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Amplification of *rbcL* DNA Barcode from Different Organs of Black Turmeric and its Herbal Medicinal Products

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Abstract
Curcuma caesia, or black turmeric, is widely used as commercial herbal medicinal products due to its therapeutic benefits. However, the increasing market demand had raised concerns over the authenticity of black turmeric herbal medicinal products (HMPs) that are susceptible to adulteration. This study aimed to identify the correct species in commercially available HMPs through DNA barcoding and targeting the *rbcL* gene. To determine high-quality gDNA, two lysis buffers (CTAB-based PL1 and SDS-based PL2) with various incubation times were studied. Annealing temperature in PCR amplification was optimized. DNA barcodes, specifically rbcL, were generated and underwent bioinformatics analysis, and phylogenetic trees were constructed for species identification. From results, genomic DNA extracted using CTAB-based PL1 with overnight incubation yielded the best DNA quality. All samples successfully amplified at optimal annealing temperature, 52ºC. Following the analysis of bioinformatics and phylogenetic tree of *rbcL*, rhizome and leaf samples were identified as *Curcuma caesia*. Tea products had shown to contain *Curcuma caesia* but coffee powder matches other species, *Paederia lanuginosa*, indicating adulteration. These studies highlight the reliability of DNA barcoding in the authentication of HMPs.
Keywords: *Curcuma caesia*, DNA extraction, PCR amplification, DNA barcoding, *rbcL*, authentication

Introduction

In recent years, the demand for herbal medicinal products (HMPs) has increased globally due to their therapeutic effects. According to the World Health Organization (WHO), around 80% of people worldwide utilize HMPs as their primary source of healthcare (Saggar et al., 2022). *Curcuma caesia* or black turmeric is widely used as commercial HMPs and traditional herbals due to its valuable phytochemical properties, therapeutic effects and medicinal application. However, improper practices during manufacturing stages may contribute to fraudulent activities, such as substitution by inferior species, undeclared ingredients or contamination. Consequently, the marketed products were counterfeit and undeclared ingredients in packaging label would raise concerns about the safety and authenticity as well as efficacy of HMPs.

Molecular approaches such as the DNA barcoding method utilize specific DNA regions such as *rbcL*, *matK*, and *ITS2* for species identification. Therefore, it is used as an effective tool for determining the presence of *Curcuma caesia* in HMPs. Nevertheless, the DNA barcoding process requires high purity and high-quality genomic DNA for a successful downstream process, including PCR amplification, DNA sequencing and species identification through a similarity-based approach using online databases such as Genbank or the BOLD system. The genetic reference databases related to *Curcuma caesia are also* limited to the region and mostly available in China and India (Sahu et al., 2022). Additionally, ineffective DNA extraction methods can lead to the reduction of accuracy and efficiency of PCR amplification in the initial steps of DNA barcoding. DNA extraction is not a universal method and should be optimized based on species due to the different content of phenolic compounds in medicinal plants. The raw materials used, which are dried/shredded or powdered, also make it difficult to identify the correct plant species. To overcome these gaps, the objectives of this study focus on optimizing the lysis step in DNA extraction to enhance the quality of genomic DNA for successful PCR amplification, thus increasing the reliability of DNA barcoding for authenticating HMPs.

Materials and methods

In this study, six samples comprising rhizome, leaf of black turmeric and HMPs, including tea and coffee powder, were selected. Figure 1 shows the plant samples collected for DNA extraction. The DNA extraction was performed using the NucleoSpin® Plant II kit (Machery-Nagel, Düren, Germany) following the manufacturer's manual. Twenty mg of the samples was ground into powder using liquid nitrogen. The lysis step was treated using CTAB-based PL1 and SDS-based PL2, respectively with various incubation times (10 minutes, 1 hour, 2 hours and overnight (16 hours). The yield and purity of genomic DNA extracted were analyzed using the Nanodrop™ Spectrophotometer with a ratio at A260/280. Meanwhile, the size and quality of genomic DNA was examined through 1.0% (w/v) agarose gel electrophoresis with 1kb DNA ladder. The gel images were visualized using a UV transilluminator. High-quality genomic DNA were selected for PCR amplification of the *rbcL* barcode using sequence-specific primers (*rbcL* forward, 5'- ATGTCACCACAAACAGAGACTAAAGC-3' and *rbcL* reverse, 5'-TCGCATGTACCTGCAGTAGC-3') (Sahu et al., 2022). A total of 5µL of 5X Green GoTaq® Flexi Buffer (Promega, Madison, WI), 2µL of 25mM MgCl2, 0.5µL of 10mM PCR dNTPs mix, 1µL of each 10mM forward and 10mM reverse primers, 0.625U of GoTaq® DNA polymerase and sterile distilled water were included in each 25µL reaction mixture, and 20ng/µL of genomic DNA template was added. A negative control was carried out by adding sterile distilled water to the PCR reaction mixture. The *rbcL* amplification profile was set as follows: 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, 51°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 5 minutes. The PCR amplification process was repeated by testing annealing temperature at 52°C, 53°C, 54°C, 55°C and 56°C. All PCR products were then examined on 1.7% (w/v) agarose gel electrophoresis with a standard 100bp DNA ladder. Successfully amplified PCR products were sent to Apical Science Sdn. Bhd (Selangor, Malaysia) to undergo DNA purification, followed by Sanger Sequencing of PCR products in both forward and reverse directions. DNA sequence results were used as query barcodes for sequence analysis and species identification by comparing query sequences with reference species in the GenBank (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Barcode of Life Database (BOLD, http://www.boldsystems.org/). A multiple sequence alignment (MSA) and phylogenetic tree was later analyzed and constructed, respectively. Lastly, the authentication of samples was performed from this analysis.

Figure 1 Sample collected for DNA extraction (S1: rhizome A, S2: leaf A, S3: black turmeric tea, S4: coffee powder, S5: rhizome B and S6: leaf B).

Results and discussion

In this study, genomic DNA (gDNA) was successfully extracted from all fresh plant samples and HMPs. The qualitative analysis of extracted gDNA from the CTAB-based lysis buffer (PL1) produced clearer and more intense bands compared to the SDS-based lysis buffer (PL2), which showed smeared and faint bands. CTAB-based buffers were more effective in breaking down the cell wall of plant cells by forming insoluble complexes with polysaccharides and other contaminants, thereby facilitating the separation of DNA from cellular components. CTAB also prevented the co-precipitation of polysaccharide and secondary metabolite, which helped to maintain the integrity of DNA (Heikrujam et al., 2020). In addition, genomic DNA extracted under prolonged lysis incubation periods, especially the overnight (16 hours) incubation, allowed more sufficient cell lysis and enhanced the release of nuclear DNA into the lysis buffer. This had significantly enhanced the visibility of DNA bands. Figure 2 illustrates the genomic DNA from the rhizome and leaf of black turmeric, as well as the tea product and coffee powder samples extracted using Buffer PL1, visualized on 1.0% (w/v) agarose gel after overnight (16 hours) incubation. On the other hand, quantitative analysis using the Nanodrop™ spectrophotometer supported these observations. Genomic DNA extracted using buffer PL1 with overnight incubation

yielded the highest DNA concentrations and A260/280 ratios within 1.8 to 2.0, indicating high-purity DNA. Table 1 summarizes the DNA yield and purity of genomic DNA extracted using Buffer PL1 under overnight (16 hours) incubation. Overall, CTAB-based Buffer PL1 with overnight incubation was the most effective condition for obtaining high-quality and high-yield gDNA.

Table 1: DNA yield and purity of genomic DNA extracted using Buffer PL1 under overnight (16 hours) incubation.

Sample	Sample Concentration	$A_{260/280}$	$A_{260/230}$
S1	28.3 ± 10.0	1.81 ± 0.06	0.61 ± 0.02
S2	39.8 ± 13.3	1.91 ± 0.17	0.67 ± 0.003
S3	14.4 ± 5.2	1.98 ± 0.03	0.51 ± 0.06
S4	10.5 ± 0.5	1.84 ± 0.12	0.55 ± 0.02
S5	27.3 ± 12.0	1.85 ± 0.10	0.60 ± 0.01
S6	17.5 ± 4.3	1.93 ± 0.08	0.71 ± 0.16

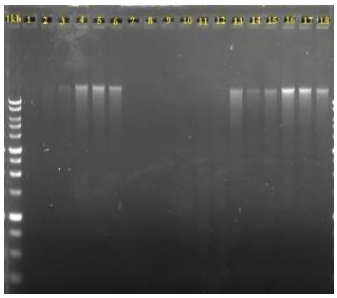

Figure 2 Genomic DNA from rhizome and leaf of black turmeric plant, as well as tea bag and coffee powder extracted using Buffer PL1, visualized on 1.0% (w/v) agarose gel after overnight (16 hours) lysis incubation time (lane 1-3: rhizome A, lane 4-6: leaf A, lane 7-9: tea, lane 10-12: coffee powder, lane 13-15: rhizome B, lane 16-18: leaf B).

All samples were successfully amplified using *the rbcL barcode across the tested annealing temperatures,* and 52°C was determined as the optimal temperature because it achieved the most intense and clearly visible bands on 1.7% (w/v) agarose gel. The sample S3 only showed a visible band when 52°C was tested, as shown in Figure 3. Theoretically, the optimal annealing temperature for PCR amplification was set approximately 5°C lower than the melting temperature (Silalahi et al., 2021). The melting temperature (Tm) of the *rbcL* forward and *rbcL* reverse primers was 57.2°C and 57.3°C, respectively. The annealing temperature of 52°C was approximately 5°C lower than the Tm of both forward and reverse *rbcL* primers, supporting the theoretical basis of annealing temperature optimization in PCR amplification.

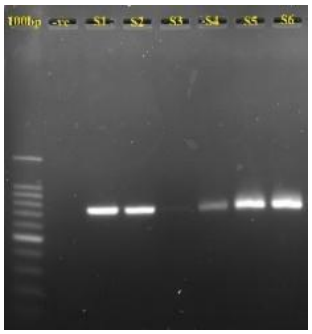

Figure 3 PCR amplification for *rbcL* barcode at 52°C annealing temperature for black turmeric organs and its HMP products run on 1.7% (w/v) agarose gel (-ve: negative control, S1: rhizome A, S2: leaf A, S3: tea, S4: coffee powder, S5: rhizome B, S6: leaf B).

The *rbcL* gene was typically used to assess phylogenetic relationships at the interfamilial level (Nurhasanah et al., 2019). Results from BLASTn analysis showed rhizomes and leaves of the black turmeric plant were matched with Curcuma *caesia*. For tea products, similarity was 100% to *Curcuma caesia* (accession number: KX758481.1), but coffee powder matched with Paederia lanuginosa from the Rubiaceae family. Figure 4 demonstrates the neighbour-joining phylogenetic trees for rhizome and leaves of black turmeric, as well as tea product and coffee powder, respectively. In a phylogenetic tree, smaller branch length values indicate a closer evolutionary relationship between clades (Paradis, 2016). Similarly, the tea products are also grouped together with *Curcuma caesia* clade, validating its authenticity, which is the same as the packaging label. In contrast, the coffee powder was separated from the Curcuma group and clustered with *Paederia lanuginose*. This indicated the occurrence of adulteration for commercially available coffee powder. Lastly, each nucleotide was assigned a specific colour in a colour-coded barcode system to visualize the nucleotide composition of the sequences generated from each DNA barcode locus. This colour-coded fingerprint accurately represented the distinct nucleotide composition encoded by each sequence and was provided in the form of barcode primers (Sahu et al., 2024). The barcode can be generated through the online tool (http://biorad-ads.com/DNABarcodeWeb/).

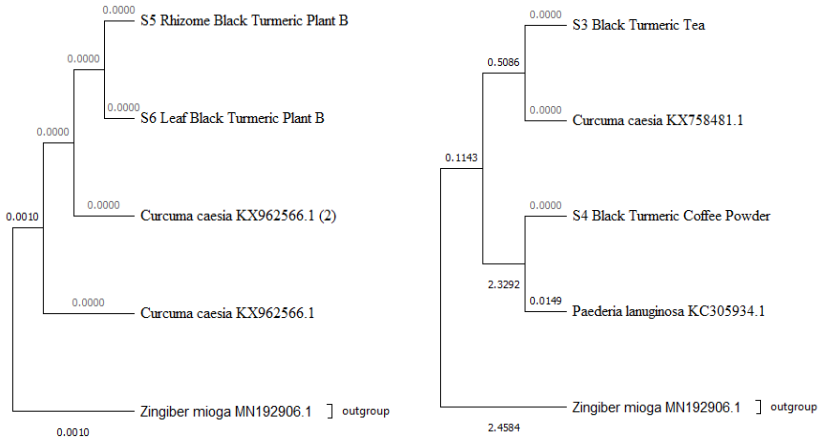

Figure 4 The neighbor-joining phylogenetic trees for rhizome and leaves of black turmeric, as well as tea product and coffee powder, respectively.

Conclusion

The findings in this study proved that rhizome and leaf samples, as well as tea samples, were identified as *Curcuma caesia*, but coffee powder matched with *Paederia lanuginosa*, indicating adulteration. The optimized DNA extraction also contributed to successful DNA barcoding in authenticating *Curcuma caesia* in HMPs. Therefore, it was suggested that a standardized DNA barcoding protocol as a routine quality control check was essential before the sale of HMPs in the market in order to ensure the authenticity, reliability and safety of HMPs.

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