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Multiplex Pcr-Based Detection of Pig Skin and Sterculia Gum Adulterants in Refined Edible Bird's Nests

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Abstract

This study presents a novel multiplex PCR technique designed to identify adulteration in refined edible bird's nests (EBNs), specifically targeting common adulterants such as pig skin and Sterculia gum. Distinctive PCR product sizes of 294 bp for pig DNA and 157 bp for EBN DNA, together with 750 bp for Sterculia gum were achieved, demonstrating a sensitivity of 1 ng/ μ L for the three of the samples, which underscores the method's specificity. Optimal conditions were established with a primer concentration of 20 μ M and an annealing temperature of 59.5°C for pig skin detection, while for Sterculia gum, a primer concentration of 10 μ M and an annealing temperature of 57°C were effective using ITS and COI primers, respectively. This method offers unprecedented precision in distinguishing between adulterants in purified EBNs, providing a significant advancement in ensuring the integrity and safety of these products in the field of oriental medicine and cuisine.

Keywords: edible bird's nests, adulterants, pig skin, Sterculia gum, multiplex PCR, detection.

INTRODUCTION

Edible bird's nests of the species *Aerodramus fuciphagus* hold a long-standing reputation in oriental medicine and cuisine for their perceived health benefits and culinary significance. Despite the esteemed status of EBNs, there has been an upsurge in counterfeit products due to their high market value, with pig skin and *Sterculia* gum being commonly used as adulterants. Detecting these sophisticated counterfeit products poses a significant challenge using traditional testing methods; thus, the development of reliable techniques for identifying adulterants in refined EBNs is crucial to uphold product quality and ensure consumer safety (Guo et al., 2014). Molecular biology techniques such as DNA sequencing, polymerase chain reaction, and DNA barcoding have proven instrumental in species identification (Cai et al., 2022). Of these techniques, multiplex PCR stands out as an innovative method that amplifies multiple targets in a single reaction while saving time and effort without compromising experimental results (Xu et al., 2008). This technique holds great promise for identifying pig skin and *Sterculia* gum adulterants within EBNs, forming the basis for the research project entitled "Multiplex PCR-Based Detection of Pig Skin and *Sterculia* Gum Adulterants in Refined Edible Bird's Nests".

The primary goal of this study is to establish a robust process for detecting pig skin and *Sterculia* gum within refined EBNs through activities such as optimizing primer concentrations in singleplex PCR reactions, investigating annealing temperature variations during multiplex PCR reactions, evaluating the sensitivity of multiplex PCR assay while maintaining specificity when testing various primer pairs. The successful

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development of such a process would make noteworthy contributions to product quality assessment procedures alongside market regulation efforts—ultimately safeguarding consumer interests while enhancing trustworthiness regarding high-quality EBN products.

This research at Can Tho University endeavors to address the critical need for dependable approaches toward identifying adulterants in refined EBNs—specifically targeting pig skin and *Sterculia* gum—and aims to offer meaningful advancements within the realm of food authenticity verification thereby ensuring overall consumer well-being.

Materials and Methods

Time and place

Study was conducted from September 2022 to May 2023 in Laboratory of Molecular Biology, Institute of Food and Biotechnology, Can Tho University.

Sampling location

Samples of *Sterculia* gum were purchased from Binh Thuan, Nha Trang city. Raw pig skin was bought from Xuan Khanh market, Xuan Khanh Ward, Ninh Kieu District, Can Tho city. DNA extract from EBNs was provided by the Laboratory of Molecular Biology, Institute of Food and Biotechnology, Can Tho University.

Equipments

PCR machine (MultiGene Optimax, USA); spectrophotometer (Nanodrop One, USA); gel imaging system (BIORAD Gel Doc XR, USA); tissue lyser (Tissue Lyser II Qiagen, Dutch); centrifugator (Eppendorf centrifuge 5430R); vacuum centrifugator (Eppendorf concentrator 5301); fume hood (Labconco, USA); electronic scale (Ohaus Corporation, USA); freezer -20°C (Sanaky, Vietnam); water bath (GFL, Germany); electrophoresis system (Embi Tec RunOne electrophoresis system, USA); microwave (National, Japan); vortex (Vortex Lab Companion, Korea); electronic scale (Ohaus Corporation, USA).

Chemicals

Chloroform (Merck, Germany); Isoamyl alcohol (Chemsol, Vietnam); Isopropanol (Chemsol, Vietnam); Proteinase K (ABT, Vietnam); Tris HCl, SDS, EDTA, NaCl (Chemsol, Vietnam); TE 0,1X; Distilled water (Laboratory of Molecular Biology).

Extracting DNA from pig skin using SDS method

DNA extraction from pig skin samples was performed using the Sodium Dodecyl Sulfate lysis method described by Alvarado et al. (2017), which facilitates the breakdown of cell membranes and ensures the release of genomic DNA. This method was chosen for its effectiveness in solubilizing proteins and disrupting lipid-lipid and lipid-protein interactions, making it particularly suitable for tough, fibrous tissues like pig skin. The procedure involved an incubation with SDS lysis buffer, followed by protease treatment to digest proteins and subsequent purification steps to yield high-quality genomic DNA suitable for PCR amplification. The process involved washing and finely chopping approximately 100 mg of pig skin, which was then placed into a 2 mL Eppendorf tube containing iron beads along with 250 µL of lysis buffer and ground in a sample grinder. The samples were then added to 750 µL of lysis buffer, vortexed for 2 minutes at 3,000 rpm until homogenized, and incubated at a temperature of 60°C for 30 minutes. After the incubation period, chloroform-isoamyl alcohol at a ratio of 24:1 was added to the samples, vortexed for 5 minutes at a speed of 3,000 rpm, and centrifuged at 14,000 rpm, 4°C for 10 minutes. The resulting supernatant was collected and supplemented with 10 µL of proteinase K. Subsequently, the samples were incubated at a temperature of 50°C for 30 minutes. After incubation, the samples were added to 600 µL of chloroform-isoamyl alcohol solution at a ratio of 24:1. The samples were then vortexed at a speed of 3,000 rpm for 5 minutes, ensuring thorough mixing. They were

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subsequently centrifuged at a speed of 14,000 rpm, 4°C for 10 minutes. The upper aqueous phase was transferred to a 1.5 mL tube, followed by the addition of 500 µL of isopropanol and gentle agitation. Next, the samples were centrifuged at a speed of 14,000 rpm, 4°C for 10 minutes. The DNA precipitate was recovered by pouring off the liquid phase and inverting the Eppendorf tube on absorbent paper. After removing excess alcohol, the DNA precipitate was dried using a vacuum centrifuge. Finally, the dried DNA precipitate was resuspended in 100 µL of 0.1X TE buffer solution. The samples were stored at a temperature of -20°C.

Extracting DNA from *Sterculia* gum using CTAB method

DNA from *Sterculia* gum were extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) process (Doyle, 1991) with some suitable adjustments. The process involved sterilization of the sample surface with 70% ethanol, followed by grinding the sample with liquid nitrogen. Then, 1 mL mixture of EB (Extraction Buffer) and 50 µL of 10% SDS was added to the ground sample and gently stirred. After that, the sample was incubated at 65°C for 30 minutes, with gentle stirring every 5 minutes. The supernatant was then transferred to a new tube, and an equivalent amount of isopropanol was added to precipitate the DNA. Next, the sample was vortexed for 5 minutes at a speed of 12,000 rpm, and centrifuged at 12,000 rpm for 10 minutes. The supernatant was removed and the sediment was kept. Then, 400 µL TE 1X was added into the tube. The sample was added with 400 µL of 2% CTAB and incubated at 65°C for 15 minutes, gently inverting every 5 minutes. Then, 700 µL of Chloroform/Isoamylalcohol (24:1) was also added and stirred several times, and centrifuged at 12000 rpm for 10 minutes. The top liquid was withdrawn (about 500 µL) to a new tube, and added 1000 µL of 95% ethanol, and the resulting DNA was incubated for 15 minutes at room temperature. Next, the sample was centrifuged at 12000 rpm for 10 minutes, discarded off the liquid, and the precipitate was washed with 700 µL of 70% ethanol (repeated 2 times), each time for 700 µL of 70% ethanol, and centrifuged at 12000 rpm for 10 minutes. After that, the liquid was removed and it was then dried with a vacuum dryer at 60°C for 10 minutes. Finally, the sample was dissolved with 100 µL TE 0.1X and the DNA was stored at -20°C for preservation.

DNA Concentration and Purity Assessment

The concentration and purity of the extracted DNA from pig skin and bird's nest were evaluated using a Nanodrop One spectrophotometer at wavelengths of 260nm and 280nm.

Performing PCR reactions for detection of pig skin, *Sterculia* gum and EBN

This study used primer sequences designed specifically for pig (Spychaj et al., 2016) and swiftlet (Chau Huu Hung., 2023) and forward primer ITS1 and reverse primer ITS4 (White et al., 1990) to detect *Sterculia*.

Table 1. Forward and reverse primer sequences for pig, *Sterculia* and swiftlet

Sequence (5' – 3')	Targeted gene	Species	Size	Source
F: GGAGCAGTGTTTCGCCATTAT R: TTCTCGTTTTGATGCGAATG	COI	Pig	294 bp	Spychaj et al. (2016)
F: CGAGCAGAACTTGGACAACC R: GCGCACCGATTATGAGGG	COI	Swiftlet	157 bp	Chau Huu Hung (2023)
F: TCCGTAGGTGAACCTGCGG R: TCCTCCGCTTATTGATATGC	ITS	<i>Sterculia</i>	750 bp	White et al. (1990)

Thermal cycles for PCR reactions of pig skin, *Sterculia* gum and EBN as presented in Table 2, Table 3 and Table 4. The components of PCR reaction is also presented in Table 5.

Table 2. Thermal cycles for PCR reaction with COI specific primer for detection of pig

Stages	Temperature (°C)	Time
Initial denaturation	95°C	9 mins.
Reaction (30 cycles)	Denaturation	1 min.
	Annealing	1 min.
	Elongation	3 mins.
Final extension	72°C	3 mins.

Table 3. Thermal cycling for PCR reaction with primer ITS1 & ITS4 for detection of *Sterculia* gum

Stages	Temperature (°C)	Time
Initial denaturation	95°C	5 mins.
Reaction (35 cycles)	Denaturation	1 min.
	Annealing	45 sec.
	Elongation	1 min 10 sec.
Final extension	72°C	5 mins.

Table 4. Thermal cycling for PCR reaction with COI specific primer for detection of swiftlet

Stages	Temperature (°C)	Time
Initial denaturation	95°C	3 mins.
Reaction (35 cycles)	Denaturation	15 sec.
	Annealing	30 sec.
	Elongation	1 min.
Final extension	72°C	7 mins.

Table 5. Components of PCR reaction

Chemicals	Volume (µL)	Concentration
BiH ₂ O	9.5	-
Master Mix 2X	12.5	X
Forward primer (F)	0.5	20 µM
Reverse primer (R)	0.5	20 µM
DNA sample	2.0	
Total volume	25	

Identifying annealing temperature in multiplex PCR of pig skin and EBN

Specific primers for each sample type were utilized and determined the appropriate primer concentration to be 10 µM. The DNA concentration of *Sterculia* leaves used was 104.4 ng/µL. The multiplex PCR reaction was carried out using a thermal cycling protocol that averaged the optimal cycles for the two specific primer pairs. Electrophoresis on a 2% agarose gel was performed at 50V for 45 minutes to visualize the PCR products. The results of this experiment were crucial for establishing the optimal conditions for detecting DNA from both pig skin, EBN and *Sterculia* gum in multiplex PCRs setup, ensuring accurate and specific identification of the target samples.

Table 6. Multiplex PCR reaction components for pig skin and EBN DNA

Chemicals	Volume (µL)	Concentration
BiH ₂ O	6.5	-

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Master Mix 2X	12.5	X
Forward primer (F) – swiftlet	0.5	20 µM
Reverse primer ® - swiftlet	0.5	20 µM
Forward primer (F) - pig	0.5	20 µM
Reverse primer (R) - pig	0.5	20 µM
DNA sample from EBN	2.0	
DNA sample from pig skin	2.0	
Total volume	25	-

Table 7. Thermal cycling of multiplex PCR reaction of *Sterculia* gum and EBN DNA

Stages	Temperature (°C)	Time
Initial denaturation	95°C	4 mins.
Reaction (30 cycles)	Denaturation	30 sec.
	Annealing	45 sec.
	Elongation	1 min.
Final extension	72°C	7 mins.

Table 8. Multiplex PCR reaction components for *Sterculia* gum and EBN DNA

Chemicals	Volume (µL)	Concentration
BiH ₂ O	6.5	-
Master Mix 2X	12.5	X
Forward primer (F) - swiftlet	0.5	20 µM
Reverse primer (R) - swiftlet	0.5	20 µM
Forward primer (F) - <i>Sterculia</i>	0.5	20 µM
Reverse primer (R) - <i>Sterculia</i>	0.5	20 µM
DNA sample from EBN	2.0	
DNA sample from <i>Sterculia</i>	2.0	
Total volume	25	-

RESULTS

DNA Quality and Concentration

The Nanodrop Spectrophotometer was utilized for quantifying and assessing the purity of DNA from pig skin, *Sterculia* gum, and EBN samples. A high DNA concentration was noted in pig skin samples, measured at 169 ng/µL with an A260/A280 purity ratio of 1.91, suggestive of good nucleic acid purity. Conversely, the purity ratios of EBN DNA, 1.39 for concentrations between 16.0 ng/µL, indicated possible protein contamination or extraction inefficiencies. Ideally, these ratios should fall between 1.8 to 2.0 to reflect pure DNA (Quek et al., 2021). However, although the A260/A280 value is outside the purity range of 1.8-2.00, the results are similar to the study of (Nguyen Le Tram Anh, 2022) on DNA samples extracted from edible bird's nest. Besides, the *Sterculia* gum exhibited a DNA concentration of 104.4 ng/µL with an A260/A280 of 1.87, which aligns with acceptable purity standards.

Table 9. Results for DNA concentration and purity measured by Nanodrop Spectrophotometer

Sample	ng/µL	A _{260/280}
Pig skin	169.0	1.91
EBN	16.0	1.39
<i>Sterculia</i> gum	104.4	1.87

PCR reactions of pig skin, *Sterculia* gum and EBN

The gel electrophoresis results showed a clear and bright band, indicating successful amplification. The size of the PCR product band was approximately 294 bp (Figure 1), consistent with the expected size. This successful amplification demonstrates the effectiveness of the COI specific primer in detecting pig DNA in the samples.

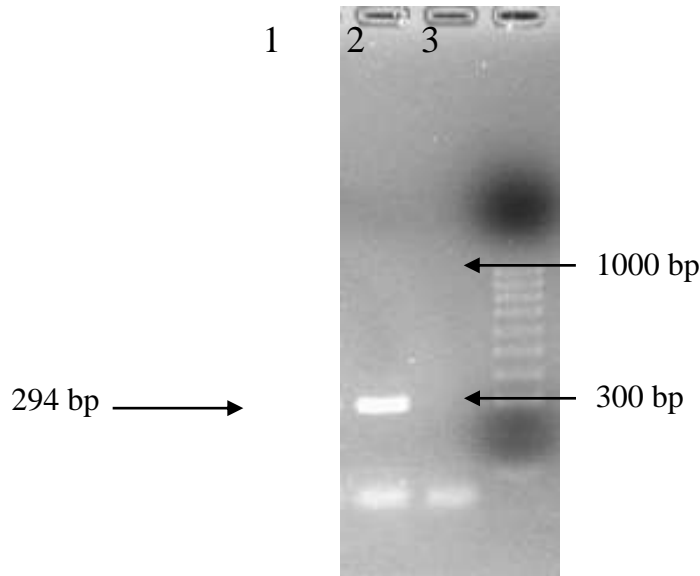
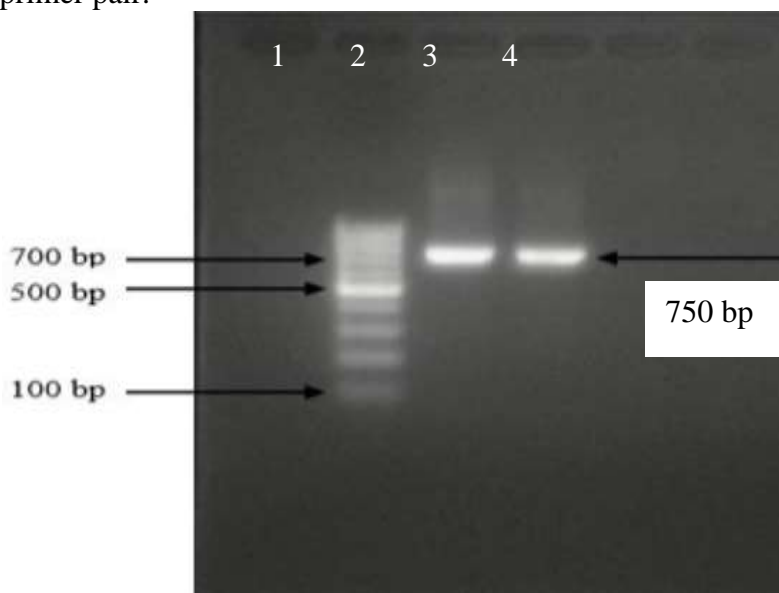


Figure 1. Gel electrophoresis results for PCR reaction of pig skin and COI specific primer
(Well 1: pig; Well 2: negative control; Well 3: Standard 100-bp DNA ladder
– Thermo Scientific)

The primer pairs ITS1 and ITS4 were used to amplify the ITS gene region of *Sterculia* gum DNA. Electrophoresis of the PCR products confirmed successful amplification, showing a clear band of approximately 750 bp in size for the *Sterculia* gum DNA sample. No amplification was observed for the EBN sample. The negative control PCR sample exhibited no secondary band, indicating the absence of contamination. The electrophoresis bands were specific, clear, and free of additional bands, demonstrating the specificity of the PCR reaction. In conclusion, the experiment successfully amplified the ITS gene region of *Sterculia* gum DNA using the ITS1 and ITS4 primer pair.



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Figure 2. Gel electrophoresis results for PCR reaction of *Sterculia* gum and primer ITS1 & ITS4

(Well 1: Standard 100-bp DNA ladder – Thermo Scientific; Well 2: positive control – Durian; Well 3: *Sterculia* gum, Well 4: negative control)

The PCR products amplified by COI primers were analyzed in the study to detect DNA from edible bird's nest samples. The electrophoresis results showed clear DNA bands for the positive control sample and the edible bird's nest samples. These bands indicated successful amplification of the COI gene region, with a size of approximately 157 bp. No extra bands appeared in the negative control sample, indicating the absence of external contamination. The results demonstrated the specificity of the COI primers for detecting DNA from edible bird's nests and their potential for accurate identification in multiplex PCR reactions.

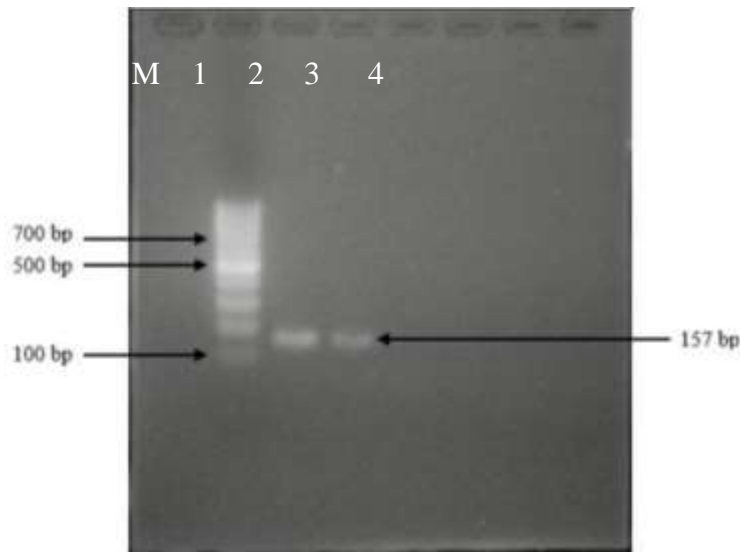


Figure 3. Gel electrophoresis results for PCR reaction of EBN and specific primer COI

(M: Standard 100-bp DNA ladder – Thermo Scientific; Well 1: positive control; Well 2: EBN sample; Well 3: *Sterculia* gum, Well 4: negative control)

Applying Multiplex PCR for Simultaneous Target Detection of Pig skin, EBN and *Sterculia* gum

Figure 4 showcases the efficacy of multiplex PCR in simultaneous DNA amplification from pig skin and EBN in a single reaction mixture. This is evidenced by two distinct bands at 294 bp for pig DNA and 157 bp for EBN DNA, confirming the successful amplification of each target sequence. The optimized annealing temperature of 59.5°C for 45 seconds yielded a highly specific and efficient multiplex PCR reaction, without the presence of non-specific bands, indicating that the reaction conditions are appropriate for detecting both pig and EBN DNA within the same sample.

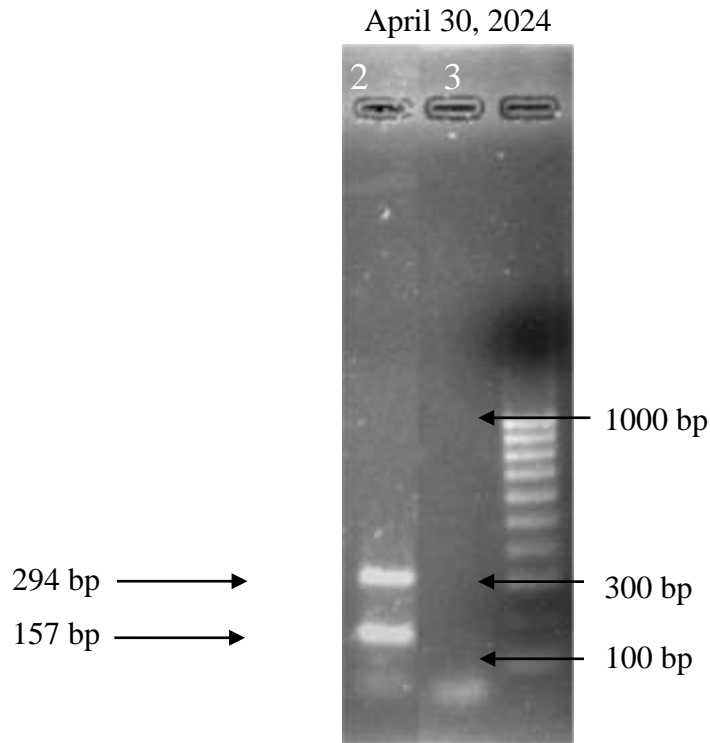


Figure 4. Gel electrophoresis results for multiplex PCR reaction of pig skin and EBN DNA mixture (Well 1: Pig skin & EBN; Well 2: negative control; M: Standard 100-bp DNA ladder – Thermo Scientific)

The optimization of the multiplex PCR reaction annealing temperature for samples containing *Sterculia* gum DNA and EBN DNA was critical. According to research by Qi *et al.* (2007), the optimal annealing temperature of the ITS gene used with the template DNA obtained from the *Brassica juncea* sample was 54°C. In addition, the primer binding temperature of COI gene at 60°C with template DNA from swiftlets resulted in the highest light intensity of the product band combined with the absence of by-products and primer-dimer (Chau Huu Hung, 2023).

The thermal cycle used in the multiplex PCR reaction is the average of 2 optimal thermal cycles for two pairs of specific primers to identify *Sterculia* leaves as ITS and edible bird's nest as COI, giving clear single-primer PCR electrophoresis results, so 57 °C is determined as the annealing temperature. This temperature consistently produced sharp bands that matched the expected sizes of 750 bp for *Sterculia* gum and 157 bp for EBN, respectively. The absence of extraneous bands confirmed the specificity and efficiency of the multiplex PCR under these conditions. These findings are pivotal for refining the PCR conditions to simultaneously detect target DNA from *Sterculia* gum and EBN, furthering the capabilities of molecular analysis in the authentication of natural products.

The experimental results to investigate the annealing temperature of the multiplex PCR reaction involved determining the appropriate annealing temperature for detecting DNA from *Sterculia* gum and edible bird's nest. The study found that using a primer concentration of 10 µM and a *Sterculia* gum DNA concentration of 104.4 ng/µL in the multiplex PCR reaction yielded successful amplification products. The DNA extracted from edible bird's nest was confirmed to be suitable as a template for PCR reactions. By optimizing the primer concentration and DNA concentration, the study aimed to prevent unequal amplification between loci and ensure accurate detection of the target DNA. The results indicated that the annealing temperature of 57°C was effective for detecting DNA from both *Sterculia* gum and edible bird's nest in the multiplex PCR reaction.

Sensitivity of the multiplex PCR

The sensitivity of the multiplex PCR method for detecting pig skin and EBN DNA was rigorously evaluated. Figure 5 illustrates that DNA concentrations as low as 1 ng/µL for both pig skin and EBN could still be

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detected, demonstrating the assay's high sensitivity. While the band intensity on the gel diminished with reduced DNA concentrations, the visibility of bands at these low concentrations confirmed the assay's capability to detect minute amounts of DNA. This sensitivity is significant for detecting potential adulteration in edible bird's nest products, underscoring the multiplex PCR's practical application in quality assurance.

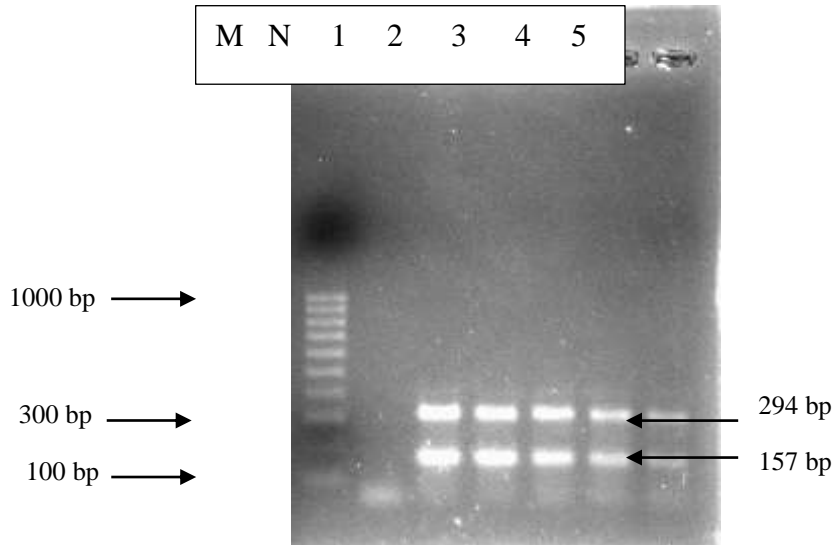


Figure 5. Gel electrophoresis results for testing sensitivity of multiplex PCR reaction of pig skin and EBN DNA

M: Standard 100-bp DNA ladder – Thermo Scientific; N: negative control; well 1: pig DNA 16 ng/μL & swiftlet DNA 16 ng/μL; well 2: pig DNA 12 ng/μL & swiftlet DNA 12 ng/μL; well 3: pig DNA 8 ng/μL & swiftlet DNA 8 ng/μL; well 4: pig DNA 4 ng/μL & swiftlet DNA 4 ng/μL; well 5: pig DNA 1 ng/μL & swiftlet DNA 1 ng/μL

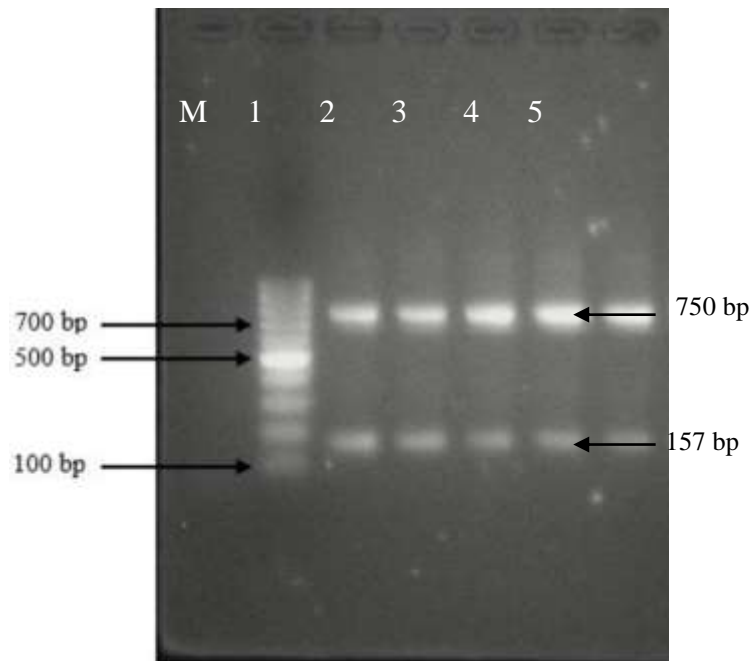


Figure 6. Gel electrophoresis results for testing sensitivity of multiplex PCR reaction of pig skin and EBN DNA

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M: Standard 100-bp DNA ladder – Thermo Scientific; well 1: *Sterculia* gum DNA 1 ng/μL & EBN DNA 1 ng/μL; well 2: *Sterculia* gum DNA 4 ng/μL & EBN DNA 4 ng/μL; well 3: *Sterculia* gum DNA 8 ng/μL & EBN DNA 8 ng/μL; well 4: *Sterculia* gum DNA 12 ng/μL & EBN DNA 12 ng/μL; well 5: *Sterculia* gum DNA 16 ng/μL & EBN DNA 16 ng/μL

Following the assessment of the pig skin and EBN, the multiplex PCR sensitivity was further tested on *Sterculia* gum DNA and EBN DNA from a separate experiment. Starting with initial DNA concentrations of 104.4 ng/μL for *Sterculia* gum and 103.8 ng/μL for EBN, serial dilutions were performed. The multiplex PCR was able to consistently detect 1 ng/μL for both *Sterculia* gum and EBN DNA. As anticipated, a decline in band intensity was observed with decreasing DNA concentrations, reinforcing the fact that optimal amplification is dependent on the delicate balance of DNA concentration in the reaction. These findings are essential for ensuring the accuracy of *Sterculia* gum and EBN DNA identification in various samples, which is particularly important given the potential for DNA loss during processing or through adulteration.

DISCUSSION

The optimized primer and DNA concentrations leading to effective amplification highlight the nuanced balance required in PCR assays, especially when dealing with a range of DNA sources. The specificity reached using the ITS1/ITS4 for *Sterculia* gum and COI primers for EBN is of particular interest. These findings underscore the potential of target-specific primers in addressing the complexities inherent in natural product matrices for accurate species identification.

The failure to amplify EBN DNA using ITS primers points to the specificity of the primer design, exemplifying the necessity of careful primer selection to avoid cross-reactivity in multiplex PCR scenarios. This specificity is further analyzed considering the differences in genomic sequences between the species tested, which may require additional primer sets for comprehensive authentication testing.

The fine-tuning of annealing temperatures to suit the thermal profiles of different DNA targets reflects the intricacies of PCR optimization. It shows that multiplex PCR can be made reliable and sensitive to the thermal characteristics of each DNA target, which is paramount for accurate amplification in a multiplexed format.

The sensitivity of the multiplex PCR assay, with the capability to detect DNA at concentrations as low as 1 ng/μL, suggests a significant advantage in detecting adulteration, even in highly processed products where DNA might be degraded or present only in trace amounts. This sensitivity has far-reaching implications for ensuring the quality and authenticity of food products, and such reliability is indispensable in regulatory frameworks.

However, the research presented is not without limitations. The concentration and quality of DNA extracted from commercial products can vary. Moreover, processing conditions may fragment DNA, affecting the efficiency of PCR. Future research could explore the use of this multiplex PCR method across a broader range of samples and consider the impact of different processing methods on DNA integrity.

Conclusion

In conclusion, the research demonstrated the efficacy of multiplex PCR in detecting and differentiating DNA from various natural product samples including pig skin, EBN, *Sterculia* gum, and edible bird's nest. Optimal annealing temperatures were obtained at 59.5°C and 57°C for multiplex PCR of pig skin and EBN; *Sterculia* gum and EBN, respectively. The assay showed high sensitivity with successful detection at low DNA concentrations (1 ng/μL) for all tested samples, affirming its potential for applications in quality control and forensic analysis in the food industry.

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