การวิเคราะห์ลายพิมพ์ดีเอ็นเอของพืชสกุลระย่อมในประเทศไทย

นางสาว สุรัชนีย์ จิตต์แจ้ง

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ปีการศึกษา 2550

DNA FINGERPRINT ANALYSIS OF *RAUVOLFIA* IN THAILAND

Miss Suratchanee Jitjeang

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacognosy

Department of Pharmacognosy
Faculty of Pharmaceutical Sciences
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(DNA FINGERPRINT ANALYSIS OF *RAUVOLFIA* IN THAILAND)
อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. สุชาดา สุขหร่อง,
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พืชสกุลระย่อมเป็นพืชสกุลหนึ่งที่ใช้เป็นยา จัดอยู่ในวงศ์ Apocynaceae ในประเทศไทยมีการรายงานถึงพืชสกุลนี้ 5 ชนิด ได้แก่ *Rauvolfia serpentina, R. cambodiana, R. micrantha, R. sumatrana* และ *R. verticillata* จากข้อมูลการศึกษาพบว่าพืชสกุลนี้มีองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพที่ต่างกัน ตำรายาไทยระบุการใช้พืช *R. serpentina* และ *R. cambodiana* โดยรากของ *R. serpentina* มีสรรพคุณลดความดันโลหิต แก้ไข้ ขับพยาธิ ช่วยให้นอนหลับ และแก้อาการทางจิต ส่วนรากของ *R. cambodiana* มีสรรพคุณใกล้เคียงกับ *R. serpentina* แต่ไม่มีฤทธิ์ลดความดันโลหิต อย่างไรก็ตามพบว่ามีการใช้รากของ *R. cambodiana* ปลอมปนกับรากของ *R. serpentina* ดังนั้นจึงมีความจำเป็นต้องใช้สมุนไพรระย่อมให้ถูกชนิดสำหรับฤทธิ์ลดความดันโลหิต การพิสูจน์เอกลักษณ์ของพืชสมุนไพรจึงมีความจำเป็นอย่างยิ่งที่จะต้องทำเป็นอันดับแรกเพื่อคุณภาพ ความปลอดภัย และประสิทธิผล วัตถุประสงค์ของงานวิจัยครั้งนี้เพื่อวิเคราะห์ลายพิมพ์ดีเอ็นเอโดยการศึกษาลำดับนิวคลีโอไทด์ในส่วน internal transcribed spacer (ITS) ของไรโบโซมอลดีเอ็นเอของพืชสกุลระย่อมในประเทศไทย และนำข้อมูลที่ได้ไปวิเคราะห์ความสัมพันธ์ทางวงศ์วานวิวัฒนาการ ประยุกต์ใช้เป็นเครื่องหมายทางพันธุกรรมชนิดพีซีอาร์-อาร์เอฟแอลพี (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) เพื่อใช้เป็นเครื่องมือในการพิสูจน์เอกลักษณ์ในการจำแนกพืชสกุลระย่อมแต่ละชนิดในประเทศไทยออกจากกัน และนำไปใช้ในการพิสูจน์เครื่องยาสมุนไพรที่ซื้อจากตลาดเครื่องยา จากการวิจัยพบว่าการใช้ลำดับนิวคลีโอไทด์ในส่วน ITS ร่วมกับการใช้เทคนิคพีซีอาร์-อาร์เอฟแอลพีสามารถจำแนกพืชสกุลระย่อมในประเทศไทยออกจากกันได้

ภาควิชา.....เภสัชเวท.....ลายมือชื่อนิสิต.....สุรัชนีย์ จิตต์แจ้ง.....
สาขาวิชา.....เภสัชเวท.....ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา.....2550.....ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4976605233 : MAJOR PHARMACOGNOSY

KEY WORD : RAUVOLFIA/ INTERNAL TRANSCRIBED SPACER OF rDNA (ITS)/ DNA FINGERPRINTING/ PCR-RFLP/ IDENTIFICATION

SURATCHANEE JITJEANG: DNA FINGERPRINT ANALYSIS OF *RAUVOLFIA* IN THAILAND. THESIS PRINCIPAL ADVISOR: ASSIST. PROF. SUCHADA SUKRONG, Ph.D., THESIS COADVISOR : ASSOC. PROF. THATREE PHADUNGCHAROEN, 87 pp.

Rauvolfia is a medicinal genus belonging to family Apocynaceae. There are five *Rauvolfia* species reported in Thailand: *Rauvolfia serpentina*, *R. cambodiana*, *R. micrantha*, *R. sumatrana*, and *R. verticillata*. Chemical constitution and biological activity in *Rauvolfia* species were studied and found that there are differences among them. Root of *R. serpentina* and *R. cambodiana* were reported as medicinal herbs in Thai herbal medicine. Root of *R. serpentina* has been used as antihypertensive, antipyretic, anthelmintic, sedative and palliative for insanity. Root of *R. cambodiana* has been used similar to *R. serpentina*, but it lacks of antihypertensive activity. Since *R. cambodiana* was reported as an adulterant for *R. serpentina*, the precise of authentication of *R. serpentina* is necessary for quality control of the use for antihypertensive purpose. The accurate identification and quality control of the plant material is, therefore, an essential prerequisite for ensuring the quality, safety, and efficacy. The goal of this study was fingerprinting the internal transcribed spacer (ITS) of rDNA of *Rauvolfia* species existing in Thailand. Phylogenetic relationship of them were analyzed. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method was applied as a molecular marker to discriminate *Rauvolfia* species in Thailand. Five crude drugs purchased from crude drug markets were identified in this study. Combination of ITS sequence and PCR-RFLP technique provide an effective and accurate identification of *Rauvolfia* species in Thailand.

Department .....Pharmacognosy..... Student's signature.....Suratchanee Jitjeang.....

Field of study .... Pharmacognosy..... Advisor's signature.....

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LIST OF ABBREVIATIONS

18s gene	a gene encoding for the small ribosomal subunit
26s gene	a gene encoding for the large ribosomal subunit
AFLP	Amplified fragment length polymorphism
AP-PCR	Arbitrarily primed-PCR
ARMS	Amplification refractory mutation system
A, T, G, C	Nucleotides containing the base adenine, thymine, guanine and cytosine, respectively
bp	Base pair
BSU	BioService Unit
°C	Degree Celcius
CAPS	Cleaved amplified polymorphic sequence
CI	Consistency index
cm	Centimeter
cyt b	*Cytochrome b* gene
DALP	Direct amplification of length polymorphism
DAMD	Directed amplification of minisatellite-region DNA
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
ddNTPs	Dideoxynucleotide triphosphates (ddATP, ddTTP, ddGTP, ddCTP)
EDTA	Ethylenediamine tetraacetic acid
HCl	Hydrochloric acid
ITS	Internal transcribed spacer
ITS 1	Internal transcribed spacer 1

ITS 2	Internal transcribed spacer 2
kb	Kilobase
KCl	Potassium chloride
*mat*K gene	Gene encoding for maturase K
$MgCl_2$	Magnesium chloride
ml	Milliliter
mm	Milimeter
nrDNA	Nuclear ribosomal DNA
ng	Nanogram
PAUP	Phylogenetic analysis using parsimony
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction- Restriction fragment length polymorphism
*rbc*L gene	Gene encoding the large subunit of the ribulose bisphosphate carboxylase
RC	Rescaled consistency index
RFLP	Restriction fragment length polymorphism
RI	Retention index
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RP-PCR	Random primed-Polymerase chain reaction
SCAR	Sequence characterized amplified regions
TAE	Tris acetate EDTA
TBR	Tree-bisection-reconnection
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
$tRNA^{Leu}$	Transfer RNA of leucine
*trn*L gene	Gene encoding for $tRNA^{Leu}$
*trn*L- *trn*F spacer	non-coding intergenic spscer between *trnL* and *trn*F in the chloroplast genome

µg	Microgram
µl	Microliter
µM	Micromolar
UV	Ultraviolet
V	Volt

CHAPTER I

INTRODUCTION

Rauvolfia is a genus in the family Apocynaceae consisting about sixty species in the world (Li *et al*., 1995; Santisuk and Larsen, 1999). In Thailand, There are five species: *R. serpentina* (L.) Benth. ex Kurz (ระย่อมน้อย), *R. cambodiana* Pierre ex Pit (ระย่อมหลวง), *R. sumatrana* Jack (ระย่อมตีนเป็ด), *R. micrantha* Hook.f. (ระย่อมใบบาง) and *R. verticillata* (Lour.) Baillon (ระย่อมใหญ่) (Smitinand, 1999).

Rauwolfia is the dried root of *R. serpentina*, a small shrub from India, Pakistan, Burma, Vietnam, Malaysia, Indonesia and Thailand (Dewick, 2002; Claus *et al*.,1970). Other species used in commerce including *R. vomitoria* from Tropical Africa and *R. canescens* from India (Dewick, 2002). Rauwolfia, which includes *R. serpentina*, *R. vomitoria*, and *R. canescens*, has been used in Africa for hundreds of years, and in India for at least 3,000 years. It was used as an antidote to snake-bite, to remove white spots in the eyes, against stomach pains, fever, vomiting, headache and to treat insanity (Dewick, 2002).

In traditional Thai herbal medicine, Roots of *R. serpentina* are used as antipyretic, appetizing agent, antihypertensive, sedative, anthelmintic and palliative for insanity (สุนทรี สิงหบุตรา, 2540; คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล, 2539). Roots of *R. cambodiana* are used as antipyretic, anthelmintic and palliative for insanity (คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล, 2539).

Most of commercial products have been collected from the wild. *R. serpentina* contains a wide range of indole alkaloids, principally reserpine, rescinnamine and deserpidine. Reserpine and deserpidine have been widely used as antihypertensives and mild tranquillizers (Samuelsson, 1992). They act by interfering with catecholamine storage, depleting levels of available neurotransmitters. Riscinnamine is an angiotensin-converting enzyme inhibitor which used as antihypertensive (Fife *et al*., 1960). Prolonged use of pure alkaloids, reserpine in particular, has been shown to lead to severe depression in some patients (Dewick, 2002). Besides, *Rauvolfia* species have

been found to have severe toxicity such as sympathetic nerve stimulation, tremor and depressant (Duke, 2002).

Rauwolfia roots are difficult to differentiate by visual examination. During the period of great demand for roots of *R. serpentina*, *R. cambodiana* was reported as an adulterant (Boonchuay and Court, 1973). However, they have different chemical constitution such as ajmalicine, an alkaloid used for the treatment of cardiac arrhythmia, which was found in *R. serpentina*, but not in *R. cambodiana* (Arthur and Loo, 1966; Boonchuay and Court, 1973; Evans, 2002; Itoh *et al*., 2005). In addition, *Rauvolfia* species have wide distribution, being found in several geographical areas of the world, so confusion of their synonyms may arise.

Boonchuay and Court, 1973 was reported that….

> " ….there is concerning the identity of synonymy of S.E. Asian *Rouvolfia* species. Malaysian botanists are confused *R. serpentina* with *R. verticillata,* and *R. verticillata* would appear to be a widespread species with consequent variability in form….".

The accurate identification and quality control of the plant material is, therefore, an essential prerequisite for ensuring the quality, safety, and efficacy of rauwolfia roots and other herbal medicines. In addition, authentication of herbal plants is also an extremely important prerequisite for chemical and pharmacological investigations of herbal medicines. General approaches to herbal identification depend on morphological, anatomical, and chemical analyses, but these characteristics are often affected by environmental and/or developmental factors during plant growth (Li *et al*., 1994). Nevertheless, the use of chromatographic techniques and marker compounds to standardize botanical preparations has limitations because of the varied sources and chemical complexity of such preparations. In particular, many extrinsic factors such as methods of cultivation, harvesting, drying and storing may affect the ultimate chemical profile of a given herb. Moreover since many associated agents and bonding agents are mixed with herbal extracts at 90-100 ºC for 2-4 hours, it is difficult to identify the plants involved in the final products (extracts, capsules, liquids) by TLC (Shim *et al*.,

2005). On the other hand, medicinal plants are processed for use as crude drugs. Through these processes, some morphological and anatomical characteristics and some chemical constituents are changed. Therefore, it is difficult to determine the botanical origins of crude drugs through anatomical and chemotaxonomical studies (Joshi *et al*., 2004).

DNA based polymorphism assay may offer an alternative method to identify herbal medicines. The analysis of DNA has the advantages of being applicable to all parts of plants and not being affected by conditions of culture (Shim *et al*., 2003). Furthermore, a number of recent studies have indicated that DNA markers are ideal tools for elucidating the molecular evolution and phylogeny of the species concerned, as well as for identifying crude herbal materials (Xue *et al*., 2006). Medicinal products whose origins are often controversial, such as *Rheum* (Yang *et al*., 2004), *Dendrobium* (Xu *et al*., 2006), Licorice (Kondo *et al*., 2007), *Bupleurum* (Shim *et al*., 2005), *Mitragyna* (Sukrong *et al*., 2007), *Derris* (Sukrong *et al*., 2005), etc. have been intensively studied and other herbal medicines have also been analyzed by DNA markers.

DNA sequencing has become one of the most utilized approaches for inferring taxonomic and phylogenetic relationships of animals and plants, and can be applied for identification of organisms (Sahin *et al*., 2007). Ribosomal RNA gene is an attractive target for molecular analysis, because a large number of copies of these genes are present in the plant genome and the regions encoding 18S-, 5.8S- and 26S rRNA are highly conserved, whereas two internal transcribed spacers (ITS 1 and ITS 2) between the ribosomal RNA genes are variable and useful as possible sources of polymorphisms for plant identification (Sahin *et al*., 2007; Baldwin *et al*., 1995; Chou and Tsai, 1999; Andreev *et al*., 2005).

There are not much data of *Rauvolfia* sequences published in National Center for Biotechnology Information (NCBI or GenBank). Those are the sequences of the ITS region of *R. serpentina*, *trn*L gene of *R. serpentina* and *R. balansae*, *trn*F-*trn*L intergenic spacer of *R. serpentina* and *R. balansae*, and *rbc*L and *mat*K gene of *R. manii*.

To date, there is only one sequence of ITS region of *R. serpentina* from Japan reported in Genbank database. There is no study which determines the ITS region sequence of *Rauvolfia* species existing in Thailand. Thus, the ITS regions of these plants

were examined in this study. Obtained results would be used for their classification and phylogenetic studies, as well as characterization of crude drug. Based on the differences among the sequences, the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis was performed in order to develop a more convenient and efficient identification method. We applied the PCR-RFLP method to the crude drug to show that the DNA-based assay is reliable for identification of them.

CHAPTER II

LITERATURE REVIEW

1. Botanical data

Rauvolfia spp. are shurbs or small trees. Leaves in whorls of 3-5, rarely opposite; usually with glands in axils. Inflorescence a terminal and/or axillary cyme or whorl of cymes. Sepals without colleters inside or only at the sepal corners. Corolla lobes overlapping to the left in bud; mature corolla salverform; lobe subsymmetrical, ovate or elliptic, apex obtuse to rounded. Stamens free from the pistil head; completely included in the corolla tube; filaments short and narrow; anthers ovate to lanceolate, base cordate, apex acute to obtuse, fertile entire length. Disk annular, shorter than the ovary. Ovary syncarpous and bilocular, of 2 carpels connate at base or of 2 free carpels, in each case united into a common style, glabrous; 2 ovules per carpel; style filiform with a collared style head. Fruit a drupe; of 2 separate endocarps within a single fleshy mesocarp, of 2 parts connate at the base or of 2 separate parts, sometimes with one not developing. Seeds ovoid, flattened. (Santisuk and Larsen, 1999).

In Thailand, there are five species; *R. cambodiana*, *R. micrantha*, *R. serpentina*, *R. sumatrana*, and *R. verticillata* (Santisuk and Larsen, 1999).

Key to the species (Santisuk and Larsen, 1999)

1. Leaf blades coriaceous; secondary veins ± straight, intramarginal nerve present; ovary syncarpous *R. sumatrana*

1. Leaf blades papery or, rarely, subcoriaceous; secondary veins arcuate ascending, no intramarinal nerve; ovary of 2 separate carpels or connate only at base

2. Corolla tube < 4 mm long; fruits connate at base, each half mango shaped *R. micrantha*

2. Corolla tube > 8 mm long; fruits connate or free at base; each half ± ovoid

3. Carpels connate at base; in florescences solitary, terminal, flowers clustered; fruits connate for up to half length *R. serpentina*

3. Carpels free from each other; inflorescences in a whorl or solitary and then axillary or terminal, flowers lax; fruits paired, free or solitary

4. Inflorescence in a whorl; peduncles not robust *R. verticillata*

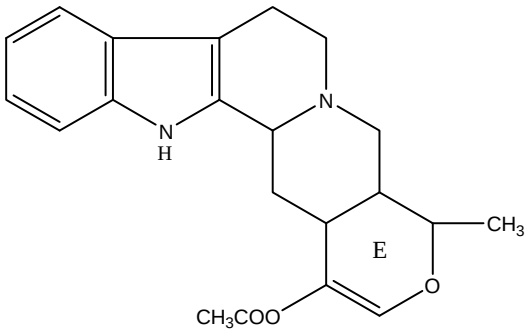
4. Inflorescence solitary, axillary or, more rarely, terminal; peduncles robust *R. cambodiana*

Table 1. Comparison of morphological characteristics and medicinal uses among *Rauvolfia* spp. existing in Thailand.

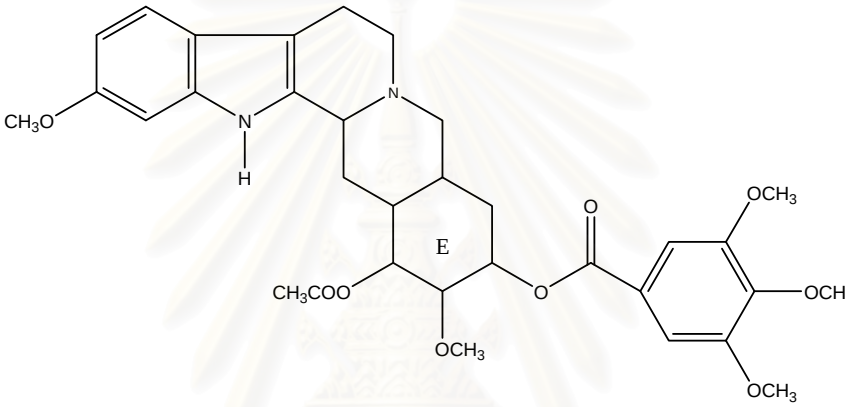
	R. cambodiana	*R. micrantha*	*R. serpentina*	*R. sumatrana*	*R. verticillata*
Habitat	Shrub to 2 m. tall	Small shrub	Low growing shrub	Tree to 10 m.	Shrub or small tree
Leaf	Blade papery to subcoriaceous, narrowly elliptic to oblanceolate, secondary veins arcuate ascending	Blade papery, elliptic, secondary veins arcuate ascending	Blade papery, secondary veins arcuate ascending	Blade coriaceous, secondary vein ± straight, anastomosing into an intramarginal nerve	Blade papery, secondary veins arcuate ascending
Inflorescence	Solitary, axillary or terminal; robust	In a whorl	Usually solitary, terminal	In a whorl, robust	In a axillary or terminal, not robust
Corolla	Tube reddish or purplish, lobe white	White	White, reddish,pinkish or purplish	White	White or white and reddish
Fruit	Paired, free from each other, ovoid	Paired, connate at base; each half mango shaped	Paired, connate at base; each half ovid	2 hard separate endocarps surrounded by single fleshy mesocarp; globose or indented on top	Paired, free from each other, or solitary; ovoid
Vernacular	Khayom luang (ขะย่อมหลวง), khayom tin ma (ขะย่อมตีนหมา) , nang yaem (นางแย้ม), rayom (ระย่อม)	Rayom bai bang (ระย่อมใบบาง)	Ko-me (กอเหม่), khemdaeng (เข็มแดง), tum khlan (ตูมคลาน), ma-ong-thee (มะโอ่งที), sa-mo-u (สะมออู), rayom (ระย่อม)	Ra yom tinpet (ระย่อมตีนเป็ด)	Chaek (แฉก), chipuk (จี้ปุก), phut noi (พุดน้อย), ya kae rhak khom (ยาแก้ฮากขม)
Thai medicinal use	Root used in preparing a febrifuge, for sedative and appetizing agent	N/A	Root used in preparing a febrifuge, for sedative, decrese blood pressure, anthelmintic,appetizing agent	Root used as antidote to poison in Indonesia and a decoction of the bark is taken to relieve malaria in the Philippines.	N/A
Reference	Santisuk and Larsen, 1999	Santisuk and Larsen, 1999.	Santisuk and Larsen. 1999., Li *et al*., 1995.	Subhadhirasakul *et al*., 1994., Santisuk and Larsen. 1999.	Santisuk and Larsen, 1999., Li *et al*., 1995.

2. Chemical constitutions

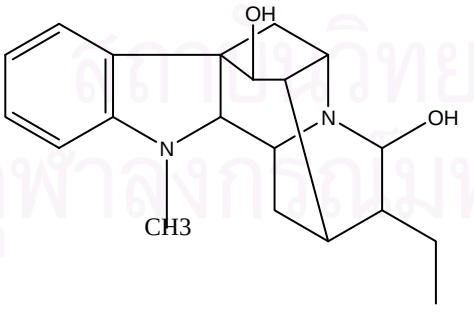
There are three series of alkaloids which have been reported in *Rauvolfia* spp.: (1) tertiary indole alkaloids, separated into two group following structures; heterocyclic ring E and carbocyclic ring E, (2) indoline alkaloids, and (3) anhydronium alkaloids (Figure 2.1) (Claus *et al*., 1970). The principal alkaloids, reserpine, rescinnamine, and deserpidine, are tertiary indole alkaloids that have a carbocyclic structure in ring E and are of the corynane type. Other tertiary indole alkaloids exhibit a heterocyclic structure in ring E: δ - yohimbine (identical to ajmalicine) tetrahydroserpentine, and raubasine and reserpiline. Ajmaline, isoajmaline, rauwolfinine, and others are listed as indoline alkaloids; however, these bases do not have a tranquilizing action. Serpentine, serpentinine, and alstonine are classed as anhydronium alkaloids. The latter type is not considered of practical therapeutic importance. From the 25 or more species of *Rauvolfia* investigated, at least 50 alkaloids have been reported (Robbers *et al*., 1996; Schlittler, 1957).



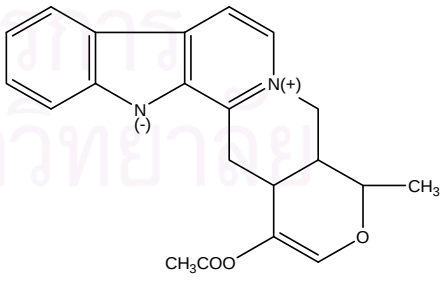
(A) heterocyclic ring E : Ajmalicine



(B) carbocyclic ring E : Reserpine



(C) indoline alkaloid : Ajmaline



(D) anhydronium alkaloid : Serpentine

Figure 2.1 Basic structure of *Rauvolfia* alkaloids. (A,B): tertiary indole alkaloids, (C): indoline alkaloid, (D): anhydronium alkaloid

Table 2. The chemistry of *Rauvolfia* alkaloids.

Chemical constitutions	*R. cambodiana*	*R. micrantha*	*R. serpentina*	*R. sumatrana*	*R. verticillata*
Total alkaloid contents	1.4%	N/A	0.7-2.4%	N/A	0.74 %
(1) Tertiary indole alkaloids					
Heterocyclic ring E	Reserpiline, isoreserpiline, aricine	Ajmalicine, aricine, reserpiline	Ajmalicine, reserpinine, reserpiline	N/A	Ajmalicine
Carbocyclic ring E	Reserpine	Reserpine, sarpagine	Reserpine, rescinnamine, deserpidine, yohimbic acid ,isorauhimbinic acid		Reserpine, yohimbine
(2) Indoline alkaoids	Ajmaline	N/A	Ajmaline, isoajmaline, rauwolfinine	N/A	Ajmaline
(3) Anhydronium alkaloids	N/A	Serpentine	Serpentine, serpentinine, alstonine	N/A	Serpentine
Reference	Boonchuay and Court, 1976.	Schlittler, 1957, Boonchuay and Court, 1976.	Evans, 2002, Schlittler, 1957, Itoh *et al*., 2005.	Subhadhirasakul *et al*., 1994.	Arthur and Loo, 1966, Boonchuay and Court,1976.

3. Type of molecular marker methods

Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphisms. In terms of the mechanisms involved, DNA methods can be classified into three types, namely hybridization-based method, PCR-based method and sequencing-based method (Yip *et al*., 2007, Joshi *et al*., 2004).

3.1 Hybridization-based method

Restriction Fragment Length Polymorphisms (RFLP) is the most widely used hybridization-based molecular marker (Semagn *et al*., 2006). The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. On an agarose gel, RFLP can be visualized using radiolabeled complementary DNA sequences. Polymorphisms are analyzed after hybridization by observing present or absent bands (Joshi *et al*., 2004). The strength points of RFLP markers are high reproducibility, codominant inheritance, no sequence information required, and relatively easy to score due to large size difference between fragments (Semagn *et al*., 2006). There are, however, some problems with the RFLP method of DNA fingerprinting. Firstly, the results do not specifically indicate the chance of a match between two organisms. Secondly, the process involves a lot of money and labor, which not many laboratories can afford (Vasudevan, 2007).

3.2 PCR-based method

PCR-based methods use amplification of particular DNA sequences or loci, with the oligonucleotide primers and the thermostable DNA polymerase enzymes (Joshi *et al*., 2004). PCR-based methods include sequence characterized amplified regions (SCAR), amplification refractory mutation system (ARMS), simple sequence repeat (SSR) analysis, PCR-restriction fragment length polymorphism (PCR-RFLP), random primed PCR (RP-PCR), direct amplification of length polymorphism (DALP), inter-simple

sequence repeat (ISSR), amplified fragment length polymorphism (AFLP) and directed amplification of minisatellite-region DNA (DAMD) (Yip *et al*., 2007).

PCR-RFLP is widely use as a molecular marker. PCR-RFLP is a combination of the PCR and RFLP (Yip *et al*., 2007). Another name of this method is cleaved amplified polymorphic sequence (CAPS) (Maeda *et al*., 1990). This technique involves amplification of a target DNA through PCR, followed by digesting with restriction enzyme (or restriction endonuclease). The distance between the locations digested by restriction enzymes (the restriction sites) varies between individuals so the length of the fragments varies, and the digested amplification products may reveal polymorphisms after separation on agarose gel (Figure 2.1). This can be used to genetically tell individuals apart. It can also show the genetic relationship between individuals. It is also used to determine relationships among and between species. This method is more reproducible than random priming methods, but it is limited by the degree of polymorphism among individuals within a species. The loss of restriction sites involved with degraded DNA, creation or deletion of restriction sites due to intra-specific variation (Lockley and Bardsley, 2000). The presence of enzyme inhibitors may lead to incomplete digestion (Yip *et al*., 2007).

Restriction enzymes are enzymes isolated from bacteria that recognize specific sequences in DNA and then cut the DNA to produce fragments, called restriction fragments. Once it encounters its specific recognition sequence, generally 4 to 6 nucleotides long, the enzyme will bond to the DNA molecule and makes one cut in each of the two sugar-phosphate backbones of the double helix. Once the cuts have been made, the DNA molecule will break into fragments. A restriction enzyme always cuts DNA at its recognition sequence, regardless of whether the DNA is from a virus, a bacterium, a plant or an animal (Avise, 2004).

Several studies used PCR-RFLP analysis for investigation of many plants. For instance, Sukrong *et al*. (2007) used PCR-RFLP for the differentiation of *Mitragyna speciosa*, a narcotic plant, from the other *Mitragyna* species existing in Thailand. Guo *et al*. (2006) applied PCR-RFLP analysis for identification of Ephedra herbs obtained in the Chinese market. Yang *et al*. (2004) developed PCR-RFLP analysis for correct identification of herbal drugs and plants of *Rheum* species. *Lum et al*. (2005) used PCR-

RFLP for identification of botanicals, alfalfa and red clover, and potential contaminants. Wang *et al.* (2007) used PCR-RFLP analysis to differentiate Bulb of Fritillaria from other species of Bulb of Fritillaria antitussive herb in China. Liu *et al*. (2007) developed PCR-RFLP a rapid and reliable method to accurately identify hybrids of *Leucadendron*.

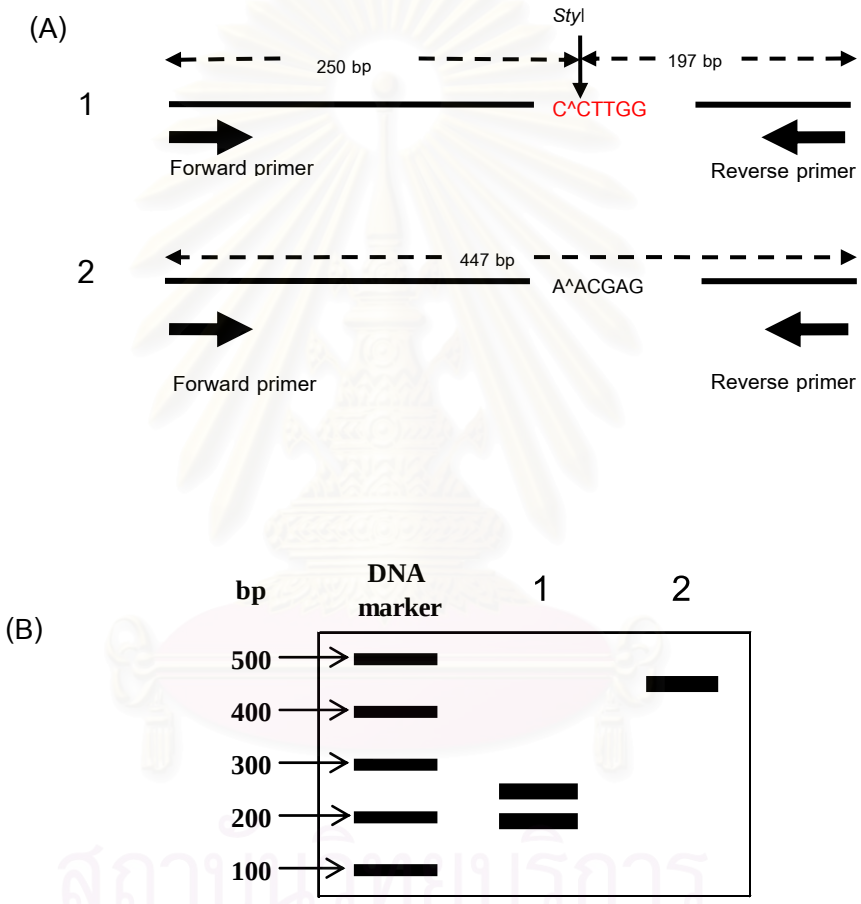


Figure 2.2 Principle of PCR-RFLP (A) Restriction site of *Sty*I is C^CWWGG (when w means nucleotide A or T). When the enzyme encounters this sequence, it cleaves each backbone between the C and the adjacent C base. (B) Once the cuts have been made and detected by agarose gel. Lane 1 indicated fragment digested with *Sty*I produced two restriction bands at 197 bp and 250 bp. Lane 2 indicated fragment undigested with *Sty*I at 447 bp.

3.3 Sequencing-based method

The DNA sequencing is one of the most techniques used for studying phylogenetic relationships among different species. DNA sequencing used to acess variation due to transversions, transitions, insertions or deletetions. Sequencing DNA has become a routine task in the molecular biology laboratory. Two basic strategies of DNA sequencing were chemical degradation method of Maxam and Gilbert and the chain termination method described by Sanger *et al*., 1977.

Nowadays, the sequencing method developed by Fred Sanger forms the basis of automated "cycle" sequencing reactions. DNA sequencing reaction is like the PCR reaction for replicating DNA. The reaction mix contains template DNA, primers, DNA polymerase, four nucleotides (dGTP, dCTP, dATP and dTTP). In addition, a second type of nucleotide called dideoxynucleotide, are added in the reaction mix. These dideoxynucleotide are labeled with fluorescent dye and can be recognized by DNA sequencer. To start the reaction, the mixture is heated until the two stands of DNA separated. Then the primer sticks to its intended location and DNA polymerase starts elongating the primer. If allowed to go to completion, a new strand of DNA would be the result. The enzyme makes no diference between dNTPs or ddNTPs. When a ddNTP is included, the synthesis stops. Because billions of DNA molecules are present in the tube, the stand can be terminated at any position, so diferrent lengths of DNA stands are emerged. Then, the reaction is transferred to polyacrylamide gel. The gel is placed into a DNA sequencer for electrophoresis and analysis. The fragments migrate according to size, and each is detected as it passes a laser beam at the bottom of gel. Each dideoxynucleotide emits colored light of a characteristic wavelength and is recorded as a colored band on a simulated gel image (Mount, 2000; Weising *et al*., 2005).

DNA sequencing provides highly robust, reproducible, and informative data set, and can be adapted to different levels of discriminatory potential by choosing appropriate genomic target regions. However, DNA sequencing can be prohibitively tedious and expensive when very large numbers of individuals have to be assayed (e.g., in population genetics, phylogeography, and marker-assisted plant breeding program)

(Weising *et al*., 2005). Different genes or parts of the genome might evolve at different rates. The selection of genes or any parts of genome depends on the taxonomic levels. (Figure 2.3)

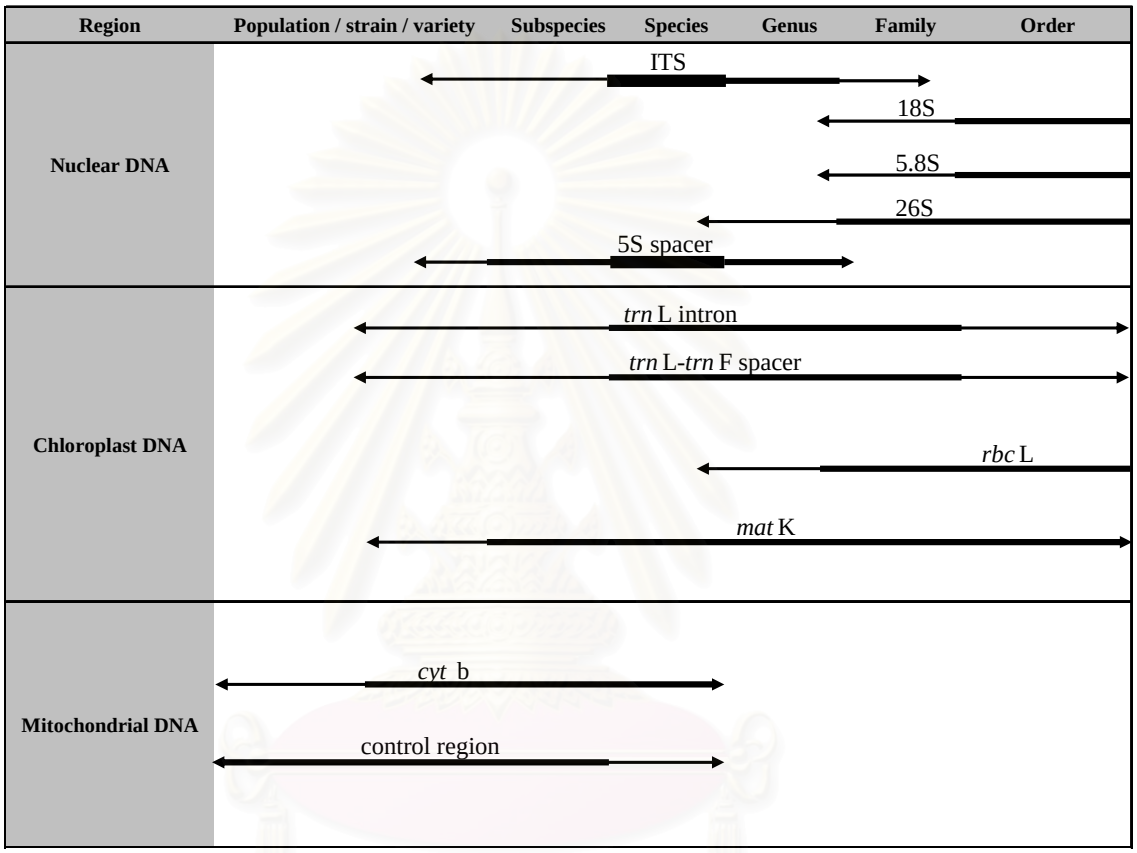


Figure 2.3 Comparison of various regions for use on various taxonomic levels. The thickness of the lines represents how frequently the regions are used for identification at the levels (Adapted from Yip *el al*., 2007).

4. The ITS region of nuclear ribosomal DNA

The internal transcribed spacer (ITS) region of 18S – 26S nuclear ribosomal DNA (nrDNA) has proven to be a useful source of characters for phylogenetic studies (Baldwin *et al*., 1995, Jiang *et al*., 2006, Alvarez and Wendel., 2003). This region includes three components as defined here (Figure 2.4): the 5.8S subunit, an evolutionarily highly conserved sequence, two spacers designated ITS 1 and ITS 2.

Several general features of the ITS region promote its use for phylogenetic analysis. First, along with the other components of the nrDNA multigene family, the ITS region highly repeat unit is present in up to many thousands of copies arranged in tandem repeats at a chromosomal locus or at multiple loci (Ding *et al*., 2002, Sahin *et al*., 2007). This high copy number promotes detection, amplification, cloning, and sequencing of nrDNA (Baldwin *et al*., 1995). Second, the small size of the ITS region (500-700 bp in angiosperm and approximately 1500-3700 bp in some gymnosperms) and the presence of highly conserved sequences flanking each of the two spacers make this region easy to amplify using universal primers design by White *et al*., 1990 (Bobola *et al*., 1992, Germano and Klein, 1999, Maggini *et al*., 2000). The presence of highly conserved sequences flanking each of the two specers make this region easy to amplify (Ding *et al*., 2002, Yang *et al*., 2007), even from herbarium material. (Baldwin *et al*., 1995; Sahin *et al*., 2007).

Well-resolved generic and specific-level phylogenies have been obtained using ITS regions in many plant families such as Asteraceae (Kim and Jansen, 1994), Apiaceae (Downie *et al*., 1994), Poaceae (Kellogg *et al*., 1994), Apocynaceae (Sper-Whitis *et al*., 1992), and Capparaceae (Hahn *et al*., 1994).

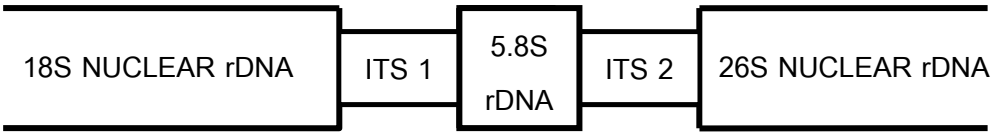


Figure 2.4 Organization of the ITS region in plants. The nrDNA units, separated by intergenic spacers, consist of the 18S, 5.8S and 26S coding regions. ITS 1 is located between 18S and 5.8S coding regions, and the ITS 2 between 5.8S and 26S coding regions.

5. Sequence Alignment

DNA sequence data is converted to characters and used in phylogenetic analyses. First, the sequences of a given length of DNA are aligned, in which homologous nucleotide positions (e.g., corresponding to the same codon position of a given gene) are arranged in corresponding column (Figure 2.5). For some genes that are relatively conserved, alignment is straightforward. For other genes or DNA segments, some taxa may have one or more additions, deletions, inversions or translocations relative to other taxa. The occurrence of these mutations, and/or the occurrence of considerable homoplasy among taxa, can make alignment of DNA sequence difficult. In addition, multiple copies of a gene can make homology assessment difficult. Various computer algorithms can be used to automatically align sequences of the taxa, but these have assumptions that must be carefully assessed. Most web-based tools such as Bioedit (www.mbio.ncsu.edu/BioEdit/page2.html) and ClustalX 2.0 program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) allow a number of input and output formats, such as FASTA format and GenBank format (Mount, 2000).

Figure 2.5 Example of the alignment of sequences by Bioedit program (www.mbio.ncsu.edu/BioEdit/page2.html). Nucleotide in same color in the column mean that they have same nucleotide sequence. Deletions of nucleotide are present in gap (-).

CHAPTER III

SEQUENCE ANALYSIS OF ITS REGIONS OF *RAUVOLFIA* SPECIES IN THAILAND

1. Materials

Fresh plants of four *Rauvolfia* species were carefully identified by Prof. Thatree Phadungjaroen and Prof. Suchada sukrong according to the definition of Flora of Thailand. The four species are: *R. cambodiana, R. serpentina, R. sumatrana* and *R. verticillata*. Herbarium specimens of *R. micrantha* and *R. cabodiana* were obtained from The Florest Herbarium, Bangkok, Thailand. The plant materials used in this study are summarized in Table 3. The voucher specimens are deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The pictures of all specimens are shown in Appendix A.

Table 3. Plant materials used in this study and their GenBank accession numbers of ITS region sequences.

Species	Code no.	Locality of voucher (province)	Date of collection	Voucher no.	GenBank accession no.
Rauvolfia cambodiana Pierre ex Pit.	RCb02	Saraburi	2006.10	TH100601	AB376674
	RCb14	Chiangmai	2007.01	TH010701	-
	RCb19*	Phetchaboon	1996	SN004821	-
	RCb20*	Chinagrai	1998	SN004827	-
	RCb21*	Nakhonratchasima	1998	SN004828	-
R. micrantha Hook.f.	RMc22*	Prachuabkerekhun	2002	SN144043	AB376677
	RMc23*	Petchaburi	2002	SN144045	-
R. serpentina (L.) Benth. Ex Kurz.	RSp05	Nakornpathom	2006.12	TH120601	AB376673
	RSp06	Songkhla	2006.10	SJ100601	-
	RSp07	Krabi	2006.10	SJ100602	-
	RSp08	Kanchanaburi	2006.03	SJ030601	-
	RSp09	Songkhla	2006.12	SS120601	-
	RSp10	Rayong	2007.05	SS050701	-
	RSp11	Rayong	2007.05	SS050702	-
	RSp17	Kanchanaburi	2006.03	SJ030602	-
	RSp29	Uttaradit	2007.11	TH110701	-
R. sumatrana Jack	RSm12	Nakornpathom	2006.12	TH120602	-
	RSm 13	Songkhla	2006.12	SS120602	AB376676
	RSm 18	Chachoengsao	2007.07	SJ070701	-
R. verticillata (Lour.) Baillon	RVt16	Bangkok	2007.11	SJ110701	-
	RVt30	Kanchanaburi	2005.03	TH030501	AB376675

* Herbarium specimens from The Forest Herbarium, Bangkok, Thailand.

2. Methods

Total DNA Extraction

Fresh or dried leaves of each sample were ground under liquid nitrogen to a fine powder using a mortar and pestle. Total DNA was extracted using a DNeasy® Plant Mini Kit (QIAGEN, Germany), following the manufacturer's protocol. For herbarium specimens, samples were rinsed with PBS-EDTA solution. Extraction was performed according to the QIAGEN protocol with a small modification. For lysis of the cells, a longer incubation time (30 min) and 450 µL (instead of 400 µL) of AP1 buffer were used to increase the final DNA concentration, 50 µL (in stead of 100 µL) of molecular water (in stead of 100 µL) and a longer elution time were employed. Finally, the total of DNA solution was purified by a Geneclean® II Kit (QBiogene Inc., U.S.A.). Total genomic DNA analysis was performed on 0.8 % agarose gel electrophoresis stained by ethidium bromide and visualized under UV light to determine quality and quantity. A Lambda DNA-*Hind* III Digest (New England BioLabs Inc., U.S.A.) was used as standard molecular size. The extracted DNA was kept at -20 °C for further use as template in PCR amplification.

Amplification of ITS Region

A pair of primers flanking ITS region, ITS1 and ITS4, was used for amplification of ITS region (White *et al*., 1990). For herbarium specimens, it was difficult to amplify the whole ITS region, so nested PCR was performed. In such cases, internal primer Rau-F01 and stmn-5S-R annealing to 5'-end and 3'-end, respectively, of 5.8S rRNA gene were used for nested PCR. Annealing positions of these PCR primers were shown in Figure 3.1 and detail of these primers are presented in Table 4.

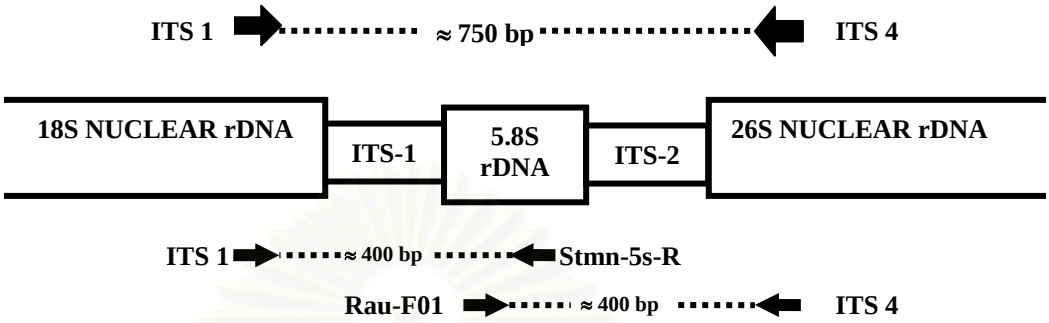


Figure 3.1 Structure of ITS region and localization of primers used for the amplification and sequencing of the ITS region.

Table 4 PCR amplification primers and sequencing primers of ITS region used in this study

Primer name	Primer sequence (5′ to 3′)	Direction
ITS1	GTC CAC TGA ACC TTA TCA TTT AG	forward
ITS4	TCC TCC GCT TAT TGA TAT GC	reverse
Rau-F01	TCG CAT CGA TGA AGA ACG TA	forward
Stmn-5s-R	GAG TTT TTG AAC GCA AGT TGC G GG	reward

PCR amplification was carried out in a 50 µl reaction mixture consisting of 50-100 ng of total DNA as a template, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.25 µM of each primer, and 2.5 U of Taq Polymerase (Promega, U.S.A.). PCR amplification was carried out in PCR Thermocycler (Eppendorf Mastercycler Personal, Eppendorf North America Inc., U.S.A.). The PCR cycling program started with an initial denaturation step at 95 °C for 3 minutes to ensure the complete separation of the DNA strands, 29 cycles of denaturation at 95 °C for 40 seconds, annealing at 55 °C for 40 seconds and elongation at 72 °C for 1 minutes 30 second, and then final elongation at 72 °C for 5 minutes to ensure that all amplicons are fully extended, then held at 4 ºC.

Quantitation and Qualitation of DNA

Quantitation and qualitation of DNA based on the UV-induced fluorescence emitted by ethidium bromide-DNA complexes was used in this study. The PCR products were run on 0.8 % agarose gel in 1XTAE buffer (Bio-Rad Laboratories, U.S.A.). The gel was prepared by adding 0.4 g of agarose to 50 ml of 1XTAE buffer (0.04 M Tris-acetate, and 1 mM EDTA pH 8.0). Agarose was solubilized by heating in a microwave oven and then allowed to cool to 60 ºC before pouring gel into plastic gel tray with the preset locations for the slots forming combs for casting the gel. After the gel was solid, the comb was carefully removed, and the gel was inserted into an electrophoresis apparatus filled with 1XTAE buffer. DNA samples were electrophoresed at 90 volts. The gel was stained with ethidium bromide solution for 15 minutes and destained in deionized water for 5 minutes. DNA was visualized under UV light and photographed using Quantity One 1-D Analysis software, Gel Doc™ XR System PC/Mac (Bio-Rad Laboratories, U.S.A.). A 1 kb plus DNA ladder (Invitrogen Corp., U. S. A.) was used as standard molecular size.

PCR Product Purification

PCR products were purified using a Qiaquick PCR Purification Kit (QIAGEN, Germany) following the manufacturer's protocol.

Nucleotide Sequencing

Nucleotide sequences of purified PCR products were determined by BioService Unit (BSU), National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. Primer for sequencing in the forward direction was ITS1 and primer ITS4 was used for the complementary strand. For herbarium specimens, primers for sequencing in the forward direction were ITS1 and Rau-F01. Primers ITS4 and Stmn-5S-R were used for the complementary strand.

Sequence Analysis and Phylogenetic Analysis

DNA sequences of ITS locus were aligned using the ClustalX 2.0 program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) and Bioedit program (www.mbio.ncsu.edu/BioEdit/BioEdit.html). The nucleotide sequence data of ITS 1-5.8S-ITS 2 region were deposited in the DDBJ, EMBL, and Genbank nucleotide sequence database with the accession numbers shown in Table 3. Sequence boundaries of ITS 1, 5.8S, ITS 2 regions were determined following the previous sequence data of *Alstonia scholaris* and *Rauvolfia serpentina* from Genbank (accession number DQ358880 and AB331857, respectively). The phylogenetic tree based on sequences of ITS1, 5.8S, and ITS2 region sequences of 21 examined samples was constructed using the computer program PAUP* (Version 4.0b10, Sinauer Addoc. Inc., U.S.A.). Parsimony analysis was performed using Branch-and-bound search, with tree-bisection-reconnection (TBR) branch-swapping, MulTrees, a random addition sequence of 100 replicates. *Mondia whitei* (accession number AJ581679) belonging to the same family as *Rauvolfia* was

used as an outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of topology of the consensus tree.

3. Results

Total DNA and PCR Products

Total genomic DNA was isolated from leaves of each specimen by using the DNeasy® Plant Mini Kit (QIAGEN, Germany), and then DNA solution was purified by Geneclean® II Kit (QBiogene Inc., U.S.A.). Purified DNA was examined on 0.8 % agarose gel electrophoresis. The size of extracted DNA in each specimen was shown in Figure 3.2. The purified DNA was stored at –20 °C until used.

Using the obtained genomic DNA as templates, complete ITS regions were amplified by PCR technique using a pair of primers, ITS1 and ITS4, for *R. serpentina, R. cambodiana*, *R. sumatrana* and *R. verticillata*. The resulting PCR products were about 750 bp in length (Figure 3.3). For herbarium specimens, *R. micrantha* and *R. cambodiana,* two pairs of primers, 1) ITS 1 and Stmn-5s-R and 2) Rau-F01 and ITS 4, were used. The resulting partial PCR products were about 450 bp in length (Figure 3.3).

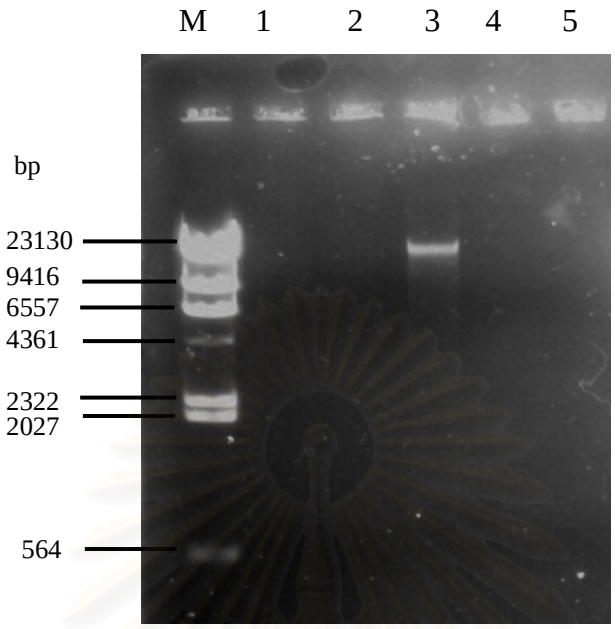


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Figure 3.2 Agarose gel electrophoretogram of genomic DNA from *Rauvolfia* species.

Lane M: Lambda DNA-*Hind* III Digest (The sizes are 564, 2027, 2322, 4361, 6557, 9416, and 23130 bp, respectively.)

Lane 1: *R. serpentina* (Rsp05, Nakornpathom)

Lane 2: *R. cambodiana* (Rcb02, Saraburi)

Lane 3: *R. verticillata* (Rvt30, Kanchanaburi)

Lane 4: *R. sumatrana* (Rsm12, Nakornpathom)

Lane 5: *R. micrantha* (Rmc22, Prachuabkerekhun)

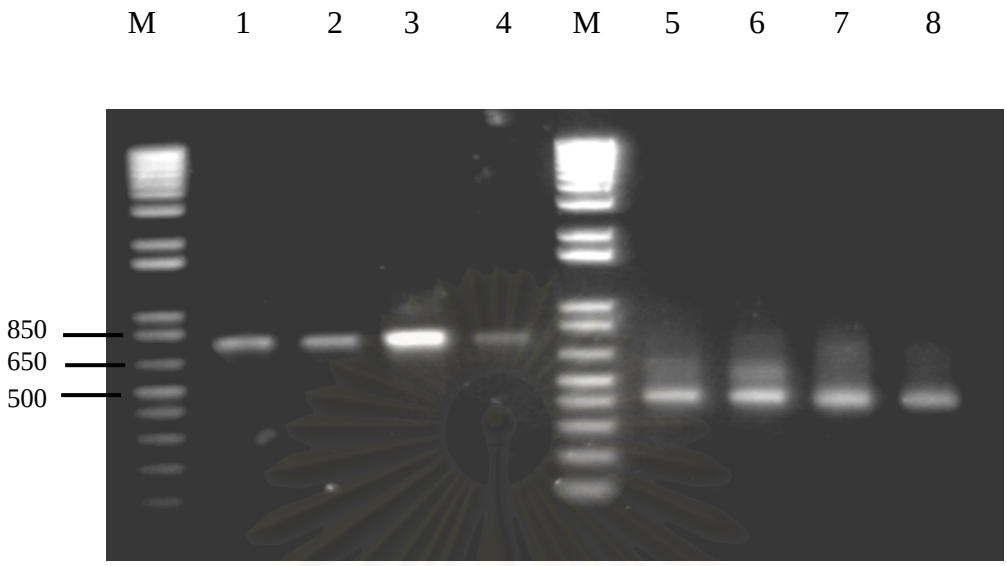


Figure 3.3 Agarose gel electrophoretogram of PCR products of ITS region generated by primers ITS1 and ITS4 (Lane 1-4), ITS1 and Stmn-5S-R (Lane 5-6), and Rau-F01 and ITS4 (Lane 7-8).

Lane M: 1 Kb plus DNA Ladder. (The sizes are 100, 200, 300, 400, 500, 650, 850, 1000, 1650, 2000, 3000, 4000, 5000, 6000,7000, 8000, 9000, 10000, 11000 and 12000 bp, respectively.)

Lane 1 : *R. serpentina* (Rsp05, Nakornpathom)

Lane 2 : *R. cambodiana* (Rcb02, Saraburi)

Lane 3 : *R. verticillata* (Rvt30, Kanchanaburi)

Lane 4 : *R. sumatrana* (Rsm12, Nakornpathom)

Lane 5: *R. micrantha* (Rmc22, Prachuabkerekhun)

Lane 6: *R. micrantha* (Rmc23, Petchaburi)

Lane 7: *R. micrantha* (Rmc22, Prachuabkerekhun)

Lane 8: *R. micrantha* (Rmc23, Petchaburi)

Sequence Analysis of Amplified the ITS Region from *Rauvolfia* Species

PCR products encompassing ITS 1, 5.8S rDNA and ITS 2 were amplified from 21 samples of five *Rauvolfia* species distributed in Thailand using a PCR primer sets and their nucleotide sequences were determined. The sequences obtained were used for designing internal PCR primers. In some herbarium samples, DNA might be highly degraded into small fragments and did not give any PCR products when DNA preparations from these samples were used as template. In such cases, ITS 1 and ITS 2 were separately amplified using internal primers, Rau-F01 and Stmn-5s-R, annealing to 5'- and 3'- end of 5.8S rDNA respectively as shown in Figure 3.1. The full nucleotide sequence of the ITS regions was determined by combination of two separate products.

All specimens of the same species showed completely identical sequence. The sequence alignment is shown in Figure 3.4 and the borders of each region are indicated. The ITS region varied in each *Rauvolfia* species, 615 bp in length in *R. micrantha*, 627 bp in length in *R. cambodiana*, 628 bp in length in *R. serpentina* and *R. sumatrana*, and 631 bp in length in *R. verticillata*. ITS 1 ranged from 228 bp to 231 bp, and ITS 2 from 195 bp to 210 bp. The length of 5.8S coding region is conserved at 189 bp for all five species. The sequence divergence among these species ranged from 1.59 % - 33.24 %. The nucleotide sequence of the ITS region of *R. micrantha* was quite different from other *Rauvolfia* species. Twenty-one positions of nucleotide additive sequence were found in sequence of *R. verticillata*.

The nucleotide sequences of ITS region of *Rauvolfia* spp. have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers as shown in Appendix B.

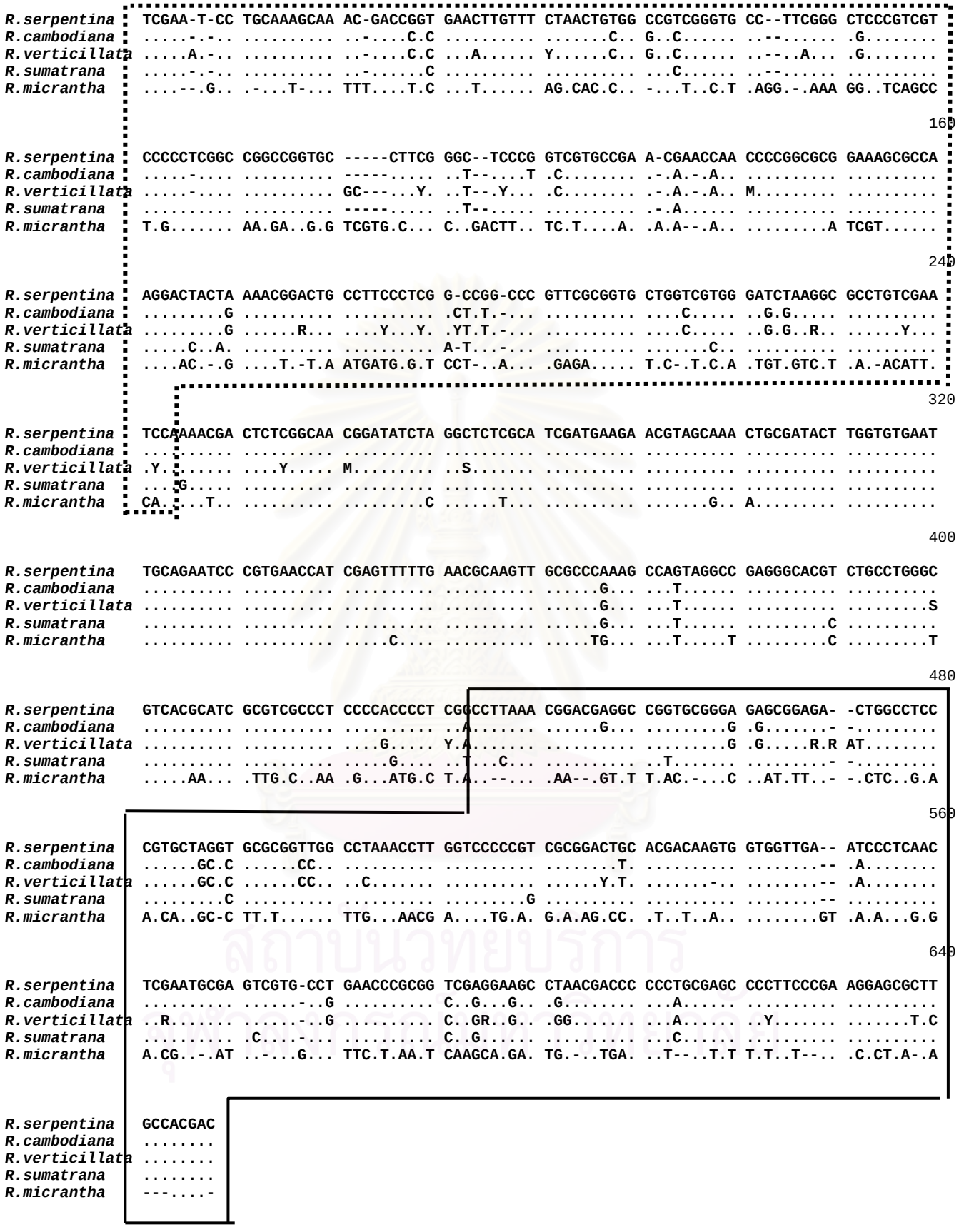


Figure 3.4 Nucleotide sequence alignments of ITS1-5.8S-ITS2 region of *R. serpentina, R. cambodiana, R. verticillata, R.sumatrana* and *R. micrantha*. Position1 to 244 is the ITS1 region (enclosed in the dash box), the 5.8S region corresponds to position 245 to 433, and The ITS 2 region corresponds to position 434 to 648 (enclosed in the black box). The same sequences are indicated by dot (.). Gaps (-) are introduced for the best alignment. The characters indicate nucleotide additives. M = A and C; R= A and G; S= C ang G; W= A and T; Y= C and T.

Phylogenetic Analysis

The phylogenetic tree was constructed on the basis of complete ITS region sequences of 21 specimens of five *Rauvolfia* spp. in Thailand. Parsimony analysis showed that the most parsimonious trees fully resolved tree with a length of 380, 364 characters constant, 226 variable characters are parsimony-uninformative, a consistency index (CI) of 0.8108 and a retention index (RI) of 0.7789. As shown in Figure 3.5, strict consensus tree divided the five *Rauvolfia* species into two groups. The four Thailand species, *R. serpentina*, *R. sumatrana*, *R. cambodiana*, and *R. verticillata*, and *R. serpentina* from Japan formed a big group. *R. micrantha* placed in the former group as an individual branch with a bootstrap value of 100%.

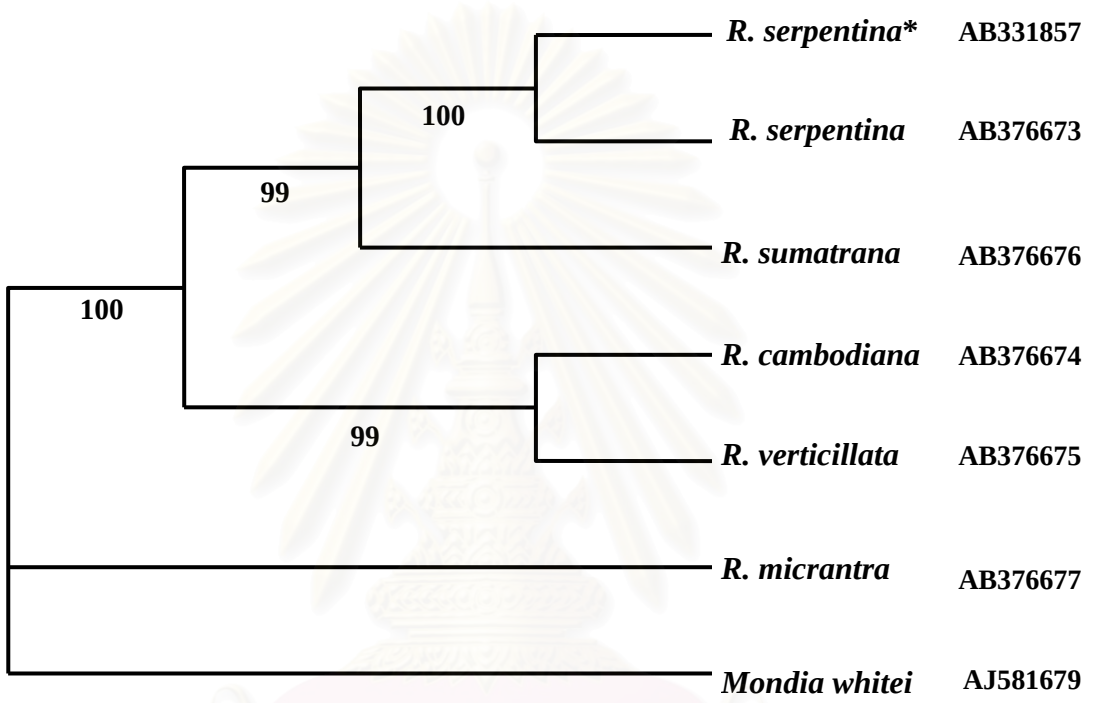


Figure 3.5 Phylogenetic tree of the most parsimonious trees reconstructed on the basis of ITS sequences from *Rauvolfia* species. Tree length = 380, CI=0.8108, RI=0.7789, RC=0.7359. Number below line is the bootstrap value with 1000 replicates. *R. serpentina* from Japan (retrived from Genbank) are shown with asterisks.

4. Discussion

The present study was designed to determine the DNA markers of all five *Rauvolfia* species in Thailand and to clarify their phylogenetic relationships. Nucleotide sequences of ITS region were used to identify *Rauvolfia* species. Herbarium specimens of *R. micrantha* and *R. cambodiana* were used in this study since it is a rare medicinal species (Sudah and Seeni, 1996). However, DNA extraction from herbarium specimens requires some modifications because of the small amount of dry herbarium tissue available (Drábková, 2002; Sahin, 2007). In this study, extraction was performed according to manufacture's protocol with some modifications. Modifications were 1) incubation time to lysate cell was prolonged, 2) lysis buffer for lysate cell was increased, 3) elution solution was decreased and 4) elution time was prolonged.

In this experiment, even though the extracted genomic DNA of some *Rauvolfia* spp. were not observed but PCR could be used successfully for the amplification of DNA.

Complete ITS sequences of *Rauvolfia* species were analyzed. For *Rauvolfia* species existing in Thailand, genetic variation was found only interspecies. The similar intraspecies difference was observed between the sequences of *R. serpentina* from Thailand and that deposited in GenBank (AB331857). There was found 10 sites of substitution. However, such phenomena have been reported in previous studies such as the sequence analyses of red cover (Lum *et al*., 2004) and *Mitragyna* species (Sukrong *et al*., 2007). The sequence variation may be due to some factors such as intraspecies polymorphism and cross-hybridization between species (Alvarez and Wendel, 2003). However, more samples of *R. serpentina* should be investigated to solve this problem.

In the sequencing results of *R. verticillata*, combination of nucleotides, that has been called "nucleotide additives" were recognized at the variable sites in the ITS sequence. Two types of nucleotides were observed on 21 positions in the ITS sequence of *R. verticillata.* Nucleotide additives are often observed in hybrids between two species (Kondo *et al*., 2007). For this study, *R. cambodiana* might be recognized as one of the parents of *R. verticillata*, another one was not identified in this study. The close relationship was supported by Flora of China in that *R. cambodiana* is a synonym of

R. verticillata. However, further studies may be required, including larger sample size and more detail in morphological characteristics of the two species.

From this study, the phylogenetic tree was constructed on the basis of ITS sequences. *R. serpentina* from Thailand and that deposited in GenBank (AB331857) are joined at the same node with high bootstrap value of 100%. *R. sumatrana* was closely related to *R. serpentina* even though they had different morphological. *R. cambodiana* and *R. verticillata* are formed the same group with high bootstrap value of 99%. Five nucleotide substitutions together with 3-bp indels were observed between two species. ITS sequence of *R. micrantha* was the most different from the other species so it was placed in the individual branch. Therefore accurate phylogenetic relationships among species could not be estimated from only few samples and few genetic markers (Kondo *et al*., 2007). To investigate genetic diversity among species, further study should be applied to analyze other part of genome such as *mat*K and *rbc*L genes.

CHAPTER IV

POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) OF *RAUVOLFIA* SPECIES IN THAILAND AND THEIR APPLICATION FOR IDENTIFICATION

1. Materials

. The leaves of five *Rauvolfia* species, *R. cambodiana*, *R. micrantha*, *R. serpentina*, *R. sumatrana* and *R. verticillata,* were used in this study. All of the collected plant materials and their localities are listed in Table 3 in Chapter III. Five Rauwolfia crude drugs were purchased from different places (Figure 4.1). Both specimens and crude drugs have been deposited in the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The pictures of all specimens are shown in Appendix A.

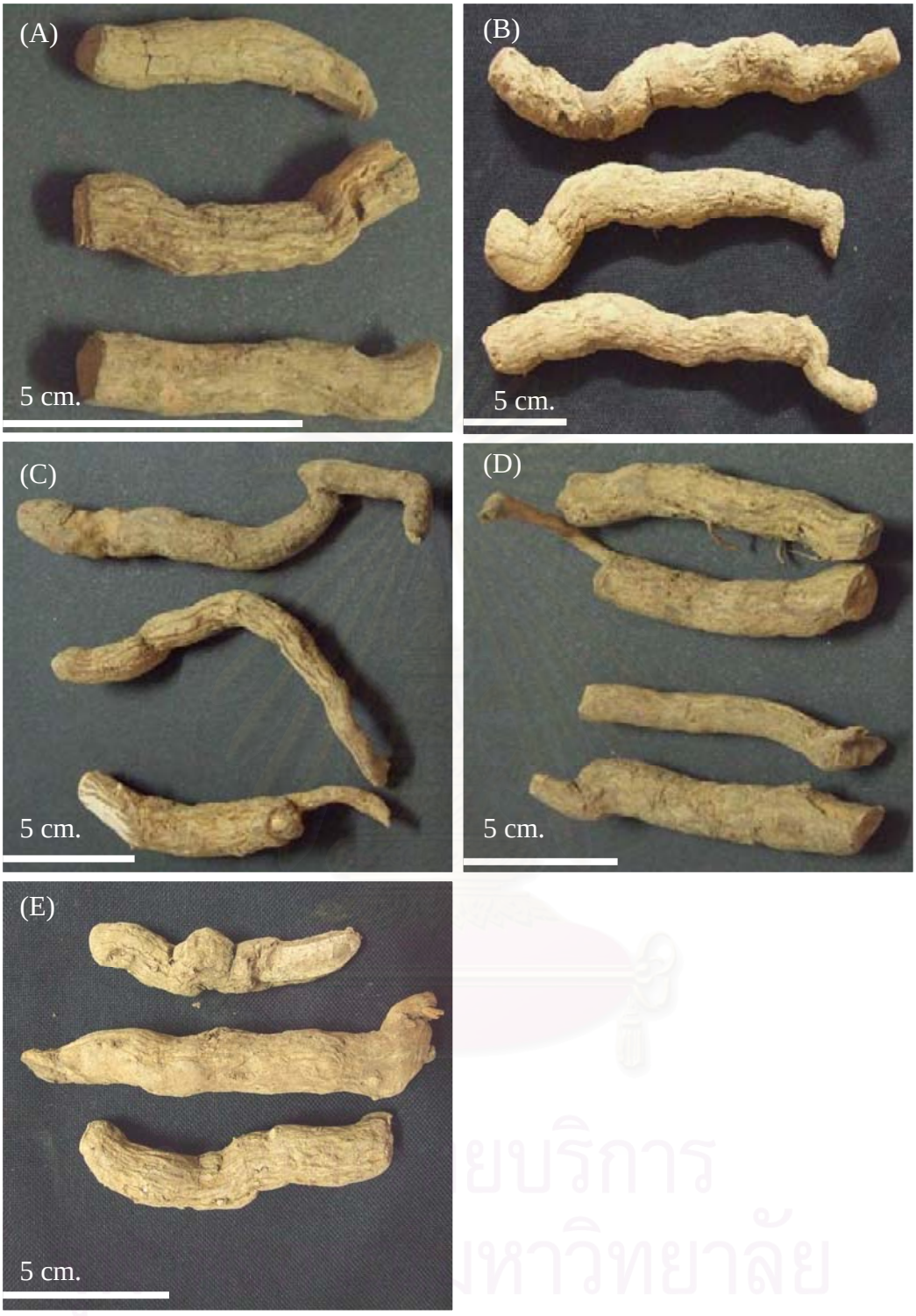


Figure 4.1 Crude drug of *Rauvolfia* species (A-E) purchased from crude drug markets in different provinces. A) Nakornpathom, B) Srisakate, C) Songkhla, D) Chiangmai, and E) Ayuttaya.

2. Methods

Total DNA Extraction

Leaves of silica gel-dried samples and herbarium specimens were ground under liquid nitrogen to a fine powder using a mortar and pestle. Fifty milligrams of plant tissue were used for total DNA extraction using the DNeasy® Plant Mini Kit (QIAGEN, Germany), following the manufacture's protocol. For herbarium specimens, extractions were performed according to the QIAGEN protocol with a small modification which described in Chapter III. The 50 µl of DNA solution was purified by a Geneclean® II Kit (QBiogene Inc., U.S.A.). For crude drugs, 1-5 miligrams of crude drug were used for total DNA extraction using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, U.S.A.) Total genomic DNA was performed on 0.8 % agarose gel electrophoresis stained by ethidium bromide and visualized under UV light to determine quality and quantity. A Lambda DNA-*Hind* III Digest (New England BioLabs Inc., U.S.A.) was used as standard DNA size. The extracted total DNA was kept at -20 °C for further use as templates in PCR amplification.

PCR-RFLP Analysis

PCR amplification of two DNA regions, 1) ITS 1 region and partial 5.8S rDNA and 2) partial 5.8S rDNA and ITS 2 region, was performed separately using 50 ng of total DNA as a template in 50 µl of reaction mixture consisting of 10 mM Tris-HCL (pH 9.0 at 25 °C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ (Promega, U.S.A), 0.2 mM of each dNTPs, 1.5 U *Taq* DNA polymerase (Promega, U.S.A), and 0.25 mM of each primer. The amplification primers used to amplify ITS 1 region and 5.8S rDNA of plant samples and crude drugs are ITS1 (5' GTC CAC TGA ACC TTA TCA TTT AG 3') and Stmn-5s-R (5' GAG TTT TTG AAC GCA AGT TGCG 3'). A pair of primers used to amplify partial 5.8S rDNA and ITS 2 region of plant samples are: Rau-F01 (5' TCGCATCGATGAAGAACGTA 3'), which was designed based on our obtained sequence of *R. serpentina*, and ITS 4 (5' TCCTCCGCTTATTGATATG C 3').

PCR amplification was carried out in a PCR Thermocycler (Eppendorf Mastercycler Personal, Eppendorf North America Inc., U.S.A.). The PCR cycling program started with an initial denaturation step at 95 ºC for 3 minutes, followed by strand denaturation at 95 ºC for 40 seconds, primer annealing at 55 ºC for 40 seconds, and primer extension at 72 ºC for 1 minute for 29 cycles, and final extension step at 72 ºC for 5 minutes, then held at 4 ºC. In the case of herbarium specimens of *R. micrantha* and crude drugs, nested PCR method was applied. The first PCR product was used as a template for the second PCR amplification.

The restriction sites of ITS 1 and ITS 2 sequence were analyzed by using NEBcutter program (http://tools.neb.com/NEBcutter2/index.php). Three restriction enzymes, *Sty*I, *Eag*I, and *Dde*I, were selected as suitable candidates for identification. The PCR products amplified by primers Rau-F01 and ITS4, were digested with 5 units of restriction enzyme, *Eag*I and *Sty*I (BioLabs, Inc., U.S.A.) at 37 ºC for 3 hrs. The PCR products which were amplified by primers ITS1 and Stmn-5s-R were digested with 5 units of restriction enzyme, *Dde*I (BioLabs, Inc., U.S.A.), at 37 ºC for 3 hrs.

The PCR products and the resulting restriction digests were detected by agarose or MetaPhor agarose gel electrophoresis and stained with ethidium bromide, visualized under UV light and photographed by Gel Doc™ XR System PC/Mac (Bio-Rad Laboratories, U.S.A.). The size of fragments was estimated by comparison with 1 kb plus DNA ladder (Invitrogen Corp., U.S.A.).

3. Results

Genomic DNA and PCR products

Genomic DNA isolated from leaves of five *Rauvolfia* species, *R. cambodiana*, *R. micrantha*, *R. serpentina*, *R. sumatrana* and *R. verticillata* (Figure 3.2 in Chapter III), and from crude drug samples (A-E) were obtained (Figure 4.2).

Using the total DNA as a template, partial 5.8 S and ITS 2 was amplified using a pair of primers, Rau-F01 and ITS4. The PCR products of *R. micrantha*, *R. verticillata*, and other species were 432, 448, and 447 bp in length, respectively (Figure 4.3A). PCR products of ITS 1 and partial 5.8S were amplified using a pair of primers, ITS1 and Stmn-5S-R. The PCR products of *R. micrantha* and *R. verticillata*, *R. cambodiana*, *R. serpentina* and *R. sumatrana* were 448, 456, and 457 bp in length, respectively (Figure 4.3B).

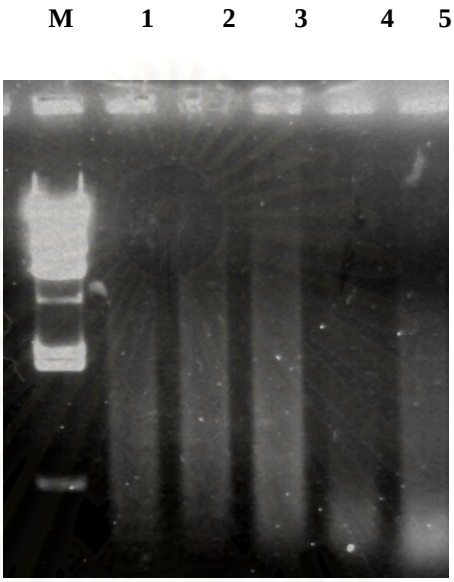


Figure 4.2 Agarose gel electrophoretogram of genomic DNA from crude drugs of *Rauvolfia* species purchased from different provinces.

Lane M : Lambda DNA-*Hind* III Digest

Lane 1 : Nakornpathom (A)

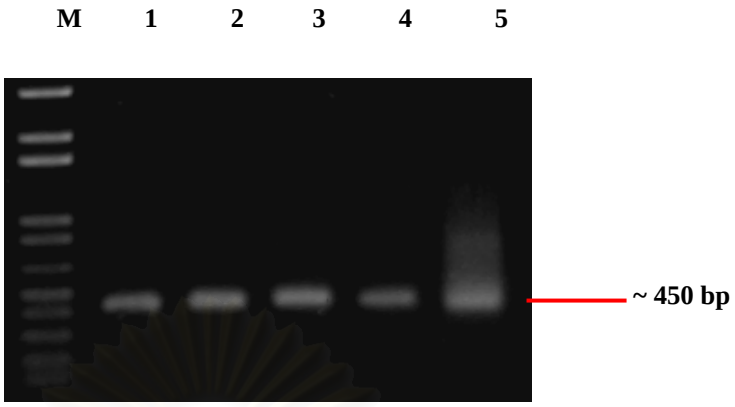
Lane 2 : Srisakate (B)

Lane 3 : Songkhla (C)

Lane 4 : Chiangmai (D)

Lane 5 : Ayutthaya (E)

(A)



(B)

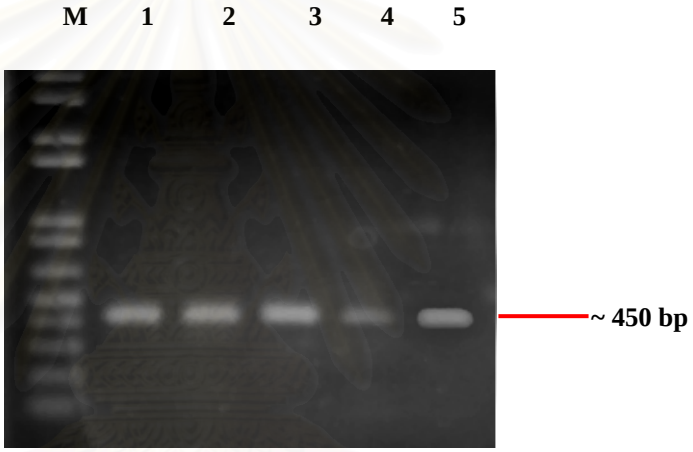


Figure 4.3 Agarose gel electrophoretogram of PCR products (A) PCR products generated from a pair of primers, Rau-F01 and ITS4, on partial 5.8S and ITS 2 region. (B) PCR products generated from a pair of primers, ITS1 and Stmn-5S-R, on ITS 1 region and partial 5.8S

Lane M : 1 Kb plus DNA Ladder.

Lane 1 : *R. serpentina* (Rsp05, Nakornpathom)

Lane 2 : *R. cambodiana* (Rcb02, Saraburi)

Lane 3 : *R. verticillata* (Rvt30, Kanchanaburi)

Lane 4 : *R. sumatrana* (Rsm12, Nakornpathom)

Lane 5 : *R. micrantha* (Rmc22, Prachuabkerekhun)

Identification of *Rauvolfia* species in Thailand by PCR-RFLP Analysis

For identification of *Rauvolfia* species in Thailand, three restriction enzymes were used in this study. The summary of data for identification are shown in Table 5 and Figure 4.4.

Table 5 Restriction fragment size (in base pairs) of ITS region of *Rauvolfia* species digested with *Sty*I, *Eag*I and *Dde*I

Rauvolfia species	*Sty*I[a]	*Eag*I[a]	*Dde*I[b]
R. micrantha	432 (undigested)	432 (undigested)	138, 321
R. serpentina	197, 250	447 (undigested)	170, 287
R. sumatrana	197, 250	119, 328	170, 287
R. cambodiana	197, 250	90, 119, 237	456 (undigested)
R. verticillata	197, 252	90, 119, 239	138, 321

[a] PCR product of partial 5.8S and ITS 2 amplified with Rau-F01 and ITS4 primers.

[b] PCR product of ITS 1 and partial 5.8S amplified with ITS1 and Stmn-5S-R primers.

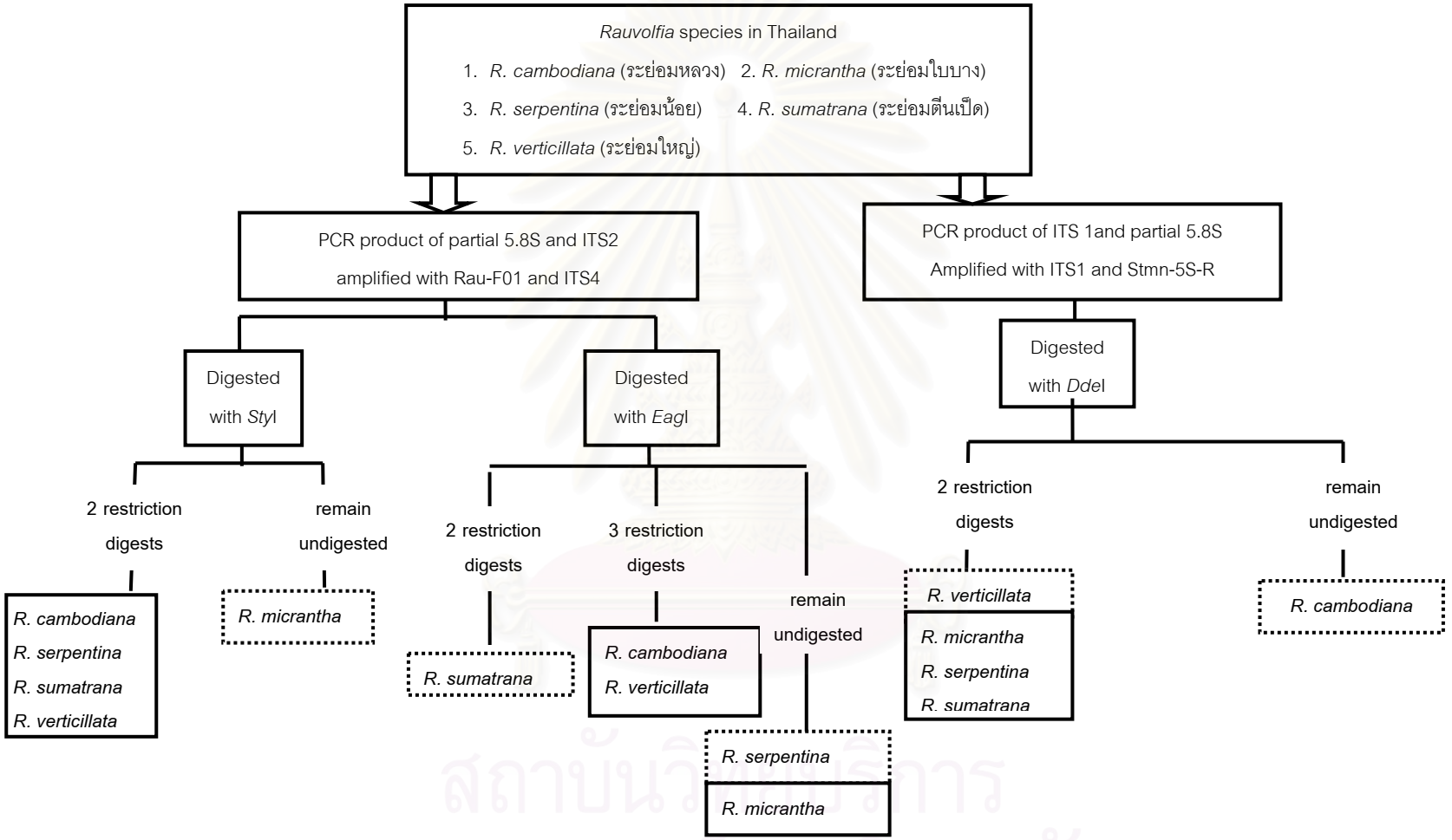


Figure 4.4 Diagram for identification of *Rauvolfia* species with three restriction enzymes. Species in the dash boxes mean the species separated from others by PCR-RFLP technique.

***Sty*I Digest**

The PCR products of *Rauvolfia* species amplified with a pair of primers, Rau-F01 and ITS4, on partial 5.8S rDNA and ITS region were 432 bp and 449 bp in length for *R.micranha* and *R. verticillata* respectively. For other species, 447 bp in length was gained. The restriction enzyme *Sty*I, which recognizes the sequence of 5′-C^CWWGG -3′ (when W means A or T), was found to give diagnostic fragments among the five species and divided *Rauvolfia* species into two groups. The amplified region of *R. cambodiana, R. verticillata, R. sumatrana* and *R. serpentina* had a *Sty*I restriction site at the nucleotide position 507 from 5-end of ITS 1 of *Rauvolfia* species (Figure 4.5A and Figure 3.4), resulting in two banding patterns of either 250 or 252 bp and 197 bp which depends on species. While *R. micrantha* did not have the *Sty*I restriction site so undigested fragment showed only one of 409 bp (Figure 4.5A and Figure 4.5B). Five crude drugs (A-E) were digested and resulted in two banding patterns similar to *R. serpentina, R. cambodiana, R. verticillata* and *R. sumatrana* (Figure 4.5B).

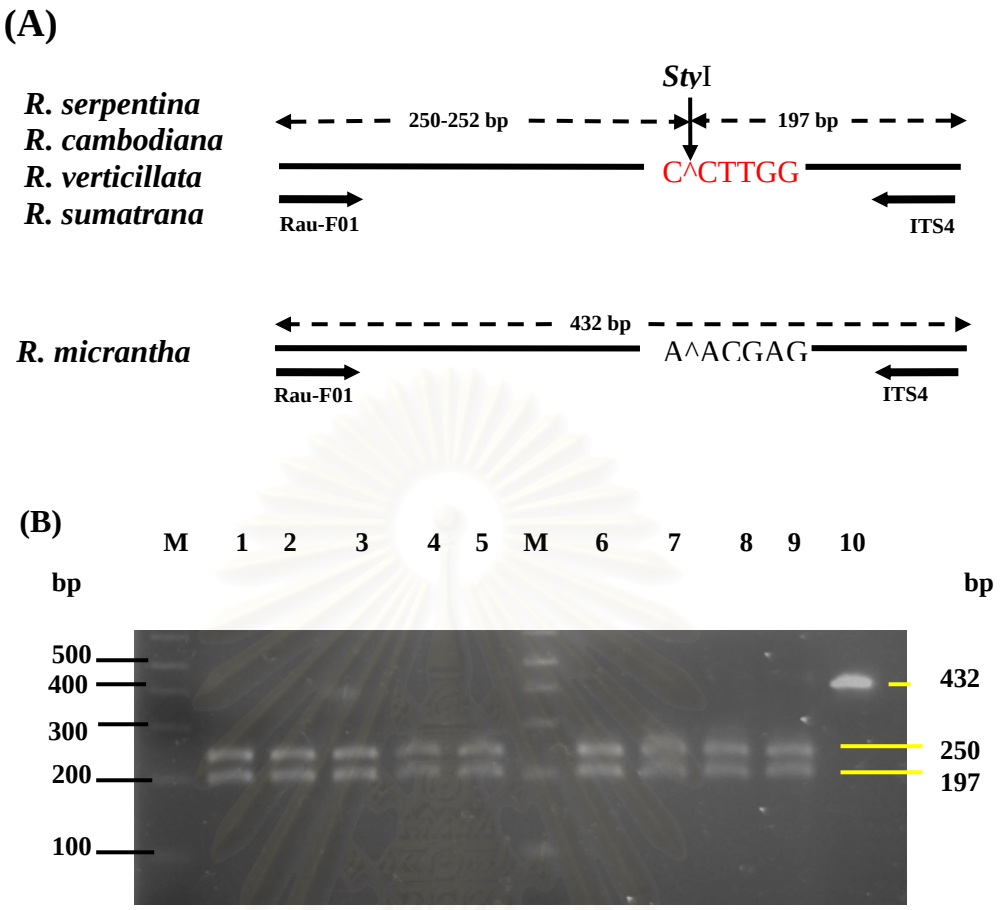


Figure 4.5 PCR-RFLP analysis of *Rauvolfia* species using the restriction enzyme *Sty*I on PCR products generated by Rau-F01 and ITS4 primers on partial 5.8S rDNA and ITS2 region.

(A) *Sty*I restriction sites in *Rauvolfia* species. Fragment digests in bp were indicated. Red nucleotides indicate the defined marker nucleotide.

(B) 2.5 % Metaphor agarose gel electrophoretograms of PCR products digested with *Sty*I.

Lane M : 1 Kb plus DNA Ladder.

Lane 1-5 : Crude drug A-E from different provinces, Nakornpathom, Srisakate, Songkhla, Chiangmai and Ayuttaya as listed in Figure 4.1

Lane 6 : *R. serpentina* (Rsp05, Nakornpathom)

Lane 7 : *R. cambodiana* (Rcb02, Saraburi)

Lane 8 : *R. verticillata* (Rvt30, Kanchanaburi)

Lane 9 : *R. sumatrana* (Rsm12, Nakornpathom)

Lane 10 : *R. micrantha* (Rmc22, Prachuabkerekhun)

***Eag*I Digest**

The PCR products of *Rauvolfia* species amplified with a pair of primers, Rau-F01 and ITS4, on partial 5.8S rDNA and ITS region were 432 bp in length for *R.micrantha*, 449 bp for *R. verticillata* and 447 bp for other species. The restriction enzyme *Eag*I, which recognizes the sequence of 5′-C^GGCCG -3′, was found to give diagnostic fragments among the five species and divided *Rauvolfia* species into three groups. The PCR products of *R. cambodiana* and *R. verticillata* had two *Eag*I restriction sites at the nucleotide position 494 and 588 from 5'-end of ITS 1 of *Rauvolfia* species (Figure 4.6A and Figure 3.4). The resulting restriction digest showed that three fragments of either 237 or 239 bp, 90 bp and 119 bp depend on species (Figure 4.6B), while in the *R. sumatrana*, they had only one *Eag*I restriction site at the nucleotide position 588 from 5′-end of ITS 1 (Figure 4.6A and Figure 3.4). The resulting restriction digests showed two fragments of 328 bp and 119 bp in electrophoretogram (Figure 4.6B). In the case of *R. serpentina* and *R. micrantha*, they were absent of this recognize sites so undigested bands present in 447 bp and 432 bp, respectively (Figure 4.6B and Figure 4.6A). Five crude drugs (A-E) showed one undigested band after digestion, similar to *R. serpentina* and *R. micrantha* (Figure 4.6B).

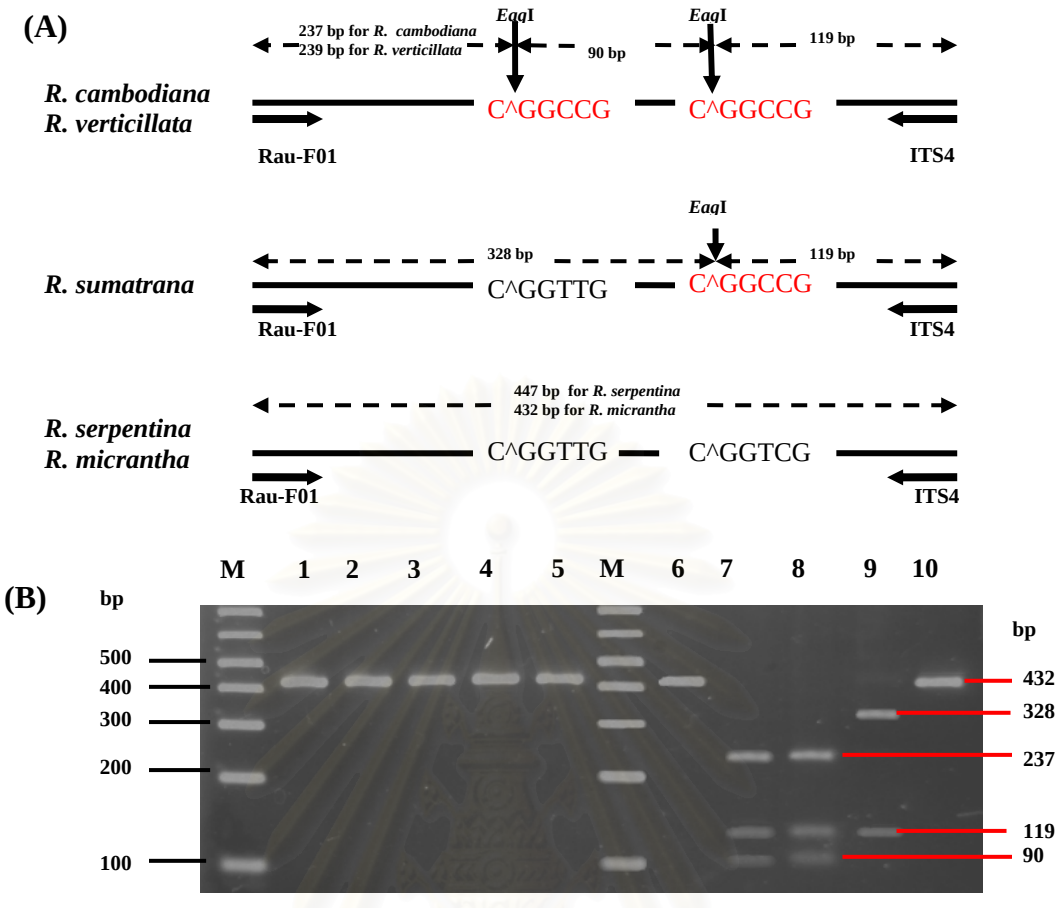


Figure 4.6 PCR-RFLP analysis of *Rauvolfia* species using the restriction enzyme *Eag*I on PCR products generated by Rau-F01 and ITS4 primers on partial 5.8S rDNA and ITS2 region

(A) *Eag*I restriction sites in *Rauvolfia* species. Fragment digests in bp were indicated. Red nucleotides indicate the defined marker nucleotide.

(B) 2.5 % Metaphor agarose gel electrophoretograms of PCR product digested with *Eag*I.

Lane M : 1 Kb plus DNA Ladder.

Lane 1-5 : Crude drug A-E from different provinces, Nakornpathom, Srisakate, Songkhla, Chiangmai and Ayuttaya as listed in Figure 4.1

Lane 6 : *R. serpentina* (Rsp05, Nakornpathom)

Lane 7 : *R. cambodiana* (Rcb02, Saraburi)

Lane 8 : *R. verticillata* (Rvt30, Kanchanaburi)

Lane 9 : *R. sumatrana* (Rsm12, Nakornpathom)

Lane 10 : *R. micrantha* (Rmc22, Prachuabkerekhun)

***Dde*I Digest**

The PCR products of *Rauvolfia* species amplified with a pair of primers, ITS1 and Stmn-5S-R, on ITS1 region and partial 5.8S rDNA were 456 bp, 457 bp and 448 bp in length for *R. cambodiana*, *R. serpentina* and *R. sumatrana*, and *R. verticillata* and *R. micrantha*, respectively. The restriction enzyme *Dde* I, which recognizes the sequence of 5′-C^TNAG -3′, was found to give diagnostic fragments among the five species and divided *Rauvolfia* species into three groups. Amplification products of *R. sumatrana* and *R. serpentina* had a *Dde*I restriction site at the nucleotide position 234 from 5′-end ITS 1 of *Rauvolfia* species (Figure 4.7A and Figure 3.4) and resulted in two banding patterns, giving rise to fragments of 287 bp and 170 bp, while in *R. verticillata* and *R. micrantha*, each had a *Dde*I restriction site at the nucleotide position 62 and 74 from 5′-end of ITS 1 of *Rauvolfia* species (Figure 4.7A and Figure 3.4). The resulting restriction digest showed two fragments of 138 or 146 and 321 or 313 bp, whereas that of *R. cambodiana* did not have the *Dde*I cleavage site
(Figure 4.7A), and only one fragment of 456 bp was present (Figure 4.7B). Five crude drugs (A-E) could be digested by *Dde* I and the results indicated that they could be *R. serpentina* or *R. sumatrana*, but absolutely not *R. cambodiana*, *R. verticillata* and *R. micrantha* (Figure 4.7B).

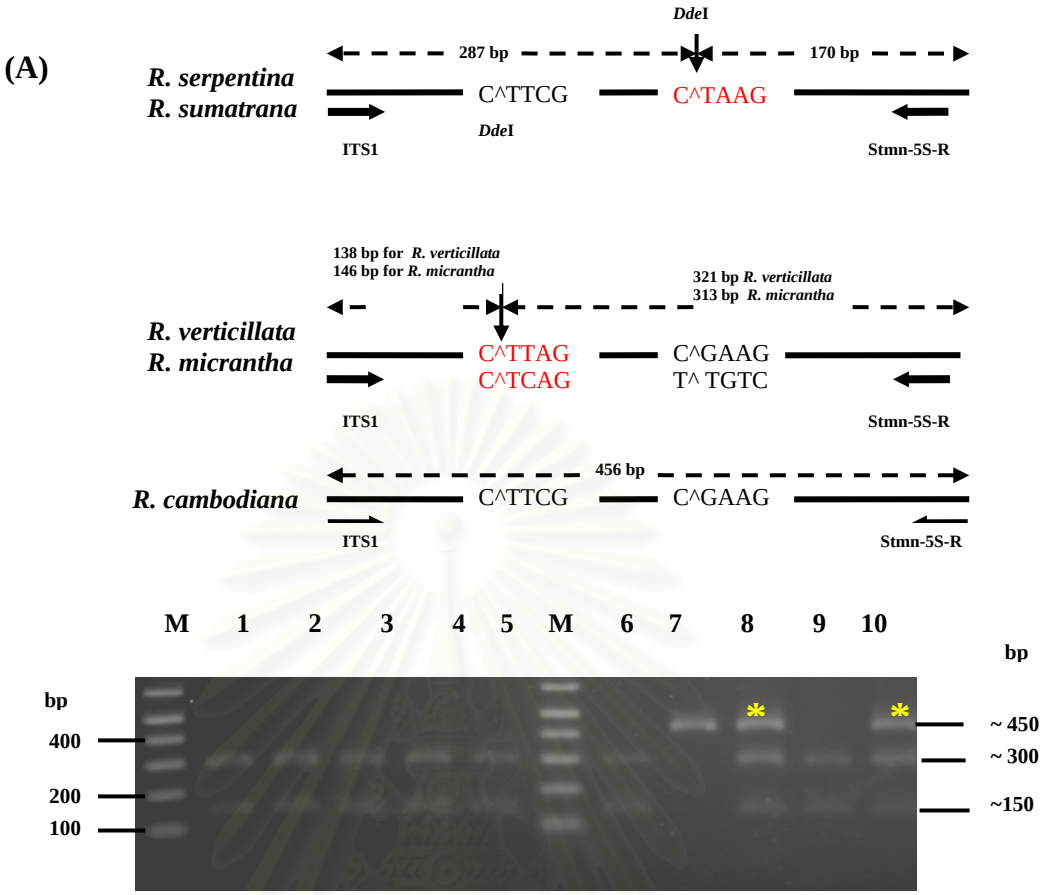


Figure 4.7 PCR-RFLP analysis of *Rauvolfia* species using the restriction enzyme *Dde*I on PCR products generated by Stmn-5S-R and ITS4 primers on ITS 1 region and partial 5.8S rDNA.

(A) *Dde*I restriction sites in *Rauvolfia* species. Fragment digests in bp were indicated. Red nucleotides indicate the defined marker nucleotide.

(B) 1.5% agarose gel electrophoretograms of PCR product digested with *Dde*I. * above the digested band means partial digestion with *Dde*I.

Lane M : 1 Kb plus DNA Ladder.

Lane 1-5 : Crude drug from different provinces, Nakornpathom, Srisakate, Songkhla, Chiangmai and Ayuttaya as listed in Figure 4.1

Lane 6 : *R. serpentina* (Rsp05, Nakornpathom)

Lane 7 : *R. cambodiana* (Rcb02, Saraburi)

Lane 8 : *R. verticillata* (Rvt30, Kanchanaburi)

Lane 9 : *R. sumatrana* (Rsm12, Nakornpathom)

Lane 10 : *R. micrantha* (Rmc22, Prachuabkerekhun)

4. Discussion

In the present study, PCR-RFLP method was performed for identification of *Rauvolfia* species. Unfortunately, it is often difficult to obtain high quality DNA from crude drugs. DNA from crude drugs may degrade into small pieces due to the oxidative and hydrolytic process during preservation (Mizukami *et al*., 2000; Yang *et al*., 2004). Therefore, new sets of primer for amplification of short fragment of PCR products were designed for identification of crude drugs. Two pairs of primer sets, 1) ITS1 and Stmn-5S-R and 2) Rau-F01 and ITS4, for amplification of ITS1 region-partial 5.8S rDNA and partial 5.8S rDNA-ITS2 region respectively, were designed and used in this study. Patterns of digestion were analyzed by using NEB cutter program and three restriction enzymes, *Eag*I, *Sty*I and *Dde*I, were selected.

For PCR products generated from Rau-F01 and ITS4 primers of partial 5.8S and ITS 2 region, *Sty*I digestion was found to identify *R. micrantha* from other species because *R. micrantha* remained undigested (Figure 4.5A and Figure 4.5B). The other four *Rauvolfia* species, *R. serpentina, R. cambodiana, R. verticillata* and *R. sumatrana*, were digested and gave two bands of 197 bp and 250 bp.

Restriction enzyme *Eag*I was also used to digest PCR products as above. From the result, *R. sumatrana* had a different digestion pattern from the others (Figure 4.6A and Figure 4.6B). *R. sumatrana* was digested at only one site and gave two fragments of product. *R. serpentina* and *R. micrantha* were not digested with *Eag*I but *R. cambodiana* and *R. verticillata* were digested into three fragments of products. If two restriction enzymes, *Sty*I and *Eag*I, were applied, *R. serpentina* could be separated from *R. micrantha.* Although *R. cambodiana* could not be distinguished from *R. verticillata* with two restriction enzymes described above.

Amplification of ITS 1 and partial 5.8S follow by the *Dde*I digestion could be used to distinguish *R. cambodiana* from *R. verticillata* (Figure 4.7A)*. Dde*I restriction site was absent in *R. cambodiana*, making the PCR product remained undigested. Although *R. serpentina*, *R. sumatrana*, *R. verticillata* and *R. micrantha* were digested at different sites, two fragments were obtained at the same position on 1.5% agarose gel due to the limitation in the separation of nearby fragments (Figure 4.7B). Form this result,

R. cambodiana was distinguished from *R. verticillata*. In addition, partial digestion was detected in the cleavage of *R. verticillata* and *R. micrantha* with *Dde*I, as shown by an asterisk above band in Figure 4.7B. Unsuitable reaction conditions were not the reason for the partial digestion because many reaction conditions such as increasing the quality of enzyme and prolonging incubation time were performed. Partial digestion probably is due to hybridization between related species and these results are similar to the previous reports on medicinal herb, Bulb of Fritillaria (Wang *et al*., 2007), bacteria (Hong *et al*., 1999) and fungi (Thomas *et al*., 1996). Nevertheless, this result should not influence the identification of *R. cambodiana* and *R. verticillata*.

In the case of rauwolfia crude drugs, PCR products were not detected at the first of amplification. Since DNA extraction from crude drugs was difficult due to the high amounts of polysaccharides and secondary metabolites that formed insoluble complexes with nucleic acids during extraction (Cai *et al*., 1996 and Pandey *et al*., 1999). So, nested PCR was performed. The first PCR product was used as a template of the second PCR amplification. From this result, PCR products were obtained successfully.

For authentication by PCR-RFLP technique, the results from this experiment indicated that all rauwolfia crude drugs investigated in this experiment had digestion patterns which fit into *R. serpentina* for all restriction enzymes. It is possible that they are *R. serpentina* and infers all crude drugs are absolutely not *R. cambodiana*, *R. verticillata*, *R. sumatrana*, and *R. micrantha*. In this study, adulterant of *R. serpentina* was not found.

PCR-RFLP method was established successfully as a powerful tool for identification of *Rauvolfia* species and rauwolfia crude drugs.

CHAPTER V

CONCLUSION

The main purpose of this study was to identify *Rauvolfia* species in Thailand, *R. serpentina, R. cambodiana, R. micrantha, R. sumatrana, and R. verticillata* by DNA fingerprinting analysis. To achieve this purpose, combination of sequencing based method and PCR-RFLP method were applied as a molecular marker. PCR-RFLP method proved to be a rapid and easy-to-perform analytical tool to achieve species authentication and identification of rauwolfia crude drugs.

To reach the objective, an investigation was performed in three steps. In the first step, sequencing based method was performed. Internal transcribed spacer (ITS) region was selected in this study. A pair of universal primer, ITS1 and ITS4, was used for PCR amplification and DNA sequencing. In the case of herbarium specimens, PCR products were not detected at the firstt amplification. A pair of internal primer was designed and nested PCR was required. Successful amplification was detected at the second round. The full length of ITS region (ITS 1, 5.8S rDNA, and ITS 2) of *Rauvolfia* species was varied among species ranging from 615 bp to 631 bp. Phylogenetic relationship analyzed by parsimony analysis showed that the four species, *R. serpentina, R. cambodiana*, *R. sumatrana,* and *R. verticillata*, formed a big group. *R. micrantha* was placed in another group as an individual branch. From the ITS sequences, *R. serpentina* was more closely related to *R. sumatrana*, *R. cambodiana*, and *R. verticillata* than to *R. micrantha*.

In the second step, PCR amplification of two small regions of rDNA was obtained as follows: 1) regions of ITS 1 and partial 5.8S rDNA with 448 - 457 bp in length and 2) partial 5.8S rDNA and ITS 2 region with 432-449 bp in length.

In the last step, restriction enzymes, *Sty*I, *Eag*I, and *Dde*I, were selected to digest the small fragments of rDNA generated as described above. Patterns of three enzyme digestions could be used to identify *Rauvolfia* species in Thailand. Combination of the second step and the final step was called PCR-RFLP. Five crude drugs in this study were identified by this method. All of them had restriction patterns similar to

R. serpentina. There is high possibility that they are *R. serpentina* but not *R. cambodiana, R. micrantha, R. verticillata*, and *R. sumatrana*.

Based on results from this study, it is reasonable to say that ITS region sequence established in this study is useful to discriminate *Rauvolfia* species in Thailand. PCR-RFLP technique developed here has been proved as a powerful tool for the identification of *Rauvolfia* species and authentication of rauwolfia crude drugs.

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APPENDICES

APPENDIX A

Figure A 1 Specimen of *Rauvolfia cambodiana* Pierre ex Pit. (RCb02) used in this study.

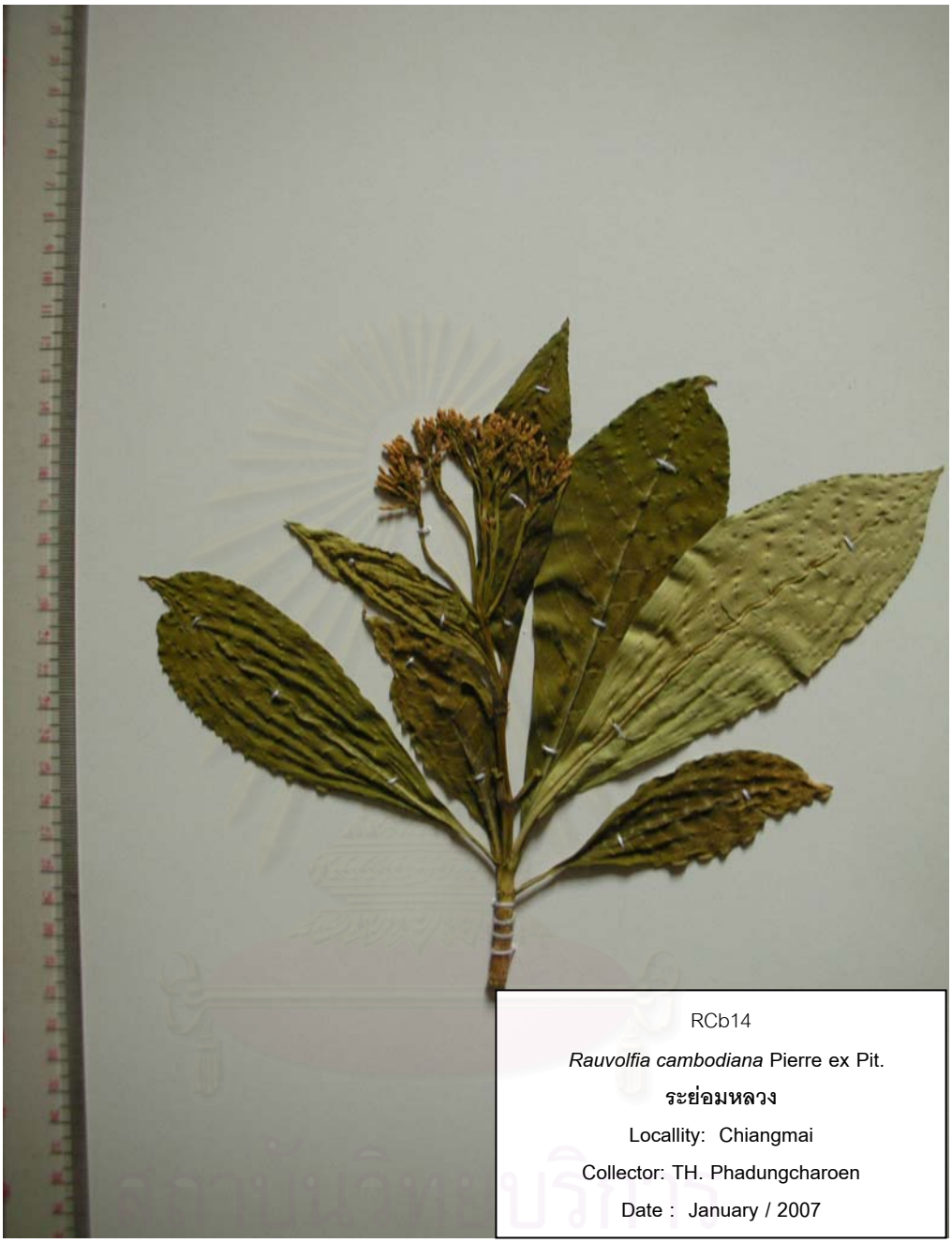

Figure A 2 Specimen of *Rauvolfia cambodiana* Pierre ex Pit. (RCb14) used in this study.

Figure A 3 Herbarium specimen of *Rauvolfia cambodiana* (RCb19) from The Forest Herbarium.

Figure A 4 Herbarium specimen of *Rauvolfia cambodiana* (RCb20) from The Forest Herbarium.

Figure A 5 Herbarium specimen of *Rauvolfia cambodiana* (RCb21) from The Forest Herbarium.

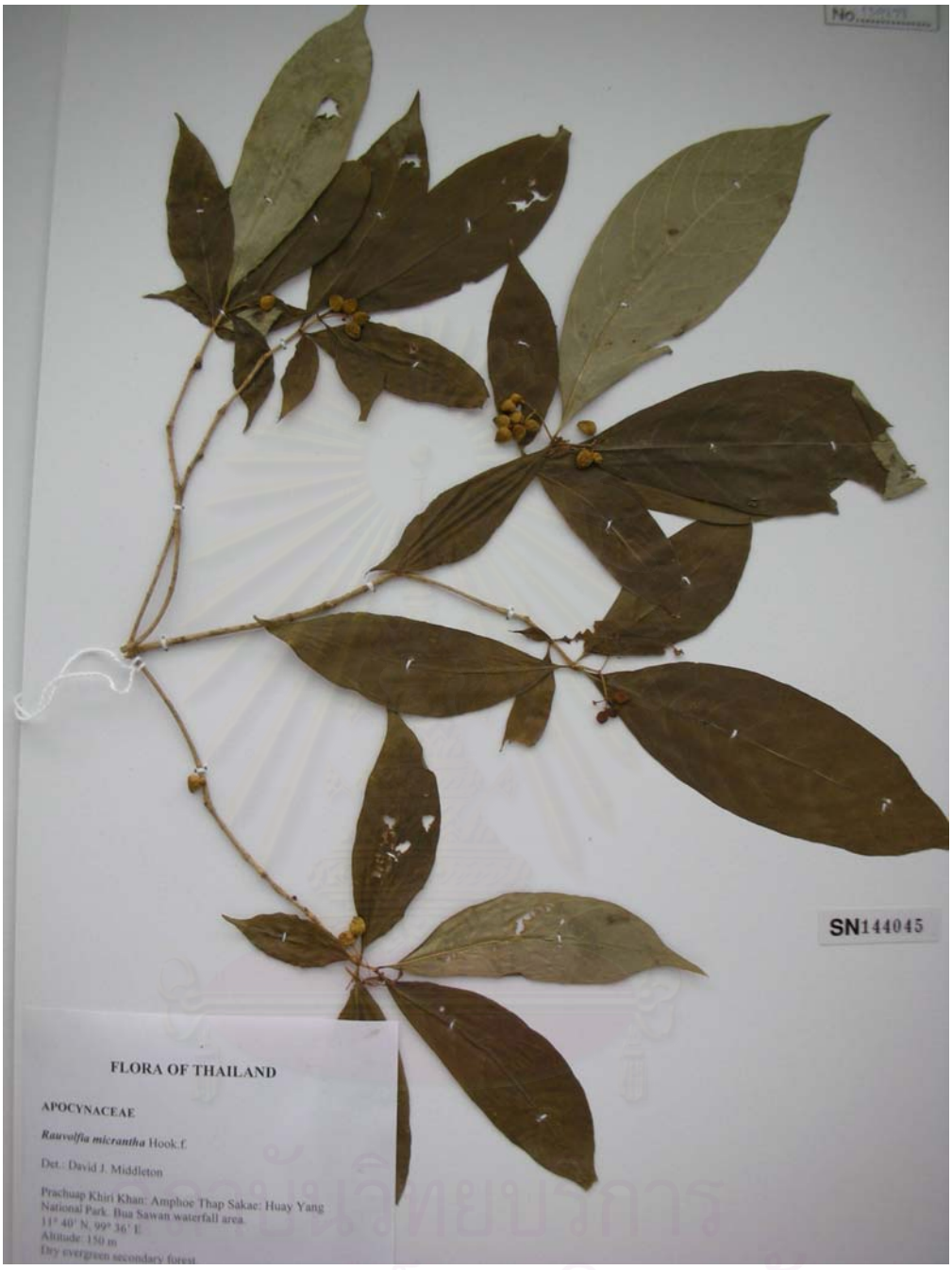

Figure A 6 Herbarium specimen of *Rauvolfia micrantha* (RMc 22) from The Forest Herbarium.

Figure A 7 Herbarium specimen of *Rauvolfia micrantha* (RMc 23) from The Forest Herbarium.

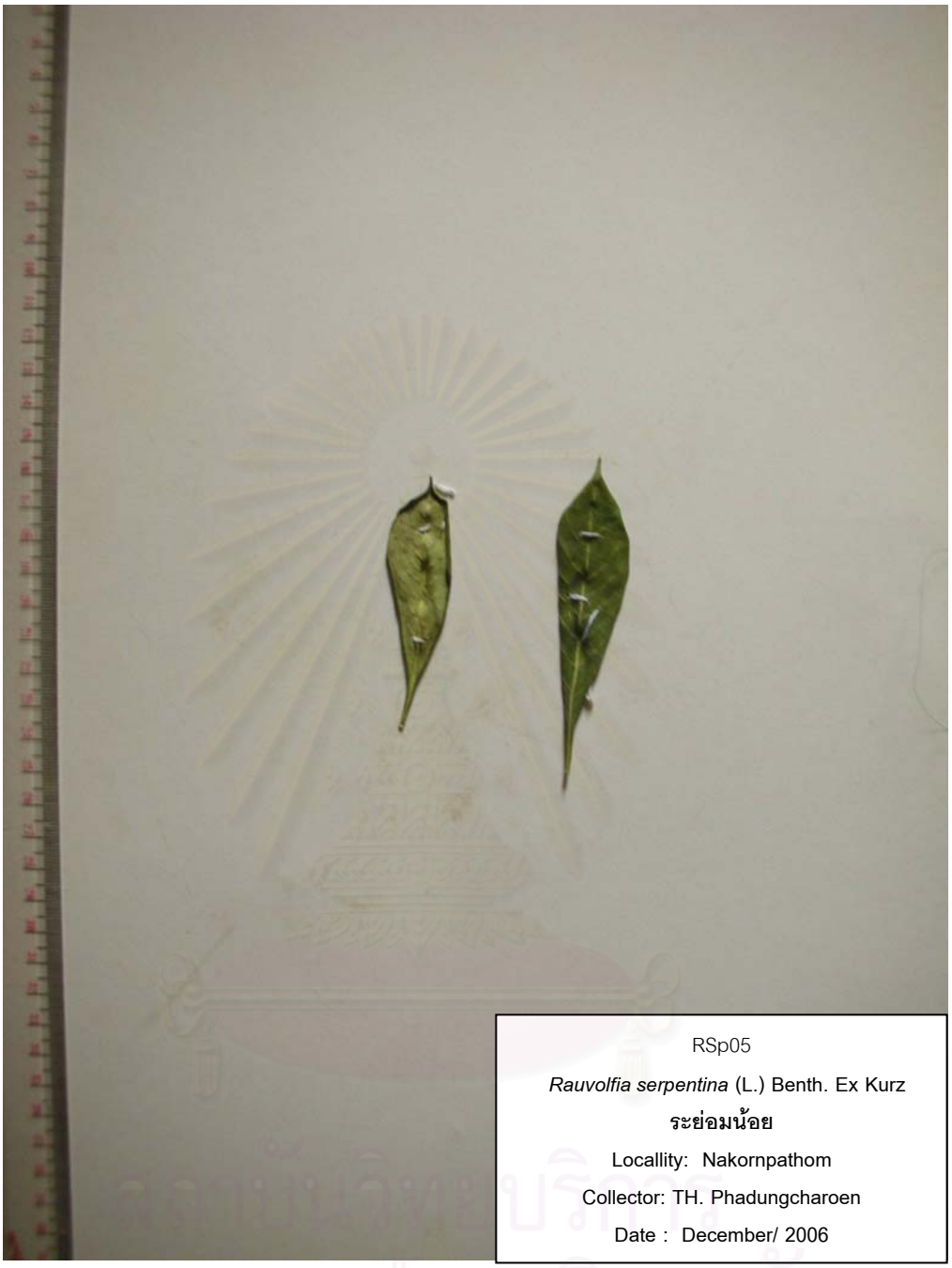

Figure A 8 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp05) used in this study.

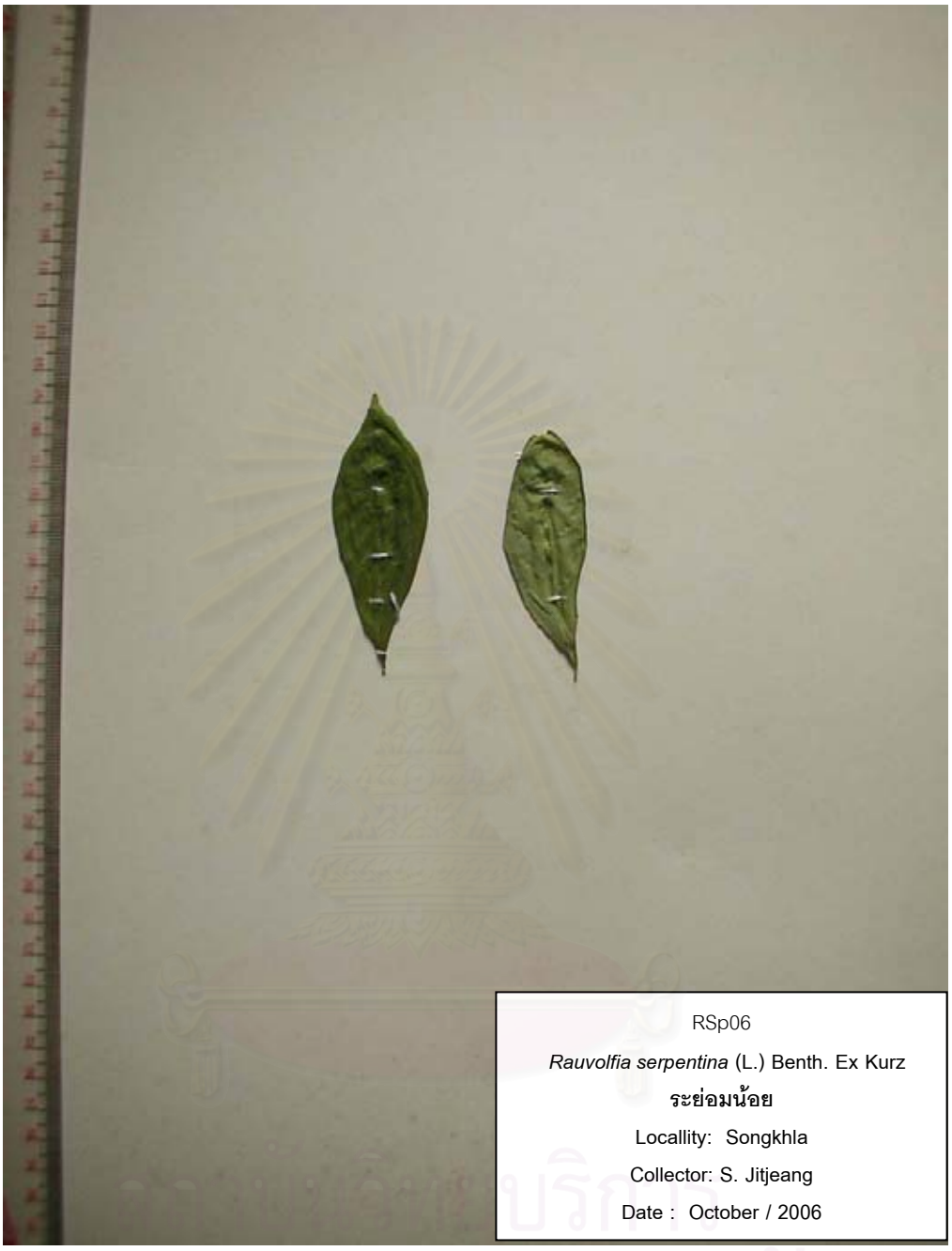

Figure A 9 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp06) used in this study.

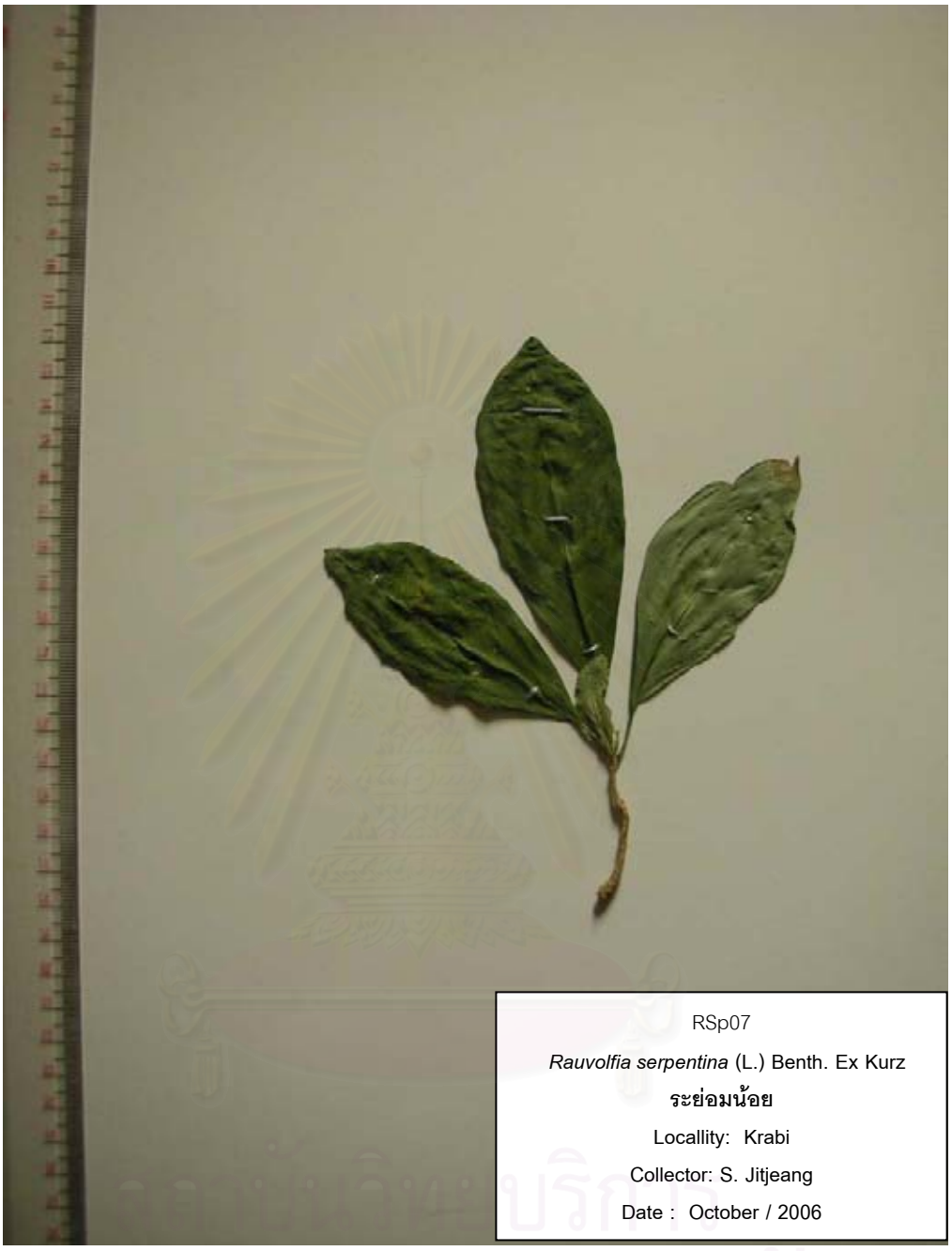

Figure A 10 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp07) used in this study.

Figure A 11 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp08) used in this study.

Figure A 12 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp09) used in this study.

Figure A 13 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp10) used in this study.

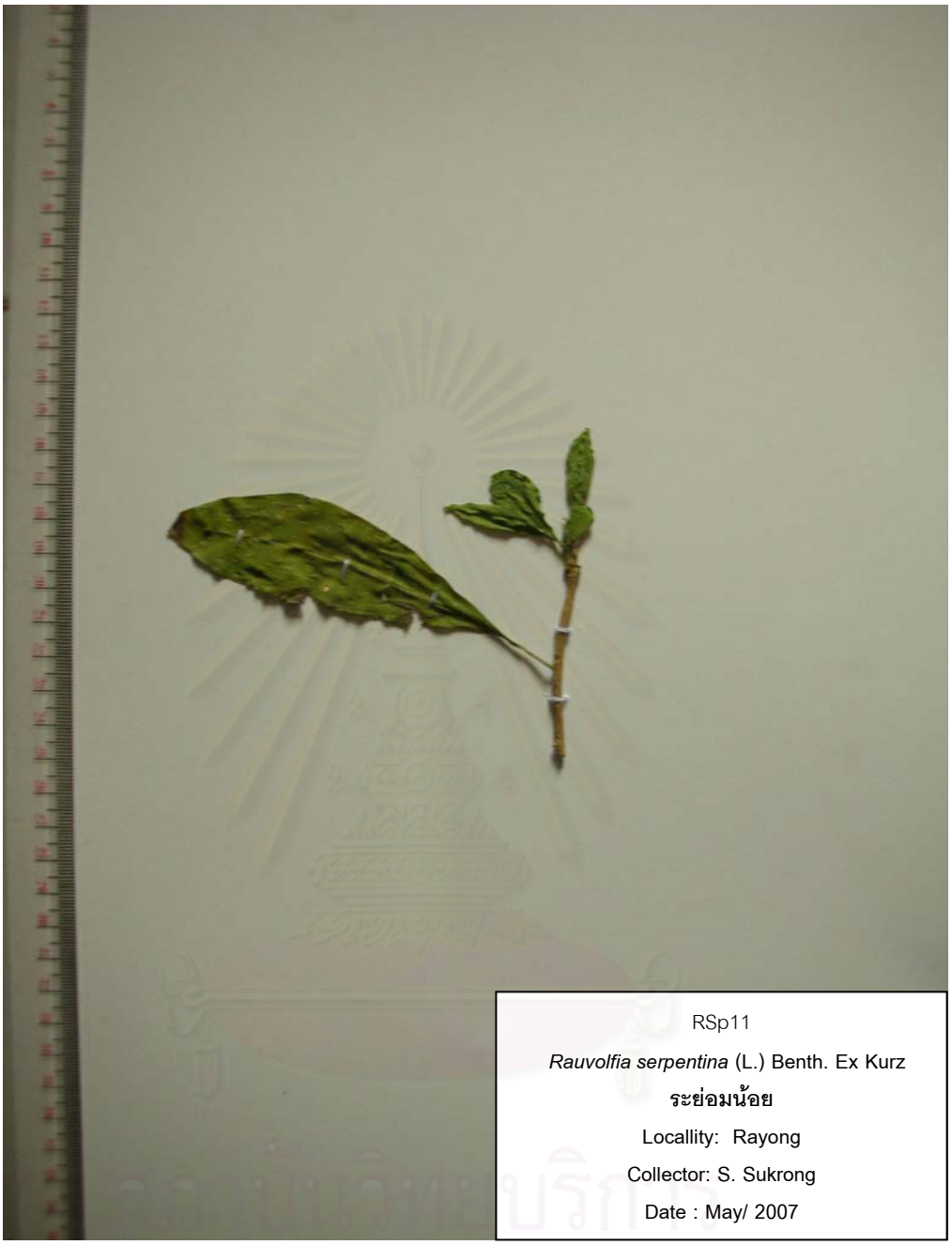

Figure A 14 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp11) used in this study.

Figure A 15 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp17) used in this study.

Figure A 16 Specimen of *Rauvolfia serpentina* (L.) Benth. Ex Kurz (RSp29) used in this study.

Figure A 17 Specimen of *Rauvolfia sumatrana* Jack (RSm12) used in this study.

Figure A 18 Specimen of *Rauvolfia sumatrana* Jack (RSm13) used in this study.

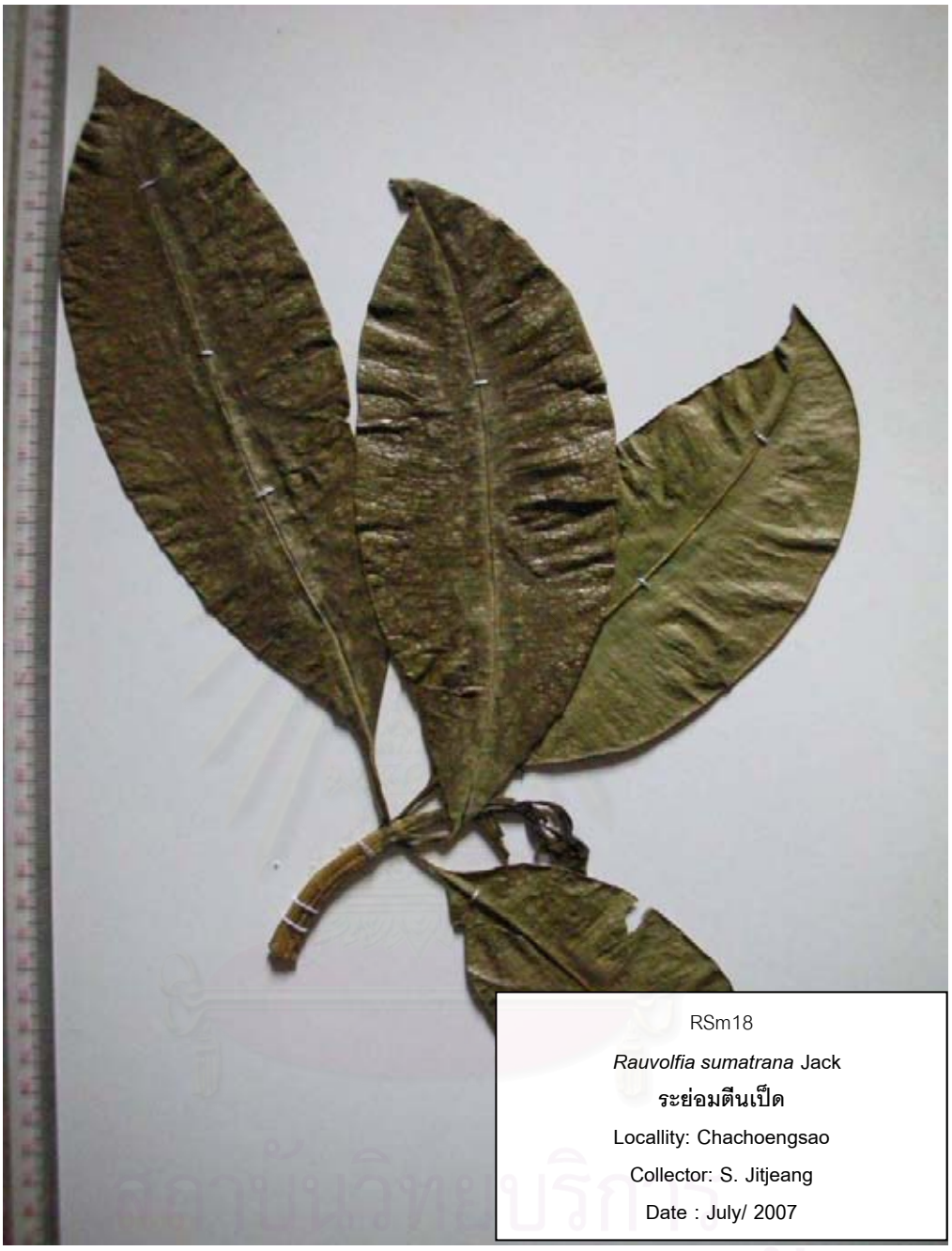

Figure A 19 Specimen of *Rauvolfia sumatrana* Jack (RSm18) used in this study.

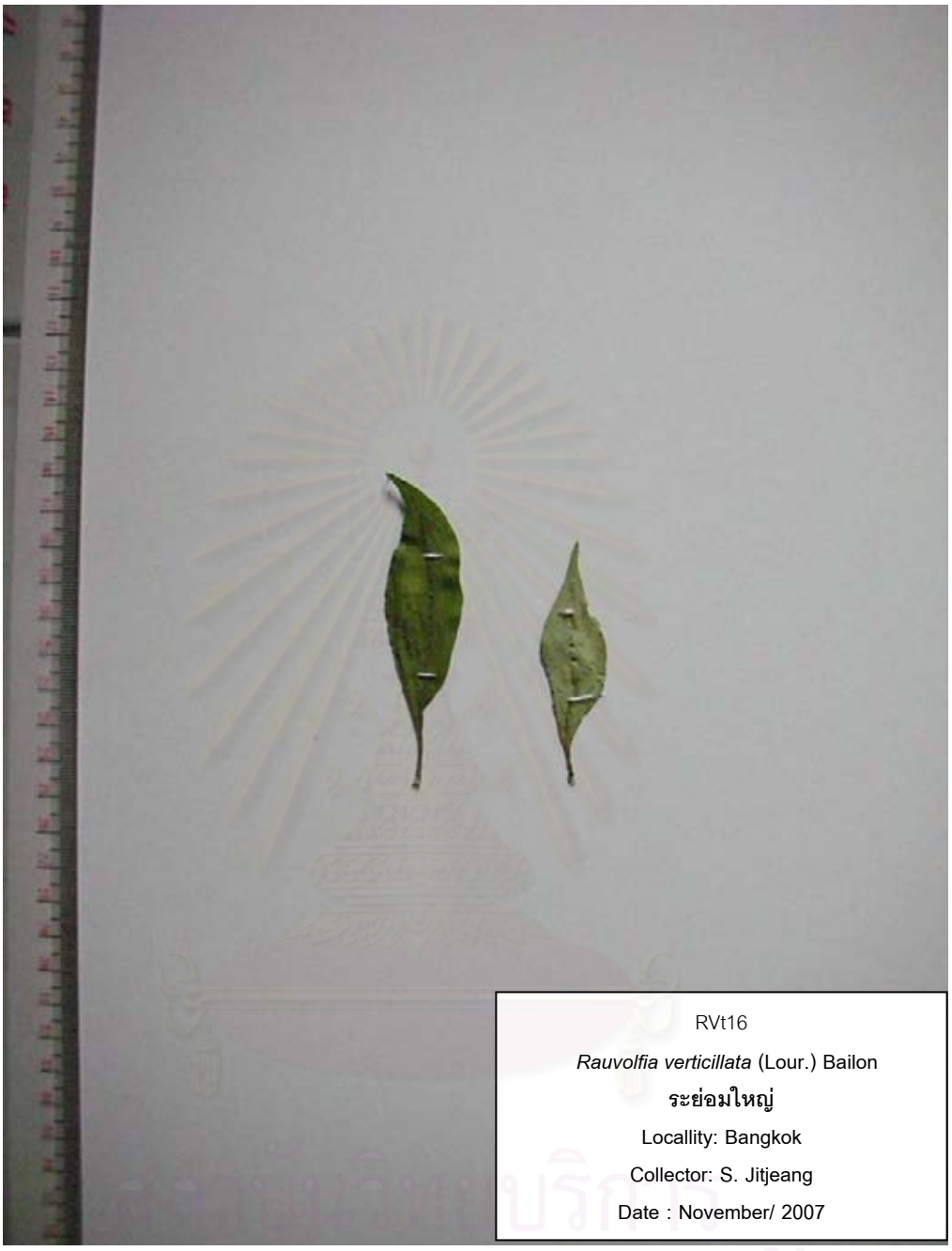

Figure A 20 Specimen of *Rauvolfia verticillata* (Lour.) Bailon (RVt16) used in this study.

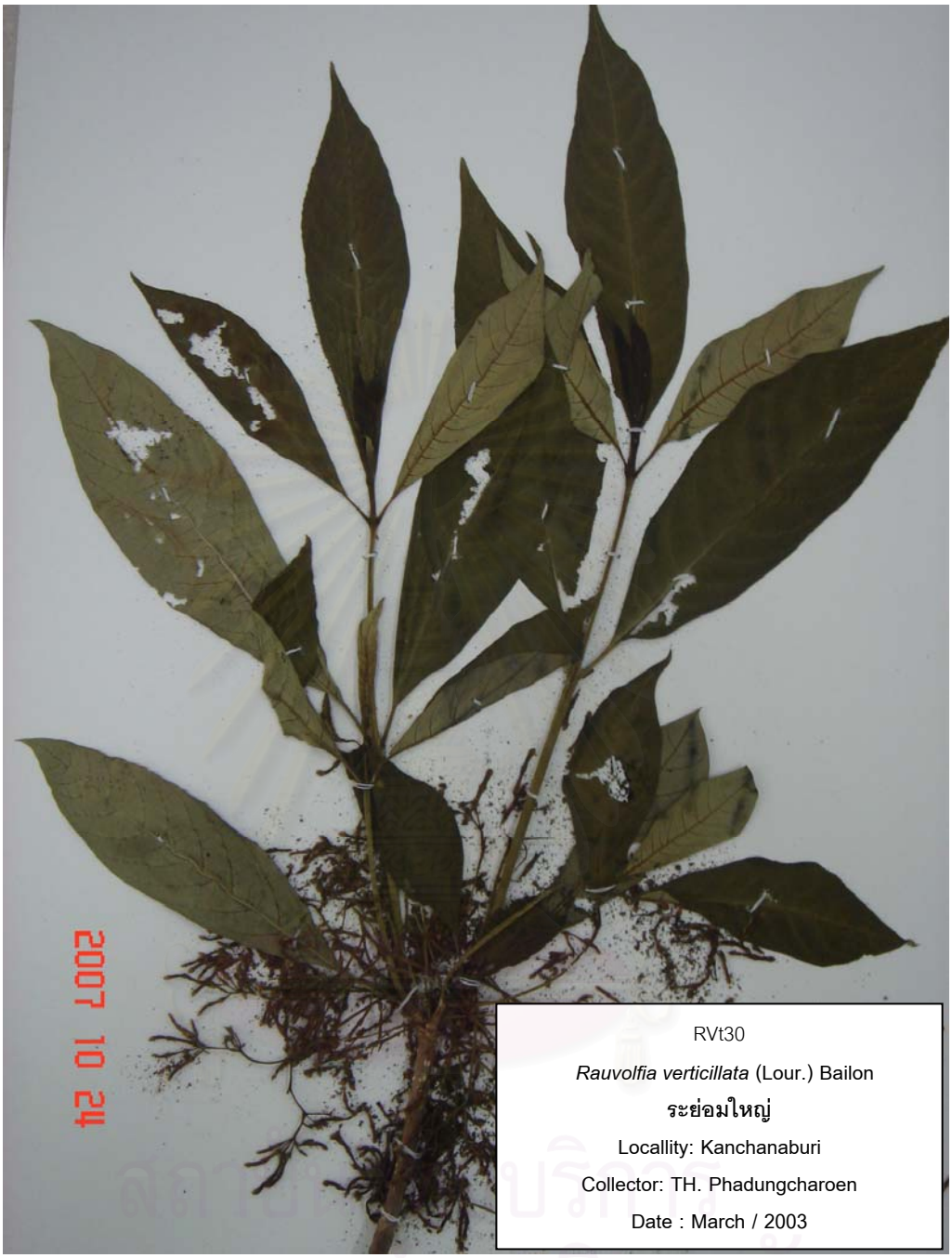

Figure A 21 *Rauvolfia verticillata* (Lour.) Bailon (RVt30)

APPENDIX B

Data of DNA sequences which were submitted to GenBank database

1. *Rauvolfia serpentina* (accession no. AB376673)

FEATURES Location/Qualifiers

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/collection_date="10-NOV-2006"

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/identified_by="Thatree Phadungcharoen"

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/organism="Rauvolfia serpentina"

misc_RNA 1..229

/note="internal transcribed spacer 1"

rRNA 230..418

/product="5.8S ribosomal RNA"

misc_RNA 419..628

/note="internal transcribed spacer 2"

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61 tcgggctccc gtcgtccccc tcggccggcc ggtgccttcg ggctcccggt cgtgccgaac

121 gaaccaaccc cggcgcggaa agcgccaagg actactaaaa cggactgcct tccctcggcc

181 ggcccgttcg cggtgctggt cgtgggatct aaggcgcctg tcgaatccaa aacgactctc

241 ggcaacggat atctaggctc tcgcatcgat gaagaacgta gcaaactgcg atacttggtg

301 tgaattgcag aatcccgtga accatcgagt ttttgaacgc aagttgcgcc caaagccagt

361 aggccgaggg cacgtctgcc tgggcgtcac gcatcgcgtc gccctcccca cccctcggcc

421 ttaaacggac gaggccggtg cgggagagcg gagactggcc tcccgtgcta ggtgcgcggt

481 tggcctaaac cttggtcccc cgtcgcggac tgcacgacaa gtggtggttg aatccctcaa

541 ctcgaatgcg agtcgtgcct gaacccgcgg tcgaggaagc ctaacgaccc ccctgcgagc

601 cccttcccga aggagcgctt gccacgac

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2. *Rauvolfia cambodiana* (accession no. AB376674)

FEATURES Location/Qualifiers

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/collection_date="10-OCT-2006"

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/note="internal transcribed spacer 1"

rRNA 229..417

/product="5.8S ribosomal RNA"

misc_RNA 418..627

/note="internal transcribed spacer 2"

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61 tcgggcgccc gtcgtccccc cggccggccg gtgccttcgg gttccctgcc gtgccgaaca

121 acaaaccccg gcgcggaaag cgccaaggac tactgaaacg gactgccttc cctcggctct

181 gcccgttcgc ggtgctggcc gtgggagcga aggcgcctgt cgaatccaaa acgactctcg

241 gcaacggata tctaggctct cgcatcgatg aagaacgtag caaactgcga tacttggtgt

301 gaattgcaga atcccgtgaa ccatcgagtt tttgaacgca agttgcgccc gaagccatta

361 ggccgagggc acgtctgcct gggcgtcacg catcgcgtcg ccctccccac ccctcgacct

421 taaacggacg gggccggtgc gggggggcgg agactggcct cccgtgctgc gcgcgcggcc

481 ggcctaaacc ttggtccccc gtcgcggact tcacgacaag tggtggttga aaccctcaac

541 tcgaatgcga gtcgtgccgg aacccgcggc cggggaggcc gaacgacccc ccagcgagcc

601 ccttcccgaa ggagcgcttg ccacgac

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3. *Rauvolfia verticillata* (accession no. AB376675)

FEATURES Location/Qualifiers

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rRNA 232..420

/product="5.8S ribosomal RNA"

misc_RNA 421..631

/note="internal transcribed spacer 2"

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1 tcgaaatcct gcaaagcaaa cgacccgcga aattgtttyt aactgcgggc gccgggtgcc

61 ttagggcgcc cgtcgtcccc ccggccggcc ggtgcgcctt ygggttyccg gccgtgccga

121 acaacaaamc ccggcgcgga aagcgccaag gactactgaa acggrctgcc ttycctyggy

181 tctgcccgtt cgcggtgctg gccgtgggag cgaargcgcc tgtygaatyc aaaacgactc

241 tyggcaamgg atatctaggs tctcgcatcg atgaagaacg tagcaaactg cgatacttgg

301 tgtgaattgc agaatcccgt gaaccatcga gtttttgaac gcaagttgcg cccgaagcca

361 ttaggccgag ggcacgtctg cctgggsgtc acgcatcgcg tcgccctccc cgcccctyga

421 ccttaaacgg acgaggccgg tgcgggggggg cggararatt ggcctcccgt gctgcgcgcg

481 cggccggccc aaaccttggt cccccgtcgc ggayttcacg acaatggtgg ttgaaaccct

541 caactcraat gcgagtcgtg ccggaacccg cggccggrga ggccggacga ccccccagcg

601 agcccyttcc cgaaggagcg ttcgccacga c

//

4. *Rauvolfia sumatrana* (accession no. AB376676)

FEATURES Location/Qualifiers

source 1..628

/collected_by="Suchada Sukrong"

/collection_date="26-DEC-2006"

/country="Thailand: Songkhla province"

/identified_by="Suchada Sukrong"

/mol_type="genomic DNA"

/organism="Rauvolfia sumatrana"

misc_RNA 1..229

/note="internal transcribed spacer 1"

rRNA 230..418

/product="5.8S ribosomal RNA"

misc_RNA 419..628

/note="internal transcribed spacer 2"

ORIGIN

1 tcgaatcctg caaagcaaac gaccggcgaa cttgtttcta actgtggccg ccgggtgcct

61 tcgggctccc gtcgtccccc tcggccggcc ggtgccttcg ggttcccggt cgtgccgaac

121 aaaccaaccc cggcgcggaa agcgccaagg accacaaaaa cggactgcct tccctcgatc

181 ggcccgttcg cggtgctggt cgcgggatct aaggcgcctg tcgaatccag aacgactctc

241 ggcaacggat atctaggctc tcgcatcgat gaagaacgta gcaaactgcg atacttggtg

301 tgaattgcag aatcccgtga accatcgagt ttttgaacgc aagttgcgcc cgaagccatt

361 aggccgaggg cacgcctgcc tgggcgtcac gcatcgcgtc gccctcccca gccctcgtcc

421 tcaaacggac gaggccgttg cgggagagcg gagactggcc tcccgtgcta ggcgcgcggt

481 tggcctaaac cttggtcccc cggcgcggac tgcacgacaa gtggtggttg aatccctcaa

541 ctcgaatgcg agccgtgcct gaacccgcgg ccggggaagc ctaacgaccc ccccgcgagc

601 cccttcccga aggagcgctt gccacgac

//

5. *Rauvolfia micrantha* (accession no. AB376677)

FEATURES Location/Qualifiers

source 1..615

/collected_by="David J. Middleton"

/collection_date="08-AUG-2002"

/country="Thailand: Phetchaburi province"

/identified_by="David J. Middleton"

/mol_type="genomic DNA"

/organism="Rauvolfia micrantha"

misc_RNA 1..231

/note="internal transcribed spacer 1"

rRNA 232..420

/product="5.8S ribosomal RNA"

misc_RNA 421..615

/note="internal transcribed spacer 2"

ORIGIN

1 tcgatgcctc aatcaatttg acctgcgaat ttgtttagac acgcggcgtt ggcttcaggt

61 caaaggcctc agcctcgcct cggcaaggag ggggtcgtgc ctcgcgcgac ttcgtccttg

121 ccaaaacaca aaccccggcg catcgtgcgc caaggaacat gaaactgtta atgatgcgct

181 cctggacccg gagacggtgt tcttgcgagt gttgtcgtga cacattacac aaaatgactc

241 tcggcaacgg atatctcggc tcttgcatcg atgaagaacg tagcgaaatg cgatacttgg

301 tgtgaattgc agaatcccgt gaaccatcga gtctttgaac gcaagttgcg cctgaagcca

361 ttaggctgag ggcacgcctg cctgggtgtc acaaatcgtt gccccaacgc caatgcctga

421 ccaaacaagg tgttgacggg gcgaatgttg acctcccgca agcactgcct tgtggttggt

481 tgaaaaacga gtcctgcatg gagagcccca tgataaatgg tggttgagta acactcgaga

541 ccgatcatgt gtggcctttc ctcaagtcaa gcaagactga cgtgaccctc gtgttctttt

601 gaacgctcat aacga

//

VITA

Miss Suratchanee Jitjeang was born on April 24, 1979 in Trang, Thailand. In 2002, she graduated with a Bachelor's Degree of Science in Pharmacy from the Faculty of Pharmaceutical Sciences, Prince of Songkhla University.

History of working

April, 2002 – May, 2003 : Pharmacist in Srisakate Hospital, Srisakate province.

June, 2003 – December, 2003: Pharmacist in Samroiyod Hospital, Prachuabkerekhun province.

January, 2004 – March, 2005 : Pharmacist in Boot retail, Thailand.

April, 2005 – May, 2006 : Pharmacist in Wattanapat hospital, Trang province.