Authentication of *Coscinium fenestratum* among the Other Menispermaceae Plants Prescribed in Thai Folk Medicines

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In Ayurveda and Thai traditional medicines, material from *Coscinium fenestratum* is commonly prescribed as active ingredients with diverse therapeutic purposes. However, *C. fenestratum* is also a seriously endangered medicinal liana. Thus, its crude material is very rare and is being substituted with substances from *Arcangelisia flava* or *Fibraurea tinctoria* (Menispermaceae), which have high morphological similarity. In this current study, nuclear 18S ribosomal RNA (rRNA) gene and nuclear ribosomal DNA internal transcribed spacer (ITS) gene sequences with the polymerase chain reaction–restriction fragment length polymorphisms (PCR–RFLPs) technique were exploited to identify these three species. The nuclear 18S rRNA gene sequences of *C. fenestratum*, *A. flava*, and *F. tinctoria* consisted of 1809, 1805, and 1809 base pairs (bps), respectively, while their ITS gene regions were 694, 622, and 631 bps in length, respectively. The 18S rRNA gene of *C. fenestratum* digested with *Sma*I restriction enzyme displayed the electrophoresis profile of 729 and 790 bps; for *A. flava* and *F. tinctoria*, the digested products showed fragments of 1519 bps. Although the ITS gene regions of *A. flava* and *F. tinctoria* had unrecognized sequences with *Sal*I, the *Sal*I-digested ITS of *C. fenestratum* exhibited fragments of approximately 599 bp. Thus, the 18S rRNA gene and ITS gene sequences with PCR–RFLPs were proven to be powerful molecular markers for identifying *C. fenestratum* and distinguishing it from the other two Menispermaceae plants.

Key words *Coscinium fenestratum*; *Arcangelisia flava*; *Fibraurea tinctoria*; 18S ribosomal RNA gene; internal transcribed spacer gene; polymerase chain reaction–restriction fragment length polymorphism

For centuries, *Coscinium fenestratum*, *Arcangelisia flava* and *Fibraurea tinctoria* belonging to the family Menispermaceae have been used as medicinal plants in most countries in Asia. For example, an alcoholic extract of the stems of *C. fenestratum* is used in Ayurveda and Sridhar medicines for treating diabetes, and is used in Sri Lanka as an antidote for tetanus.^{1,2)} The major components of this plant are berberine and its relative compounds. Berberine has strong antibacterial activity.³⁾ Recently, berberine was also found to have anti-proliferative activities against human cervix HeLa adenocarcinoma, human lung A549 adenocarcinoma, murine colon 26-L5 carcinoma, murine Lewis lung carcinoma (LLC), and murine B16-BL6 melanoma cells.⁴⁾ *A. flava* was used as an important component of folk medicines for the treatment of jaundice, smallpox and aphtha, and as a stomachic and anti-helminthic.⁵⁾ The chemical compounds in aqueous extracts of this species, such as palmatine, berberine, jatrorrhizine and dihydroberberine, had significant inhibitory effects on *Babesia gibsoni* in a culture system.⁵⁾ An experiment using berberine to treat HepG2 cells indicated that the viability was increased to more than 90% after treatment and the secretion of alpha-fetoprotein by HepG2 cells was also inhibited by berberine.⁶⁾ Interestingly, berberine from *A. flava* exhibited anti-malarial activity by inhibiting telomerase in a dose-dependent manner.⁷⁾ Likewise, *F. tinctoria* was found to possess strong antiplasmodial activity.⁸⁾

Although in the Thai Pharmacopoeia the crude drugs from these three medicinal plants are individually prescribed for diverse therapeutic purposes, *C. fenestratum* is the most valuable based on its reputation of high and broad therapeutic efficacy. Unfortunately, it is also a critically endangered

species. Therefore, the raw material from *C. fenestratum* is often substituted with cheaper comparable samples from *A. flava* or *F. tinctoria*. Since they share morphological similarity, botanical classification is required using different parts such as leaf, stem, flower and fruit. Nevertheless, it is impractical to identify individual plant materials from crude drugs needed in quality control.

The 18S ribosomal RNA (rRNA) genes are found in the nuclear genome of all eukaryotes and have been utilized in inferring phylogenetic relationships. So far, many phylogenies derived from small subunit rRNA sequences are useful in examining the relationships among families of angiosperms.⁹⁾ Similarly, the internal transcribed spacer (ITS) gene sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 28S rRNA coding regions (ITS2) are routinely used for phylogenetic analysis of closely related species in a wide range of organisms.^{10,11)}

Due to the constraint of classical botanical classification, the present study was conducted to utilize the genetic marker systems of the 18S rRNA genes and ITS gene regions for the ultimate identification of *C. fenestratum* from *A. flava* and *F. tinctoria*. The data confirmed that with the polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) technique, the 18S rRNA genes and ITS gene regions were powerful genetic markers with which to easily identify the Menispermaceae plants prescribed in Thai folk medicine.

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Table 1. Plant Materials Collected in Thailand

Species	Locality of voucher	Collection date	Plant part	Voucher No.
<i>Coscinium fenestratum</i>	NongKai	Jan. 2004	Leaves	CNK 0401
<i>C. fenestratum</i>	NongKai	Jan. 2004	Stem	CNK 0402
<i>C. fenestratum</i>	NongKai	Oct. 2005	Stem	CNK 0501
<i>Fibraurea tinctoria</i>	NongKai	Jan. 2004	Leaves	FNK 0401
<i>F. tinctoria</i>	NongKai	Jan. 2004	Stem	FNK 0402
<i>F. tinctoria</i>	NongKai	Oct. 2005	Stem	FNK 0501
<i>Arcangelisia flava</i>	Songkhla	Oct. 2005	Leaves	ASK 0501
<i>A. flava</i>	Songkhla	Oct. 2005	Stem	ASK 0502
<i>A. flava</i>	Nakorn Prathom	May 2005	Stem	ANP 0501

MATERIALS AND METHODS

Plant Materials The three Menispermaceae species used were as follows; *C. fenestratum*, *A. flava*, and *F. tinctoria*. All plant materials were collected in Thailand, identified by Ms. Leena Phuphatanapong, a botanist affiliated with the National Parks of Thailand, and stored at the Faculty of Pharmacy, Srinakharinwirot University, Thailand. The data collected are summarized in Table 1.

Isolation of Total DNA, PCR Amplification, and Sequencing Reaction Total DNA was extracted from 100–200 mg of plant leaves or stems using a DNeasy Plant Mini Kit (QIAGEN, Germany) with some modifications. The complete 18S rRNA gene and ITS gene region amplifications by the polymerase chain reaction (PCR) were performed using 10–100 ng of total DNA as a template in 50 µl of reaction mixture consisting of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 1.5 U *Taq* polymerase (Promega, U.S.A.), and 0.25 mM of each primer. Two pairs of the primers flanking the 18S rRNA gene and the ITS gene sequence were as follows; 18S-5'F (5'-CAACCTGGTTGATCCTGCCAGT-3') and 18S-3'R (5'-CTGATCCTTCTGCAGGTTTCACCTAC-3'), and ITS-5'F (5'-GTAG GTGAACCTGCAGAAGGATAC-3') and ITS-3'R (5'-CCATGCTTAACTCAGCGG GT-3') for the 18S rRNA gene and the ITS gene sequence, respectively. All the primers used here, the amplification conditions, and the sequencing reaction were performed as previously described.^{12–14)}

Briefly, the PCR amplifications were carried out in a Thermal Controller PTC-100 (MJ Research Inc., U.S.A.) under individual cycling conditions. For the 18S rRNA gene, each cycle consisted of 94 °C for 3 min and 65 °C for 8 min, followed by 30 cycles of 94 °C for 40 s and 65 °C for 8 min, and a final extension at 72 °C for 30 min. For the ITS gene regions, the hot start was required at 95 °C for 3 min, followed by 40 cycles 95 °C for 30 s, 52 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 10 min. The 1/10 volume of PCR product was resolved by 1.0% agarose gel electrophoresis and then the remaining part was purified using a QIAquick PCR Purification Kit (QIAGEN, Germany). Sequencing of the PCR product was performed using the Big Dye Terminator Kit Version 1.1 (Applied Biosystems, U.S.A.) with an ABI Prism 3100 Avant Genetic Analyzer (HITACHI, Japan). The nucleotide sequence data of the 18S rRNA gene and the ITS regions were recorded in GenBank.

Sequencing Analysis DNA sequencing data were analyzed using DNA sequencing analysis software (version 5.1,

Table 2. DNA Sequence Homology of the 18S rRNA Genes in the Three Menispermaceae Plants

Species (GenBank accession number)	<i>A. flava</i>	<i>C. fenestratum</i>	<i>F. tinctoria</i>
<i>A. flava</i> (FJ603106)	—	98.67%	98.67%
<i>C. fenestratum</i> (FJ603107)	98.67%	—	99.34%
<i>F. tinctoria</i> (FJ603108)	98.67%	99.34%	—

Applied Biosystems, U.S.A.). Multiple sequence alignments were determined with Clustal X version 1.8.

PCR-RFLP Analysis Fifteen microliters of the PCR products of the 18S rRNA gene and the ITS gene sequence were individually digested with 5 units of the restriction enzymes, *Sma*I and *Sal*I (BioLabs, England), respectively, at 37 °C for 1.5 h. The digested-PCR fragments were then detected by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining under UV.

RESULTS

18S rRNA Gene Sequences The leaves or stems were utilized as the sources of genomic DNA. The 18S rRNA genes of *C. fenestratum*, *A. flava*, and *F. tinctoria* amplified with a pair of primers, 18S-5'F and 18S-3'R, were 1809, 1805, and 1809 bp in length, respectively. The genes were further sequenced and the data were deposited in GenBank (accession numbers FJ603107, FJ603106, and FJ603108, respectively).

Sequence alignment comparison indicated that the three 18S rRNA genes shared 98–99% sequence homology (Table 2) and there were a total of 40 positions in base variation (Table 3).

PCR-RFLP Analysis on the 18S rRNA Gene The sites of the restriction enzyme for the PCR-RFLP analysis were sought on the basis of each sequence of the 18S rRNA gene from the three Menispermaceae species. The results of analysis of sites of restriction *Sma*I enzyme gave distinctive diagnostic fragments of *C. fenestratum* from the other two species. The *Sma*I enzyme recognizes the sequence 5' CC-CGGG 3'. All three 18S rRNA genes had a *Sma*I restriction site at the nucleotide position 1517–1522, and only the 18S rRNA gene of *C. fenestratum* contained an additional *Sma*I restriction site at the nucleotide position 727–732 (Fig. 1). In the electrophoresis profile, the PCR product on the 18S rRNA gene of *C. fenestratum* with the *Sma*I enzyme displayed the fragments of 729 and 790 bps, while both in *A. flava* and *F. tinctoria*, the fragment of 1519 bp was detected

Table 3. Variation in DNA Sequences of the 18S rRNA Gene of the Three Menispermaceae Species Collected in Thailand^{a)}

Species	Nucleotide positions																													
	2	2	2	2	3	3	4	5	5	1	2	3	4	4	5	5	5	6	6	6	6	7	7	7	1	1	1	1	1	
	4	5	6	9	1	2	2	2	3	3	8	4	9	9	6	9	9	2	4	4	5	1	2	9	3	3	7	7	7	
										2	3	8	7	9	3	0	6	1	0	9	4	1	7	4	5	6	0	1	5	
																								1	6	6	0	4	8	
<i>C. fenestratum</i>	G	T	C	A	G	C	A	G	C	A	G	C	A	T	C	G	A	T	—	T	C	C	C	G	C	C	C	C	T	G
<i>F. tinctoria</i>	G	T	C	A	G	C	A	G	C	A	A	T	T	G	C	G	A	T	—	T	T	T	G	T	A	T	T	T	T	G
<i>A. flava</i>	C	A	T	T	T	G	T	T	G	—	A	T	A	T	—	A	—	G	G	G	C	C	G	G	C	T	T	T	—	—

a) Numbering starts from the beginning of the 18S rRNA gene.

Fig. 1. The Restriction Site of Enzyme *Sma*I on the 18S rRNA Gene of *C. fenestratum* Fig. 2. PCR-RFLP Analysis with the Restriction Enzyme *Sma*I

The PCR products of individually 18S rRNA genes were digested with the restriction enzyme *Sma*I and visualized by ethidium bromide staining under UV; Lane M, 1 kb DNA ladder (GIBCO, U.S.A.); C, *C. fenestratum* (CNK 0401); F, *F. tinctoria* (FNK 0401); A, *A. flava* (ASK 0501).

(Fig. 2). These restriction sites were located on the variable regions at V4 (645—857) and V8 (1475—1530) on the 18S rRNA gene previously defined.¹⁵⁾

PCR-RFLP Analysis on the ITS Region The complete ITS gene regions (containing ITS1, ITS2 and 5.8S genes) of *C. fenestratum*, *A. flava*, and *F. tinctoria* were of 694, 622, and 631 base pairs in length, respectively (Fig. 3A). Sites of the restriction enzyme for the PCR-RFLP analysis were sought on the basis of each sequence of the ITS gene from the three Menispermaceae species. The result gave distinctive diagnostic fragments of *C. fenestratum* from the other two species using the specific *Sal*I enzyme cutting site. The *Sal*I enzyme recognizes the sequence 5' GTCGAC 3'. The ITS gene region of *C. fenestratum* has a *Sal*I restriction site at the nucleotide position 598—603 (Fig. 4), while both *A. flava* and *F. tinctoria* showed unrecognized sequences with *Sal*I enzyme at these positions. The ITS fragment of *C. fenestratum* was digested into smaller fragment sizes of about 599 bp by *Sal*I (Fig. 3B).

Sequence Analysis of the ITS Regions The ITS gene

Fig. 3. PCR-RFLP Analysis Using Restriction Enzyme *Sal*I

The PCR product of the ITS gene region was digested with restriction enzyme *Sal*I and visualized by ethidium bromide staining under UV; A, ITS region; B, ITS gene region was digested with restriction enzyme *Sal*I; Lane M, 100 bp DNA ladder (BioLabs, England); A, *A. flava* (ASK 0501); C, *C. fenestratum* (CNK 0401); F, *F. tinctoria* (FNK 0402).

regions of *C. fenestratum*, *A. flava*, and *F. tinctoria* were sequenced and the data were recorded in GenBank (accession numbers FJ603110, FJ603109, and FJ603111, respectively). Interestingly, the data from sequence alignment indicated that the ITS1 and ITS2 regions of each species were quite unique and different from each other. However, the sequences of 5.8S genes shared high homology (Table 4).

DISCUSSION

In the Thai Pharmacopoeia, the crude drugs from the three Menispermaceae medicinal plants are individually prescribed in anticipation of different therapeutic outcomes. Although

Fig. 4. The Restriction Site of Enzyme *SalI* on the ITS Gene Region of *C. fenestratum*

Table 4. The Homology Score in the ITS Gene Region of Three Menispermaceae Plants

		<i>A. flava</i>			<i>C. fenestratum</i>			<i>F. tinctoria</i>		
		ITS1	5.8S	ITS2	ITS1	5.8S	ITS2	ITS1	5.8S	ITS2
<i>A. flava</i>	ITS1	—	—	—	56.22	—	—	64.32	—	—
	5.8S	—	—	—	—	90.18	—	—	98.16	—
	ITS2	—	—	—	—	—	52.19	—	—	74.27
<i>C. fenestratum</i>	ITS1	70.43	—	—	—	—	—	61.23	—	—
	5.8S	—	90.18	—	—	—	—	—	88.34	—
	ITS2	—	—	57.43	—	—	—	—	—	56.85
<i>F. tinctoria</i>	ITS1	78.49	—	—	59.66	—	—	—	—	—
	5.8S	—	98.16	—	—	88.34	—	—	—	—
	ITS2	—	—	71.89	—	—	50.00	—	—	—

all three Menispermaceae plants here contain high contents of berberine and are widely used in folk medicines, *C. fenestratum* is the most valuable based on its reputation of high and broad therapeutic efficacy. Unfortunately, the plant is also a critically endangered species. Thus, the raw material from *C. fenestratum* is often substituted with cheaper comparable samples. Due to their morphological similarities, the crude material of *C. fenestratum* is easily changed or contaminated with those from *A. flava* or *F. tinctoria*, which have a lower value. Therefore, precise quality control during drug formulation is needed and usually requires an experienced botanist for plant identification.

In the present study, we applied the nuclear 18S rRNA gene and ITS gene sequences with PCR-RFLPs for the rapid and reliable identification of *C. fenestratum* from *A. flava* or *F. tinctoria*. As expected, the nuclear 18S rRNA gene and ITS gene sequences of *C. fenestratum* are unique and different from those of *A. flava* and *F. tinctoria*. In addition, the 18S rRNA gene of *C. fenestratum* has a unique *SmaI* recognition site at position 729. Likewise, the result from DNA analysis on the ITS region of *C. fenestratum* also shows the specific location of a unique *SalI* recognition at position 599. Therefore, PCR-RFLP analysis of the nuclear 18S rRNA gene and ITS gene sequences is an effective tool for the crude drug identification of *C. fenestratum* from *A. flava* or *F. tinctoria*. Moreover, we found that the method could be applied to specimens from either leaves or stems. The result clearly indicated that no intraspecific variation in the nuclear 18S rRNA gene and ITS gene sequences within the same plant species was detected (data not shown). Thus, the discovery confirmed the power of the nuclear 18S rRNA gene and ITS gene sequences coupled with PCR-RFLPs for the easy and reliable authentication of *Coscinium fenestratum* among other Menispermaceae plants that are prescribed in Thai folk medicine.

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