Genetic Analysis of Plants in the Genus *Mitragyna* and Development of SNP Marker in the ITS2 Region for the Identification of *M. speciosa*

การวิเคราะห์ทางพันธุกรรมของพืชสกุล *Mitragyna* และการพัฒนาเครื่องหมาย SNP ของดีเอ็นเอบริเวณ ITS2 เพื่อการพิสูจน์เอกลักษณ์กระท่อม

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ABSTRACT

Plants in the genus *Mitragyna* have been used as traditional medicines. There are four species, *M. speciosa*, *M. diversifolia*, *M. hirsuta*, and *M. rotundifolia* existing in Thailand. *M. speciosa*, known as "Kratom" in Thai, is a narcotic plant and has particular medicinal importance. Consumption of *M. speciosa* is illegal in Thailand. This study aimed to develop molecular marker for detection and identification of *M. speciosa*. The patterns of intragenomic variation were detected in nucleotide sequences of internal transcribed spacer 2 (ITS2) region from the four *Mitragyna* species. Based on the nucleotide polymorphism, single nucleotide polymorphism (SNP) was developed. Species-specific primers were designed for identification and differentiation of *M. speciosa* from the closely related species.

บทคัดย่อ

พืชสกุล *Mitragyna* มีการใช้เป็นสมุนไพรพื้นบ้าน ในประเทศไทยพบ 4 ชนิด ได้แก่ กระท่อม (*M. speciosa*) กระทุ่มนา (*M. diversifolia*) กระทุ่มโคก (*M. hirsuta*) และกระทุ่มเนิน (*M. rotundifolia*) กระท่อมเป็นพืชที่มีฤทธิ์ทางยาที่สำคัญและเป็นพืชเสพติด การบริโภคพืชกระท่อมถือว่าผิดกฎหมายในประเทศไทย งานวิจัยนี้จึงได้ทำการพัฒนาเครื่องหมายโมเลกุลสำหรับการตรวจสอบและพิสูจน์เอกลักษณ์กระท่อม ซึ่งได้มีการตรวจสอบรูปแบบ intragenomic variation จากลำดับนิวคลีโอไทด์บริเวณ internal transcribed spacer 2 (ITS2) ของพืชสกุล *Mitragyna* ทั้ง 4 ชนิด พบตำแหน่งที่เป็น polymorphism จึงนำไปพัฒนาเป็นเครื่องหมายดีเอ็นเอชนิด single nucleotide polymorphism (SNP) โดยออกแบบไพรเมอร์ที่จำเพาะต่อกระท่อมเพื่อการพิสูจน์เอกลักษณ์และแยกพืชกระท่อมออกจากพืชชนิดอื่นในสกุลเดียวกัน

Key Words: ITS2 region, *M. speciosa*, SNP marker
คำสำคัญ: ดีเอ็นเอบริเวณไอทีเอส 2 กระท่อม เครื่องหมายเอสเอ็นพี

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Introduction

The genus *Mitragyna* belongs to the Rubiaceae family. There are four species existing in Thailand including *M. speciosa* (Korth.) Havil., *M. diversifolia* (Wall. ex G.Don) Havil., *M. hirsuta* Havil. and *M. rotundifolia* (Roxb.) O. Kuntze. The genus *Mitragyna* has a history of use as a medicinal plant for a wide variety of disease such as fever, malaria, diarrhea, cough and muscular pains (Gong *et al.*, 2012). *M. speciosa*, commonly known as "Kratom", is a narcotic plant and have particular medicine importance such as antinociceptive and anti-inflammatory (Shaik Mossadeq *et al*., 2009), antidiarrheal (Chittrakarn *et al*., 2008), anticancer (Ghazali *et al*., 2011), antidiabetic (Purintrapiban *et al*., 2011) and antidepression (Idayu *et al*., 2011). Leaves have been used as a stimulant by Thai laborers and farmers to reduce the strain and fatigue of hard work (Ahmad and Aziz, 2012). However, the consumption of *M. speciosa* is illegal due to its narcotic effects (Saingam *et al.*, 2013).

In previous research, microscopic examination cannot be used to identify of *M. speciosa* because of the anatomical fragmentation of the material similarities between species within the same genus. Various species of genus *Mitragyna* can be difficult to distinguish by anatomically microscopic method (Kowalczuk *et al.*, 2013). The chemical identification has been developed using different techniques (Chan *et al.*, 2005; Kikura-Hanajiri *et al.*, 2009; Parthasarathy *et al.*, 2013). However, chemical composition of *M. speciosa* may be affected by environmental conditions (Takayama, 2004).

In the past few decades, DNA-based markers have been applied to authenticate important medicinal plants materials (Boonsom *et al.*, 2012). DNA molecular markers using the internal transcribed spacer (ITS) region can differentiate *M. speciosa* from the other species by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Sukrong *et al.*, 2007). Besides, the ITS2 region has been frequently used for molecular analysis because sufficient variations in its sequence among different species (Song *et al.*, 2012). It can potentially be used as a molecular marker to identify medicinal plants and their closely related species (Xin *et al.*, 2013).

In this study, the patterns of intragenomic variation in the ITS2 region of four *Mitragyna* species were analyzed. The single nucleotide polymorphism (SNP) marker was developed for a rapid and accurate identification and differentiation of *M. speciosa* from the other *Mitragyna* species.

Objective of the study

The aim of this study was to develop a SNP marker in the ITS2 region for identification and discrimination of a narcotic species, *M. speciosa*, from the closely related species.

Methodology

Plant materials and DNA extraction

Fresh leaves of four *Mitragyna* species, including *M. speciosa*, *M. diversifolia*, *M. hirsuta*, and *M. rotundifolia* were obtained from various locations in Thailand (Table 1) and preserved at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. All samples were identified by Assoc. Prof. Nijsiri Ruangrungsi, Ph.D. of Chulalongkorn University.

The fresh leave of plant specimens were frozen in liquid nitrogen and ground into fine powders. Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's procedure. In brief, the ground tissue was added lysis buffer, the lysate was loaded into QIAshredder spin column and centrifuged to remove precipitates. The flow-through fraction was applied to a DNeasy mini spin column. The column was washed with buffer and centrifuged to dry the membrane. The DNA was eluted using 100 µl of double deionized water. All DNA extracts were stored at -20 °C until real-time PCR analysis.

Table 1 Plant samples used in this study

Sequence analysis in the ITS2 region

Samples	Code	Place of collection (Thailand, Province)	Voucher no.
M. speciosa (Roxb.) Korth.	MS-01	Bangkok	MUS-5512-1
	MS-02	Bangkok	MUS-5512-2
	MS-03	Chumporn	MUS-5602-1
	MS-04	Nonthaburi	MUS-5602-2
	MS-05	Chachoengsao	MUS-5603-1
M. diversifolia (Wall. ex G.Don) Havil.	MD-01	Bangkok	MUS-5512-4
	MD-02	Khon kaen	MUS-5602-2
	MD-03	Nakorn pathom	MUS-5603-3
M. hirsuta Havil.	MH-01	Bangkok	MUS-5512-5
	MH-02	Kampaengpet	MUS-5603-5
	MH-03	Nakhon ratchasima	MUS-5604-3
M. rotundifolia (Roxb.) Kuntze	MR-01	Bangkok	MUS-5512-6
	MR-02	Nakhon ratchasima	MUS-5601-5
	MR-03	Nakhon ratchasima	MUS-5604-5

Sequences alignment of the ITS1, 5.8S and ITS2 regions of *M. speciosa*, *M. diversifolia*, *M. hirsuta*, and *M. rotundifolia* (GenBank accession numbers AB249645.1, AB249646.1, AB249647.1 and AB249648.1, respectively) were constructed using CLUSTALW program.

Design of species-specific primer

To identify *M. speciosa*, the Ms-F2 specific forward primer was designed based on the SNP sites detected in ITS2 region of *M. speciosa* (Figure 1). The two common primers, Ms-F3 forward and Ms-R2 reverse were also designed to amplify the ITS2 region of all species. The sequences of primers are listed in Table 2.

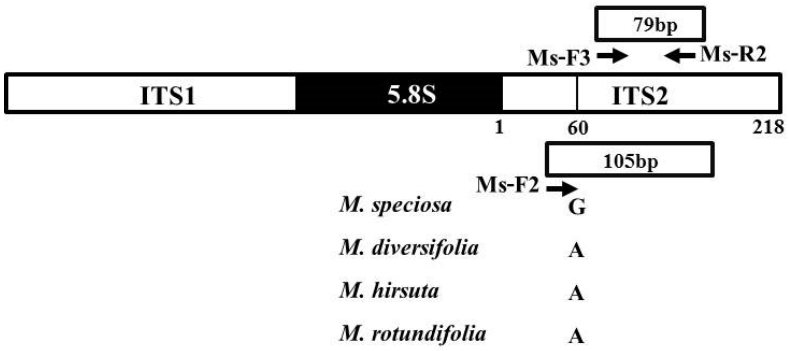

Figure 1 Structure of ITS1, 5.8S and ITS2 region. Sharp arrows indicate orientation and approximate position of species-specific primer (Ms-F2) and common primers (Ms-F3 and Ms-R2). PCR products were 105 and 79 bp, respectively.

Table 2 Primer used in this study.

Primer name	Orientation	Primer sequence (3'-5')
Ms-F2	Forward	TGG CCT CCC GTG CCC TG
Ms-F3	Forward	CGG CCT AAA TGC GAG TCC TC
Ms-R2	Reverse	CGG CAC GAC AGA AAT CGA GTC

Multiplex PCR amplification

SNP-based multiplex PCR was performed for identification of *M. speciosa* using species-specific primers. For multiplex PCR, amplification were carried out in a 25 µl reaction mixture containing: 10 ng of template DNA, 5 µl 5X PCR reaction buffer (Promega, USA), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µl of each primer, and 1 U of *Taq* polymerase (Promega, USA).

Annealing temperature was determined by gradient PCR with temperature increasing from 58 to 68°C. The optimal PCR condition were carried out in a C1000™ Thermal Cycler (Bio-Rad, USA) using cycling conditions start at 94°C for 3 min, followed by extension at 72°C for 10 min. PCR conditions were 35 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, extension at 72°C for 90s, and final extension at 72°C for 10 min. PCR products were separated by 2.5% agarose gel electrophoresis with the addition of ethidium bromide into the gel. The gels were run at 80 volt for 40 min in 1X TAE buffer, and visualized using a UV transilluminator.

Results

Analysis of SNPs in ITS

The fragments of ITS2 sequence from the four *Mitragyna* species were examined for SNPs at the interspecies level. The ITS2 sequence of *M. speciosa* was 218 bp while that of *M. diversifolia*, *M. hirsuta*, and *M. rotundifolia* were 217 bp in length. Eleven nucleotides at position 60, 120, 124, 126, 136, 174, 175, 187, 196, 207 and 216 are polymorphism sites (Table 3).

The identification of *M. speciosa* is essential for both forensic and medicinal usage. SNP analysis can distinguish *M. speciosa* from the other *Mitragyna* species. The interspecific

Table 3 Eleven SNPs in ITS2 sequence alignment of four *Mitragyna* species

	SNP location										
Plant species	**60**	**120**	**124**	**126**	**136**	**174**	**175**	**187**	**196**	**207**	**216**
M. speciosa	G	T	T	A	A	C	A	C	T	T	C
M. diversifolia	A	-	C	C	A	T	G	C	T	T	A
M. hirsuta	A	-	C	C	A	T	G	C	T	T	A
M. rotundifolia	A	-	C	C	T	T	G	T	A	C	C

nucleotide diversity of *M. speciosa* and the other is represented by six SNPs at nucleotide position 60, 120, 124, 126, 174, and 175.

In addition, the nucleotide at position 136, 187, 196 and 207 can be used as a unique marker to discriminate *M. rotundifolia* from the others, whereas *M. diversifolia* and *M. hirsuta* have the same ITS2 sequence.

SNP marker of *M. speciosa*

According to the analysis of sequence alignments of ITS2 regions from four *Mitragyna* plants, the nucleotide at position 60 was chosen to design species-specific primer to identification of *M. speciosa*. The 3' end of Ms-F2 primer was G which specific for *M. speciosa*. The common forward primer Ms-F3 and common reverse primer Ms-R2 were also designed for the internal amplification control for four *Mitragyna* plants.

In order to determine the specific of this method, an annealing temperature range from 58 to 68°C was tested (data not shown). To confirm the reproducibility of the method, the experiment was repeated three times.

In the multiplex PCR reaction, the combination of specific primer and common forward primer generated different fragment patterns to discriminate *M. speciosa* from the other species. The specific PCR product of 105 bp was amplified from the primer Ms-F2 and Ms-R2. The two common primers, Ms-F3 and Ms-R2, were also designed to amplify a 79 bp fragment as an internal amplification control (figure 2). The size of PCR products was examined by 2.5% agarose gel electrophoresis and visualized by UV transillumination.

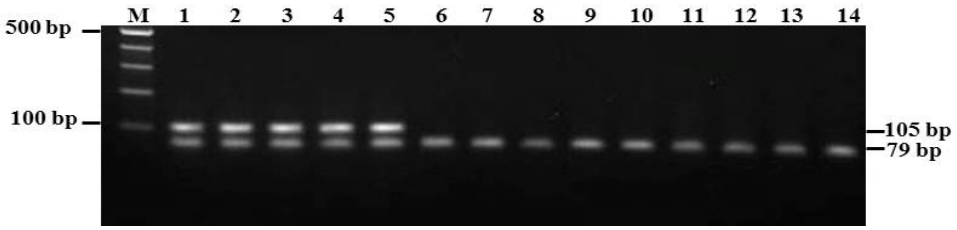

Figure 2 Species-specific identification of *M. speciosa* by SNP marker. M: VC 100 bp plus DNA marker; lane 1-5: *M. speciosa* (MS-01-MS-05), lane 6-8: *M. diversifolia* (MD-01-MD-03), lane 9-11: *M. hirsuta* (MH-01-MH-03), lane 12-14: *M. rotundifolia* (MR-01-MR-03)

Discussion and Conclusions

The section of internal transcribed spacer (ITS) includes the ITS1, 5.8S, and ITS2 regions. The short ITS2 sequence serves as an efficient taxonomic sequence tag in comparison with the full-length ITS (Han *et al*., 2013). The usage of the variation in ITS2 region is sufficient for species determination in most cases (Song *et al*., 2012). According to the sequence difference of ITS2 region among four *Mitragyna* plants indicated that *M. speciosa* processes high interspecific polymorphism. Therefore, the ITS2 region appeared to be suitable DNA regions for molecular identification of *M. speciosa*.

Previous studies have demonstrated identification of *M. speciosa* DNA using the PCR-RFLP (Sukrong *et al.*, 2007). However, PCR-RFLP method is necessary strict reaction and time consuming process. The present study was the first to develop SNP position into effective tools for the rapid and accurate identification of a narcotic plants of the *Mitragyna* genus; *M. speciosa.* The SNP marker is potentially useful for the analysis of genetic diversity in plants, particularly in closely related species (Chen *et al.,* 2013). Moreover, it has been used for authentication studies in several plant species (Sun *et al.,* 2011; Bielsa *et al.,* 2014). The SNP sites were exploited for *M. speciosa* in ITS2 region. The 105 bp amplicon specific to *M. speciosa* was amplified by Ms-F2/ Ms-R2 primer pair. The results of this study confirmed that SNP markers based on ITS2 region is an efficient, specific and rapid method for authentication and discrimination of *M. speciosa* from *M. diversifolia*, *M. hirsuta,* and *M. rotundifolia.* Additionally, *M. rotundifolia* could be discriminated from the related species of *Mitragyna* genus by this SNP site. However, *M. diversifolia* and *M. hirsuta* could not be differentiated by SNP site in the ITS2 region. Therefore, the study of DNA sequences in other regions may be useful for development of species specific molecular marker to distinguish all plants in the genus *Mitragyna*.

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