การวิเคราะห์ทางพันธุกรรมของพืชสกุล *Mitragyna* และการพิสูจน์เอกลักษณ์พืชกระท่อม

โดยพีซีอาร์ ณ เวลาจริง

นางสาวสุปิตา อวชัย

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GENETIC ANALYSIS OF PLANTS IN THE GENUS *MITRAGYNA* AND THE IDENTIFICATION OF *MITRAGYNA SPECIOSA* BY REAL-TIME PCR

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สุปิตา อวชัย : การวิเคราะห์ทางพันธุกรรมของพืชสกุล *Mitragyna* และการพิสูจน์เอกลักษณ์พืชกระท่อมโดยพีซีอาร์ ณ เวลาจริง (GENETIC ANALYSIS OF PLANTS IN THE GENUS *MITRAGYNA* AND THE IDENTIFICATION OF *MITRAGYNA SPECIOSA* BY REAL-TIME PCR) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ร.ต.อ.หญิง ดร.สุชาดา สุขหร่อง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภก. ดร.ธงชัย สุขเศวต, 94 หน้า.

พืชในสกุล *Mitragyna* มีการนำมาใช้เป็นสมุนไพรพื้นบ้าน ประเทศไทยสามารถพบได้ 4 ชนิด ได้แก่ กระทุ่มนา (*M. diversifolia*) กระทุ่มโคก (*M. hirsuta*) กระทุ่มเนิน (*M. rotundifolia*) และกระท่อม (*M. speciosa*) พืชในสกุลนี้มีแต่กระท่อมเท่านั้นที่จัดเป็นพืชเสพติด การบริโภคพืชกระท่อมถือว่าผิดกฎหมายในประเทศไทย แต่อย่างไรก็ตามยังพบว่ามีการบริโภคในหมู่ผู้ใช้แรงงานเพื่อจุดประสงค์ให้ทำงานได้ทนขึ้น ในปัจจุบันพบว่ามีการนำกระท่อมมาใช้ในทางที่ผิดโดยการเตรียมเป็นรูปแบบสูตรผสมที่เรียกว่า “สี่คูณร้อย” ซึ่งส่งผลเสียต่อสุขภาพของประชาชน ดังนั้นการพิสูจน์เอกลักษณ์ของกระท่อมจึงมีประโยชน์ต่อการนำไปประยุกต์ใช้ในงานวิจัยทางสมุนไพร เภสัชวิทยาและนิติวิทยาศาสตร์

ในการศึกษาครั้งนี้จึงได้ทำการพัฒนาเครื่องหมายโมเลกุลสำหรับการพิสูจน์เอกลักษณ์กระท่อมอย่างถูกต้อง ซึ่งได้มีการตรวจสอบรูปแบบ interspecific variation จากลำดับนิวคลีโอไทด์บริเวณ ITS2 ของพืชสกุล *Mitragyna* ทั้ง 4 ชนิด พบตำแหน่งที่เป็น nucleotide polymorphism จึงนำไปพัฒนาเป็นเครื่องหมายดีเอ็นเอโดยออกแบบไพรเมอร์ที่จำเพาะต่อกระท่อมเพื่อการพิสูจน์เอกลักษณ์และแยกพืชกระท่อมออกจากพืชชนิดอื่นในสกุลเดียวกัน และยิ่งไปกว่านั้นได้มีการพัฒนาการใช้เทคนิคพีซีอาร์ ณ เวลาจริง ซึ่งเป็นการวิเคราะห์ melting curve ของผลผลิตพีซีอาร์ ที่มี melting temperature แตกต่างกัน การวิเคราะห์ melting curve เป็นวิธีที่มีความจำเพาะและมีความไวสูง ทำให้สามารถพิสูจน์เอกลักษณ์และแยกกระท่อมออกจากพืชในสกุลเดียวกันได้ วิธีนี้มีประสิทธิภาพร้อยละ 95.6 นอกจากนี้ยังได้นำมาประยุกต์ใช้ในการตรวจสอบและวิเคราะห์ปริมาณดีเอ็นเอของกระท่อมจากตัวอย่างสี่คูณร้อย

ภาควิชา	เภสัชเวทและเภสัชพฤกษศาสตร์	ลายมือชื่อนิสิต	.....
สาขาวิชา	เภสัชเวท	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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The genus *Mitragyna* has been used as local traditional medicines. There are four species, *M. diversifolia*, *M. hirsuta*, *M. rotundifolia and M. speciosa*, existing in Thailand. Only *M. speciosa*, known as kratom, is officially classified as a narcotic plant among the genus *Mitragyna*. Consumption of *M. speciosa* is illegal in Thailand. However, this plant is popularly consumed by construction workers for the purpose of work harder. Recently, the pattern of consumption has increasingly changed toward a drink called “kratom cocktail”. The abuse of *M. speciosa* can affect of the consumer health. Thus, correct identification of *M. speciosa* is essential for both traditional medicinal and pharmacological studies as well as forensic investigation.

This study aimed to develop molecular markers for the accurate identification of *M. speciosa*. The patterns of interspecific variation were detected in the nucleotide sequences of the ITS2 region in the four *Mitragyna* species. Based on the nucleotide polymorphism, species-specific primers were designed for the identification and differentiation of *M. speciosa* from the other closely related species. Moreover, a real-time PCR technique based melting curve analysis was developed for the detection and discrimination of *M. speciosa* from other *Mitragyna* species. Based on the distinct melting temperatures of amplification products, the melting curve was highly specific for the detection of *M. speciosa* DNA with the amplification efficiency value of 95.6%. Additionally, this assay can be used to detection and quantification of *M. speciosa* DNA in kratom cocktail samples.

Department:	Pharmacognosy and Pharmaceutical Botany	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Pharmacognosy	Co-Advisor's Signature .....
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LIST OF ABBRAVATION

A	adenine
AFLP	amplified fragment length polymorphism
bp	base pair
C	cytosine
Ct	threshold cycle
°C	degree Celsius
DNA	deoxyribonucleic acid
dsDNA	double strand DNA
E	efficiency
EST	external transcribed spacer
G	guanine
Indels	insertions/deletions
IGS	intergenic spacer
ITS	internal transcribed spacer
ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
LOD	limit of detection
$MgCl_2$	magnesiem chloride
mM	millimolar
µl	microliter
ng	nanogram
R^2	correlation coefficients
*rbc*L	large subunit of ribulose-bisphosphate carboxylase
rDNA	ribosomal DNA
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length Polymorphism
pg	picrogram
S	Svedberg unit

SNP	single nucleotide polymorphism
T	thymine
TAE	tris-acetate-EDTA buffer
Tm	melting temperature
*trn*T-F	non-coding intergenic spacer between *trn*T and *trn*F in the chloroplast genome
U	unit
UV	ultraviole

CHAPTER I

INTRODUCTION

The genus *Mitragyna*, which belongs to Rubiaceae family, is widely found in Africa and Asia (Gong et al. 2012). Four species, *M. diversifolia* (Wall. ex G.Don) Havil., *M. hirsuta* Havil., *M. rotundifolia* (Roxb.) O. Kuntze, and *M. speciosa* (Korth.) Havil. are distributed throughout Thailand (Smitinand, 2001). *M. speciosa,* widely known as kratom, has been used as traditional medicine with a specific medicinal importance. It has been reported for a psychoactive effect. *M. speciosa* could be classified into red veined leaf type and green veined leaf type (Saingam *et al.*, 2013). The red veined type is supposed to have a stronger effect (Chittrakarn *et al.*, 2012). *M. speciosa* can be used to treat the opioid addiction because of its narcotic property (Vicknasingam *et al.*, 2010). The plant also has other medicinal properties. It is used to treat diarrhea (Chittrakarn *et al.*, 2008), inflammation (Utar *et al.*, 2011) and diabetes (Purintrapiban *et al.*, 2011). Although the other species such as *M. diversifolia* can be used substitutive to *M. speciosa* in Thai folklore medicine, it is not as effective as *M. speciosa* (Sukrong *et al.*, 2007).

In 1979, the Thai government announced the Narcotics Act B.E. 2522, assigning *M. speciosa* to Category V of a five-category classification. Therefore, any activities concerning buying, selling, importing, and possessing of *M. speciosa* are illegal. However, the villagers in the south of Thailand popularly consume this plant by chewing fresh leaves or drinking boiled leaves. This is due to a belief that *M. speciosa* can help maintain their working capacity and give them a longer endurance. Recently, a “Kratom cocktail” called “4×100” formula, an illegal product, has emerged and been widely used among teenage users in the southern provinces of Thailand. It consisting of three main components in general: boiled *M. speciosa* leaves, cola soft drink and codeine- or diphenhydramine- cough syrup which the powder peeled from the inside of fluorescent light bulbs, mosquito coils, herbicide

and tranquilizer add (Tungtananuwat and Lawanprasert, 2010). There are many forms of *M. speciosa* sold on the internet such as dried leaves, powdered and drink. Abuse of this plant is easily accessible worldwide (Ahmad and Aziz *et al.*, 2012), which can affect the health of consumers (Nelsen *et al.* 2010; Tungtananuwat and Lawanprasert, 2010; Neerman *et al.*, 2013). Therefore, the identification of *M. speciosa* is an important part not only for medicinal purpose but also forensic investigation.

Several techniques have been developed for identification of mitragynine, which is found exclusively in *M. speciosa* leaves. The chemical identification has been developed using different techniques such as gas chromatography (GC) (Chan *et al.*, 2005), liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) (Kikura-Hanajiri *et al.*, 2009) and high performance liquid chromatography with diode array detection (HPLC-DAD) (Parthasarathy *et al.*, 2013). However, chemical identification may be limited because the chemical compositions of *M. speciosa* vary according to among seasons and growing areas (Takayama, 2004). Therefore, DNA-based method has been recently applied to identify medicinal plants and herbal drugs (Manissorn *et al.*, 2010; Boonsom *et al.*, 2012) on account of the stability of the DNA under different environmental conditions, farming and production techniques (Madesis *et al.*, 2014). DNA molecular markers using the internal transcribed spacer (ITS) region can differentiate *M. speciosa* from psychoactive plants and also from closely related species by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Sukrong *et al.*, 2007; Maruyama *et al.*, 2009). However, PCR-RFLP method requires strict reaction and therefore is not suitable for identification of processed samples due to the degradation of genomic DNA.

Recently, Real-time PCR techniques have been widely used for molecular authentication of organisms. It enables both detection and quantification for specific sequences in a DNA sample. Melting curve analysis of real-time PCR product provide rapid, sensitive and specific method for identification of many organisms (Xue *et al.*, 2009; Traunsek *et al.*, 2011; Serradilla *et al.*, 2013). Different sequences can be discriminated by measurement of their specific melting temperature (Tm) of PCR products, leading to disposal of post-PCR gel electrophoresis.

The purposes of this study were to develop molecular marker from the internal transcribed spacer 2 (ITS2) region of ribosomal DNA that could distinguish *M. speciosa* from three other closely related species; *M. diversifolia*, *M. hirsuta* and *M. rotundifolia*. Based on the nucleotide polymorphism, species-specific primers of *M. speciosa* were designed. Moreover, melting curve analysis based real-time PCR method was developed for detection of *M. speciosa*. In addition, this assay was applied for detection and quantification of *M. speciosa* DNA present in samples of kratom cocktail.

CHAPTER II

LITERATURE REVIEWS

2.1 Plant materials

2.1.1 Botanical description of the genus *Mitragyna*

The genus *Mitragyna* belongs to the Rubiaceae family and is found in swampy territory in the tropical and subtropical regions of Asia and Africa. Six species, *M. diversifolia* (Wall. ex G. Don) Havil. *M. speciosa* (Korth.) Havil., *M. tubulosa* (Arn.) Havil., *M. parvifolia* Korth., *M. hirsuta* Havil., and *M. rotundifolia* (Roxb.) O. Kuntze, widely grow in India and Asia. Other four species, *M. ciliate*, *M. inermis* (Willd) Kuntze, *M. stipulosa* Kuntze and *M. africanus,* widely grow in west African (Gong *et al.*, 2012). In Thailand, Four species namely *M. diversifolia*, *M. hirsuta*, *M. rotundifolia* and *M. speciosa* are found (Smitinand, 2001) (Table 1).

Table 1 The list of *Mitragyna* species exsisting in Thailand.

No.	Scientific name	Thai name
1	*M. speciosa* (Korth) Havil.	**กระท่อม**, อีถ่าง, ท่อม
2	*M. diversifolia* (Wall. ExG.Don) Havil.	**กระทุ่มนา**, กระท่อมขี้หมู, กระทุ่มดง, ตำ, กระทุ่มน้ำ, กาตูม, ตุ้มแซะ, ตุ้มน้อย, ตุ้มน้ำ, ถ่มพาย, ท่อมขี้หมู, ท่อมนา, โทมน้อย
3	*M. hirsuta* Havil.	**กระทุ่มโคก**, ตุ้มเขา, ทุ่มพาย
4	*M. rotundifolia (Roxb.) O. Kuntze*	**กระทุ่มเนิน**, แก่นเหลือง

The Rubiaceae of Thailand (Puff *et al.*, 2005) describes the characteristics of the genus *Mitragyna* as follows (Figure 1);

"Small to relatively large trees. Leaves opposite, petiolate, blades chartaceous to subcoriaceous; stipules interpetiolar, entire, with a central keel, often large and ovate to lanceolate, caducous. Inflorescences consisting of globose flowering heads terminal on lateral branches and on their (sometimes repeatedly branched) side shoots, often arranged like simple or compound dichasia, like thyrses, or ± like pseudo-umbels; young heads subtended by a pair of leaves with enlarged, sometimes ± colored deciduous stipules, or only the involucre-like enlarge stipules (Figure 1 (B), (H)); flowering heads with spathulate interfloral bracteoles (Figure 1 (F)). Flowers 5-merous, hermaphrodite, (sub)sessile; calyx with a basal tubular part and variously shaped lobes; corolla yellowish, infundibular to narrowly hypocrateriform, tube hairy around the throat, lobes valvate in bud, ascending in open flowers; stamens around the throat, filaments short, at least partially exserted; style with mitriform to elongate clavate stigma exserted; ovary 2-celled, each locule with numerous ovules on an elongated placenta attached to upper part of septum; ovaries of adjoining flowers of a head not fused with each other. Fruitlets of a fruiting head free from one another, crowned by the persistent calyx, ellipsoid to ovoid, capsular, dehiscing septicidally and then loculicidally from the apex downwards. Seeds small, numerous, slightly winged at both ends, the lower wing ± bifid."

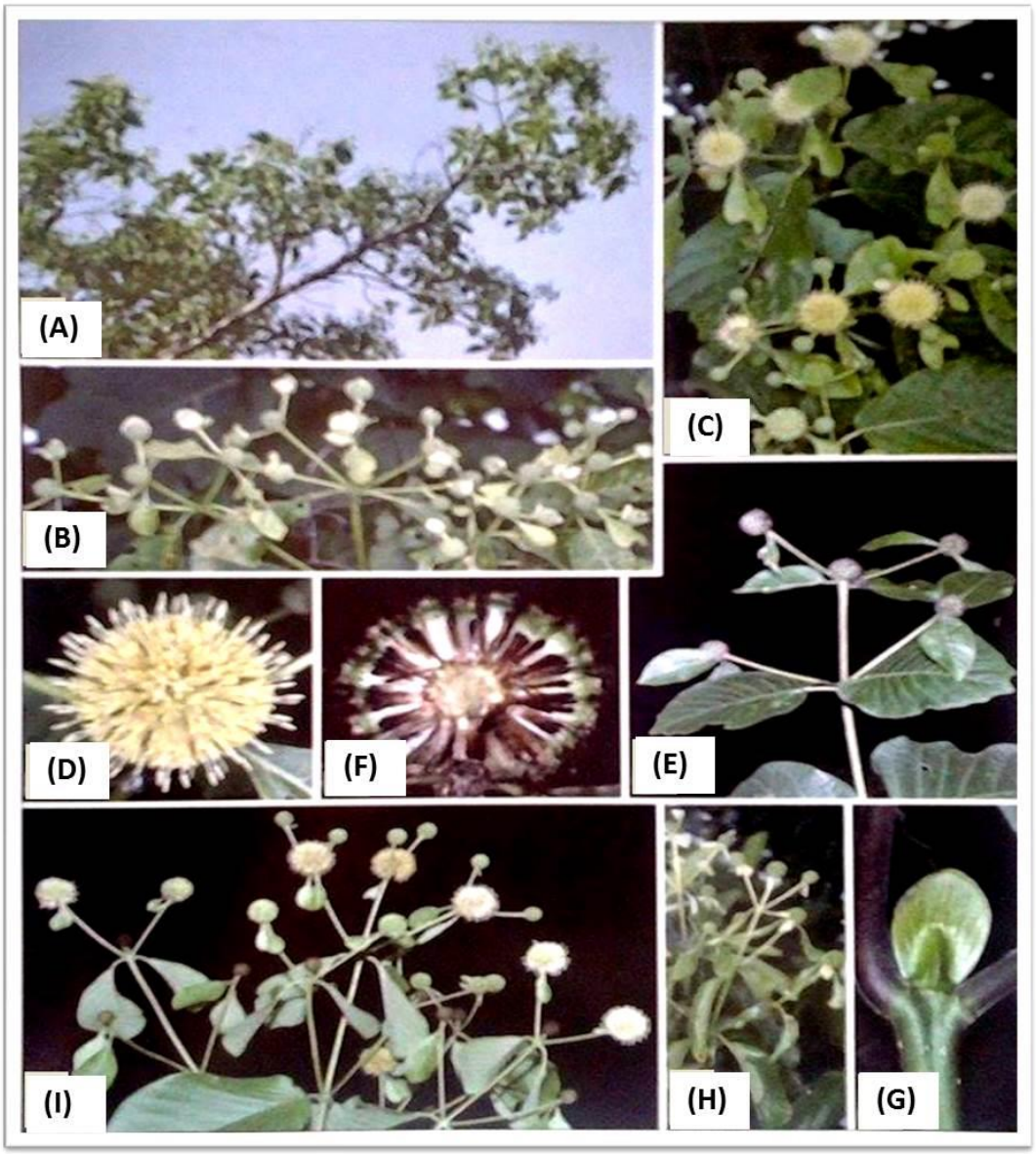


Figure 1 *Mitragyna* plant description: (A)-(G) *M. rotundifolia*; (A) part of crown of old tree; (B) young flowing heads (note enlarged, whitish involucre-like stipules enveloping the heads); (C)-(D) flowing heads; (E) immature fruiting heads; (F) section of immature fruiting head, note blackish bracteoles between individual fruits; (G) stipule. (H)-(I) *M. diversifolia*; (H) young heads (note enlarged stipules; compare with (B); (I) inflorescence system with heads in bud, flower and young fruit (Puff et al., 2005).

The flora of Thailand (The forest herbarium, 2014) describes the characteristics of the *M. speciosa* (Figure 2) as follows;

***M. speciosa* (Korth.) Havil.**

"Small to medium sized tree, up to 25 m tall. Bark smooth, greyish. Interpedicel stipule chartaceous, caduceus. Leaves opposite decussate; elliptic to ovate or obovate, 5-11 x 12-17 cm; apex shortly pointed; base usually rounded to cordate, sometimes cuneate; glabrous above, slightly hairy below; secondary vein 8-17 pairs, with pale-hairy domatia at the axils; tertiary veins fine, ladder-like; petioles 2.5-4 cm long. Inflorescence a solitary head, terminal, globose, densely covered with flowers and spathulate interfloral bracteoles. Flowers 5-merous; calyx tube about 2 mm. long; corolla tube 3.5-5 mm. long, lobes 2.5-3 mm. long. Stamens 5; anthers 1-1.5 mm long; filaments 0.5 mm long. Ovary 2-celled; ovules numerous. Fruiting heads globose, 2-3 cm in diam.; fruitlets 7-9 x 4-5 mm. Seeds about 1 mm long, with papery wing at each end, 1-2 mm long."

The flora of China (Flora of China, 2012) describes the characteristics of the *M. diversifolia* (Figure 3), *M. hirsuta* (Figure 4) *and M. rotundifolia* (Figure 5) as follows;

***M. diversifolia* (Wall. ExG.Don) Havil.**

"Trees, perhaps deciduous, to 15 m tall; branches angled becoming terete, pilosulous to glabrescent. Petiole 5–15 mm, glabrous, puberulent, or pilosulous; leaf blade drying papery, ovate-oblong to elliptic-ovate, 6–14 × 3–9 cm, adaxially glabrous, abaxially sparsely to densely pilosulous or tomentulose, base rounded to cordulate, apex obtuse to shortly acuminate; secondary veins 8 or 9 pairs, strongly ascending, sometimes with pilosulous domatia in abaxial axils; stipules elliptic-oblong to ovate, ca. 2.5 cm, strigillose to glabrous, abaxially weakly to strongly keeled and pilosulous, apex obtuse to rounded. Inflorescence densely pilosulous or strigillose to glabrescent; peduncles 1–3 mm (i.e., portion above articulation of subtending leaves

Figure 2 *M. speciosa (Korth.)* Havil. The plant (A); leaves (B); and flowers (C).

Figure 3 *M. diversifolia* (Wall. ExG.Don) Havil. The plant (A); leaves (B); and flowers (C).

but not including entire growth of branch); flowering heads 3 to numerous, 8–10 mm in diam. across calyces, 13–20 mm in diam. across corollas; bracteoles linear-spatulate, ca. 3 mm, glabrous to sparsely ciliolate. Calyx glabrous; ovary portion obconic, ca. 1.5 mm; limb ca. 1.5 mm, subtruncate to lobed for up to ca. 1/2; lobes triangular, obtuse. Corolla yellowish white, outside glabrous, inside densely pilosulous in throat and on lobes; tube ca. 3 mm; lobes triangular, ca. 2.5 mm, acute. Fruiting heads 8–10 mm in diam. Capsules 3–4 mm, with persistent calyx limb markedly thickened; seeds 1–2 mm."

***M. hirsuta* Havil.**

"Trees, deciduous, to 20 m tall; branches angled to terete, densely pilosulous to glabrescent. Petiole 5–30 mm, glabrous to densely pilosulous; leaf blade drying stiffly papery, suborbicular to broadly elliptic or ovate, 8–18(–30) × 2–12(–20) cm, adaxially glabrous, abaxially sparsely to densely pilosulous or rarely glabrescent, base broadly obtuse to cordulate, apex rounded to acute; secondary veins 6–12 pairs, spreading, sometimes with pilosulous domatia in abaxial axils; stipules ellipticoblong to ovate, 10–20 × 8–15 mm, pilosulous and weakly keeled, apex obtuse to rounded. Inflorescences densely puberulent to pilosulous; flowering heads sessile, 7 to numerous, 10– 12 mm in diam. across calyces, 20–25 mm in diam. Across corollas; bracteoles linear-spatulate, 2.5–3.5 mm, glabrous to sparsely pubescent and/or ciliolate. Calyx glabrous; ovary portion obconic, 1.2–2 mm; limb deeply lobed; lobes oblanceolate to spatulate, 1.5–2.5 mm, entire to ciliolate. Corolla yellow, outside glabrous, inside densely hairy; tube 5–6 mm; lobes narrowly elliptic, 2–2.5 mm, acute. Fruiting heads 15–20 mm in diam. Capsules 5–8 mm, weakly ridged; seeds ca. 1 mm."

***M. rotundifolia* (Roxb.) O. Kuntze**

"Trees, perhaps deciduous, to 30 m tall; branches angled to subterete, glabrous to glabrescent. Petiole 15–60 mm, glabrous to densely pilosulous; leaf blade drying papery, suborbicular to broadly elliptic or ovate, 9–25 × 6–20 cm (to 75 cm on seedlings and sprouts), adaxially glabrous to puberulent, abaxially sparsely to

densely pilosulous or tomentulose, base rounded to cordate, apex rounded to obtuse; secondary veins 5–7 pairs, spreading, sometimes with pilosulous domatia in abaxial axils; stipules elliptic-oblong to ovate, 13–50 × 5–30 mm, pilosulous, keeled, apex obtuse to rounded. Inflorescences densely puberulent to pilosulous; peduncles 1–3 mm (i.e., portion above articulation of subtending leaves but not including internode below node bearing inflorescence); flowering heads 1–5, 7–10 mm in diam. across calyces, 15–20 mm in diam. across corollas; bracteoles linear-spatulate, 1–1.5 mm, glabrous or sparsely pubescent. Calyx glabrous; ovary portion 1.5–3 mm; limb ca. 0.5 mm, subtruncate to lobed for ca. 1/2; lobes triangular, obtuse. Corolla yellowish white, outside glabrous, densely hairy inside; tube 2–3 mm; lobes narrowly oblanceolate, 4–5 mm, acute. Fruiting heads 10–16 mm in diam. Capsules 3–5 mm, weakly ridged, with persistent calyx thickened; seeds ca. 1 mm."

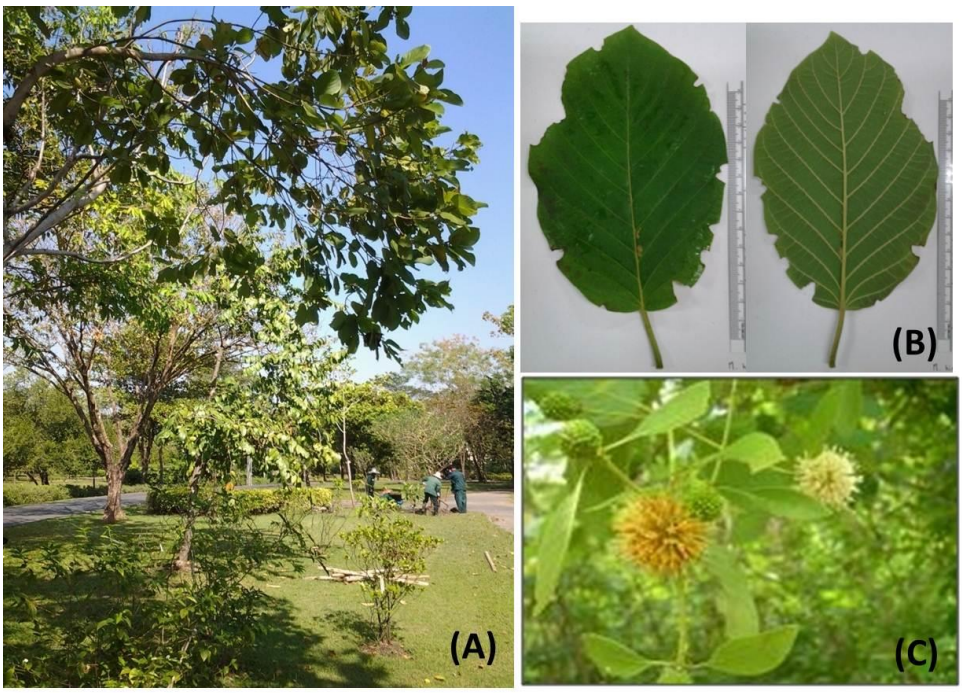


Figure 4 *M. hirsuta* Havil. The plant (A); leaves (B); and flowers (C).

Figure 5 *M. rotundifolia* (Roxb.) O. Kuntze. The plant (A); leaves (B); and flowers (C).

2.1.2 Pharmacological studies of the genus *Mitragyna*

Since the genus *Mitragyna* have been used as medicinal plants for a wide variety of diseases, many pharmacological studies have been carried out to confirm its therapeutic effect. The advances in pharmacological activities of *Mitragyna* genus are summarized in Table 2.

Table 2 Pharmacology studies of the genus *Mitragyna.*

Pharmacology	Plant	Part	Chemical	Reference
Antinociceptive and anti-inflammatory activities	*M. ciliata*	stem bark	-	Dongmo *et al*., 2003
	M. speciosa	Leaf	mitragynine pseudoindoxyl, 7-hydroxymitragynine	Takayama, 2004
	M. speciosa	leaf	7-hydroxymitragynine, mitragynine, speciogynine, speciociliatine, paynantheine	Horie *et al*., 2005
	M. speciosa	leaf	-	Reanmongkol *et al*., 2007
	M. parvifolia	stem bark	-	Gupta *et al*., 2009
	M. speciosa	leaf	-	Shaik Mossadeq *et al*., 2009
	M. speciosa	leaf	mitragynine	Utar *et al*., 2011
Anticancer activities	*M. ciliata*	stem bark	arjunolic acid	Diallo *et al*., 1995
	M. inermis	bark	inermiside I and II	Cheng *et al*., 2002
	M. speciosa	leaf	-	Ghazali *et al*., 2011
	M. diversifolia	stem bark	triterpenes	Cao *et al*., 2014
Cardiovascular activities	*M. ciliata*	stem bark	-	Dongmo *et al*., 2004
	M. inermis	stem bark	-	Ouédraogo *et al*., 2004

Table 2 (continued).

Pharmacology	Plant	Part	Chemical	Reference
Antioxidant	*M. speciosa*	leaf	-	Parthasarathy *et al*., 2009
Antispasmodic	*M. inermis*	bark		Sy *et al*., 2004
Antidiarrheal activities	*M. speciosa*	-	7-hydroxymitragynine	Mutsumoto *et al*., 2006
	M. speciosa	leaf	-	Chittrakarn *et al*., 2008
	M. diversifolia	stem bark	-	Jebunnessa *et al*., 2009
Acetylcholinesterase inhibition	*M. diversifolia*	-	mitradiversifoline	Cao *et al*., 2013
Antiplasmodial	*M. ciliata*	leaf	-	M´enan *et al*., 2006
	M. inermis	stem and root	-	Mustofa *et al*., 2000
Working memory effects	*M. speciosa*	leaf	mitragynine	Apryani *et al*., 2010
Anthelmintic	*M. parvifolia*	Leaf	-	Sahu *et al*., 2009
Antimalarial	*M. ciliata*	Leaf	-	Adjétey *et al*., 2007
Antidiabetic activities	*M. rotundifolia*	Leaf and bark	-	Kang et al., 2010

2.1.3 A narcotic plant; *Mitragyna speciosa*

In Thailand, *M. speciosa* is commonly known as kratom. It grows wild mainly in southern Thailand, northern Malaysia, Indonesia and Papua New Guinea. There are two types of *M. speciosa* as distinguished by the color of veins in the leaf; red and green (Figure 6) (Saingam *et al.*, 2013). *M. speciosa* has been used by natives of Thailand and other regions of Southeast Asia as an herbal drug for decades. Traditionally, *M. speciosa* was mostly used as a stimulant by Thai and Malaysian laborers and farmers to reduce the strain and fatigue of hard work (Ahmad and Aziz, 2012). *M. speciosa* constituents acting on the central nervous system has been attributed to various indole alkaloids such as mitragynine [1] (the major constituent of this plant) and 7-hydroxymitragynine [2] (a minor constituent of this plant) which exhibits opioid agonistic activities and produce effects similar to morphine (Figure 7) (Takayama, 2004).

In 1979, the Thai government announced the control of *M. speciosa* by enacted the Narcotics Act B.E. 2522 in the same enforcement as cannabis, opium and hallucinogenic mushrooms. It was classified in Category V of a five-category classification of narcotic substances. Therefore, it is illegal to buy, sell, import, or possess *M. speciosa*. However, the villagers in the south of Thailand popularly consume this plant by chewing fresh leaves or drinking boiled leaves. According to a belief that can help maintain their working capacity with more efficiency and giving them a longer endurance. Recently, the Narcotic Control Technology Center (NCTC) reported on an illegal Kratom cocktail named “4×100” formula which was emerged and widely used among teenage users in southern provinces of Thailand for providing mental and physical stimulation. Kratom cocktail is a three main ingredients of mixture of boiled *M. speciosa* leaves, cola soft drink and codeine-or diphenhydramine- cough syrup which the powder peeled from the inside of fluorescent light bulbs, mosquito coils, herbicide and tranquilizer add (Chittrakarn *et al.*, 2012; Tungtananuwat and Lawanprasert, 2010; Hassan *et al.*, 2013). Consumtion of this plant in long term can cause health problems (Chittrakarn *et al.*, 2012; Lesiak *et al.*, 2014). Moreover, there are the fatal case reports from abuse of *M. speciosa*

(Tungtananuwat and Lawanprasert, 2010; Neerman *et al.*, 2013) Therefore, the correct and accurate identification of *M. speciosa* is an important issue not only for medicinal purpose but also forensic investigation.

In a previous research, use of microscopic examination cannot identify *M. speciosa* because of similarities of the anatomical fragmentation of the plant materials among species within the same genus. Various species of the genus *Mitragyna* can be difficult to distinguish by anatomically microscopic method (Kowalczuk *et al.*, 2013). Mitragynine is a major psychoactive substance which occurs exclusively in *M. speciosa* (Takayama *et al.*, 2002). The chromatographic methods previously reported for identification of mitragynine in plant materials and *M. speciosa* preparations include gas chromatography (GC) (Chan *et al.*, 2005), liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) (Kikura-Hanajiri *et al.*, 2009), high performance liquid chromatography (HPLC) (Chittrakarn *et al.*, 2012), and high performance liquid chromatography with diode array detection (HPLC-DAD) (Parthasarathy *et al.*, 2013). Moreover, the analysis of mitragynine in biological fluids has been reported in rat plasma by LC–MS/MS (De moraes *et al.*, 2009), in rat serum by high-performance liquid chromatographic method (Janchawee *et al.*, 2007), in human urine by high performance liquid chromatography–tandem mass spectrometry (Lu *et al.*, 2009) and in rat and human urine using liquid chromatography–linear ion trap mass spectrometry (Philipp *et al.*, 2009). Besides, DNA molecular markers by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the internal transcribed spacer (ITS) region can differentiate *M. speciosa* from other psychoactive plants and also from other closely related species (Sukrong *et al.*, 2007; Maruyama *et al.*, 2009).

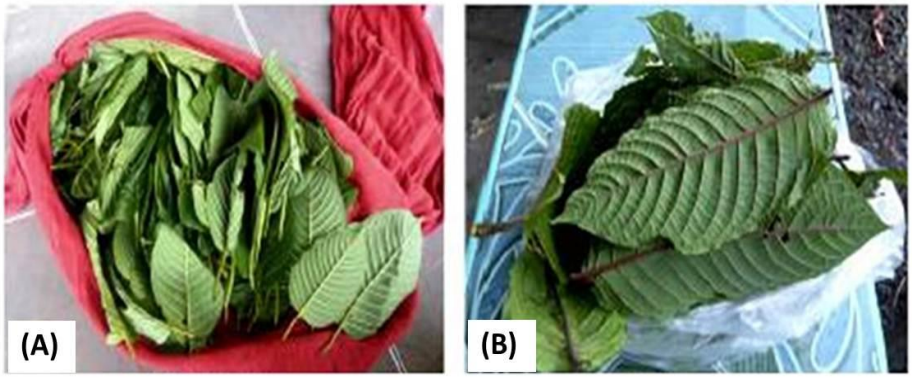
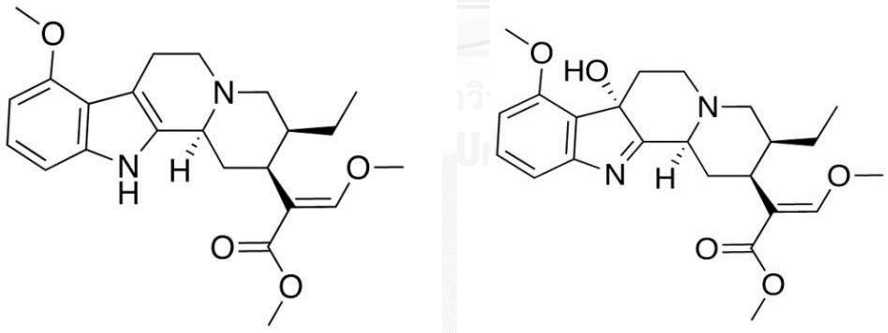


Figure 6 Green veined type (A) and red veined type (B) of *M. speciosa* (Saingam *et al.*, 2013).



[1] mitragynine **[2]** 7-hydroxymitragynine

Figure 7 Chemical structures of mitragynine [1] and 7-hydroxymitragynine [2].

2.2 Genetic-based technique for the identification of plants

Correct and accurate plant identification is essential to ensure safety and quality of herbal medicines. In practice, the identification of plant materials at the species level relies on the morphological features and chemical analyses. However, the number of specially trained experts in plant identification may be limited. Besides, the morphology method is not suitable for identifying the processed plant materials. Regarding chemical analysis, chemical properties are influenced by both genetic and environmental factors. Therefore, molecular markers are considered to provide more reliability for the authentication of plant materials at the DNA level.

In the past few decades, DNA-based markers have been applied to authenticate several important medicinal plants materials (Manissorn *et al.*, 2010; Boonsom *et al.*, 2012). DNA can be recovered from fresh, dried and even processed plant materials and is not affected by the age of the plant or external factors. Moreover, a small amount of sample is sufficient for analysis.

The internal transcribed spacer (ITS) regions have been widely used as a powerful tool for plant identification. This section of the genome includes the 18S, 5.8S and 25S genes which encode for ribosomal RNA (rRNA). The intervention for the spacer between the 18s and 5.8s genes is ITS1, the intervention for the spacer between the 5.8s and 25s genes is ITS2. A third spacer, the large intergenic spacer (IGS), is found between the 3' end of the 25s and the 5' external transcribed spacer (ETS). The rRNA coding regions have low rate of polymorphism among species which conserve nucleotide sequences throughout plant species, whereas the ITS regions show higher rates of sequence divergence between closely related species (Ghada *et al.*, 2009). Therefore, the ITS region has been frequently used for molecular analysis. It has also been used for molecular identification of medicinal plants such as *Dendrobium officinale* (Ding *et al.*, 2002), *Panax* species (Kim *et al.*, 2007), *Plantago* herb (Sahin *et al.*, 2007), *Hypericum perforatum* (Howard *et al.*, 2009) and *Phyllanthus amarus* (Manissorn *et al.*, 2010).

In addition, the ITS2 region (Figure 8), a section of ITS, has become an important nuclear locus for molecular authentication of plants because it is easy to

amplify even from small quantities of DNA and has a high degree of variation even between closely related species. Moreover, the interspecific variation of the ITS2 sequence can be useful in phylogenetic and barcoding applications (Song *et al.*, 2012; Xin *et al.*, 2013).

There are various types of molecular markers that are based on polymerase chain reaction (PCR). Conventional PCR techniques involve amplification of particular DNA amplicon by specific oligonucleotide primers that allow qualitative detection of the different organisms. Molecular techniques such as DNA barcoding, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphisms (SNP) have recently been used for plant identification studies.

Lately, real-time PCR has greatly applied to the molecular authentication such as in agricultural analysis, food research, clinical diagnoses and forensic analysis. Real-time PCR was developed in the early 1990s and allows the PCR amplification using fluorescent chemistries to be monitored in real time (Soliman and Othman, 2009). It is used to fast detect and quantify specific sequences in a DNA sample with accuracy and high sensitivity over a wide dynamic range. The use for detection and quantitation of a product can be divided into two categories; sequence specific probe based assays and non-specific dsDNA intercalating dye based assays (Sharma and Dasgupta, 2012). The main advantages of real-time PCR over conventional PCR are that real-time PCR reactions are run and data are evaluated in a closed-tube, thus contamination are reduced and post-amplification manipulation is eliminated.

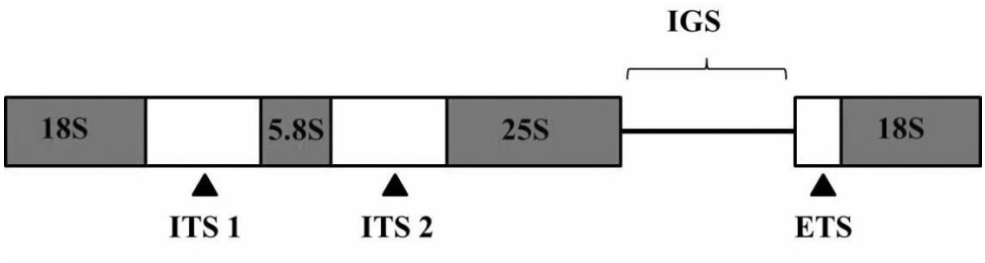


Figure 8 Schematic diagram of the nucler rDNA contains 18S, 5.8S and 25S genes. ITS1 and ITS2 fragment are located between 18S and 5.8S rRNA subunits and between 5.8S and 25S rRNA subunits. IGS is composed between the 3' end of the 25S and the 5' ETS.

2.2.1 Single nucleotide polymorphism

Single nucleotide polymorphism (SNP) is the basis for most molecular markers both in animal and plant genomes. Single nucleotide polymorphism is a DNA sequence variation occurring when a single bases (A, T, G or C) within the genome differs among members of a species. These sequence differences are likely SNPs or insertions/deletions (indels) (Komar, 2009; Arif *et al.*, 2010). SNP can serve as valuable biological markers. Thus, genetic tests and methods allowing for rapid and accurate determine of a SNP marker. There are several SNP markers that are used for the identification of plants such as tomato cultivate (Gómez and Maloo, 2009), ginseng (Sun *et al*., 2011; Wang *et al.*, 2011; Chen *et al.*, 2013), Pericarpium Citri Reticulatae and Citri Unshius Pericarpium (Wang *et al.*, 2012).

2.2.2 Melting curve analysis

Melting curve analysis is an assessment of the dissociation-characteristics of double-stranded DNA during heating. As the temperature is increased, the double strand begins to dissociate leading to an increase in the absorbance intensity. The temperature at which 50% of DNA is denatured is known as the melting point. Different sequences can be detected and discriminated according to measurement of their specific melting temperature (Tm). The shape and position of melting peak is a function of the GC/AT ratio, length and nucleotide sequences (Ririe *et al.*, 1997). DNA-binding dyes in real-time PCR are employed to indicate the emission of dye fluorescence that is to perform a melting curve analysis to identify the specific amplification reaction (Figure 9).

Melting curve analysis provides a rapid detection and quantification of specific DNA with high sensitivity method (Jiang *et al.*, 2014). It has been used to identify and differentiate many closely related plant species, such as *Cimicifuga foetida* (Xue *et al.*, 2009), *Prunus avium* (Serradilla *et al.*, 2013) and *Prunus persica* (Shang *et al.*, 2014).

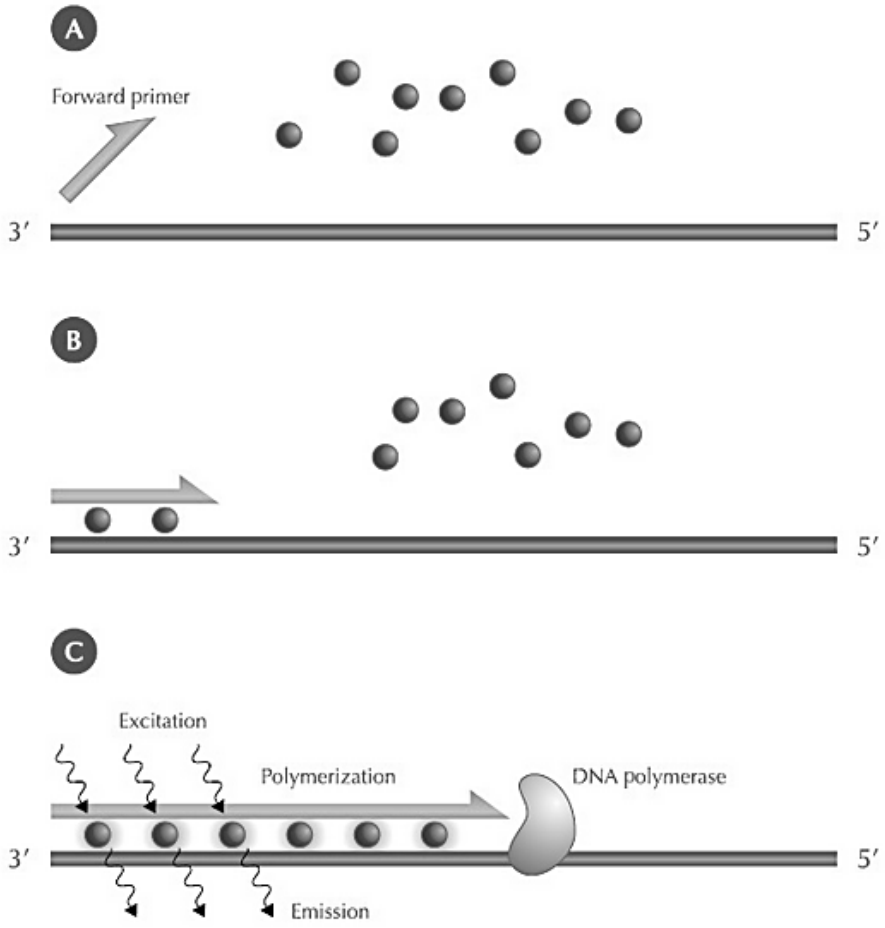


Figure 9 Principle of DNA-binding dyes in real-time PCR. (A) DNA is denatured and dye molecules are free in the reaction mix. (B) Primers anneal and dye molecules bind to the dsDNA. (C) DNA polymerase elongates the template and more dye molecules bind to the product formed resulting in exponential increase in the fluorescence level. (Life technology, 2014).

CHAPTER III

MOLECULAR ANALYSIS OF PLANTS IN THE GENUS MITRAGYNA AND DEVELOPMENT OF SPECIES-SPECIFIC MARKER IN THE ITS2 REGION FOR THE IDENTIFICATION OF *M. SPECIOSA*

3.1 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. *M. speciosa*, commonly known as Kratom, is a narcotic plant and has particular medicinal importance such as antinociceptive and anti-inflammatory (Shaik Mossadeq *et al*., 2009), antidiarrheal (Chittrakarn *et al*., 2008), anticancer (Ghazali *et al*., 2011) and antidiabetic (Purintrapiban *et al*., 2011). Its 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 a previous research, microscopic examination cannot be used to differentiate *M. speciosa* from other *Mitragyna* species because of the similarities of the plant materials from the same genus (Kowalczuk *et al.*, 2013). Several chemical methods have been developed using different techniques (Chan *et al.*, 2005; Kikura-Hanajiri *et al.*, 2009; Parthasarathy *et al.*, 2013). However, the 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 several 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 chapter, the patterns of interspecific variation in the ITS2 region of four *Mitragyna* species were analyzed. The species-specific marker was developed for a rapid and accurate identification and differentiation of *M. speciosa* from the other *Mitragyna* species.

3.2 Materials and methods

3.2.1 Plant materials

Samples of four *Mitragyna* plants were obtained from various locations in Thailand (Table 3) and preserved at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. All samples were identified by Assoc. Prof. Dr. Nijsiri Ruangrungsi and Assoc. Prof. Thatree Padungcharoen of Chulalongkorn University.

3.2.2 DNA extraction

The fresh leaves 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) following the manufacturer's procedure. Briefly, the lysis buffer was added to ground tissue, and then 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 used.

Table 3 Plant samples used in this study.

Samples	Code	Place of collection (Thailand, Province)	Voucher no.
M. speciosa (Roxb.) Korth.	MS-01	Bangkok	MUS-5512-1
	MS-03	Chumporn	MUS-5601-1
	MS-06	Bangkok	MUS-5512-3
	MS-09	Nonthaburi	MUS-5602-2
M. diversifolia	MD-01	Bangkok	MUS-5512-4
(Wall. ex G.Don) Havil.	MD-03	Khon kaen	MUS-5604-1
	MD-05	Nakorn pathom	MUS-5603-2
M. hirsuta Havil.	MH-01	Bangkok	MUS-5512-5
	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

3.2.3 Sequence analysis of ITS2

Sequences alignment of the retrieved 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) from GenBank were constructed using CLUSTALW program.

3.2.4 Design of species-specific primers

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 10). 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 4.

3.2.5 Multiplex PCR amplification

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.

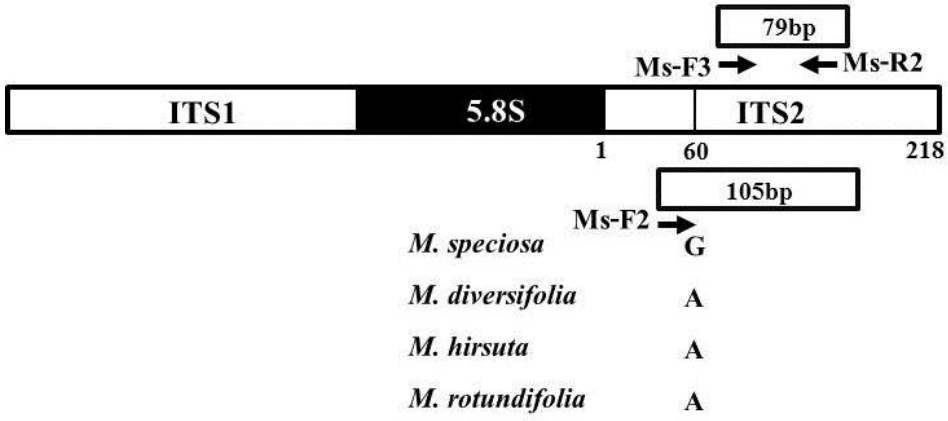


Figure 10 Structure of ITS1, 5.8S and ITS2 region. Sharp arrows indicate orientation and approximate position of species-specific primer (Ms-F2) and common primers (Ms-F3 and Ms-R2). PCR products generated from Ms-F2 and Ms-R2 primer pair, and Ms-F3 and Ms-R2 primer pair were 105 and 79 bp, respectively.

Table 4 The sequence of primers used in this study.

Primer name	Orientation	Primer sequence (5'-3')	Position in ITS2 region	Tm (°C)
Ms-F2	Forward	TGG CCT CCC GTG CCC TG	514-533	57.3
Ms-F3	Forward	CGG CCT AAA TGC GAG TCC TC	436-452	62.4
Ms-R2	Reverse	CGG CAC GAC AGA AAT CGA GTC	462-481	61.4

3.3 Results

3.3.1 Analysis of nucleotide polymorphism in ITS2 region

The fragments of ITS2 sequences from the four *Mitragyna* species were examined for nucleotide polymorphism at the interspecies level (Table 5). 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.

The identification of *M. speciosa* is essential for both forensic and medicinal usage. Nucleotide polymorphism analysis can distinguish *M. speciosa* from the other *Mitragyna* species. The interspecific nucleotide diversity of *M. speciosa* and the others is represented by six nucleotides at 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.

3.3.2 Species-specific marker of *M. speciosa*

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

In order to determine the specificity of this method, an annealing temperature range from 58 to 68 ℃ 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 that can be used as a

Table 5 Nucleotide polymophisms in ITS2 of each *Mitragyna* species.

	Position of nucleotide polymorphism										
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

tool to discriminate *M. speciose* 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 11). The size of PCR products was examined by 2.5% agarose gel electrophoresis and visualized by UV transillumination.

3.4 Discussion

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). 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 a suitable DNA region for molecular identification of *M. speciosa*.

The present study was to find and develop nucleotide polymophism position into effective tools for accurate identification of *M. speciosa.* The species-specific 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 nucleotide polymorphism 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 species-specific markers based on ITS2 region is a specific method for authentication and discrimination of *M. speciosa* from *M. diversifolia*, *M. hirsuta,* and *M. rotundifolia.* Additionally, *M. rotundifolia* could be discriminated from the other related species of *Mitragyna* genus by this nucleotide polymorphism site. However, *M. diversifolia* and *M. hirsuta* could not be differentiated by the ITS2 region. Therefore, the study of DNA sequences in the other regions is needed for development of species specific molecular marker to distinguish all plants in the genus *Mitragyna*.

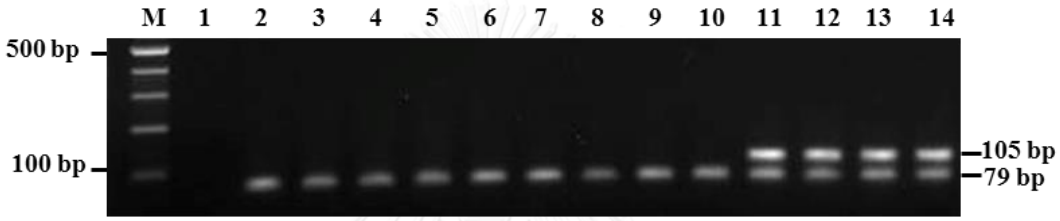


Figure 11 Species-specific identification of *M. speciosa*. M: VC 100 bp plus DNA marker; lane 1-5: *M. speciosa* (MS-01-MS-05), lane 6-8: *M. diversifolia* (MD-01-MD-03), lane 9-11: *M. hirsuta* (MH-01-MH-03), lane 12-14: *M. rotundifolia* (MR-01-MR-03).

CHAPTER IV

A MELTING CURVE ANALYSIS OF PLANTS IN THE GENUS *MITRAGYNA*

4.1 Introduction

M. speciosa is used for psychoactive properties and medicinal purposes for its opium-like effect. The authentication of *M. speciosa* is essential for both medicinal and forensic investigation. DNA molecular markers could differentiate *M. speciosa* from psychoactive plants and also from closely related species by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Sukrong *et al.*, 2007; Maruyama *et al.*, 2009). However, conventional PCR requires detection by gel electrophoresis, which is time consuming, is prone to contamination and can discriminate from size-based only.

Recently, the development of real-time PCR has been extensively applied in agricultural and food research, gene expression analysis, clinical diagnoses and forensic analysis. It can also identify and differentiate the species by melting curve analysis (Xue *et al.*, 2009; Traunšek *et al.*, 2011; Serradilla *et al.*, 2013). Melting curve analysis provides a rapid detection and quantification of specific DNA with high sensitivity and specificity (Jiang *et al.*, 2014). Different sequences can be discriminated according to the measurement of their specific melting temperature (Tm), leading to the disposal of post-PCR gel electrophoresis. A small amplicon size was shown to facilitate discrimination by distinct Tm of the real-time PCR product (Mouillesseaux *et al.*, 2003); thus DNA fragment can be detected from processed samples (Soares *et al.*, 2013).

In this chapter, a melting curve analysis by real-time PCR, which is a specific and sensitive technique, was exploited for the identification and discrimination of the narcotic plant, *M. speciosa*, from its closely related species; *M. diversifolia*, *M. hirsuta* and *M. rotundifolia*.

4.2 Materials and methods

4.2.1 Plant materials and DNA extraction

Twenty-four specimens of four *Mitragyna* species were obtained from various habitats in Thailand (Table 6) and examined by Assoc. Prof. Dr. Nijsiri Ruangrungsi and Assoc. Prof. Thatree Padungcharoen of Chulalongkorn University. Herbarium voucher specimens were preserved at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The extraction of the total DNA from the fresh leaves of plant specimens were performed using a DNeasy Plant Mini Kit (Qiagen, Germany) as described previously. The quantity was determined by measuring the absorbance at 260 using a SpectramaxM5 spectrophotometer (Molecular Devices, Sunnyvale, CA). All DNA extracts were stored at -20 ℃ until used.

4.2.2 Primer design

The sequences of primers are listed in Table 7. PCR amplification was run in three different combinations, including a primer pair of Ms-F1/Ms-R2, Ms-F3/Ms-R2 and a multiplex primer of Ms-F2/Ms-F3/ Ms-R1. These primer pairs generated PCR products of 538bp, 98bp, 78bp in length and multiplex primer generated PCR products of 98bp and 70bp in length, respectively. A schematic diagram of the primers used for the real-time PCR analyses is shown in Figure 12.

4.2.3 Melting curve analysis

The real-time PCR and subsequent melting step was applied on a CFX 96 Real-Time System (Bio-Rad, USA). The optimized protocol was performed with a final volume of 20 µL, consisting of 10 ng of template DNA, 10 µL of EvaGreen Supermix (Bio-Rad, USA), and 0.8 µl of each forward and reverse primer. The annealing

Table 6 Twenty-four samples of *Mitragyna* species.

Samples	Code	Place of collection (Thailand, Province)	Voucher no.	Notes
M. speciosa	MS-01	Bangkok	MUS-5512-1	
(Roxb.) Korth.	MS-02	Bangkok	MUS-5512-2	
	MS-03	Chumporn	MUS-5601-1	red-veined
	MS-04	Pattani	MUS-5601-2	leaf
	MS-05	Yala	MUS-5601-3	
	MS-06	Bangkok	MUS-5512-3	
	MS-07	Bangkok	MUS-5601-4	
	MS-08	Nonthaburi	MUS-5602-1	green-veined
	MS-09	Nonthaburi	MUS-5602-2	leaf
	MS-10	Chachoengsao	MUS-5603-1	
M. diversifolia	MD-01	Bangkok	MUS-5512-4	
(Wall. ex G.Don)	MD-02	Bangkok	MUS-5602-2	
Havil.	MD-03	Khon kaen	MUS-5604-1	
	MD-04	Khon kaen	MUS-5604-2	
	MD-05	Nakorn pathom	MUS-5603-2	
	MD-06	Nakorn pathom	MUS-5603-3	
M. hirsuta Havil.	MH-01	Bangkok	MUS-5512-5	
	MH-02	Sukothai	MUS-5603-4	
	MH-03	Kampaengpet	MUS-5603-5	
	MH-04	Nakhon ratchasima	MUS-5604-3	
M. rotundifolia	MR-01	Bangkok	MUS-5512-6	
(Roxb.) Kuntze	MR-02	Bangkok	MUS-5601-5	
	MR-03	Nakhon ratchasima	MUS-5604-4	
	MR-04	Nakhon ratchasima	MUS-5604-5	

Table 7 The sequences of primers used in the PCR amplifications.

Primer name	Orientation	Primer sequence (5'-3')	Tm (°C)
Ms-F1	Forward	GAA TCC TGC AAA ACG CAC G	56.7
Ms-F2	Forward	TGG CCT CCC GTG CCC TG	62.4
Ms-F3	Forward	CGG CCT AAA TGC GAG TCC TC	61.4
Ms-R1	Reverse	ACA GAA ATC GAG TCG GGT CA	57.3
Ms-R2	Reverse	CGG CAC GAC AGA AAT CGA GTC	61.8

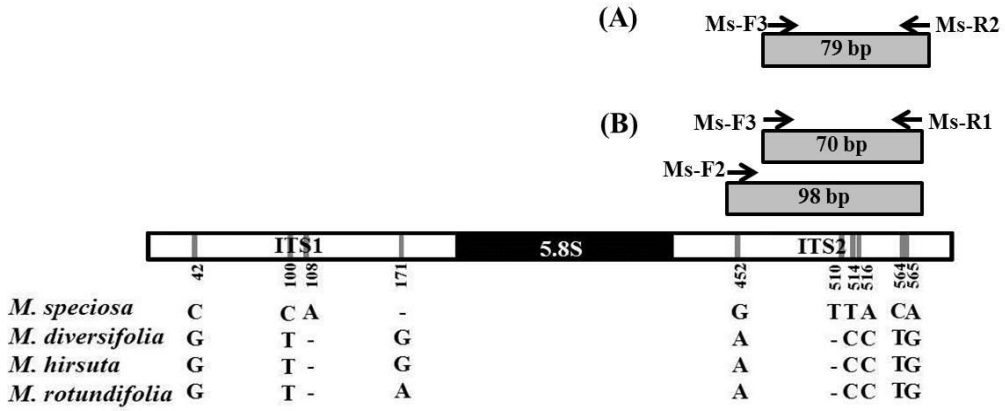


Figure 12 Structures of ITS1, 5.8S and ITS2 region. Sharp arrows indicate orientation and approximate position of primers. (A) PCR product of *M. speciosa* from Ms-F3/Ms-R2 primer pair. (B) PCR product of *M. speciosa* from Ms-F2/ Ms-F3/Ms-R1 multiplex primers.

temperature of real-time PCR method is the important parameters for test specificity. To find the optimal annealing temperature of reaction, a range of temperatures were tested. The following thermal cycling conditions were used: an initial denaturation step at 98 ℃ for 2 min, and 35 cycles of denaturation at 98 ℃ for 40 s, annealing at 55-63 ℃ for 30 s. After final PCR cycle, a melting curve analysis of the PCR products was performed by heating to 70-95 ℃ with continuous measurement of the fluorescence to verify the PCR products. Four *Mitragyna* species were tested and a control sample without DNA template was also included in the runs. The size of PCR products was examined by 2.5% agarose gel electrophoresis and visualized by UV transillumination.

4.2.4 Specificity and sensitivity of the real-time PCR

The specificity of the real-time PCR assay was estimated by testing the amplification of DNA from twenty different samples (Table 6) including two kinds of *M. speciosa*, 5 samples of red veined leaf and 5 samples of green veined leaf, 6 samples of *M. diversifolia*, 4 samples of *M.hirsuta* and 4 samples of *M. rotundifolia*, were tested with the melting curve analysis in section 4.2.3. Each sample was amplified with Ms-F3/Ms-R2 primer pair in triplicate.

Ten-fold serial dilutions (from 10 ng μL^{-1} to 1 pg μL^{-1}) of the *M. speciosa* DNA were determined. The standard curve for *M. speciosa* was obtained by plotting the threshold cycle (Ct) values against the log values of DNA concentration of standard samples. The slopes of the standard curves (S) and correlation coefficients (R^2) were calculated to evaluate the real-time PCR efficiency (E) and the linear range of detection with the formula $E = (10^{-1/s} - 1) \times 100$ (Serradilla *et al.*, 2013). The limit of detection (LOD) was established as the minimum concentration of *M. speciosa* DNA which evaluate the sensitivity of the real-time PCR.

4.3 Results

4.3.1 Melting curve analysis of plant in the genus *Mitragyna*

The melting curves of the real-time PCR products of four *Mitragyna* species; *M. speciosa*, *M. diversifolia*, *M. hirsuta* and *M. rotundifolia* using Ms-F1/Ms-R2, Ms-F3/Ms-R2, and Ms-F2/Ms-F3/Ms-R1 primer pairs are plotted and summarized in Table 8.

For 538 bp amplicon, a PCR fragment generated from *M. speciosa* DNA had different nucleotide polymorphisms at positions 42, 100, 108, 171, 452, 510 and 516 (Figure 12). However, the melting curve analysis of four *Mitragyna* species had same Tm range at 90.4 ± 0.2 ℃. The Tm values of 538 bp amplicon were higher than 78 bp amplicon and could not discriminate *M. speciosa* from other *Mitragyna* species (Table 8).

The melting peak of 78 bp amplicons enabled the discrimination of *M. speciosa* from other *Mitragyna* species with distinct peaks at 83.8 ± 0.2 ℃ and 85.4 ± 0.2 ℃, respectively (Figure 13A). Accordingly of, there were nucleotide polymorphisms at positions 510, 514 and 516 of *M. speciosa* as compared with other species.

In addition, two forward primers (Ms-F2, Ms-F3) and one reverse primer (Ms-R2) generated overlapping two PCR products of 98 bp and 70 bp for *M. speciosa* and 70 bp for the other *Mitragyna* plants. Thus, the amplification products of *M. speciosa* showed two distinct melting peaks at 86.6 ± 0.2 ℃ and 83.0 ± 0.2 ℃ whereas the amplification products of other *Mitragyna* plants showed only one specific melting peak at 83.0 ± 0.2 ℃ (Figure 13B).

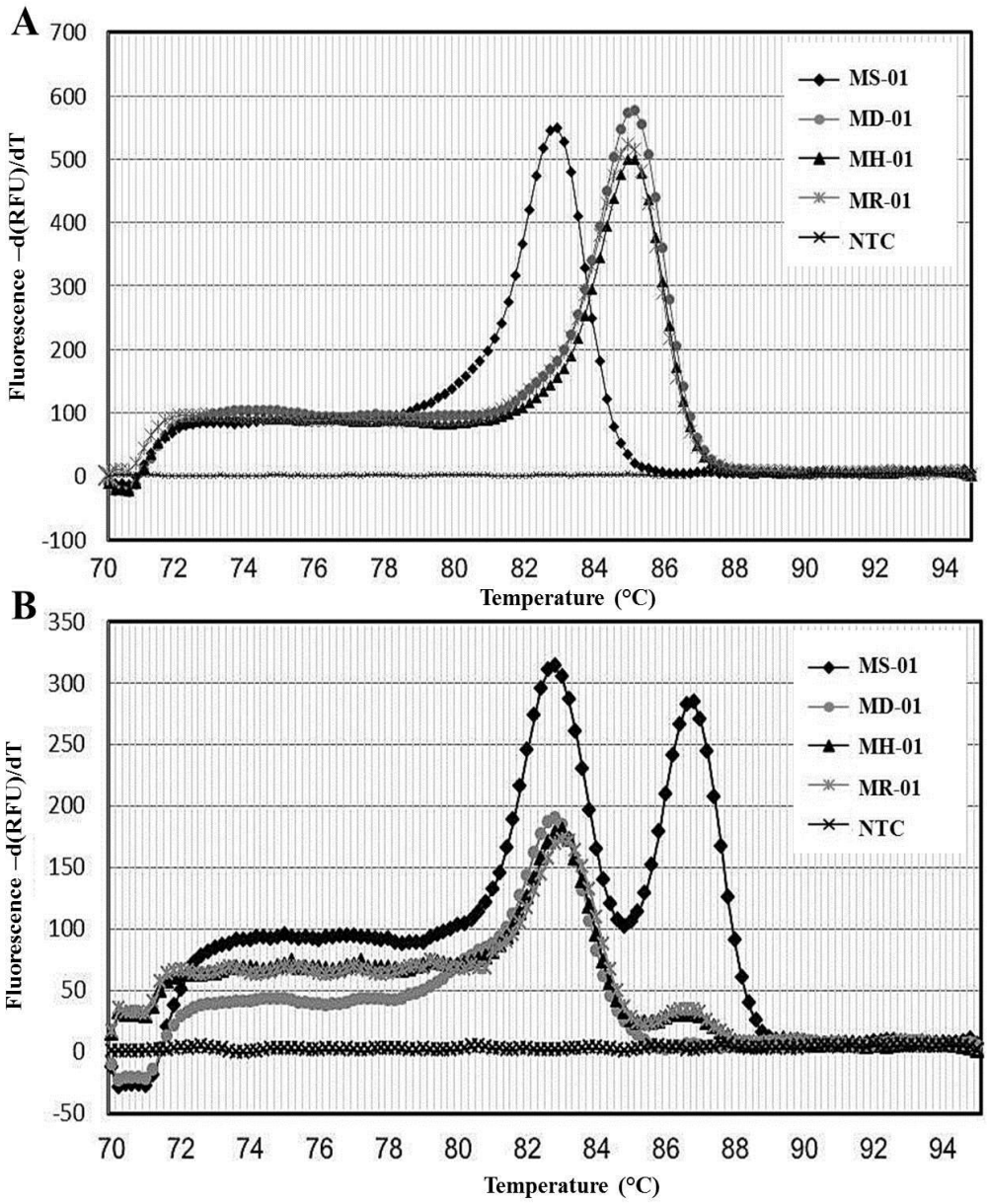


Figure 13 Melting curve analysis profiles of *Mitragyna* plants DNA from the 2 tested of primers (A) Ms-F3/Ms-R2. (B) Ms-F2/Ms-F3/Ms-R1.

Table 8 Tm values obtained with the different primer pairs for each *Mitragyna* species.

Primer pair	***Mitragyna* plants**	**Amplicon (bp)**	**Tm (C°)**
Ms-F1/Ms-R2	*M. speciosa*	538	90.4
	M. diversifolia	537	90.4
	M. hirsuta	537	90.4
	M. rotundifolia	537	90.4
Ms-F3/Ms-R2	*M. speciosa*	79	83.2
	M. diversifolia	78	85.4
	M. hirsuta	78	85.4
	M. rotundifolia	78	85.4
Ms-F2/Ms-F3/Ms-R1	*M. speciosa*	98, 70	86.6, 83.0
	M. diversifolia	69	83.0
	M. hirsuta	69	83.0
	M. rotundifolia	69	83.0

4.3.2 Specificity and sensitivity of the real-time PCR

The specificity of the Ms-F3/Ms-R2 primer pair for *M. speciosa* was confirmed using genomic DNA from *Mitragyna* plants from different geographical areas. Melting peak experiments gave specific results for the *M. speciosa* with Tm value at 83.8 ± 0.2 °C, unlike the Tm values of the other *Mitragyna spp*. (85.4 ± 0.2 °C) (Figure 14).

The sensitivity range of the assay was determined by using 10-fold dilutions of *M. speciosa* DNA template (Figure 15), and the linearity was maintained from 4 orders of magnitude. The efficiency of the method developed was calculated on the basis of the slope of the standard curve obtained using the Ms-F3/Ms-R2 primer pair with equation of y = -3.432x+31.157 which corresponding to a PCR amplification efficiency of 95.6% where R^2 = 0.999 (Figure 16). The detection limit of the real-time PCR assay was revealed at least 1 pg of the target DNA.

4.4 Discussion

The real-time PCR technique offers rapid and specific assays with melting curve analysis that generated Tm from temperature at which 50% of DNA is denatured. It depends on the product size as well as the GC content of the sequence (Renouf, *et al.*, 2006). Targeted product with a distinct sequence can be differentiated from each other according to their specific Tm (Serradilla *et al.*, 2013), thus dispensable of any post-PCR gel electrophoresis and sequencing (Chomic *et al.*, 2011).

This chapter was aimed at the development and evaluation of melting curve analysis of real-time PCR products for detection and discrimination of *M. speciosa* from the three closely related species. The Ms-F3/Ms-R2 primer pair amplified 78 bp product showed a specific Tm for *M. speciosa* at 83.2 ± 0.2 °C which discriminate it from the other species which showed a specific Tm at 85.4 ± 0.2 °C. In comparison, the 538 bp amplicons failed to provide a specific Tm for identification of *Mitragyna* species. PCR product size was a critical factor for discrimination organisms (Chomic *et*

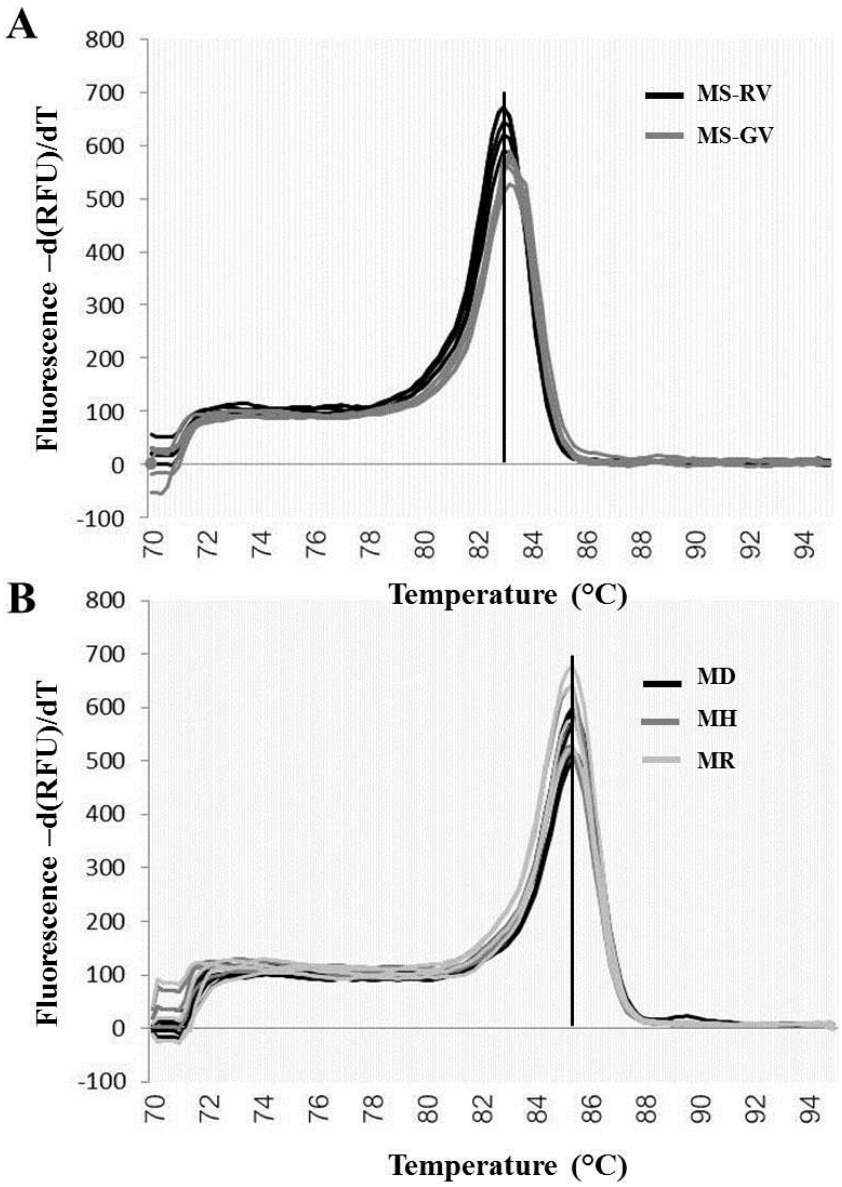


Figure 14 Specificity of the real-time PCR assay (A) melting peak of PCR product generated from *M. speciosa* including red-veined leaf (MS-RV) and green-veined leaf (MS-GV), with Tm value of 83.2 ± 0.2 °C. (B) melting peak of PCR product generated from other *Mitragyna* species including *M.diversifolia* (MD), *M. hirsuta* (MH) and *M. rotundifolia* (MR), with Tm value of 85.4 ± 0.2 °C.

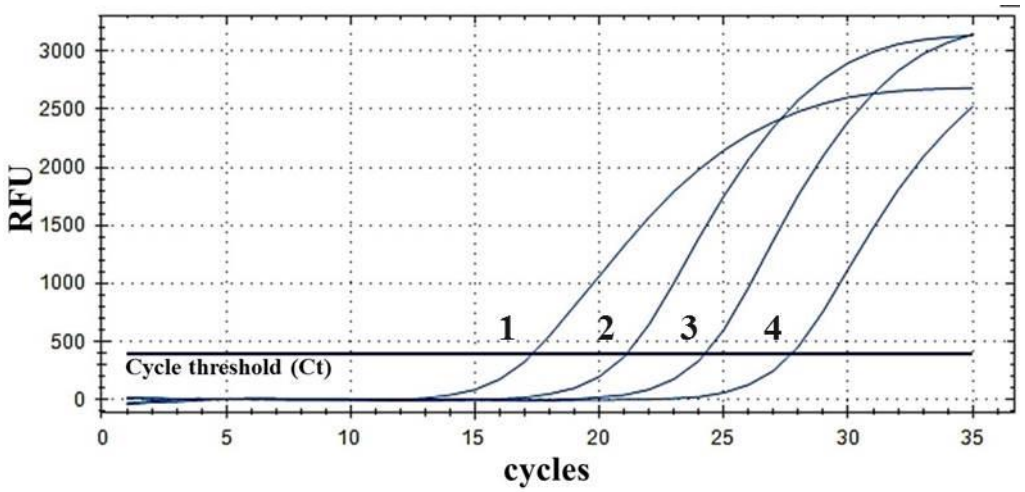


Figure 15 Amplification plot for 10-fold serial dilutions ranging from 10 ng μL^{-1} to 0.01 ng μL^{-1} of *M. speciosa* DNA (1-4).

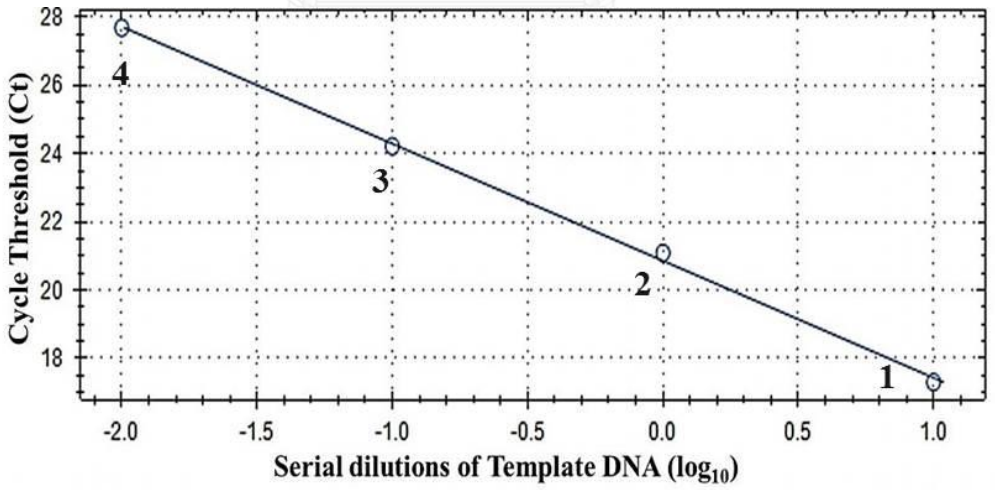


Figure 16 Standard curves of *M. speciosa* DNA based on 10-fold dilutions obtained by plotting average Ct values versus the log concentration DNA.

al., 2011). Compared to the multiplex PCR reaction, primers Ms-F2/Ms-F3/Ms-R1 generated two distinct peaks for 98 bp and 70 bp products while the other species showed only a single peak of 70 bp product. Therefore, the Ms-F3/Ms-R2 primer pair and multiplex primers Ms-F2/Ms-F3/Ms-R1 allowed us to discriminate *M. speciosa* from the other species. However, the Ms-F3/Ms-R2 primer pair gave a more simplified result.

There are two kinds of *M. speciosa*, the red-veined and the green-veined leaf. The red veined variety is supposed to have the stronger biological activities (Chittrakarn *et al*., 2008). Based on rDNA, ITS sequences showed 100% similarity for the red-veined and green-veined leaf (Srisiri *et al*., 2007). However, sequences of *rbc*L gene and *trn*T-F spacer showed polymorphism between red-veined and green-veined leaf of *M. speciosa* (Srisiri *et al*., 2007). Therefore, the study of DNA sequences of other regions may be suitable for develop molecular marker to distinguish *M. speciosa* type.

Melting curve was used to evaluate the specific PCR product (Xue *et al*., 2009). The specificity of the primer pair to amplify *M. speciosa* was verified using genomic DNA from *Mitragyna* species from different geographical regions. The efficiency of the method developed was calculated on the basis of the slope of the standard curve obtained using the I2MsF/I2MsR primer pair for *M. speciosa* DNA dilutions as templates for real-time PCR. A good linear relationship between the Ct values and the $\log_{10}$ DNA concentration was obtained with this method, with an R^2 value close to 0.99. The slope of the standard curve, Ct values against $\log_{10}$ DNA concentration, should range between -3.1 and -3.6 which corresponding to a PCR efficiency of 80% and 110%, respectively. R^2 value should be ≥ 0.98 (Serradilla *et al*., 2013). These values of Ct and efficiency demonstrated the utility of the real-time PCR system to identify *M. speciosa*.

CHAPTER V

THE APPLICATION OF A MELTING CURVE ANALYSIS TO DETECT *M. SPECIOSA* IN FORENSIC SAMPLES

5.1 Introduction

Currently, the plant materials of *M. speciosa* are widely commercially available on the internet resulting in the increase of abuses (Kowalczuk *et al.*, 2013). There was a case report that described a popular preparation named "Krypton", which contained a blend of powdered *M. speciosa* leaves and *O*-desmethyltramadol and caused unexpected the death (Kronstrand *et al.*, 2011). In the south of Thailand, the spread of kratom cocktail has become a terrible local issue (Tungtananuwat and Lawanprasert, 2010). Kratom cocktail is a mixture of boiled *M. speciosa* leaves, cola soft drink and codeine-or diphenhydramine-cough syrup (Hassan *et al.*, 2013). It was used for mental and physical stimulation. The formula depends on individual kratom users. To identify the samples that contain *M. speciosa*, an assay for the analysis of mitragynine was detected by high performance liquid chromatography (HPLC) was proposed (Chittrakarn *et al*., 2008). However, no rapid and suitable methods for the detection and quantification of *M. speciosa* in any raw materials or extracts were reported prior to this study.

In this chapter, we aim to apply the melting curve analysis based on real-time PCR method for the detection of *M. speciosa* DNA in forensic samples.

5.2 Materials and methods

5.2.1 Preparation of *Mitragyna* samples

The water extracts of *M. speciosa, M. diversifolia, M. hirsuta* and *M. rotundifolia* were prepared. Fresh leaves were washed with water and dried in the oven at 40 °C. The dried leaves were pulverized and boiled in water for 30 min. Negative control was a mixture of cola soft drink and cough syrup without any of *Mitragyna* spp. Four samples of kratom cocktail were obtained from various locations in Thailand (Figure 17). All samples were stored at 4 °C until analysis.

5.2.2 DNA extraction

Additional steps described in Kazi et al., 2013 were done before DNA extraction. Two hundred µl of sample was placed in a 1.5 ml microcentrifuge tube and 200 µl of 95% (v/v) ethanol was added. The solution was shaken for 5 min, centrifuged for 10 min at 12,000 rpm and the supernatant was discarded. *M. speciosa* DNA was extracted from approximately 200 µl of the residue using a DNeasy Plant Mini Kit (Qiagen, Germany).

5.2.3 Analysis of *M. speciosa* DNA in samples

The water extracts of *Mitragyna* plants were evaluated using Ms-F3/Ms-R2 primers by real-time PCR analysis for authenticate samples. The species-specific marker and melting curve analysis were applied for the identification of *M. speciosa* in 4 samples of kratom cocktail. Protocol was described in section 3.2.5 and 4.2.3, respectively. For species-specific marker, each sample was amplified with multiplex

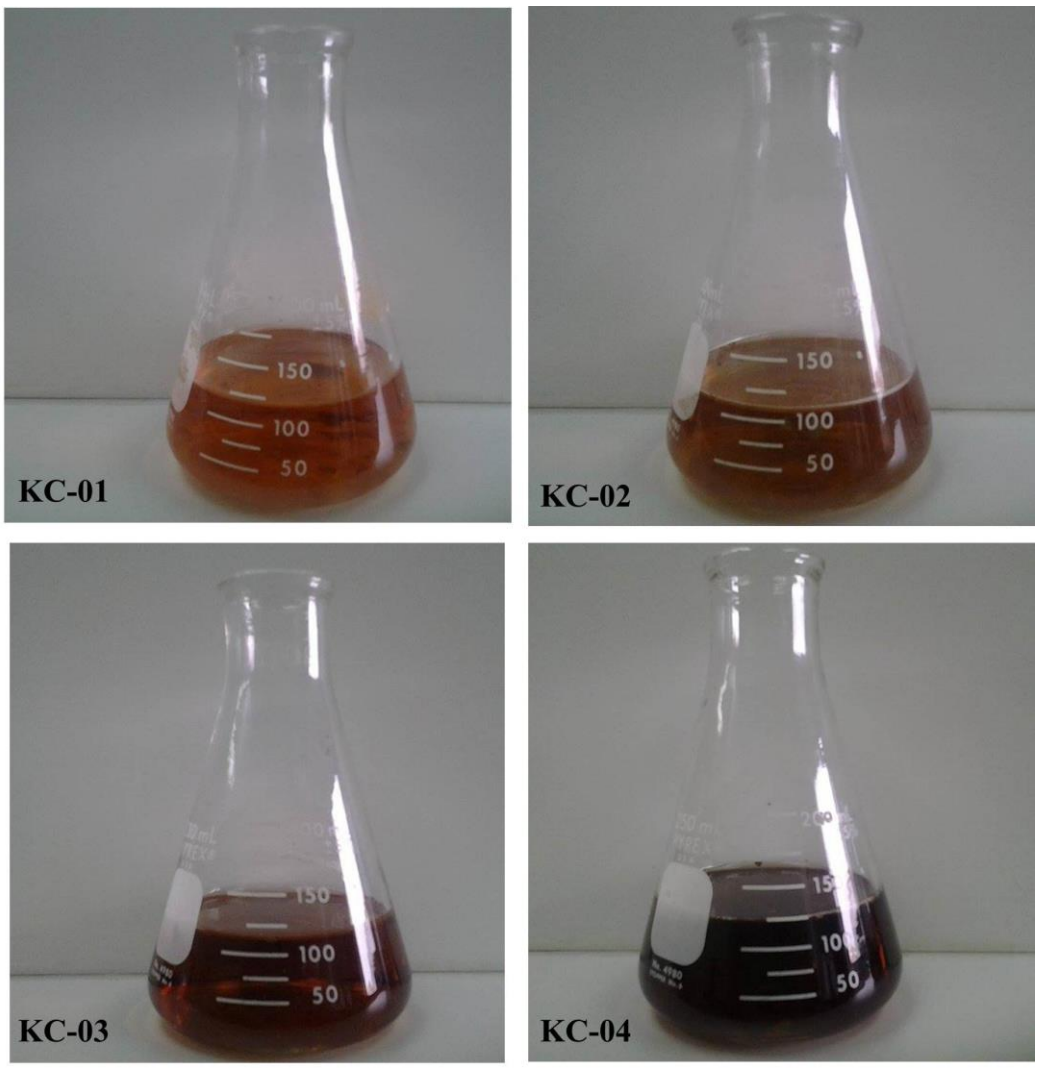


Figure 17 Samples of kratom cocktail (KC-01-KC-04).

primers, Ms-F2/Ms-F3/Ms-R1. For melting curve analysis, each sample was amplified with Ms-F3/Ms-R2 primers. The amount of *M. speciosa* DNA isolated was estimated using a standard curve.

5.3 Results

PCR products amplified from Ms-F3/Ms-R2 primers of four water extracts from *Mitragyna* plants were detected by real-time PCR method. Apart from water extracts, the application of the real-time PCR method was used to identify *M. speciosa* DNA in kratom cocktail. Kratom cocktail samples were obtained from four different sources in Thailand. Melting curve analysis of products generated by Ms-F3/Ms-R2 primer pair was compared with species-specific marker of products generated from multiplex primer, Ms-F2/Ms-F3/Ms-R1. *M. speciosa* DNA can be detected in all samples of kratom cocktail by melting curve analysis (Figure 18) but not by gel based species-specific marker (Table 9).

The quantification of *M. speciosa* DNAs recovered from 200 µl samples of KC-01, KC-02, KC-03 and KC-04 were estimated using the standard curve in chapter 4.3.2 with corresponding to Ct value of 4.36 pg, 4.95 pg, 50.14 pg and 64.27 pg, respectively (Table 9).

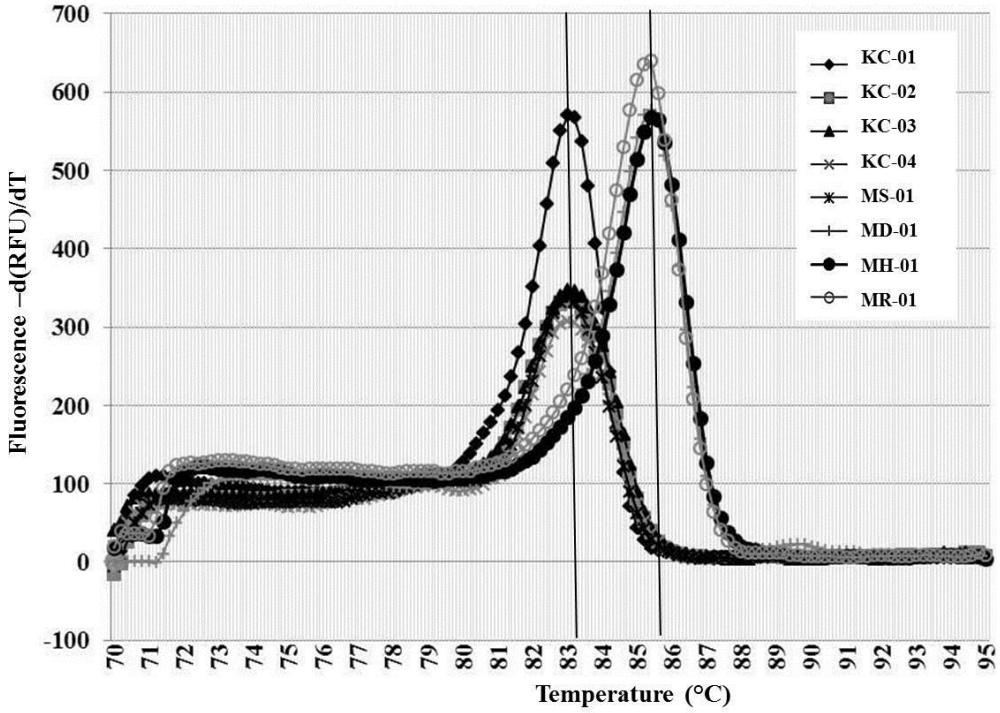


Figure 18 Melting curve analysis of M. speciosa provided the peaks with Tm of 83.2 ± 0.2 ℃ for detected kratom cocktail.

Table 9 Comparative evaluation of kratom cocktail samples using species-specific marker with primer Ms-F2/M-F3/Ms-R1 and real-time PCR with primer Ms-F3/Ms-R2.

Sample number	Location (Thailand, Province)	Species-specific marker	Real-time PCR (Ct)
KC-01	Bangkok	-	25.53
KC-02	Bangkok	-	25.34
KC-03	Chumporn	-	21.89
KC-04	Pattani	-	21.52

5.4 Discussion

kratom cocktail is illegal in Thailand; however it is mostly used as a stimulant by teenage users. There is still a lack of suitable methods for identifying *M. speciosa* in materials, extracts and preparations. In chemical methods, the low quantity of mitragynine was found in the water extract because of its poor solubility in water (Parthasarathy *et al.*, 2013). Recently, molecular analysis has been applied to identify the degraded DNA from over-the-counter herbal medicines and plant extracts (Novak *et al.*, 2006; Kazi *et al.*, 2013). PCR allows the amplification of DNA in small amounts of plant materials that DNA might be damaged during the manufacturing and storage processes. A small amplicon size with melting curve analysis based on real-time PCR was shown to facilitate discrimination by distinct Tm (Mouillesseaux *et al.*, 2003). Thus DNA fragment can be detected in processed samples (Soares *et al.*, 2013). The real-time PCR method can also be used for the quantification of *M. speciosa* DNA present in kratom cocktails.

CHAPTER VI

CONCLUSION

Genetic techniques could be effective tools to discriminate closely related plant species. The present studies provide genetic analysis for the identification of the narcotic plant, *M. speciosa,* from the three closely related species including *M. diversifolia*, *M. hirsuta* and *M. rotundifolia*. The nucleotide polymorphism site in ITS2 region of four *Mitragyna* plants can be used to identify *M. speciosa* by a species-specific primer. *M. rotundifolia* could be discriminated from others by interspecific variation in ITS2 fragment. Unfortunately, there were no nucleotide difference in the ITS2 region between *M. diversifolia* and *M. hirsuta*.

The proposed real-time PCR assay demonstrated that melting curve analysis can be used for detection of *M. speciosa*. The Ms-F3/Ms-R2 primer pair amplifies 78 bp products with high specificity and sensitivity. Melting cure analysis based on 78 bp products can differentiate *M. speciosa* from other species.

The melting curve analysis, a fast and specific real-time PCR method, has been successfully developed for the detection and quantification of plants in the genus *Mitragyna* including *M. speciosa* DNA in forensic specimens, kratom cocktail, while species-specific marker failed to detect. The melting curve analysis method described in this study could be applied for high levels of quality control of any plant raw materials in the future.

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APPENDICES

APPENDIX A

Melting curve analysis of *Mitragyna* plants DNA

Table 10 Amplification data of PCR product obtained with the Ms-F1/Ms-R2 primer pair for each *Mitragyna* species.

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
70	-23.9	-27.4	-22.7	-24.6	1.46
70.2	-51.8	-59.3	-49.2	-53.4	3.15
70.4	-47.8	-54.7	-45.5	-49.3	2.91
70.6	-47.8	-54.7	-45.5	-49.3	2.91
70.8	-47.8	-54.7	-45.5	-49.3	2.91
71	-49.8	-55.8	-47.1	-51	3.06
71.2	-37.6	-49.1	-37.1	-40.3	2.08
71.4	-15.6	-34.9	-17.8	-19.4	0.534
71.6	-2.62	-24.1	-4.21	-5.27	-0.409
71.8	6.48	-16.6	6.22	4.29	-0.82
72	14.3	-10.5	14.9	11.4	-0.983
72.2	19.6	-3.73	21.9	16.9	-0.145
72.4	22.9	1.9	26.4	20.8	1.31
72.6	26.3	6.42	30	23.9	1.62
72.8	30.6	11.1	32.6	26.2	1.47
73	33.6	16.2	35	28.7	0.962
73.2	35.6	20.5	37.5	32	0.054
73.4	40	23.4	40.4	34.8	-0.708
73.6	44.3	26.2	42.5	37	-0.366
73.8	45.5	28.8	44.2	40	0.488
74	47	29.6	45.6	42.5	1.51
74.2	49.7	30.1	46	43.1	2.41
74.4	50	31.3	45.4	44.1	2.87
74.6	48.1	31.7	45.8	45.5	3.63
74.8	48.6	32.9	46.8	45.8	3.43
75	49.2	35.4	46.5	46.1	2.58
75.2	47.5	37.3	47.1	47.7	2.01
75.4	46.3	38.1	48.7	48.7	1.31
75.6	47	39.4	48.4	49.4	-0.139
75.8	46.2	39.3	48.1	50.3	-1.04
76	44.7	38	49.5	51.1	-0.665
76.2	45.9	38	49.3	51.9	-0.656
76.4	46.8	39.1	48.5	52.7	0.0556
76.6	47.3	39.8	48.9	53	0.89
76.8	49.8	41.2	48.4	54.7	1.4

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
77	52.3	42.2	46.6	56.5	1.61
77.2	53.3	41.4	46.7	56.7	1.86
77.4	54.8	40.2	47.5	57	1.66
77.6	55.8	39	47.2	58.7	1.5
77.8	55.7	37.5	47.9	59.2	1.45
78	55.1	37	49.3	58.1	0.97
78.2	53.6	37.3	48.9	58.3	1.59
78.4	52.4	37.5	47.9	57.5	2.49
79.2	46.8	35.9	44.9	45.7	4.61
79.4	45.8	35.1	44.5	43.2	2.89
79.6	44.2	34.2	44.3	41.5	2.07
79.8	44	34	44.7	39.4	1.33
80	44.9	34.5	44	37.3	-0.48
80.2	44.7	34.9	42.5	35.9	-1.58
80.4	44.3	33.6	41.5	35.8	-0.274
80.6	45	31.1	40.2	36.1	0.777
80.8	44.2	29.4	39.2	36.6	1.32
81	41.8	27.5	38.9	37	2.71
81.2	39.9	25.9	39.2	36.2	3.73
81.4	38.8	26.9	40.6	33.9	2.48
81.6	38.4	28.8	42.1	32	1.12
81.8	39.5	29.5	42.4	31.7	0.812
82	40.2	29.6	42.3	31.1	0.159
82.2	40.6	29	41.6	31.1	0.23
82.4	41	27.3	40	32	2.09
82.6	39.7	25.8	38.9	32.3	3.61
82.8	38.1	25.5	38.1	31	3.68
83	37.4	26.5	37.4	30	4.15
83.2	36.9	27.5	37.2	29.4	4.09
83.4	36.9	27.9	36.9	27.8	2.97
83.6	37.3	28	35.8	26.7	1.96
83.8	36	27	35.1	26.5	0.894
84	35.5	25.2	34.2	26.7	0.0596
84.2	36.6	24.2	33.4	26.6	0.341
84.4	36	24.2	34.3	27	0.619
84.6	35.5	24.9	35.9	27	0.909
84.8	37.4	26.4	36.6	27.5	2.4
85	37.6	27.5	37.7	28.1	3.17
85.2	36.4	27.8	38.4	28.5	1.99
85.4	36.4	27.8	36.6	29.5	1.48

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
85.6	36.3	26.5	34.9	30.5	2.03
85.8	36.7	25.7	34.9	30.5	2.55
86	38.2	26.3	35.3	30	2.88
86.2	39.1	26.6	35.9	30.2	4.08
86.4	40.4	27.3	38.2	31.2	4.55
86.6	42.4	29.9	40.8	32.7	3.52
86.8	43.6	31.5	42.8	34.9	1.9
87	47	33.3	47.1	39	0.69
87.2	53.1	38	53.1	44	-0.262
87.4	60.2	43.1	59.8	48.7	-0.417
87.6	68.8	48.3	67.6	54.8	0.942
87.8	77	54.4	75	60.9	2.44
88	80.3	59.1	79.2	64	3.96
88.2	78.8	59.7	79.6	65.1	5.22
88.4	76.3	58.7	78.8	65.5	5.79
88.6	71.7	56.7	76.4	63.2	5.1
88.8	66.5	53.1	73	59.8	4.85
89	65.2	49.8	70.3	58	4.39
89.2	69.2	49.4	71.3	57.7	3.44
89.4	74.7	51.5	74.3	57.6	3.52
89.6	83.7	55.3	80.8	61.3	4.18
89.8	100	62	94.1	70.8	3.4
90	120	72.2	113	82.4	2.96
90.2	140	85	134	95.9	4.17
90.4	156	95.9	151	108	3.74
90.6	161	103	160	112	2.84
90.8	151	104	155	105	3.71
91	128	96.6	136	90.7	4.55
91.2	96.2	80.3	105	68.2	3.73
91.4	64.3	60.6	73.9	45.2	3.32
91.6	38.5	41	46.5	27.7	3.29
91.8	20.5	24.2	25.4	15	3.06
92	9.89	12.5	13.4	7.42	3.19
92.2	4.8	5.55	8.53	6.27	3.89
92.4	3.68	3.06	6.68	6.81	4.96
92.6	4.46	3.61	6.33	6.05	5.68
92.8	5.22	4.35	6.66	6.01	5.13
93	6.48	5.79	6.89	7.1	4.34
93.2	7.98	8	7.04	7.33	3.76
93.4	8.59	8.93	7.01	7.17	3.34

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
93.6	8.9	9.14	7.25	8.68	3.64
93.8	8.82	9.56	7.75	9.52	4.45
94	8.32	9.19	7.83	8.63	4.71
94.2	7.27	8.14	7.73	8.06	4.79
94.4	6.29	6.68	6.38	7.84	5.94
94.6	5.45	5.87	5.52	6.83	5.64
94.8	5.63	3.76	2.18	6.68	8.31
95	2.76	0.473	-1	3.17	5.2

Table 11 Amplification data of PCR product obtained with the Ms-F3/Ms-R2 primer pair for each *Mitragyna* species.

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
70	-5.4	-11.8	-11.1	4.91	1.37
70.4	-10.8	-23.7	-22.2	9.81	2.97
70.8	-10.8	-23.7	-22.2	9.81	2.74
71	-12.5	-25.6	-24.1	7.87	2.74
71.2	-2.2	-13.8	-12.2	19.6	2.74
71.4	19.3	9.84	12.8	45	2.74
71.6	37.1	28.8	34.3	64.5	2.85
71.8	50.6	47.4	51.6	78.6	2.07
72	60.2	63.9	65	87.8	0.595
72.2	69.1	75.2	75.1	92.9	-0.00338
72.4	76.2	82.2	81.6	95.6	-0.0319
72.6	79.8	86.5	85.1	96.6	-0.377
72.8	83.1	89.2	86.5	96.6	-0.165
73	85.1	91.1	87.2	96.9	0.885
73.2	84.4	93.8	88	97.6	1.14
73.4	83.5	97.4	89.2	96.9	1.33
73.6	84.2	100	90	95.3	1.8
73.8	83.2	102	91.3	95.5	1.33
74	82.2	103	92.1	95.2	0.142
74.2	83.5	104	92.2	93.3	0.011
74.4	84.8	104	91.6	92.8	0.413
74.6	85.8	103	91.4	93.5	0.584
74.8	88.4	103	91.3	92.7	1.11
75	90.6	104	90.9	91.9	2.26
75.2	91	103	90.3	93.1	2.89
75.4	91.4	102	90.1	93	1.8
75.6	90.5	101	89.2	91.6	1.21
75.8	89.4	99.6	88.4	90.1	1.74
76	89.4	97.9	88.8	89	0.914
76.2	89.7	96.7	89.5	88	-0.395
76.4	89.2	94.8	90	87.4	0.75
76.6	89.8	93.5	90.4	87.9	1.55
76.8	90.2	93.2	90.2	89.7	0.393
77	89.8	92.8	89.2	90.7	0.859
77.2	90.3	93	87.3	90.5	2.23
77.4	91.6	94.5	86.6	90	1.83

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
77.4	92.0	95.5	85.7	90.5	1.83
77.6	92.1	95.7	85.6	90.4	1.45
77.8	92.4	96.8	86	88.7	2.26
78	92.9	97.1	86.6	88.1	2.65
78.2	93.4	96.1	87.7	88.4	2.35
78.4	94.2	95.2	88.7	87.4	2.2
78.6	96	94.3	88.8	86.7	1.95
78.8	98.9	93.4	88.2	87.4	1.31
79	102	93	86.9	88.2	0.448
79.4	111	94.3	84.8	89	1.25
79.6	116	94.8	83.2	90.4	1.6
79.8	123	95.1	81.8	89.9	2.41
80	130	95.8	81.6	88.6	2.95
80.2	138	95.8	82.6	89.4	2.94
80.4	147	95.4	83.2	89.9	2.84
80.6	158	96.1	84.1	89.4	2.83
80.8	169	96.1	85.9	91.4	2.92
81	182	96.4	87.4	94.6	2.68
81.2	197	98	88.4	97.2	1.76
81.4	217	100	90.7	101	1.21
81.6	242	104	93.9	106	0.612
81.8	275	109	97.4	112	0.0567
82	317	118	103	118	0.646
82.2	366	127	108	126	0.905
82.4	420	137	115	135	1.24
82.6	473	147	124	144	2.55
82.8	518	158	133	154	2.27
83	546	169	143	165	1.62
83.2	549	182	155	179	2.43
83.4	527	200	170	196	2.36
83.6	479	224	190	219	1.15
83.8	410	255	218	250	1.75
84	329	294	253	287	2.35
84.2	250	341	295	331	1.61
84.4	181	395	344	381	1.86
84.6	122	450	394	431	2.61
84.8	79	503	439	474	2.4
85	52	547	476	507	2.39
85.2	33.9	574	499	523	3.59
85.4	21.3	577	499	515	3.52

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
85.6	15.1	555	478	482	2.93
85.8	12.2	508	437	430	2.81
86	8.88	441	376	362	1.43
86.2	6.14	361	306	288	-0.123
86.4	5.24	279	237	218	-0.176
86.6	4.97	205	172	156	0.453
86.8	4.46	142	118	107	1.36
87	4.76	93.9	77.8	70.3	3.24
87.2	5.97	60.9	49.3	45.7	4.57
87.4	6.26	39.5	30.3	30.4	5.02
87.6	5.69	26	20.3	21.5	5.05
87.8	5.23	17.8	14.3	16.2	4.06
88	4.53	13.1	10.2	13	3
88.2	4.29	10.8	8.69	11.1	2.86
88.4	4.82	9.82	7.7	9.48	3.02
88.6	5.68	8.54	6.16	8.34	2.87
88.8	6.67	6.93	5.34	7.72	3.55
89	7.22	6.69	5.59	8.4	4.17
89.2	6.58	6.25	5.81	8.6	2.97
89.4	6.02	5.06	5.78	8.22	1.41
89.6	5.7	4.9	5.47	7.74	1.28
89.8	4.78	5.82	6.02	7.38	1.14
90	4.72	5.53	6	5.9	1.33
90.2	5.92	5.33	4.71	4.89	3.3
90.4	6.71	6.17	4.59	5.16	5.1
90.6	7.14	6.1	5.91	5.35	5.51
90.8	7.64	5.44	6.4	5.64	5.5
91	7.33	5.27	6.14	6.16	5.29
91.2	6.23	4.96	6.98	7.05	3.97
91.4	5.47	4.68	7.51	7.08	2.84
91.6	4.25	5.03	6.72	6.47	2.74
91.8	3.45	5.48	5.94	6.56	2.73
92	4.38	6.13	5.82	7.38	3.03
92.2	5.64	6.75	5.06	7.06	4.03
92.4	6.11	6.3	4.56	7.31	5.21
92.6	7.59	5.89	4.71	8.55	5.2
92.8	8.56	6.41	5.1	7.92	5.16
93	8.14	6.57	6.18	6.71	5.07
93.2	7.45	6.49	7.85	6.4	4.71
93.4	6.99	6.75	8.68	5.21	4.19

Temperature	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
93.6	6.09	7.35	9.12	4.11	4.53
93.8	5.67	7.58	9.04	5.2	5.64
94	5.74	6.89	8.42	6.41	5.03
94.2	5.63	7.27	7.91	7.4	3.98
94.4	6.87	6.33	6.5	8.47	4.56
94.6	7.26	5.25	6.17	9.3	4.24
94.8	10.2	1.54	4.4	9.02	4.75
95	5.87	-1.31	0.77	3.47	6.35

Table 12 Amplification data of PCR product obtained with the Ms-F2/Ms-F3/Ms-R1 multiplex primer for each *Mitragyna* species.

Temperature (C°)	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
70	-13.6	-10.5	14.4	16	0.148
70.2	-29.5	-22.7	31.3	34.6	0.32
70.4	-27.2	-21	28.9	31.9	0.295
70.6	-27.2	-21	28.9	31.9	0.295
70.8	-27.2	-21	28.9	31.9	0.295
71	-28.7	-22.2	27.6	30.5	0.236
71.2	-19.5	-14.8	35.5	39.7	0.685
71.4	0.605	0.686	49.2	56.2	0.828
71.6	19.3	13	56.1	62.9	0.788
71.8	36.3	22.1	60.1	64.7	2
72	50.4	28.7	62.2	65.7	2.76
72.2	62.1	33	62.1	64.6	2.31
72.4	71.4	35.9	61.6	62.4	3.1
72.6	76.8	37.4	62.2	62.3	3.79
72.8	80.6	38.9	63	62.9	2.9
73	83.9	39.5	63.4	63.8	2.23
73.2	85.4	39.7	65.3	66.2	1.47
73.4	87.3	40.1	67	68.7	-0.115
73.6	89.7	40.7	66.9	65.7	-1.04
73.8	89.8	41.3	67	64.6	-0.82
74	89.7	41.6	67.3	62.4	-0.673
74.2	91.8	42.6	65.8	62.3	-0.0858
74.4	92.3	43.6	65	62.9	1.36
74.6	91.1	43.8	66.7	63.8	2.09
74.8	92.5	43.1	68.4	66.2	2.11
75	94.3	42.9	69.8	68.7	2.1
75.2	92.5	42	72.5	66.2	1.78
75.4	91.5	40.5	67	68.7	1.46
75.6	92.1	39.6	66.9	65.7	1.35
75.8	91.3	39.4	67	64.6	0.834
76	90.6	38.5	67.3	62.4	1.01
76.2	92	38.4	65.8	62.3	1.86
76.4	92.7	39	65	62.9	1.5
76.6	93.2	39.1	66.7	63.8	1.06
76.8	93.8	40	68.4	66.2	1.57
77	92.9	42.1	69.8	68.7	1.25

Temperature (C°)	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
77.2	92.5	43.2	72.5	65.7	0.453
77.4	92.3	43.1	67	64.6	0.865
77.6	91.2	43.4	66.9	62.4	1.98
77.8	90.6	43.2	67	62.3	2.16
78	90.7	42.3	67.3	62.9	1.71
78.2	88.5	42.2	65.8	63.8	2.02
78.4	87.2	43.8	65	66.2	1.88
78.6	87.9	46.1	66.7	68.7	0.55
78.8	87.7	47.6	68.4	69.9	0.543
79	88.5	49.7	69.8	67.5	1.67
79.2	92.3	52.8	72.5	73.8	1.14
79.4	95	56.3	71.8	70.6	0.612
79.6	96.2	59.8	70.8	69.9	0.921
79.8	98.9	64.8	69.9	67.5	1.32
80	102	70.6	69.6	66.9	1.53
80.2	103	74.8	70.4	65.7	2.35
80.4	107	78.3	71.6	69.9	3.65
80.6	113	82	73.3	67.5	4.25
80.8	121	85	75.8	66.9	3.1
81	131	87.8	80.2	81.4	2.25
81.2	145	93.3	86	84.3	2.26
81.4	165	101	91.9	88.7	1.4
81.6	188	112	100	94.8	1.24
81.8	215	127	112	104	2.13
82	245	144	125	116	2.01
82.2	273	162	140	130	1.2
82.4	295	177	156	143	1.35
82.6	310	187	170	157	1.4
82.8	313	190	177	168	0.936
83	305	185	179	172	1.19
83.2	286	173	172	171	1.92
83.4	260	154	157	163	1.97
83.6	229	132	138	150	2.22
83.8	196	107	117	131	2.82
84	164	82.5	94.2	109	2.61
84.2	139	61.4	72.8	86.6	2.31
84.4	120	45.2	55.6	66.5	1.84
84.6	106	33.3	41.7	48.7	0.511
84.8	101	24.7	31.1	35.4	-0.0811
85	105	18.7	24.7	27.4	1.2

Temperature (C°)	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
85.2	113	14.1	23	22.9	1.67
85.4	128	9.58	22.9	21	2.3
85.6	151	5.56	23.2	21.9	3.94
85.8	178	3.18	23.9	24.2	4.48
86	209	2.48	26.6	27.4	3.13
86.2	240	2.74	28.8	31	2.59
86.4	265	4.25	29.1	33.2	2.6
86.6	282	5.87	29.5	33.6	1.23
86.8	284	6.01	30	33.3	1.13
87	270	5.25	27.7	30.9	2.26
87.2	244	4.56	22.9	25.3	2.93
87.4	207	3.78	18.9	19.8	3.54
87.6	166	3.02	15.5	16	5.31
87.8	125	3.7	11.4	11.6	4.76
88	89.8	5.03	7.16	7.28	3.62
88.2	60.1	5.37	4.97	6.75	3.36
88.4	38.5	5.37	4.35	7.46	2.38
88.6	24.4	5.45	3.88	6.13	1.49
88.8	15.1	5.02	4.47	6.09	2.48
89	10.3	4.48	6.75	7.58	2.94
89.2	8.23	4.59	8.91	7.12	2.34
89.4	7.58	4.33	9.6	7.38	2.58
89.6	7.57	3.98	8.94	8.57	2.45
89.8	7.42	4.05	8	8.13	2.26
90	6.81	3.89	7.04	7.08	2.9
90.2	6.22	4.2	5.42	7.5	3.77
90.4	5.68	5.14	4.17	6.46	4.03
90.6	5.41	6.16	5.48	5.08	4.71
90.8	5.28	6.52	6.8	5.83	4.43
91	5.19	5.86	5.91	6.34	3.53
91.2	5.43	4.78	5.82	4.94	4.23
91.4	5.51	3.95	6.7	4.3	4.6
91.6	5.38	3.58	5.17	4.54	3.45
91.8	6.08	3.96	3.4	3.65	3.59
92	7.41	5.62	4.43	3.75	5.06
92.2	7.84	7.28	4.72	4.96	4.29
92.4	8.11	7.56	3.47	5.64	3.71
92.6	8.23	7.21	4.22	6.77	5.06
92.8	7.08	7	5.26	8.06	5.47
93	5.44	6.81	5.38	7.62	4.46

Temperature (C°)	*M. speciosa*	*M.diversifolia*	*M. hirsuta*	*M. rotundifolia*	NTC
93.2	5.52	6.33	6.15	7.35	4.22
93.4	5.32	6.75	6.97	7.62	4.75
93.6	4.68	7.58	6.71	6.11	4.16
93.8	5.75	7.09	6.78	4.88	4.05
94	6.57	5.88	6.11	5.26	4.51
94.2	5.85	4.74	5.07	5.05	4.95
94.4	6.46	4.91	4.31	4.36	4.34
94.6	7.12	4.21	3.5	4.91	4.23
94.8	9.08	6.97	2.01	5.01	3.15
95	5.14	4.85	0.133	1.81	0.531

APPENDIX B

Specificity of the real-time PCR assay

Table 13 Amplification data of PCR product obtained with the Ms-F3/Ms-R2 primer pair for *M. speciosa*.

Temp (C°)	MS-01	MS-02	MS-03	MS-04	MS-05	MS-06	MS-07	MS-08	MS-09	MS-10
70	21.9	19.8	32.1	1.39	57.2	31.4	29.5	11.3	-16.7	-53.3
70.2	20.2	18.3	29.6	1.29	52.8	29	27.2	24.6	-15.4	-53.3
70.4	20.2	18.3	29.6	1.29	52.8	29	27.2	22.7	-15.4	-55.8
70.6	20.2	18.3	29.6	1.29	52.8	29	27.2	20.2	-15.4	-40.4
70.8	18.7	16.4	28	-1.14	51.2	27.2	25.6	35.6	-17.6	-9.01
71	28.1	27.9	37.8	13.9	60.9	38.8	35.4	64.4	-4.41	17.3
71.2	46.5	51.6	58	42.7	78.9	58.7	54.3	80.3	24.1	41.1
71.4	61.4	67.5	73.2	62.1	89.2	70.6	67.2	89	48	59.7
71.6	72.7	78.8	86.2	76.6	95.8	79.3	75.7	93.3	68.4	69.5
71.8	80.5	87	95.5	86.4	101	85.8	80.6	94.1	82.6	74.6
72	88	92.3	102	91.9	103	89.4	83.3	93.9	91.3	78.6
72.2	92.8	95.6	106	95	104	91.2	85.3	94.8	97	80.2
72.4	94.4	97.6	108	96.2	106	92.7	86.9	97	97.9	81.4
72.6	96.7	97.6	111	96.5	107	92.2	87.4	98.8	96	83.5
72.8	98	97.7	112	95.9	108	91.4	88.3	100	94	85.7
73	97.2	100	112	95.2	108	92.2	90.1	101	92.1	87.3
73.2	97.7	101	114	95.4	106	92.7	90.2	102	91.2	88.9
73.4	98	101	116	95.7	104	92.2	89.4	102	93.2	89.2
73.6	97	103	114	94.7	103	92.9	89.4	100	95.4	89.3
73.8	97.3	104	111	92.8	101	91.9	89.5	100	97.9	90
74	97.3	103	111	92.8	98.9	89.8	89	99.5	101	89.2
74.2	96.3	103	108	94	99.4	89.6	89.9	97.5	102	87.6
74.4	96.4	104	106	93.8	101	89.3	91.3	97.3	101	87.8
74.6	97.1	104	105	93.7	102	88.4	90.9	98.3	101	87.8
74.8	97.1	104	105	96.2	103	89.3	90	97.5	101	87.1
75	97.2	105	106	97.3	106	90.5	89.9	96.7	98.8	87.8
75.2	98.3	105	106	94.7	107	91.4	88.8	96.8	97.4	88.5
75.4	98.4	104	106	94.2	107	92.1	87.1	96.1	96.2	88.3
75.6	97.3	104	107	95.2	107	92.3	87.8	94.7	94.5	88.7
75.8	96.7	104	108	93.5	107	92.2	89.2	93.6	92.9	88.8
76	96.4	103	106	92.1	106	91.6	88.6	93.3	92.6	88.7
76.2	95.7	105	108	92.7	105	89.5	88.1	93.1	93	89.5
76.4	95.4	107	110	92.8	105	88.4	88.1	92.3	92.4	90.1
76.6	96.1	109	110	92.4	105	88.9	87.3	92.2	91.3	92.1
76.8	96.5	110	111	93.4	104	89	86.3	93.2	91.7	93.6
77	96.7	111	113	94.7	103	89.9	86.3	93.9	91.5	94.1

Temp (C°)	MS-01	MS-02	MS-03	MS-04	MS-05	MS-06	MS-07	MS-08	MS-09	MS-10
77.2	97.2	110	111	96.7	102	91.3	87.6	94	91.2	96.2
77.4	97.2	108	109	97.8	101	92.8	89	94.6	92.2	97.6
77.6	97	106	110	97.9	101	93.6	89.6	95.3	93.6	97.3
77.8	97.3	105	109	98.4	102	94.2	91.4	94.6	93.6	98.5
78	97.6	105	107	98.8	104	95.2	94.4	93.6	93.9	101
78.2	98.2	108	110	99.3	107	97.6	96.1	94.1	94.1	103
78.4	101	112	115	102	111	100	98.4	95.2	94.1	105
78.8	110	122	122	111	120	106	104	101	98.7	111
79	116	127	128	115	124	109	106	105	101	113
79.2	122	131	134	120	129	111	110	107	104	114
79.4	127	137	136	125	135	113	114	110	108	117
79.6	132	144	142	130	141	118	118	114	110	119
79.8	139	154	152	138	148	124	126	118	113	120
80	146	165	162	149	158	132	133	124	119	124
80.2	155	177	174	158	169	141	142	132	126	129
80.4	166	190	189	169	181	151	153	140	133	134
80.6	178	201	204	181	194	163	165	150	142	143
80.8	191	215	221	193	208	174	178	163	154	153
81	206	232	238	206	225	187	192	177	167	164
81.2	225	254	258	222	244	203	208	194	181	177
81.4	250	284	288	244	271	223	226	215	199	194
81.6	283	326	326	273	307	246	250	241	223	215
81.8	327	377	374	310	354	278	280	271	252	244
82	381	436	435	356	407	316	319	309	288	282
82.2	441	500	501	409	467	360	364	355	333	328
82.4	502	564	564	465	529	410	414	407	385	378
82.6	559	620	621	519	585	463	465	460	442	430
82.8	601	659	661	563	625	511	510	510	487	477
83	620	672	672	590	644	578	562	549	545	510
83.2	612	654	652	584	636	573	557	568	590	527
83.4	577	605	602	571	596	572	546	560	569	523
83.6	514	528	524	519	529	544	509	524	542	495
83.8	430	435	431	446	445	492	451	464	527	446
84	338	339	334	361	352	420	375	388	461	383
84.2	250	249	244	274	262	336	293	304	381	311
84.4	173	173	169	197	184	253	217	225	298	239
84.6	114	115	110	134	123	180	151	158	222	173
84.8	73.5	73	69.6	87.1	78.1	121	98.7	106	158	117
85	48.2	44	43.8	55.1	48	77.4	63.3	68	108	75.2
85.2	31.6	25.6	26.9	35.4	29.5	49.3	40.4	43.6	73	45.3

Temp (C°)	MS-01	MS-02	MS-03	MS-04	MS-05	MS-06	MS-07	MS-08	MS-09	MS-10
85.4	20.9	15.2	17.7	23	18.9	32.4	26	27.8	49.5	24.7
85.6	14.2	10.5	14	15.5	13.5	22.1	18.3	17.5	35	12.3
85.8	9.61	9.65	11.4	11.9	11.5	15.6	14.1	11.9	28.2	5.88
86	6.19	9.86	9.36	10.5	10.9	11.7	11.2	9	24.6	2.28
86.2	4.49	10.2	9.13	9.96	9.68	9.35	9.42	7.8	22.1	-0.034
86.4	4.49	10.1	8.89	8.92	8.2	7.75	8.44	7.24	21.5	-0.085
86.6	5.8	8.98	7.39	8.65	7.28	7.15	7.89	7.08	20.7	0.856
86.8	6.79	7.37	6.73	8.39	5.58	6.84	7.15	6.9	17.9	0.0196
87	6.51	6.46	7.26	7.44	4.02	6.44	7	6.76	15.8	-1.15
87.2	7.01	6.41	7.14	6.06	3.45	6.01	6.48	6.77	14.4	-1.18
87.4	7.44	6.88	5.83	6.01	3.46	5.21	5.33	6.82	12.2	-1.53
87.6	6.21	7.22	5.41	5.81	3.79	4.41	5.56	6.44	10.5	-2.77
87.8	5.31	6.96	6.09	5.05	4.69	4.42	6.61	6.81	10.9	-1.42
88	6.26	6.99	5.53	5.73	4.91	4.56	6.36	7.4	12.1	0.861
88.2	6.1	6.62	5.25	6.42	5.65	5.07	7.07	6.98	12.6	1.71
88.4	5.65	5.68	7.06	6.57	6.79	6.65	9.05	6.68	14.4	2.56
88.6	6.44	5.44	8.37	7.07	6.55	7.76	8.69	6.71	16.4	3.58
88.8	6.4	5.51	7.78	7.75	6.24	7.95	7.41	5.7	16.4	2.59
89	5.93	5.55	7.57	6.91	7.4	8.65	8.27	5.51	15.2	0.832
89.2	5.95	6.29	8.32	6.35	8.16	8.59	9.02	6.55	15.5	0.114
89.4	5.94	6.56	7.34	6.02	6.85	6.53	8.15	7.7	14.5	-0.637
89.6	5.49	6.56	5.87	4.78	6.35	5.26	8.29	8.66	12.1	-2
89.8	5.52	7.41	6.1	3.99	6.83	5.27	9.47	9.05	11.3	-2.7
90	4.79	7.37	6.71	4.45	5.95	4.79	9.44	8.36	11.6	-2.04
90.2	4.71	6.23	5.73	5.34	4.66	4.23	7.88	6.99	10.1	-1.34
90.4	5.45	6.32	6.01	6.3	5.97	5.41	7.02	5.36	9.19	-1.46
90.6	5.37	6.61	7.46	7.61	7.25	6.52	7.32	3.74	10	-0.406
90.8	5.87	5.83	7.49	8.7	7	6.37	6.69	3.77	9.44	0.659
91	7.34	5.4	6.76	8.95	7.05	6.45	5.47	4.26	8.98	0.379
91.2	7.12	5.78	6.98	8.19	6.61	7.29	6	4.22	10.7	-0.2
91.4	5.6	5.97	6.07	6.77	5.46	7.02	6.86	4.62	10.8	-0.437
91.6	5.14	6.06	4.01	6.32	4.51	6.41	6.2	6.28	9.47	-0.891
91.8	4.67	6.32	3.5	6.5	3.67	6.92	6.18	7.01	10.1	-1.66
92	3.23	6.6	3.99	5.68	4.08	6.98	6.99	7	9.95	-2.6
92.2	3.32	6.05	4.05	5.34	5.72	6.31	7.07	7.61	8.19	-2.94
92.4	4.99	5.11	4.57	6.5	6.11	6.41	6.48	7.94	8.99	-2.16
92.6	6.12	4.44	6.14	6.42	6.59	6.72	6.43	6.98	10.6	-2.68
92.8	6.41	4.7	6.45	5.37	8.48	5.73	7.13	6.47	10.3	-2.73
93	7.37	5.57	6.08	6.16	8.62	4.94	7.28	6.43	10.1	-0.976
93.2	7.74	6.32	6.02	6.96	7.2	5.42	7.11	6.87	10.6	-0.49

Temp (C°)	MS-01	MS-02	MS-03	MS-04	MS-05	MS-06	MS-07	MS-08	MS-09	MS-10
93.4	6.6	7.41	6.97	6.89	7.47	5.77	7.88	7.33	9.99	-1.67
93.6	5.9	7.78	7.77	7.68	7.49	5.13	8.57	7.69	8.89	-0.493
93.8	5.82	7.42	8.01	8	6.01	5.42	7.53	8.3	9.32	0.567
94	5.84	7.19	8.88	8.14	6.22	5.67	7.48	8.71	9.6	-0.351
94.2	7.42	8.74	8.45	6.58	7.99	5.29	8.57	8.81	9.72	0.0677
94.4	8.57	9.1	7.57	5.46	8.09	5.19	8.04	9.24	10	0.487
94.6	13.3	13.2	5.02	1.29	10.5	6.71	9.95	4.3	11.5	-0.351
94.8	8.14	7.94	0.886	-1.75	5.88	3.74	5.5	5.3	5.41	0.0677
95	8.14	7.94	0.886	-1.75	5.88	3.74	5.5	4.3	5.41	0.487

Table 14 Amplification data of PCR product obtained with the Ms-F3/Ms-R2 primer pair for *M. diversifolia*.

Temperature (C°)	MD-01	MD-02	MD-03	MD-04	MD-05	MD-06
70	-7.54	0.183	-2.43	5.38	18.1	6.29
70.2	-16.3	0.397	-5.26	11.7	39.3	13.6
70.4	-15.1	0.367	-4.85	10.8	36.3	12.6
70.6	-15.1	0.367	-4.85	10.8	36.3	12.6
70.8	-15.1	0.367	-4.85	10.8	36.3	12.6
71	-17.5	0.367	-7.78	7.87	33.4	9.32
71.2	-2.54	-1.56	-7.78	7.87	33.4	9.32
71.4	26.9	10.4	10.2	25.8	51.6	29.7
71.6	49.1	33.4	46.8	60.4	85.9	67.6
71.8	68	50	71	82.2	105	92.8
72	81.9	64.6	85.9	93.9	115	109
72.2	90.6	76.9	94.5	99.7	119	115
72.4	96.1	86	97.5	101	121	117
72.6	100	93.8	96.7	101	121	117
72.8	103	100	96.1	100	120	114
73	103	104	96.6	101	118	113
73.2	104	106	95.2	101	117	113
73.4	104	107	95.5	102	117	113
73.6	105	107	97.6	102	117	113
73.8	104	106	98.1	102	116	114
74	106	106	98.2	102	117	114
74.2	106	106	101	101	119	114
74.4	106	105	103	99.6	117	113
74.6	106	106	102	99.6	116	112
74.8	106	106	102	99.5	116	113
75	105	105	102	97.3	113	112
75.2	104	105	97.8	96.9	109	111
75.4	104	105	95.2	97.7	109	112
75.6	103	104	94.7	96	108	112
75.8	102	103	93.1	93.7	108	112
76	102	103	92.3	93.5	110	112
76.2	101	103	93.6	92.6	110	114
76.4	99.4	102	94.3	90.2	110	112
76.6	100	100	92.8	89.5	109	110
76.8	100	100	92.3	91.7	107	109
77	100	99.6	93.2	92.5	105	107

Temperature (C°)	MD-01	MD-02	MD-03	MD-04	MD-05	MD-06
77.2	100	98.4	92.3	92.6	105	105
77.4	98.9	98	90.7	94.9	105	105
77.6	97.5	98	91.1	97	105	106
77.8	96.1	97.2	91.3	96.1	106	107
78	94.3	96.2	89.7	96.9	106	109
78.2	93.9	95.2	90.1	97.2	107	109
78.4	95.1	94.7	92.3	94.3	106	107
78.8	96.8	94.3	94.1	91.8	105	104
79	98.7	95.5	95.6	90.6	105	102
79.2	99.9	98.4	95.3	90.9	104	102
79.4	99.7	101	93.7	93.2	105	104
79.6	99.6	102	92.7	94.3	107	105
79.8	99.9	103	91.5	95.5	107	105
80	99.9	104	90.6	95.2	107	107
80.2	100	103	90.8	95.3	109	108
80.4	102	102	91.2	96.4	109	108
80.6	105	104	93.5	97.3	109	109
80.8	106	105	95.6	97.9	112	111
81	108	106	97.7	101	115	113
81.2	111	110	101	105	116	115
81.4	113	115	106	108	119	119
81.6	117	119	109	111	125	126
81.8	124	125	115	117	129	134
82	132	133	123	123	134	142
82.2	140	141	130	127	143	151
82.4	151	149	137	134	152	161
82.6	162	161	146	141	161	170
82.8	172	174	155	147	172	179
83	185	186	164	156	185	190
83.2	200	201	175	168	197	204
83.4	216	220	191	182	212	222
83.6	239	240	208	199	231	242
83.8	269	266	229	221	256	271
84	305	302	258	249	288	307
84.2	349	345	294	282	328	350
84.4	400	394	334	321	373	398
84.6	452	447	379	365	421	452
84.8	503	499	428	411	470	506
85	548	542	470	452	514	553
85.2	578	570	501	484	549	586

Temperature (C°)	MD-01	MD-02	MD-03	MD-04	MD-05	MD-06
85.4	589	577	517	501	568	599
85.6	577	561	511	497	564	587
85.8	537	518	479	470	535	548
86	475	454	429	423	482	486
86.2	399	377	363	363	411	409
86.4	316	297	289	293	331	326
86.6	237	222	218	224	253	246
86.8	169	157	157	164	183	177
87	114	109	107	114	126	121
87.2	73.8	73.1	69	75.6	84	81.1
87.4	46	47.6	43.9	49.6	55.8	53.8
87.6	28	32.8	28.4	33.1	37.9	35.6
87.8	17.8	25.1	19.1	22.2	26.2	24.7
88	12.3	19.3	13.8	16.7	19.2	18.3
88.2	10.2	16.7	12.1	14.5	16.2	14.9
88.4	9.59	16.8	11.6	12.9	13.7	13.2
88.6	9.21	16.3	9.77	11.8	11	11.9
88.8	9.14	16.3	8.17	11.2	10.6	11.8
89	8.86	18.4	7.09	10.1	9.94	11.7
89.2	8.18	20.6	5.54	9.26	8.1	11
89.4	8.29	22	4.27	9.29	7.06	10.6
89.6	8.81	22.8	4.76	8.74	6.32	11.1
89.8	8.09	22.2	5.71	8.43	5.06	10.5
90	7.28	19.5	6.28	8.37	5.38	9.55
90.2	7.05	16.7	6.87	7.96	5.98	9.5
90.4	6.05	15	7.15	7.42	5.92	8.76
90.6	4.85	13.6	7.87	7.07	6.59	8.05
90.8	5.37	12.3	7.75	6.98	7.07	8.43
91	6.75	12.4	6.88	7.31	6.68	9.09
91.2	6.99	12.4	6.72	7.39	6.6	8.62
91.4	6.99	10.8	7.04	7.15	6.97	9.05
91.6	7.72	8.94	6.19	7.74	6.61	8.98
91.8	7.41	8.7	5.93	7.44	5.97	7.7
92	6.22	7.89	7.45	6.49	5.82	6.83
92.2	6.3	6.52	8.14	6.97	5.64	7.21
92.4	7.26	6.49	7.77	7.33	5.18	6.93
92.6	7.64	7.57	7.18	6.78	5.15	5.89
92.8	7.77	7.38	6.69	6.73	5.76	6.87
93	8.28	6.82	5.56	6.92	6.37	7.9
93.2	9.14	7.14	5.42	6.49	7.07	7.19

Temperature (C°)	MD-01	MD-02	MD-03	MD-04	MD-05	MD-06
93.4	9.89	6.46	6.5	6.29	7.52	7.05
93.6	9.82	5.51	7.89	6.44	7.69	8.8
93.8	9.4	5.15	8.56	6.95	8	8.48
94	8.76	5.49	8.93	7.69	7.6	7.47
94.2	5.99	6.15	8.15	7.92	6.95	8.74
94.4	3.55	7.78	6.27	8.23	7.06	9.51
94.6	2.44	9.2	4.4	8.32	7.38	8.97
94.8	2.74	10.1	2.89	8.23	7.07	9.89
95	1.77	5.83	1.18	3.54	3.81	4.94

Table 15 Amplification data of PCR product obtained with the Ms-F3/Ms-R2 primer pair for *M. hirsuta*.

Temperature (C°)	MH-01	MH-02	MH-03	MH-04
70	35.8	1.67	35.5	18.3
70.2	77.6	3.62	77	39.6
70.4	71.6	3.34	71.1	36.6
70.6	71.6	3.34	71.1	36.6
70.8	71.6	3.34	71.1	36.6
71	69	0.393	68.8	33.1
71.2	86.3	18.8	83.9	55
71.4	113	53.4	107	94.4
71.6	119	73.2	114	115
71.8	120	83.9	114	123
72	118	91.5	114	125
72.2	118	97.4	113	126
72.4	118	100	111	125
72.6	117	102	110	126
72.8	117	105	109	128
73	117	106	107	129
73.2	117	106	105	130
73.4	116	107	105	129
73.6	116	106	105	128
73.8	114	108	105	127
74	113	109	106	126
74.2	112	107	106	125
74.4	110	107	105	124
74.6	109	110	104	123
74.8	109	110	103	120
75	110	108	103	118
75.2	110	108	103	118
75.4	111	106	101	117
75.6	111	102	101	118
75.8	110	99.7	99.5	119
76	109	98.2	96.5	119
76.2	107	97.8	95.6	119
76.4	106	99.2	96.2	118
76.6	105	100	95.4	118
76.8	105	99.3	95.6	118
77	104	99.3	97.6	117

Temperature (C°)	MH-01	MH-02	MH-03	MH-04
77.2	103	98.8	97.4	116
77.4	104	96.3	96.1	116
77.6	106	95.4	96.3	114
77.8	104	94.8	96.4	113
78	104	93.7	95.7	113
78.2	106	93.9	95	114
78.4	104	95.2	95.5	115
78.8	100	98.3	97.9	115
79	100	102	97.9	115
79.2	99.5	102	99	115
79.4	99.5	102	101	113
79.6	101	102	99.7	113
79.8	103	101	98.1	115
80	103	100	98.8	115
80.2	105	102	99.4	116
80.4	108	103	98.9	120
80.6	111	104	101	121
80.8	113	106	105	123
81	115	108	107	126
81.2	117	109	110	130
81.4	120	113	114	134
81.6	125	118	117	141
81.8	132	122	122	149
82	141	129	128	157
82.2	151	137	137	167
82.4	162	144	148	178
82.6	171	152	158	190
82.8	182	162	168	204
83	193	174	180	220
83.2	206	188	194	238
83.4	224	207	208	260
83.6	247	232	229	288
83.8	275	260	256	325
84	309	294	290	369
84.2	348	334	330	419
84.4	390	377	374	474
84.6	433	422	421	529
84.8	474	465	464	577
85	506	499	495	615
85.2	526	518	513	635

Temperature (C°)	MH-01	MH-02	MH-03	MH-04
85.4	527	517	519	639
85.6	503	494	485	598
85.8	456	449	439	539
86	392	387	377	461
86.2	317	316	306	373
86.4	242	245	235	285
86.6	175	181	171	207
86.8	122	126	117	145
87	81.8	85.7	76.2	97.9
87.2	53.4	57.5	48.9	63.4
87.4	36	37.9	31.3	41.2
87.6	25.3	25.9	20.8	27
87.8	16.5	19.1	16.3	17.8
88	11.7	15	13.9	12.7
88.2	10.3	12.3	11	11.2
88.4	8.68	10.9	9.06	11
88.6	8.04	10.6	7.96	11
88.8	10	10.5	7	11.1
89	11	10.2	7.59	11.5
89.2	9.99	10.1	9.44	10.7
89.4	9.36	9.74	10.4	8.83
89.6	8.71	9.13	10.3	7.77
89.8	6.59	8.74	10.1	6.97
90	5.3	8.03	8.63	6.77
90.2	6.08	7.62	7.14	7.18
90.4	6.94	7.74	6.6	7.34
90.6	7.14	7.41	7.01	6.8
90.8	8.42	6.44	7.5	6.5
91	9.15	5.98	7.42	5.71
91.2	7.61	6.17	6.94	5.68
91.4	6.55	6.26	6.47	6.65
91.6	6.45	6.55	6.01	7.49
91.8	5.22	7.02	5.35	8.09
92	4.01	7.4	6.07	8.25
92.2	5.04	7.45	7.07	7.59
92.4	6.15	6.67	7.26	6.99
92.6	6.7	6.01	6.66	7.39
92.8	8.25	6.15	6.75	7.5
93	9.44	6.57	6.1	7.94
93.2	9.74	6.74	5.23	8.92

Temperature (C°)	MH-01	MH-02	MH-03	MH-04
93.4	9.46	7.31	5.93	8.99
93.6	8.5	8.29	7.19	7.96
93.8	6.26	8.89	7.56	7.41
94	4.66	8.7	7.66	6.96
94.2	3.28	8.35	8.32	6
94.4	3.26	7.47	6.69	7.21
94.6	3.57	6.61	5.73	7.89
94.8	7.27	4.79	3.08	12.1
95	5.27	1.2	-0.126	7.53

Table 16 Amplification data of PCR product obtained with the Ms-F3/Ms-R2 primer pair for *M. rotundifolia*.

Temperature (C°)	MR-01	MR-02	MR-03	MR-04
70	9.17	42.5	-11.6	-11.8
70.2	19.9	92.1	-25.1	-25.6
70.4	18.3	85	-23.2	-23.7
70.6	18.3	85	-23.2	-23.7
70.8	18.3	85	-23.2	-23.7
71	15.2	83	-27	-25.6
71.2	34.6	96.2	-3.42	-13.8
71.4	70.7	116	-3.42	9.84
71.6	93.5	122	43.3	28.8
71.8	108	122	74.9	47.4
72	115	121	96.1	63.9
72.2	121	120	108	75.2
72.4	126	118	112	82.2
72.6	127	116	112	86.5
72.8	127	114	112	89.2
73	127	114	113	91.1
73.2	126	113	116	93.8
73.4	124	113	120	97.4
73.6	122	113	124	100
73.8	120	113	128	102
74	119	110	129	103
74.2	117	109	129	104
74.4	116	109	128	104
74.6	114	109	127	103
74.8	114	108	125	103
75	115	110	124	104
75.2	116	111	123	103
75.4	115	111	121	102
75.6	117	110	120	101
75.8	117	110	119	99.6
76	115	109	117	97.9
76.2	114	107	117	96.7
76.4	114	106	117	94.8
76.6	113	106	116	93.5
76.8	112	105	115	93.2
77	111	104	116	92.8

Temperature (C°)	MR-01	MR-02	MR-03	MR-04
77.2	111	103	115	93
77.4	110	101	115	94.5
77.6	110	101	116	95.7
77.8	110	100	116	96.8
78	110	99.4	114	97.1
78.2	110	99.3	113	96.1
78.4	110	101	112	95.2
78.6	109	101	111	94.3
78.8	109	101	111	93.4
79	110	101	112	93
79.2	110	102	113	93.4
79.4	110	102	115	94.3
79.6	110	102	116	94.8
79.8	109	103	117	95.1
80	108	103	118	95.8
80.2	109	103	120	95.8
80.4	110	104	121	95.4
80.6	113	106	121	96.1
80.8	117	107	124	96.1
81	121	111	127	96.4
81.2	128	115	128	98
81.4	135	118	131	100
81.6	141	123	137	104
81.8	150	130	144	109
82	160	136	153	118
82.2	169	145	167	127
82.4	179	156	181	137
82.6	192	165	194	147
82.8	204	176	208	158
83	218	187	222	169
83.2	239	201	238	182
83.4	262	218	257	200
83.6	292	239	284	224
83.8	333	265	319	255
84	381	297	363	294
84.2	433	333	416	341
84.4	490	373	475	395
84.6	547	416	537	450
84.8	592	458	594	503
85	622	495	641	547

Temperature (C°)	MR-01	MR-02	MR-03	MR-04
85.2	633	519	671	574
85.4	645	524	678	577
85.6	570	507	655	555
85.8	503	467	604	508
86	421	408	530	441
86.2	333	336	440	361
86.4	249	261	344	279
86.6	178	192	255	205
86.8	122	135	179	142
87	81	89.9	120	93.9
87.2	54.7	58.7	77.7	60.9
87.4	37.4	38.1	50.2	39.5
87.6	26.2	25.6	34.1	26
87.8	20.9	18.8	24.1	17.8
88	17.2	14.8	17.9	13.1
88.2	13.8	13.6	14.7	10.8
88.4	12.5	13.8	12.5	9.82
88.6	11.6	12.4	10.1	8.54
88.8	10.3	11.1	9.5	6.93
89	10.5	10.7	9.62	6.69
89.2	11.8	9.2	9.39	6.25
89.4	11.8	7.44	9.83	5.06
89.6	11.5	7.48	10.4	4.9
89.8	11.3	6.91	10.1	5.82
90	10.6	6.55	9.38	5.53
90.2	9.88	7.91	8.1	5.33
90.4	9.84	7.96	6.6	6.17
90.6	10.1	6.86	6.11	6.1
90.8	9.82	7.02	5.37	5.44
91	8.65	7.05	5.8	5.27
91.2	7.07	6.01	7.45	4.96
91.4	6.98	7.11	8.51	4.68
91.6	6.43	9.04	8.58	5.03
91.8	6.47	9.31	8.51	5.48
92	8.47	8.86	7.09	6.13
92.2	9.95	8.44	5.83	6.75
92.4	9.28	6.87	5.38	6.3
92.6	9.13	5.34	4.92	5.89
92.8	9.04	5.99	5.45	6.41
93	8.06	7.28	7.03	6.57

Temperature (C°)	MR-01	MR-02	MR-03	MR-04
93.2	7.24	7.77	7.91	6.49
93.4	7.25	8.17	7.99	6.75
93.6	8.58	8.76	9.32	7.35
93.8	9.36	7.4	9.66	7.58
94	9.02	5.86	9.3	6.89
94.2	9.84	6.13	9.02	7.27
94.4	11.2	7.28	8.16	6.33
94.6	10.1	7.38	5.84	5.25
94.8	11.7	10.1	4.63	1.54
95	5.99	5.75	2.46	-1.31

APPENDIX C

The sensitivity range of the real-time PCR assay

Table 17 Amplification data of 10-fold serial dilutions ranging from 10 ng μL^{-1} to 0.01 ng μL^{-1} of *M. speciosa* DNA.

	DNA concentration (ng)			
Cycle	10 (C°)	1 (C°)	0.1 (C°)	0.01 (C°)
1	7.58	17.7	-31.2	-41.9
2	11.5	9.55	-12.3	-27.1
3	2.02	7.51	-4.09	-20.3
4	4.65	4.83	7.63	-6.41
5	1.47	-1.01	5.6	4.57
6	1.53	1.97	0.579	10.6
7	-2.1	0.673	3.04	8.91
8	-1.27	-2.21	-1.24	7.24
9	-2.32	-2	1.31	6.27
10	-4.69	-1.1	2.23	5.29
11	-3.51	-2.05	-3.75	3.87
12	-1.05	-8.54	-1.56	-0.489
13	12.1	-8.17	-3.55	0.963
14	38.2	-4.57	-1.5	-0.154
15	84.1	-0.648	-3.86	2.97
16	175	4.7	-3.5	-4.92
17	326	16.7	-6.24	-3.03
18	551	48	-4.33	-6.34
19	808	97.4	1.23	-3.11
20	1066	197	19.8	-5.1
21	1326	368	38.2	0.153
22	1571	649	86	0.555
23	1794	1007	176	8.88
24	1982	1393	335	21.7
25	2146	1752	600	58.4
26	2281	2072	968	127
27	2390	2348	1375	247
28	2480	2582	1764	457
29	2549	2760	2102	762
30	2602	2900	2395	1124
31	2635	2995	2634	1477
32	2657	3061	2831	1804
33	2670	3099	2976	2086
34	2678	3121	3075	2322
35	2683	3135	3147	2524

Table 18 Standard curves of *M. speciosa* DNA based on 10-fold dilutions.

DNA concentration (ng)	Ct value
10	27.7
1	24.22
0.1	21.09
0.01	17.3

VITA

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Poster presentation

Supita Awachai, Thongchai Sooksawate, Suchada Sukrong. Genetic Analysis of Plants in the Genus Mitragyna and Development of SNP Marker in the ITS2 Region for the Identification of M. speciosa. Proceeding of the 34th National Graduate Research Conference, March 27, 2015. Khon Kaen, Thailand.