การประเมินทางพันธุกรรมและพฤกษเคมีเพื่อพิสูจน์เอกลักษณ์ของพืชสกุล *Mucuna* และสมุนไพรกลุ่มกวาวเครือ

นางสาวสุชยา วิริยะการุณย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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GENETIC AND PHYTOCHEMICAL ASSESSMENT FOR IDENTIFICATION OF *MUCUNA* PLANTS AND KWAO KHRUEA HERBS

Miss Suchaya Wiriyakarun

A Dissertation Submitted in Partial Fulfillment of the Requirements

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สุชยา วิริยะการุณย์ : การประเมินทางพันธุกรรมและพฤกษเคมีเพื่อพิสูจน์เอกลักษณ์ของพืชสกุล *Mucuna* และสมุนไพรกลุ่มกวาวเครือ (GENETIC AND PHYTOCHEMICAL ASSESSMENT FOR IDENTIFICATION OF *MUCUNA* PLANTS AND KWAO KHRUEA HERBS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.สุชาดา สุขหร่อง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.นิจศิริ เรืองรังษี, 144 หน้า.

พืชสกุล *Mucuna* จัดอยู่ในวงศ์ Fabaceae การอนุกรมวิธานของพืชสกุลนี้ยังมีความสับสนเนื่องจากการมีชื่อพ้องหรือชื่อซ้ำกันมาก และมีลักษณะทางสัณฐานวิทยาที่คล้ายคลึงกัน ทุกส่วนของพืชมี สรรพคุณทางยา เมล็ดเป็นแหล่งของ L-Dopa ซึ่งเป็นสารตั้งต้นของสารสื่อประสาท ที่มีผลใช้ในการ รักษาโรคพาร์กินสัน ในประเทศไทยมีรายงานการพบพืชสกุลนี้ 13 ชนิด ส่วนใหญ่ใช้เป็นยาพื้นบ้าน ถึงแม้ว่าพืชในสกุลนี้เป็นแหล่งของสาร L-Dopa แต่พืชแต่ละชนิดมีคุณสมบัติในการนำไปใช้ประโยชน์ต่างกัน พืชสกุล *Mucuna* ที่ศึกษาจำนวน 6 ชนิด ได้แก่ *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens* และ *M. warburgii* เนื่องจากลักษณะทางสัณฐานวิทยาที่คล้ายกันทำให้การพิสูจน์เอกลักษณ์ของพืชสกุล *Mucuna* เป็นไปได้ค่อนข้างยากโดยเฉพ าะเมื่ออยู่ในรูปของผงยา ส่วนหัวของ *M. macrocarpa* รู้จักทั่วไปในนามกวาวเครือดำ กวาวเครืออีกสองชนิด ได้แก่ กวาวเครือขาว (*Pueraria candollei*) และกวาวเครือแดง (*Butea superba*) จัดอยู่ในวงศ์ Fabaceae เช่นกัน หัวกวาวเครือขาว มีผลต่อฮอร์โมนเอสโตรเจนในเพศหญิง ส่วน หัวกวาวเครือแดงและกวาวเครือดำ มีผลต่อฮอร์โมนแอนโดรเจนในเพศชาย อย่างไรก็ตามการจำแนกชนิดพืชจากหัวที่มีชื่อเรียกเดียวกันว่า “กวาวเครือ” และมีลักษณะภายนอกที่คล้ายกัน อาจจะเกิดปัญหาในตลาดยาเครื่องยาสมุนไพร การควบคุมคุณภาพสมุนไพรและผลิตภัณฑ์จากสมุนไพรให้มีความปลอดภั ยและเกิดประสิทธิผลจึงมีความจำเป็น การศึกษานี้ใช้การประเมินทางพันธุกรรม ร่วมกับการประเมินทาง พฤกษเคมีเพื่อพิสูจน์เอกลักษณ์ของพืชสกุล *Mucuna* และสมุนไพรกลุ่มกวาวเครือ การประเมินทางพันธุกรรม ได้พัฒนาวิธีมัลติเพล็ กซ์พีซีอาร์ในการพิสูจน์เอกลักษณ์พืชสกุล *Mucuna* ด้วยลำดับนิวคลีโอไทด์บริเวณ internal transcribed spacers (ITS) การประเมินทางพฤกษเคมี โดยการใช้ วิธีทีแอลซี เดนซิโทเมท รีในการวิเคราะห์เชิงเปรียบเทียบปริมาณสาร L-Dopa นอกจากนี้ได้ใช้เทคนิคพีซีอาร์-อาร์เอฟแอลพี โดยการใช้เอนไซม์ตัดจำเพาะ *Dde*I และ *Taq*I ในการจำแนกความแตกต่างระหว่างกวาวเครือขาว กวาวเครือแดง และกวาวเครือดำของยีน *mat*K เทคนิคนี้ยัง ถูกนำไปตรวจ เครื่องยาสมุนไพรกวาวเครือชนิดต่างๆ ที่ ซื้อจากตลาดเครื่องยา สมุนไพรด้วย เทคนิค cycleave PCR ที่มีความจำเพาะและมีความไวสูง ได้ถูกนำมาใช้และประสบผลสำเร็จในการจำแนกกวาวเครือ เหล่านี้ด้วยเช่นกัน ยิ่งไปกว่านั้นได้มีการทดสอบเพื่อยืนยันความจำเพาะของเทคนิคนี้ด้วยการนำไปใช้ในการแยก *M. macrocarpa* ออกจากพืชในสกุล *Mucuna* ชนิดอื่นอีก 5 ชนิดที่มีความสัมพันธ์ใกล้ชิดกัน ผลจากการศึกษาในครั้งนี้แสดงให้เห็นว่า การประเมินทางพันธุกรรมร่วมกับการประเมินทางพฤกษเคมีสามารถพิสูจน์เอกลักษณ์ของพืชสกุล *Mucuna* และสมุนไพรกลุ่มกวาวเครือได้

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชเวท ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : L-DOPA / MULTIPLEX PCR / PCR-RFLP / CYCLEAVE PCR / FABACEAE / *MUCUNA* / KWAO KHRUEA

SUCHAYA WIRIYAKARUN: GENETIC AND PHYTOCHEMICAL ASSESSMENT FOR IDENTIFICATION OF *MUCUNA* PLANTS AND KWAO KHRUEA HERBS. ADVISOR: ASSOC. PROF. SUCHADA SUKRONG, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, 144 pp.

The genus *Mucuna* belongs to the Fabaceae family. The taxonomy of the plants in this genus is confused with several synonyms at the species and the morphological features of some species are often similar. All parts of the plants have medicinal properties. The seeds have been employed as a source of L-Dopa, a neurotransmitter precursor which provides an effective remedy for the treatment of Parkinson's disease. There are thirteen species of *Mucuna* found in Thailand report. Most of them were used as folk medicine. Although the plants in this genus are known as source of L-Dopa, each species has been used in different properties. Six *Mucuna* plants, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*, were used in this study. According to the similar morphological features, the authentication of *Mucuna* plants is quite difficult, especially when they are in the form of powders. The tuberous root of *M. macrocarpa* was commonly known as "Black Kwao Khruea". The other two Kwao Khruea, White (*Pueraria candollei*) and Red (*Butea superba*) Kwao Khruea, also belong to the Fabaceae family. The tuberous root of White Kwao Khruea shows oestrogenic effects in the females, whereas Red and Black Kwao Khruea show androgenic effects in males. However, the identification of these roots bearing the name "Kwao Khruea" and have similar features might cause problems in the crude drug market. Quality control for safety and efficacy of medicinal plants and herbal products is necessary. In this study, genetic assessment combining with phytochemical assessment was used to identify *Mucuna* plants and Kwao Khruea herbs. For genetic assessment, the multiplex PCR was developed for species identification based on ITS region. For phytochemical assessment, TLC densitometric method was used for comparative L-Dopa content. In addition, PCR-RFLP using restriction enzymes *Dde*I and *Taq*I was utilised to differentiate White, Red, and Black Kwao Khruea based on *mat*K gene. This technique was also conducted to authenticate crude drugs sold as various types of Kwao Khruea in the crude drug markets. For rapid detection and highly sensitive, cycleaved PCR was also performed to discriminate these Kwao Khruea species. Moreover, the specificity of this technique was confirmed by its ability to distinguish *M. macrocarpa* from five related *Mucuna* plants. The results from these studies indicated that the combination of genetic and phytochemical assessment would be useful for the identification and discrimination of *Mucuna* plants and Kwao Khruea herbs.

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Field of Study : Pharmacognosy Advisor's Signature .....

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LIST OF ABBREVIATIONS

AFLPs	=	amplified fragment length polymorphisms
AP-PCR	=	arbitrarily primed polymerase chain reaction
ARMS	=	amplification-refractory mutation system
AS-PCR	=	allele-specific polymerase chain reaction
AU	=	absorption units
AUC	=	area under the curve
bp	=	base pair
cAMP	=	cyclic adenosine monophosphate
°C	=	degree Celsius
CPT	=	cycling probe technology
CTAB	=	cetyltrimethylammonium bromide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyribonucleotide triphosphate
EDTA	=	ethylenediaminetetraacetic acid
FAM	=	6-carboxy-fluorescine
HCl	=	hydrochloric acid
H_2O	=	water
ITS	=	internal transcribed spacer
Kb	=	kilobase
L-Dopa	=	levodopa
LOD	=	limit of detection
LOQ	=	limit of quantitation
MAMA-PCR	=	mismatch amplification mutation polymerase chain reaction
*mat*K	=	maturase K
MGB	=	minor groove binder
nrDNA	=	nuclear ribosomal DNA
PCR	=	polymerase chain reaction
PCR-RFLP	=	polymerase chain reaction-restriction fragment length polymorphism
r	=	correlation coefficient

*rbc*L	=	large subunit of ribulose-bisphosphate carboxylase
Rf	=	retention factor
RAPD	=	random amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism
ROX	=	6-carboxy-X-rhodamine
rDNA	=	ribosomal deoxyribonucleic acid
RNA	=	ribonucleic acid
RSD	=	relative standard deviation
S	=	slope
SCAR	=	sequence characterised amplified region
SD	=	standard deviation
SNP	=	single nucleotide polymorphism
sp.	=	species
SSCP-PCR	=	single-strand conformation polymorphism polymerase chain reaction
TLC	=	thin-layer chromatography
U	=	unit
UV	=	ultraviolet
V	=	voltage
var.	=	variety

CHAPTER I

INTRODUCTION

The genus *Mucuna*, belonging to the Fabaceae family, is native to Southern Asia (Wilmot-Dear, 1993). All parts of the plants have high medicinal properties (Caius, 1989). The *Mucuna* seeds have been employed as a source of L-3,4-dihydroxy phenylalanine (L-Dopa), a neurotransmitter precursor which provides an effective remedy for the treatment of Parkinson's disease (Nagashayana and Sankarankutty, 2000). In addition, the seeds have also been reported to contain hallucinogenic tryptamines and antinutritional factors such as phenols and tannins (Ravindran and Ravindran, 1988). *Mucuna* plants have traditionally been used as an aphrodisiac for a long time. They have been pharmacologically studied for various activities like anti-Parkinson, antioxidant, antidiabetic, antibacterial, antiaging activities, and erectile-dysfunction (Natarajan *et al*., 2012). Thirteen common species of *Mucuna* are native to Thailand and have been recorded in Thai Forest Bulletin (Wilmot-Dear, 1993). Most of them have been used as Thai folk medicines. However, the uses of *Mucuna* vary among its different species and locations.

Among of these six *Mucuna* plants, the velvet bean *M. pruriens* is one of the most world-famous because of its very high amount of L-Dopa (Manyam and Parikh, 2002). It has been used for a long time as an aphrodisiac for males in traditional Ayurvedic medicines (Shaw and Bera, 1993; Amin, 1996) and has been used to treat nervous disorders and arthritis (Wijeyaratne, 1987). Since *M. pruriens* contains very high levels of L-Dopa, it is the only species which is used as a commercial source of L-Dopa for the treatment of Parkinson's disease (Shaw and Bera, 1993). Besides, velvet bean has been consumed to increase testosterone levels (Amin, 1996), leading to muscle protein deposition and to increase muscle mass and strength (Bhasin *et al.*, 1996). Chen *et al.* have reported that *M. macrocarpa* has also been examined as a source of L-Dopa and it has become one of the most popular sources (Chen *et al.*, 1993).

After the discovery that the *Mucuna* seeds contain L-Dopa, which provides a powerful remedy for the treatment of Parkinson's disease, the demand for *Mucuna*

seeds in international market has increased (Farooqi *et al.*, 1999). In addition to the treatment of Parkinson's disease, *Mucuna* plants have been traditionally used for other purposes. *M. macrocarpa* has been known as the rejuvenating herb (Suntara, 1931). *M. monosperma* showed wound healing activity (Manjunatha *et al.*, 2006a) and antibacterial activity (Manjunatha *et al.*, 2006b). The seeds of *M. interrupta* have been used as an antipyretic (Chuakul, 2009) and those of *M. gigantea* have been used to treat skin problems. *M. warburgii* has been grown as an ornament plant (Wilmot-Dear, 1993). However, the taxonomy of the plants in the genus *Mucuna* is quite complicated due to synonymous scientific names (Duke, 1981) and similar morphological features of some different species such as *M. macrocarpa* and *M. monosperma*, resulting in misidentification (Wilmot-Dear, 1993). Due to their similar morphological features, the authentication of *Mucuna* plants is quite difficult, especially when they are reduced into powders.

Among six *Mucuna* plants used in this study, *M. macrocarpa* is the only one known as the rejuvenating "Black Kwao Khruea" herb. Kwao Khruea is known to Thai people for its rejuvenation properties and has been used in traditional medicine for centuries (Suntara, 1931). The other two Kwao Khruea, White (*Pueraria candollei* Graham ex Benth.) and Red (*Butea superba* Roxb.), belong to the family Fabaceae (Suntara, 1931; Niyomdham, 1992). Although all Kwao Khruea herbs have an indication for rejuvenation, each type is used for specific purposes and effects (Kerr, 1932; Chukeatirote and Saisavoey, 2009). White Kwao Khruea has been used for oestrogen replacement therapy in menopausal women (Cain, 1960; Chedshewasart *et al*., 2004). In contrast, Red and Black Kwao Khruea promote male sexual activity with their androgenic effects and function as cAMP phosphodiesterase inhibitors (Sookkongwaree, 1999; Roengsamran *et al.*, 2000). However, the identification of these roots as "Kwao Khruea" can cause confusion as they have similar physical features, especially when they are in the form of shredded pieces or powder form. Misidentification might cause inconsistent results because of the different therapeutic effects of these species. Quality control for the efficacy and safety of herbal products and medicinal plants is necessary because misidentification by similar appearance may lead to ineffective treatment. Therefore, genetic assessment with a convenient method for the identification of *Mucuna* plants and Kwao Khruea herbs is required.

Genetic assessment is believed to be a reliable tool for identifying medicinal materials (Shaw *et al.*, 1997; Kaplan *et al.*, 2004). Several genetic or DNA-based marker techniques, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), amplification-refractory mutation system (ARMS), arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD), sequence characterised amplified region (SCAR) analysis and DNA sequencing, have recently been used for the authentication and standardisation of medicinal plants (Feng *et al.*, 2010). In recent years, DNA barcodes have also been used to reliably identify herbal medicinal species (Li *et al.*, 2011). The sequences of the standard DNA barcodes, the nuclear ribosomal internal transcribed spacer (nrITS) region and the chloroplast region has high variation and interspecific divergence, and are easy to amplify even from small amount of DNA. Among the candidate DNA barcodes, the maturase K (*mat*K) gene is one of the most rapidly evolving coding regions for the identification of angiosperms at the family, genus, and species levels (Fazekas *et al.*, 2008; Lahaye *et al.*, 2008). Besides genetic assessment, the phytochemical assessment has been important for species identification. Chemically, comparative analysis between the five *Mucuna* plants regarding marker compound such as L-Dopa content need to be done. Therefore, a rapid and simple method for simultaneous detection of qualitative and quantitative analysis is required.

In the present study, genetic assessment by PCR-based methods, including PCR-RFLP, multiplex PCR, and cycleave PCR, combined with phytochemical examination using TLC densitometric method, were performed to identify *Mucuna* plants and Kwao Khruea herbs.

CHAPTER II

LITERATURE REVIEWS

2.1 Plant Samples

2.1.1 *Mucuna* plants

2.1.1.1 Morphology of *Mucuna* plants

The taxonomic description of *Mucuna* was reported by Wilmot-Dear (1993) as follows.

"*Mucuna* is a genus of around 100 species of herbaceous or woody climbers of the Fabaceae family, found worldwide in the tropics and subtropics. Leaves trifoliate, lateral leaflets ± asymmetrical, stipules and often stiples caduceus. Inflorescences axillary on leafy shoots or on old branches, mostly a pseudo-raceme through reduction of ultimate branchlets (secondary axes) or sometimes subumbellate; bract and bracteoles caduceus. Flowers conspicuous, purple, red, greenish, yellow or white. Like other "*Mucuna* plants bear pods".

According to Wilmot-Dear (1993), thirteen *Mucuna* plants have been identified in Thailand.

1) *Mucuna macrocarpa* Wall. กวาวเครือดำ สะบ้าลิง สะบ้าลิงดำ

2) *M. thailandica* Niyomdham & Wilmot-Dear พวงมรกต

3) *M. gigantea* (Willd.) DC. หมามุ่ยช้าง หมามุ่ย สะบ้าลิงลาย

4) *M. oligoplax* Niyomdham & Wilmot-Dear

5) *M. monosperma* DC. ex Wight หมาหมุ้ยใหญ่ สะบ้าลิงลาย

6) *M. stenoplax* Wilmot-Dear

7) *M. hainanensis* Hayata กลั๊ะอื้อซา

8) *M. revoluta* Wilmot-Dear สะบ้าลาย หมามุ่ย

9) *M. interrupta* Gagnep. สะบ้าลิงลาย

10) *M. gracilipes* Craib หมามุ่ยขน

11) *M. pruriens* (L.) DC. หมามุ้ย กลออื้อแซ หมาเหยือง

12) *M. bracteata* DC. ex Kurz หมาเยื่อง บ่าเหยื่อง

13) *M. warburgii* K. Schum. & Lauterb. พวงโกเมน

Among these *Mucuna* plants, six of them including, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, and *M. pruriens*, have been chosen as plant materials in this study because they are common and widely distributed. In addition, most of them have been used as medicinal plants. The other species are excluded because they are very rare or completely absent. The botanical descriptions of the six *Mucuna* species were reported by Wilmot-Dear (1993) as follows.

***M. gigantea* (Willd.) DC.** is called as "Ma Mui Chang" or "หมามุ่ยช้าง" in Thai. The plant is a large sprawing climber. The stems, petioles, and leaflets are glabrous or sparsely fine-adpressed-hairy. The leaves have a terminal leaflet, which is elliptic-ovate (sometimes elliptic or rhombic) in shape. The flowers in inflorescences arranged in axillary with 8-25 cm in length. The bracts are narrowly ovate to elliptic in shape with 3-5 mm in length. The calyx is pubescent like pedicels and abundant in irritant bristles. The flowers are pea-like with white, tinged green, yellow or pink corolla. The fruit is asymmetrically oblong or elliptic-oblong in shape and have 1-4 seeds inside. Fruit surface is brown pubescent and scattered bristles but glabrous when mature. The seeds are dark brown or black (Appendix A3).

***M. interrupta* Gagnep.** is known in Thai as "Sa Ba Lai" or "สะบ้าลาย". The stems are glabrous or sparsely covered with adpressed or abundant spreading fine hairs. The leaves have terminal leaflet with elliptic or ovate in shape, covered with hair on both sides. Inflorescences are approximately 10-14 cm in length. The main axis of which is unbranched and densely covered with adpressed and pale hairs. The

calyx is narrow and covered with hairs and irritant red bristles. The corolla is white or cream and has tinged purple at base. The fruit is large and has 3 orange-brown seeds inside. Fruit surface was covered with abundant, fine, spreading, red brown hairs and irritant bristles (Appendix A4).

***M. macrocarpa* Wall.** is known as "Black Kwao Khruea" or "กวาวเครือดำ" in Thai. It is a woody climber (up to 70 m) which the stems and petioles are densely covered with light brown or red-brown fine pubescent (sometimes later glabrous). The leaves have terminal leaflets with elliptic to ovate (or obovate) in shape. The young leaflets are covered with hairs like the stem and often glabrous later. The inflorescences with unbranched main axis are 5-23 cm in length. The calyx is covered with hairs. The corolla is large and has two-coloured, standard greenish or pinkish white. The woody fruit is linear-oblong in shape and has around 6-15 seeds inside. The seeds are very large with black or dark brown in colour (Appendix A5).

***M. monosperma* DC.** is called in Thai as "Ma Mui Yai" or "หมามุ่ยใหญ่". The plant is a climber which the stems and petioles are rarely glabrescent and usually covered with abundant red-brown hairs. The leaves have terminal leaflet with elliptic or ovate in shape. The main axis of inflorescence is very short with 3-6 cm in length and sparsely covered with hairs and irritant bristles. They are often branched once or more close to base. The calyx has abundant irritant bristles. The corolla is dark purple. The leathery fruit with asymmetrically oblong to elliptic in shape has 1 redish-brown seed (rarely 2 seeds) (Appendix A6).

***M. pruriens* (L.) DC.** has the common name as "Velvet bean". The plant is locally known in Thai as "Ma mui" or "หมามุ่ย". It is a slender climber and often climb up to the tree. The leaves and leaflets are very variable in size. They are elliptic to rhombic-ovate in shape. The main axis of inflorescence is slender and usually long up to 40 cm. The calyx densely covered with adpressed hairs, silvery or brownish pubescent and often also irritant red bristles. The corolla is dark purple (rarely white). The fruit is narrow and linear-oblong in shape and has 3-6 seeds inside. It often curved into "S-shape". Fruit surface densely covered with irritant bristles or

silky hairs, sometimes ornamented with partial longitudinal ridges. The seed is small and ellipsoid in shape (Appendix A7).

***M. warburgii* Lauterb. & K. Schum.** has the common name as "Newguinea Creeper" or "Red Jade Vine" and known as "พวงโกเมน" in Thai. It is very different from all native species in flower colour and shape. The flowers are large with bright orange-red in colour. The fruit is large linear-oblong in shape with at least 20 cm in length. It is native to Indonesia (Sulawesi, Moluccas, Papua New Guinea, and Irian Jaya). This plant is occasionally cultivated for ornamental purposes (Appendix A8).

2.1.1.2 L-Dopa content in *Mucuna* plants

The plants in the genus *Mucuna* are the best source of L-Dopa due to the large amounts of L-Dopa, the aromatic non-protein amino acid, found in the seeds (Daxenbichler *et al*., 1971) (Figure 2.1)

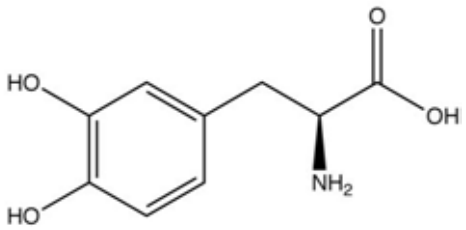

Figure 2.1 Structure of L-Dopa

The seeds of all *Mucuna* species were found to contain L-dopa (Ratnawati *et al*., 2011). Many studies have shown that the level of L-Dopa percentages widely varied in different *Mucuna* plants. *M. holtonii*, *M. urens*, and *M. aterrima* have been found to contain 6.7%, 5.2%, and 5.0%, respectively (Daxenbichler *et al*., 1971). *Mucuna* plants located in Sri Lanka, including *M. nivea*, *M. deeringiana*, *M. utilis*, and *M. aterrima*, have been found to contain the amount of L-Dopa 2.5, 2.7, 3.8, and 4.4%, respectively (Amarasekera and Jansz, 1980). L-Dopa contents of 1.5% and 4.56% were observed in *M. gigantea* (Rajaram and Janardhanan, 1991) and *M. monosperma* (Arulmozhi and Janardhanan, 1992), respectively. The

amount of L-Dopa in *M. pruriens* seed was higher than other tribal pulses, *M. utilis* and *M. monosperma* (Mohan and Janardhanan, 1995). L-Dopa in *M. gigantea* seed has been found to be very low (1.7-2%) compared to the other *Mucuna* plants (Janardhanan, 1982).

There are no reports on the L-Dopa content of Thai *Mucuna* plants.

2.1.1.3 Bioactivities of *Mucuna* plants

Mucuna plants possess valuable traditional and medicinal properties. This genus is known as a natural source of L-dopa (Daxenbichler *et al*., 1971), which has been used to treat people with Parkinson's diseases, who lack dopamine in the brain, which is a direct precursor for the neurotransmitter 3,4-dihydroxyphenyl-ethylamine (dopamine) (Haq, 1983; Nagashayana and Sankarankutty, 2000). After L-Dopa has entered the brain, it is rapidly decarboxylated to dopamine by the enzyme aromatic L-amino acid decarboxylase (Calne, 1970).

Besides L-Dopa, many species of *Mucuna* plants have been found to have other compounds that have different properties. *M. gigantea* has been used as traditional medicine and utilized as a fertilizer (Onweiluzoan and Eilitta, 2003). The protein fractions in seed, albumins and globulins, have been also utilized to strengthen blood. The seeds of *M. gigantea* have been used for the treatment of dermatological diseases or skin disorders (วงศ์สถิตย์ ฉั่วกุล, 2543). Seed powder of *M. gigantea* has been used as a purgative in Hawii. The root has been used to treat gonorrhoea and schistosomiasis. The bark has been used for the treatment of rheumatic complaints in India (Brink, 2006). *M. macrocarpa* has been known as a rejuvenating herb in Thai traditional medicine for the promotion of sexual potency in males (Wiriyakarun *et al.*, 2012). *Mucuna monosperma* seeds have been used for treating cough and asthma. It has been found to have expectorant properties. In addition, this plant is also used to treat acute spasm (Janardhanan *et al*., 2003). All parts of *M. pruriens* possess important medicinal properties (Caius, 1989), including anti-inflammatory, diuretic, antibacterial, antidiuretic, aphrodisiac, anti-neoplastic, properties (Sathiyanarayanan *et al*., 2007; Bala *et al*., 2011; Majekodunmi *et al*., 2011). It has been used in

Ayurvedic Indian medicine to provide symptomatic relief in Parkinson's disease (Haq, 1983). Moreover, the use as a fertility agent for males has been reported by Buckles *et al*. (1995).

2.1.2 Kwao Khruea herbs

2.1.2.1 Morphology of Kwao Khruea herbs

There are three types of Kwao Khruea: White (*Pueraria candollei*), Red (*Butea superba*), and Black (*Mucuna macrocarpa*) (Suntara, 1931; Niyomdham, 1992).

White Kwao Khruea or *P. candollei* belongs to the family Fabaceae, sub-family Papilionoideae. In general, this species is a strong perennial woody climber. Many of *Pueraria* species are found throughout Asia, Australia, Africa, and North, Central and South America. Approximately 76 different sub-species of *Pueraria* have been identified (Maeson, 1985). *P. candollei* is found in the Northern, Western, and Northeastern of Thailand at elevations between 300 and 800 meters (Niyomdham, 1992). Two varieties of *P. candollei*, var. *mirifica* and var. *candollei*, are very similar in morphological features. Length of the inflorescence, flowers, and calyx of *P. candollei* var. *mirifica* are approximately 30-80 cm, 13-15 mm, and 5-8 mm, respectively. Length of the inflorescence, flowers, and calyx of *P. candollei* var. *candollei* are around 30 cm, 18-20 mm, 8-12 mm, respectively (Niyomdam, 1992).

Red Kwao Khruea or *B. superba* is a member of the family Fabaceae, sub-family Papilionoideae. It is a perennial twinning shrub. The elongated tuberous root released red sap when cut. The leaves are pinnately trifoliate, with equal sized oblong-ovate leaflets. The petiole is long. The flowers are of a yellowish orange color, found in clusters. The petals are three times longer than the calyx. Pods are oblong shaped with silvery silky short hair and around 3-4 inches in length (Suntara, 1931; Niyomdham, 1992).

Black Kwao Khruea or *M. macrocarpa* is the only *Mucuna* species known as a rejuvenating "Black Kwao Khruea" herb. The morphology of this plant is described in 2.1.1.1.

2.1.2.2 Chemical constituents of Kwao Khruea herbs

The tuberous roots of all of the three Kwao Khruea herbs contain the commonly formed flavonoids such as daidzin, daidzein, genistin, genistein (Ingham *et al.*, 1989; Chukeatirote and Saisavoey, 2009), and other isoflavones (Chansakaow *et al.*, 2000) that are known for their rejuvenating properties. Although all of these species have indications for rejuvenation, each species contains additional and different active constituents that could be related to this property (Kerr, 1932; Chukeatirote and Saisavoey, 2009). White Kwao Khruea contains miroestrol, deoxymiroestrol, and isoflavone puerarin (Bounds and Pope, 1960; Tayler *et al.*, 1960; Jones and Pope, 1961; Ingham *et al.*, 1986; Chansakaow *et al.*, 2000). Red Kwao Khruea contains high levels of flavonoids and flavonoid glycosides (Roengsamran *et al.*, 2000). The bioactive substances in Black Kwao Khruea include quercetin, kaempferol, and hopeaphenol (Sookkongwaree, 1999).

2.1.2.3 Bioactivities of Kwao Khruea herbs

White Kwao Khruea has strong effects similar to oestrogen replacement therapy in females, especially in menopausal women (Cain, 1960; Cherdshewasart *et al.*, 2004; Yusakul *et al.*, 2011). Red Kwao Khruea has been used in mature males for the treatment of erectile dysfunction and the maintenance of sexual performance (Roengsamran *et al.*, 2000). Black Kwao Khruea is also used to promote male sexual activity like Red Kwao Khruea with its androgenic effects. Quercetin found in Black Kwao Khruea shows stimulating effects on sperm quality and reproductive organs in adult male rats (Taepongsorat *et al.*, 2008).

2.2 Assessment for Identification of the Plants

2.2.1 Genetic assessment

Genetic assessment by PCR-based techniques has been widely used for the identification and authentication of plant species of medicinal importance (Joshi *et al.*,

2004). It involves amplification of partial DNA of interest by using specific or arbitrary oligonucleotide primers and thermostable DNA polymerase. PCR techniques have the main advantages that can analyse DNA even from a very small amount of starting material and can be performed without prior information of the sequence. Several genetic markers can be performed within a short time (Heubl, 2010).

DNA regions recommended for molecular identification of herbal drugs are ITS region, *mat*K gene, and the other plastid gene regions (Li *et al*., 2011; Sukrong *et al*., 2007). The ITS region has high interspecific divergence, and are easy to amplify even from small amount of DNA. The entire ITS region of nuclear ribosomal DNA (nrDNA) comprises two non-coding regions, ITS1 and ITS2 intergenic spacers, which flank the conserved coding region of 5.8S ribosomal subunit. Of these, the sequences of ITS1 and ITS2 are highly variable, whereas the sequence of 5.8S rDNA is highly conserved. The complete sequence of ITS region ranges from 400 to over 1000 bp in length (Nagy, 2012) (Figure 2.2).

Figure 2.2 Schematic diagram of the nucler rDNA ITS region. It composes of ITS1 fragment between 18S and 5.8S rRNA subunits and ITS2 fragment between 5.8S and 28S rRNA subunits

Due to high species discrimination, ITS region has been frequently used for investigation of plant phylogenetic relationships between species (Howard *et al.*, 2009) and for genetic diversity assessment of herbal species (Mondini *et al*., 2009). Similarlity, it has also been frequently used for molecular authentication of medicinal plants (Zhang *et al.*, 2007). The ITS region was successfully used to distinguish the authentic species of the antitumor herbal medicine "*Baihuasheshecao*" derived from *H. diffusa* Willd. from its adulterant, *H. corymbosa* (L.) Lam (Li *et al.*, 2010). Furthermore, it is also useful for the discrimination of *Baihuasheshecao* from adulterants derived from the other species, such as *Sagina japonica* (SW.) Ohwi,

Stellaria alsine Grimm, *Arenaria serpyllifolia* L., and the *Mollugo* L. species (Liu and Hao, 2005).

Several plastid regions, including *atp*F-H, *mat*K, *psb*K-I, *rbc*L, *rpo*B, *rpo*C1 and *trn*H-*psb*A have been widely used for development of candidate markers for plant DNA barcoding. The *matK* coding region is the most rapidly evolving chloroplast gene which has a simple and stable genetic structure. It has been found to be suitable DNA barcodes because of high universality, quality and coverage of sequence, and discrimination power. The *mat*K gene, located in the intron of the *trn*K, coding for the maturase, is approximately 1,500 bp in length (Neuhaus and Link, 1987) (Figure 2.3). It is useful for identification at family, genus, and even species levels due to its highsubstitution rates. There are no recombinations along the *mat*K gene because it is generally uniparentally transmitted.

Moreover, the *mat*K gene sequence is a powerful marker for identifying the botanical origins of certain medicinal herbs (Fushimi *et al.*, 1996). In recent years, *mat*K gene barcodes were employed to identify ginseng drugs (Fushimi *et al.*, 1997) as well as *Rheum* (Yang *et al.*, 2004), *Agastache* (Heubl, 2010), and *Dioscorea* species (Sun *et al.*, 2012).

Figure 2.3 Schematic diagram of the chloroplast *mat*K gene

2.2.1.1 PCR-RFLP

PCR-RFLP is a PCR amplification of specific DNA combined with digestion of PCR products using restriction enzyme (Heubl, 2010). This technique is generated in two steps. Firstly, a sequence of interest is amplified using specific primers. Secondly, the amplified product is degested with a restriction enzyme which

usually recognizes a 4 base-pair sequence of DNA. The digested fragments are separated according to their lengths by agarose gel electrophoresis (Heubl, 2010). PCR-RFLP has been successfully used for the discrimination of various medicinal plants from related species or adulterants, including *Fritillaria pallidiflora* (Wang *et al.*, 2005), *Alisma orientale* (Li *et al.*, 2007), *Actinidia macrosperma* (Zhao *et al.*, 2007), *Stemona tuberosa* (Vongsak *et al.*, 2008; Fan *et al.*, 2009), and *Panax ginseng* (Diao *et al.*, 2009).

2.2.1.2 Multiplex PCR technique

Multiplex PCR is a powerful technique that enables simultaneous amplification of two or more targets using multiple primers in a single reaction mixture (James *et al.*, 2003). It has been widely used for species identification of several organisms, including microorganisms (Settanni *et al.*, 2005; Koh *et al.*, 2012), genetically modified crops (James *et al.*, 2003), and medicinal plants (Lin *et al.*, 2006). However, multiplex PCR for authentication of medicinal plants in the genus *Mucuna* has not been reported. In recent year, single nucleotide polymorphisms (SNPs), one class of DNA markers, has become widely used as molecular marker for genomic studies in various laboratories (Jehan and Lakhanpaul, 2006). In 2008, multiplex PCR was used for microsatellites and SNPs analysis by Hayden *et al.* (Hayden *et al.*, 2008). Multiplex PCR based on SNPs exhibits high specificity for the identification of medicinal plants when compared with the other common molecular techniques such as RAPD, restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphisms (AFLPs), and simple sequence repeat (SSR) (Kim *et al.*, 2012). Recently, SNP-based multiplex PCR has been developed for medicinal plants authentication base on ITS sequences (Jigden *et al.*, 2010; Lee *et al.*, 2012).

2.2.1.3 Cycleave real-time PCR

Cycleave PCR is a combination of Cycling Probe Technology (CPT) and real-time PCR (Ogino *et al.*, 2011). The assay can detect SNPs using a cycling

probe (chimeric DNA-RNA-DNA probe labelled with a fluorescent dye and quencher at each end) and RNase H (Duck *et al.*, 1990; Bekkaoui *et al.*, 1996; Hou *et al.*, 2011). This cycling probe is generally 10-14 bases in length (Itahashi *et al.*, 2010). The RNA part of the probe is cleaved by RNase H after hybridisation with the complementary sequence in the amplified product, which results in strong fluorescence emission by the separation of the fluorescent dye from the quencher (Figure 2.4) (Esaki *et al.*, 2004; Yatabe *et al.*, 2006; Urasaki *et al.*, 2008). Cleavage will not occur if there is a mismatch within the probe-binding region (Yabutani *et al.*, 2009). By measuring the fluorescence emission intensity, the amplification of the target region can be monitored (Esaki *et al.*, 2004).

Most studies involving cycleave PCR are used for quantitative analysis and are focused on the detection and identification of microorganisms in clinical samples and plant diseases (Itahashi *et al.*, 2010; Suzuki *et al.*, 2010; Hou *et al.*, 2011). Recently, cycleave PCR has been successfully used for the qualitative detection of point mutations in many organisms (Esaki *et al.*, 2004; Urasaki *et al.*, 2008).

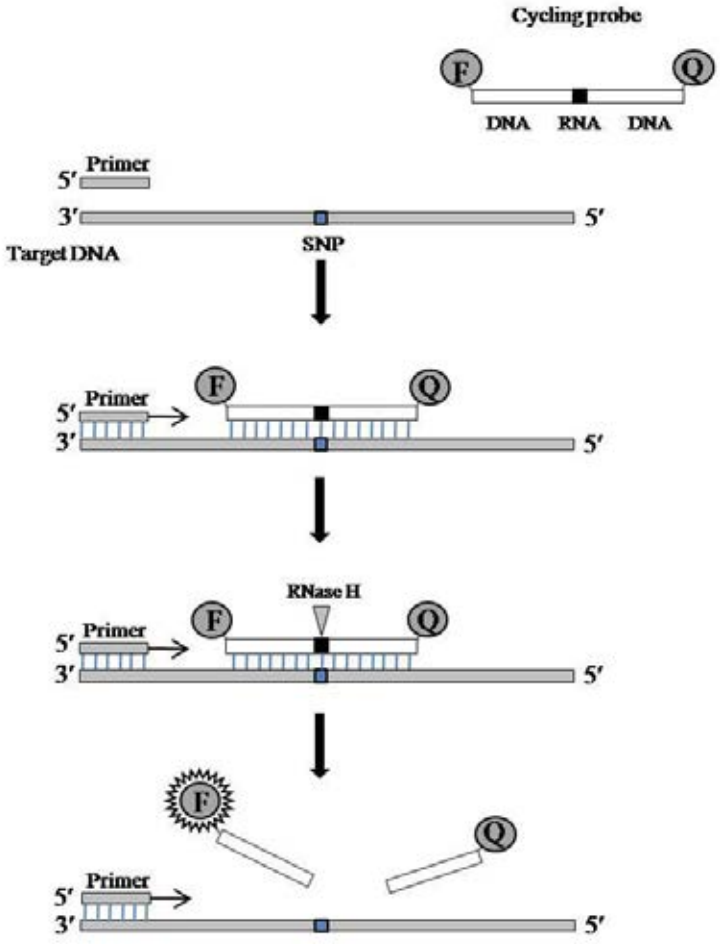

Figure 2.4 Principle of cycleave PCR. The cycling probe (generally 10-14 nucleotides) contains RNA corresponding to the SNP of the target sequence. The probe hybridises to its complementary target DNA and forms a segment of DNA/RNA duplex. The RNA part of the probe is cleaved by RNase H at the RNA linkage after hybridisation. When the fluorescence and quencher molecules separate from each other, a fluorescent signal is emitted. F and Q indicate the fluorescence molecule and quencher molecule, respectively

2.2.1.4 DNA sequencing

DNA sequencing techniques are the tools widely used in many fields such as archaeology, anthropology, genetics, biotechnology, molecular biology, and forensic sciences. It involves the process of determining the order of the four bases, adenine, guanine, cytosine, and thymine, in DNA sample. In addition, this technique can be used to determine the sequence of individual genes, clusters of genes or entire genomes (Franca-Lilian *et al*., 2002).

2.2.2 Phytochemical assessment

2.2.2.1 Thin-layer chromatography (TLC)

TLC is a simple, reliable, and rapid analytical technique of chromatography. It is widely used as a screening tool and routinely used for qualitative assessment of chemical constituents of medicinal materials (Mohammad *et al*., 2010). TLC methods are successfully used in many fields of laboratory research such as biochemistry, clinical medicine, and pharmaceutical analysis (Dickson *et al*., 2004). This method is usually the method of choice for preliminary phytochemical screening of L-Dopa in *Mucuna pruriens* seeds (Misra and Wagner, 2007; Krishnaveni *et al*., 2009). In addition, TLC can also be used for quantitative analysis by combining with the other detection methods of varying degrees of sensitivity and specificity (Heftmann, 2004).

2.2.2.2 TLC densitometry

TLC densitometric method is TLC method combined with UV densitometry. This combination technique can be used for accurate qualitative and quantitative analysis. Quantitative TLC measurements are determined UV by densitometric scanning (Najar *et al*., 2007). This technique has been successfully used for the analysis of active constituents in medicinal plants such as *Strychnos* spp. (Dhalwal *et al*., 2007), *Clerodendrum phlomidis* (Raja and Mishra, 2009), *Mucuna*

pruriens (Jegadeesan and Saravana, 2010), and *Cassia fistula* (Chewchinda *et al*., 2012)**.**

CHAPTER III

DNA SEQUENCES OF ITS REGION AND *MAT*K GENE OF SIX *MUCUNA* PLANTS

3.1 Introduction

In recent years, DNA sequences unique to a species have also been used to reliably identify herbal medicinal species (Barthelson, 2009). Among the candidate DNA regions, the *mat*K gene is one of the most rapidly evolving coding regions for the identification of angiosperms at the family, genus, and even species levels due to its highsubstitution rates (Fazekas *et al.*, 2008; Lahaye *et al.*, 2008). The ITS region of nuclear ribosomal DNA is the most widely used genetic markers in plants due to its high variability (Tippery and Les, 2008). In this study, the *mat*K gene and ITS region were used as suitable DNA markers for the identification and differentiation of different species in the genus *Mucuna* using DNA sequencing technique.

3.2 Materials and Methods

3.2.1 Plant materials

Samples of six *Mucuna* plants, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*, considered as authentic samples were collected from various locations from Thailand (Table 3.1). Voucher specimens have been deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3.2.2 DNA extraction

Total genomic DNA was extracted from 100 mg of leaves from each sample, and was frozen using liquid nitrogen and ground with a mortar and pestle to obtain a fine powder. The isolation of the total DNA from the powder was performed using a modified CTAB method (Doyle *et al.*, 1991). The genomic DNA was separated using

1% agarose gel electrophoresis, stained with ethidium bromide and visualised under UV light. The quantity and quality of genomic DNA were assessed using UV spectrophotometry and gel electrophoresis. The DNA concentrations were adjusted to 50-80 μg/ml using water. The extracted DNA samples were stored at -20 °C until further use.

Table 3.1 Plant materials used in this study

Species	Location (Province)	Sample size	Voucher No.	Accession No. (ITS/*mat*K)
Mucuna gigantea (Willd.) DC.	Bangkok	3	MUS-H3868	AB775134/ AB627860
M. interrupta Gagnep.	Chiang Rai	3	MUS-H3867	AB775135/ AB627862
M. macrocarpa Wall.	Chiang Mai	5	MUS-H3847	AB775133/ AB627858
M. monosperma Wight	Phang Nga	3	MUS-H3865	AB775136/ AB627859
M. pruriens (L.) DC.	Kanchanaburi	5	MUS-H3864	AB775137/ AB627857
M. warburgii K. Schum. & Lauterb.	Nakornratchasrima	4	MUS-H3866	AB775138/ AB627861

3.2.3 Primer design

A pair of the universal primers, ITS1 (forward): 5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS4 (reverse): 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White *et al*., 1990), positioned on the conserve regions (18S and 28S), were used to amplify the complete ITS regions of the genus *Mucuna* (Figure 3.1). To amplify and sequence the *mat*K gene of *Mucuna* plants, six primers were designed based on the sequences of *trn*K-*mat*K regions obtained from GenBank. The *trn*K-*mat*K sequences of related species in Fabaceae family, including *Glycine max* (L.) Merr. (accession number

AF142700), *Pseudovigna argentea* (Willd.) Verdc. (accession number EU717423), *Apios americana* Medik. (accession number EU717426), *Galactia striata* (Jacq.) Urb. (accession number AF142704), and *Mucuna* sp. (accession number EU717422) were aligned and flanking conserved regions were selected. The locations of amplification primers, *mat*K-MUF and *mat*K-MUR, and the sequencing primers on *mat*K region are shown in Figure 3.2. Details of these primers are presented in Table 3.2.

Figure 3.1 Map of ITS region (ITS1-5.8S-ITS2) with the positions of the universal primers ITS1 and ITS4. The arrows represent the direction of the primers

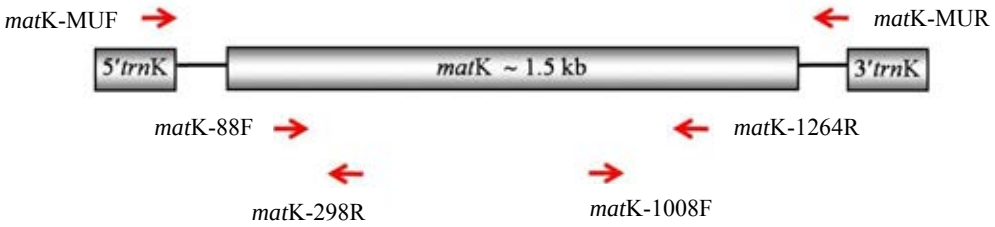

Figure 3.2 Schematic diagram of the chloroplast *mat*K gene and relative positions of the PCR amplification primers and sequencing primers used in this study. The arrows represent the directions of the primers

Table 3.2 PCR amplification primers and sequencing primers of *mat*K gene used in this study

Primer name	Nucleotide sequence (5′ to 3′)	Direction	T_m (°C)
*mat*K-MUF*	GTC CGT TGA TGR DTT TTA CTT G	forward	48.5
*mat*K-MUR	TTA ATG AAT CCC GAA TCC TG	reverse	49.3
*mat*K-88F	GGA CTC GCT TAT GGT CAT GG	forward	59.4
*mat*K-1264R	GAG GAT CCT TTG TAA TAA TGA GAA	reverse	55.9
*mat*K-1008F	TCG GCT AAA TCT TTC AGT GGT	forward	55.9
*mat*K-298R	CCA CGA CGA CAA TAA AAC CTT C	reverse	58.4

*Degenerate primer which each letter represents a combination of one or several nucleotides: R = (G, A) and D = (T, A, G)

3.2.4 PCR amplification of the ITS region and the *mat*K gene

A pair of the universal primers, ITS1 and ITS4, was used to amplify the complete ITS regions of the genus *Mucuna*. The PCR amplification was conducted in a total volume of 50 µl. The PCR reaction mixture contained 1 µl of DNA template, 2.0 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of each dNTP, and 1 U *Taq* polymerase (Promega, U.S.A.). The amplification was performed using a DNA thermal cycler (Bio-Rad, USA) with an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, primer annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 7 min.

The obtained genomic DNAs were used as templates for the amplification of *mat*K gene. The amplification primers and sequencing primers (Table 3.2) were used to amplify and sequence the complete *mat*K gene of six *Mucuna* plants. The amplified products of ITS region and *mat*K gene were detected using 1% agarose gel electrophoresis, stained with ethidium bromide. The obtained fragments were visualized and photographed using a UV transilluminator and analyzed with a gel documentation system (Bio-Rad, USA).

3.2.5 DNA sequencing of the ITS region and the *mat*K gene and phylogenetic tree analysis

The PCR products of the ITS region and the *mat*K gene were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA), and then the purified products were sequenced directly at the BioService Unit (BSU) of the National Centre for Genetic Engineering and Biotechnology (BIOTEC, Klong Luang Pathumthani, Thailand). The obtained ITS and *mat*K gene sequences were aligned using ClustalW software. The phylogenetic tree was created by the Lagergene Megalign program (DNASTAR, Inc., USA). *M. hainanensis* and *B. superba* were included as outgroup for ITS and *mat*K gene sequences, respectively. The sequences were submitted to GenBank, and the accession numbers are listed in Table 3.1.

3.3 Results

3.3.1 Sequence analysis of ITS region and *mat*K gene of six *Mucuna* plants and phylogenetic tree

The ITS regions of six *Mucuna* plants were amplified using the ITS1 and ITS4 universal primers. The fragments approximately 800 bp of ITS region were obtained (Figure 3.3). The purified products were sequenced individually. The ITS sequences of six *Mucuna* plants, *M. pruriens*, *M. warburgii*, *M. interrupta*, *M. monosperma*, *M. gigantea*, and *M. macrocarpa* were 747, 756, 736, 750, 755, and 742 bp in length, respectively. The sequence divergence among six *Mucuna* plants varied from 1.3% to 15.9%. A pairwise comparison between *M. gigantea* and *M. pruriens* showed the highest nucleotide sequence divergence at 15.9%. Whereas, a pair of *M. gigantea* and *M. monosperma* showed the lowest nucleotide sequence divergence at 1.3%. The percentage identity and the ITS sequence divergence between six *Mucuna* plants were shown in Table 3.3. The obtained ITS sequences have been deposited in GenBank (Appendix B) and the accession numbers are listed in Table 3.1.

The phylogenetic trees shown as dendrogram were created based on the percent divergence of ITS region and *mat*K gene from six *Mucuna* plants. Based on

the dendrogram, the six *Mucuna* species on the basis of ITS region were categorized into four groups (Figure 3.4). The first group was comprised of *M. macrocarpa* and *M. interrupta*. *M. gigantea* and *M. pruriens* were individually separated from the other species and were arranged in the second and the third group, respectively. The forth group consisted of the *M. warburgii* and *M. monosperma*. In the dendrogram generated from the *mat*K gene sequence, the six *Mucuna* species were devided into two groups (Figure 3.5). The first group, *M. warburgii*, *M. gigantea M. monosperma*, *M. interrupta*, and *M. macrocarpa*. In the second group was comprised of *M. pruriens* which was clearly separated from the other species.

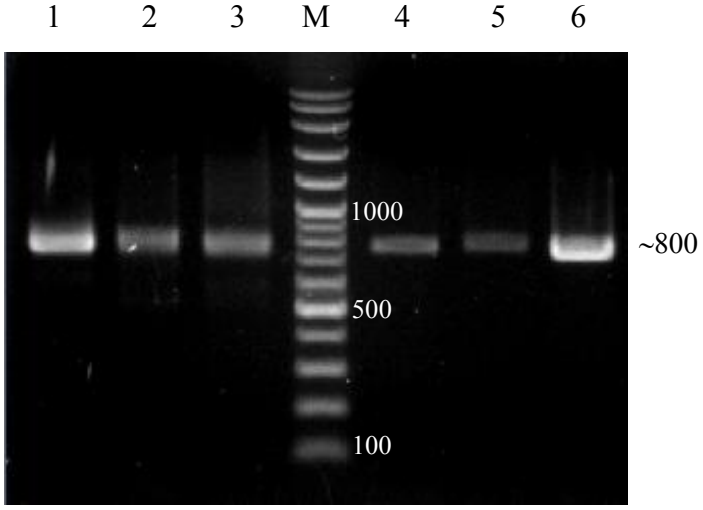

Figure 3.3 Agarose gel electrophoretogram of PCR products of complete ITS region**.** Lane 1: *M. pruriens*; Lane 2: *M. warburgii*; Lane 3: *M. interrupta*; Lane M: DNA marker VC 100 bp plus; Lane 4: *M. monosperma*; Lane 5: *M. gigantea*; Lane 6: *M. macrocarpa*

Table 3.3 Pairwise percent identity and sequence divergence in the ITS region among six species in the genus *Mucuna*

Percent identity

Divergence

	1	**2**	**3**	**4**	**5**	**6**	
1		89.9	90.8	89.7	92.6	88.9	1 *M. macrocarpa*
2	11.0		95.7	98.7	85.8	96.3	2 *M. gigantea*
3	9.9	4.5		95.2	87.8	94.0	3 *M. interrupta*
4	11.2	1.3	4.9		86.3	96.1	4 *M. monosperma*
5	7.8	15.9	13.5	15.3		86.2	5 *M. pruriens*
6	12.2	3.8	6.3	4.0	15.5		6 *M. warburgii*
	1	**2**	**3**	**4**	**5**	**6**	

The complete *mat*K gene of six *Mucuna* plants was amplified using the amplification primers *mat*K-MUF and *mat*K-MUR by PCR technique. The fragments of *mat*K gene about 1,500 bp in length were obtained. The purified products were sequenced individually using the sequencing primers list in Table 3.2. The complete *mat*K gene sequences of *M. warburgii*, *M. interrupta*, *M. monosperma*, *M. gigantea*, and *M. macrocarpa* were 1,518 bp in length except *M. pruriens* was 1,524 bp. Sequence distance (percent identity and divergence) were calculated by using the program ClustalW in the LASERGENE software. The *mat*K sequences from all samples of the same species showed completely identical sequence. The sequence divergence among six *Mucuna* plants varied from 1.3% to 3.6%. A pairwise comparison between *M. pruriens* and *M. warburgii* showed the highest nucleotide sequence divergence at 3.6%. Whereas, a pair of *M. macrocarpa* and *M. monosperma* and a pair of *M. warburgii* and *M. interrupta* showed the lowest nucleotide sequence divergence at the same percentage of 1.3%. The percentage identity and the nucleotide sequence divergence between six *Mucuna* plants were shown in Table 3.4. The obtained *mat*K gene sequences have been deposited in GenBank (Appendix B) and the accession numbers are listed in Table 3.1.

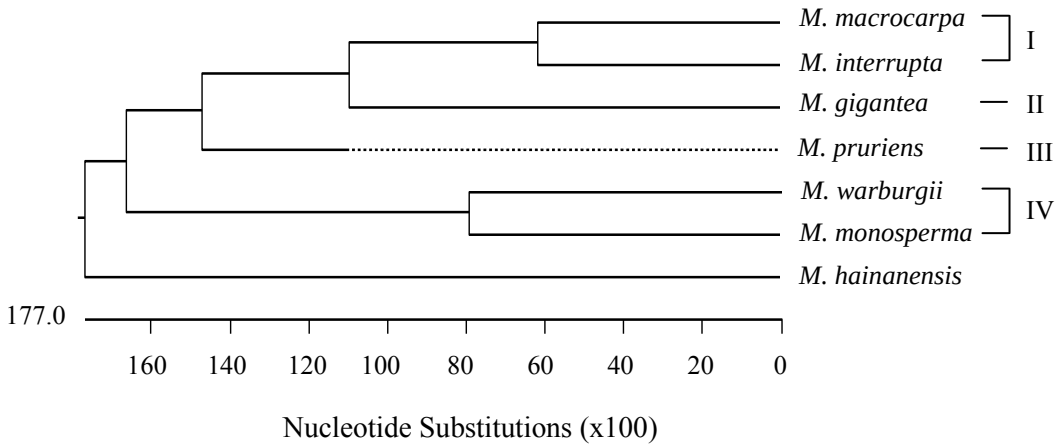

Figure 3.4 The phylogenetic tree of six *Mucuna* species generated from the ITS region**.** The length in each branch represents the distance between sequence pairs. Scale bar measures the distance between sequences, proportional to the number of nucleotide substitutions. The dotted lines indicate that the sequence distance based on ITS region is not proportional to the scale. *M. hainanensis* was included as an outgroup

Table 3.4 Pairwise percent identity and sequence divergence in the *mat*K gene among six species in the genus *Mucuna*

Percent identity

Divergence

	1	**2**	**3**	**4**	**5**	**6**	
1		98.5	97.5	98.7	97.0	97.0	1 *M. macrocarpa*
2	1.5		98.1	99. 7	96.8	98.6	2 *M. gigantea*
3	2.5	1.9		98.0	96.6	98.7	3 *M. interrupta*
4	1.3	0.3	2.1		97.0	98.3	4 *M. monosperma*
5	3.0	3.2	3.4	3.1		96.4	5 *M. pruriens*
6	3.0	1.5	1.3	1.7	3.6		6 *M. warburgii*
	1	**2**	**3**	**4**	**5**	**6**	

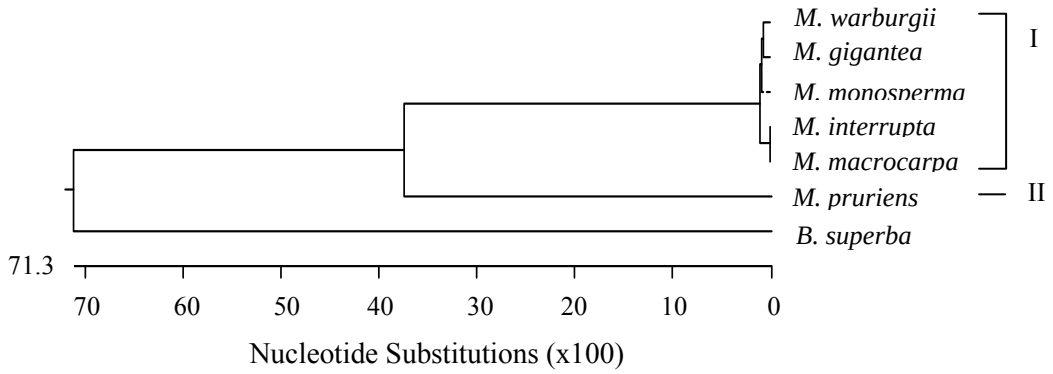

Figure 3.5 The phylogenetic tree of six *Mucuna* species generated from the *mat*K gene sequence**.** The length in each branch represents the distance between sequence pairs. Scale bar measures the distance between sequences, proportional to the number of nucleotide substitutions. *B. superba* was included as an outgroup

3.4 Discussion

DNA sequencing technique was used for the identification of *Mucuna* plants in this study. ITS region and the chloroplast *mat*K gene were used as suitable DNA regions. The ITS regions of six *Mucuna* plants were amplified easily with the ITS1 and ITS4 universal primers. The obtained fragments were found to be approximately 750 bp in length. These product sizes were consistent with the previous report showing that the ITS regions range from 400 to over 1,000 bp in length in general (Nagy, 2012). The *mat*K gene was amplified using primers *mat*K-MUF and *mat*K-MUR designed based on the sequences of *trn*K-*mat*K regions. The complete *mat*K gene of six *Mucuna* plants were about 1,518-1,524 bp in length, which is consistent with a previous report showing that the *mat*K coding region in the most angiosperms is aound 1.5-1.6 kb in length (Neuhaus and Link, 1987). Interestingly, the DNA sequences of six *Mucuna* plants have two different sizes, 1) the size of 1,518 bp in *M. warburgii*, *M. interrupta*, *M. monosperma*, *M. gigantea*, and *M. macrocarpa*; and 2) the size of 1,524 bp in *M. pruriens*. The result was consistent with the previous report by Wilmot-Dear (1993) showing that *Mucuna* has been divided into two subgenera, subgenus *Mucuna* and *Stizolobium* (P.Br.) Prain. *M. pruriens* was arranged in

subgenus *Stizolobium* whereas the other five species were arranged in subgenus *Mucuna* (Wilmot-Dear, 1993). Moreover, the result from the phylogenetic study based on *mat*K gene showed that *M. pruriens* was clearly separated from the other species which are very closely related. This result indicated that the *mat*K gene sequence was correlated with morphological features. In addition, according to the sequence divergence of ITS region and *mat*K gene among six *Mucuna* plants, the results indicated that the *mat*K gene sequences was more conserved than ITS region. Indeed, DNA sequence is unique that can be used to identify individual species. However, there are the alternative molecular methods as well as PCR-RFLP or the other techniques that provide rapid and simple process, and can also be applied for species identification.

3.5 Conclusion

To identify medicinal plant species in the genus *Mucuna*, six species of which, including *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*, were examined by using DNA sequencing technique. With highly conserved, the *mat*K gene was chosen as first priority of DNA markers for identification of *Mucuna* plants. However, the highly variable ITS region is also a valuable marker for phylogenetic studies and identification at the genus and species level. The ITS region and the *mat*K gene appeared to be suitable DNA regions for the species identification and provided the simple and rapid tools for further study.

CHAPTER IV

AUTHENTICATION OF SIX *MUCUNA* PLANTS USING PCR-RFLP AND MULTIPLEX PCR

4.1 Introduction

Molecular technology is a reliable tool for the identification of medicinal materials (Shaw *et al.*, 1997; Kaplan *et al.*, 2004). DNA-based method such as PCR-RFLP, ARMS, AP-PCR, RAPD, SCAR analysis and DNA sequencing, have recently been used for the authentication and standardisation of medicinal plants (Feng *et al.*, 2010). Recentlyr, AFLP analysis have been used to study genetic diversity in *Mucuna* from various geographical regions in the world and to study genetic diversity among Indian *Mucuna* accessions (Sathyanarayana *et al.*, 2011). However, the disadvantage of AFLP is difficulty to score the presence or absence of AFLP fragments (Savelkoul *et al*., 1999). Therefore, more simple technique than AFLP as well as PCR-RFLP is required. However, the use of PCR-RFLP for the differentiation of medicinal plants in the genus *Mucuna* has not been reported.

In this study, PCR-RFLP technique base on *mat*K gene and the multiplex PCR using diagnostic primers via SNPs analysis of the sequences of ITS region was developed for the convenient and rapid identification of six closely related *Mucuna* plants, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*. This is the first study to distinguish six different species in the genus *Mucuna* by using PCR-RFLP and a multiplex PCR.

4.2 Materials and Methods

4.2.1 Plant materials and DNA extraction

Plant specimens of *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens pruriens*, and *M. warburgii* collected from various locations were used in this study. Total genomic DNA was extracted from 100 mg of leaves from each individual plant specimen and was frozen using liquid nitrogen and

ground with a mortar and pestle to obtain a fine powder. The isolation of the total DNA from the powder was performed using a modified CTAB method as described previously.

4.2.2 PCR-RFLP fingerprinting of the *mat*K gene

4.2.2.1 Sequence analysis of *mat*K gene

The *mat*K gene sequences of *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii* (accession no AB775134, AB775135, AB775133, AB775136, AB775137, and AB775138, respectively) were obtained from GenBank. The sequences were aligned using ClustalW software (Appendix C).

Restriction maps of *mat*K gene were obtained using CLC Sequence Viewer version 6.4 (CLC bio, Denmark). The restriction enzymes *Hinf*I (5′ G*ANTC 3′) which generated unique restriction profiles among *Mucuna* plants, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*, was applied for PCR-RFLP analysis. The fragments about 844-850 bp in length were generated using a primer pair, *mat*K-Mu-327F (5′- CTC TTT CTT AAA GGA GTT AGA AAT-3′) and *mat*K-Mu-1179R (5′- CGG CTT ACT AAT GGG ATG AC- 3′) (Figure 4.1). The PCR products were amplified in 50-µl reaction mixtures containing 2 µl template DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate, 0.5 µM of each primer, and 0.75 unit/reaction *Taq* DNA polymerase (Invitrogen, USA). The amplification was performed using a thermal cycler with an initial denaturing step at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 52 °C for 1 min, and extension at 72 °C for 45 sec, followed by a final extension at 72 °C for 7 min. The PCR products (5 µl) were examined using electrophoresis on a 1% agarose gel in 0.5X Tris-borate-EDTA buffer at 100 V for 35 min, stained with ethidium bromide and visualised under UV light.

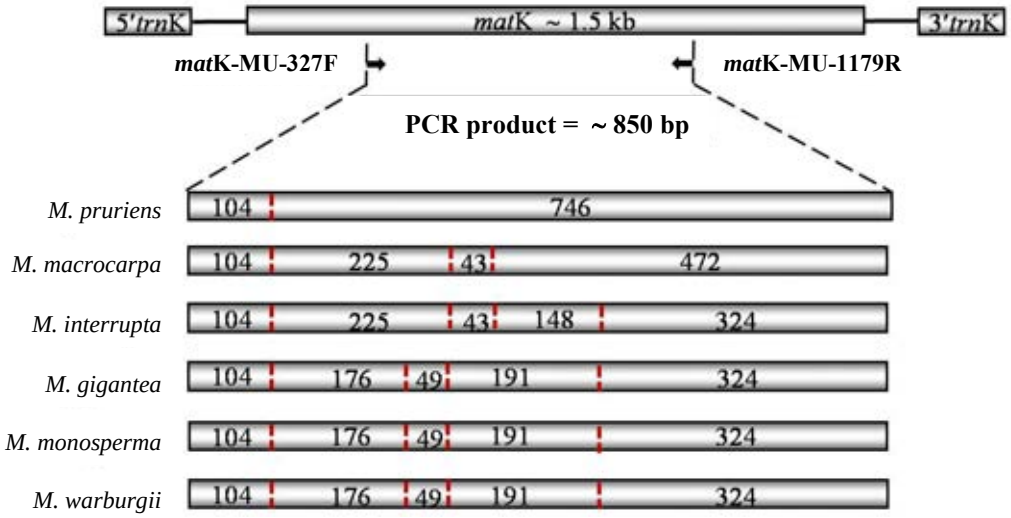

Figure 4.1 Position of amplification primers the *mat*K-MU-327F and *mat*K-MU-1179R primers on *mat*K gene. The arrows indicate the directions of the primers. The partial *mat*K gene of each *Mucuna* plants correspond to the PCR product digested with restriction enzyme *Hinf*I. The numbers in the bars indicate the fragment sizes after digestion

4.2.2.2 PCR-RFLP analysis

According to the restriction maps, the appropriate restriction enzymes *Hinf*I (New England Biolabs, England) was selected as suitable candidates for digestion of the PCR products amplified from six species of the genus *Mucuna*. The PCR products from all samples were completely digested in separate reactions (20 µl) using 5 units of *Hinf*I at 37 ºC for 3 hours. An aliquot of each digested PCR fragment was separated using 2.5% agarose gel electrophoresis. The gels were run at a low voltage (60 V) for 1.30 h in 0.5X TBE buffer, stained with ethidium bromide and visualised under UV light. A total of *Mucuna* samples were analysed and authenticated based on the resultant restriction patterns.

4.2.3 Multiplex PCR technique of the ITS region

4.2.3.1 Sequence analysis of ITS region

The DNA sequences of ITS region from six *Mucuna* plants were aligned using ClustalW software. According to the multiple sequence alignment, SNP polymorphic sites were detected on the ITS region. The diagnostic reverse primer was designed to be complementary to a region of the PCR product where SNP of interest occurs (Figure 4.2). The primer pairs that would produce different sizes of PCR products were designed. Each primer was designed to anneal to a specific region of each *Mucuna* plants. The two common primers, forward and reverse, were also designed to amplify an endogenous DNA sequence as an internal amplification control.

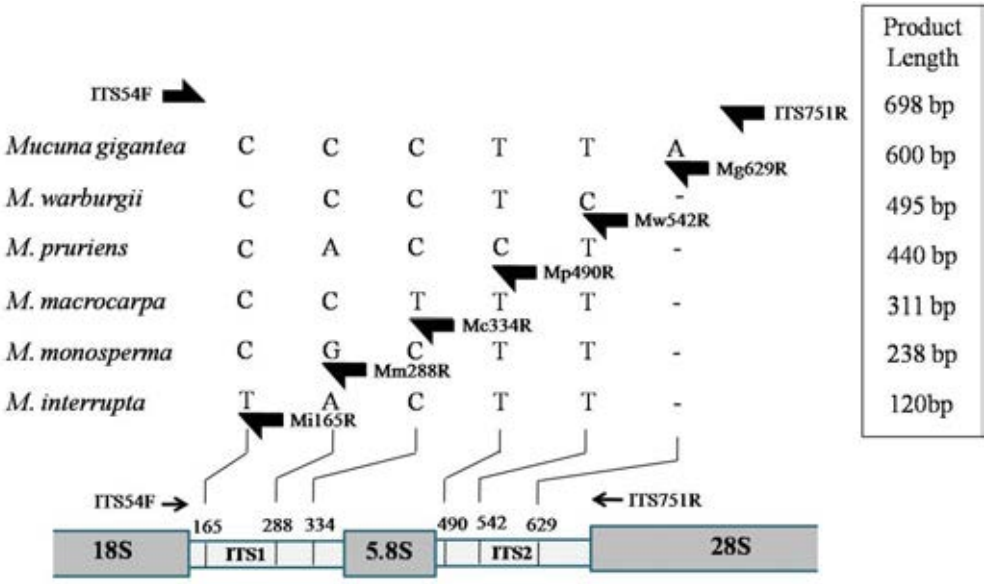

Figure 4.2 Positions of diagnostic primers for multiplex PCR

4.2.3.2 Multiplex PCR analysis

SNP-Based multiplex PCR was performed to authenticate six *Mucuna* plants using species-specific primers. Before starting the multiplex PCR, single-plex

PCR should be first examined to assess the specificity of diagnostic primers (Sint *et al*., 2012). Each diagnostic primer pair was conducted separately using genomic DNA of each species as template (Zhu *et al.*, 2004). With the multiplex PCR system, six diagnostic primers and two common primers were included in the multiplex PCR reaction using genomic DNA of each species as template. The amplification reaction in a total volume of 20 μl contained DNA template 1 μl, 1× PCR buffer (Mg^{2+} free), 2.0 mM $MgCl_2$, 0.5 μM of each primer, 0.2 mM of each dNTP, and 1 U *Taq* polymerase (Promega, U.S.A.).

Annealing temperatures were determined by gradient PCR with temperatures increasing from 62 to 72 ºC. The optimal PCR condition obtained was established as follows: hot start at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 30 s and combined annealing and extension at 69.5 ºC for 1 min, and a final extension at 72 ºC for 5 min.

An aliquot of each amplified product was separated using 2% agarose gel electrophoresis at 100 V for 35 min. Separated DNA fragments were stained with ethidium bromide and the image was observed under UV light. Fragment sizes were estimated by comparison with DNA marker. This experiment was repeated three times to verify the stability and reproducibility of banding patterns.

4.3 Results

4.3.1 The *mat*K gene sequence

The *mat*K gene sequences of *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii* were 1,518-1,524 bp in length. The restriction enzymes *Hinf*I and the *mat*K-MU-327F and *mat*K-MU-1179R primers were used to develop PCR-RFLP analysis for the discrimination of six *Mucuna* plants. The PCR analysis revealed a single 850-bp band in the electrophoretic profile (Figure 4.3). The restriction enzymes *Hinf*I generated specific fragments for these species. The PCR-RFLP profiles of six *Mucuna plants* showed four restriction patterns after digestion with *Hinf*I (Table 4.1).

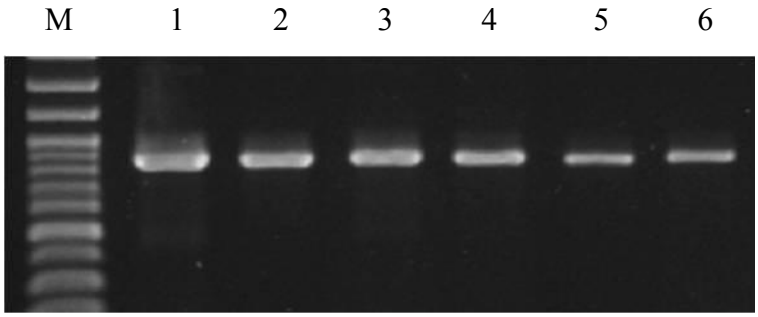

Figure 4.3 PCR products of six *Mucuna* plants. Lanes 1: *M. pruriens*; Lanes 2: *M. macrocarpa*; Lanes 3: *M. warburgii*; Lanes 4: *M. interrupta*; Lane 5: *M. monosperma*; Lane 6: *M. gigantea*; Lane M: VC 100-bp plus DNA Ladder

Table 4.1 PCR-RFLP fragments of the PCR products (amplified using primers *mat*K-MU-327F and *mat*K-MU-1179R) digested with *Hinf*I

Species	PCR product size (bp)	Enzymes *Hinf*I Fragment sizes (bp)
M. pruriens	850	104, 746
M. macrocarpa	844	(43)[a], 104, 225, 472
M. warburgii	844	(49)[a], 104, 176, 191, 324
M. interrupta	844	(43)[a], 104, 148, 225, 324
M. monosperma	844	(49)[a], 104, 176, 191, 324
M. gigantea	844	(49)[a], 104, 176, 191, 324

[a]The numbers in parentheses represent small fragments that are not consistently detected using agarose gel electrophoresis

4.3.2 PCR-RFLP analysis

A unique *Hinf*I restriction site was located within the 850-bp PCR product of *M. pruriens*, and two fragments of 104 and 476 bp were obtained. There were three *Hinf*I restriction sites in the sequence of *M. macrocarpa*. As a result, four fragments of 43, 104, 225, and 472 bp were shown. The partial *mat*K gene of *M. interrupta* contained four *Hinf*I restriction sites within the nucleotide sequence as well as *M. gigantea*, *M. monosperma*, and *M. warburgii*, but different in locations Consequently, the *Hinf*I PCR-RFLP profiles of *M. interrupta* showed five fragments of 43, 104, 148, 225, and 324 bp. Whereas, PCR-RFLP profiles of *M. gigantea*, *M. monosperma*, and *M. warburgii* showed 49, 104, 176, 191, and 324 bp (Figure 4.4). The fragments of 43 bp from *M. macrocarpa* and *M. interrupta* and the 49-bp fragments from *M. gigantea*, *M. monosperma*, and *M. warburgii* were not visualized due to the limited resolution of the electrophoresis gel.

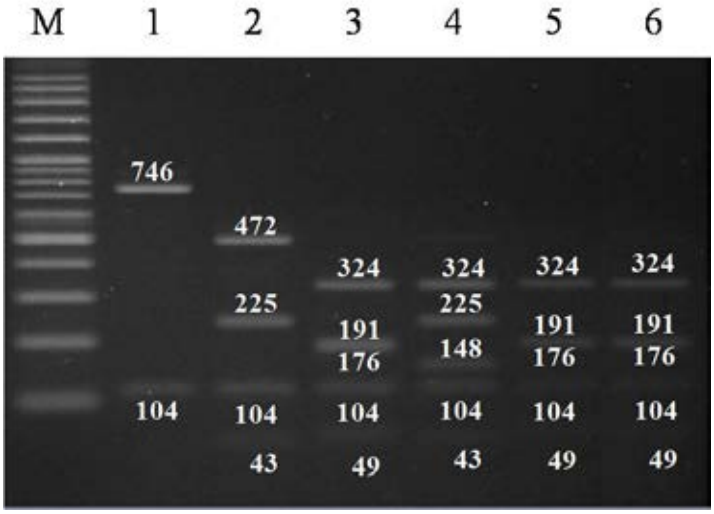

Figure 4.4 PCR-RFLP analysis of the partial *mat*K gene of *Mucuna* plants using the restriction enzymes *Hinf*I. Lanes 1: *M. pruriens*; Lanes 2: *M. macrocarpa*; Lanes 3: *M. warburgii*; Lanes 4: *M. interrupta*; Lane 5: *M. monosperma*; Lane 6: *M. gigantea*; Lane M: VC 100-bp plus DNA Ladder

4.3.3 ITS sequences and species-specific primers for multiplex PCR

According to the multiple sequence alignments of ITS regions from six *Mucuna* plants, six SNP sites specific to each of *M. interrupta*, *M. macrocarpa*, *M. pruriens*, *M. warburgii*, *M. monosperma*, and *M. gigantea* were detected. These SNPs were chosen to design diagnostic primers. The nucleotide at position 165 and 334 was C in all *Mucuna* plants, but T in *M. interrupta* (165^{th}) and *M. macrocarpa* (334^{th}). The nucleotide at position 490 and 542 was T in all *Mucuna* plants, whereas in *M. pruriens* (490^{th}) and *M. warburgii* (542^{th}) was C. The 288^{th} nucleotide in *M. monosperma* was G, but not in the other species. The 629^{th} nucleotide in *M. gigantea* was A, whereas in the same position an insertion/deletion (indel) present in the other species.

Six diagnostic reverse primers with complementary at 3′ end were designed for specific amplification using the available SNP sites on the ITS region of six *Mucuna* plants. The primers Mi165R, Mm288R, Mc334R, Mp490R, Mw542R, and Mg629R were used to produce the fragments of 120, 238, 311, 440, 495, and 600 bp specific for *M. interrupta*, *M. monosperma*, *M. macrocarpa*, *M. pruriens*, *M. warburgii*, and *M. gigantea*, respectively. The common forward primer ITS54F was determined to be common to all *Mucuna* plants for PCR amplification, and the common reverse primer ITS751R was also designed for the internal amplification control of 698 bp fragment (Table 4.2).

Table 4.2 Diagnostic primers for multiplex PCR

Diagnostic primer	Nucleotide sequence (5′ to 3′)	Approximate size of PCR products (bp)	T_m (°C)
ITS54F [a]	TGC GGA AGG ATC ATT GTC GTT GTC T		69.8
ITS751R	CCG CCT GAC CTG AGG TCT CG	698	67.9
Mi165R	CGG AGG AAG GAC GGG GTC GA [b]	120	71.0
Mm288R	GGG TCC GCG AAA ATT GCA CCC [b]	238	72.5
Mc334R	GAG AGT CAT TTT GTA TCG TGT GTC GTG A [b]	311	66.5
Mp490R	CAG GCA GGC GTG CCC TCG [b]	440	71.1
Mw542R	CCA CCC TGC ACA CGC ACA TGG [b]	495	72.4
Mg629R	ACG CTC ATC CAC CAT TTT ATC ACG GT [b]	600	69.2

[a]The forward primers ITS54F of six diagnostic primer pairs and one internal primer pair are of the same sequences.

[b]Underlined nucleotide at 3′ end of each specific primer is complementary with its target sequence.

4.3.4 Multiplex PCR analysis

The specificity of each primer was tested using single-plex PCR. DNA template from each species was amplified individually with six pairs of diagnostic primers. Only product that specific to each primer was amplified. Each species generated specific fragments with different size. For example, only *M. interrupta* generated specific fragment of 120 bp. In the same manner, *M. monosperma*, *M. macrocarpa*, *M. pruriens*, *M. warburgii*, and *M. gigantea* generated their specific fragments of 238, 311, 440, 495, and 600 bp, respectively. These results indicated that each primer was specific to each species (data not shown).

In the multiplex PCR reaction, each specific fragment was amplified specifically from its target species with the combination of the diagnostic reverse primer and common forward primer. The PCR result was observed in gel electrophoresis. Six individual species-specific fragments were shown with different size. The fragments of 120, 238, 311, 440, 495, and 600 bp amplified with the primer pairs Mi165R/ ITS54F, Mm288R/ ITS54F, Mc334R/

ITS54F, Mp490R/ ITS54F, Mw542R/ ITS54F, and Mg629R/ ITS54F were observed specifically for *M. interrupta*, *M. monosperma*, *M. macrocarpa*, *M. pruriens*, *M. warburgii*, and *M. gigantea*, respectively, while the 698-bp fragment generated from common primer pair ITS54F/ITS751R could be used as an internal control was present in all target species. Thus, two different-sized fragments, one fragment used as an internal control and one additional specific fragment used as identify fragment for each species (Figure 4.5), were simultaneously amplified in all of individuals of species. To confirm the reproducibility of the method, the experiment was repeated three times.

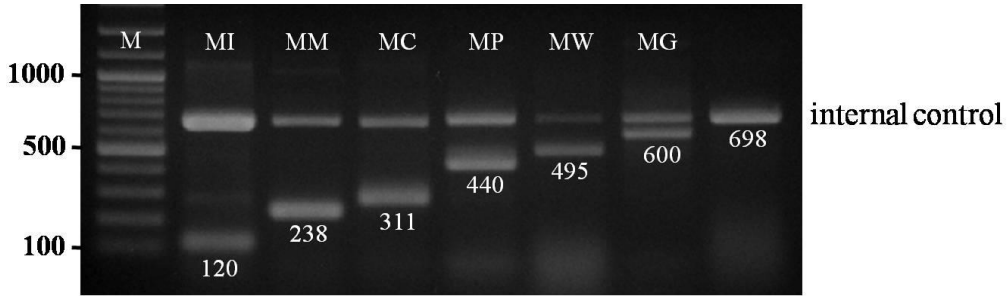

Figure 4.5 Specific authentication of six *Mucuna* plants by multiplex PCR. All samples containing the internal control DNA show a band of 698 bp. M: VC100 bp plus DNA marker; MI: *M. interrupta*; MM: *M. monosperma*; MC: *M. macrocarpa*; MP: *M. pruriens*; MW: *M. warburgii*; MG: *M. gigantea*

4.4 Discussion

The genus *Mucuna*, including *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*, are known as a source of L-Dopa. However, these species differ in traditional treatment applications. The discrimination of *Mucuna* plants is difficult when they are in shredded pieces or in powder form. To ensure the true therapeutic efficacy and safety, the development of a simple method for the discrimination of *Mucuna* plants is necessary. In this study, the *mat*K gene was examined to discriminate six species in the genus *Mucuna* because of its high

substitution rates and variability. Recently, PCR-based methods have become widely employed for the simple and rapid identification of herbal medicines. PCR-RFLP is the appropriate method to use for detecting an adulterant in herbal medicine (Hon *et al*., 2003). Herein, the *mat*K gene sequences were analysed to produce PCR-RFLP restriction maps, and the enzymes *HinfI* was chosen for this analysis. The PCR-RFLP profiles showed four groups of polymorphic fingerprints of six *Mucuna* plants. This result showed that PCR-RFLP using restriction enzyme *Hinf*I based on *mat*K gene could be used for the discrimination of these *Mucuna* plants, but it is not the best technique. This result indicated that *mat*K gene sequence of *Mucuna* plants was highly conserved. It was not suitable DNA marker for this study because it could not be used to distinguish between individual species within the genus *Mucuna*. Therefore, a new method based on a new DNA marker that could be used for the discrimination of six *Mucuna* plants is required.

For this, a multiplex PCR assay was developed for the discrimination of six *Mucuna* plants using species-specific primers. The ITS region was chosen as a new suitable DNA marker for the analysis. The PCR products of ITS region from six *Mucuna* plants were amplified with the universal primers ITS1 and ITS4, and were sequenced individually. The obtained sequences were deposited in GenBank. For sequence analysis, ITS sequences from six *Mucuna* plants were aligned using ClustalW software. To form distinct banding profiles, the amplicon sizes of each specific fragment should be different when detected by gel electrophoresis (Hao *et al.*, 2010). Based on species-specific SNP sites found at ITS region of six *Mucuna* plants, each diagnostic primer was designed specifically to each *Mucuna* plants. To test the specificity of each diagnostic primer before starting the multiplex PCR, single-plex PCR was examined under optimized condition.

The optimal annealing temperatures that work for all primer pairs must be optimized for efficient and specific amplification within a single reaction (Hao *et al.*, 2010). For this, a gradient PCR was required. The results showed that the annealing temperature at 69.5 ºC was the best condition for all primers (data not shown). Since the optimal annealing temperature obtained was close to the extension temperature (72°C), a two-step PCR protocol combining primer annealing and extension step was

determined. In this condition, PCR amplification was conducted under stringent condition lead to enhance specificity of the method.

With the multiplex PCR, the authentication of each *Mucuna* plants was determined by mixing eight primers, six species-specific primers generated different sizes of PCR products and two common primers targeting the internal amplification control, in the multiplex PCR reaction. In general, the internal amplification control should be included in the experimental design to ensure that DNA samples were amplified and detected successfully (Rosenstraus, 1998). The fragment of internal control should always be generated even though there is no target DNA sequence of interest. Successful amplification of the internal control indicated that genomic DNA was suitable for PCR. The multiplex PCR assay was easy to observe based on the presence of PCR products on agarose gel after electrophoresis. The result indicated that multiplex PCR simultaneously amplified two fragments, one corresponding to target sequence for each species and another to an endogenous sequence as an internal control. Interestingly, only one specific fragment of each species was amplified by its specific primer. This result indicated that the each species-specific primer designed was highly specific to its target sequence.

According to the related location of distinguishable bands, the specific fragments could be identified and differentiated obviously from each other. Six *Mucuna* plants could be discriminated individually between species with their specific primers. The results from this study are consistent with previous studies showing that the multiplex PCR could be applied to effectively authenticate medicinal plants (Jigden *et al.*, 2010; Kim *et al.*, 2012).

4.5 Conclusion

PCR-RFLP based on *mat*K gene was performed to discriminate these six *Mucuna* plants. This technique could be used to discriminate 6 *Mucuna* plants into 4 groups; 1) *M. pruriens* 2) *M. macrocarpa* 3) *M. interrupta* and 4) *M. gigantea, M. monosperma,* and *M. warburgii*. The results indicated that PCR-RFLP of the the *mat*K gene failed to discriminate individual species. It confirmed that the *mat*K gene of six *Mucuna* plants was highly conserved and might not be suitable for the

discrimination of these species. Therefore, a novel multiplex PCR based on ITS region was examined and it successfully applied for the differentiation of six *Mucuna* plants. The results confirmed that multiplex PCR is a convenient, efficient and specific method for species identification. This is the first report of the authentication of six species in the genus *Mucuna*, and this developed assay could be adapted for identification of other medicinal plant species in a simple, accurate, time-saving, and inexpensive method.

CHAPTER V

ANALYSIS OF L-DOPA IN SIX *MUCUNA* PLANTS

5.1 Introduction

The seeds of the plants in the genus *Mucuna* are the best natural source of L-Dopa (Daxenbichler *et al*., 1971) used in the treatment of Parkinson's disease (Nagashayana and Sankarankutty, 2000). However, each species has its own chemical composition which can cause different effects and benefits. In order to ensure efficacy, selection of a suitable method for species identification is needed. For genetic assessment, a multiplex PCR technique could be successfully used to distinguish these six *Mucuna* plants. However, the routine chemotaxonomic method providing qualitative of major compounds for phytochemical assessment is still required. For quality control, measurement of the amount of active phytochemicals may be required in the use of materials for pharmaceutical purposes (Joshi *et al*., 2004). TLC densitometric method is one of the suitable methods widely used for the simultaneous qualitative and quantitative analysis of active constituents (Najar *et al*., 2007).

L-Dopa can be used as a chemical marker for the quality control of the *Mucuna* plants and it can be determined by measuring and comparing the concentrations between species. In the present study, the TLC densitometric method was performed to simultaneously detect L-Dopa and measure L-Dopa content of eleven samples from five *Mucuna* plants, including *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, and *M. pruriens*. The comparative L-Dopa content in five *Mucuna* plants was conducted. *M. warburgii* was not included in the analysis because its seeds are very rare and it has not been used as medicinal plant.

5.2 Materials and Methods

5.2.1 Seed materials

Mucuna seeds, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, and *M. pruriens*, were purchased from various locations from Thailand (Figure 5.1, Table 5.1). The botanical identity was confirmed with the comparison of voucher specimens available in the Forest Herbarium – BKF, Bangkok, Thailand. All samples were deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Figure 5.1 *Mucuna* seeds **A**: *M. macrocarpa* **B**: *M. gigantea* **C**: *M. monosperma* **D**: *M. interrupta* **E**: *M. pruriens*

Table 5.1 *Mucuna* seeds used in this study

Species	Local name	Location (Province)	Sample no.
Mucuna gigantea (Willd.) DC.	หมามุ่ยช้าง	Bangkok	M1
M. interrupta Gagnep.	สะบ้าลาย	Bangkok	M2
		Chiang Mai	M3
M. macrocarpa Wall.	กวาวเครือดำ	Bangkok	M4
		Chiang Mai	M5
		Chiang Rai	M6
M. monosperma Wight	หมามุ่ยใหญ่	Phang Nga	M7
M. pruriens (L.) DC.	หมามุ่ย	Bangkok	M8
		Chaiyaphum	M9
		Kanchanaburi	M10
		Mahasarakham	M11
M. warburgii K. Schum. & Lauterb.	พวงโกเมน	Nakornratchasrima	-

5.2.2 Extraction of L-Dopa

The content of L-Dopa in seed powder of *Mucuna* samples was determined by sonication method with modification (St. Laurent *et al*., 2002) The seed powder of *Mucuna* samples approximately 5-10 g (excluding seed coat) was initially defatted with petroleum ether (100 ml three times) by shaking for 24 h at room temperature (Ketkar *et al*., 2011). The organic solvent was then removed by filtration at room temperature. The defatted powder materials were dried at room temperature and then extracted by sonication with methanol for 20 minutes. To separate powder material from solution, the tube was centrifuged and the supernatant was directly used as sample extracts in subsequent steps.

5.2.3 Optimization of mobile phase

The mobile phase compositions for TLC were optimized by testing different solvent mixtures of varying polarity as follows, n-butanol–acetic acid–water (4:1:1,

v/v) (Raina and Khatri, 2011), phenol–water (8:2, v/v) (Sundaram and Gurumoorthi, 2012), and n-butanol–methanol–water (4:1:1, v/v). Standard L-Dopa was purchased from Sigma-Aldrich (Missouri, USA). Other chemicals and solvents used in the experiments were of analytical grade. Silica gel $60F_{254}$ TLC plates (20×20 cm with 0.2 mm thickness) were purchased from Merck (Darmstadt, Germany). The standard L-Dopa and the *Mucuna* seed extract solutions were spotted on the TLC plate and these developing solvents were tried to get a good separation. TLC co-spotting technique was used to confirm the identity of L-Dopa.

5.2.4 Instruments and chromatographic conditions

Densitometric analysis of L-Dopa was determined using a CAMAG Linomat 5 automatic sample spotter (Muttenz, Switzerland) under a flow of N_2 gas. The solution samples were spotted in the form of bands of width 6 mm with a CAMAG microlitre syringe on a precoated silica gel plate 60 F_{254} (20×10 cm). The plate was developed in a CAMAG glass twin-through chamber (20×20 cm) which was presaturated with 10 ml mobile phase of n-butanol–methanol–water (4:1:1, v/v) for 30 min at room temperature. The development distance was 8 cm from the base. Subsequently, developed TLC plates were air dried and scanned with a CAMAG TLC scanner 3 in absorbance at 280 nm and integrated winCATS software version 1.4.4 was used for the analysis. Each sample was prepared and analyzed in triplicate.

5.2.5 Method validation

The TLC densitometric method was validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and specificity according to International Conference on Harmonization (ICH) guidelines (ICH, 2005).

5.2.5.1 Linearity

A stock solution of L-Dopa (1 mg/ml) was prepared by dissolving an accurately weighed 5 mg of L-Dopa standard in 5 ml of 0.1 N HCl in a volumetric flask. Standard working solution was prepared by diluting stock solution with 0.1 N HCl with concentration of 1 μg/μl. Different volumes of standard working solution 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 μl were spotted on TLC plate to obtained final concentration range of 1000, 1500, 2000, 2500, 3000, and 3500 ng/spot of L-Dopa. The data of average areas under the curve (AUC) were plotted against the corresponding concentrations. The experiment was repeated in triplicate. Regression equation and co-efficient of correlation (r^2) was derived.

5.2.5.2 Precision

The precision was determined in terms of intra- and inter-day precision. The different concentrations of standard L-Dopa, 1000, 1500, 2000, 2500, 3000, and 3500 ng, were spotted onto a TLC plate on the same day for intra-day precision and on two different days for inter-day precision. The AUC was recorded and L-Dopa content was determined. The precision was expressed as the standard deviation (SD) and percent relative standard deviation (RSD).

5.2.5.3 Accuracy

The accuracy of the analytical method was done using the recovery studies. The *Mucuna* seed extract (150 μl) was spiked with 10, 20, 30, 40, and 50 μl of standard L-Dopa solution (1 mg/ml). After spiking, five microliters of each solution was applied onto a TLC plate and analyzed. Three determinations were performed for each concentration of L-Dopa. The average recoveries were calculated as percentage recovery (%) = 100 × (experimental content/theoretical content).

5.2.5.4 LOD and LOQ

LOD is the lowest amount of analyte of interest which can be detected but not necessarily quantitated as an exact value. LOQ is the lowest amount of analyte of interest which can be quantitatively determined with acceptable precision and accuracy. LOD and LOQ values were determined by standard deviation method. Blank methanol samples were spotted in triplicate and the peak areas of these blank samples were calculated for the standard deviation. LOD and LOQ were calculated from SD of the blank response and the slope (S) of the calibration curve (y = 21948x+4272) according to the formula: LOD = 3.3(SD/S) and LOQ = 10(SD/S), respectively (ICH, 2005).

5.2.5.5 Specificity

The specificity of the method was verified by analyzing the standard L-Dopa and the seed extract samples from *Mucuna* plants. The extract sample containing L-Dopa was confirmed by comparing Rf values and UV spectra of standard. The peak purity of L-Dopa was assessed by comparing the overlay spectra of standard L-Dopa and seed extract at three different positions, peak start, peak apex, and peak end of the spot detected at 280 nm.

5.2.6 Measurement of L-Dopa in seed extracts of *Mucuna* samples

Approximately 50 mg of defatted seed powder was weighed and placed into a separate 1.5-ml microcentrifuge tube, shaken to mix with 1 ml of methanol, and sonicated for 20 min (St. Laurent *et al*., 2002). The extract solution was centrifuged at 8,000 rpm for 2 min and the filtered supernatant solution was used for L-Dopa content analysis. An aliquot of 3 µl of the filtered solution was applied on TLC plate, followed by development and scanning as described in the section of instrumentation. All samples were freshly prepared and analyzed in triplicate. The amount of L-Dopa was calculated using the calibration curve.

5.3 Results

5.3.1 The optimum mobile phase for TLC method

Developing TLC plate with different solvent systems, n-butanol–acetic acid–water (4:1:1), phenol–water (8:2), and n-butanol–methanol–water (4:1:1), were tried to optimize the mobile phase. The result showed that there were more than one compound have the same Rf value of L-Dopa developed with the mobile phase n-butanol–acetic acid–water (4:1:1) (Figure 5.2). Whereas, the mobile phase phenol–water (8:2) and n-butanol–methanol–water (4:1:1) gave good resolution (Figures 5.3 and 5.4). The profile of L-Dopa was shown in all *Mucuna* samples, although some habitat differences were observed (Figure 5.4). The selected mobile phase, n-butanol–methanol–water (4:1:1) showed one compound of test solution at the same Rf value 0.39 identified as L-Dopa (Figure 5.5).

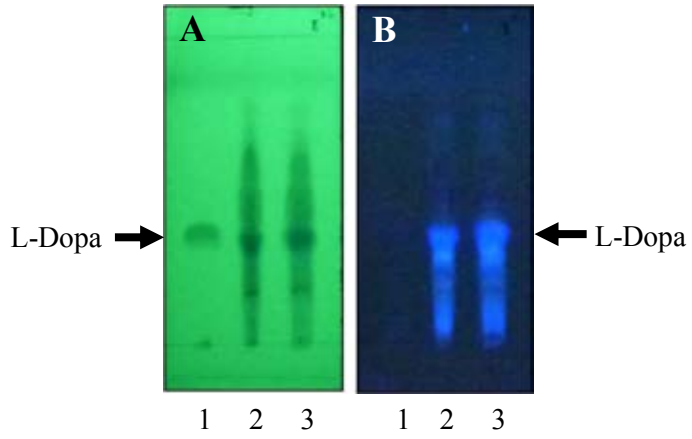

Figure 5.2 TLC profile of standard L-Dopa and seed extract of *M. pruriens* observed **A**: under UV at 254 nm, and **B**: under UV at 365 nm. Track 1: standard L-Dopa; track 2: seed extract; track 3: co-spot of both standard L-Dopa and seed extract, mobile phase: n-butanol–acetic acid–water (4:1:1, v/v).

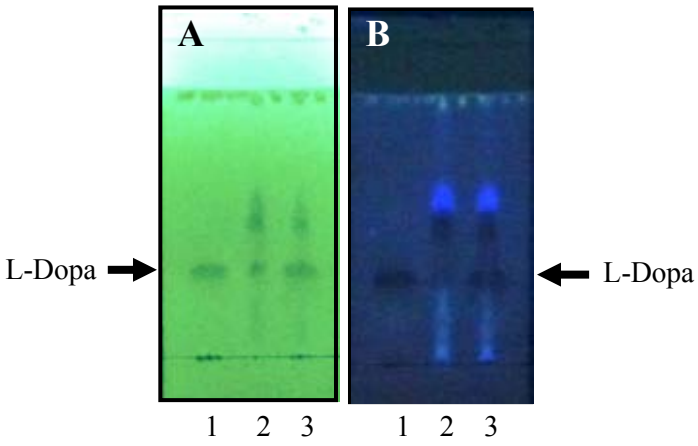

Figure 5.3 TLC profile of standard L-Dopa and seed extract of *M. pruriens* observed **A**: under UV at 254 nm, and **B**: under UV at 365 nm. Track 1: standard L-Dopa; track 2: seed extract; track 3: co-spot of standard L-Dopa and seed extract, mobile phase: phenol–water (8:2, v/v).

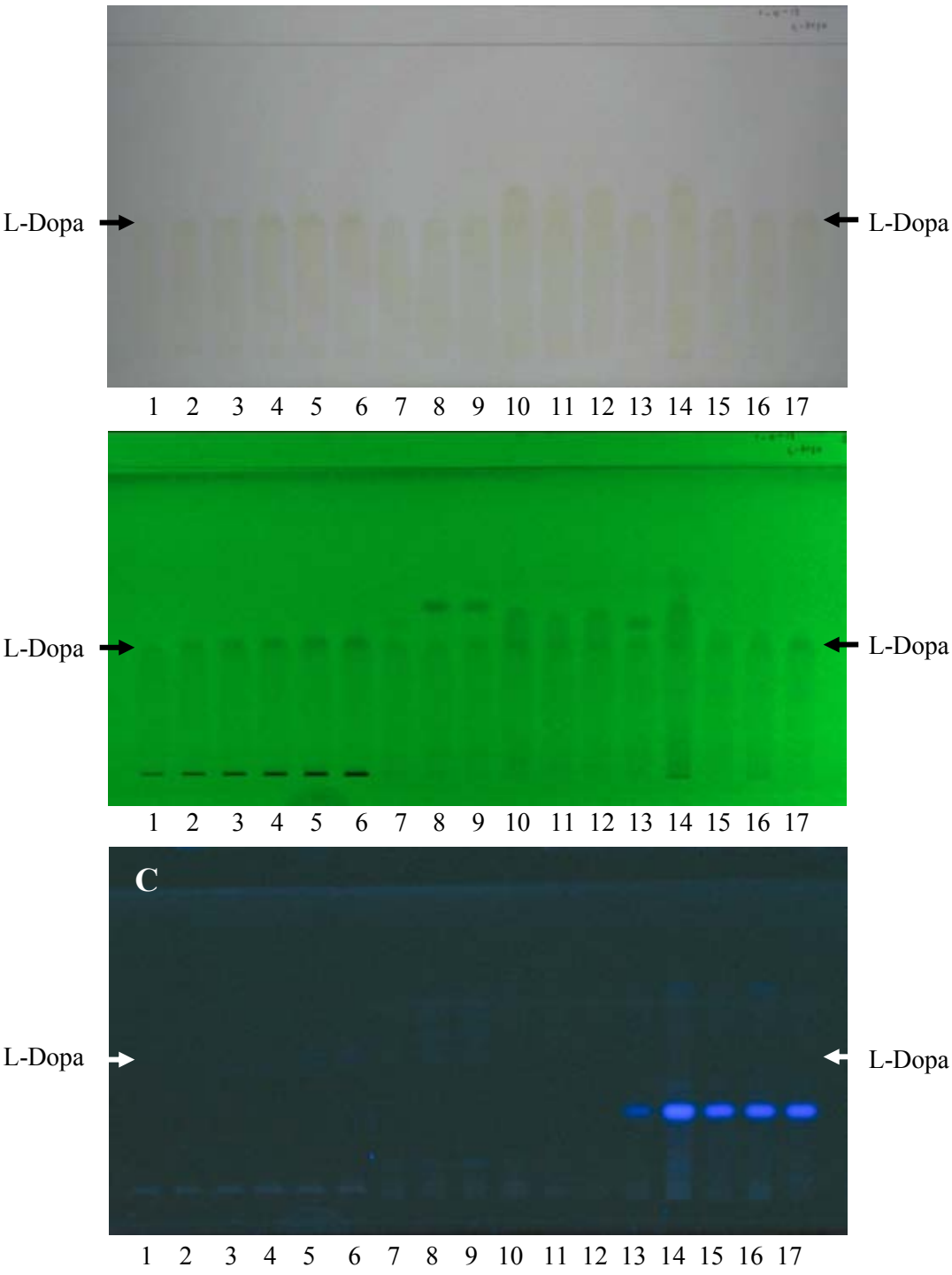

Figure 5.4 Images of TLC plates of different concentration of L-Dopa (track 1-6) and seed extract of *Mucuna* samples (track 7-17) observed **A**: in visible light, **B**: under UV at 254 nm, and **C**: under UV at 365 nm, mobile phase: n-butanol–methanol–water (4:1:1, v/v).

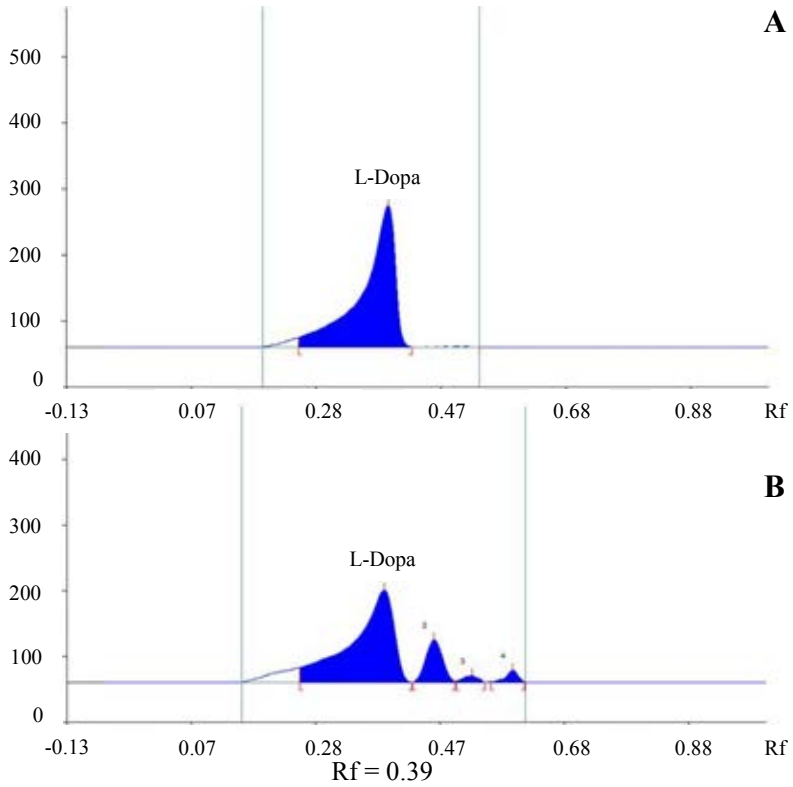

Figure 5.5 TLC chromatogram of **A**: standard L-Dopa (3,000 ng/spot) **B**: seed extract of *Mucuna* plants, mobile phase: n-butanol–methanol–water (4:1:1, v/v)

5.3.2 Validated TLC densitometric method

The method was validated for its linearity, intra- and inter-day precision, accuracy, LOD, LOQ, and specificity. The linear calibration curves of L-Dopa were within the concentration range of 1,000-3,500 ng/spot. A linear calibration equation, $y = 21948x+4272$ was obtained with a correlation coefficient of 0.9975 (Figure 5.6 and Table 5.2). The intra- and inter-day precisions were expressed as the %RSD, with values of 0.40-2.65% and 0.57-2.95%, respectively (Table 5.3). The accuracy was performed by spiking standard solution at five levels, and the percentage recovery values of 99.59, 99.60, 101.09, 99.81, and 97.54% were obtained (Table 5.4). LOD and LOQ values were 0.22 and 0.67 µg/spot and LOQ, respectively. These values were calculated according to the formula: LOD = 3.3(SD/S) and LOQ = 10(SD/S),

respectively, where, SD of the response and S values were 1465.93 and 21948, respectively. For the specificity assessment, three different levels, peak start, peak apex, and peak end of L-Dopa peak in *Mucuna* samples were consistent with the peak of standard L-Dopa (Figure 5.7).

5.3.3 L-Dopa content in seed extracts of *Mucuna* samples

A single spot at Rf value 0.39 was observed in the densitogram of L-Dopa extracted from all *Mucuna* seed samples (Figure 5.8). The amount of L-Dopa was determined by six-point standard curve of L-Dopa. The L-Dopa content of seed extracts from five *Mucuna* plants, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, and *M. pruriens*, ranged from 1.14% to 3.12% w/w. The amount of L-Dopa in *M. macrocarpa*, *M. pruriens*, and *M. interrupta* ranged from 2.63% to 3.12% (average 2.82%), 1.73% to 2.47% (average 1.97%), and 1.18% to 1.24% (average 1.21%), respectively. By comparison, *M. macrocarpa* contain the higher amount of L-Dopa than any other species, which have been found to contain less than 2% L-Dopa (Table 5.5).

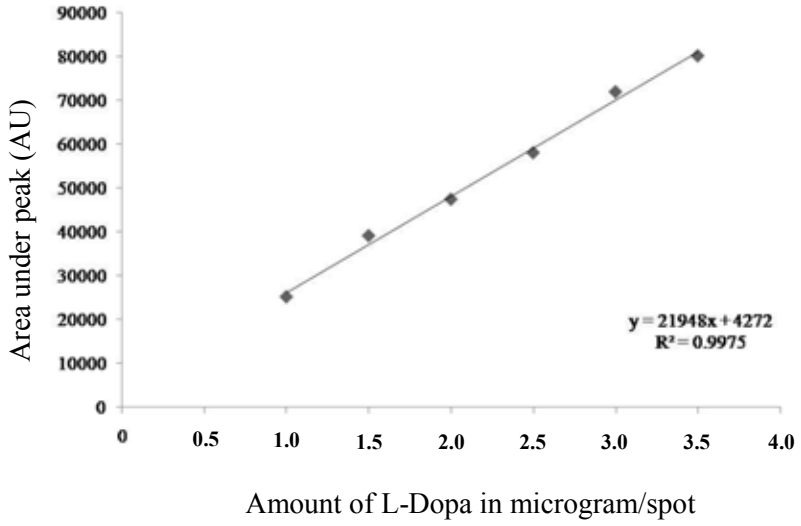

Figure 5.6 Calibration curve of L-Dopa by TLC-densitometric method

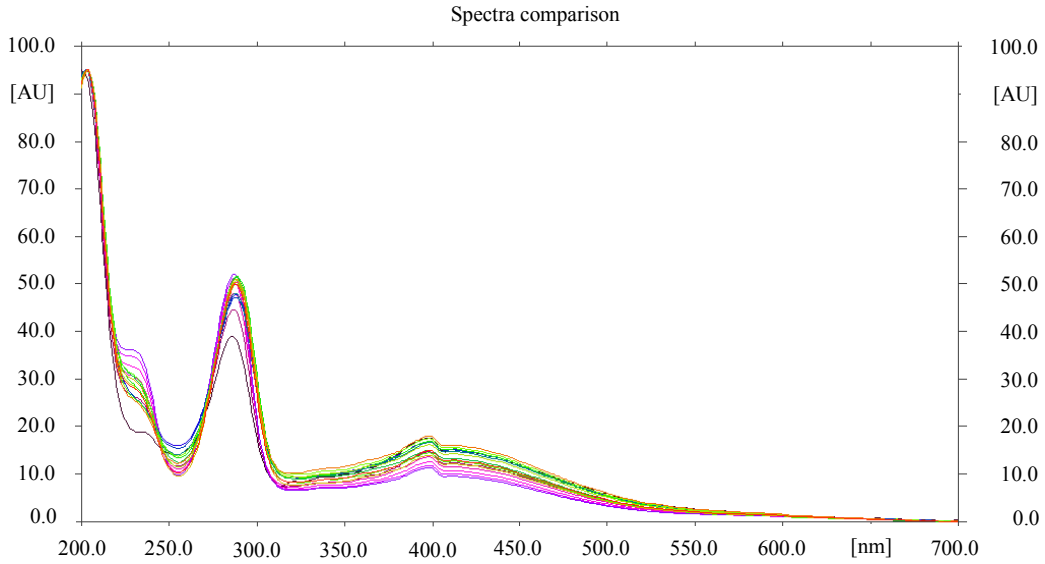

Figure 5.7 UV spectral comparison of standard L-Dopa and seed extract from *Mucuna* samples. Detection at 280 nm

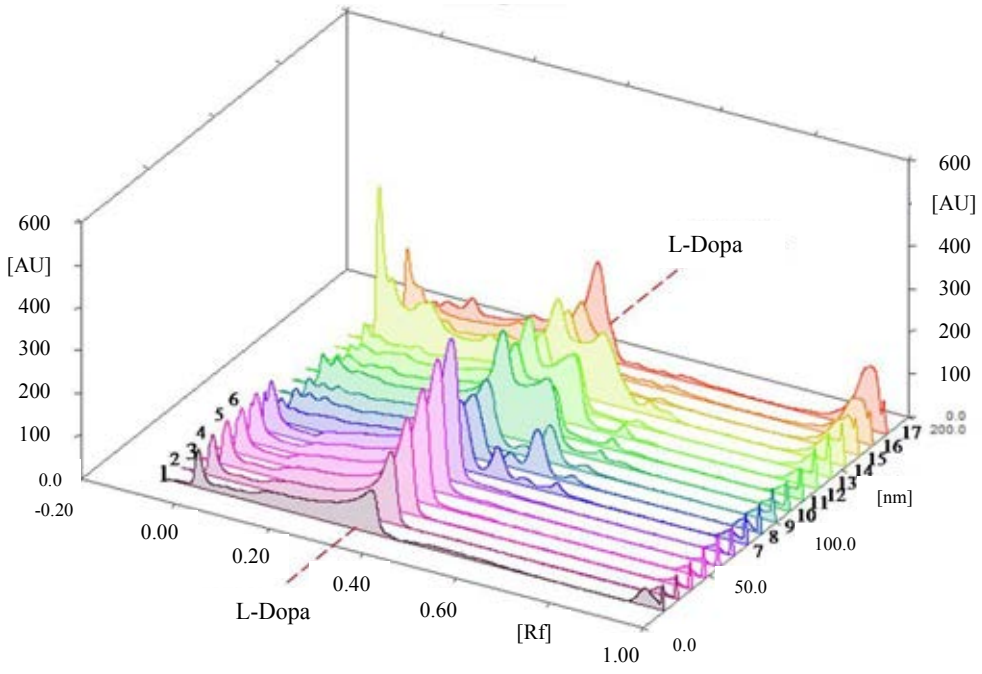

Figure 5.8 Densitogram of standard L-Dopa (track 1-6) and seed extracts from *Mucuna* samples (track 7-17), mobile phase: n-butanol–methanol–water (4:1:1, v/v).

Table 5.2 Method validation parameters for the measurement of L-Dopa by TLC densitometric method

No.	Parameter	Result
1	Linearity range (ng/spot)	1,000-3,500
2	Correlation coefficient	0.9975
3	Rf	0.39
4	Limit of detection (µg/spot)	0.22
5	Limit of quantification (µg/spot)	0.67
6	Accuracy (%)	97.54-101.09

Table 5.3 Intra- and Inter-day precision

Amount (ng/spot)	Intra-day precision			Inter-day precision		
	Mean area	SD (±)	%RSD	Mean area	SD (±)	%RSD
1000	20211.60	536.4608	2.65	20375.60	231.9405	1.14
1500	31984.66	351.0417	1.10	32489.28	713.6428	2.20
2000	40839.38	358.7764	0.88	41708.79	1229.534	2.95
2500	49276.81	267.5702	0.54	50093.21	1154.552	2.30
3000	56080.84	346.5971	0.62	56827.69	1056.201	1.86
3500	62170.61	249.2202	0.40	62416.36	347.5430	0.57

Table 5.4 Accuracy determined for the TLC densitometric method

Sample no.	L-Dopa added (µg/spot)	Theoretical content (µg/spot)	Experimental* content (µg/spot)	Recovery* (%)
1	0.25	1.9203	1.9123±0.0145	99.59±0.76
2	0.50	2.1703	2.1617±0.0301	99.60±1.39
3	0.75	2.4203	2.4467±0.0566	101.09±2.34
4	1.00	2.6703	2.6653±0.0096	99.81±0.35
5	1.25	2.9203	2.8483±0.0315	97.54±1.08

*Mean±SD, n=3

Table 5.5 Percentage of L-Dopa in raw seeds of *Mucuna* samples

Sample no.	Species/Sample name	% of L-Dopa*
1	*M. gigantea*/M1	1.14±0.15
2	*M. interrupta*/M2	1.18±0.08
3	*M. interrupta*/M3	1.24±0.05
4	*M. macrocarpa*/M4	3.12±0.22
5	*M. macrocarpa*/M5	2.63±0.16
6	*M. macrocarpa*/M6	2.72±0.10
7	*M. monosperma*/M7	1.31±0.16
8	*M. pruriens*/M8	1.73±0.21
9	*M. pruriens*/M9	2.47±0.50
10	*M. pruriens*/M10	2.04±0.22
11	*M. pruriens*/M11	1.64±0.20

*Mean±SD, n=9

5.4 Discussion

A TLC densitometric method was developed for comparison of L-Dopa content in different *Mucuna* seeds. The seed powders of *Mucuna* samples from five species, *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, and *M. pruriens*, were initially defatted with petroleum ether and then extracted with methanol. Different systems of mobile phase were tested on silica plate. Good resolution of L-Dopa was obtained with the mobile phase phenol–water (8:2) and n-butanol–methanol–water (4:1:1). However, a good analytical method requires a safe and simple solvent system to separate the L-Dopa from other compounds in the extract. In the candidate solvent system phenol–water (8:2), phenol is considered to be quite toxic, which can cause skin irritation as well as skin burn (Leitao, 2009). To avoid the danger of phenol poisoning, n-butanol–methanol–water (4:1:1) solvent system was chosen as a suitable mobile phase for TLC method.

After developing TLC plate with the mobile phase n-butanol–methanol–water (4:1:1), a spot with Rf value of 0.39 identified as L-Dopa was obtained. The spot of L-Dopa of *Mucuna* seed extract was further confirmed by comparing its spectral characteristics with those of standard L-Dopa. The calibration curve was constructed by plotting area under peak and different concentrations of L-Dopa. The correlation coefficient was 0.9975, which confirmed the high linearity. For intra- and inter-day precision, the results showed acceptable precision values, with RSD less than 3%. LOD and LOQ values were 0.22 and 0.67 µg/spot. The LOQ value confirmed that the lowest concentration of standard L-Dopa (1.0 µg/spot) used in this stydy was suitable. The proposed method showed the satisfactory values of percent recovery ranged from 97.54-101.09% confirming the accuracy of the method.

The validated TLC densitometric method was used to measure the amount of L-Dopa in the seeds of five *Mucuna* plants. All seed extracts were found to contain L-Dopa, with a low yield ranging from 1.14% to 3.12% w/w. By comparison, *M. macrocarpa* contain higher amount of L-Dopa than *M. gigantea*, *M. interrupta*, *M. monosperma*, and *M. pruriens*. Although, *M. pruriens* was commonly known as a commercial source of L-Dopa due to its high concentration, our results indicate that the L-Dopa content in *M. macrocarpa* is higher than that of *M. pruriens*. This finding

suggests that *M. macrocarpa* may be used as an alternative source of L-Dopa. The range of L-Dopa content in *Mucuna* plants obtained in this study (1.14-3.12%) was found to be lower than previous reported (2.3 to 9.0%) (Bell and Janzen, 1971; Daxenbichler *et al*., 1971; Amarasekera and Jansz, 1980). The difference of percentage of L-Dopa may be due to the atmosphere, environment, or methods of extraction and determination. These results indicated that L-Dopa could be used as a chemical marker for the quality control of the plants in the genus *Mucuna*.

5.5 Conclusion

The developed TLC densitometric method was found to be simple, rapid, and accurate for the measurement of L-Dopa content in different *Mucuna* seeds. The amount of L-Dopa in each plant was also compared. Among the five *Mucuna* plants, *M. macrocarpa* is the richest source of L-Dopa. These data should be useful for finding a richer L-Dopa source of *Mucuna* plants existing in Thailand. TLC densitometric method is a useful technique for standardization of plant raw materials because it can be used for simultaneous qualitative and quantitative analysis. In addition, this method can also be used to evaluate a large number of material samples. Therefore, it could be applied for assessment of L-Dopa content in marketed herbal formulations.

CHAPTER VI

DISCRIMINATION OF THE WHITE, RED, AND BLACK KWAO KHRUEA USING PCR-RFLP

6.1 Introduction

There are three types of Kwao Khruea: White (*Pueraria candollei* Graham ex Benth.), Red (*Butea superba* Roxb.), and Black (*Mucuna macrocarpa* Wall.) (Suntara, 1931; Niyomdham, 1992). Although all Kwao Khruea herbs are used for rejuvenation, they are used for different purposes (Chukeatirote and Saisavoey, 2009). Two varieties of *P. candollei*, var. *mirifica* and var. *candollei*, have been used as sources of White Kwao Khruea for oestrogen replacement therapy in menopausal women (Cain, 1960; Cherdshewasart *et al.*, 2004; Yusakul *et al.*, 2011). *B. superba* has been used in mature males for the treatment of erectile dysfunction and the maintenance of sexual performance (Roengsamran *et al.*, 2000). *M. macrocarpa* promotes more effective reproductive function for males compared to *B. superba* (Suntara, 1931). White Kwao Khruea was initially mistakenly identified as Red Kwao Khruea owing to its taxonomical misidentification within the species and confusion among dried tuberous root types (Kerr, 1932). Such misidentification is particularly common for species that share a similar name or are similar but significantly vary in their medicinal properties. Therefore, the proper identification of Kwao Khruea herbs is needed, especially when these herbs appear in the form of a powder, shredded material, or a formulated mixture. To ensure the correct species of these herbs, a simple, species-specific method for the discrimination of *P. candollei*, *B. superba*, and *M. macrocarpa* is warranted.

In this study, a novel PCR-RFLP technique was developed based on a partial *mat*K gene sequence for the discrimination of White, Red, and Black Kwao Khruea, and was used to authenticate crude Kwao Khruea drugs purchased from various local markets. Moreover, experimental mixtures of Kwao Khruea were characterized to test the accuracy of this method.

6.2 Materials and Methods

6.2.1 Plant materials and crude drugs “Kwao Khruea”

Plant specimens of *P. candollei* var. *mirifica*, *P. candollei* var. *candollei*, *B. superba*, and *M. macrocarpa* were collected from various locations in Thailand (Table 6.1) and identified by Dr. Charan Ditchaiwong, a Horticultural Scientist at the Department of Agriculture at the Ministry of Agriculture. Eight commercial products of Kwao Khruea (C1-C8) were purchased from various local markets. All samples were deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

6.2.2 DNA extraction

Total genomic DNA was extracted from 100 mg of leaves and 50-100 mg of crude drug samples using a modified CTAB method as mentioned in Material and Methods of Chapters III and IV.

6.2.3 Sequence analysis and PCR-RFLP analysis

The *mat*K gene sequences of *P. candollei*, *B. superba*, and *M. macrocarpa* (accession no EU106108, EU106111, and AB627858, respectively) were obtained from GenBank. The sequences were aligned for PCR-RFLP analysis using ClustalW software. The restriction enzymes *Dde*I and *Taq*I, which generated unique restriction profiles among the varieties *P. candollei*, *B. superba*, and *M. macrocarpa*, were applied for the analysis. A small 500-bp fragment was generated using a new primer pair, *mat*K-BMP1 (5′- TTC TAC GTA ACA AAT CCT CTC AG- 3′) and *mat*K-BMP2 (5′-CGG CTT ACT AAT GGG ATG AC- 3′). The PCR products were amplified in 50-μl reaction mixtures containing 2 μl template DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.5 μM of each primer, and 0.75 unit/reaction *Taq* DNA polymerase (Invitrogen, USA). The

amplification was performed using a thermocycler with an initial denaturing step at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 45 sec, and extension at 72 °C for 45 sec, followed by a final extension at 72 °C for 7 min. The PCR products (5 µl) were separated using electrophoresis on a 1% agarose gel in 0.5X Tris-borate-EDTA buffer at 100 V for 35 min, stained with ethidium bromide and visualised under UV light.

According to the restriction maps, the appropriate restriction enzymes *Dde*I and *Taq*I (New England Biolabs, England) were selected as suitable candidates for digestion of the PCR products amplified from *P. candollei*, *B. superba*, and *M. macrocarpa.* Eight commercial products of Kwao Khruea were also authenticated based on the resultant restriction patterns. The PCR products from all samples were completely digested in separate reactions (20 µl) using 5 units of *Dde*I and *Taq*I for 4 hours at 37 ºC and 65 ºC, respectively. An aliquot of each digested PCR fragment was fractionated using 2.5% agarose gel electrophoresis, stained with ethidium bromide and visualised under UV light.

6.2.4 The analysis of DNA admixtures

DNA admixtures were used to verify of the accuracy of the PCR-RFLP technique. The desired mixtures, containing DNA from two or three different Kwao Khruea species (White Kwao Khruea: WY081205, TH090505; Red Kwao Khruea: TH130206; Black Kwao Khruea: MUS-H3847) as shown in Table 6.1, were prepared from pooled DNA stocks of each species. Equal amounts of DNA templates from each variety were prepared for *P. Candollei*. The DNA of each species present in the experimental mixtures was added in equal amounts in the following combinations prior to PCR amplification: 1) *P. candollei* and *B. superba*; 2) *P. candollei* and *M. macrocarpa*; 3) *B. superba* and *M. macrocarpa*; and 4) *P. candollei*, *B. superba*, and *M. macrocarpa*. The PCR reaction mixtures were amplified using the primer pair *mat*K-BMP1 and *mat*K-BMP2 and subjected to PCR-RFLP analysis.

Table 6.1 List of plant materials used in PCR-RFLP analysis

Species	Vernacular names	Collection site (Province)	Voucher no.	Accession no.
P. candollei Graham ex Benth. var. *mirifica* (Airy Shaw & Suvat.) Niyomdham	White Kwao Khruea	Nonthaburi	TH090505	EU106108
		Lampang	MUS-H3852	
		Saraburi	MUS-H3853	
		Chiang Mai	MUS-H3854	
		Loei	MUS-H3855	
		Tak	MUS-H3856	
		Kanchanaburi	MUS-H3857	
		Prachuap Khiri Khan	MUS-H3858	
P. candollei Graham ex Benth. var. *candollei*	White Kwao Khruea	Bangkok	WY081205	EU106106
		Bangkok	MUS-H3859	
		Kanchanaburi	MUS-H3860	
		Bangkok	WY090505	
		Kanchanaburi	CC290905	
B. superba Roxb.	Red Kwao Khruea	Chachoengsao	TH130206	EU106111
		Lampang	MUS-H3861	
		Prae	MUS-H3862	
		Kanchanaburi	MUS-H3863	
		Kanchanaburi	TH230306	
		Bangkok	TH050706	
		Kalasin	BR010807	
M. macrocarpa Wall.	Black Kwao Khruea	Bangkok	MUS-H3847	AB627858
		Bangkok	MUS-H3848	
		Chiang Mai	MUS-H3849	
		Chiang Mai	MUS-H3850	
		Chiang Mai	MUS-H3851	

6.3 Results

6.3.1 The *mat*K gene sequence and PCR-RFLP analysis with the restriction enzymes *Dde*I and *Taq*I

The *mat*K gene sequences of three rejuvenating herbs *P. candollei*, *B. superba*, and *M. macrocarpa* were 1521, 1527, and 1518 bp in length, respectively. The *mat*K sequences from all specimens of the same species showed completely identical sequence despite of different locations. The nucleotide sequence divergence between *P. candollei* and *B. superba*, *P. candollei* and *M. macrocarpa*, and *B. superba* and *M. macrocarpa*, was 4.9%, 5.8%, and 5.7%, respectively. The restriction enzymes *Dde*I and *Taq*I and the primer pair *mat*K-BMP1 and *mat*K-BMP2 were used to develop a novel PCR-RFLP analysis technique for the discrimination of these species (Figure 6.1). The PCR analysis revealed a single 500-bp band in the electrophoretic profile. The restriction enzymes *Dde*I and *Taq*I generated diagnostic fragments for *P. candollei*, *B. superba*, and *M. macrocarpa*. The PCR-RFLP profile of each species showed distinct restriction patterns after digestion with *Dde*I and *Taq*I (Figure 6.2 and Table 6.2). A unique *Dde*I restriction site (5′ C*TNAG 3′) was located within the 500-bp PCR product of both varieties of *P. candollei,* and two fragments measuring 19 and 481 bp were obtained. The partial *mat*K gene of both *B. superba* and *M. macrocarpa* contained two *Dde*I restriction sites in different locations within the nucleotide sequence. As a result, the *Dde*I PCR-RFLP profiles of *B. superba* and *M. macrocarpa* showed three fragments of 9, 84, and 397 bp and 19, 202, and 279 bp, respectively (Figure 6.3A). The 19-bp fragment from each species was not visualized due to the limited resolution of the electrophoresis gel. The PCR products of both varieties of *P. candollei* and *M. macrocarpa* contained two and one restriction sites (5′ T*CGA 3′), respectively, from the restriction digestion with *Taq*I. In contrast, this restriction site was absent from the *B. superba* sequence. Consequently, PCR products from both varieties of *P. candollei* could be cleaved into 79, 187, and 234-bp fragments, and that of *M. macrocarpa* generated two fragments of 220 and 280 bp in length. Cleavage was not observed in PCR product from *B. superba* (Figure 6.3B).

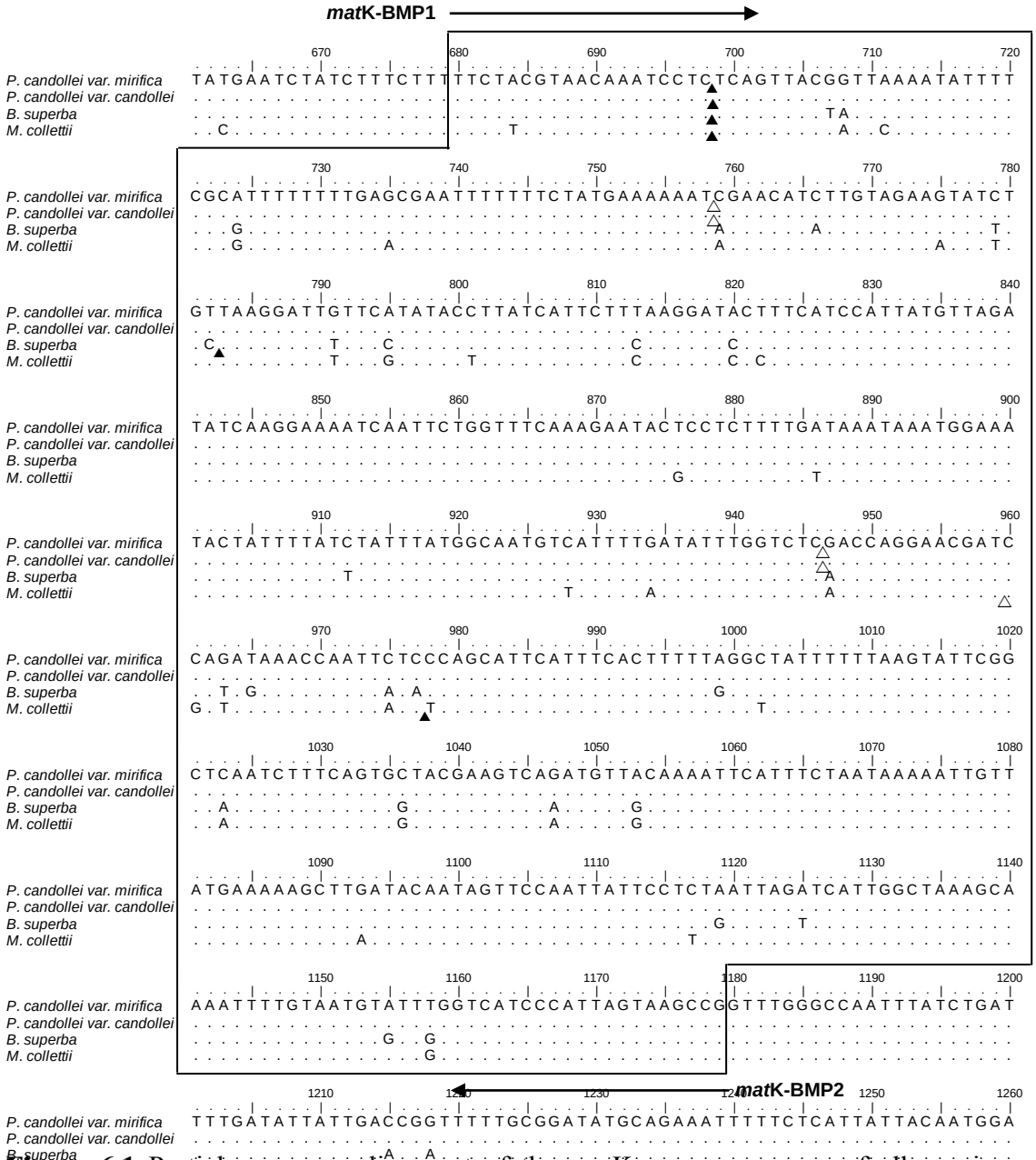

Figure 6.1 Partial sequence alignment of the *mat*K gene sequences of all species. Position 661 corresponds to position 655, 655, 661, and 652 of *mat*K gene of *P. candollei* var. *mirifica*, *P. candollei* var. *candollei*, *B. superba*, and *M. macrocarpa*, respectively. Consensus sequences are indicated with dots. The altered bases indicate the sequence differences. The arrows indicate the directions of the *mat*K-BMP1 and *mat*K-BMP2 primers. The region corresponding to the PCR amplicon within the *mat*K gene sequence is outlined with an empty square box. The *Dde*I sites are indicated by shaded triangles (▲), and the *Taq*I sites are indicated by open triangles (△)

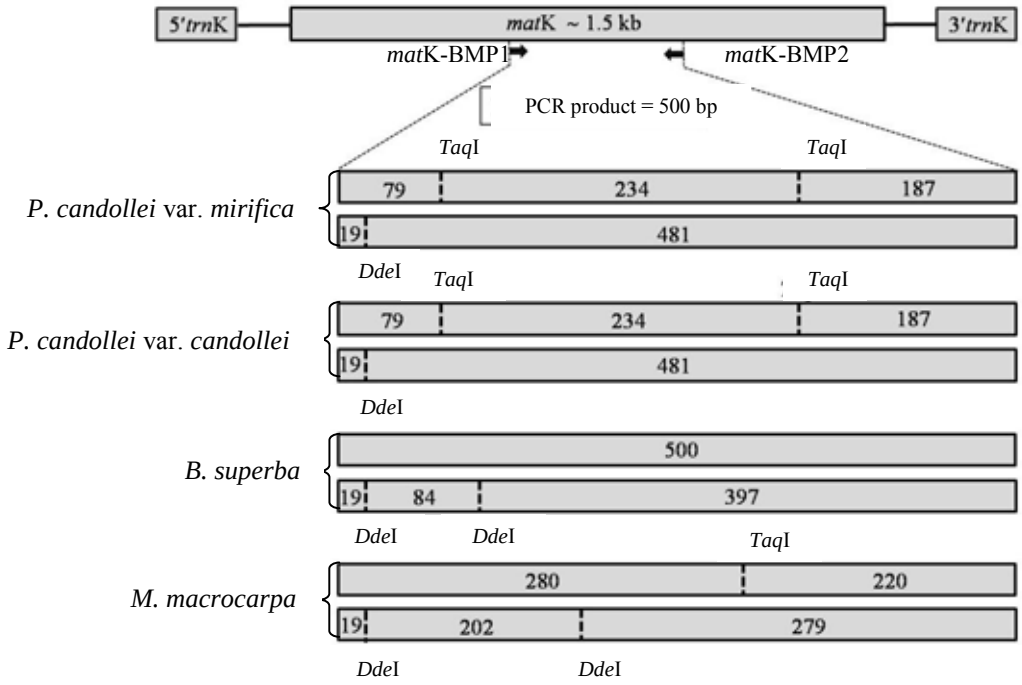

Figure 6.2 Position of the amplification primers *mat*K-BMP1 and *mat*K-BMP2 primers on *mat*K gene. The arrows indicate the directions of the primers. The partial *mat*K gene of each species corresponds to the PCR amplicon (500 bp) digested with *Dde*I and *Taq*I. The numbers in the bars indicate the fragment sizes after digestion

Table 6.2 PCR-RFLP fragments of the PCR products (amplified using primers *mat*K-BMP1 and *mat*K-BMP2) digested with *Dde*I and *Taq*I

Species	PCR product size (bp)	Enzymes	
		*Dde*I fragment sizes (bp)	*Taq*I fragment sizes (bp)
P. candollei var. *mirifica*	500	(19)[a], 481	79, 187, 234
P. candollei var. *candollei*	500	(19)[a], 481	79, 187, 234
B. superba	500	(19)[a], 84, 397	500
M. macrocarpa	500	(19)[a], 202, 279	220, 280

[a]The numbers in parentheses represent small fragments that are not consistently detected using agarose gel electrophoresis

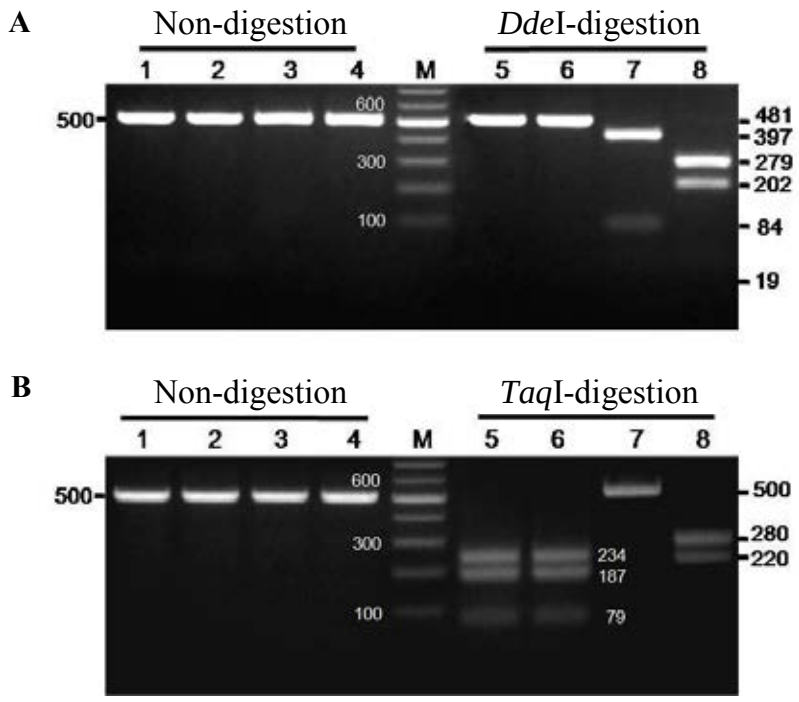

Figure 6.3 PCR-RFLP analysis of the partial *mat*K gene using the restriction enzymes **A**: *Dde*I and **B**: *Taq*I. The PCR products before (Lane 1-4) and after (Lane 5-8) digestion, Lanes 1 and 5: *P. candollei* var. *mirifica*; Lanes 2 and 6: *P. candollei* var. *candollei*; Lanes 3 and 7: *B. superba*; Lanes 4 and 8: *M. macrocarpa*; M: VC 100-bp plus DNA Ladder

6.3.2 The analysis of DNA admixtures

The experimental DNA admixtures containing the genomic DNA of two or three species of Kwao Khruea were prepared and subjected to PCR-RFLP analysis to test the accuracy of this technique. After digestion, the combined electrophoresis patterns were resolved, thereby providing evidence of the presence of different species in the mixtures. Each fragment exhibited the unique characteristic of the species. The DNA admixture containing *P. candollei* and *B. superba* presented a combined pattern of four fragments: a 19-bp fragment from *P. candollei* and *B. superba*, a 481-bp fragment from *P. candollei*, and two 84- and 397-bp fragments from *B. superba*. A combined pattern of four fragments of 19, 202, 279, and 481 bp was also observed in the restriction profile of the DNA admixture containing *P.*

candollei and *M. macrocarpa*. A single 19-bp fragment belonged to both *P. candollei* and *M. macrocarpa*. A fragment of 481 bp and two fragments of 202 and 279 bp were unique to *P. candollei* and *M. macrocarpa*, respectively. The combined restriction profile of *B. superba* and *M. macrocarpa* DNA comprised five fragments after digestion with *Dde*I: one fragment (19 bp) was derived from both *P. candollei* and *M. macrocarpa*, two fragments (84 and 397 bp) were unique to *B. superba*, and two different fragments (202 and 279 bp) were specific to *M. macrocarpa*. The *Dde*I digestion of the *P. candollei*, *B. superba*, and *M. macrocarpa* DNA admixture generated six fragments (19, 84, 202, 279, 397, and 481 bp) as described above (Figure 6.4A). The PCR-RFLP profiles of all experimental DNA admixtures generated with *Taq*I also generated the expected combined restriction patterns. However, PCR-RFLP fingerprints of the mixtures containing *P. candollei* and *M. macrocarpa* were unclear due to similar sizes of four digested fragments (280, 234, 220, and 187 bp) (Figure 6.4B).

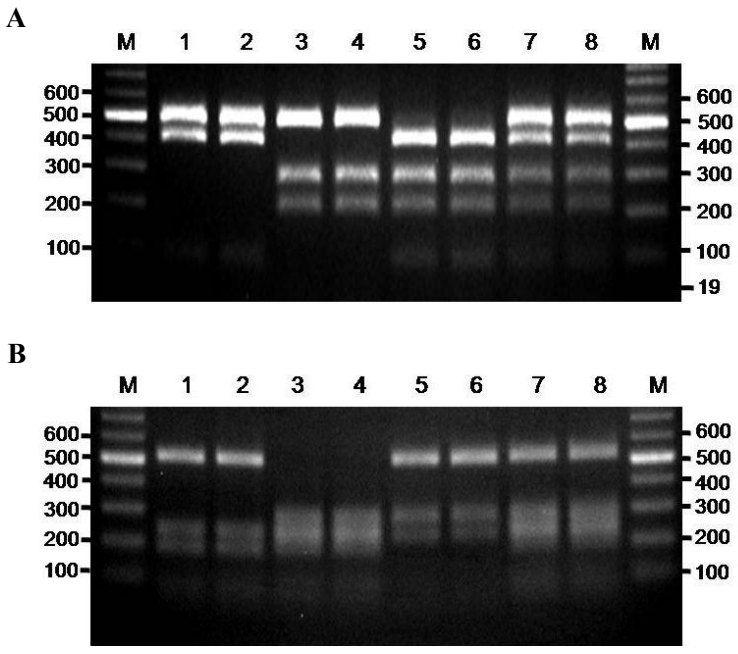

Figure 6.4 PCR-RFLP profiles generated from DNA admixtures containing equal amounts of DNA of two or three species of Kwao Khruea. Digestion with **A**: *Dde*I and **B**: *Taq*I. Lanes 1-2: *P. candollei* and *B. superba*; Lanes 3-4: *P. candollei* and *M. macrocarpa*; Lanes 5-6: *B. superba* and *M. colletti*; Lanes 7-8: *P. candollei, B. superba,* and *M. macrocarpa*; M: VC 100-bp plus DNA Ladder

6.3.3 PCR-RFLP of the commercial Kwao Khruea products

We also applied PCR-RFLP analysis to examine eight crude commercially available Kwao Khruea drugs (Table 6.3 and Figure 6.5).

Table 6.3 Commercial herbal "Kwao Khruea" drugs purchased from crude drug markets

Herbal drug name	Code	Purchased location (Province)	Purchase date	Voucher no.	Claimed original plant (Species)
White Kwao Khruea	C1	Bangkok	2011.04.28	SW280411	*P. candollei*
White Kwao Khruea	C2	Nongkhai	2010.11.05	SW051110	*P. candollei*
White Kwao Khruea	C3	Phetchaboon	2010.12.18	SW181210	*P. candollei*
White Kwao Khruea	C4	Lampang	2010.09.20	SW200910	*P. candollei*
Red Kwao Khruea	C5	Nongkhai	2010.11.06	SW061110	*B. superba*
Red Kwao Khruea	C6	Bangkok	2011.04.29	SW290411	*B. superba*
Red Kwao Khruea	C7	Lampang	2010.09.21	SW210910	*B. superba*
Black Kwao Khruea	C8	Phitsanulok	2011.02.15	SW150211	*M. macrocarpa*

After the PCR reaction, the products were digested using *Dde*I and *Taq*I, and the unique restriction profiles were observed. Seven of the eight samples (C1-C7) were correctly identified as their original plants. Two fragments of 19 and 481 bp were theoretically generated from the PCR products of White Kwao Khruea (C1-C4) digested with *Dde*I, whereas the same enzyme produced three fragments of 19, 397, and 84 bp from the PCR products of Red Kwao Khruea (C5, C6, and C7). The RFLP pattern of the remaining sample (C8), which was marketed as Black Kwao Khruea, showed two 397- and 84-bp fragments corresponding to Red Kwao Khruea (Figure 5.6A). The PCR products of White Kwao Khruea (C1-C4) generated three fragments (234, 187, and 79 bp) after digestion with *Taq*I, whereas the 500-bp Red Kwao Khruea (C5, C6, and C7) PCR product remained uncleaved. An undigested fragment

of 500 bp was also observed in the sample that was marketed as Black Kwao Khruea (C8) (Figure 6.6B).

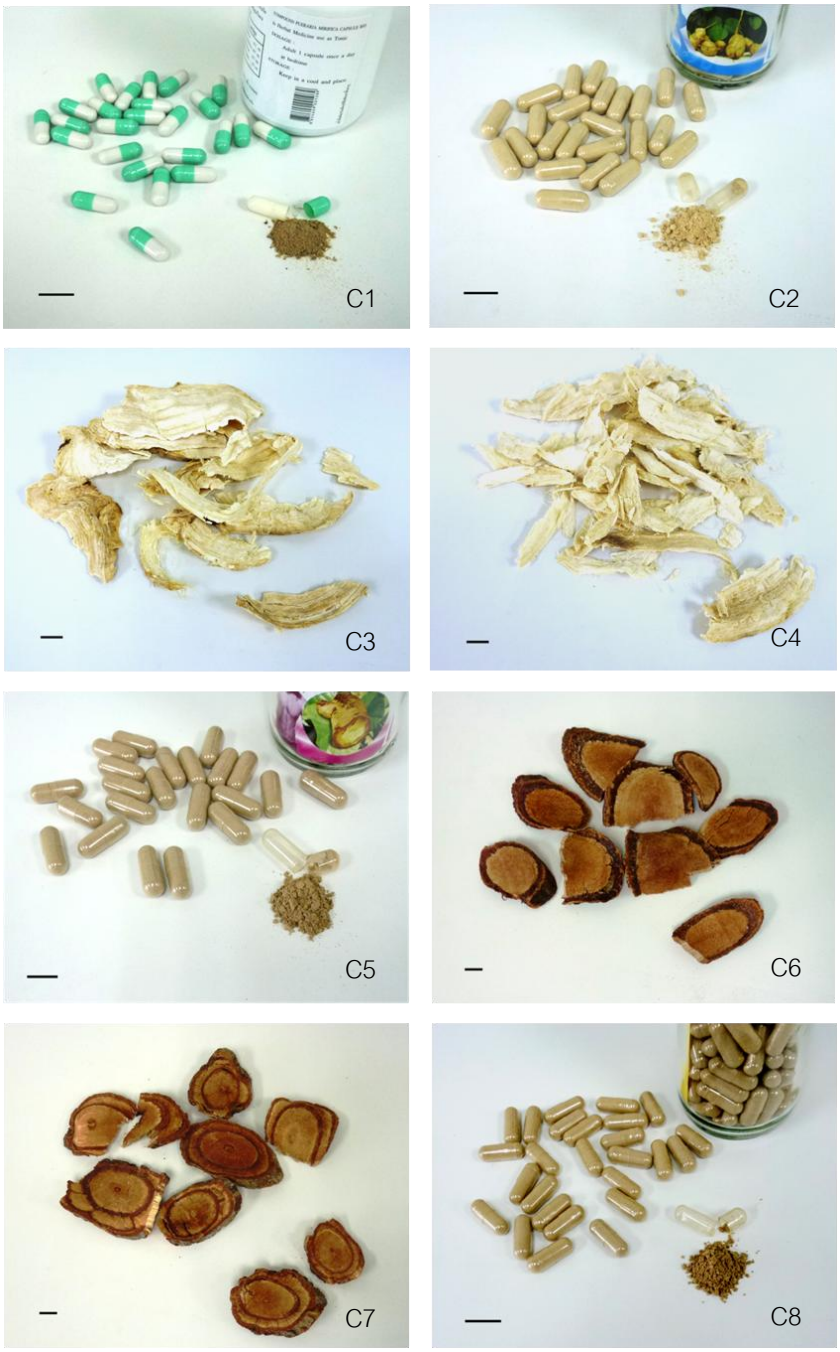

Figure 6.5 Samples of commercial products and crude drug preparations of Kwao Khruea. White (C1-C4), Red (C5-C7), and Black Kwao Khruea (C8). Scale bar = 1 cm

These results indicated that the four White Kwao Khruea samples (C1-C4) and three Red Kwao Khruea samples (C5, C6, and C7) were authentic *P. candollei* and *B. superba* species, respectively. The results of this work confirmed that the species corresponding to samples C1-C7 were correctly identified as claimed. Interestingly, sample C8 generated a different restriction pattern from that previously described for *M. macrocarpa* (Figure 6.6A and 6.6B). Therefore, the results showed that sample C8 was not *M. macrocarpa* as originally claimed. After further investigation using nucleotide sequencing, sample C8 was identified as *B. superba*.

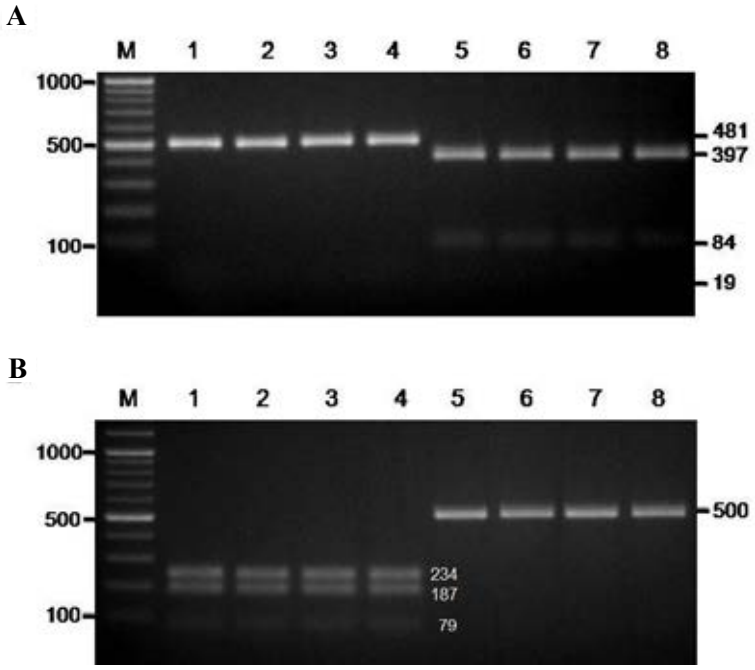

Figure 6.6 PCR-RFLP profiles of the eight crude drugs (C1-C8) using the restriction enzymes **A**: *Dde*I and **B**: *Taq*I. Lanes 1-4: "White Kwao Khruea" samples (C1-C4); Lanes 5-7: "Red Kwao Khruea" samples (C5-C7); Lane 8: "Black Kwao Khruea" samples (C8); M: VC 100-bp plus DNA Ladder

6.4 Discussion

The rejuvenating herbs *P. candollei*, *B. superba*, and *M. macrocarpa* share a similar vernacular name in Thai as White, Red, and Black Kwao Khruea, respectively. However, these species differ in their sources and traditional treatment applications (Chukeatirote and Saisavoey, 2009). The discrimination of Kwao Khruea herbs is challenging when they are presented as a powder or formulated mixture. Therefore, consumers must be aware that they are selecting the correct species to ensure the true therapeutic efficacy and safety. Hence, the development of a simple and accurate method for the discrimination of Kwao Khruea herbs is important.

The confusion of herbal medicines as a result of name sharing is one of the most common causes of medication errors in many countries. PCR-RFLP analysis has been successfully used to solve this problem in several case studies. For example, this technique was used to distinguish authentic "qin-jiu" (*Gentiana macrophylla* Pall.), which is primarily distributed throughout China and Siberia (Ho *et al.*, 1995). Many *Gentiana* species were used as "qin-jiu" in traditional Chinese medicines, although these species demonstrated a low efficacy of antirheumatic therapy relative to *G. macrophylla* (Tan *et al.*, 1996). "Dijincao" is another important traditional Chinese medicine that is used for treating dysentery and colitis in many Asian countries. Only two species in the genus of *Euphorbia* are listed as the origin of "dijincao", namely *E. humifusa* and *E. maculata*. However, there are at least 3 adulterants on the market bearing the name "dijincao" (Xue *et al.*, 2008).

Recently, several sequences in the chloroplast genome, such as *trn*K and *mat*K, have been frequently used to identify and discriminate between closely related species at the molecular level (Singh, 2012). Due to its high substitution rates and variability, the *mat*K gene was used to discriminate Kwao Khruea herbs in the present study. The complete *mat*K gene sequences were 1,518-1,527 bp in length, which is consistent with a previous report showing that the *mat*K coding region in most angiosperms is 1.5-1.6 kb in length (Neuhaus and Link, 1987). Recently, PCR-based methods have become widely employed for the simple and rapid identification of herbal medicines. Because the DNA of plant materials is often degraded after commercial processing or drying, it is necessary to develop methods that use short

amplicons to increase the probability of a successful application (Heubl, 2010). PCR-RFLP is an effective discrimination method because it requires only one specific site difference between the primers (Wang *et al.*, 2007). Furthermore, PCR-RFLP has also been applied for the authentication of certain medicinal plants in previous studies. For example, Sukrong *et al.* (Sukrong *et al.*, 2007) used this methodology to differentiate the narcotic plant *Mitragyna speciosa* from various substitutes, and Manissorn *et al.* (Manissorn *et al.*, 2010) showed that three important medicinal *Phyllanthus* species, *P. amarus*, *P. debilis*, and *P. urinaria*, could be discriminated. To our knowledge, our study is the first to use PCR-RFLP to discriminate the rejuvenating herbs *P. candollei* (White Kwao Khruea), *B. superba* (Red Kwao Khruea), and *M. macrocarpa* (Black Kwao Khruea). Herein, the *mat*K gene sequences were analysed to create restriction maps, and the enzymes *Dde*I and *Taq*I were chosen for use in subsequent restriction analyses. The PCR-RFLP profiles clearly showed distinct and polymorphic fingerprints of *P. candollei*, *B. superba*, and *M. macrocarpa*, strongly suggesting that *Dde*I and *Taq*I are suitable restriction enzymes for the discrimination of these rejuvenating herbs. The two varieties of *P. candollei* could not be distinguished. However, both of them have the same therapeutic properties and have been used as sources of White Kwao Khruea (Yusakul *et al.*, 2011).

The PCR-RFLP technique has been successfully used in previous studies for the detection of authentic species in admixtures containing DNA samples from multiple species (Quinteiro, 2001; Dooley *et al.*, 2005). In the present study, PCR-RFLP was applied to detect the presence of individual Kwao Khruea species within the intended mixtures. An equal amount of DNA from each species was mixed and subjected to PCR-RFLP analysis. The results showed the combination of various banding patterns of each species using gel electrophoresis, thereby providing evidence of the presence of different species within the mixed DNA samples. The presence of the combined fragment pattern was consistent with a previous study that demonstrated the suitability of the PCR-RFLP technique for confirming the presence of target species in DNA admixtures (Quinteiro, 2001; Dooley *et al.*, 2005). In our study, it was difficult to clearly identify the presence of each species in the experimental mixture digested with *Taq*I due to the similar sizes of the four digestion products and the inability to clearly discern these fragments using 2.5% agarose gel electrophoresis.

However, the result from *Dde*I digestion clearly confirmed the presence of different species within the mixtures.

This technique was also applied to eight crude commercial "Kwao Khruea" drugs purchased from various markets in Thailand. Only one sample of Black Kwao Khruea was included in the analysis because this species was not easy to find in the drug market. After digestion with *Dde*I and *Taq*I, seven out of eight Kwao Khruea samples showed PCR-RFLP patterns that clearly corresponded to the originally claimed species. However, according to the *Dde*I digestion pattern, the Black Kwao Khruea (C8) sample, which was claimed as *M. macrocarpa*, was confirmed to be *B. superba* based on a restriction pattern that was similar to that of *B. superba.* The confusion regarding the botanical characteristics of Black and Red Kwao Khruea may have occurred due to their similar appearances. To confirm the result of the PCR-RFLP analysis using *Dde*I, another enzyme, *Taq*I, was investigated. The result clearly showed that the *Taq*I fragment pattern of the C8 sample also matched that of *B. superba*, and as a result, sample C8 was properly reclassified as Red Kwao Khruea. These results suggested that PCR-RFLP was not only able to confirm the presence of correctly identified species, but could also detect incorrectly labeled plant material. Furthermore, the use of at least two restriction enzymes is necessary for the adequate discrimination between isolates using the PCR-RFLP technique (Deborah *et al.*, 2009). Finally, the *mat*K gene PCR product from sample C8 was sequenced to ensure the correct identification. The sequencing result showed that the *mat*K gene sequence of C8 was identical with that reported for *B. superba* in GenBank. Although the majority of the crude herbal drugs were properly authenticated and confirmed as "Kwao Khruea", the misidentification of crude drugs remains a problem in drug markets. Thus, the availability of an accurate PCR-RFLP technique will be valuable for the verification of genuine crude drug species.

6.5 Conclusion

Among six *Mucuna* plants investigated in this study, only *M. macrocarpa* was known as a rejuvenating herb "Black Kwao Khruea". Misidentification with the other two Kwao Khruea herbs, such as White (*P. candollei*) and Red (*B. superba*) Kwao Khruea could occur due to their similar morphological features. PCR-RFLP technique used in this study was successfully applied to discriminate these rejuvenating Kwao Khruea herbs, *P. candollei*, *B. superba*, and *M. macrocarpa*. Furthermore, PCR-RFLP technique can be applied to authenticate commercial herbal drugs. The results presented here strongly suggest that the PCR-RFLP method developed in this investigation will be helpful for maintaining quality control and identifying Kwao Khruea herbs in the drug market. Furthermore, this work can be modified for the identification of individual Kwao Khruea plants in herbal drug formulations by using more sensitive, rapid, accurate, and simple techniques such as real-time PCR technique.

CHAPTER VII

THE USE OF CYCLEAVE PCR FOR THE DIFFERENTIATION OF THE REJUVENATING HERB, WHITE, RED, AND BLACK KWAO KHRUEA, AND THE SIMULTANEOUS DETECTION OF MULTIPLE DNA TARGETS IN A DNA ADMIXTURE

7.1 Introduction

A previous study using PCR-RFLP to discriminate the Kwao Khruea herbs, White (*P. candollei*), Red (*B. superba*), and Black (*M. macrocarpa*) found that a crude drug made of Red Kwao Khruea was often misidentified as Black Kwao Khruea because of the misidentification of dried tuberous roots in crude drug markets (Wiriyakarun *et al.*, 2012). Although Red and Black Kwao Khruea are used for male rejuvenation, Red Kwao Khruea is less effective (Suntara, 1931) and less expensive than Black Kwao Khruea. The misidentification of crude Kwao Khruea drugs is a continuing problem in the drug market. A species-specific method for authenticating Kwao Khruea species is needed. Although PCR-RFLP is a simple, specific, and accurate method, it takes time to complete the digestion and requires post-PCR analysis by gel electrophoresis. For large sample sizes, a more rapid and specific method for identification is required. A recent advance in DNA technology, cycleave PCR has considerable advantages in the detection of SNPs because it is highly specific, sensitive, rapid, and reproducible compared to other available techniques, such as DNA sequencing, allele-specific PCR (AS-PCR), single-strand conformation polymorphism PCR (SSCP-PCR), mismatch amplification mutation assay (MAMA-PCR), PCR-RFLP, and TaqMan minor groove binder (MGB) (Hou *et al.*, 2011). In this study, the *mat*K gene was used as a suitable region because of its high substitution rate and variability. Based on sequence variations in the *mat*K gene, the cycleave PCR technique was developed to differentiate the Thai rejuvenating Kwao Khruea herbs. The specificity of this method was evaluated using a multiplex cycleave PCR assay for the simultaneous detection of two similar herbs, Red Kwao Khruea (*B. superba*) and Black Kwao Khruea (*M. macrocarpa*), in a DNA admixture.

7.2 Materials and Methods

7.2.1 Plant materials

Plant specimens of *P. candollei*, *B. superba*, and *M. macrocarpa* were collected from various locations in Thailand (Table 7.1) and identified by Dr. Charan Ditchaiwong, a Horticultural Scientist, at the Department of Agriculture at the Ministry of Agriculture. All samples were deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

7.2.2 DNA extraction and species-specific probe design

Genomic DNA was extracted from 100 mg of leaves with a Dneasy Plant Mini Kit (Qiagen, Valencia, CA). DNA quantity and quality were determined using UV spectrophotometry and gel electrophoresis. The concentration of DNA was adjusted to 50–80 ng/ml using water. All of the extracted DNA samples were stored at -20°C for further use.

7.2.3 Primers and cycling probe design

To design the primers and cycling probes, a multiple alignment of the *mat*K gene sequences of *P. candollei*, *B. superba*, and *M. macrocarpa* as well as closely related species was performed using ClustalW. The *matK* gene sequences from *P. candollei*, EU106108; *P. montana*, AY582972; *P. phaseoloides*, EU717404; *B. superba*, EU106111; *M. macrocarpa*, AB627858; *M. gigantea*, AB627860; *M. interrupta*, AB627862; *M. monosperma*, AB627859; *M. pruriens*, AB627857; and *M. warburgii*, AB627861 were obtained from GenBank. Real-time PCR and cycling probe technology were used for SNP typing. Primers and cycling probes were designed and synthesised by TaKaRa Bio Inc. (Japan). For SNP typing, the probes specific to *P. candollei* and *M. macrocarpa* were labeled with the fluorescent label ROX and quencher eclipse, whereas that of *B. superb* was labeled with the fluorescent

label FAM and eclipse. Each probe harbored RNA corresponding to the target sequence at the SNP positions 214, 95, and 235 of *P. candollei*, *B. superba*, and *M. macrocarpa*, respectively (Figure 7.1).

Table 7.1 Plant materials used in cycleave PCR technique

Species	Local names	Locality (Province)	Specimen no.	Voucher no.	Accession no.
Pueraria candollei Graham ex Benth.	White Kwao Khruea	Nonthaburi	WK01	TH090505	EU106108
		Lampang	WK02	MUS-H3852	
		Saraburi	WK03	MUS-H3853	
		Chiang Mai	WK04	MUS-H3854	
		Loei	WK05	MUS-H3855	
		Tak	WK06	MUS-H3856	
		Kanchanaburi	WK07	MUS-H3857	
Butea superba Roxb.	Red Kwao Khruea	Chachoengsao	RK01	TH130206	EU106111
		Lampang	RK02	MUS-H3861	
		Prae	RK03	MUS-H3862	
		Kanchanaburi	RK04	MUS-H3863	
		Kanchanaburi	RK05	TH230306	
		Bangkok	RK06	TH050706	
Mucuna macrocarpa Wall. (syn. *M. collettii* Lace)	Black Kwao Khruea	Bangkok	BK01	MUS-H3847	AB627858
		Bangkok	BK02	MUS-H3848	
		Chiang Mai	BK03	MUS-H3849	
		Chiang Mai	BK04	MUS-H3850	
		Chiang Mai	BK05	MUS-H3851	

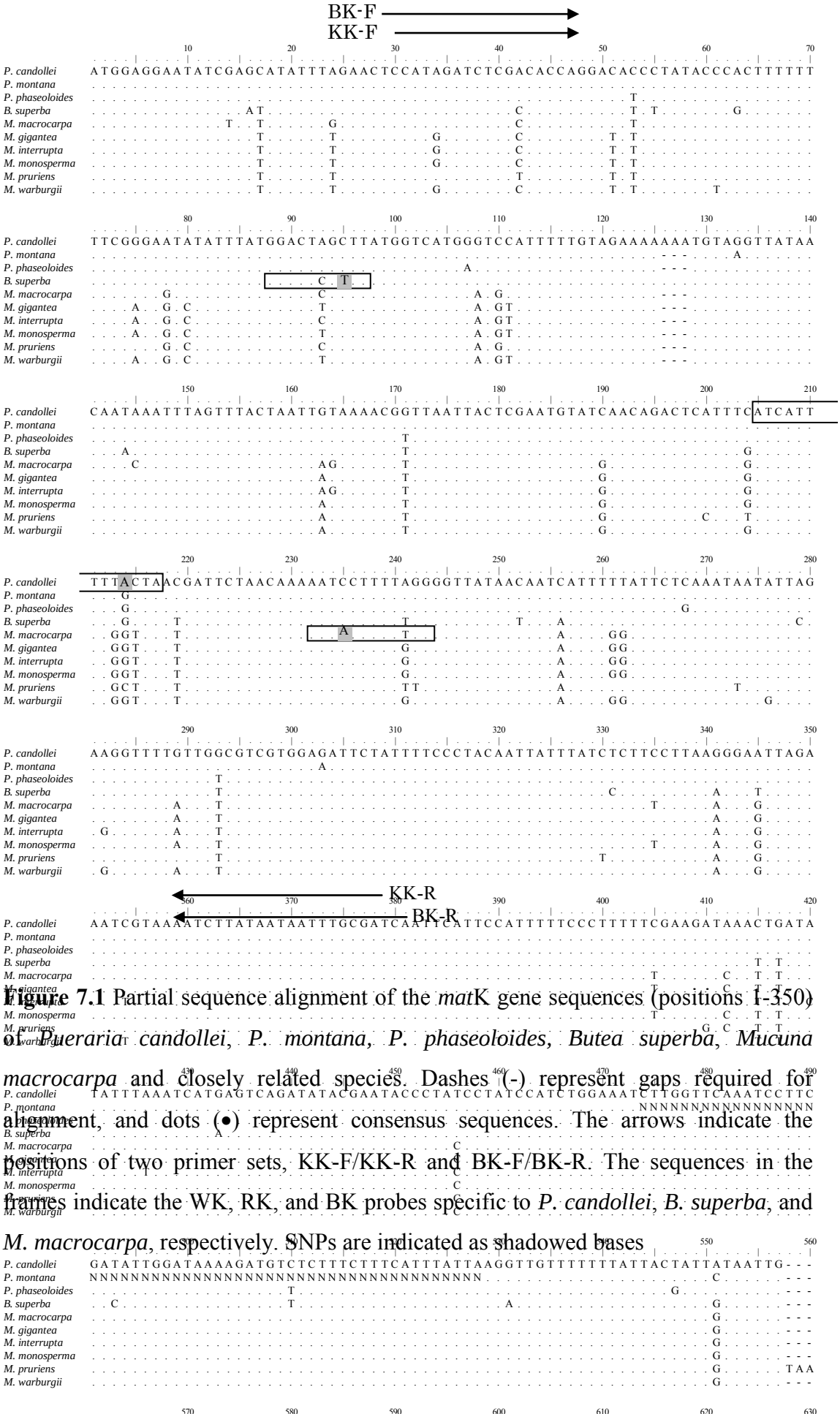

Figure 7.1 Partial sequence alignment of the *mat*K gene sequences (positions 1-350) of *Pueraria candollei*, *P. montana*, *P. phaseoloides*, *Butea superba*, *Mucuna macrocarpa* and closely related species. Dashes (-) represent gaps required for alignment, and dots (•) represent consensus sequences. The arrows indicate the positions of two primer sets, KK-F/KK-R and BK-F/BK-R. The sequences in the frames indicate the WK, RK, and BK probes specific to *P. candollei*, *B. superba*, and *M. macrocarpa*, respectively. SNPs are indicated as shadowed bases

7.2.4 Cycleave PCR

Cycleave real-time PCR was performed using a cycleave PCR core kit (TaKaRa Bio Inc., Japan). The partial *mat*K gene sequences (280 bp) of *P. candollei*, *B. superba*, and *M. macrocarpa* were amplified with two pairs of primers, set A (KK-F and KK-R) for *P. candollei* and *B. superba* and set B (BK-F and BK-R) for *M. macrocarpa*, in the presence of species-specific probes (Table 7.2). The reaction mixture consisted of 10 μl of 2× cycleave PCR reaction mixture, 10 μM of each primer, 5 μM of each probe, and 0.5 μl of DNA template. The final volume of the reaction mixture was adjusted to 20 μl with H_2O. The real-time PCR assay was performed using a Stratagene Mx3000P (Stratagene; La Jolla, CA). The PCR cycling conditions were 10 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 15 s at 55 °C, and 20 s at 72 °C. A total of 18 Kwao Khruea rejuvenating herb specimens were analysed. DNA amplification was monitored by measuring the fluorescence emission intensity when the amplicon and the probe could be completely complementary with each other. The PCR assay was completed in one to two hours.

7.2.5 Multiplex cycleave PCR for the simultaneous detection of two targets in a DNA admixture

A DNA admixture that contained equal amounts of *B. superba* and *M. macrocarpa* DNA was examined for the simultaneous detection of both species. Two sets of primers, set A and set B, were added to the cycleave reaction mixture in one tube along with the real-time PCR reaction. The species-specific probes were s with different fluorescent reporters: the RK probe (labelled with FAM) for detecting *B. superba* and BK probe (labelled with ROX) for detecting *M. macrocarpa*. The reaction mixture consisted of 10 μl of 2× cycleave PCR reaction mixture, 10 μM of each primer, 5 μM of each probe, and 0.3 μl of each DNA template. The final volume of the reaction mixture was adjusted to 20 μl with H_2O. The cycleave PCR assay was performed under the same PCR conditions as described above.

Table 7.2 The primers and probes used in cycleave PCR

Primers/Probes	Sequence (5′-3′)	Tm (ºC)	Location[a]
Primer Set A			
KK-F (forward)	-CCATAGATCTCGCCACCAG-	59.2	30-48
KK-R (reverse)	-TAGAATCTCCACGACGACAAC-	57.8	289-309
Primer Set B			
BK-F (forward)	-TCCATAGATCTCGCCACCAG-	61.2	29-48
BK-R (reverse)	-AATAGAATCTCCACGACGACAAT-	59.0	289-311
Probes[b]			
WK probe	-(Eclipse[c])-ATCATTTTT***A***CTA-(ROX[d])-	25.4	205-217
RK probe	-(Eclipse[c])-AA***A***CGAGTCC-(FAM[d])-	28.6	88-97
BK probe	-(Eclipse[c])-AAT***A***CTTTTTGG-(ROX[d])-	26.4	232-243

[a]Location of primers and probes in the *mat*K gene

[b]Fluorescence and quencher-labelled DNA-RNA chimeric probe. The boldface italic letters in the sequences indicate the SNP position that was synthesised as RNA

[c]Quenching molecules

[d]Fluorescent molecules

7.2.6 Reliability of cycleave PCR to distinguish *M. macrocarpa* from related *Mucuna* plants using highly specific cycling probes

The reliability of cycleave PCR was confirmed by detecting different species using a highly specific cycling probe. *M. macrocarpa* was selected to conduct the test. The primers BK-F and BK-R and species-specific BK probe were used to distinguish *M. macrocarpa* from five related species in the same genus (*M. gigantea*, *M.*

interrupta, *M. monosperma*, *M.pruriens*, and *M. warburgii*). The cycleave PCR assay was performed using the same protocol as previously described.

7.3 Results

7.3.1 Primers and species-specific probe

The primers and species-specific probes used in the present study were designed based on their *mat*K gene sequences, including sequences of closely related *Mucuna* plants obtained from GenBank (Figure 7.1). According to sequence alignment of the *mat*K gene, SNP sites specific to each Kwao Khruea species were detected. The species-specific probe was designed to be complementary to a region of the amplified product where SNP of interest was located.

Optimisation of the PCR conditions, primers, and species-specific probes enabled the detection of target species with high specificity. The 280-bp fragments of the *mat*K gene amplification products of the samples were obtained from the two primer pairs set A (KK-F and KK-R primers) for *P. candollei* and *B. superba* and set B (BK-F and BK-R primers) for *M. macrocarpa*.

7.3.2 Differentiation of "Kwao Khruea" species by cycleave PCR

Cycleave PCR was performed using the highly species-specific probes WK, RK, and BK in the presence of *P. candollei*, *B. superba*, and *M. macrocarpa* DNA, respectively. Each probe was found to be specific and gave a signal only with its target sequence. The fluorescent reporters ROX (6-carboxy-X-rhodamine) and FAM (6-carboxyfluorescein) were detected during the PCR reactions. The ROX signal was detected when genomic DNA of *P. candollei* or *M. macrocarpa* was used as a template for the WK probe or BK probe, respectively. The WK probe failed to detect *B. superba* and *M. macrocarpa*, and the BK probe failed to detect *P. candollei* and *B. superba* (Figure 7.2). There are several base pair mismatches within the *B. superba* and *M. macrocarpa* amplicons for the WK probe and several base pair mismatches within the *P. candollei* and *B. superba* amplicons for the BK probe (Figure 7.1).

Consequently, the probes could not form complexes that could dissociate during hybridisation; therefore, they could not be cleaved, and the ROX signal could not be detected. A similar result was obtained for the detection of *B. superba.* A FAM signal was detected with the RK probe when genomic DNA from *B. superba* was used as a template, and no signal was detected with the WK and BK probes (Figure 7.2).

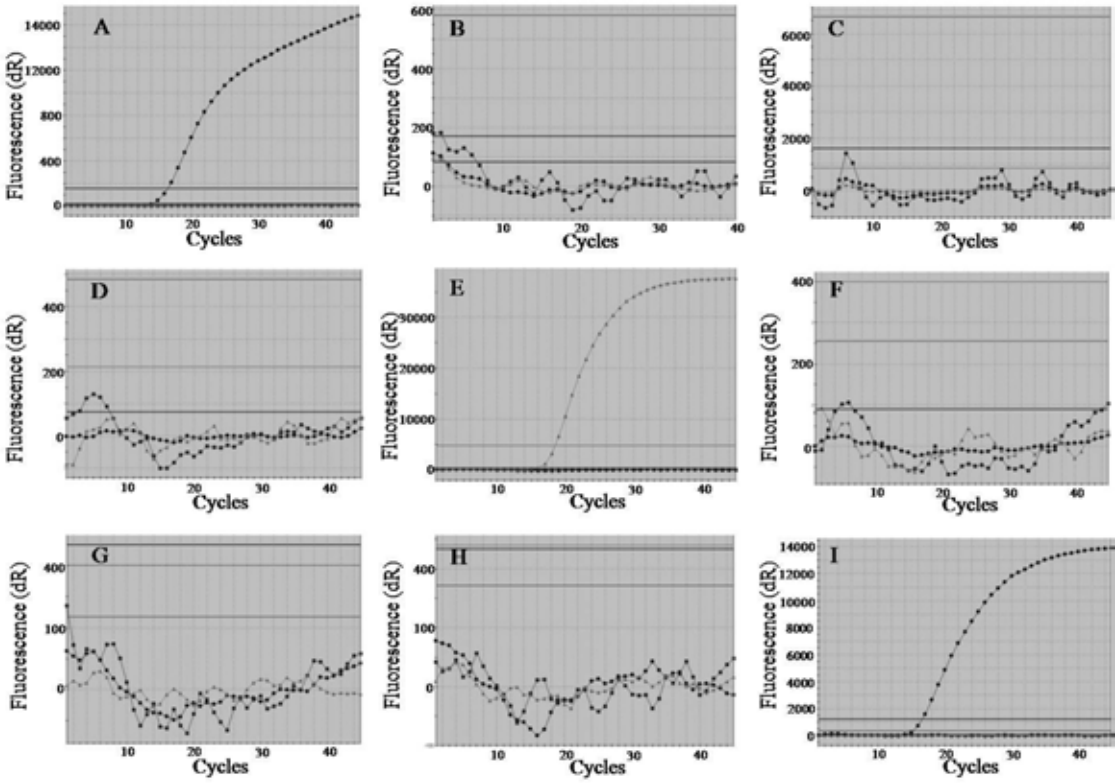

Figure 7.2 ROX and FAM signals detected during the cycleave PCR reaction. **A-C** Specificity of the WK probe labeled with ROX. **D-F** Specificity of the RK probe labeled with FAM. **G-I** Specificity of the BK probe labeled with ROX. **A, D, G** *P. candollei*; **B, E, H** *B. superba*; and **C, F, I** *M. macrocarpa*

7.3.3 Simultaneous detection of DNA admixtures using multiplex cycleave PCR

Multiplex cycleave PCR technique was performed to simultaneously detect *B. superba* and *M. macrocarpa* in a DNA admixture. A DNA admixture was prepared by mixing the DNA of *B. superba* and *M. macrocarpa* in equal amounts. A multiplex

cycleave PCR with differently labeled probes was examined in the DNA admixture. Each probe was specific and could accurately identify *B. superba* and *M. macrocarpa* in the same reaction. The RK probe labeled with FAM was used to detect *B. superba*, whereas the BK probe labeled with ROX was used to detect *M. macrocarpa*, and the RK and BK probes were added to a single cycleave PCR reaction.

The results showed that DNA identification of two species in a mixture could be performed simultaneously by multiplex cycleave PCR. *B. superba* and *M. macrocarpa* were amplified, and FAM and ROX signals were detected simultaneously (Figure 7.3).

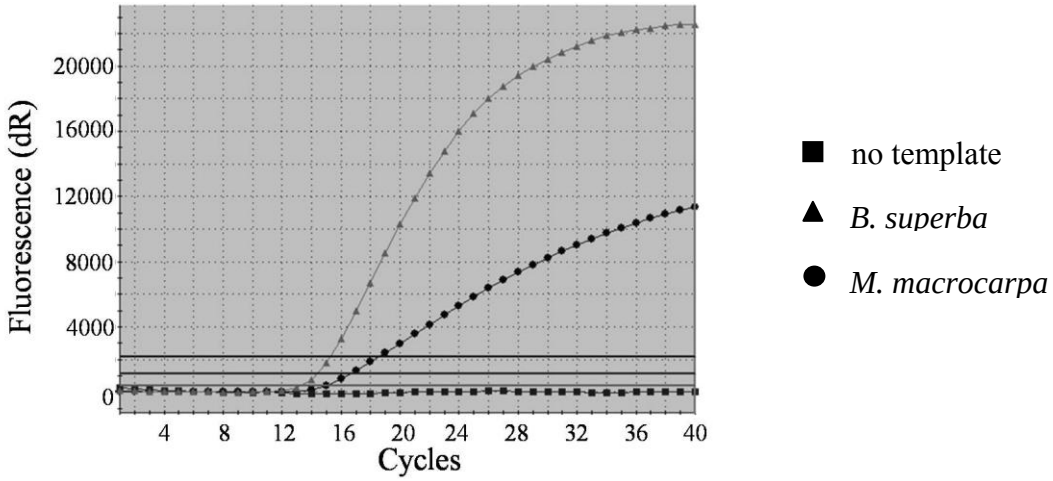

Figure 7.3 Multiplex cycleave PCR for the simultaneous detection of mixed DNA samples of *B. superba* and *M. macrocarpa*. The RK and BK probes are specific to *B. superba* and *M. macrocarpa*, respectively

7.3.4 Authentication of *M. macrocarpa* from five related *Mucuna* plants using a highly specific probe

The specific BK probe was designed based on this SNP site to amplify only *M. macrocarpa*. To confirm the species specificity of cycleave PCR, the specific BK probe was used to distinguish *M. macrocarpa* from other *Mucuna* plants. The ROX signal of the BK probe was detected when the amplification product was obtained from *M. macrocarpa*, and there was no signal for the other five species (Figure 7.4).

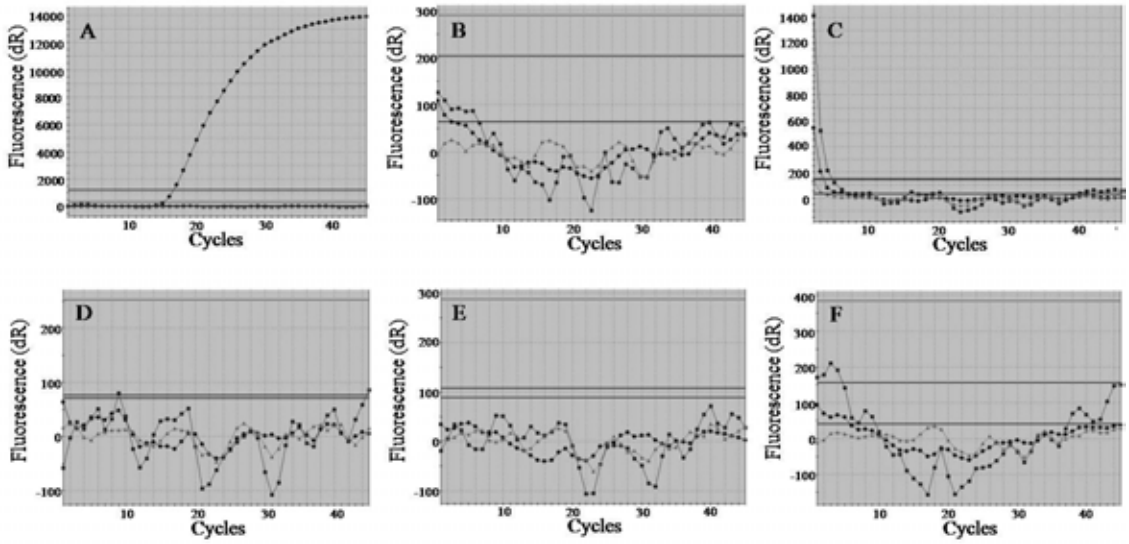

Figure 7.4 Cycleave PCR assay. Even when amplified products were obtained from species other than *M. macrocarpa*, they were not detected by the BK probe labeled with ROX. **A** *M. macrocarpa*; **B** *M. gigantea*; **C** *M. interrupta*; **D** *M. monosperma*; **E** *M. pruriens*; and **F** *M. warburgii*

This result confirmed that the BK probe is highly specific and is suitable for the accurate detection of the *M. macrocarpa* SNP. This highly specific probe could be used to authenticate *M. macrocarpa* from five similar *Mucuna* plants.

7.4 Discussion

In the present study, the *mat*K gene sequence was used as target for development of cycleave PCR analysis to differentiate three rejuvenating herb species, *P. candollei* (White Kwao Khruea), *B. superba* (Red Kwao Khruea), and *M. macrocarpa* (Black Kwao Khruea). This gene sequence has been widely used to identify the botanical origins of medicinal plants, such as plants in the genus *Panax* (Zhu *et al.*, 2003), *Asparagus* (Boonsom *et al.*, 2012), and *Dioscorea* (Sun *et al.*, 2012). Typically, cycling probes contain RNAs corresponding to a unique SNP of its target sequence and were labeled with different fluorescent dyes.

The optimal amplicons for real-time PCR are typically short (they should not exceed 400 bp) because long products do not amplify as efficiently as shorter products (Keohavong and Grant, 2005). Because of the high amplification efficiency of cycleave real-time PCR, this technique was suitable for the identification of crude Kwao Khruea samples with partially degraded genomic DNA.

In recent years, the use of real-time PCR has improved the molecular identification of organisms (Xue *et al.*, 2008). Various techniques based on real-time PCR are used to differentiate medicinal plants, including Scorpion probe PCR (Xue *et al.*, 2008), TaqMan probe PCR (Xue *et al.*, 2008), and melting curve analysis (Xue *et al.*, 2009). To our knowledge, this is the first study in which cycleave PCR has been used to differentiate medicinal plant species. The obtained results indicated that cycleave PCR with specific probes could be used to differentiate individual Kwao Khruea species. The WK, RK, and BK probes are highly specific to *P. candollei*, *B. superba*, and *M. macrocarpa*, respectively. Because cycleave PCR is monitored in real time, it does not require post-PCR steps, such as agarose gel electrophoresis (Fraga *et al.*, 2008). The entire process can be completed within one to two hours. In addition, it enables a large number of samples to be simultaneously analyzed using a 96-well plate.

The misidentification of crude Kwao Khruea drugs, such as Red (*B. superba*) and Black (*M. macrocarpa*) Kwao Khruea, is a problem in drug markets (Wiriyakarun *et al.*, 2012). The identification of two species in a mixture could be performed simultaneously by multiplex cycleave PCR. In this study, *B. superba* and *M. macrocarpa* were successfully amplified, and FAM and ROX signals were detected simultaneously. The amplitudes of the fluorescence curves showed unequal PCR efficiencies, although the mixture was prepared from equal amounts of DNA. Similar results were found by Henegariu *et al.* (1997) and Shokoples *et al.* (2009). Because the two target sequences were amplified in the same reaction, they compete for the same reagents (dNTPs and polymerase) (Henegariu *et al.*, 1997). In addition, the amplification of two targets can inhibit each other through interactions among the two primer pairs, specific probes, amplified products, or a combination of PCR components (Henegariu *et al.*, 1997; Shokoples *et al.*, 2009). Therefore, optimising

the concentrations of primers and probes might be necessary to avoid competition in a multi-target amplification reaction (He *et al.*, 2010).

According to traditional Thai medicine, only *M. macrocarpa* is considered as botanical source of Black Kwao Khruea (Suntara, 1931). However, other *Mucuna* plants, including *M. gigantea*, *M. interrupta*, *M. monosperma*, *M. pruriens*, and *M. warburgii*, belong to the same genus and often have similar features. According to a multiple sequence alignment, the nucleotide at position 235 is cytosine I for all species except *M. macrocarpa*, which has an adenine (A) at that position. Thus, the specific BK probe was designed based on this SNP site to amplify only *M. macrocarpa*. To confirm the species specificity of cycleave PCR, the specific BK probe was used to distinguish *M. macrocarpa* from other *Mucuna* plants. The ROX signal of the BK probe was detected when the amplification product was obtained from *M. macrocarpa*, and there was no signal for the other five species. This result confirmed that the BK probe is highly specific and is suitable for the accurate detection of the *M. macrocarpa* SNP. This highly specific probe could be used to authenticate *M. macrocarpa* from five similar *Mucuna* plants.

7.5 Conclusion

In this study, cycleave real-time PCR was also performed and successfully used to differentiate the rejuvenating Kwao Khruea herb species *P.candollei*, *B. superba*, and *M. macrocarpa*. This technique provides a rapid and specific method for detecting specific DNA sequences, even sequences that differ by a single SNP. Cycleave PCR was also used to determine the presence or absence of a target-specific DNA sequence in an artificial mixture. In the present study, the specific sequences were amplified and simultaneously detected for *B. superba* and *M. macrocarpa* in a DNA admixture. Moreover, the results showed that the cycling probe is highly specific to its target sequence. For example, the species-specific BK probe could be used to distinguish Black Kwao Khruea, *M. macrocarpa*, from two other Kwao Khruea species, *P. candollei* and *B. superba*, and from five related *Mucuna* plants. The results indicate that cycleave PCR is useful for qualitative analysis. Future research should be focused on the use of cycleave PCR in quantitative analysis.

CHAPTER VIII

CONCLUSION

The present studies provide genetic and phytochemical assessment for identification of *Mucuna* plants and Kwao Khruea herbs. Genetic assessment could be an effective way to discriminate different or confused plant species in the same genus. PCR-based methods based on the ITS region and the *mat*K gene was used for the identification of *Mucuna* plants, including *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*. PCR-RFLP of the *mat*K gene failed to discriminate individual species because of the low level of *mat*K gene sequence divergence in six *Mucuna* plants, while a multiplex PCR of the ITS region was successfully used for the identification of these plants. Moreover, PCR-RFLP based on *mat*K gene was successfully used to discriminate rejuvenating Kwao Khruea herbs, White (*P. Candollei*), Red (*B. superba*), and Black (*M. macrocarpa*) Kwao Khruea. This technique can be applied to authenticate commercial herbal drugs. A sophisticated novel cycleave real-time PCR was also successfully used to differentiate these rejuvenating Kwao Khruea herb species. It was also used to determine the presence of a target-specific DNA sequence in an artificial mixture. This technique was also used to distinguish *M. macrocarpa* from five related *Mucuna* plants.

These results confirmed that multiplex PCR of the ITS region is a convenient, efficient and specific method for identification of *M. gigantea*, *M. interrupta*, *M. macrocarpa*, *M. monosperma*, *M. pruriens*, and *M. warburgii*. PCR-RFLP and cycleave real-time PCR based on *mat*K gene are suitable techniques for the discrimination of Kwao Khruea herbs. They are simple, rapid, sensitive, accurate, and useful for qualitative analysis. The developed methods used in this study could be applied for the identification of other several pharmaceutical herbs or individual species in herbal drug formulations

Phytochemical assessment used in this study could be an effective method for identification at the genus level. In order to ensure the efficacy, selection of the valuable compound of the plant as chemical marker is necessary. L-Dopa determined

by TLC densitometric method could serve as a chemical marker for quality control of the *Mucuna* plants. The amounts of L-Dopa in seeds of each *Mucuna* plants varied according to different genetic profiles or environmental factors. The proposed TLC densitometric method was found to be simple, rapid, and accurate for detection and comparison of L-Dopa content in different *Mucuna* plants. This method can be used to examine very large numbers of samples. In addition, this method can also be applied for the quantitative determination of L-Dopa in herbal extracts or these product formulations in the market.

The results from our studies indicated that the combination of genetic and phytochemical assessments is useful and could be used as suitable tools for genus-level and species-level identification of *Mucuna* plants.

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APPENDICES

APPENDIX A

Plant morphology of *Mucuna* plants

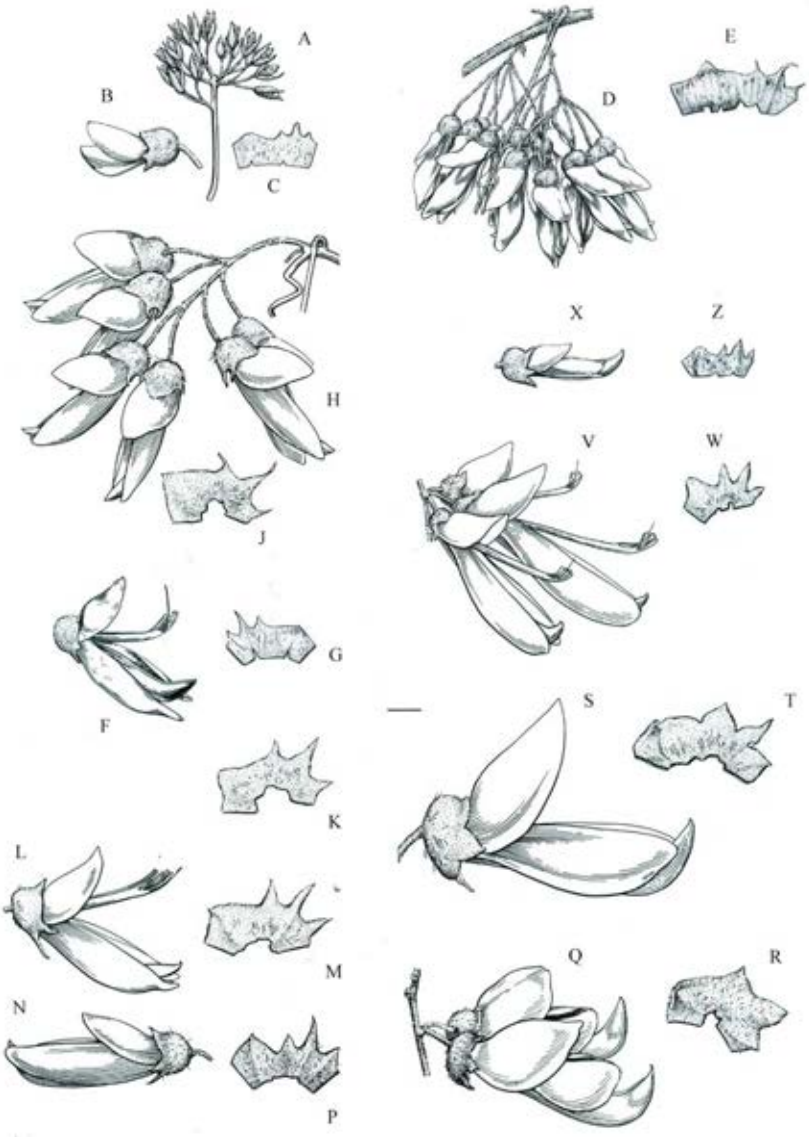

Figure A1 *Mucuna* flowers, calyx (opened out) and inflorescences. *M. gigantea*: A. inflorescence in bud stage; B. flower; C. calyx; *M. oligoplax*: D. inflorescence; E. calyx; *M. monosperma*: F, G. *M. stenoplax*: H, J; *M. hainanensis*: K; *M. revolute*: L, M; *M. interrupta*: N, P; *M. macrocarpa*: Q, R; *M. thailandica*: S, T; *M. gracilipes*: V, W; *M. bracteata*: X, Z. Scale bar for A, D, H = 1.5 cm, for all others = 1 cm (Wilmot-Dear, 1993)

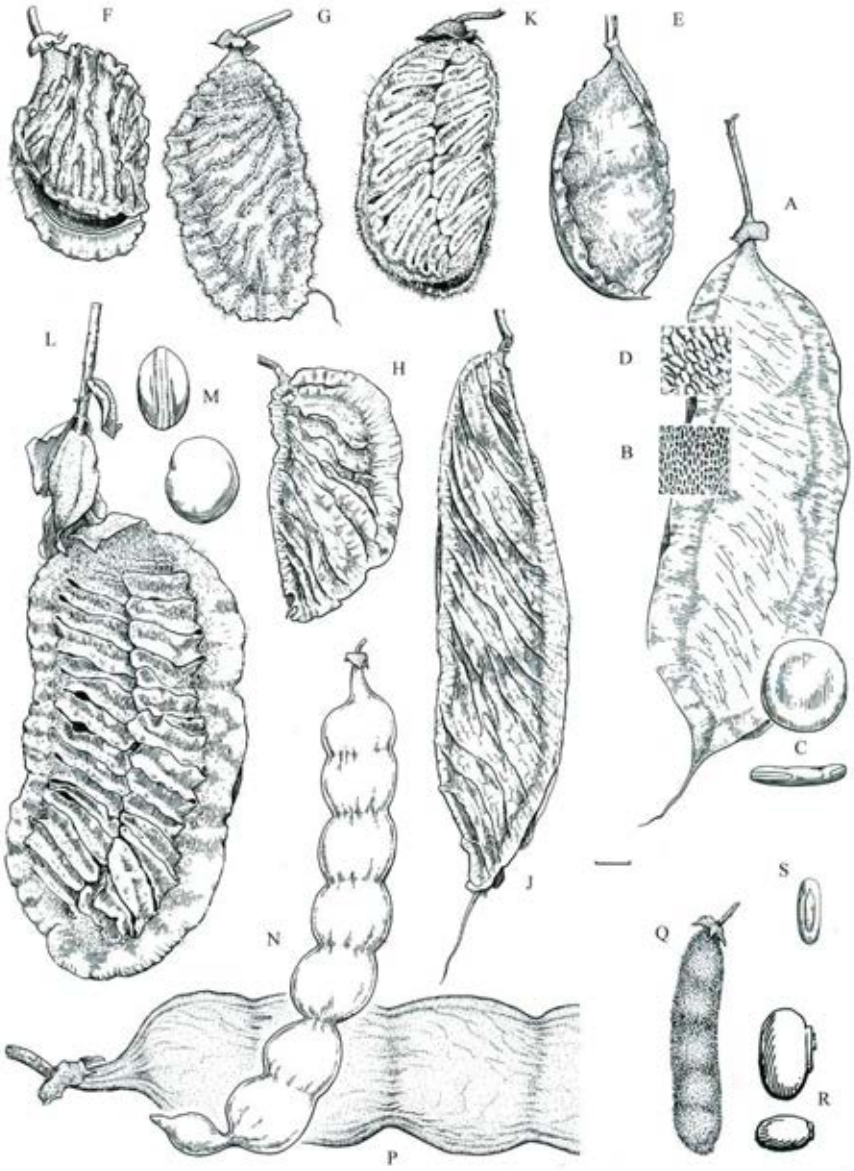

Figure A2 *Mucuna* fruits and seeds. *M. gigantea*: A. fruit; B. detail of fruit surface view; *M. acuminata*: D. detail of fruit surface; *M. oligoplax*: E. fruit; *M. monosperma*: F. fruit; *M. stenoplax*: G. fruit; *M. hainanensis*: H, J. fruits; *M. revoluta*: K. fruit; *M. interrupta*: L. fruit; M. seeds side and surface view; *M. macrocarpa*: N. young fruit; P. part of fruit; *M. bracteata*: Q. fruit; R. seed, apical and lateral view; *M. pruriens* var. *hirsuta*: S. seed side view showing aril. Scale bar for R, S = 0.5 cm, for all others = 1 cm (Wilmot-Dear, 1993)

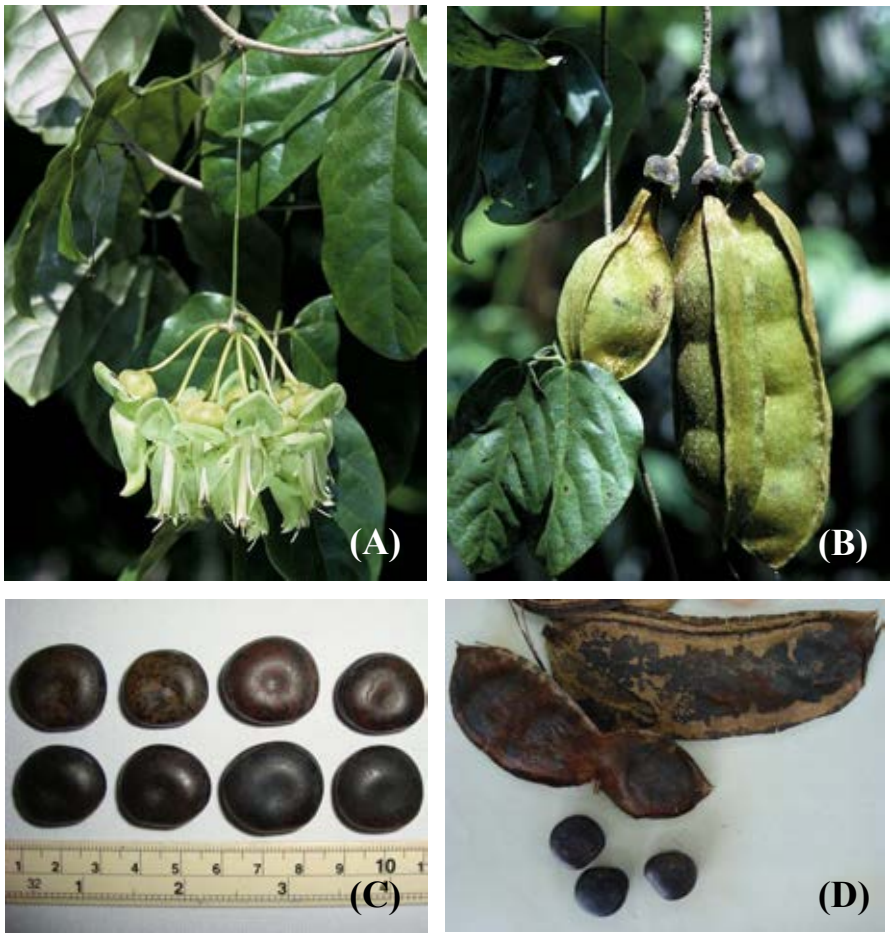

Figure A3 *Mucuna gigantea* (Willd.) DC. Flowers (A); fruits (B) [cited 2012 November 23] Available from: http://keys.trin.org.au/key-server/data/0e0f0504-0103-430d-8004-060d07080d04/media/Html/taxon/Mucuna_gigantea.htm; seeds (C); and dry fruits and seeds (D)

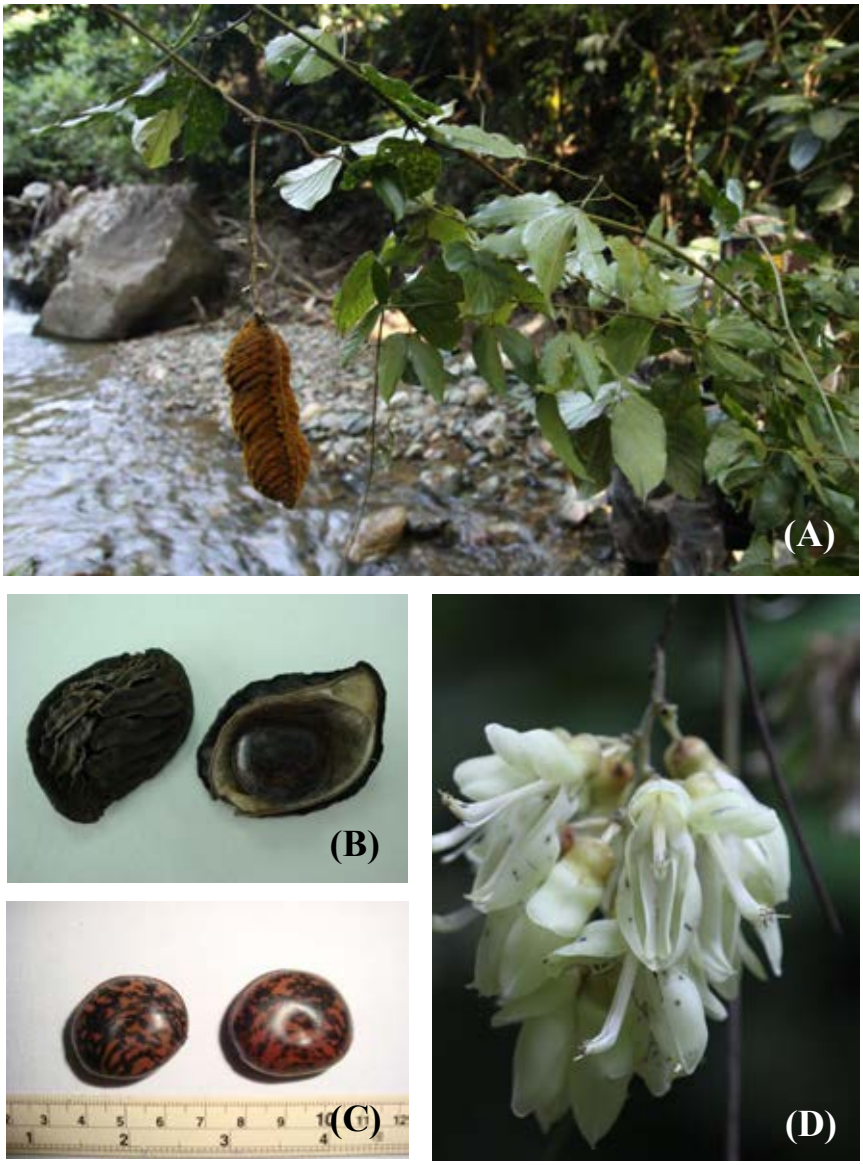

Figure A4 *Mucuna interrupta* Gagnep. The plant with fruits (A); dry fruit with one seed (B); seeds (C); and flowers (D)

Figure A5 *Mucuna macrocarpa* Wall. The plant (A); trifoliate leaves (B); dry fruit and seed (C); seeds (D); and flowers (E) [cited 2012 November 23] Available from: http://www.kinmatsu.idv.tw/show.php?f=plant/Rosidae/Mucuna.macrocarpa

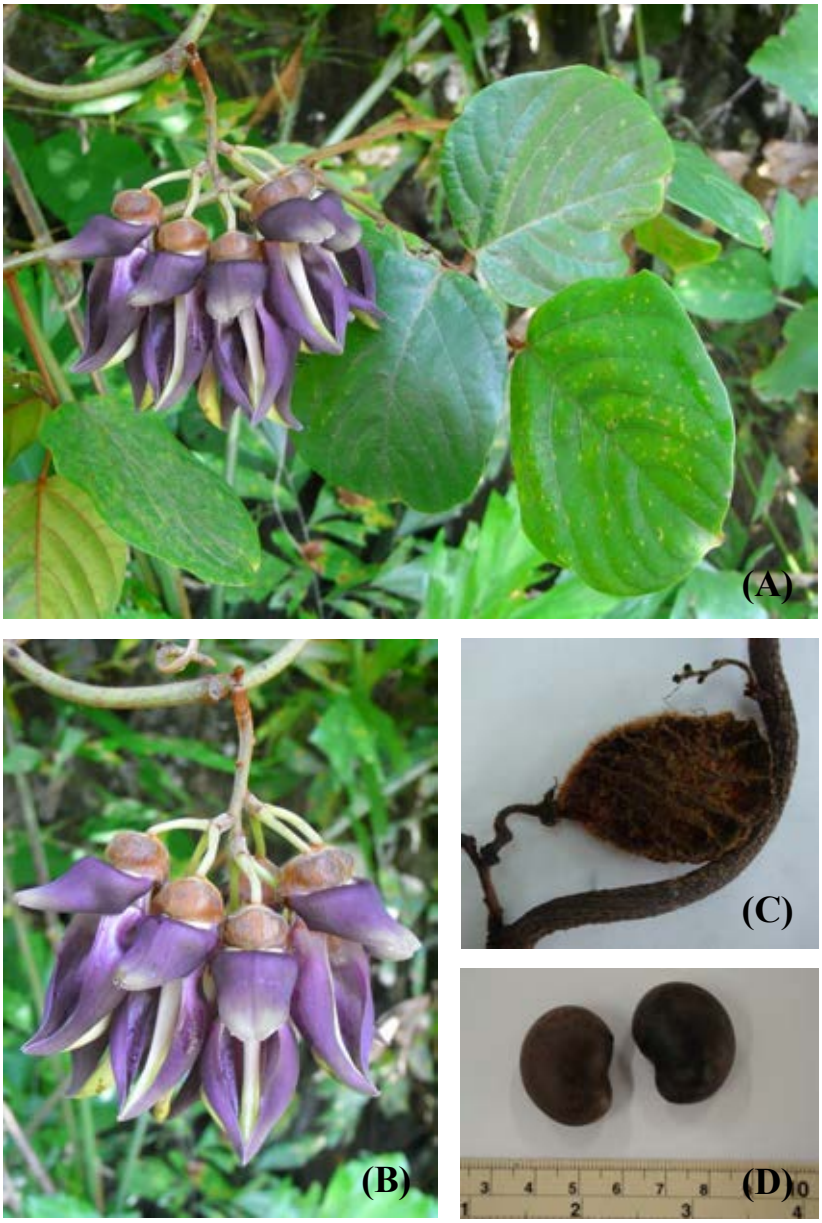

Figure A6 *Mucuna monosperma* DC. The plant with flowers (A); flowers (B); dry fruit (C); and seeds (D)

Figure A7 *Mucuna pruriens* DC. Flowers (A); fruits (B); trifoliate leaves (C); and seeds (D)

Figure A8 *Mucuna warburgii* Lauterb. & K. Schum. The plant (A); trifoliate leaves (B); and flowers (C)

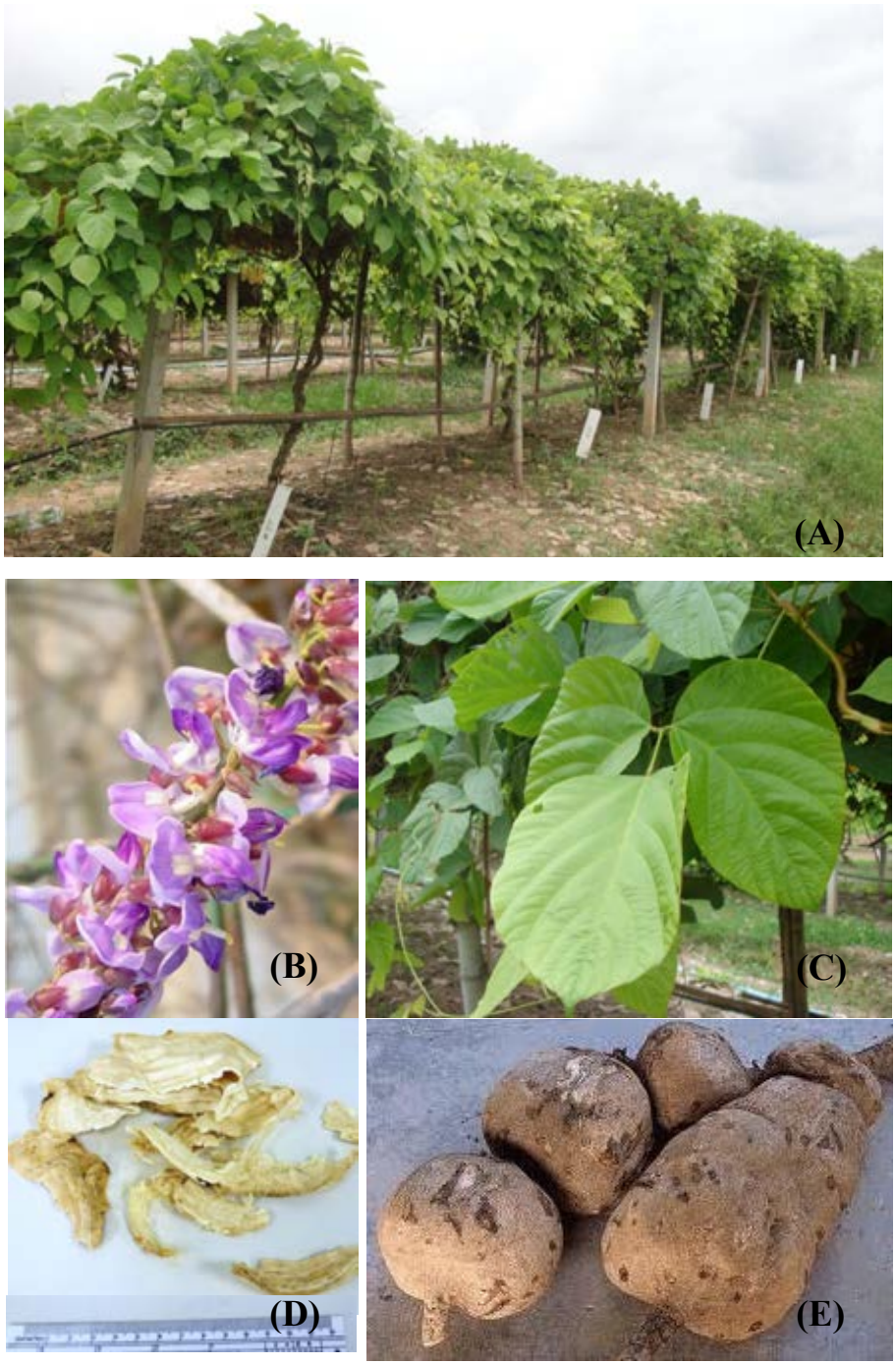

Figure A9 *Pueraria candollei* Graham ex Benth. The plant (A); flowers (B); trifoliate leaves (C); crude drug (D); and tuberous roots (E) [cited 2012 December 12] Available from: http://thaiherbonly.blogspot.com/2012/01/pueraria-candollei-grah.html

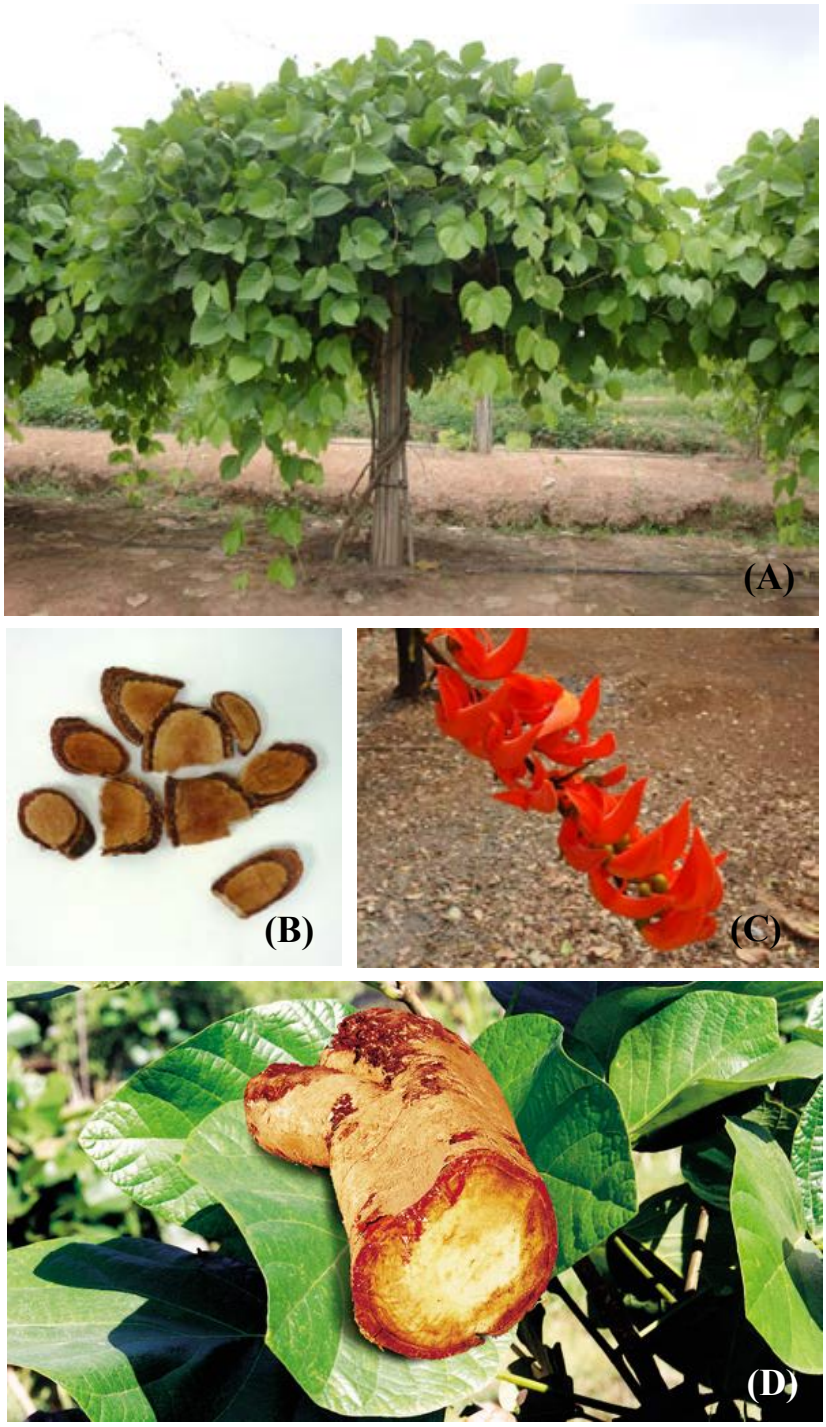

Figure A10 *Butea superba* Roxb. The plant (A); crude drug (B); flowers (C) and tuberous root (D) [cited 2012 December 12] Available from: http://www.puerariathai.com/butea-superba-extract/butea-superba-mix-powder-extract.htm

APPENDIX B

DNA sequences of six *Mucuna* plants deposited in GenBank

Data of *mat*K gene sequences

1. *Mucuna pruriens* (L.) DC.

LOCUS AB627857 1524 bp DNA linear PLN 10-JAN-2013
DEFINITION Mucuna pruriens chloroplast matK gene for maturase K, complete cds, specimen_voucher: personal: Suchaya Wiriyakarun :SW260710.
ACCESSION AB627857
VERSION AB627857.1 GI:441418547
KEYWORDS .
SOURCE chloroplast Mucuna pruriens
ORGANISM Mucuna pruriens
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1
AUTHORS Wiriyakarun, S., Yodpetch, W., Ruangrungsi, N. and Sukrong, S.
TITLE Identification of Thai Medicinal Plants White Kwao Khruea (Pueraria candollei), Red Kwao Khruea (Butea superba), and Black Kwao Khruea (Mucuna macrocarpa) and Application for Detection of Kwao Khruea in Admixtures by Using PCR-RFLP Analysis
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1524)
AUTHORS Sukrong, S.
TITLE Direct Submission
JOURNAL Submitted (29-APR-2011) Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Road, Bangkok, Patumwan 10330, Thailand
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ACCESSION AB627858
VERSION AB627858.1 GI:441418549
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ORGANISM Mucuna macrocarpa
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REFERENCE 1
AUTHORS Wiriyakarun, S., Yodpetch, W., Ruangrungsi, N. and Sukrong, S.
TITLE Identification of Thai Medicinal Plants White Kwao Khruea (Pueraria candollei), Red Kwao Khruea (Butea superba), and Black Kwao Khruea (Mucuna macrocarpa) and Application for Detection of Kwao Khruea in Admixtures by Using PCR-RFLP Analysis
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1518)
AUTHORS Sukrong, S.
TITLE Direct Submission
JOURNAL Submitted (29-APR-2011) Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Road, Bangkok, Patumwan 10330, Thailand
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ACCESSION AB627859
VERSION AB627859.1 GI:441418551
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ORGANISM Mucuna monosperma
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REFERENCE 1
AUTHORS Wiriyakarun, S., Yodpetch, W., Ruangrungsi, N. and Sukrong, S.
TITLE Identification of Thai Medicinal Plants White Kwao Khruea (Pueraria candollei), Red Kwao Khruea (Butea superba), and Black Kwao Khruea (Mucuna macrocarpa) and Application for Detection of Kwao Khruea in Admixtures by Using PCR-RFLP Analysis
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1518)
AUTHORS Sukrong, S.
TITLE Direct Submission
JOURNAL Submitted (29-APR-2011) Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Road, Bangkok, Patumwan 10330, Thailand
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4. *Mucuna gigantea* (Willd.) DC.

LOCUS AB627860 1518 bp DNA linear PLN 10-JAN-2013
DEFINITION Mucuna gigantea chloroplast matK gene for maturase K, complete cds, specimen_voucher: personal: Suchaya Wiriyakarun :SW020610.
ACCESSION AB627860
VERSION AB627860.1 GI:441418553
KEYWORDS .
SOURCE chloroplast Mucuna gigantea
ORGANISM Mucuna gigantea
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1
AUTHORS Wiriyakarun, S., Yodpetch, W., Ruangrungsi, N. and Sukrong, S.
TITLE Identification of Thai Medicinal Plants White Kwao Khruea (Pueraria candollei), Red Kwao Khruea (Butea superba), and Black Kwao Khruea (Mucuna macrocarpa) and Application for Detection of Kwao Khruea in Admixtures by Using PCR-RFLP Analysis
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1518)
AUTHORS Sukrong, S.
TITLE Direct Submission
JOURNAL Submitted (29-APR-2011) Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Road, Bangkok, Patumwan 10330, Thailand
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181 atgtatgaac agactcattt gatcattttg gttaatgatt ctaacaaaaa tccttttggg
241 ggttataaca ataatttgga ttctcaaata atattagaag gttttattgt cgtcgtggag
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421 ttaaatcatg agtcagatat acgaataccc taccctatcc atctggaaat cttggttcaa
481 atccttcgat attggataaa agatgtctct ttctttcatt tattaaggtt gttttttat
541 tactattgta attggaatag tcttttttact ccaaaaaaat cgatttctac ttttttttca
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5. *Mucuna warburgii* Lauterb. & K. Schum.

LOCUS AB627861 1518 bp DNA linear PLN 10-JAN-2013
DEFINITION Mucuna warburgii chloroplast matK gene for maturase K, complete cds, specimen_voucher: personal: Suchaya Wiriyakarun :SW031010.
ACCESSION AB627861
VERSION AB627861.1 GI:441418555
KEYWORDS .
SOURCE chloroplast Mucuna warburgii
ORGANISM Mucuna warburgii
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1
AUTHORS Wiriyakarun, S., Yodpetch, W., Ruangrungsi, N. and Sukrong, S.
TITLE Identification of Thai Medicinal Plants White Kwao Khruea (Pueraria candollei), Red Kwao Khruea (Butea superba), and Black Kwao Khruea (Mucuna macrocarpa) and Application for Detection of Kwao Khruea in Admixtures by Using PCR-RFLP Analysis
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1518)
AUTHORS Sukrong, S.
TITLE Direct Submission
JOURNAL Submitted (29-APR-2011) Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Road, Bangkok, Patumwan 10330, Thailand
FEATURES Location/Qualifiers
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/organism="Mucuna warburgii"
/organelle="plastid:chloroplast"
/mol_type="genomic DNA"
/specimen_voucher="personal: Suchaya Wiriyakarun:SW031010"
/db_xref="taxon:1032650"
/country="Thailand"
/note="MUS-H3866; preserved in Museum of Natural Medicines, Chulalongkorn University, Bangkok, Thailand."
gene 1..1518
/gene="matK"
CDS 1..1518
/gene="matK"
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/protein_id="BAM74465.1"
/db_xref="GI:441418556"
/translation="MEEYRVYFELHGSRHQDILYSLFFRESIYGLAY
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KSYNNLRSIHSIFPFFEDKFIYLNHESDIRIPYPIHLEILVQILR
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LFLFLYNLYVWEYESIFLFLRNKSSQLRLKYFRVFFERIFFYE
KIEHLVEIFVKGFSYTLSFFKDPFIHYVRYQGKSILVSKNTPL
LINKWKYYFIYIWQCHFNIWSQPGTIDIKQLSQHSFHLLGYF
LSIRLNLSVVRSQMLQNSFLIQIVMKKVDTIVPIIPLIRSLAK
AKFCNVLGHPISKPVWANLSDFDIIYRFLRICRNFAHYYKGS
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HA"

ORIGIN

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1381gaagaattct ttacagaaga agaagatatt ttttctttga tttttccaag aacttctttt
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1501ttggtcaatc atgcataa
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6. *Mucuna interrupta* Gagnep.

LOCUS AB627862 1518 bp DNA linear PLN 10-JAN-2013
DEFINITION Mucuna interrupta chloroplast matK gene for maturase K, complete cds, specimen_voucher: personal: Suchaya Wiriyakarun :SW080910.
ACCESSION AB627862
VERSION AB627862.1 GI:441418557
KEYWORDS .
SOURCE chloroplast Mucuna interrupta
ORGANISM Mucuna interrupta
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1
AUTHORS Wiriyakarun, S., Yodpetch, W., Ruangrungsi, N. and Sukrong, S.
TITLE Identification of Thai Medicinal Plants White Kwao Khruea (Pueraria candollei), Red Kwao Khruea (Butea superba), and Black Kwao Khruea (Mucuna macrocarpa) and Application for Detection of Kwao Khruea in Admixtures by Using PCR-RFLP Analysis
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1518)
AUTHORS Sukrong, S.
TITLE Direct Submission
JOURNAL Submitted (29-APR-2011) Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Road, Bangkok, Patumwan 10330, Thailand
FEATURES Location/Qualifiers
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/organism="Mucuna interrupta"
/organelle="plastid:chloroplast"
/mol_type="genomic DNA"
/specimen_voucher="personal: Suchaya Wiriyakarun:SW080910"
/db_xref="taxon:1032651"
/country="Thailand"
/note="MUS-H3867; preserved in Museum of Natural Medicines, Chulalongkorn University, Bangkok, Thailand."
gene 1..1518
/gene="matK"
CDS 1..1518
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/transl_table=11
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/protein_id="BAM74466.1"
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/translation="MEEYRVYFELHGSRHQDILYPLFFRESIYGLAY
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SYNNLRSIHSIFPFFEDKFIYFNHESDIRIPYPIHLEILVQILRY
WIKDVSFFHLLRLFFYYYCNWNSLFTPKKSISTFFSKSNPRL
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KIEHLVEIFIKDFSYTLSFFKDPFIHYVRYQGKSILVSKNTPLL
INKWKYYFIYIWQCHFNIWSQPGTIDIKQLSQHSFHLLGYFL
SIRLNLSVVRSQMLQNSFLIQIVMKKLDTIVPIIPLIRSLAKA
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ORIGIN

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241 ggttataaca ataatttgga ttctcaaata atattagagg gttttattgt cgtcgtggag
301 attctatttt ccctacaatt atttatctct tccttaaagg agttagaaat tgtaaaatct
361 tataataatt tgcgatcaat tcattccatt tttccctttt tcgaagataa atttatctat
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541 tactattgta attggaatag tcttttttact ccaaaaaaat cgatttctac ttttttttca
601 aaaagtaatc caagattatt cttgttccta tataatttat atgtatggga atacgaatct
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Data of ITS sequences of *Mucuna*

1. *Mucuna macrocarpa* Wall.

ACCESSION	AB775133
KEYWORDS	.
ORGANISM	Mucuna macrocarpa Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE	1 (bases 1 to 742)
AUTHORS	Sukrong, S. and Wiriyakarun, S.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2013) to the DDBJ/EMBL/GenBank databases. Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Rd., Bangkok, Patumwan 10330, Thailand
REFERENCE	2
AUTHORS	Wiriyakarun, S., Zhu, S., Komatsu, K., Ruangrungsi, N. and Sukrong, S.
TITLE	Evaluation of Octaplex PCR for Rapid Differentiation of Six Mucuna plants
JOURNAL	Unpublished (2013)
FEATURES	Location/Qualifiers
source	1..742 /country="Thailand" /mol_type="genomic DNA" /organism="Mucuna macrocarpa" /PCR_primers="fwd_name: ITS1, fwd_seq: tccgtaggtgaacctgcgg, rev_name: ITS4, rev_seq: tcctccgcttattgatatgc"
misc_RNA	<1..>742 /note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

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121 ccgggggccgg ctcgggggcg ctgttcgttc tcgaacaccg accccgtcct ccctcgggtt
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241 cgccaaggaa ctcgaaattg ttcagtgcaa tcttcgcggg cccggagacg gcgatcccgc
301 ggaccttgtc acgacacacg atacaaaatg actctcggca acggatatct cggctcttgc
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361 atcgatgaag aacgtagcga aatgcgatac ttggtgtgaa ttgcagaatc ccgtgaacca
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481 tgtcacacat cgttacccca aagcaaacgc ctcacgtgcg tgcgcagggt ggaagctgac
541 ctcccgcggg caacgactcc cgcggctggt tgaaaatcga gttcgtggcc gggtgcgccg
601 cgataaaatg gtggatgagc gacgctcgag accaatcgcg cgcgaccccg tcagcgtcgg
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721 acccgctgag tttaagcata tc
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2. *Mucuna gigantea* (Willd.) DC.

ACCESSION	AB775134
KEYWORDS	.
ORGANISM	Mucuna gigantea Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE	1 (bases 1 to 755)
AUTHORS	Sukrong, S. and Wiriyakarun, S.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2013) to the DDBJ/EMBL/GenBank databases. Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Rd., Bangkok, Patumwan 10330, Thailand
REFERENCE	2
AUTHORS	Wiriyakarun, S., Zhu, S., Komatsu, K., Ruangrungsi, N. and Sukrong, S.
TITLE	Evaluation of Octaplex PCR for Rapid Differentiation of Six Mucuna plants
JOURNAL	Unpublished (2013)
FEATURES	Location/Qualifiers
source	1..755 /country="Thailand" /mol_type="genomic DNA" /organism="Mucuna gigantea" /PCR_primers="fwd_name: ITS1, fwd_seq: tccgtaggtgaacctgcgg, rev_name: ITS4, rev_seq: tcctccgcttattgatatgc"
misc_RNA	<1..>755 /note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

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3. *Mucuna interrupta* Gagnep.

ACCESSION AB775135
KEYWORDS .
ORGANISM Mucuna interrupta
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1 (bases 1 to 736)
AUTHORS Sukrong, S. and Wiriyakarun, S.
TITLE Direct Submission
JOURNAL Submitted (04-JAN-2013) to the DDBJ/EMBL/GenBank databases. Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Rd., Bangkok, Patumwan 10330, Thailand
REFERENCE 2
AUTHORS Wiriyakarun, S., Zhu, S., Komatsu, K., Ruangrungsi, N. and Sukrong, S.
TITLE Evaluation of Octaplex PCR for Rapid Differentiation of Six Mucuna plants
JOURNAL Unpublished (2013)
FEATURES Location/Qualifiers
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/organism="Mucuna interrupta"
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fwd_seq: tccgtaggtgaacctgcgg, rev_name: ITS4,
rev_seq: tcctccgcttattgatatgc"
misc_RNA <1..>736
/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

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181 ggaggtggtc gcctcgtgct gctcctcccg tcgaacaaaa accccggcgc ttcgtgcgtc
241 aaggaatttg aaattgttag gtgcaatttt cgcggacccg gagacggtga tctcgcggac
301 cttgccacga cacacgatac aaaatgactc tcggcaacgg atatctcggc tcttgcatcg
361 atgaagaacg tagcgaaatg cgatacttgg tgtgaattgc agaatcccgt gaaccatcga
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421 gtctttgaac gcaagttgcg cccgaagcca ttaggttgag ggcacgcctg cctgggtgtc
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541 cgtgggccac gactcgcggc tggttgaaaa tggagttcat ggttgagaat gccgtgataa
601 aatggtggat gagcgttgct cgagaccaat cgcgtgcgac tcggtcaatt ttggactctt
661 cgacccaatt gcgtcgatgg acgctccgaa cgagacctca ggtcaggcgg ggccacccgc
721 tgagtttaag catatc
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4. *Mucuna monosperma* DC.

ACCESSION AB775136
KEYWORDS .
ORGANISM Mucuna monosperma
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1 (bases 1 to 750)
AUTHORS Sukrong, S. and Wiriyakarun, S.
TITLE Direct Submission
JOURNAL Submitted (04-JAN-2013) to the DDBJ/EMBL/GenBank databases. Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Rd., Bangkok, Patumwan 10330, Thailand
REFERENCE 2
AUTHORS Wiriyakarun, S., Zhu, S., Komatsu, K., Ruangrungsi, N. and Sukrong, S.
TITLE Evaluation of Octaplex PCR for Rapid Differentiation of Six Mucuna plants
JOURNAL Unpublished (2013)
FEATURES Location/Qualifiers
source 1..750
/country="Thailand"
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/PCR_primers="fwd_name: ITS1,
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rev_seq: tcctccgcttattgatatgc"
misc_RNA <1..>750
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181 tgggttggcg ggaggtggtt gcctcgtcct cctcctcccg tcgaactaaa accccggcgc
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361 ctcttgcatc gatgaagaac gtagcgaaat gcgatacttg gtgtgaattg cagaatcccg
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541 aagctgacct cccgtggggc acgactctcg cggctggttg aaaatggagt tcacggttga
601 gaatgccgtg ataaaatggt ggatgagcgt tgctcgagac caatcgcgtg ctactcagtt
661 aattttggac tctttgaccc agatgcgtcc tcggacgctc ccaacgagac ctcaggtcag
721 gcggggctac ccgctgagtt taagcatatc
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5. *Mucuna pruriens* (L.) DC.

ACCESSION AB775137
KEYWORDS .
ORGANISM Mucuna pruriens
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1 (bases 1 to 747)
AUTHORS Sukrong, S. and Wiriyakarun, S.
TITLE Direct Submission
JOURNAL Submitted (04-JAN-2013) to the DDBJ/EMBL/GenBank databases. Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Rd., Bangkok, Patumwan 10330, Thailand
REFERENCE 2
AUTHORS Wiriyakarun, S., Zhu, S., Komatsu, K., Ruangrungsi, N. and Sukrong, S.
TITLE Evaluation of Octaplex PCR for Rapid Differentiation of Six Mucuna plants
JOURNAL Unpublished (2013)
FEATURES Location/Qualifiers
source 1..747
/country="Thailand"
/mol_type="genomic DNA"
/organism="Mucuna pruriens"
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fwd_seq: tccgtaggtgaacctgcgg, rev_name: ITS4,
rev_seq: tcctccgcttattgatatgc"
misc_RNA <1..>747
/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

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181 ctggcgagag gcggtcgccc cgcgcacctc ctctcgccaa aacacaaacc ccggcgcttc
241 gtgcgccaag gaactcgaaa ctgttaagtg caatgttcgc gggcccggag acggcgaccc
301 cgcggacctt gccacgacac acaacataca aaatgactct cggcaacgga tatctcggct
361 cttgcatcga tgaagaacgt agcgaaatgc gatacttggt gtgaattgca gaatcccgtg
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421 aaccatcgag tctttgaacg caagttgcgc ccgaagccat taggccgagg gcacgcctgc
481 ctgggtgtca cacatcgtta ccccaaatgc aaacgcctca cgtgcgtgcg cagggtggat
541 gctgacctcc cgcgagcatc gtctcgtggc tggttgaaaa tcgagtccgc ggccgagctc
601 gtcgcgacaa aatggtggat gagcgatgct cgagaccagt cgcgccggac ccggccaagg
661 tcggactccc cgaccctaca cgcgtccacg gacgctccca acgagacctc aggtcaggcg
721 gggctacccg ctgagtttaa gcatatc
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6. *Mucuna warburgii* Lauterb. & K. Schum.

ACCESSION AB775138
KEYWORDS .
ORGANISM Mucuna warburgii
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Mucuna.
REFERENCE 1 (bases 1 to 756)
AUTHORS Sukrong, S. and Wiriyakarun, S.
TITLE Direct Submission
JOURNAL Submitted (04-JAN-2013) to the DDBJ/EMBL/GenBank databases. Contact: Suchada Sukrong Chulalongkorn University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences; Phyathai Rd., Bangkok, Patumwan 10330, Thailand
REFERENCE 2
AUTHORS Wiriyakarun, S., Zhu, S., Komatsu, K., Ruangrungsi, N. and Sukrong, S.
TITLE Evaluation of Octaplex PCR for Rapid Differentiation of Six Mucuna plants
JOURNAL Unpublished (2013)
FEATURES Location/Qualifiers
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rev_seq: tcctccgcttattgatatgc"
misc_RNA <1..>756
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ORIGIN

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121 ccgggggctag gtcggggctg ttgttgttct ccaacaccta ccccgtcctt cctcgggttg
181 gcgggaggtg gttgctcagc tcttgtgcta tgctcctcct cctgtcgaac taaaaccccg
241 gcgcttcgtg tgccaaggaa tctgaaaatt gttcggtgca attttcgcgg acccggacac
301 ggtgatctcg cggaccttgc cacacgacac acgatacaaa atgactctcg gcaacggata
361 tctcggctct tgcatcgatg aagaacgtag cgaaatgcga tacttggtgt gaattgcaga
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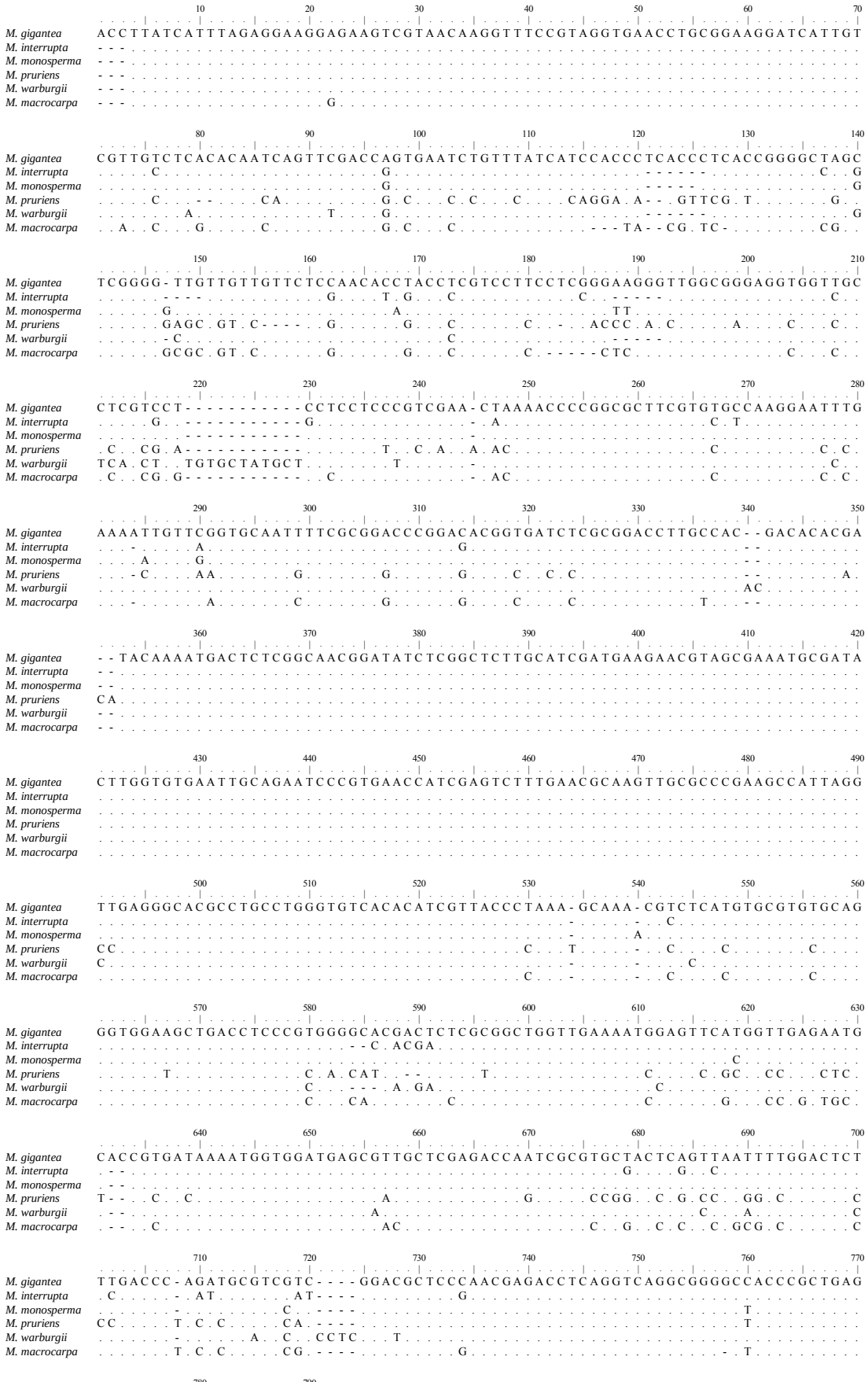
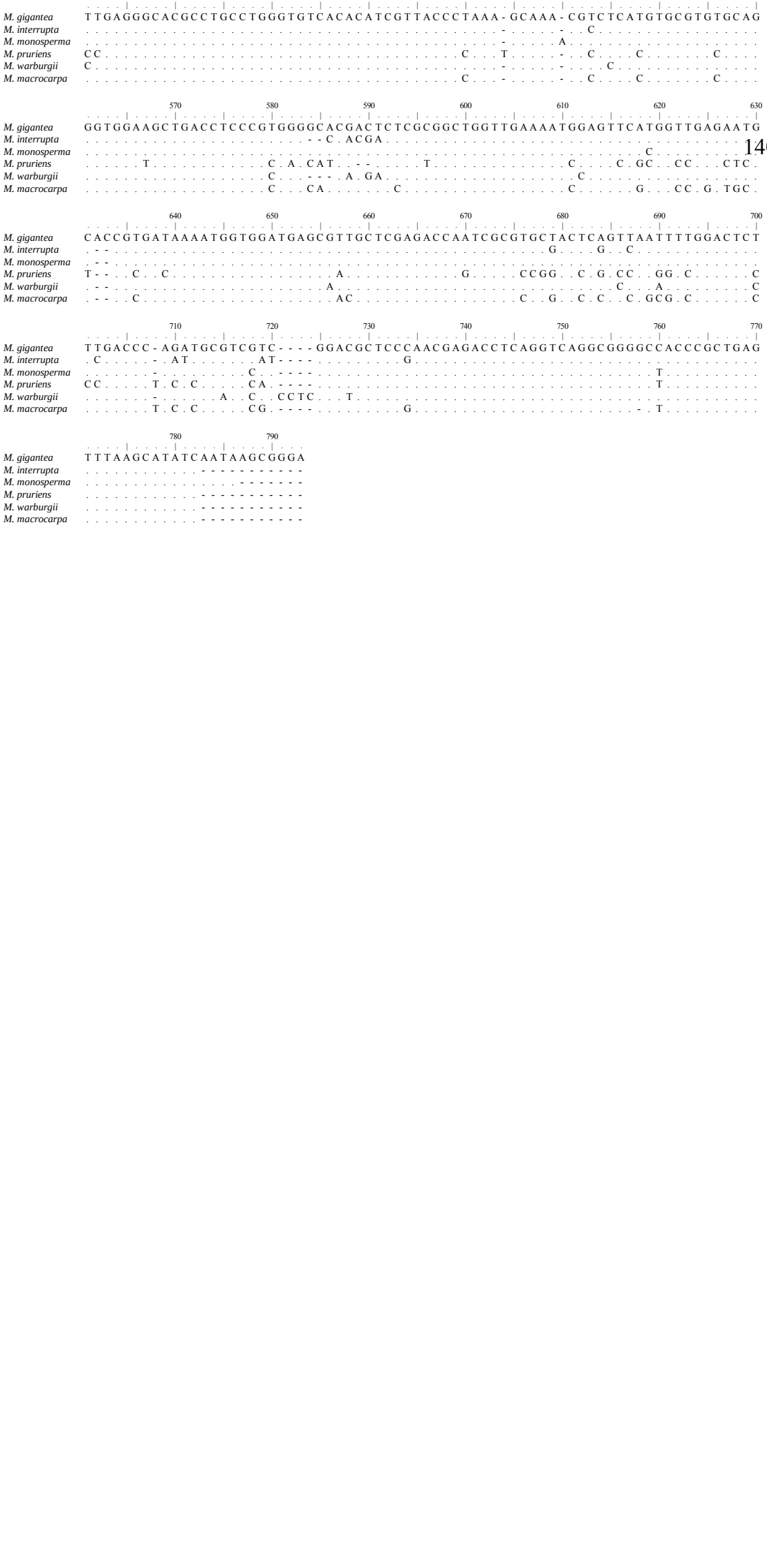
421 atcccgtgaa ccatcgagtc tttgaacgca agttgcgccc gaagccatta ggctgagggc
481 acgcctgcct gggtgtcaca catcgttacc ctaaagcaaa cgtcccatgt gcgtgtgcag
541 ggtggaagct gacctcccgc gggcaagact cgcggctggt tgaaaatgca gttcatggtt
601 gagaatgccg tgataaaatg gtggatgagc attgctcgag accaatcgcg tgctactcag
661 ctaaatttgg actccttgac ccagatgcat cctccctcgg atgctcccaa cgagacctca
721 ggtcaggcgg ggccacccgc tgagtttaag catatc
//
```

APPENDIX C

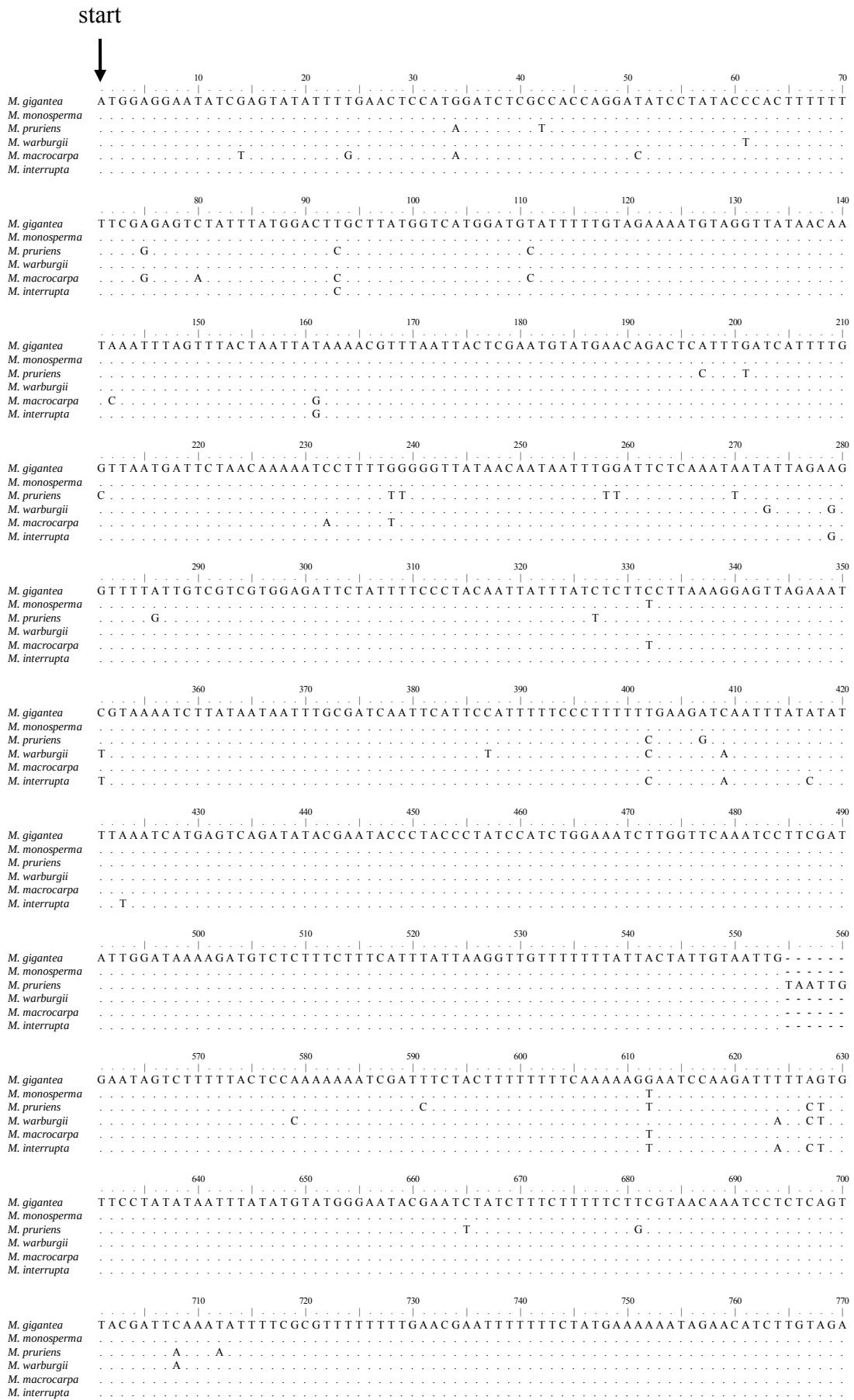
DNA sequence alignments of six *Mucuna* plants

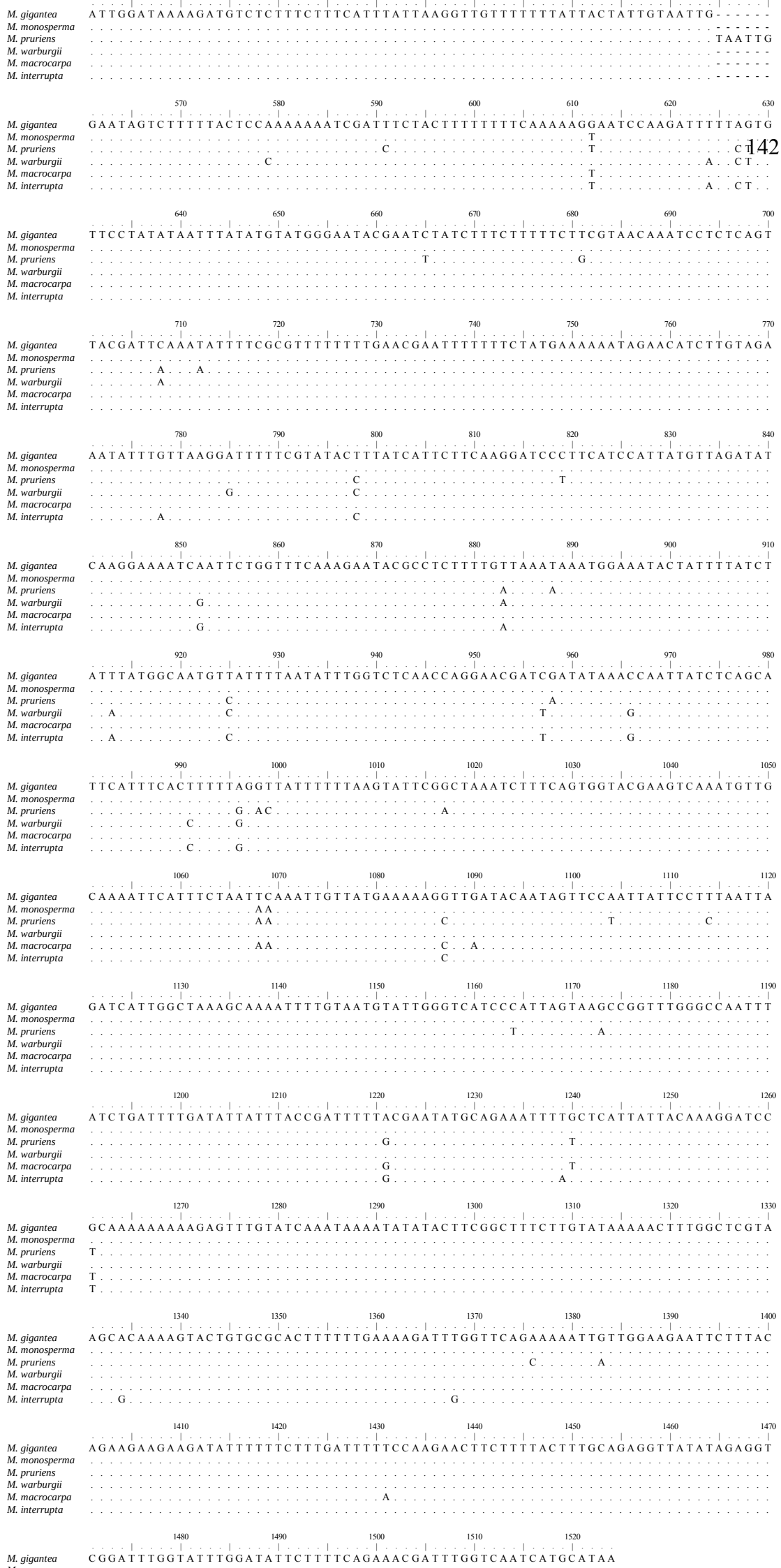
Sequence alignment of ITS region of six *Mucuna* plants. Consensus sequences are indicated with dots. The altered bases indicate the sequence differences.

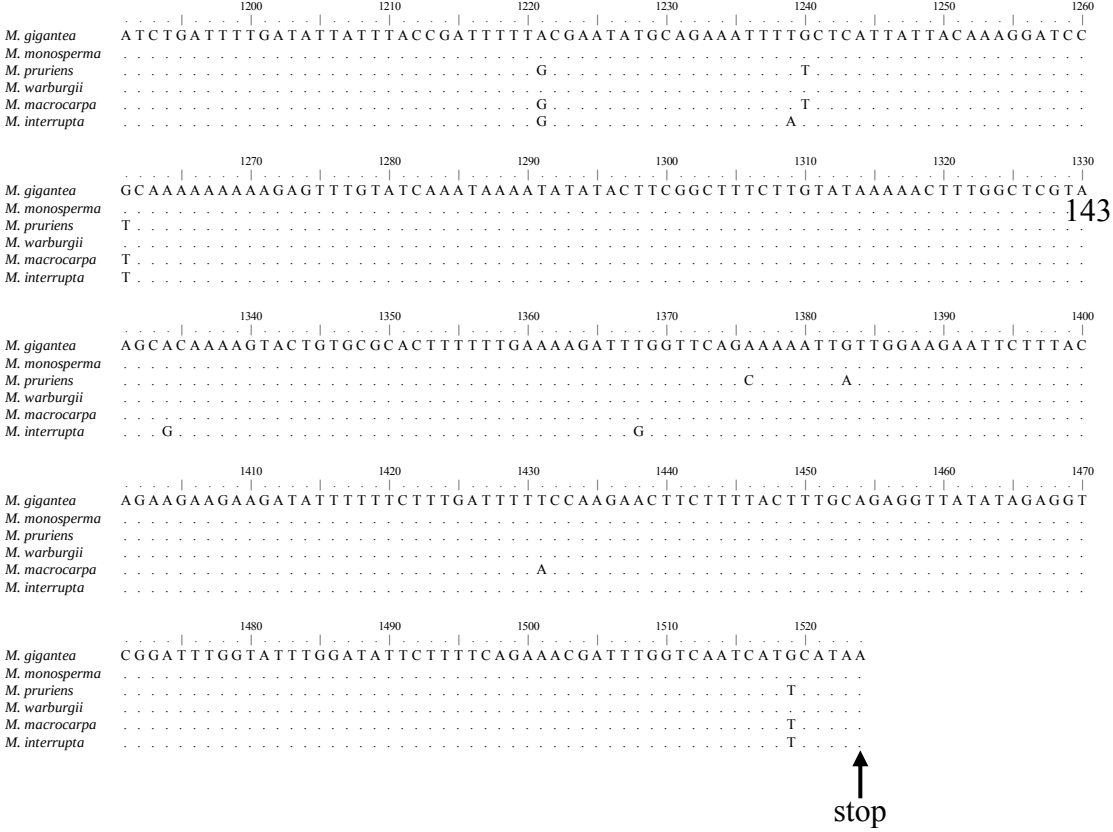



M. gigantea TTGAGGGCACGCCTGCCTGGGTGTCACACATCGTTACCCTAAA-GCAAA-CGTCTCATGTGCGTGTGCAG
M. interrupta ...-...-C...
M. monosperma ...-...A...
M. pruriens CC...C...T...-...C...C...C...
M. warburgii C...-...-...C...
M. macrocarpa ...C...-...-...C...C...C...
570 580 590 600 610 620 630
M. gigantea GGTGGAAGCTGACCTCCCGTGGGGCACGACTCTCGCGGCTGGTTGAAAATGGAGTTCATGGTTGAGAATG
M. interrupta ...---C.ACGA...
M. monosperma ...C...
M. pruriens ...T...C.A.CAT...---...T...C...C.GC..CC...CTC.
M. warburgii ...C...---.A.GA...C...
M. macrocarpa ...C...CA...C...C...G...CC.G.TGC.
640 650 660 670 680 690 700
M. gigantea CACCGTGATAAAATGGTGGATGAGCGTTGCTCGAGACCAATCGCGTGCTACTCAGTTAATTTTGGACTCT
M. interrupta .--...G...G..C...
M. monosperma .--...
M. pruriens T----C..C...A...G.....CCGG..C.G.CC..GG.C...C
M. warburgii .--...A...C...A...C
M. macrocarpa .----C...AC...C..G..C.C..C.GCG.C...C
710 720 730 740 750 760 770
M. gigantea TTGACCC-AGATGCGTCGTC----GGACGCTCCCAACGAGACCTCAGGTCAGGCGGGGCCACCCGCTGAG
M. interrupta .C.....-.AT.....AT----...G...
M. monosperma .....-.....C.....T...
M. pruriens CC.....T.C.C.....CA.....T...
M. warburgii .....-.....A..C..CCTC...T...
M. macrocarpa .....T.C.C.....CG.....G...-.T...
780 790
M. gigantea TTTAAGCATATCAATAAGCGGGA
M. interrupta ...-----------
M. monosperma ...-------
M. pruriens ...-----------
M. warburgii ...-----------
M. macrocarpa ...-----------

Sequence alignment of full length *mat*K gene of six *Mucuna* plants. Consensus sequences are indicated with dots. The altered bases indicate the sequence differences.




M. gigantea
M. monosperma
M. pruriens
M. warburgii
M. macrocarpa
M. interrupta
570 580 590 600 610 620 630
640 650 660 670 680 690 700
710 720 730 740 750 760 770
780 790 800 810 820 830 840
850 860 870 880 890 900 910
920 930 940 950 960 970 980
990 1000 1010 1020 1030 1040 1050
1060 1070 1080 1090 1100 1110 1120
1130 1140 1150 1160 1170 1180 1190
1200 1210 1220 1230 1240 1250 1260
1270 1280 1290 1300 1310 1320 1330
1340 1350 1360 1370 1380 1390 1400
1410 1420 1430 1440 1450 1460 1470
1480 1490 1500 1510 1520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M. gigantea ATCTGATTTTGATATTATTTACCGATTTTTACGAATATGCAGAAATTTTGCTCATTATTACAAAGGATCC
M. monosperma
M. pruriens G T
M. warburgii
M. macrocarpa G T
M. interrupta G A
1270 1280 1290 1300 1310 1320 1330
M. gigantea GCAAAAAAAAAGAGTTTGTATCAAATAAAATATATACTTCGGCTTTCTTGTATAAAAACTTTGGCTCGTA
M. monosperma
M. pruriens T
M. warburgii
M. macrocarpa T
M. interrupta T
1340 1350 1360 1370 1380 1390 1400
M. gigantea AGCACAAAAGTACTGTGCGCACTTTTTTGAAAAGATTTGGTTCAGAAAAATTGTTGGAAGAATTCTTTAC
M. monosperma
M. pruriens C A
M. warburgii
M. macrocarpa
M. interrupta G G
1410 1420 1430 1440 1450 1460 1470
M. gigantea AGAAGAAGAAGATATTTTTTCTTTGATTTTTCCAAGAACTTCTTTTACTTTGCAGAGGTTATATAGAGGT
M. monosperma
M. pruriens
M. warburgii
M. macrocarpa A
M. interrupta
1480 1490 1500 1510 1520
M. gigantea CGGATTTGGTATTTGGATATTCTTTTCAGAAACGATTTGGTCAATCATGCATAA
M. monosperma
M. pruriens T
M. warburgii
M. macrocarpa T
M. interrupta T
stop

VITA

Miss Suchaya Wiriyakarun was born on May 12, 1977 in Ubonratchathani, Thailand. She received her Bachelor's Degree of Science in Biology in 1999 from the Faculty of Sciences, Khon Khean University, Thailand and Master's Degree of Science in Biology in 2003 from the Faculty of Sciences, Khon Khean University, Thailand. She was a recipient of Strategic Scholarships Fellowships Frontier Research Networks under Office of the Higher Education, Thailand since 2009.

Publications

1. Wiriyakarun, S., Yodpetch, W., Komatsu, K., Zhu, S., Ruangrungsi, N., and Sukrong, S. 2012. Discrimination of the Thai rejuvenating herbs *Pueraria candollei* (White Kwao Khruea), *Butea superba* (Red Kwao Khruea), and *Mucuna collettii* (Black Kwao Khruea) using PCR-RFLP. J. Nat. Med. (in press).

Poster presentations

1. Wiriyakarun, S., Yodpetch, W., Ruangrungsi, N., and Sukrong, S. 2012. Discrimination of Thai rejuvenating herbs *Pueraria candollei* (White Kwao Khruea), *Butea superba* (Red Kwao Khruea), and *Mucuna collettii* (Black Kwao Khruea) using PCR-RFLP. CHE-USDC Congress V, September, 14-16, 2012, Pattaya, Chonburi, Thailand.