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Original article

Specific, sensitive and rapid *Curcuma longa* turmeric powder authentication in commercial food using loop-mediated isothermal nucleic acid amplification

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ABSTRACT

Turmeric (*Curcuma longa*) is a rhizomatous plant of the ginger family *Zingiberaceae* that is usually dried and ground into powder for use as a seasoning. Because turmeric has become increasingly popular in the functional food market, adulteration of *C. longa* by other turmeric species is becoming an increasingly significant problem. In this study, loop-mediated isothermal amplification (LAMP) was developed for the detection of *C. longa* DNA for turmeric authentication. ITS2-26S rDNA was used for the LAMP primer designation. The results demonstrated that the specific primers exhibited high specificity, authenticated *C. longa* DNA within 30 min at 65 °C isothermally and had no cross-reaction with other adulterants. LAMP was sensitive to 0.1 ng of turmeric *C. longa* DNA, and only 0.01% of *C. longa* turmeric powder in the sample was required for DNA amplification. The sensitivity of LAMP was 10-fold higher than that of PCR (0.1%) from a previous report. Moreover, all the collected commercial turmeric products were positively detected by LAMP and RtF-LAMP (real-time fluorescence LAMP). The developed LAMP assay not only had higher specificity and rapidity than that of other methods but could also be applied to authenticate turmeric to prevent adulteration in food products.

1. Introduction

Turmeric (*Curcuma longa*), belonging to *Zingiberaceae*, is an important medicinal plant, and it is well known as a spice and coloring agent for food; it is popularly used in daily diets in China and East-South Asia. Recently, curcumin in turmeric has been proven to have several physiological activities that prevent a variety of human diseases, such as antioxidant, anti-inflammatory, antimicrobial, antiseptic, and anticancer activities and the attenuation of Alzheimer's disease (Karimi et al., 2018; Hayakawa et al., 2011; Venigalla et al., 2015). Thus, turmeric has great potential and has attracted the attention of researchers for the development of functional products in the food, cosmetic and pharmaceutical industries. Traditionally, apart from *C. longa*, turmeric powders are manufactured from other Curcuma species, such as *C. aromatica*, *C. zedoraria* and *C. aliismatifolia,* or other adulterants with similar morphological features. Among them, *C. longa,* with a high curcumin concentration, is preferred by consumers and has become mostly utilized in spice markets for consumption. Moreover, *C. longa* turmeric is frequently sold at a higher price than other curcuma species. In particular, the adulteration of *C. longa* by *C. aromatica* frequently occurs, and this adulteration is done intentionally because of their highly similar morphological features (Hayakawa et al., 2011). At present, several techniques are applied for the botanical identification of *C. longa,* including immunological assays, HPLC and TLC chromatographic analysis. In addition, DNA-based molecular techniques such as RADP, RFLP and PCR have also been established by various studies (Galimberti et al., 2013; Sasikumar et al., 2004; Oh and Jang, 2020). These identification techniques have been further developed depending on their effectiveness, sensitivity and specificity.

Loop-mediated isothermal amplification (LAMP) is a well-developed DNA amplification method that has been widely used

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for the authentication of various bioresources, such as bacteria, viruses, animals or plants (Notomi et al., 2000; Huang et al., 2010; Chen et al., 2015; Lee et al., 2017; Lee et al., 2019). Theoretically, at least four LAMP primers, including two outer primers (denoted by F3 and B3) and two inner primers (denoted by FIP and BIP), were needed to perform the LAMP reaction. Loop primers (denoted by LF and LB) are also frequently involved in the reaction for the improvement of sensitivity (Notomi et al., 2000). LAMP is performed by the catalysis of *Bst* DNA polymerase under isothermal conditions. Thus, there is no need to use a thermal cycler to denature DNA at high temperatures. Only 40–60 mins without any temperature change is required for running the LAMP reaction. These is very convenient for developing a LAMP-based point of care testing (POCT) method for biomaterial authentication (Notomi et al., 2000; Huang et al., 2010; Chen et al., 2015; Lee et al., 2017; Lee et al., 2019).

In this study, a rapid, sensitive and specific LAMP assay for detecting the DNA of *C. longa* turmeric was developed. The partial sequence of ITS2-26S ribosomal RNA (rRNA) of the genomic DNA was applied to design LAMP primers to evaluate the primer specificity, sensitivity and reactivity. Different commercial turmeric-related products were also used and examined to evaluate the feasibility of the assay. Finally, to the best of our knowledge, this specific LAMP assay established herein is more sensitive and faster than the previous literature reporting real-time PCR for the detection of *C. longa* DNA to prevent adulteration (Oh and Jang, 2020).

2. Materials and methods

2.1. Turmeric samples

Turmeric samples, *C. longa*, *C. aromatica*, and two adulterants, *Zingiber officinale* and *Alpinia galanga*, were collected from a local supermarket (Pingtung, Taiwan) and are depicted in Fig. 1. The botanical origin of turmeric samples was verified by Professor Wen-Te Chang of the China Medical University (Taichung, Taiwan). Commercial turmeric food items including Taiwan turmeric powder (Nahua Dist. Farmers' Association, Tainan, Taiwan), Malaysia turmeric powder (Brand: BABA, Lot No. R0606 V-LN, Malaysia), turmeric tea (Pukka Herbs Ltd, UK; Lot No. L8074), turmeric noodles (Brand: FONG-MAN FARM, Nantou, Taiwan) and turmeric energy drinks (Brand: House, House Foods Group Inc, Japan) were collected from a local supermarket (Pingtung, Taiwan). All the above samples were deposited at the Department of Food Science of National Pingtung University of Science and Technology.

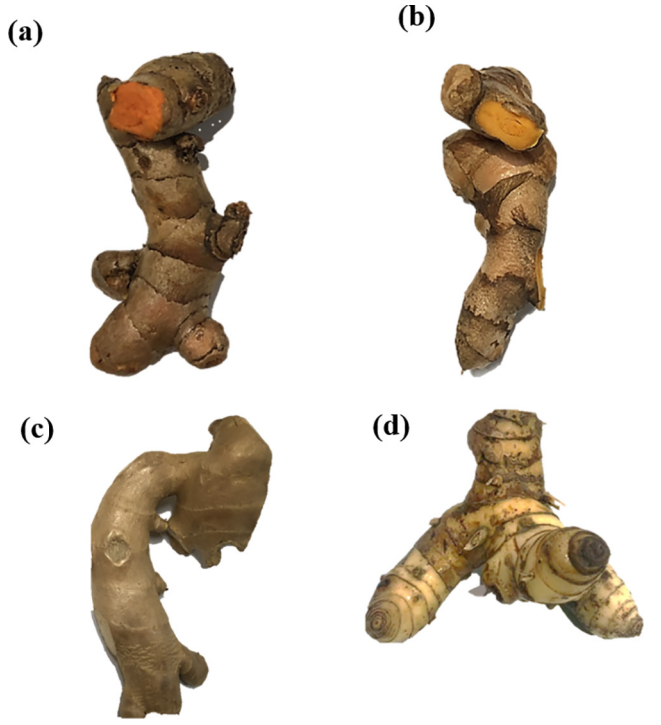

Fig. 1. Samples used in this study. (a) *C. longa*; (b) *C. aromatica*; (c) *Z. officinale*; (d) *A. galanga*.

2.2. DNA extraction

Total genomic DNA from turmeric and adulterant samples was extracted by a modified method from a previous report by Swetha et al. (2014). Briefly, 1 g of turmeric sample was frozen using liquid nitrogen and ground in a glass mortar by pestle grinding then stored at − 80 °C for further experiments. One hundred milligrams of turmeric powder was completely dissolved in 1 mL of 65 °C preheated hexadecyl trimethylammonium bromide (CTAB) extraction buffer (100 mM Tris base, 20 mM ethylenediaminetetraacetic acid (EDTA), 3 M sodium chloride, 5% CTAB (Sigma Aldrich, CA), 1% polyvinylpyrrolidone (PVP), and 0.3% β-mercaptoethanol). After incubation at 65 °C for 2 hrs, the resulting product was treated with an equal volume of a chloroform:isoamyl alcohol (24:1, v/v) solution for extraction. The top layer of extraction was harvested by centrifugation at 13200*g* for 15 min at 4 °C, and then a 1/10 vol of CTAB/NaCl buffer was added, followed by incubation for 15 min at room temperature. Then, an equal volume of chloroform/isoamyl alcohol was added again for extraction. The collected top layer of the sample was extracted by adding an equal volume of 98% isoamyl alcohol and incubating at −20 °C overnight for DNA precipitation. After centrifugation (13200*g*, 4 °C) for 10 min, precipitated DNA was collected as a pellet. The pelleted DNA was washed once with 70% ethanol. Then, the extracted DNA was air-dried and stored at −20 °C until required. The concentration of obtained genomic DNA was measured and determined by a spectrophotometer (NanoVue™, GE Healthcare, Piscataway, NJ, USA).

2.3. LAMP primers

Six primers (the outer primers, F3 and B3; inner primers, FIP and BIP; and loop primers, LF and LB) for the LAMP reaction were used for authenticating *C. longa* DNA, as listed in Table 1. The target position of LAMP primers is demonstrated and depicted in Fig. 2. The above primers were designed based on the ITS2-26S consensus sequence of nuclear ribosomal DNA (rDNA) obtained from GenBank (http://www.ncbi.nlm.nih.gov) using commercial Primer Explorer V4 software (http://primerexplorer.jp; Eiken Chemical Co., Ltd., Tokyo, Japan). The accession numbers of ITS2-26S of the *C. longa* and *C. aromatica* rDNA sequences were used for consensus sequence alignment, such as KX832017.1, DQ445151.1, JQ409956.1, KX832043.1, KX832027.1, KF694817.1, JQ409910.1,

Table 1
Sequences of the LAMP primers for the authentication of *C. longa*.

Primer	Sequence
F3	5′ GTCGCGAGCGAGAAC 3′
B3	5′ GGCTGATCCCGGTTCACT 3′
FIP (F2 + F1C)	5′ AATGATTGACGCGGCGCTTTC-GTTTTGGGATGAGCCCTCAA 3′
BIP (B2 + B1C)	5′ AGACCACCCGCCGAGTTTAAG-GCCGTTACTAGGGGAATCCT 3′
LF	5′ CATCAATCACACAGGGTCTCTTTA3′
LB	5′AAATAAGCGGAGGAGGAGAAACTTA3′

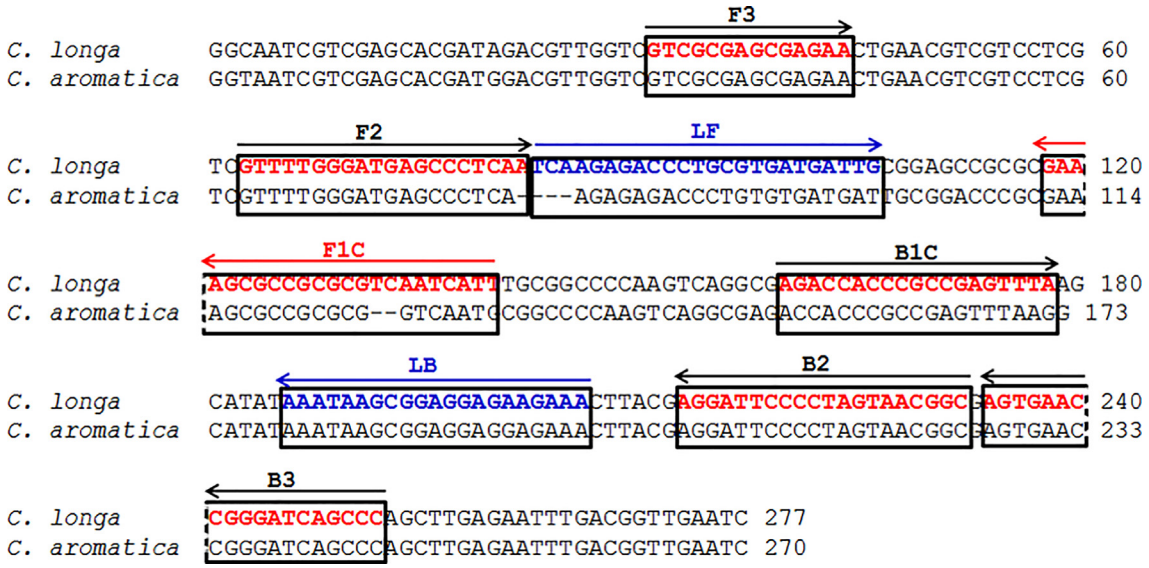

Fig. 2. Alignment of the sequence variation between *C. longa* and *C. aromatica* for the designed LAMP primers annealed on the positions of the partial ITS2 and 26S ribosomal DNA. The DNA regions depicted in boxes (F3, F2, LF, F1C, B1C, LB, B2 and B3) were used for the designation of outer primers (F3 and B3), inner primers (FIP and BIP) and loop primers (LF and LB), as shown in Table 1.

DQ438049.1 and KY129779.1, as illustrated in supplementary Fig. 1.

2.4. Authentication of turmeric C. Longa via the LAMP reaction

The LAMP reaction was performed as described by Lee et al. (2017). Briefly, various quantities of the genomic DNA of *C. longa* were added for each LAMP reaction. A 25 μL LAMP reaction mixture containing 2.5 μL of 10 × *Bst* DNA polymerization buffer, 200 mM dNTPs, 10 μM of each primer (F3, B3, FIP, BIP, LF and LB) and 8 U of *Bst* DNA polymerase (New England Biolabs, Frankfurt, Germany) was prepared. Then, the mixtures were incubated isothermally at 65 °C for 60 min using a thermal cycler (Thermo Fisher Scientific, Massachusetts, USA), and the reaction was terminated by heating at 80 °C for 5 min. The LAMP products were detected by 2% agarose gel electrophoresis. After electrophoresis, the ethidium bromide-stained gel was observed for ladder-like DNA patterns under UV excitation. The negative control was performed using a LAMP reaction without template DNA. Double distilled water and the 100 bp DNA ladder (Protech, Kaohsiung, Taiwan) was used as a negative control and DNA marker, respectively.

2.5. Sensitivity of the LAMP assay

Different amounts of *C. longa* genomic DNA (100, 10, 1, 0.1 and 0.01 ng, prepared by serial dilution) were used as template DNAs to measure the sensitivity of LAMP.

2.6. DNA preparation of turmeric C. Longa combined with other adulterants in different ratios

To prepare the experimental samples containing various percentages of the turmeric powder of *C. longa*, *A. galanga* dried powder was mixed with *C. longa* turmeric powder. Briefly, a total of 100 mg dried powder was prepared for each experimental assay. Then, different amounts of turmeric *C. longa* powder were weighed and combined with various amounts of dried *A. galanga* powder in different percentages at 50%, 10%, 5%, 1%, 0.1% and 0.01%. A total of 100, 50, 10, 5, 1, 0.1 and 0.01 mg of *C. longa* powder was weighed and mixed with 0, 50, 90, 95, 99, 99.9 and 99.99 mg of *A. galanga* dried powder to set up each experimental sample, and the resulting samples were frozen with liquid nitrogen then ground in a glass mortar by pestle grinding. All ground samples were saved for the further extraction of genomic DNA according to the above procedure.

2.7. Real-time fluorescence LAMP (RtF-LAMP) for the authentication of C. Longa in commercial turmeric food products

Five commercial turmeric food products, including Taiwan turmeric powder, Malaysia turmeric powder, turmeric tea, turmeric noodles and the turmeric energy drinks described previously, were used in DNA extraction tests following the DNA extraction method. For the turmeric powder, turmeric tea and turmeric noodle samples, 1 g of ground powder prepared by the liquid nitrogen treatment was used for DNA extraction. For the turmeric energy drink, 100 μL of sample liquid was taken and mixed with 1 mL of CTAB extraction buffer for further DNA extraction. The RtF-LAMP method was performed according to the above LAMP reaction procedure. Specifically, 25 μL of the prepared LAMP reaction mixture containing 2.5 μL of 10 × *Bst* DNA polymerization buffer (New England Biolabs, Frankfurt, Germany), 200 mM dNTPs, 10 μM of each primer (F3, B3, FIP, BIP, LF and LB, respectively), 8 U of *Bst* DNA polymerase (New England Biolabs, Frankfurt, Germany) and 1 μL of 5 × SYBR Green I (Sigma Aldrich, CA) were prepared, and then distilled water was added to the volume of 25 μL for each reaction. Isothermal DNA amplification was carried out at 65 °C for 60 min using a real-time thermal cycler (LightCycler 96, Roche, Germany), which was set to gather the fluorescence signal at 1-minute intervals. The real-time LAMP plot obtained using the RtF-LAMP method is shown in Fig. 4(b).

3. Results

3.1. Development of the LAMP assay for the authentication of turmeric C. Longa

To develop a LAMP assay for the authentication of turmeric *C. longa*, six of the specifically designed LAMP primers illustrated in

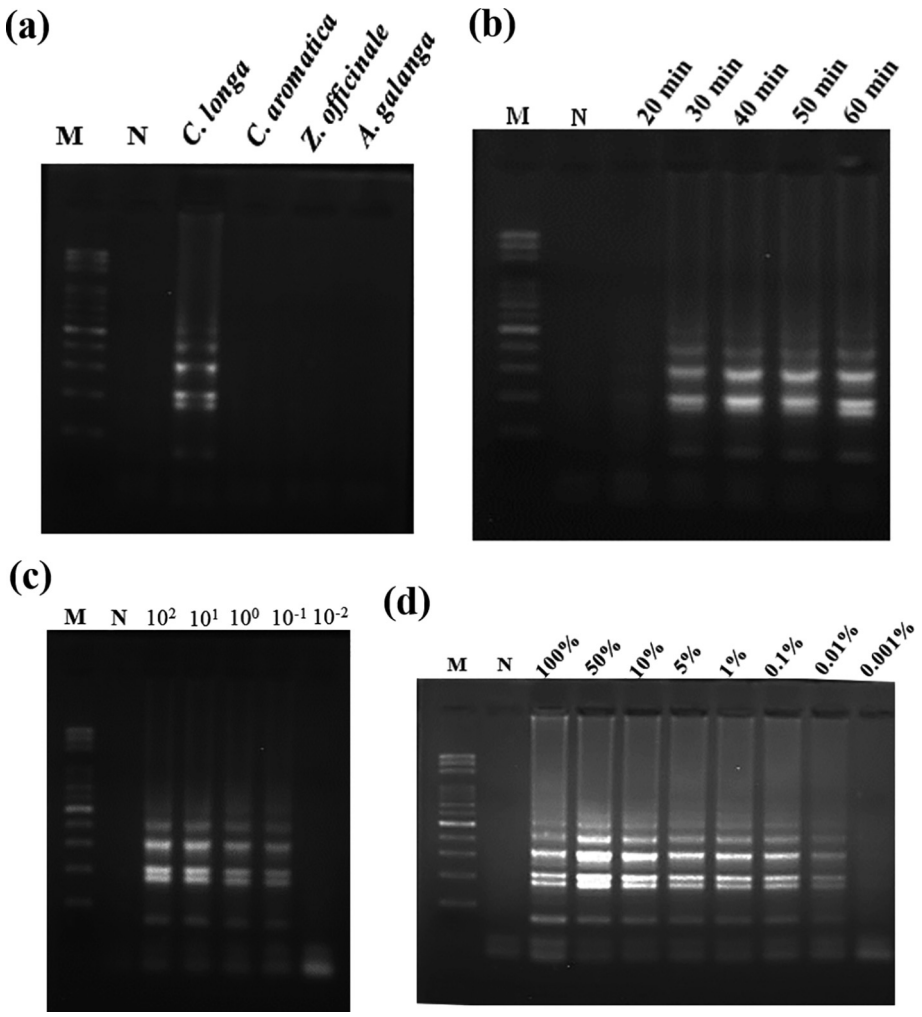

Fig. 3. Primer specificity, time for reaction, sensitivity and reactivity of the LAMP assay for the identification of *C. longa* DNA. The specificity of LAMP primers used for the detection of *C. longa* DNA was determined **(a)**. The genomic DNAs from purified various curcuma-adulterated species (*C. aromatica, Z. officinale and A. galanga*) and *C. longa* were used to perform the LAMP reactions. One hundred nanograms of genomic DNA was used as a template for each reaction. Various times for performing the LAMP reaction were used to validate the limit of minimal reaction time **(b)**. Different lanes represent different reaction times ranging from 20 to 60 min. The sensitivity of specific primers used in LAMP for the detection of *C. longa* DNA (**c**). Different amounts (nanograms) of *C. longa* DNA were added to the reaction mixtures to perform LAMP. The reactivity of the LAMP primers for the identification of *C. longa* was analyzed **(d)**. *C. longa* DNA was purified from raw *C. longa* samples mixed with different percentages of *A. galanga* samples by weight ranging from 50% to 10%, 5%, 1%, 0.1%, and 0.01%. Lanes M and N represent 100 bps of the DNA ladder marker and the negative control, respectively.

Table 1 were employed to amplify the genomic DNA of *C. longa*. After the LAMP reactions were performed, the LAMP products were amplified, and they demonstrated a pattern of ladder-like DNA fragments on the agarose gel (Fig. 3a). This result showed that the amplicon of *C. longa* existed in the sample. In contrast, no LAMP product was shown on the agarose gel in the adulterant genomic DNA samples, such as *C. aromatica*, *Z. officinale* and *A. galanga* (Fig. 3a). Moreover, only 30 min was required to amplify the amplicon DNA of *C. longa* (Fig. 3b). In summary, these results demonstrated that the LAMP primers in the set developed herein for the authentication of *C. longa* DNA are specific and rapid in practical use for amplifying the specific amplicons in turmeric samples.

3.2. Sensitivity and reactivity of LAMP for the detection of C. Longa DNA

To evaluate the LAMP reaction sensitivity, *C. longa* template DNAs with various quantities were used in the reaction. At least 0.1 ng of *C. longa* genomic DNA was required for performing LAMP (Fig. 3c). Additionally, the LAMP reaction was not influenced by the genomic DNA extracted from adulterants combined with *C. longa* DNA at different percentages (Fig. 3d). While the genomic DNA was extracted from only 0.01% *C. longa* raw material in the test sample, the LAMP reaction displayed sensitivity during DNA amplification (Fig. 3d). Thus, the LAMP reaction was sensitive to a minimal amount of 0.1 ng of *C. longa* DNA during authentication.

3.3. Authentication of C. Longa in commercial turmeric foods by LAMP and RtF-LAMP

To evaluate whether the LAMP assay developed in this study is suitable as a tool for authenticating commercial food products containing turmeric *C. longa*, five commercial turmeric products were collected from different local markets. Total DNA was extracted from the commercial food products, including Taiwan turmeric powder, Malaysia turmeric powder, turmeric tea, turmeric noodles and turmeric energy drinks, for use in the LAMP reaction. In particular, turmeric tea and turmeric noodles were also ground into fine powder by liquid nitrogen pretreatment, and 100 μL of the turmeric energy drink was taken before DNA extraction. After total DNA was extracted, 20 ng of template DNA was used for each LAMP reaction. As illustrated in Fig. 4a, all five commercial tur-

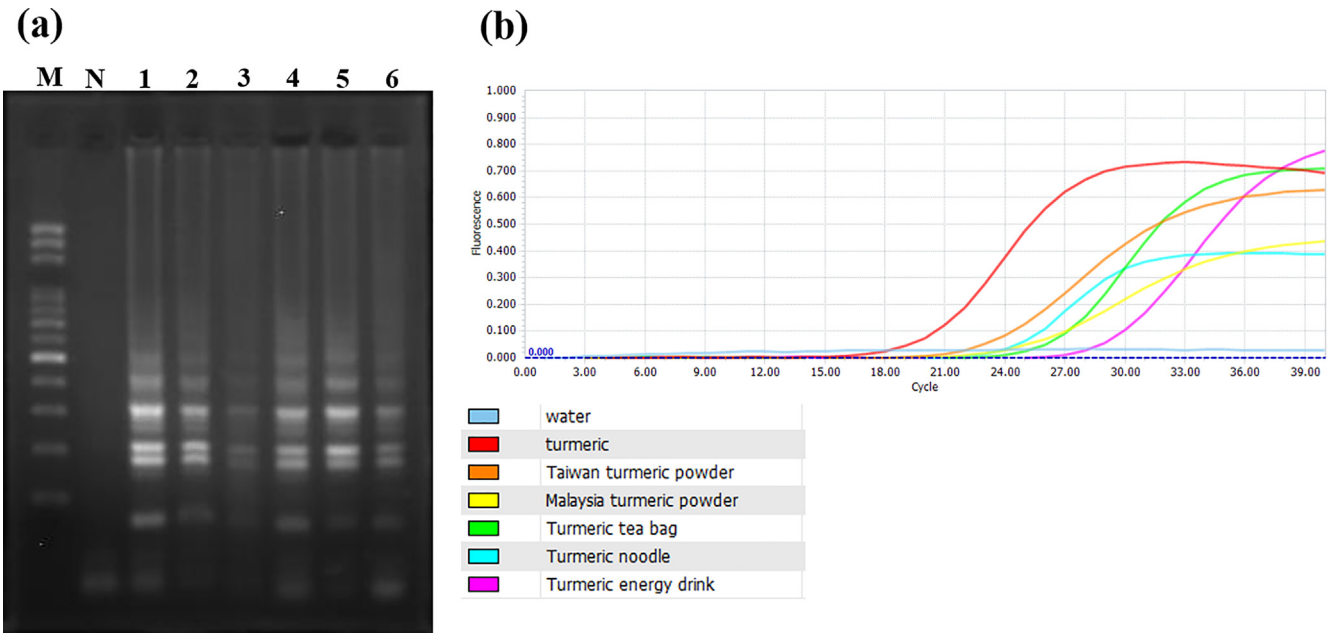

Fig. 4. Application of the specific LAMP primers for the authentication of *C. longa* turmeric in commercial turmeric food. Agarose gel electrophoresis (2%) was analyzed for the LAMP product (**a**). Twenty nanograms of genomic DNA was used as a template for each reaction. Lane M, 100 bp DNA ladder marker; Lane N, negative control; lane 1, positive control; and lanes 2–6, Taiwan turmeric powder, Malaysia turmeric powder, turmeric tea, turmeric noodle and turmeric energy drink, respectively. Analysis of the turmeric primers used in RtF-LAMP for the authentication of commercial turmeric products (**b**). In this plot, the Y-axis shows the fluorescence absorption during the reaction, and the X-axis (Cycle) denotes the time in minutes for initiation of the reaction.

meric foods were identified and exhibited typical DNA-ladder patterns with positive LAMP assay results. For the RtF-LAMP assay, all five commercial turmeric products were consistent with those from the LAMP detection after the fluorescence signals during DNA amplification were collected (Fig. 4b). It is worth noting that these various commercial turmeric products showed different initiation times for the Rtf-LAMP reaction, as illustrated in Table 2. The results obtained from the Rtf-LAMP assay not only confirmed the typical LAMP results but can also be used for the *C. longa* authentication of various turmeric food products.

4. Discussion

In recent years, adulteration of the turmeric powder of *C. longa* on the market has become increasingly serious because these adulterants exhibit similar morphological features to the powder. To resolve this problem, it is necessary to develop a convenient and efficient method to authenticate turmeric *C. longa* to protect the interests of consumers and lower the uncertainty during transactions. In this work, the specific aim was to establish an LAMP-based method with high specificity and the ability to rapidly authenticate *C. longa* DNA to avoid the adulteration of turmeric products. LAMP, a DNA molecular technique, has proven its potential and demonstrated several advantages in biological applications, such as high specificity, sensitivity and rapidity. Thus, various biomaterials or organisms have been authenticated by LAMP in previously published literature (Notomi et al., 2000; Huang et al., 2010; Chen et al., 2015; Lee et al., 2017; Lee et al, 2019; Sheu et al., 2018; Sheu et al., 2020). LAMP is an isothermal method for nucleic acid amplification, and it was first reported by Notomi et al. in 2000. Thus, LAMP is quite easy to perform for on-site identification using a simple heating device instead of a more complicated thermal cycler (Notomi et al., 2000). Herein, the procedure authenticating *C. longa* DNA by LAMP using specific LAMP primers was successfully developed. This primer set containing six primers can specifically discriminate *C. longa* from other adulterants (Fig. 3a). Despite the fact that the ITS2-26S rDNA sequences between *C. longa* and *C. aromatica* showed higher similarity, this LAMP primer set still exhibited specificity when identifying the specific amplicon of *C. longa* DNA. In a previous study, LAMP was applied to identify single nucleotide polymorphisms in the amplicon (Gill and Hadian Amree, 2020). This means that once the designed primers are specific, there is still enough power for the species identification. In addition, the *Bst* DNA polymerase used in the LAMP reaction can catalyze DNA amplification using a broader temperature range between 60 and 65 °C. In our preliminary study, it was determined that 63 °C was the optimal reaction temperature for obtaining more intense DNA bands during DNA amplification (supplementary Fig. 2). The specificity decreased when the temperature was below 63 °C. Typically, LAMP was performed completely within 60 min, and then the DNA-ladder pattern was observed (Fig. 3b). However, only 30 min is enough to initiate DNA amplification by LAMP. Over time, the accumulated DNA intensities were higher and saturated (Fig. 3b). Theoretically, the higher the content of amplicon DNA is in the sample, the shorter the initiation time for DNA amplification. This phenomenon was also reflected in our results. In Fig. 4b and Table 2, during the authentication of turmeric ingredients in commercial turmeric products by Rtf-LAMP, the various initiation times for DNA amplification varied. The time needed for the initiation of DNA amplification was correlated to the content of amplicon DNA. Thus, these results might be applied to quantify the content of biomaterials (Chen et al., 2015).

Table 2
Initiation time for DNA amplification by RtF-LAMP for the various commercial turmeric products.

Commercial product	Initiation time (min)
Taiwan turmeric powder	26.3
Malaysia turmeric powder	29.6
Turmeric tea	28.6
Turmeric noodle	27.4
Turmeric energy drink	31.4

Herein, the consensus sequences of the internal transcribed spacer (ITS) of the ribosomal DNA of *C. longa* and *C. aromatica* were used. The ITS has recently been recognized as a potential marker DNA for species identification in previous studies (Lee et al., 2017; Yan et al., 2016; Lee and Hxiao, 2019; Lai et al., 2015). In our work, the ITS was used for the designation of LAMP primers for turmeric authentication. In fact, other DNA regions, such as matK, atpF, and ycf2, have also been chosen for designing primers for DNA amplification (Oh and Jang, 2020). After the validation of the LAMP primer, the specificity of LAMP was elucidated, this was sufficiently strong enough for the identification of turmeric DNAs (Fig. 3a). Moreover, the sensitivity of LAMP improved by involving loop primers, and it is higher than that of PCR in a previous report (Oh and Jang, 2020). Only 0.01% of *C. longa* turmeric in samples is authenticated by LAMP, and 0.1% sensitivity is obtained by PCR. It should be also noted that LAMP exhibits a high efficiency for reactions less than 1 hr. In contrast, PCR frequently needs 2 to 3 hrs. to amplify DNA during the reaction (Sheu et al, 2018 & 2020). Accordingly, compared to PCR, LAMP is more appropriate and flexible for the on-site authentication of *C. longa* DNA in a small time period.

5. Conclusion

In this study, the isothermal DNA amplification method for the authentication of *C. longa* turmeric was developed; this method can potentially be applied as a rapid authentication tool with specificity and sensitivity that targets amplicons enclosed in turmeric-related foods. The DNA molecular method that was established can be useful to verify the turmeric *C. longa* content in commercial foods.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.06.057.

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