

Duplex proofman-LMTIA assay for rapid identification of *Dendrobium fimbriatum* and *Dendrobium chrysotoxum*

Dan Zhou^{1,2}, Yanwu Zhao³, Linqing Guo⁴, Yue Chen¹, Jianping Li⁴, Xiaodong Zhang², Bailing Yin⁵, Yao Wang¹, Deguo Wang², Bo Tian⁴

¹ School of Food and Biological Engineering, Henan University of Science and Technology, Luoyang, Henan, 471000, China

² Key Laboratory of Biomarker Based Rapid-detection Technology for Food Safety of Henan Province, Food and Pharmacy College, Xuchang University, Xuchang 461000, China

³ Henan Medical and Health Technician College, Kaifeng, Henan, 475000, China

⁴ Food College, Northeast Agricultural University, Harbin 150000, China

⁵ Yuzhou Houshengtang Traditional Chinese Medicine Co., Ltd, Xuchang 452570, China

Corresponding authors: Yao Wang (1278090342@qq.com); Deguo Wang (wangdg666@126.com); Bo Tian (tianyubo1995@163.com)

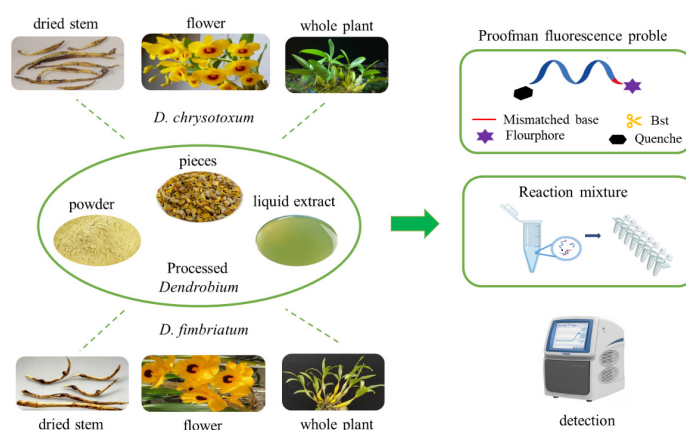
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Abstract

A rapid analytical method based on ladder-shape melting temperature isothermal amplification (LMTIA) and proofreading enzyme-mediated probe cleavage (Proofman) was developed to simultaneously identify *Dendrobium fimbriatum* and *Dendrobium chrysotoxum*. Detection was achieved by the duplex Proofman-LMTIA method within 20 min at 64 °C using specific primers targeting their internal transcribed spacer (ITS) sequences and Proofman fluorescent probes, with a sensitivity of 1 pg/μL genomic DNA and a detection limit of 5% (w/w). The method showed good specificity for five common *Dendrobium* species. Commercial *Dendrobium* samples from various sources were successfully tested using this method, and the results were consistent with first-generation gene sequencing results. This technique enables rapid and accurate detection, providing a valuable tool for identifying the two species and supervising the traditional Chinese medicine market.

Graphical abstract:



Keywords

Dendrobium chrysotoxum, *Dendrobium fimbriatum*, dual detection, Proofman-LMTIA

Introduction

Over 1,000 *Dendrobium* species have been identified globally, and they are predominantly distributed across Asia (Burzacka-Hinz et al. 2022). The medicinal utilization of *Dendrobium* can be traced back more than 1,000 years, as noted in *Shennong's Classics of Materia Medica*. *Dendrobium* is prescribed in traditional Chinese medicine hospitals for nourishing yin, promoting fluid production, clearing heat, and tonifying the stomach (Fu et al. 2023; Zhong et al. 2026). Traditional Chinese medicines, including *Dendrobium fimbriatum* Hook. (*D. fimbriatum*), *Dendrobium chrysotoxum* Lindl. (*D. chrysotoxum*), *Dendrobium officinale* (*D. officinale*), *Dendrobium huoshanense* (*D. huoshanense*), and *Dendrobium nobile* Lindl. (*D. nobile*), were listed in the 2025 edition of the Chinese Pharmacopoeia (Dai et al. 2024). Modern pharmacological studies have demonstrated that alkaloids and polysaccharides represent the major active constituents of *Dendrobium* (Zhao et al. 2023), which exert immunoregulatory, anti-tumor, antiviral, hypoglycemic, and hypolipidemic effects (Zhang et al. 2020; Wang et al. 2023). However, *Dendrobium* species vary greatly in chemical composition and medicinal efficacy. Closely related species share highly similar morphological characteristics and are difficult to distinguish by visual observation, resulting in widespread adulteration of medicinal herbs. Therefore, accurate species identification is essential to ensure the safety and efficacy of traditional Chinese medicine (TCM) products.

Currently, modern *Dendrobium* identification techniques mainly include microscopic identification (Anon 2025), thin-layer chromatography (Wang et al. 2011), chromatography and spectroscopy combined with chemometric analysis (Li et al. 2022), and molecular biology methods. Microscopic identification is strongly affected by subjective judgments, whereas chromatographic and spectroscopic techniques require costly equipment and skilled personnel, which limits their practical application. Molecular biology methods (e.g., single-nucleotide variants (SNVs) (Niu et al. 2018), large single-copy (LSC) regions (Li et al. 2020), and DNA barcoding (Chen et al. 2022)) are complex and time-consuming, requiring gene sequencing and traditional polymerase chain reaction (PCR), making them unsuitable for on-site rapid detection. Although LAMP enables on-site detection (Yang et al. 2019), it has limitations, such as complex primer design and false-positive issues. Additionally, while distinguishing *D. officinale* and *D. huoshanense* has been widely reported, simultaneous discrimination of *D. fimbriatum* and *D. chrysotoxum* is scarce.

The core principle of the LMTIA method is based on the ladder-shape melting temperature sequence of target

DNA. This structure enables stepwise local denaturation, primer binding, and isothermal amplification, in which a polymerase achieves strand displacement of the remaining double-stranded DNA (Wang et al. 2021). When integrated with Proofman probes, this approach enhances detection specificity and sensitivity. Primer design for target sequences longer than 60 nucleotides can be performed using conventional PCR primer design software. Compared with conventional PCR, LMTIA avoids complex heating and cooling cycling and significantly shortens the detection duration. Theoretically, this method does not require a fluorescent PCR system. Currently, only 8-well simple thermal cyclers are commercially available; therefore, 96-well PCR instruments are preferentially utilized in practical applications. Meanwhile, the LMTIA reaction system only requires Bst DNA polymerase, commercial master mix, primers, and probes, which simplifies experimental procedures and reduces total experimental costs. To date, numerous studies have utilized the LMTIA method, including the identification of starch (Zhang et al. 2022), edible oil (Xiao et al. 2024), and *Bupleurum* (Liu et al. 2024).

In the present study, a rapid dual-detection technique for *D. fimbriatum* and *D. chrysotoxum* was developed based on the Proofman-LMTIA method. With the internal transcribed spacer (ITS) gene sequence as the target, species-specific primers were designed and paired with Proofman probes labeled with cyanine 5 (Cy5) or carboxyfluorescein (FAM) (Wang et al. 2020). The temperature of the dual Proofman-LMTIA system was optimized, and its specificity, sensitivity, and limit of detection were examined. Furthermore, the practical utility of the dual Proofman-LMTIA system was assessed by detecting commercially available *Dendrobium* samples. This work provides technical support for raw material quality control, market supervision, and product quality assurance of *D. fimbriatum* and *D. chrysotoxum*.

Materials and methods

Materials

D. officinale and *D. huoshanense* were acquired from the National Institutes for Food and Drug Control of China. *D. chrysotoxum*, *D. fimbriatum*, and *D. nobile* were supplied by Houshengtang Traditional Chinese Medicine Co., Ltd., and all commercial samples were sourced from Bozhou Chinese Medicinal Materials Market, Anhui Province. The plant genomic DNA extraction kit for polysaccharide- and polyphenol-rich samples was purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd.

The LMTIA reaction mixture was prepared in-house. The dNTPs were provided by Nanjing Vazyme Biotech Co., Ltd., Bst DNA polymerase (8 U/ μ L) from Kelin Biological Technology (Beijing) Co., Ltd., and DEPC-treated water from Hefei Leier Biotechnology Co., Ltd. All other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd., unless otherwise specified.

Primer and probe design

Three criteria were adopted for primer design: ladder-shaped melting temperature profiles, GC content ranging from 40% to 80%, and high specificity to target DNA. ITS sequences of *D. fimbriatum* [AF362041.1] and *D. chrysotoxum* [AB873185.1] were retrieved from NCBI GenBank and aligned using DNAMAN 9.0. Sequences with ladder-shaped melting curves were identified by Oligo7 (Molecular Biology Insight, Colorado Springs, CO, USA). Primers for *D. fimbriatum* and *D. chrysotoxum* were designed with Primer3Plus (<http://www.primer3plus.com>). Proofman probes labeled with a 3' fluorophore and a 5' quencher were designed based on LF primer sequences. All primers and probes were synthesized by Anhui General Biotechnology Co. The primers and probes are shown in Table 1.

DNA extraction

D. chrysotoxum, *D. fimbriatum*, *D. officinale*, *D. huoshanense*, and *D. nobile* were supplied as powder; the other six samples were ground into powder under liquid nitrogen. Genomic DNA was extracted using a commercial kit for polysaccharide- and polyphenol-rich samples and quantified with the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only DNA with A260/A280 ratios of 1.8–2.0 was used for LMTIA analysis. All DNA samples were stored at –20 °C.

LMTIA primer screening

Preliminary primer screening was conducted using the SYBR Green I fluorescent dye-based assay. Each reaction was prepared in a 10 μ L volume containing 5 μ L of premix (including Bst DNA polymerase, dNTPs, and Bst buffer), 0.13 μ L of F primer (100 μ mol/L), 0.13 μ L of B primer (100 μ mol/L), 0.06 μ L of LF primer (100 μ mol/L), 0.06 μ L of LB primer (100 μ mol/L), 2 μ L of DNA (1 ng/

μ L), and DEPC-H₂O to bring the total volume to 10 μ L. Amplification was performed on a Gentier 96E Medical PCR Analysis System (Tianlong Technology Co., Ltd., Xi'an, China) at isothermal temperatures of 52 °C, 54 °C, 56 °C, and 58 °C. Fluorescence signals were collected every 90 s for a total of 40 cycles. Genomic DNA (1 ng/ μ L) was used as the positive control, while DEPC-H₂O served as the negative control. Primers with the earliest amplification onset cycle were selected as optimal for subsequent experiments.

Proofman-LMTIA reaction system

The Proofman-LMTIA reaction system (10 μ L total) was prepared by combining 2 μ L DNA template, 4.4 μ L LMTIA mix (Dege Biotechnology (Shandong) Co., Ltd.), 0.1 μ L Bst DNA polymerase (8 U) (Shandong Meide Biotechnology Co., Ltd.), 0.16 μ L each of primers F/B (100 μ mol/L), 0.04 μ L each of primers LF/LB (100 μ mol/L), and 0.1 μ L Proofman probe (10 μ mol/L), with DEPC-H₂O to bring the volume to 10 μ L. Reaction temperature optimization, specificity, sensitivity, limit of detection, and authentic sample detection were performed on a Gentier 96E Medical PCR System (Xi'an Tianlong Science and Technology Co. Ltd., China). Fluorescence signals were collected every 30 s over 40 cycles; each experiment was repeated three times with two parallel replicates. The reaction system is shown in Table 2.

Single-plex temperature optimization

Based on the reaction system in Table 1, optimal single-target temperatures were screened to provide a reference for the dual Proofman-LMTIA system. Temperatures for *D. chrysotoxum* and *D. fimbriatum* were separately optimized at 62 °C, 63 °C, 64 °C, and 65 °C, with their genomic DNA as positive controls and DEPC-H₂O as the negative control. Each reaction contained 2 μ L (100 pg/ μ L) DNA template, and single-target amplification curves were analyzed and evaluated.

Single-plex specificity testing

To verify the specificity of the designed ITS sequences for *D. fimbriatum* and *D. chrysotoxum*, specificity assays were performed using the Proofman-LMTIA system (Table 1). *D. fimbriatum* DNA was used as the positive control, with

Table 1. Proofman-LMTIA primers and fluorescence probes.

Primers and probes	Sequence (5' to 3')
Guc-F(100 μ M)	CCAGCCCATCCATCGATTTTCGTC AAGCGTTACGTC
Guc-B(100 μ M)	GGATGGGCTGGCGATTTTCCGAGGGGCACGAGGA
Guc-LF(100 μ M)	CGGGTGGCTGGACAC
Lius-LF(100 μ M)	GGGATGACTTGACACG
Guc-Probe(10 μ M)	BHQ2- TCGGATGTGCAG-6-FAM
Lius-Probe(10 μ M)	BHQ2- TCGGATGTGCAC-Cy5

Guc: *D. chrysotoxum* Lius: *D. fimbriatum*

Table 2. Proofman-LMTIA reaction system of *D. chrysotoxum* and *D. fimbriatum*.

Single reaction system	One tube (μL)	duplex reaction system	One tube (μL)
premix buffer (5X)	4.4	premix buffer (5X)	4.4
Bst Polymerase (8U)	0.1	Bst Polymerase (8U)	0.1
Guc/Lius-F(100 μM)	0.16	Guc-F (100 μM)	0.16
Guc/Lius-B (100 μM)	0.16	Lius-B (100 μM)	0.16
Guc/Lius-LF (100 μM)	0.04	Guc-LF (100 μM)	0.04
Guc-probe(10 μM)	0.1	Lius-LF (100 μM)	0.04
Lius-probe(10 μM)	0.1	Guc-probe(10 μM)	0.1
Guc/Lius-DNA	2	Lius-probe(10 μM)	0.1
DEPC-H ₂ O	Add to 10/μL	Guc/Lius-DNA	1
		DEPC-H ₂ O	Add to 10/μL

Guc: *D. chrysotoxum* Lius: *D. fimbriatum*

D. officinale, *D. chrysotoxum*, *D. huoshanense*, *D. nobile*, and DEPC-H₂O as negative controls; *D. chrysotoxum* DNA was used as the positive control, with *D. fimbriatum*, *D. officinale*, *D. huoshanense*, *D. nobile*, and DEPC-H₂O as negative controls. A 2 μL (100 pg/μL) DNA template was used per reaction, and single-plex amplification curves were analyzed and evaluated.

Specificity verification of dual-system temperature

To verify the specificity of the dual reaction system at the optimal temperature, the specificity of *D. fimbriatum* and *D. chrysotoxum* was assessed at 64 °C using the Proofman-LMTIA reaction system (Table 1). Genomic DNA of *D. fimbriatum* and *D. chrysotoxum* was employed as positive controls, while DNA from *D. huoshanense*, *D. officinale*, *D. nobile*, and DEPC-H₂O was used as negative controls. In positive control reactions, 1 μL (100 pg/μL) of genomic DNA from each of *D. fimbriatum* and *D. chrysotoxum* was mixed per tube. The amplification curves of the dual Proofman-LMTIA assay were then analyzed and evaluated.

Dual sensitivity detection

To determine the sensitivity of the dual Proofman-LMTIA system, genomic DNA of *D. fimbriatum* and *D. chrysotoxum* was separately diluted to 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, and 100 fg/μL. Detection groups were set with different concentrations of their genomic DNA templates, with DEPC-H₂O as the negative control. For the test group, 1 μL of each concentration of both templates was mixed per tube. Amplification was conducted at the optimized temperature for 20 min, and dual sensitivity was then determined by amplification efficiency and fluorescence intensity.

Dual limit of detection

To determine the detection limit, *D. fimbriatum* powder was mixed with *D. chrysotoxum* powder at proportions of 20%, 10%, 5%, 1%, and 0.1% (w/w), and the reverse mixing (*D. chrysotoxum* powder mixed with *D. fimbriatum* powder) was also performed. Genomic DNA was extract-

ed using the kit described in Section 2.3. For each reaction, 2 μL of genomic DNA was added per tube as the positive control, while DEPC-H₂O was used as the negative control. Amplification was carried out at the optimized temperature for 20 min, and the dual detection limit was determined based on amplification efficiency and fluorescence signals.

Actual sample detection

The established dual Proofman-LMTIA rapid detection method was applied to the detection of actual samples. Six commercial samples labeled as *D. fimbriatum* or *D. chrysotoxum* were purchased from Bozhou, Anhui, for detecting the presence of *D. fimbriatum* and *D. chrysotoxum* components. The gDNA of the six samples was extracted using the kit described in Section 2.3. According to the established dual Proofman-LMTIA reaction system, each reaction mixture included 2 μL of DNA template from the commercial samples and was run at the optimized temperature. The genomic DNA templates of *D. fimbriatum* and *D. chrysotoxum* were used as positive controls, and DEPC-H₂O was used as the negative control. The components contained were determined according to the amplification efficiency and fluorescence signal.

For comparison, the positive samples were analyzed using first-generation sequencing technology. The DNA of the commercial samples was subjected to PCR amplification (F primer: GAGAAGTCGTAACAAGGTTTCC, B primer: GACGCTTCTCCAGACTACAATT; reaction conditions: 34 cycles of 98 °C for 5 s, 60 °C for 8 s, and 72 °C for 5 s). After the amplified bands were confirmed by gel electrophoresis, the samples were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing.

Results

Target sequence analysis and Proofman-LMTIA primer design

Comparison of the ITS sequences of *D. fimbriatum* and *D. chrysotoxum* showed 91.11% target sequence similarity (Fig. 1) and eight single-nucleotide polymorphisms (SNPs).

Lius.txt	CGTCAAGCGTTACGTC ACT CCGTGTCAAGTCATCCCATCG	40
Guc.txt	CGTCAAGCGTTACGTC CT CCGTGTCCAGCCA.CCCGTCG	39
Consensus	cgteaagcgttacgtc ctcctgtgtc ag ca ccc tcg	
Lius.txt	ATGGATGGGCTGGCGAGT GCT CGGATGTGCAGAGTGGCTC	80
Guc.txt	ATGGATGGGCTGGCGAT GGCT CGGATGTGCACAGTGGCTC	79
Consensus	atggatgggctggcga gctcggatgtgca agtggctc	
Lius.txt	CTCGTGCCC	89
Guc.txt	CTCGTGCCC	88
Consensus	ctcgtgccc	

Figure 1. Alignment of target sequences of *D. chrysotoxum* and *D. fimbriatum*.

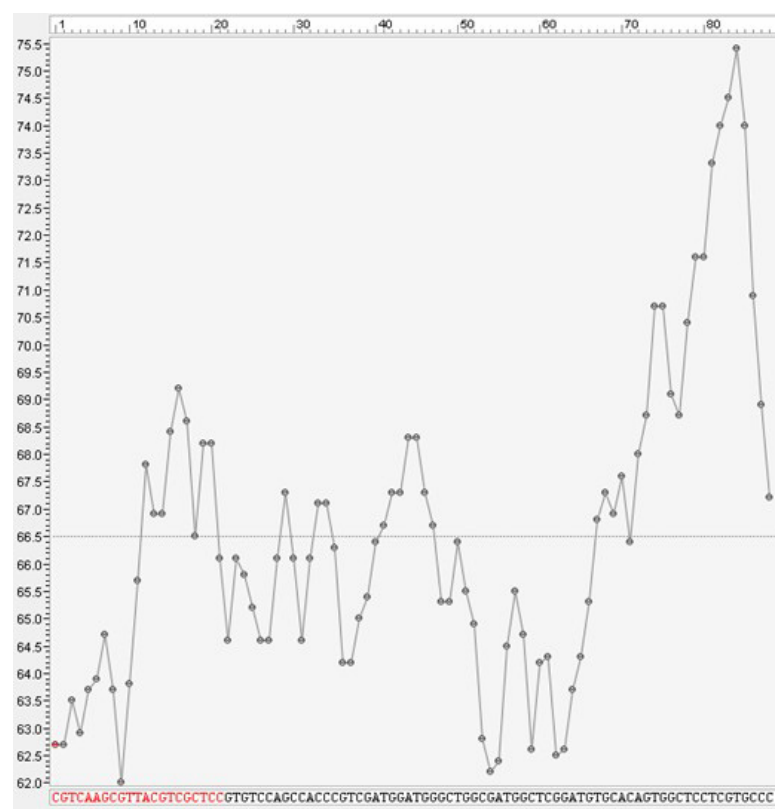


Figure 2. Target sequence with ladder-shaped melting temperature curves for detection of *D. chrysotoxum* and *D. fimbriatum*. Note: The ordinate represents temperature (°C); the abscissa represents the nucleic acid base sequence (nt).

The No. 1 site with a typical ladder-shaped melting curve (Fig. 2) was selected as the key differential site for the design of Proofman-LMTIA primers and Proofman probes.

Results of primer screening for fluorescent dye-based LMTIA

Primer screening was performed using SYBR Green I fluorescence-based LMTIA. As shown in Fig. 3, both primer sets F1 and F2 yielded successful amplification at 54 °C, 56 °C, and 58 °C, whereas no amplification was detect-

ed at 52 °C or in the negative control. Notably, amplification with F2 was initiated at 12.35 cycles, earlier than F1 at 16.76 cycles. Accordingly, the F2 primer set exhibited higher amplification efficiency and was selected for subsequent experiments.

Single-plex temperature optimization

The results for single-plex temperature optimization are shown in Fig. 4. *D. fimbriatum* was amplified at 62 °C, 63 °C, 64 °C, and 65 °C, with the highest efficiency at 64 °C, while

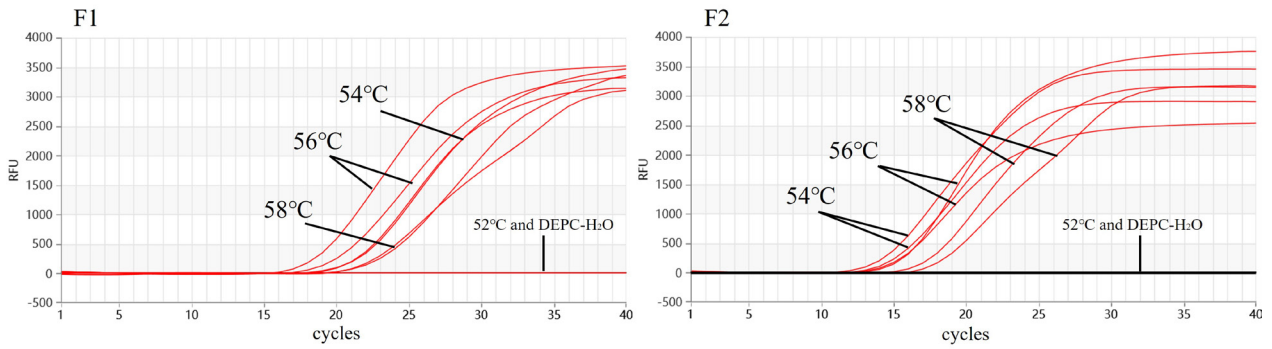


Figure 3. Fluorescent dye-based LMTIA amplification curves at different temperatures. **A.** Primer F1; positive controls: *D. chrysotoxum* or *D. fimbriatum*; negative control: DEPC-H₂O; **B.** Primer F2; positive controls: *D. chrysotoxum* or *D. fimbriatum*; negative control: DEPC-H₂O; reaction temperatures: 52 °C, 54 °C, 56 °C, and 58 °C.

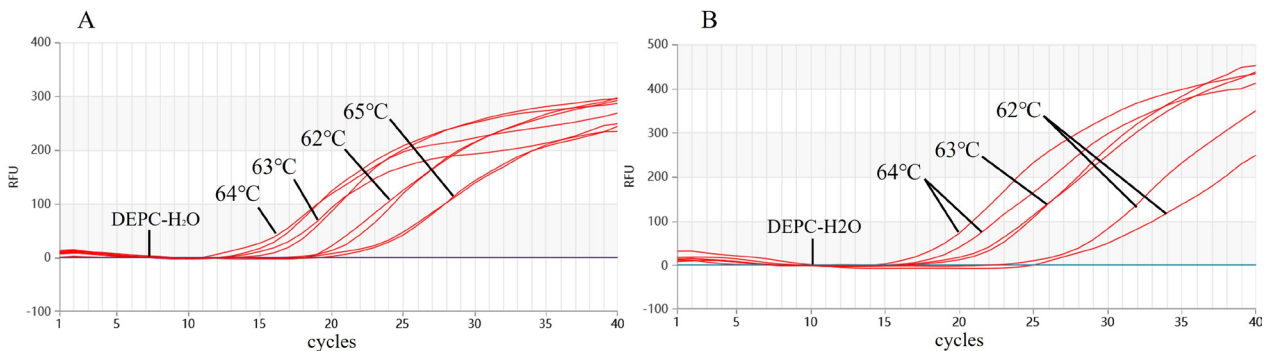


Figure 4. Single Proofman-LMTIA amplification curves at different temperatures. **A.** Positive control: *D. fimbriatum*; negative control: DEPC-H₂O; **B.** Positive control: *D. chrysotoxum*; negative control: DEPC-H₂O; reaction temperatures: 62 °C, 63 °C, 64 °C, and 65 °C.

D. chrysotoxum was amplified at 62 °C, 63 °C, and 64 °C, but not at 65 °C, with the highest efficiency at 64 °C. No amplification was detected in the negative controls. Thus, the optimal single-reaction temperature ranges were 62 °C, 63 °C, and 64 °C, and the duplex LMTIA system temperature was set at 64 °C for the best overall amplification effect.

Single-plex specificity

The results are shown in Fig. 5. At 64 °C, *D. fimbriatum* and *D. chrysotoxum* exhibited good amplification efficiency, while *D. officinale*, *D. huoshanense*, *D. nobile*, and the negative control were not amplified. This indicated that the designed ITS primers for *D. fimbriatum* and *D. chrysotoxum* were specific, capable of distinguishing the other three medicinal *Dendrobium* species and providing a scientific basis for subsequent experiments.

Dual temperature specificity

Dual-specificity results (Fig. 6) showed different fluorescence signals for *D. fimbriatum* and *D. chrysotoxum* at 64 °C. No amplification was detected in *D. officinale*, *D. huoshanense*, *D. nobile*, or the negative control (DEPC-H₂O), indicating good specificity. Thus, the optimal dual Proofman-LMTIA temperature was 64 °C, which was used for subsequent experiments.

Dual sensitivity

For the dual Proofman-LMTIA system sensitivity (Fig. 7), amplification curves were observed for *D. fimbriatum* and *D. chrysotoxum* genomic DNA at 1 ng/μL, 100 pg/μL, 10 pg/μL, and 1 pg/μL. No amplification was detected at 100 fg/μL or in the negative control (DEPC-H₂O), so their genomic DNA sensitivity was determined to be 1 pg/μL.

Dual limit of detection

The results of the dual detection limit are shown in Fig. 8. Amplification curves were observed for DNA extracted from 20%, 10%, and 5% mixed powders of *D. fimbriatum* and *D. chrysotoxum*, while no amplification was detected in 1% or 0.1% mixed powders or the negative control (DEPC-H₂O). The detection limits for both species in the dual Proofman-LMTIA reaction system were concluded to be 5% (w/w).

Actual sample detection

Six commercial samples were tested (Fig. 9A). Amplification was observed in *Dendrobium* Sample 1 but was absent in Samples 2–6, indicating that Sample 1 was *D. fimbriatum*, while Samples 2–6 belonged to neither *D. fimbriatum* nor *D. chrysotoxum*. Furthermore, Sample 1

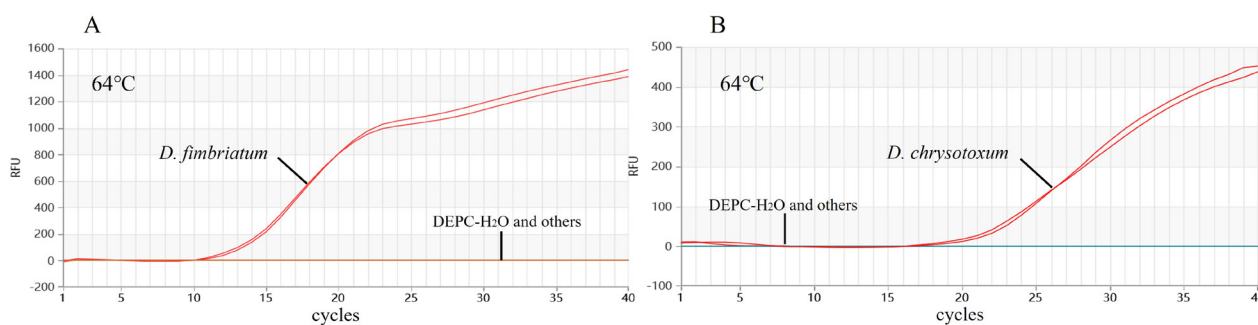


Figure 5. Specificity validation results of the single Proofman-LMTIA assay for different DNA types at 64 °C. **A.** Positive control: *D. fimbriatum* (Lius-DNA); negative controls: DNA templates of *D. officinale*, *D. huoshanense*, *D. chrysotoxum*, *D. nobile* Lindl., and DEPC-H₂O; **B.** Positive control: *D. chrysotoxum* DNA template (Guc-DNA); negative controls: DNA templates of *D. officinale*, *D. fimbriatum*, *D. huoshanense*, *D. nobile* Lindl., and DEPC-H₂O.

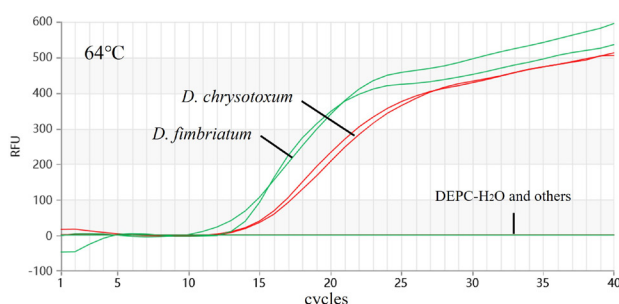


Figure 6. Specificity validation results of the dual Proofman-LMTIA assay for different DNA types at 64 °C. Positive controls: *D. fimbriatum* (Lius-DNA) and *D. chrysotoxum* (Guc-DNA); negative controls: DNA templates of *D. officinale*, *D. huoshanense*, *D. nobile* Lindl., and DEPC-H₂O.

was sequenced, and BLAST analysis of the sequence on NCBI followed by DNAMAN alignment with the highest-scoring gene sequence (Fig. 9B) showed 100% identity. In conclusion, the established dual Proofman-LMTIA system can simultaneously detect *D. fimbriatum* and *D. chrysotoxum*, improving detection efficiency and supporting rapid authenticity identification of medicinal materials in various markets.

Discussion

As representative and valuable medicinal herbs, *D. fimbriatum* and *D. chrysotoxum* are widely used in TCM. Driven by excessive economic interests (Dai et al. 2024), high-value *Dendrobium* materials are frequently adulterated or substituted with other related species, which seriously impairs their authentic medicinal quality and clinical efficacy. With the continuous advancement of *Dendrobium* research, numerous identification strategies have been developed, including near-infrared spectroscopy coupled with chemometrics (Li et al. 2022), two-dimensional correlation spectroscopy (2DCOS) integrated with residual network (ResNet) (Ding et al. 2021), metabolomics (Hao et al. 2020), electronic nose (Dai et al. 2024), and PCR-RFLP (Hu et al. 2020). De-

spite their effectiveness in species discrimination, most of these approaches involve complex operations and rely heavily on professional personnel and sophisticated instruments. With the rapid industrialization and market expansion of *Dendrobium* products, there is an increasing demand for rapid, simple, sensitive, stable, and cost-effective identification methods. Therefore, developing a rapid, field-adaptable, and highly specific method for the authentication of *D. fimbriatum* and *D. chrysotoxum* is of great practical significance.

Accordingly, the present study established a duplex Proofman-LMTIA system targeting the ITS sequences of *D. fimbriatum* and *D. chrysotoxum*, which enables complete detection within 20 min. It is noteworthy that high levels of polysaccharides and polyphenols in *Dendrobium* plants often impede efficient DNA extraction. Nevertheless, the Proofman-LMTIA assay established in this study exhibited outstanding sensitivity, permitting effective amplification even from low-concentration DNA templates. Moreover, given the rich and diverse germplasm resources of *Dendrobium*, the use of specifically designed primers and Proofman probes ensured high specificity against three other *Dendrobium* species, thus markedly reducing the risk of false-positive and false-negative results.

The reliability and applicability of the established Proofman-LMTIA method were systematically verified by temperature optimization, specificity evaluation, sensitivity assessment, and detection limit determination. The assay achieved a sensitivity of 1 pg/μL for the genomic DNA of both *D. fimbriatum* and *D. chrysotoxum*, with a detection limit of 5% (w/w) in mixed powder samples. When applied to six commercial *Dendrobium* samples, only one was identified as *D. fimbriatum*, while the other five belonged to neither of the two target species. These findings indicate that the tested commercial *Dendrobium* samples present obvious species adulteration, which may compromise their medicinal quality and safety. To date, the Proofman-LMTIA technique has been reliably applied in food analysis (Cui et al. 2024b), traditional Chinese medicine authentication (Cui et al. 2024a), and virus detection (Wang et al. 2022), demonstrating its broad applicability and stability.

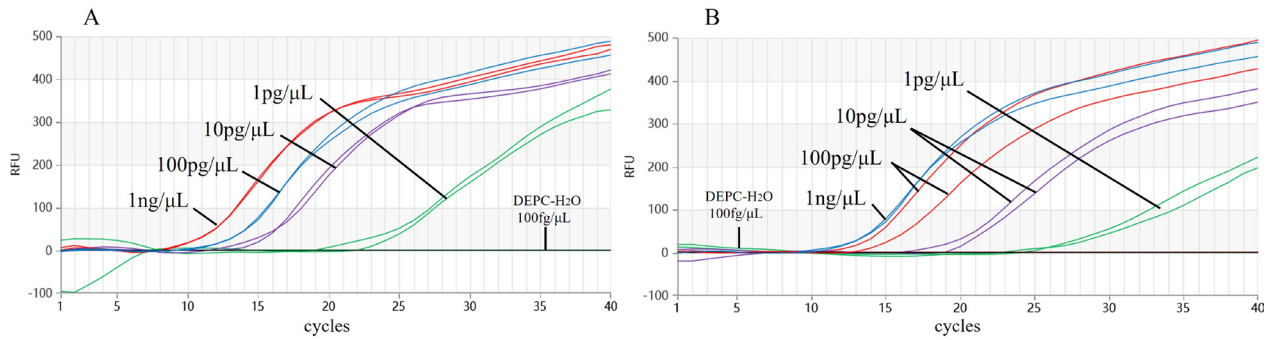


Figure 7. Sensitivity detection results of the dual Proofman-LMTIA reaction with serial dilutions of *D. fimbriatum* and *D. chrysotoxum* DNA templates. **A.** Serial dilutions of *D. fimbriatum* DNA template: 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, and 100 fg/μL; **B.** Serial dilutions of *D. chrysotoxum* DNA template: 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, and 100 fg/μL.

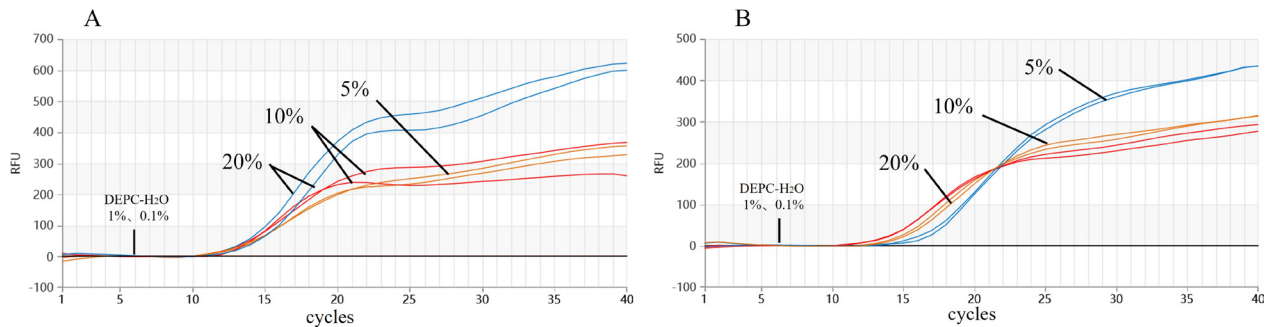


Figure 8. Limit of detection for *D. fimbriatum* and *D. chrysotoxum* in the duplex Proofman-LMTIA reaction system at 64 °C. **A.** *D. fimbriatum* was spiked into *D. chrysotoxum* at different mass fractions: 0.1%, 1%, 5%, 10%, 20%, and 50%; **B.** *D. chrysotoxum* was spiked into *D. fimbriatum* at different mass fractions: 0.1%, 1%, 5%, 10%, 20%, and 50%. Positive controls: genomic DNA of *D. fimbriatum* or *D. chrysotoxum*; negative control: DEPC-H₂O.

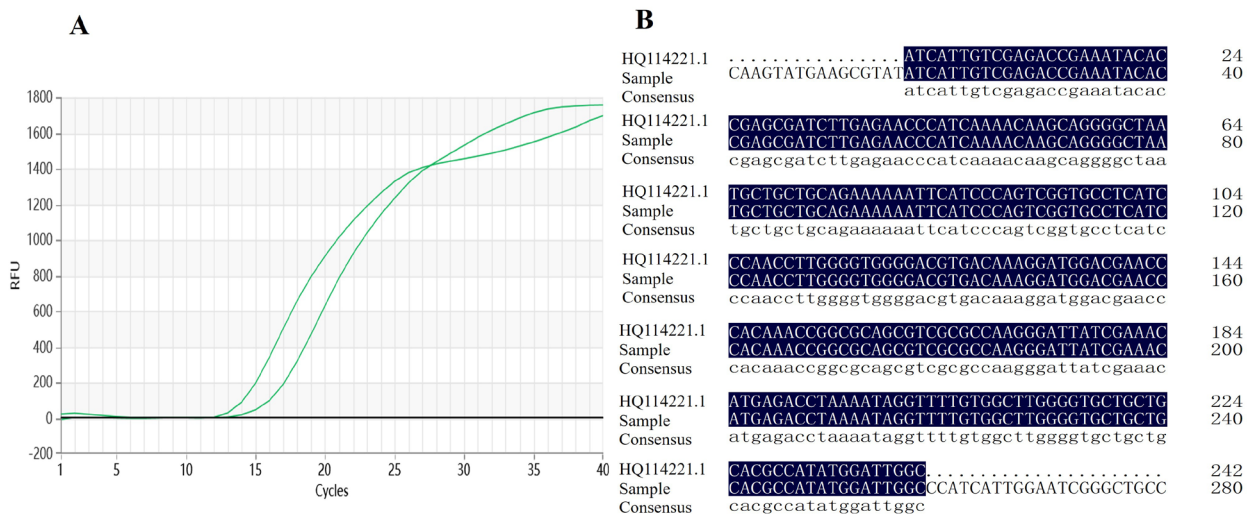


Figure 9. Detection diagrams of actual samples. **A.** Detection results of the dual Proofman-LMTIA reaction system; **B.** Comparison diagram of the first-generation sequencing results.

Although the duplex Proofman-LMTIA method established in this study exhibits multiple superior characteristics, it is currently restricted to qualitative analysis. Future research should focus on constructing a quantitative LMTIA system to meet the requirements of quantitative detection in diverse practical scenarios. In addition, de-

veloping a multiplex detection platform is warranted to realize the simultaneous and efficient identification of multiple *Dendrobium* species.

In conclusion, a duplex Proofman-LMTIA assay was successfully established for the rapid, sensitive, and specific identification of *D. fimbriatum* and *D. chrysotoxum*.

based on ITS sequences. This method allows simultaneous, accurate, and high-throughput discrimination of the two medicinal *Dendrobium* species, greatly improving the efficiency of quality control and the feasibility of on-site detection. Its widespread application will contribute to standardizing the *Dendrobium* market, ensuring the authenticity and quality of medicinal materials, and safeguarding the clinical efficacy and safety of *Dendrobium*-related products.

Conclusion

In this study, specific primers and Proofman probes were designed targeting the ITS sequences of *D. fimbriatum* and *D. chrysotoxum*, and a duplex Proofman-LMTIA assay was successfully established. This method enables simultaneous identification of the two *Dendrobium* species at 64 °C within 20 min, with a sensitivity of 1 pg/μL genomic DNA and a detection limit of 5% (w/w) for both species. When applied to commercial *Dendrobium* samples, the results were consistent with those obtained by DNA sequencing.

In summary, the established duplex Proofman-LMTIA method is rapid, simple, cost-effective, specific, and sensitive. It provides a reliable technical approach for the authentic identification of closely related *Dendrobium* species and offers important reference value for the quality control of traditional Chinese medicines.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

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Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

The authors declared that no experiments on animals were performed for the present study.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.

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Author contributions

Dan zhou: Writing-original draft, Validation, Methodology, Investigation, Conceptualization. Linqing Guo: Validation, Methodology, Investigation, Data curation, Conceptualization. Yanwu zhao: Validation, Methodology, Investigation, Funding, Data curation. Xiaodong Zhang: review & editing, Methodology, Investigation, Data curation. bailing yin: Methodology, Data curation. Yue Chen: Methodology, Data curation. Jianping li: Validation, Methodology. Yao Wang: review & editing, Methodology. Deguo Wang: Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. Yao Wang: review & editing, Methodology.

Data availability

All of the data that support the findings of this study are available in the main text.

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