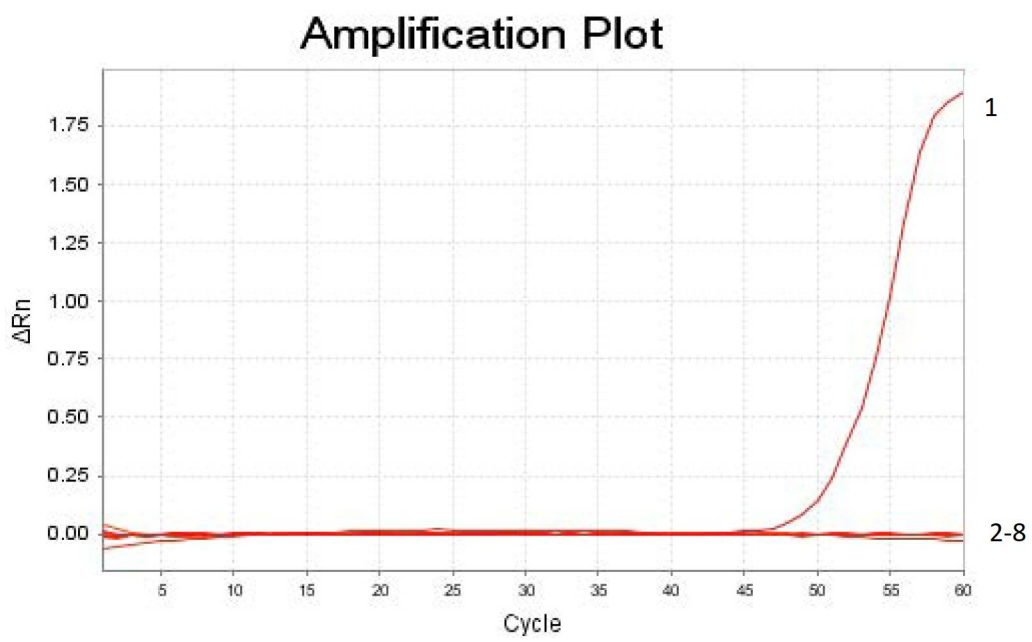156   experiment were specific and suitable for rapid identification of *D. officinale.*

Amplification Plot

157

158   **Figure 3. Specificity of LAMP by Real-time PCR.** 1, *Dendrobium officinale*; 2, *Dendrobium fimbriatum*; 3,
159   *Dendrobium nobile*; 4, *Dendrobium chrysotoxum*; 5, *Dendrobium stuposum*; 6, *Dendrobium ellipsophyllum*; 7,
160   *Flickingeria calocephala*; 8, Negative control.

161   *2.5. Sensitivity of LAMP*

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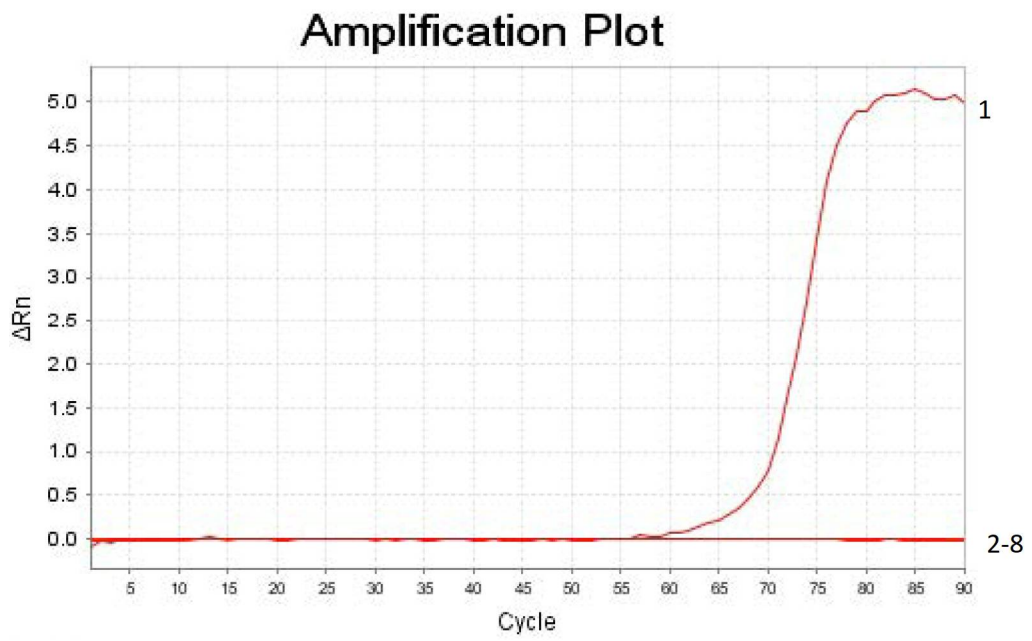166   introduced (Figure 4).

Amplification Plot

167

168   **Figure 4. Sensitivity of LAMP.** 1, 100ng/μl; 2, 10ng/μl; 3, 1ng/μl; 4, 100pg/μl; 5, 10pg/μl; 6, 1pg/μl; 7,

169   100fg/μl; 8, 10fg/μl.

170   *2.6. Visual inspection of LAMP-initiated fluorescence*

171   One of the characteristics of LAMP is its ability to produce results visible with naked eye

172   due to the color change of the LAMP product when it binds to the fluorescent SYBR Green I

173   dye. As expected, DNA samples of *D. officinale* turned green, while the negative control

174   remained orange (Figure 5).
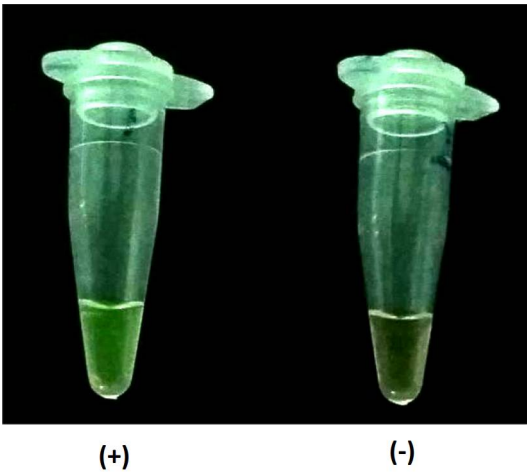

(+)   (-)

175

176   **Figure 5. Visual assessment of DNA amplification by LAMP.** The solution turned green (+) in the

177   presence of a LAMP amplification, while it remained orange (-) with no amplification.

178   The LAMP reaction mixture containing amplified fragments turned green immediately

179   after the addition of SYBR Green I, whereas solutions without amplicons retained the original

180   orange color of SYBR Green I. The samples used in the fluorescence curve analysis were also

181   used to investigate the feasibility of the fluorescent visual inspection method, and the results

182   were identical to that of real-time LAMP analysis (Figure 3); only *D. officinale* samples changed

183   color, while the other *Dendrobium* species and the negative control retained the original orange
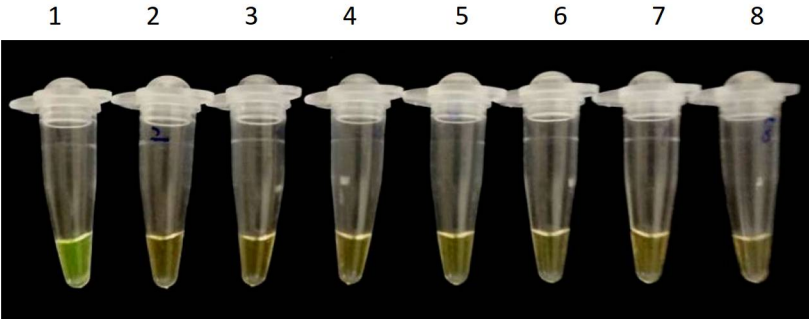
184   color (Figure 6).

185

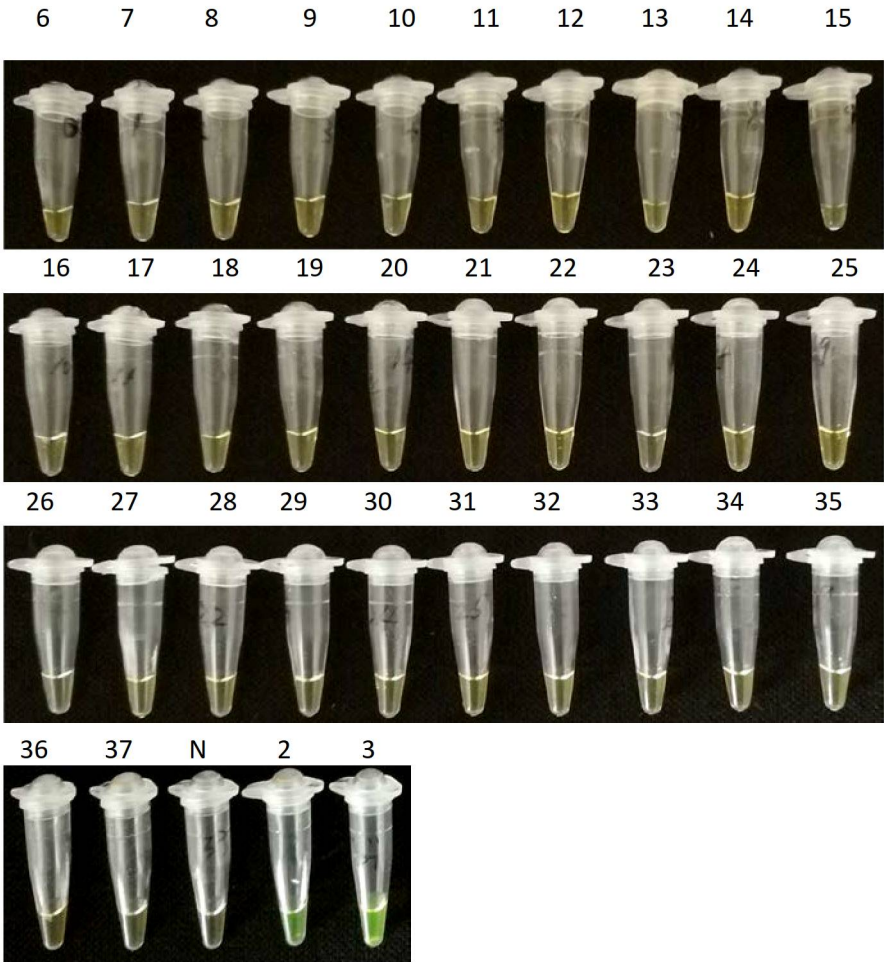186   **Figure 6. Application of fluorescent visual method.** 1, *Dendrobium officinale*; 2, *Dendrobium fimbriatum*;

187   3, *Dendrobium nobile*; 4, *Dendrobium chrysotoxum*; 5, *Dendrobium stuposum*; 6, *Dendrobium ellipsophyllum*; 7,

188   *Flickingeria calocephala*; 8, Negative control.

189   To confirm that this method can be used to distinguish *D. officinale* from its congeners,

190   DNA samples from other 32 *Dendrobium* species were tested using this method (Figure 7). Our
191   results indicated that the LAMP method using the specific primers designed in the present
192   study were able to rapidly discriminate *D. officinale* from its common adulterants under
193   isothermal conditions within 40 min after the addition of SYBR Green I.

194

Figure 7. Specificity of LAMP detection of fluorescent visual method. Tubes2-37 correspond to 2–37
listed in Table 4. Tube N, negative control.

3. Discussion

198   Among the 1000 *Dendrobium* species identified globally, 74, plus two varieties, are known
199   from China [9]. Due to its popularity and effectiveness as an herbal medicine, a rapid and
200   precise diagnostic method of *D. officinale* is urgently required. At present, the most commonly
201   used *D. officinale* detection and identification methods involve amplification of a specific DNA
202   fragment via PCR, and the most common technique to demonstrate the presence of amplified
203   DNA sequences is gel electrophoresis, which also allows estimating the quantity and size of
204   the DNA molecule. The identity of the amplified DNA can be determined by sequencing or by
205   digesting it with restriction enzymes followed by fragment analysis to determine the sizes of
206   the resulting fragments. However, all these methods have some limitations, such as including
207   relatively long and complex operations. In contrast to conventional PCR, LAMP is suitable for
208   onsite detection in the field because of its speed and robustness, and it can also be applied by

209   laymen without the need for elaborate laboratory equipment [32]. To date, LAMP has been

210   used to detect various bacteria and viruses [33, 20], as well as to identify several valuable and

211   toxic Chinese medicines [25, 29, 28]. However, no LAMP-based method for the detection of

212   *Dendrobium* species had been developed so far.

213   In the present study, we developed a highly practical and valid LAMP-based method for

214   detecting *D. officinale*. We achieved this by designing a four-primer set consisting of F3, B3, FIP,

215   and BIP, which recognized six distinct sequences on the target DNA. The reactions can be

216   completed in a water bath, as opposed to requiring expensive instruments, and the amplified

217   products can be detected visually by the naked eye within 1 hour.

218   In previous studies, DNA extraction was performed using the Kit [34], CTAB [35], and

219   modified CTAB methods [36] to obtain high quality DNA. However, because preliminary

220   experiments showed that the high content of polysaccharides in *D. officinale* negatively affected

221   its DNA extraction efficiency [11], in the present study we applied the modified CTAB method

222   for DNA extraction as it uses the CTAB precipitation solution to separate polysaccharides,

223   resulting in a higher concentration and purity of the DNA template compared to other methods.

224   The core of the LAMP process is primer design, which is also the key problem that hinders

225   the development of LAMP technology. *Dendrobium officinale* and related species are very similar

226   genetically thus making it difficult to design specific primers. In this study, LAMP technology

227   was first used to identify *D. officinale* through the analysis of several DNA sequences and then

228   to design LAMP primers. The design of specific LAMP primers for *D. officinale* was a major

229   difficulty in the present study. Although we tried using several target DNA regions, including

230   ITS, *mat*K, *rbc*L, *trn*H-*psb*A, *trn*L-*trn*F, and *nad*1, we ultimately selected the most distinct ITS

231   sequence as the target because it presented the highest number of DNA polymorphisms among

232   screened sequences. Moreover, Dong et al. (2017) have used ITS sequences to successfully

233   identify *D. officinale* [15]. The multicopy ITS sequence has good amplification efficiency, so even

234   processed *D. officinale* products with a high degree of DNA degradation can be effectively

235   identified [37]. While designing primers, we artificially introduced a mismatch (C modified to

236   A) at the second to last base position at the 5' end of the inner primer, B1c. Thus, amplified ITS

237   fragments of the several *Dendrobium* species differed by more than five nucleotides at this

238   location. The experiment showed that the introduction of this mismatch might have

239   considerably increased the specificity of LAMP primers allowing easily distinguishing *D.*

240   *officinale* from related species. In the present study, the detection-limit of template DNA was

241   100 ng/μL using the LAMP primers targeting the introduced mismatch, and LAMP sensitivity

242   was not as high as that observed in other studies [33]. This difference might be due to the

243   increase in specificity caused by the introduced mismatch, i.e., some sensitivity was offset.

244   However, this method was as sensitive as PCR. The method of primer mismatch applied here

245   provides reference for future LAMP experiments.

246   Another advantage of LAMP for molecular identification is that there are several simple

247   methods available for detecting the LAMP product. It can be directly determined whether the

248   reaction occurred or not. Because LAMP reactions produce many bands of different sizes

249   forming a ladder of DNA fragments of 100 bp or more, it is common to verify the amplification

250   results with gel electrophoresis. However, this step increases the risk of amplicon

251   contamination. Because the concentration of LAMP amplification products is extremely high,

252   once we open the tube, the reaction products can easily create aerosol pollution, which would

253   result in higher false positive rates [23]. In addition, the time and energy required for gel
254   electrophoresis reduce the applicability of the LAMP method in the field. Although
255   amplification processes are visible by gel electrophoresis, its detection costs are greatly
256   increased. A relatively simple approach to detect LAMP amplicons is to observe the change in
257   turbidity due to the formation of a white magnesium pyrophosphate precipitate. However, this
258   result is not obvious and is easily influenced by subjective observation. Thus, we used the low
259   cost and easy to operate visual fluorescence method.

260   In the present study, 1 μL of 1/10 diluted original SYBR Green I was added to the inner lid
261   of every centrifuge tube before reactions were initiated. Because color change depends on the
262   amount of DNA in the tube, solutions containing LAMP amplicons turn green after brief
263   centrifugation. Using this method, the aerosol pollution caused by opening the lids is avoided,
264   visual LAMP detection is improved, and the time and cost of the analysis are reduced. The
265   established LAMP-based method performs well in a thermocycler, in a water bath, on a heating
266   block, and even in an insulated mug [33], thus allowing on site fast detection.

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

4. Materials and methods

271   *4.1. Plant samples*

272   The 17 samples of *D. officinale* and 146 samples of 32 other *Dendrobium* species used in this
273   study were collected from Zhejiang, Anhui, and Yunnan Provinces, China, and were identified
274   based on morphological characters by Wenru Wu at the School of Pharmaceutical Sciences,
275   Guangzhou University of Chinese Medicine, Guangzhou, Guangdong Province, China. The
276   number of samples per species and the origin of all samples used in the present study are
277   summarized in Table 4. All materials were stored at the Guangzhou University of Chinese
278   Medicine.

279   **Table 4.** Plant materials used in this study

No.	Species	Sample Number	Origin
1	*Dendrobium officinale*	2	Bozhou, Anhui province, China
2	*Dendrobium officinale*	3	Huoshan, Anhui province, China
3	*Dendrobium officinale*	10	Kunming, Yunnan province, China
4	*Dendrobium officinale*	1	Pu'er, Yunnan province, China
5	*Dendrobium officinale*	1	Zhejiang province, China
6	*Dendrobium polyanthum*	3	Yunnan province, China
7	*Dendrobium gratiosissimum*	3	Pu'er, Yunnan province, China
8	*Dendrobium stuposum*	3	Pu'er, Yunnan province, China
9	*Dendrobium trigonopus*	3	Pu'er, Yunnan province, China
10	*Dendrobium falconeri*	3	Pu'er, Yunnan province, China
11	*Dendrobium wardianum*	3	Yunnan province, China
12	*Dendrobium denneanum*	3	Pu'er, Yunnan province, China
13	*Dendrobium cucullatum*	3	Yunnan province, China

14	*Dendrobium capillipes*	3	Pu'er, Yunnan province, China
15	*Dendrobium ellipsophyllum*	3	Yunnan province, China
16	*Dendrobium findlayanum*	9	Pu'er, Yunnan province, China
17	*Dendrobium chrysotoxum*	21	Pu'er, Yunnan province, China
18	*Dendrobium williamsonii*	3	Pu'er, Yunnan province, China
19	*Flickingeria calocephala*	3	Pu'er, Yunnan province, China
20	*Dendrobium dixanthum*	3	Pu'er, Yunnan province, China
21	*Dendrobium tosaense*	3	Pu'er, Yunnan province, China
22	*Dendrobium heterocarpum*	3	Pu'er, Yunnan province, China
23	*Dendrobium acinaciforme*	3	Pu'er, Yunnan province, China
24	*Dendrobium nobile*	9	Pu'er, Yunnan province, China
25	*Dendrobium crystallinum*	3	Yunnan province, China
26	*Dendrobium fimbriatum.*	3	Pu'er, Yunnan province, China
27	*Dendrobium crepidatum*	3	Yunnan province, China
28	*Dendrobium loddigesii* Rolfe	3	Pu'er, Yunnan province, China
29	*Dendrobium thyrsiflorum* Rchb.	3	Yunnan province, China
30	*Dendrobium gibsonii* Lindl.	3	Pu'er, Yunnan province, China
31	*Dendrobium chrysanthum*	3	Pu'er, Yunnan province, China
32	*Dendrobium furcatopedicellatum*	3	Pu'er, Yunnan province, China
33	*Dendrobium harveyanum*	3	Pu'er, Yunnan province, China
34	*Dendrobium moniliforme*	3	Yunnan province, China
35	*Dendrobium hancockii*	3	Pu'er, Yunnan province, China
36	*Dendrobium exile*	3	Yunnan province, China
37	*Dendrobium falconeri*	3	Pu'er, Yunnan province, China

280   *4.2. Methods*

281   4.2.1. Primer design

282   Based on the bioinformatics analysis, primers for LAMP analysis of *D. officinale* were
283   designed to target the ITS gene. The GenBank sequence HQ114245.1 was used for primer
284   design on Primer Explorer (http://primerexplorer.jp/lampv5e/index.html), a LAMP primer
285   designing software. After screening several primers, we chose the subset for which the
286   mismatch site was located in the 5' end of the B1c primer (PCR start site). In the second-to-last
287   position on the 5' end, the C base was artificially changed to A. Thus, the difference between *D.*
288   *officinale* and other species of *Dendrobium* was expanded to increase the specificity of LAMP
289   primers. Figure 1 and Table 3 show the design process and primer sequences, respectively.

290   4.2.2. DNA preparation

291   Leaf and stem tissues (100-mg samples) were obtained from each accession and either
292   immediately used to extract DNA or stored at -80 °C for later DNA isolation. To extract genomic
293   DNA, samples were high-speed shaken in 2-mL microcentrifuge tubes with stainless steel
294   beads in a Qiagen TissueLyser LT (QIAGEN, Germany). After pulverization, 750 μL of 65 °C
295   cetyltrimethylammonium bromide (CTAB) precipitation solution [2% CTAB, 10 mM EDTA, 100
296   mM Tris-HCl (pH 8.0)] was added to the extraction mixture and placed in a 65 ºC water bath
297   for 40 min. The mixture was centrifuged at 5000 rpm for 5 min, and the supernatant was

298   discarded. This step was repeated twice and DNA from the material remaining in the tube was
299   then extracted using the modified CTAB method as described by Cui et al. (2006) [36]. The
300   above steps aimed to increase the amount of polysaccharides removed from *Dendrobium* sp.
301   samples, thereby improving the success rate of the extraction. Subsequently, extracted
302   templates were examined in the Ultramicro UV spectrophotometer (Thermo Fisher Scientific,
303   USA) at A_{260}/A_{280} and A_{260}/A_{230}.

304   4.2.3. LAMP reaction

305   LAMP reactions were performed in a total volume of 25 μL, and the reaction mix consisted
306   of primers FIP and BIP (1.6 mM each), primers F3 and B3 (0.2 mM each), 1.4 mM dNTPs
307   (TaKaRa, Japan), 10 × Isothermal Amplification Buffer (New England Biolabs, USA), 6 mM
308   $MgSO_4$ (New England Biolabs), 8 U of *Bst* 2.0 WarmStart® DNA Polymerase (New England
309   Biolabs), and 2 μL of template DNA. Sterile deionized water was used as the template for the
310   negative control. These reactions were carried out in 0.2-mL microtubes, and 1 μL of 1/10
311   diluted original SYBR® Green I (Invitrogen, USA) was added to the inner lid of every
312   microtube before incubating the reactions in the Arktik™ Thermal Cycler (Thermo Scientific,
313   USA). Since color change depends on the amount of double-stranded DNA in a sample,
314   solutions with LAMP amplicons present would turn green after brief centrifugation.

315   4.2.4. Optimal reaction in real time

316   Time of positivity (T_p, expressed in min) and melting temperature (T_m, expressed in °C) are
317   two basic parameters for determining positive fluorescence signals and correspond to the time
318   at which the second derivative of fluorescent amplification reaches its peak above the baseline
319   value, and to the temperature at which amplification products melt into two single-stranded
320   DNA molecules, respectively. To determine the optimal T_p and T_m, the fluorescence of five
321   samples was measured in real time at temperatures ranging from 63 to 67 °C. For real-time
322   LAMP analysis, ROX reference dye II (Thermo, USA) (0.05 μL) and 1/500 diluted original SYBR
323   Green I (Thermo, USA) (1.0 μL) were added to the LAMP reaction. The real-time thermal cycler
324   ABI 7500 (Applied Biosystems, USA) operating version 2.3 was used to amplify and measure
325   fluorescence at different reaction temperatures and different cycles. The program was set to the
326   following conditions: 1 s to react and 40 s to collect data for either 60 or 90 cycles (1 cycle is 40
327   s).

328   4.2.5. Specificity and sensitivity of LAMP reaction

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

335   4.2.6. Visual detection of the LAMP reaction

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

339   light, with naked eye.

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

5. Conclusion

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

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

361   **Conflict of Interest:** The authors declare no conflict of interest.

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