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The rapid and sensitive detection of edible bird's nest (*Aerodramus fuciphagus*) in processed food by a loop-mediated isothermal amplification (LAMP) assay

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ABSTRACT

Edible bird's nest (EBN) is a well-known and precious traditional Chinese herbal material (CHM). Because of this, preventing the adulteration of EBN efficiently and precisely is crucial to protect consumers' interests and health. In this study, a loop-mediated isothermal amplification (LAMP) assay was developed for the detection of EBN using specifically designed LAMP primers. The results demonstrated that the identification of EBN by LAMP assay was specific and rapid (within 1 h). It had no cross-reaction with EBN adulterants, including white fungus, egg white and pig skin, in different ratios. The relative detection limit was 0.01% EBN in the adulterants. Moreover, the sensitivity of LAMP in authenticating EBN was 10^{-8} μg, it showed higher sensitivity than that of conventional PCR with 10^5 fold. When genomic DNAs extracted from boiled or steamed EBN samples were used as templates, LAMP for EBN detection was not affected and was reproducible after heat processing. In conclusion, the LAMP assay established herein could be applicable for authenticating EBN and for identifying commercial EBN products in herbal markets.

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1. Introduction

Edible bird's nest (EBN) is a well-known traditional Chinese herbal material (CHM) called *Nidus collocaliae* (known in Chinese as "yan wo") that has been used in Asia, especially China, for a long time. EBN not only has medical uses but also is an expensive food ingredient, even though some of cases of EBN-induced anaphylaxis have been reported [1,2]. Because of its low productivity, high price, large consumption and claimed benefits, EBN is also named the "Caviar of the East" [3]. EBN is an animal-derived CHM that mainly consists of regurgitated saliva from swiftlets of several species of the *Aerodramus* genus in the Apodidae family, such as *Aerodramus fuciphagus* and *Aerodramus maximus*. Among them, *A. fuciphagus* is one of the predominate species used as an EBN source, as this EBN is more valuable and often used to manufacture EBN-related products [3,4]. Previous studies of EBN extracts have revealed that it is rich in mucinous glycoproteins such as sialylglycoconjugates or nonsulfated chondroitin glycosaminoglycans and epidermal growth-like factor [5–7]. Other bioactive components, including salicylic acid, amino acids, fatty acids, glucosamine, minerals and lactoferrin, have also been found by chemical analysis [3,8,9]. Three EBNs with different colours are found in the CHM market. Based on colour, EBN can be divided into white EBN, yellow EBN and red EBN. The EBN colour might result from the differences in the nutritional contents of the swiftlets, including mineral and iron contents [10,11]. In general, red EBN is most costly, and it yields greater health benefits than white EBN [12]. Therefore, EBN has been administered *in vivo* or *in vitro* to investigate its health benefits, which are thought to be derived from its stimulation of cell proliferation, anti-influenza virus activity, improvement of bone strength and dermal thickness, antioxidative stress effects, anti-inflammation, neuroprotection, and insulin sensitisation [7,13–17]. Since the scientific evidence for certain biological characteristics or health-promoting activities of EBN is accumulating, the problem of the authenticity and quality of EBN has become more prominent. For instance, fake EBN or adulterants of EBN have appeared in the CHM or food market. Thus, it has become important to ensure the effectiveness of EBN products and to alleviate consumers' anxiety. Some examples of reported EBN adulterants are gum karaya, sterculia, red seaweed, porcine gelatin and tremella fungus [3]. They are frequently used to commit fraud in the CHM or food market.

Currently, to ensure the presence of the EBN component in food or related products, numerous practically applicable and well-developed detection methods have been reported in the previous studies. Some authentication techniques for EBN detection were performed by conventional physical examination, such as the specific observation of the appearance and structural characteristics of EBN using scanning electron microscopy or X-ray microanalysis [3,18]. However, these physical examinations only provide information on the quality of EBN rather than its authenticity. Other methods, based on either protein or DNA targets, have been applied for EBN identification. Specific electrophoresed proteins or particular glycoproteins in the EBN have been identified by mass spectrometry or using specific antibodies during the authentication of EBN [18–20]. Unfortunately, the limited protein database of EBN is still a bottleneck in EBN authentication. As for DNA molecular techniques, using DNA sequencing or DNA-based PCR of specific DNA regions has also been demonstrated in previous reports [21,22]. Because the stability of DNA is higher than protein under severe extraction conditions, DNA molecules have a higher potential for the development of more efficient, sensitive and specific methods to confirm the authenticity of EBN.

A highly effective method for nucleic acid amplification termed loop-mediated isothermal amplification (LAMP) has been applied for the detection of biomaterial such as microorganism, animals and plants [23–25]. LAMP has been comprehensively applied for the detection or diagnosis of biomaterial sensitively and rapidly. In principle, at least four primers that simultaneously recognize six distinct DNA sequences specifically are used to anneal to a target gene for nucleic acid amplification when the LAMP reaction is performed under isothermal conditions. Since *Bst* DNA polymerase is used for the LAMP reaction, it is not necessary to use a thermal cycler to denature DNA at high temperatures. Therefore, the LAMP method can be used for the rapid, sensitive and specific point-of care detection of bio-samples.

In this study, a rapid, sensitive and specific LAMP assay for the identification of EBN (*A. fuciphagus*) was developed. The sequence of cytochrome *b* (cyt *b*) in the mitochondrial DNA (mtDNA) was used as the target to design LAMP primers for the evaluation of primer specificity. Commercially processed EBN was examined to evaluate the feasibility of the assay under the various integrities of the DNA in EBN or in EBN-related products found on the market. To the best of our knowledge, this is the first report of a LAMP assay for the verification of EBN authenticity.

2. Materials and methods

2.1. Samples

Samples of EBN (*A. fuciphagus*) were purchased from New Century Herbs Company (Pingtung, Taiwan) at December 2016, and its authenticity was identified by professor Wen-Te Chang of China Medical University, Department of Chinese Pharmaceutical Science and Chinese Medicine Resources (Taichung, Taiwan). Nine commercial EBN products manufactured from Taiwan and Hong Kong were collected from the local supermarket (Pingtung, Taiwan) at June 2017. The EBN and EBN-related samples were deposited at the department of food science of National Pingtung University of Science and Technology.

2.2. DNA extraction

Total genomic DNA from the EBN samples was extracted as described by Lin et al. [21] with modification. Briefly, EBN

samples were ground in a glass mortar by freezing using liquid nitrogen and pestle grinding and then stored at −80 °C for further experiments. One gram of EBN powder was completely dissolved with extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 40 mM DTT, 2 M NaCl, 1% SDS, pH 8.0). Then, 20 mg/mL of protease K was added for digestion for 6 h at 65 °C incubation. After centrifugation (12,000× *g*, 4 °C) for 10 min, undigested debris was pelleted, and the supernatant was incubated with RNase L for 1 h at 37 °C. The resultant product was treated with an equal volume of chloroform/isoamyl alcohol for extraction. The top layer of extraction was harvested after centrifugation (12,000× *g*, 4 °C) for 10 min, and then 1/10 volume of CTAB/NaCl buffer (3% cetyltrimethyl ammonium bromide (CTAB), 28% of 5 M NaCl, 4% of 0.5 M EDTA (pH 8.0), 10% of 1 M Tris-Cl (pH 8.0), 3% polyvinylpyrrolidone, 02% β-Mercaptoethanol) 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 mixing an equal volume of 95% ethanol and incubating at −20 °C for 1 h for DNA precipitation. After centrifugation (12,000×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 the extracted DNA was measured by spectrophotometer (N60, Implen, Munich, Germany). The integrity of DNA was determined by observation in HealthView™ Nucleic acid stain (Genomics, Taipei, Taiwan) under ultraviolet light. Regarding the genomic DNA extraction of commercial EBN products, total 5 g of each sample was taken and then subjected to dehydrate using drying oven under 50 °C for 12 h. The resultant of dehydrated EBN product was harvested and then used to extract genomic DNA by following the above procedure of total DNA extraction.

2.3. Primer design for LAMP

The LAMP primers (EBN-F3, EBN-B3, EBN-FIP and EBN-BIP) (Table 1) for the detection of EBN were designed based on the consensus sequence of cytochrome *b* (cyt *b*) in mitochondrial DNA (mtDNA) obtained from GenBank (http://www.ncbi.nlm.nih.gov) using the commercial software Primer Explorer V4 (http://loopamp.eiken.co.jp/e/index.html; Eiken Chemical Co., Ltd., Tokyo, Japan). The accession numbers of the cyt *b* sequences of EBN mtDNA were KJ671391, AY135627, AY135630, AY135631, AY135632, AY294427, AY294429, DQ463130, U49993, U49994 and U49995 for the alignment of consensus sequences. The primers' position was depicted in the alignment of consensus sequence of cyt *b* as illustrated in supplementary figure 1.

2.4. LAMP reaction for EBN detection

The LAMP reaction was performed according to Lee et al. [24]. Briefly, a different amount of genomic DNA of EBN was used for each LAMP reaction. The reaction mixture contained 8 U of *Bst* DNA polymerase (New England Biolabs, Frankfurt, Germany), 1 × *Bst* DNA polymerization buffer, 4 μM inner primers (EBNFIP and EBNBIP primers each), 0.5 μM outer primers (EBNF3 and EBNB3 primers each), and 200 μM aliquots of each dNTP. The mixtures were incubated at 61 °C for 60 min in a PCR Express Thermal Cycler (TP600, Takara, Japan) for isothermal heating. The reaction was terminated by 80 °C heating for 5 min. The mixture was stored at 4 °C until the analysis of LAMP products.

2.5. Detection of LAMP products

The formation of LAMP products was detected by DNA electrophoresis with ethidium bromide staining. A 2% agarose gel was used for electrophoresis and for observation of the DNA banding. Alternatively, SYBR green I (>1 μM) was used to add to stain the amplified DNA from LAMP reaction. When the positive LAMP reaction was presented, the colour of SYBR green I added in the reaction tube was turned to green immediately under UV excitation.

2.6. PCR

PCR was carried out in a PCR Express Thermal Cycler (TP600, Takara, Japan) using two outer primers (EBNF3 and EBNB3) designed from the consensus sequence of cyt *b* of the mtDNA-based LAMP primers, as shown in Table 1. Different amounts of extracted genomic DNA of EBN were used as templates in the PCR mixtures. All amplifications were performed in a final volume of 25 μL containing 1 U *Taq* DNA polymerase, 0.4 μM aliquots of each outer primer, and 200 μM aliquots of each dNTP. The reaction was initiated with a denaturation (95 °C, 10 min), followed by 35 cycles of denaturation (95 °C, 30 s), annealing (61 °C, 30 s) and extension (72 °C, 30 s), and a final extension step (72 °C, 10 min) to complete the reaction. A 2% agarose gel for DNA electrophoresis was used to detect PCR products with ethidium bromide staining.

2.7. Preparation of boiled and steamed EBN

Five grams of each EBN sample was prepared and treated by boiling and steaming. EBN samples were immersed in water for swelling. Swollen EBN was put into a 50 mL test tube in boiling water for 20, 40 or 60 min, followed by cooling in an ice bath. For the steaming process, an autoclave was used. Swollen EBN was autoclaved at a pressure of 15 psi at 121 °C for 20, 40 or 60 min.

Table 1 – The sequences of LAMP primers for detection of edible bird's nest (EBN).

DNA regions	Primers	Sequences
Cyt *b*	EBNF3	5′CCTCCCATGAGGCCAGAT3′
	EBNB3	5′GGCCTGCGATTAGGAAGG3′
	EBNFIP	5′ACCATTACTGACAACGC GG-TGTAGCCGGTTTGGGAGCATCT3′
	EBNBIP	5′GAGTAGGTGGAGTGGGAGG-CACATCCCGCTTCTTAGCCCAA3′

After autoclaving, the steamed EBN was put in an ice bath cooling. The resultant boiled and steamed EBN was used to extract the genomic DNA according to a previously described procedure. Then, the purified DNA obtained from boiled or steamed EBN was subjected to both LAMP and PCR.

2.8. *Specificity of LAMP assay*

Three common EBN adulterants, egg white, white fungus, pig skin and red algae were purchased from a local supermarket (Pingtung, Taiwan). After DNA extraction, purified DNA of each EBN adulterant was subjected to the LAMP reaction as the template DNA for specificity determination.

2.9. *Sensitivity of LAMP assay*

Different amounts of EBN genomic DNA (10^{-1} μg, 10^{-2} μg, 10^{-3} μg, 10^{-4} μg, 10^{-5} μg, 10^{-6} μg, 10^{-7} μg, 10^{-8} μg and 10^{-9} μg, prepared by serial dilution) were used as template DNAs to measure the sensitivity of LAMP.

2.10. *Preparation of EBN with adulterants in different ratios*

The EBN adulterants egg white, white fungus, pig skin and red algae were ground in a glass mortar by freezing using liquid nitrogen and pestle grinding. All three grinded samples were taken in equal amounts and then mixed completely. The adulterant mixture was added to EBN powder at different ratios (50%, 10%, 5%, 1%, 0.1%, 0.01% and 0.001% by serial dilution). Genomic DNA was extracted from the different resultant mixtures according to the above procedure.

3. Results

3.1. *Development of LAMP assay for the detection of EBN*

To develop a LAMP assay to detect EBN DNA, LAMP primers, including two outer primer (EBN-F3 and EBN-B3) and two inner primers (EBN-FIP and EBN-BIP), were designed for specific identification of the cyt *b* gene in mtDNA sequences available in GenBank (http://www.ncbi.nlm.nih.gov) (Table 1). When LAMP primers were used for the detection of EBN DNA, the LAMP products revealed a typical pattern of ladder-like DNA fragments on the agarose gel irrespective of the sample's identity as white EBN, yellow EBN or red EBN sample (Fig. 1A, lane 1–3). This result demonstrated that LAMP primers annealed to their target DNA sequences for DNA amplification. In contrasting, no LAMP products were detected when the non-target genomic DNA samples, such as the EBN adulterants white fungus, egg white, pig skin, and red algae were used in the reactions (Fig. 1A, lane 4–7). Additionally, the LAMP reaction was not influenced by the mixing of EBN with adulterants' genomic DNA. At least 0.01% of the total DNA had to be EBN genomic DNA to achieve a specific detection, regardless of whether it was white EBN, yellow EBN or red EBN (Fig. 1B–D). These results show that the LAMP primers developed for the identification of EBN in this work are specific for EBN authentication and could be used in the amplification of EBN target DNAs.

3.2. *Sensitivity of LAMP for EBN detection*

To determine the sensitivity of LAMP, different amount of EBN genomic DNA were added to the LAMP reaction. As illustrated in Fig. 2, at least 10^{-8} μg of EBN genomic DNA was required for detection. Although LAMP products were not produced abundantly when 10^{-8} μg of red EBN genomic DNA was used as template (Fig. 2C), a clear LAMP pattern was observed on the gel when 10^{-8} μg of template DNA from white EBN or yellow EBN was used in the reaction (Fig. 2A and B). With the current primers, 10^{-3} μg EBN genomic DNA was needed for PCR (Fig. 2D–F). A specific DNA band at approximately 178 bp was seen on the gel when 10^{-3} μg genomic DNA of white EBN, yellow EBN or red EBN was added to the PCR mix (Fig. 2D–F). Taken together, these results indicate that the sensitivity of the LAMP method developed herein was higher than the PCR assay for the identification of EBN DNAs.

3.3. *Authentication of the heat-processed EBN by LAMP assay*

To examine the effect of heat processing, including boiling and steaming, on EBN identification by our LAMP assay, EBN samples were boiled in a water bath and steamed by autoclaving. Boiling for 20, 40 or 60 min did not impact the LAMP reaction on white, yellow or red EBN genomic DNA; LAMP products were amplified completely (Fig. 3A). All three kinds of EBN samples treated with boiling were still authenticated by the LAMP assay. After steaming by autoclaving for 20, 40 or 60 min, LAMP products were still produced from EBN DNA when the LAMP reaction was performed for EBN identification (Fig. 3B). When PCR was used for EBN detection, the results were similar to the LAMP results, regardless of whether the DNA template used in the reaction was from boiled or autoclaved EBN (Fig. 3C and D). Even so, it is noteworthy that the specific PCR products in the 60-min-steamed EBN samples showed less intense DNA binding than the 60-min-boiled EBN samples (Fig. 3C and D). Taken together, these results show that boiling or steaming did not influence the outcome of LAMP or PCR (Fig. 3A–D). Thus, LAMP was well-matched with PCR for the identification of EBN DNAs (Fig. 3).

3.4. *Application of LAMP in the detection of EBN in commercial EBN products*

To assess the applicability of the LAMP assay to EBN detection as a practical tool, nine commercial products containing EBN were purchased for EBN identification by LAMP. The nine commercial EBN products were prepared to purify total DNA for use in the LAMP reaction. When LAMP primers for EBN detection were used to authenticate the EBN ingredients, six of nine commercial EBN products had detectable EBN, and the typical DNA ladder-like patterns were demonstrated after performing the LAMP reaction (Fig. 4A). Additionally, above positive reactions of LAMP assay were also confirmed by direct

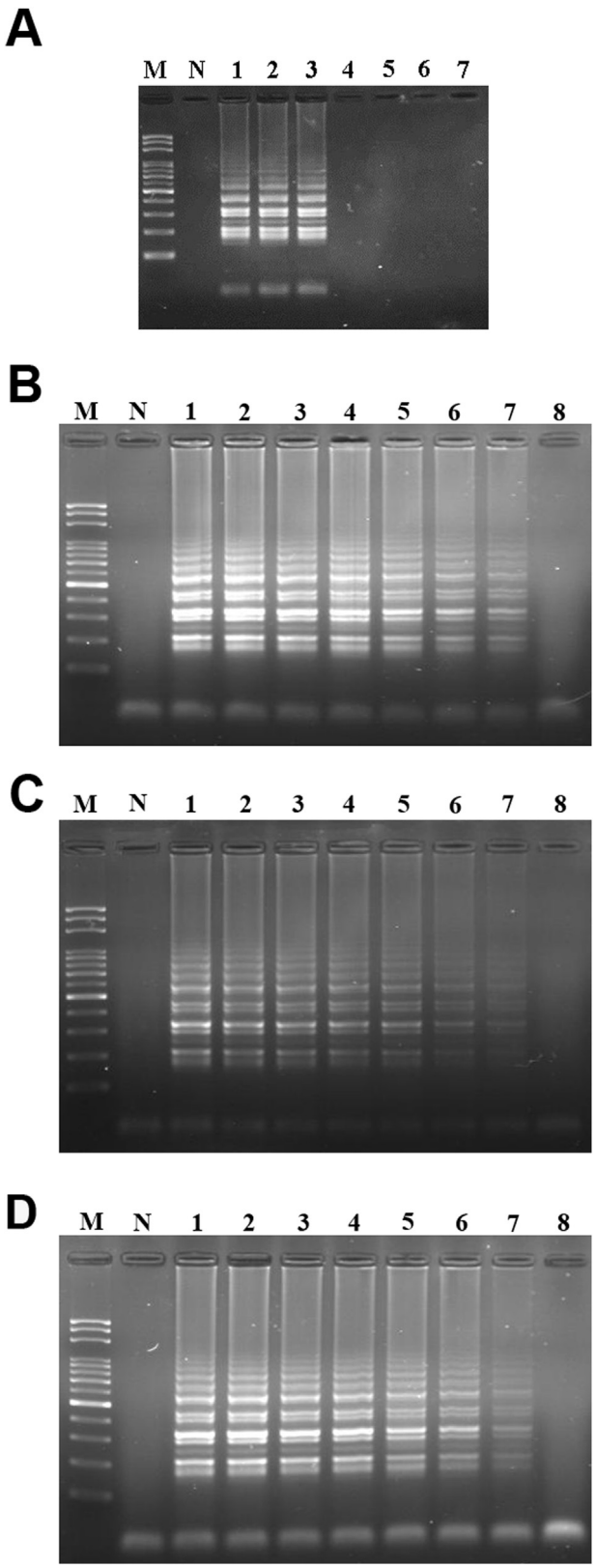

Fig. 1 – Analysis of the specificity of LAMP primer and its detection limit for EBN identification. The specificity of primers used in the LAMP assay for EBN identification (A). The specific cyt *b*-based LAMP primers were used for the evaluation of detection specificity for EBN identification. Purified 10^{-1} μg of white, yellow and red EBN genomic DNAs were used as templates for LAMP reactions. The amount of 10^{-1} μg genomic DNA of adulterants including white fungus, egg white, pig skin and red algae were also used. Lanes M and N, respectively, represent the 100 bp DNA ladder and the negative control. Lanes 1–7 represent different DNAs: 1, white EBN; 2, yellow EBN; 3, red EBN; 4, white fungus; 5, egg white; 6, pig skin; 7, red algae DNA. The detection limit of the LAMP primers for the EBN identification from mixtures of EBN and adulterants were determined. Three kinds of EBN samples, white EBN (B), yellow EBN (C) and red EBN (D), were prepared. The LAMP primers were used for the detection of EBN mixed with different percentages of DNA from EBN adulterants. Lanes M and N, respectively, represent the 100 bp DNA ladder and the negative control, respectively. Lane 1: positive control; Lanes 2–8: 50%, 10%, 5%, 1%, 0.1%, 0.01% and 0.001% EBN DNA mixed with DNAs from the three EBN adulterants. Total 13 discrete DNA fragments contained in the 100 bp DNA Ladder s ranging in size from 3000, 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 to 100 bp, sequentially.

visualization and consistent with the result of electrophoresis after SYBR green I staining. When sample turned green by adding SYBR green I under UV excitation, it indicated positive reaction of EBN authentication. In contrast, negative sample was not significant showed green of emission (Fig. 4B). The conventional PCR results were consistent with the LAMP results (Fig. 4C). These results suggest that our LAMP assay can be used for rapid and sensitive authentication of EBN-containing products. However, our LAMP results did not agree with the manufacturer-labelled formulations of all nine commercial EBN foods. Therefore, based on the specificity, sensitivity and validation of the LAMP primers, LAMP has practical applications for further authentication of EBN to provide precise information for CHM authentication.

4. Discussion

EBN is an animal-derived and precious CHM in the herbal market. Not only is EBN popular as a medicine, but it is also added as a functional ingredient to food products. Because of this, conventional histological microscopy for the authentication of botanically derived CHM is not completely suitable for the authentication of EBN. In a previous study, histology combined with spectrophotometry provided useful information for the differentiation of EBN from its adulterants [26]. In spite of the fact that current available assays can be used to EBN authentication, performing these methods rely on sophisticated operator and examiner's experience. Thus, the issue of effectiveness, reproducibility and standardization of detection would be a critical concern to develop an alternative assay for EBN authentication. In addition, many dietary supplements are manufactured by adding dried or powdered EBN, so judging the EBN content by morphological or appearance-based examination would be challenging. In contrast, development of DNA-based authentication has several

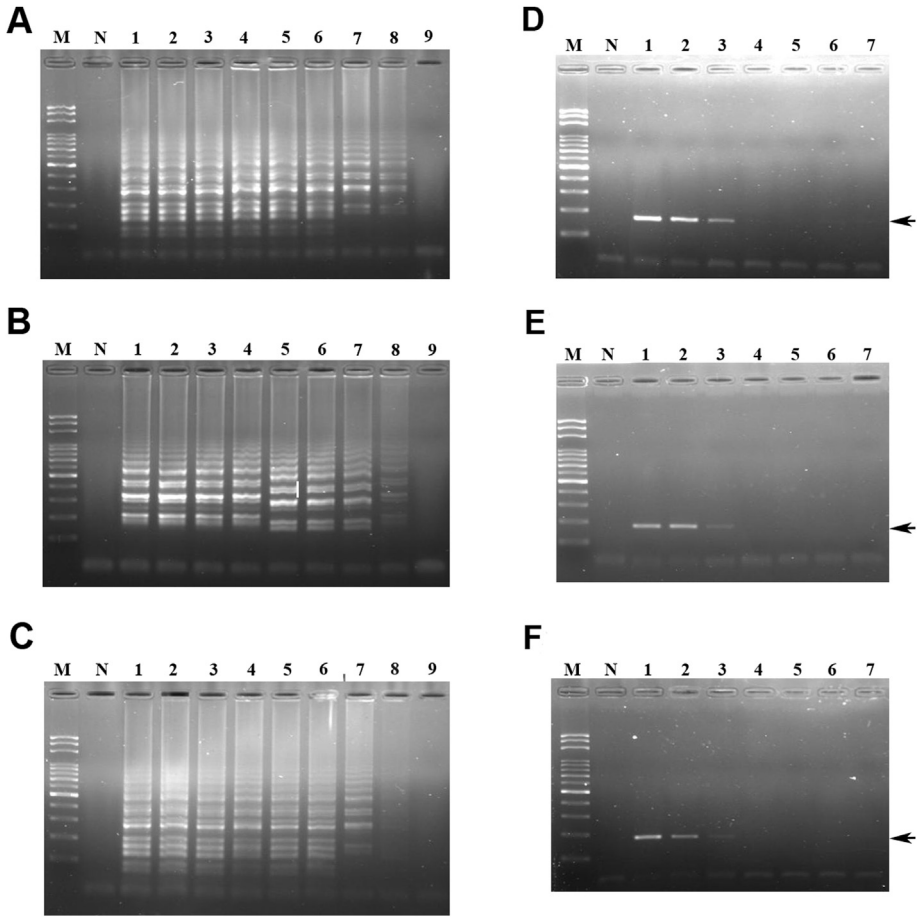

Fig. 2 – The sensitivity of specific LAMP and PCR primers used for the detection of EBN. Three kinds of EBN samples, white EBN (A), yellow EBN (B) and red EBN (C), were prepared for LAMP assay. Lanes M and N represent the 100 bp DNA ladder and the negative control, respectively. Lanes 1–9 represent the different amounts of EBN DNA added to the LAMP reaction: 1, 10^{-1} μg; 2, 10^{-2} μg; 3, 10^{-3} μg; 4, 10^{-4} μg; 5, 10^{-5} μg; 6, 10^{-6} μg; 7, 10^{-7} μg; 8, 10^{-8} μg; and 9, 10^{-9} μg. As to PCR primers used for the detection of EBN, also three kinds of EBN samples, white EBN (D), yellow EBN (E) and red EBN (F), were prepared for PCR assay. Lanes M and N represent the 100 bp DNA ladder and the negative control, respectively. Lanes 1–7 represent the different amounts of EBN DNA added to the reaction: 1, 10^{-1} μg; 2, 10^{-2} μg; 3, 10^{-3} μg; 4, 10^{-4} μg; 5, 10^{-5} μg; 6, 10^{-6} μg; 7, 10^{-7} μg. The black arrow represents the specific amplified PCR product in the agarose gel. Total 13 discrete DNA fragments contained in the 100 bp DNA Ladder s ranging in size from 3000, 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 to 100 bp, sequentially.

superiorities, including effectiveness, sensitivity and reliability, compared to other ways.

Recently, an increasing number of DNA techniques, including PCR-based methods or next-generation sequencing, have been applied to authenticate CHM rapidly and precisely [27,28]. During the past decade, several studies have used LAMP as the detection method for rapidly identifying microorganism, animals and plants or other materials [23–25]. LAMP is quite easy to perform for the on-site detection of biomaterial. Because *Bst* DNA polymerase is used in the LAMP reaction, DNA can be amplified in a simple water bath at constant temperature; no thermal cycler is needed. In this study, the specific LAMP primers were designed for an assay to specifically, sensitively and rapidly detect EBN DNA. LAMP assay significant improved 10^3 fold of the sensitivity of reaction higher than previous established real-time PCR for EBN authentication [29]. Not only the improvement of sensitivity, but also the time-effectiveness for detection is an advantage of LAMP assay during performing assay. Thus, a competitive and alternative method such as LAMP assay is still needed to handle various situations, such as less pure or lower-integrity DNA during the authentication of EBN DNA.

In this work, in contrast to the usual of the authentication of plant-derived CHM, the cyt *b* gene of swiftlets instead of the ribosomal internal transcribed spacer (ITS) in plants was used for designing specific LAMP primers. The cyt *b* gene of mtDNA is the most popular molecular marker for sequence alignment or barcoding in the identification of animal

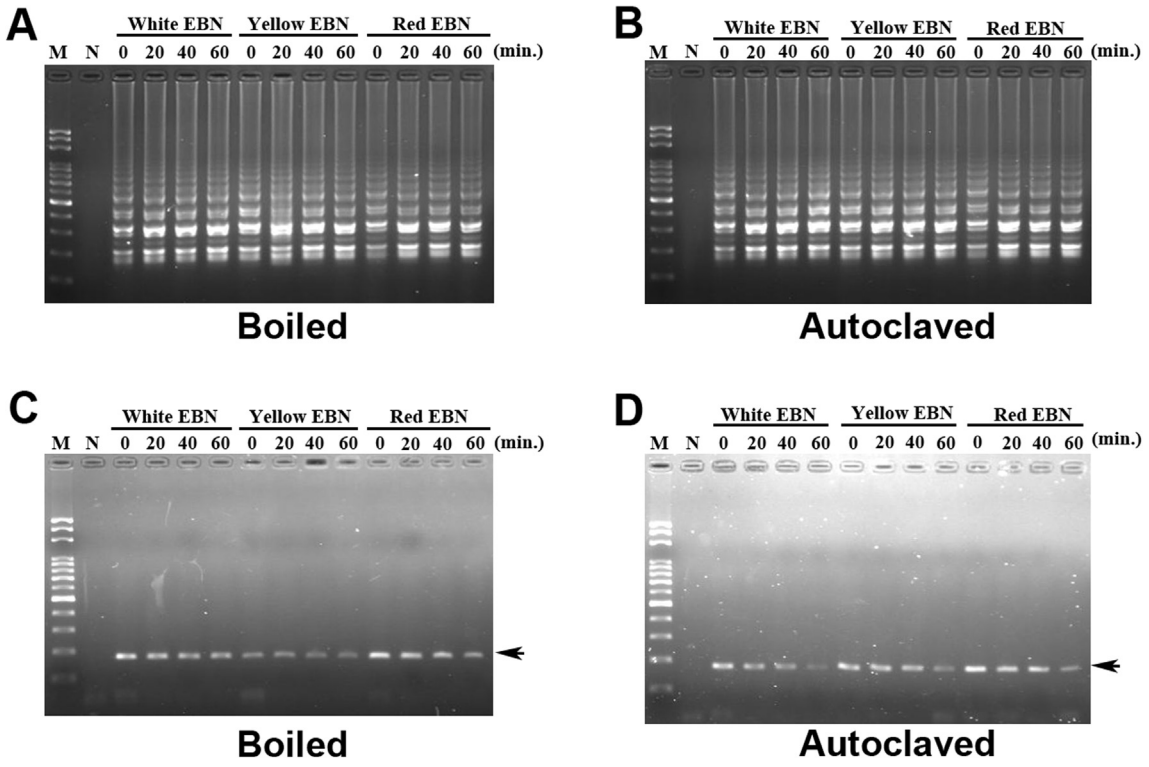

Fig. 3 – Analysis of LAMP (A, B) and PCR products (C, D) derived from EBN DNA after the boiling and autoclave-steaming procedures. When LAMP and PCR primers were used, the performance of DNA amplification was examined using purified 10^{-1} μg of EBN genomic DNAs as template. Lane M: 100 bp DNA ladder, Lane N: negative control. For the boiling treatment, the EBN DNA was boiled for 20, 40 or 60 min. For the autoclave-steaming procedure, the EBN DNA was steamed for 20, 40 or 60 min. The black arrow represents the specific amplified PCR product in the agarose gel. Total 13 discrete DNA fragments contained in the 100 bp DNA Ladder s ranging in size from 3000, 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 to 100 bp, sequentially.

authenticity [30]. Therefore, when evaluating the relevance of LAMP primers for EBN identification, we successfully confirmed the authenticity of EBN specifically from EBN adulterants (Fig. 1). The specificity of LAMP was comparable in strength to regular PCR for the identification of EBN DNAs. Moreover, the sensitivity of EBN detection by LAMP was 10^{-8} μg, which was 10^5-fold higher than the sensitivity of conventional PCR. Because of this, high amounts of template DNA are usually not required; the LAMP reactivity was not affected when a small concentration of intact amplicons of the EBN DNA existed in the reaction. Notably, the LAMP assay we developed in this work exhibited better reproducibility when LAMP was performed at a 60 °C–61 °C reaction temperature (data not shown). This temperature of primer annealing for LAMP was higher than that of the PCR primers. The higher annealing temperature for LAMP primers might increase the specificity of LAMP compared to PCR during DNA amplification [31].

In general, a single ingredient is seldom used in the formula of a processed commercial food or related product. Thus, the application of LAMP to EBN DNA identification would pose a challenge when more complicated ingredients are present in the processed food [24]. The LAMP assay established herein was specific and reproducible, and 0.01% EBN was the relative detection limit when typical EBN adulterants were added. When mixed DNAs obtained from other ingredients, such as EBN adulterants, were analysed, the specificity of LAMP did not decrease. This indicated that the identification of EBN in commercial products can be performed sensitively and stably using the LAMP method established herein. Moreover, the heating treatments did not affect the performance of LAMP. Conventional PCR for EBN detection yielded similar results to those of LAMP (Fig. 3C and D). This means that LAMP was well matched in strength with PCR for the identification of EBN DNAs (Fig. 3). However, the lower intensities of DNA banding of PCR products in the steamed EBN samples might reflect that the amplified DNA of EBN was damaged during the 60-min autoclaving procedure (Fig. 3D). Thus, this LAMP assay is more applicable and effective for the detection of EBN adulteration in commercial EBN products than PCR. Second, the performance of the detection assay is a critical issue when determining whether food formulae

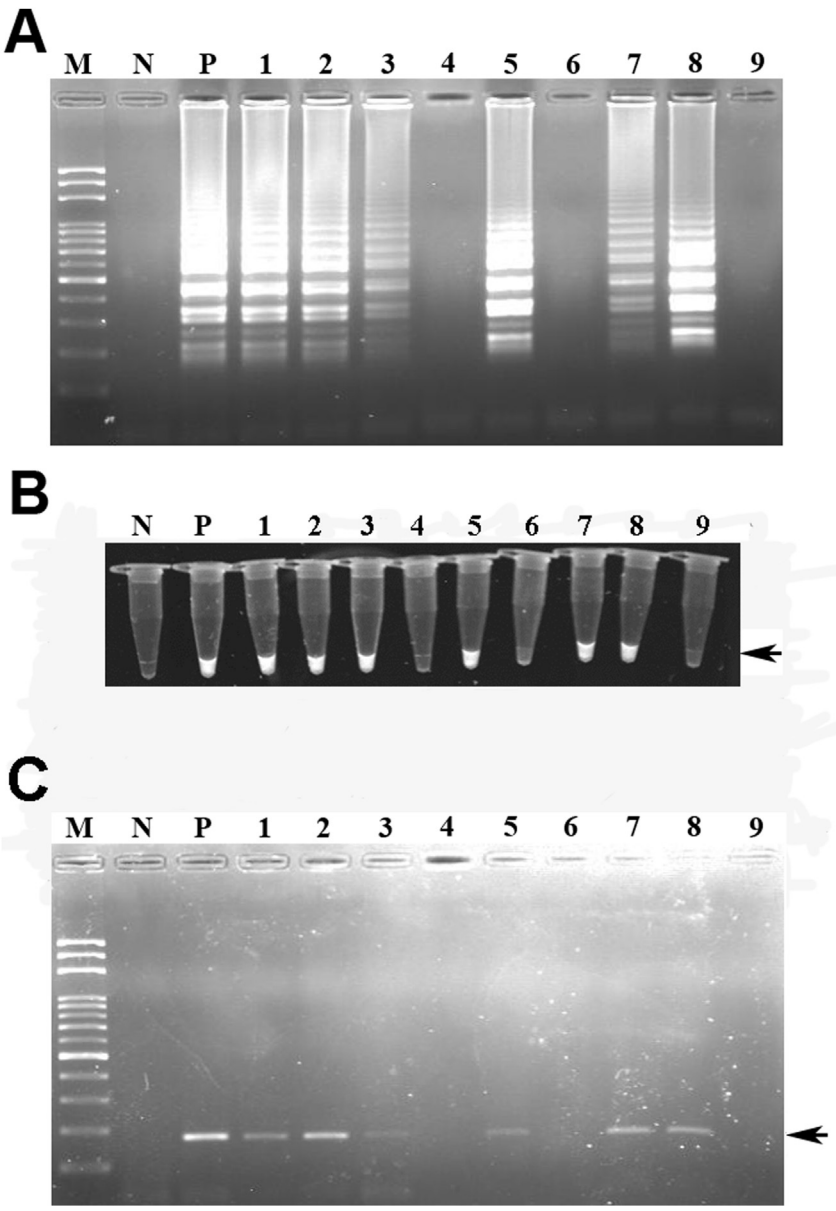

Fig. 4 – Analysis of LAMP (A, B) and PCR products (C) from commercial EBN products. LAMP and PCR assays were performed on EBN samples from nine purchased commercial products. Each sample containing purified 10^{-1} μg of total genomic DNAs as template was used for LAMP and PCR assay, respectively. The LAMP (A) and PCR (C) product was detected by electrophoresis, respectively. Alternatively, LAMP product with fluorescent was observed by SYBR green I staining under UV excitation (B). Lane M represents the 100 bp DNA ladder; lane N and lane P represent negative and positive controls, respectively. Lanes 1 to 9 represent the sample DNAs of house bird's nest, cave bird's nest, nonpareil bird's nest, Wang Tong bird's nest with rock sugar, Lohongka bird's nest with rock sugar, red date bird's nest, Brands bird's nest with rock sugar, Beauty bird's nest, and Yen premium bird's nest, respectively. Total 13 discrete DNA fragments contained in the 100 bp DNA Ladder s ranging in size from 3000, 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 to 100 bp, sequentially. The black arrow represents the fluorescent emission from the tubes (B) and the specific amplified PCR product in the agarose gel (C), respectively.

containing EBN comply with the food labels provided by the manufacturers. Our results demonstrate that LAMP performed well in the identification of EBN DNA in this context as well. This assay not only affords a possible tool for internal checking to prevent contamination by the producer, but it can also to be used for routine inspections for allergen labelling by government authorities [24]. To the best of our knowledge, this is the first report to verify EBN effectively and systematically using a LAMP assay. This could become an authentication model for rapid and sensitive determination of the authenticity of EBNs or other animal-derived CHM by LAMP methods in the future.

5. Conclusion

An isothermal DNA amplification was developed for the specific, sensitive and rapid identification of EBN. This assay not only can authenticate EBN but also can detect EBN DNA to reveal adulteration of commercial or processed EBN products.

Author contribution

M.-S. Lee, J-Y. Huang, Y-Y Lien and S-C Sheu designed the experiments. M.-S. Lee, J-Y. Huang and Y-Y Lien performed the experiments and data analysis. M.-S. Lee and S-C Sheu wrote the manuscript. All of authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.08.003.

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