การจำแนกบัวบก พรมมิ และแว่นแก้วด้วยลายพิมพ์ดีเอ็นเอที่สร้างจากวิธีพีซีอาร์อาร์เอฟแอลพี และการประยุกต์กับผลิตภัณฑ์สมุนไพร

Differentiation of *Centella asiatica*, *Bacopa monnieri*, and *Hydrocotyle umbellata* and Their Commercial Products using DNA Fingerprint Generated by PCR-RFLP

นิพนธ์ต้นฉบับ

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Original Article

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บทคัดย่อ

วัตถุประสงค์: จำแนกบัวบก พรมมิ และแว่นแก้วด้วยลายพิมพ์ดีเอ็นเอที่สร้างด้วยเทคนิค PCR-RFLP **วิธีการศึกษา:** สืบค้นข้อมูลลำดับนิวคลีโอไทด์ของยีน maturase K (*mat*K) ของบัวบก พรมมิ และแว่นแก้วจากฐานข้อมูลยีน เพื่อนำมาใช้ออกแบบไพรเมอร์และทำนายตำแหน่งเอนไซม์ตัดจำเพาะ แล้วสร้างลายพิมพ์ดีเอ็นเอต้นแบบของพืชบัวบก พรมมิ และแว่นแก้วโดยสังเคราะห์ดีเอ็นเอบริเวณยีน *mat*K ด้วยปฏิกิริยาลูกโซ่พอลีเมอเรส จากนั้นสร้างรูปแบบลายพิมพ์ดีเอ็นเอของพืชแต่ละชนิดด้วยเอนไซม์ตัดจำเพาะ และตรวจสอบรูปแบบลายพิมพ์ดีเอ็นเอด้วยอะกาโรสเจลอิเล็กโทรโฟรีซิส นำวิธีดังกล่าวมาตรวจสอบผลิตภัณฑ์สมุนไพร **ผลการศึกษา:** ลายพิมพ์ดีเอ็นเอที่มีรูปแบบจำเพาะกับบัวบกสร้างจากการตัดดีเอ็นเอด้วยเอนไซม์ตัดจำเพาะ *Bam*H1 สำหรับพรมมิใช้เอนไซม์ *Sph*1 และสำหรับแว่นแก้วใช้ *Bsp*D1 ผลการตรวจสอบผลิตภัณฑ์สมุนไพรจำนวน 8 ตัวอย่าง ด้วยวิธีที่กำหนดขึ้นพบว่ามีผลิตภัณฑ์สมุนไพร 2 ตัวอย่างที่ชนิดสมุนไพรไม่ตรงตามที่ระบุในฉลาก ได้แก่ ชาสมุนไพรบัวบก 1 ตัวอย่างที่ผลการทดสอบพบว่าเป็นแว่นแก้ว และชาชงพรมมิ 1 ตัวอย่างที่ผลการทดสอบพบว่าเป็นบัวบก **สรุป:** ลายพิมพ์ดีเอ็นเอที่สร้างด้วยเทคนิค PCR-RFLP สามารถจำแนกสมุนไพรทั้งสามชนิดได้ และสามารถนำมาใช้ตรวจสอบผลิตภัณฑ์สมุนไพร

คำสำคัญ บัวบก; พรมมิ; แว่นแก้ว; ลายพิมพ์ดีเอ็นเอ; พีซีอาร์อาร์เอฟแอลพี

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Abstract

Objective: Differentiation of *Centella asiatica* (L.) Urban (CA), *Bacopa monnieri* (L.) Pennell (BM), and *Hydrocotyle umbellata* L. (HU) using DNA fingerprints generated by PCR-RFLP technique. **Methods:** The Genbank database was used to acquire nucleotide sequence information from the maturase K (*mat*K) genes of CA, BM, and HU to design primers and predict specific enzyme cutting sites. The patterns of DNA fingerprints of CA, BM, and HU were then created using polymerase chain reaction amplification of the *mat*K gene. The fingerprint pattern was then constructed using restriction enzymes and was analysed using agarose gel electrophoresis. **Results:** Cutting DNA using the enzyme *Bam*H1 generated a distinctive fingerprint pattern for CA, while cutting DNA with *Sph*1 yielded a unique pattern for BM and *Bsp*D1 yielded a specific pattern for HU. The examination of eight samples of herbal products using the established procedure revealed that two samples did not match to labels, including one sample of CA herbal tea that was discovered to be HU and one sample of BM brewed tea that was found to be CA. **Conclusion:** DNA fingerprints created by the PCR-RFLP technique were able to identify the three herbal species and can be used to inspect herbal products.

Keywords: *Centella asiatica*; *Bacopa monnieri*; *Hydrocotyle umbellata*; DNA fingerprints; PCR-RFLP

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Introduction

Centella asiatica (L.) Urban (CA), also known as Asiatic pennywort or Indian pennywort, is called “Buabok” in Thai. It is a medicinal plant that belongs to the Family Apiaceae. *Hydrocotyle umbellata* L. (HU), on the other hand, is known as “Wankaew” or “Buabok,”[1] and is often confused with CA. Both plants have similar names in the Thai language. Besides their names, the comparable morphology of CA and HU might lead to confusion. Both plants have green stems and leaves and are herbaceous creepers. They are almost the same size. However, CA and HU can be recognized by a closer examination of their leaves. CA features bean-shaped leaves with crenate margins, whereas HU has long cylindrical petioles connected to the center of peltate-shaped leaves.[2] In addition to CA and HU, *Bacopa monnieri* (BM) or Brahmi is a plant that has been confused with CA since they share the common name Brahmi in certain regions of India[3,4], and both CA and BM are known as neuroprotective agents that can improve memory.[5] While CA and BM exhibit the same activity, they differ in active constituents. Triterpenoids, which include asiaticoside, madecassoside, asiatic acid, and madasiatic acid, are reported to respond to CA activity[6], whereas triterpenoid bacosides are claimed to respond to BM.[5] CA has also been demonstrated to have wound-healing action by improving cellular proliferation and collagen production at the wound site.[3] This

action, however, was not reported for BM. HU is not regarded as a medicinal plant in Thailand. It is ornamental and was found to contain flavonoids such as quercetin, avicularin, quercitrin, hyperoside, and neochlorogenic acid[7]. It has been reported to have anti-inflammatory activity.[7] Although the protective effects on memory in sleep-impaired female mice have been reported, the study was performed in *Hydrocotyle umbellata* var. *bonariensis*.[8]

It is critical to use the correct type of herb when employing herbs as raw materials because it is the main factor in getting products that are effective in performing the desired effects. However, herbal raw materials may undergo processing, such as shredding or grinding into powder. This makes it difficult to identify the species. Identification of processed herbs can be done by examining herb characteristics. This includes examining the tissue under a microscope, analyzing the chemical composition, and performing DNA tests. The use of DNA as a marker to identify plant species is based on the principle that the sequence of nucleotides on the DNA strand of each plant species is different.[9] Various methods have been employed to create DNA markers, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), which has been well-accepted to generate a DNA marker for plant identification at both species and subspecies levels. This method relies on the investigation of species-specific band patterns through agarose gel electrophoresis.[10]

This work attempted to develop a DNA marker for distinguishing BM, CA, and HU using the PCR-RFLP approach. The DNA fragments of the maturase K (*mat*K) gene were amplified from each sample with a set of specific primers and then digested with a restriction enzyme to generate DNA fragmentation patterns specific to each plant species. Furthermore, the method was used to study the botanical origin of commercial products sold on the Internet by Thai merchants.

Methods

Collection of the samples

Whole plant of Bacopa monnieri (L.) Pennell (Scrophulariaceae) or BM, *Centella asiatica* (L.) Urban (Apiaceae) or CA, and *Hydrocotyle umbellata* L. (Apiaceae) or HU were collected from the botanical garden of the Faculty of Pharmacy, Srinkharinwirot University, Nakonnayok, Thailand. The herbarium specimen was deposited at the faculty herbarium (Table 1). Commercial products have been purchased from Thai vendors on the internet during April 2023. They are dry *C. asiatica* powder in capsules (samples 1-2), *C. asiatica* herbal tea (samples 3-5), *C. asiatica* herbal powder (sample 6), *B. monnieri* herbal tea (sample 7) and *B. monnieri* dried herb (sample 8).

Table 1 Plants that used in this study.

Sample	Specimen number	Part used for DNA extraction
Bacopa monnieri (L.) Pennell	WSBM01	Fresh young leaves
Centella asiatica (L.) Urban	WSCA01	Fresh young leaves
Hydrocotyle umbellata L.	WSHU01	Fresh young leaves

In Silico Analysis

The nucleotide sequences of the maturase K (*mat*K) gene from CA, BM, and HU were obtained from the Genbank database. These sequences were then aligned using ClustalW (https://www.genome.jp/tools-bin/clustalw) for sequence similarity. Primers were designed manually based on nucleotide sequence similarity. In preparation for RFLP analysis, the aligned sequences were further checked for restriction enzyme digestion sites using Webcutter 2.0 (https://heimanlab.com/cut2.html).

DNA Extraction

Plant tissues were disrupted with beating-based homogenization (FastPrep-24 Instrument, MP Biomedicals), samples were put in a 2 mL RNase DNase-free tube containing 1.4 mm zirconium-silicate spheres (MP biomedicals) and 300 µL of RLT buffer + β-mercaptoethanol. Homogenization was performed on FastPrep-24 at 6 m/s for 20 and 30 s for fresh and dry tissue, respectively. DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Germany), according to the manufacturer's instructions. DNA concentration and quality were assessed using a microplate reader (Molecular Devices Spectramax M2) and run on 1% agarose gel. DNA of high quality (260/280 ratio 1.8–2.0 and 260/230 ratio 2.0–2.2) was used in downstream applications.

PCR-RFLP

A fragment of 500 bp of *mat*K gene was amplified using primers CENMATKS (5'-TATGTGAATACGAATCCATCTTCGTC-3') and CENMATKR (5'-TTACAAAATTTCGCTTTAGCCAATGA-3'). PCR reactions were performed in T-Gradient Thermoblock (Biometra), using the AccuStart II PCR SuperMix (QuantaBio, USA). Total DNA was used as a template and PCR reactions were performed as follows: initial denaturation at 94 °C for 3 min, followed by 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min for 30 cycles, and final extension at 72°C for 5 min. After purification with a QIA quick PCR Purification Kit, the PCR products were digested with *Bam*HI, *Sph*1, and *Bsp*D1, (Biolab, New England, USA). Restriction fragments were separated by electrophoresis in 2% agarose gel and stained with SYBR Safe DNA gel stain (Invitrogen, USA).

Results

The Genbank database contained twenty, ten, and four accessions of the *mat*K gene sequences from CA, BM, and HU, respectively. Alignment with ClustalW revealed that the sequences were identical within the same species. The similarity between CA and BM was **80.48%,** while the similarity between BM and HU was **79.28%.** Finally, the similarity between CA and HU was **88.65%**. CENMATKS and CENMATKR primers were constructed to amplify 500-bp fragments for PCR-RFLP analysis. According to Webcutter 2.0, the restriction sites for *Bam*H1 were determined to be restricted to CA, *Sph*1 to BM, and *Bsp*D1 to HU within the 500-bp fragments amplified.

The *mat*K gene fragments from CA, BM, and HU were amplified successfully using CENMATKS and CENMATKR primers generated in this study. A sequence comparison using the Blast search engine revealed that the nucleotide was similar to matching sequences in the GenBank database. The nucleotide sequences for CA, BM, and HU were submitted to the GenBank database as accession numbers LC778269, LC778271, and LC778270, respectively (Figure 1).

When the DNA amplicons were digested with *Bam*H1, only DNA fragment from CA was digested, generating 350 and 150 bp fragments. However, this enzyme did not affect BM or HU. When DNA fragment from BM was digested with *Sph*1, DNA fragments of 200 and 150 bp were produced. This enzyme, on the other hand, did not affect the other two plants. Similarly, *Bsp*D1 digestion produced fragments of 400 and 150 bp from only HU (Figure 2). PCR-RFLP analysis of commercial products acquired from Thai merchants on the internet found that a sample of CA herbal tea originated from HU and a sample of BM herbal tea originated from CA (Figure 3, Table 2).

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BMMATK      TATGTGAATACGAATCCATTTTCGTCTTTCTACGTGCACAATCTTATCATTTACGATCAA        60
CAMATK      TATGTGAATACGAATCCATCTTCGTCTTTTTCCGCAACCAATCTTCTCATTTACGCTCAA        60
HUMATK      TATGTGAATACGAATCCATCTTCGTCTTTCTCCGTAACCAATCTTCTCGTTTACGCTCAA        60
            ******************** ********* * **    ******* ** ****** ****

BMMATK      TATCTTCTCGACTTCTTCTTGAACGAATCTATTTCTATGCCAAAATAGAGCGTCTTGTTA        120
CAMATK      CATCTTCTAGAACCCTTCTTGAACGAGTATATTTCTATGGAAAAATAGAACATCTTGGAG        120
HUMATK      CATCTTCTGGAACCCTTCTTGAACGAATATATTTCTATGGAAAAATAAACTATCTTGTAG        120
             ******* **   ************ * **********  ****** *   *****

BMMATK      ACCTTTTTATTAAGGTTAAGCATTTTCAGGTGAATCTATGGTTGGTCAAGGAGCCTTGCA        180
CAMATK      AAGTCTTTGCT------AAGGCTTTTCAAGTCAATCTATGGTTATTGAAGGATCCTTTCA        174
HUMATK      AAGTCTTTGTT------AAGGTTTTTCAGGTCAATCTATTCTTGTTGAAGGACCCTTTCA        174
            *  * ***  *      ***  ****** ** *******  **  * ***** **** **

BMMATK      TGCTTTATGTTAGGTATCAAAAAAAATTGATTCTGGCTTCAAAAGGGACGTCTTTTTTGC        240
CAMATK      TGCATTACGTTAGGTATCAAGGAAAATCAATTCTCACATCAAAAGGGACGCCTCTTTTGA        234
HUMATK      TGCATTATGTTAGGTATCAAGGAAAATCGATTCTCGCTTCAAAAGAGACGCCCCTTTTGA        234
            *** *** ************  *****  *****  * ******* **** *  *****

BMMATK      TAAAGAAATGGAAGTCTTACCTTGTCGCTTTTTGGCAATCGCACTTTTTAATGTGGTTTC        300
CAMATK      TGAAAAAATGGGTATATTACTTTGTTAATTTATGGCAATGTCATTTTTACCTGTGGTCTC        294
HUMATK      TGAAAAAATGGACATATTATTTTGTTAATTTCTGGCAATGTCATTTTTATCTGTGGTCTC        294
            * ** ******   * ***  ****   *** *******  ** ****   ****** **

BMMATK      ATCCAAAAAGGATTTCTATAAACCAATTATCCAACTATTCTCTTGAATTTTTGGGCTATC        360
CAMATK      AACCGAGAAGGATCTGTATAAACCAATTATCTAATTATTCGCTCGACTTTCTGGGCTATC        354
HUMATK      AACCGGGACGTATCTATATAAACCAATTCTACAACCATTCCCTTGATCTTTTGGGCTATT        354
            * **   * * ** * ************ *  **  **** ** **  ** ********

BMMATK      TTTCAAGTGTGCGAATGAACCCTTCAGTAGTACGGAGTCAAATTCTAGAAAATTCATTTC        420
CAMATK      TATCAAGTGCACGGCTAAACCCTTCAATGGTACGCGGTCAAATGCTAGAAAATTCATTTC        414
HUMATK      TATCAAGTGCGCGGTTAAACCCTTCAATGATACGTGGTCAAATGCTAGAAAATGTATTTC        414
            * *******  **  * ********* *  ****  ******* *********  *****

BMMATK      TAATTAATAATCCTATTAAGAAATTCGATACCTTTGTTCCAATTATTCCTCTGATTGCGT        480
CAMATK      TAATTGATAATACTATTAATAAGTTCGATACTCTTATTCCAATTATTCCTCTGATTGCAT        474
HUMATK      TAATTGATAATGCTATTAAAAAGGTTGAGACTATTGTTCCAATTAGTCCTCTGATTGGAT        474
            ***** ***** ******* **  * ** **  ** ********* ***********  *

BMMATK      CATCATTAGCTAAAGCGAAATTTTGTAA508
CAMATK      CATCATTGGCTAAAGCGAAATTTTGTAA502
HUMATK      CATCATTGGCTAAAGCGAAATTTTGTAA502
            ******* ********************
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Figure 1 Alignment of *mat*K nucleotide sequence of BM (BMMATK), CA (CAMATK), and HU (HUMATK). Bolded showed forward and reverse primers. Underlined and bolded showed digestion sites for *Bam*H1 (G/GATCC), *Sph*1 (GCATG/C), and *Bsp*D1 (AT/CGAT). The * showed locations that have the same bases in all three plant species.

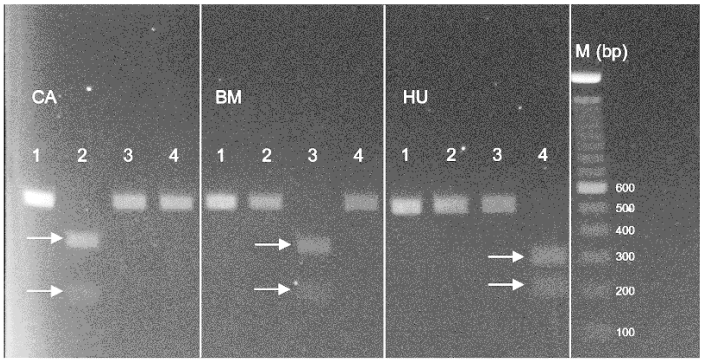

Figure 2 The PCR-RFLP patterns of the *mat*K gene fragment of CA, BM, and HU. Intact PCR products (1); PCR products after digestion with *Bam*H1(2), *Sph*1(3), and *Bsp*D1 (4). Molecular (M) sizes are in bp.

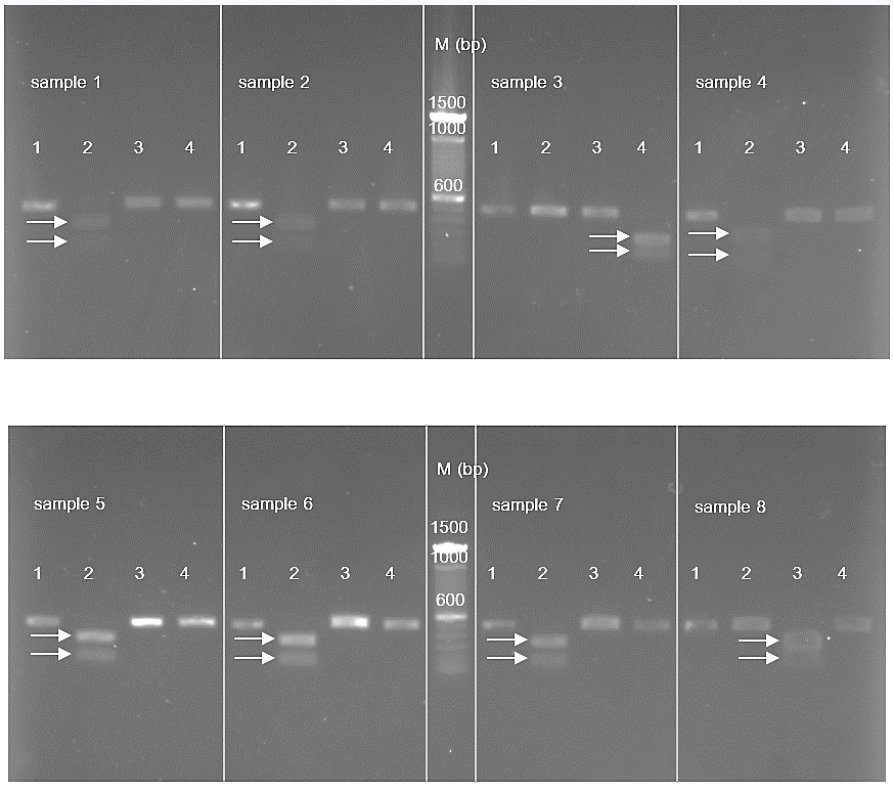

Figure 3 The PCR-RFLP patterns of the *mat*K gene fragment of the selected commercial products; CA powder in capsules (samples 1-2), CA herbal tea (samples 3-5*)*, CA herbal powder (sample 6), BM herbal tea (sample 7) and BM dried herb (sample 8). Intact PCR products (1); PCR products after digestion with *Bam*H1(2), *Sph*1(3), and *Bsp*D1 (4). Molecular (M) sizes are in bp.

Table 2 summary of PCR-RFLP analysis of commercial products acquired from Thai merchants.

Commercial product	formulation	Labeled species	PCR-RFLP result
sample 1	powder in capsule	CA	CA
sample 2	powder in capsule	CA	CA
sample 3	herbal tea	CA	HU
sample 4	herbal tea	CA	CA
sample 5	herbal tea	CA	CA
sample 6	herbal powder	CA	CA
sample 7	herbal tea	BM	CA
sample 8	dried herb	BM	BM

Discussions and Conclusion

DNA markers have become a common technique for identifying and authenticating medicinal plant materials, as DNA does not change with age, physiological conditions, or environmental conditions. The chloroplast *mat*K gene is involved in group II intron splicing and is situated within the intron of trnK.[11] The *mat*K gene has a high level of species discrimination among angiosperms and is often employed to identify medicinal plants.[12] Therefore, the *mat*K gene was selected as a DNA marker. In this study, DNA primers were designed based on the

sequence homology among CA, BM, and HU using the chloroplast *mat*K gene sequences available from Genbank. The aligned sequences were further checked for restriction enzyme digestion sites using Webcutter 2.0. The restriction enzyme digesting site unique to each plant was discovered. Then, PCR-RFLP was performed to generate 500-bp fragments of *mat*K DNA by PCR with the obtained primers. The second step was the RFLP, by which the fragmentation with the restriction enzyme could result in a DNA pattern capable of differentiating CA, BM, and HU. Because CA, BM, and HU might be misidentified by name or plant feature, an analysis of products sold on the internet in Thailand revealed that out of 8 products, there were two products derived from herbs that did not correspond to the label. Therefore, quality control of herbal raw materials is necessary. The PCR-RFLP approach proposed in this study was successful in distinguishing CA, BM, and HU. However, other DNA regions, such as the *rbc*L gene, the interval between both *trn*H (H-GUG) sequence ends and both sides of the *psb*A gene (*psb*A-*trn*H), and the *trn*L–*trn*F region should be utilized to confirm the results. A previous study using a DNA marker based on the internal transcribed spacer (ITS) region[13] demonstrated the presence of CA in BA products. As a result, the ITS sequence can also be used to confirm the substitution of CA and BA products. While DNA is a reliable method for identifying plant species, it may be unable to detect adulteration in herbs due to the small sample size used by the DNA technique. If the sample does not represent the full population, the error may go undetected.

In conclusion, DNA fingerprints created by PCR-RFLP technique using the *mat*K gene were able to differentiate between CA, BM, and HU, and can be used to inspect herbal products. The method has the advantage of preventing misidentification and misuse of plant materials and can be applied in industrial quality control for the production of herbal products.

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