การวิเคราะห์ลายพิมพ์ดีเอ็นเอของพืชสกุลสเตโมนาในประเทศไทย

นายบุญดิศย์ วงศ์ศักดิ์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเวท ภาควิชาเภสัชเวท
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

DNA FINGERPRINT ANALYSIS OF *STEMONA* IN THAILAND

Mr. Boonyadist Vongsak

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
Chulalongkorn University
Academic Year 2007
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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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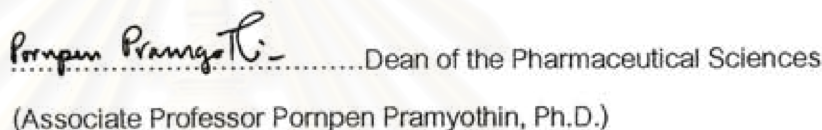
Accepted by the Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree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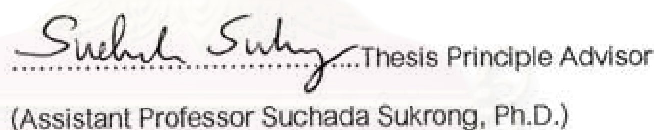
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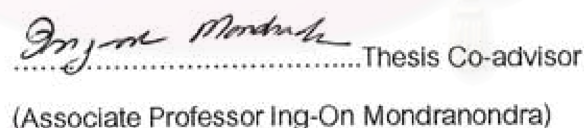
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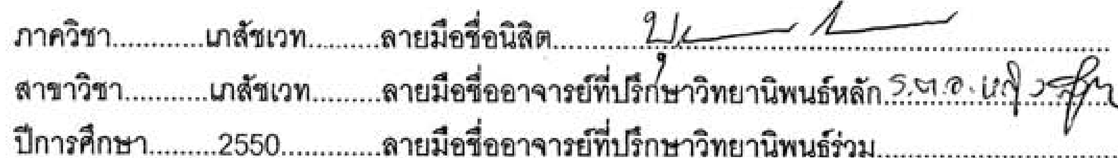
บุญดิศย์ วงศ์ศักดิ์ : การวิเคราะห์ลายพิมพ์ดีเอ็นเอของพืชสกุลสเตโมนาในประเทศไทย
(DNA FINGERPRINT ANALYSIS OF *STEMONA* IN THAILAND)
อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ร.ต.อ.หญิง ดร. สุชาดา สุขหร่อง,
อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. อิงอร มันทรานนท์, 127 หน้า.

ในประเทศไทยมีการรายงานถึงพืชในสกุลสเตโมนาจำนวนหลายชนิด ซึ่งมีฤทธิ์ทางชีวภาพที่แตกต่างกันไปในการฆ่าหนอนแมลง ไล่หนอนแมลง และการต้านมะเร็ง ดังนั้นการพิสูจน์เอกลักษณ์อย่างถูกต้องแม่นยำของพืชสกุลสเตโมนาจึงจำเป็นอย่างยิ่งต่อการประกันประสิทธิผลของสมุนไพร การพิสูจน์เอกลักษณ์ของสเตโมนาแต่ละชนิดโดยอาศัยลักษณะทางสัณฐานวิทยาเพียงอย่างเดียวกระทำได้ยากเนื่องจากพืชในสกุลนี้มีลักษณะคล้ายกันและมักจะถูกขายเป็นเครื่องยาที่สูญเสียลักษณะดั้งเดิมไป วัตถุประสงค์ของงานวิจัยชิ้นนี้จึงเป็นการวิเคราะห์ลายพิมพ์ดีเอ็นเอของพืชสกุลสเตโมนาในประเทศไทยซึ่งได้แก่ *Stemona tuberosa* Lour., *S. collinsae* Craib, *S. phyllantha* Gagnep., *S. burkillii* Prain, *S. aphylla* Craib และ *Stemona* sp. นอกจากนี้ยังพัฒนาเป็นเครื่องหมายทางพันธุกรรมชนิดพีซีอาร์-อาร์เอฟแอลพี (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) เพื่อใช้ประโยชน์ในการพิสูจน์เอกลักษณ์

ลำดับนิวคลีโอไทด์ของดีเอ็นเอสามบริเวณได้แก่ แม็ทเค, ทรานเอ็ชถึงพีเอสบีเอ และไอทีเอส1 จึงถูกนำมาใช้เพื่อพิสูจน์เอกลักษณ์พืชสกุลสเตโมนาทั้งหกชนิดในประเทศไทย จากผลการเปรียบเทียบลำดับนิวคลีโอไทด์ของยีนแม็ทเคบางส่วน สามารถจำแนกพืชสกุลสเตโมนาออกเป็นสองกลุ่มคือ กลุ่มของ *S. tuberosa* และกลุ่มของ *S. collinsae* ซึ่งสัมพันธ์กับลักษณะทางสัณฐานวิทยาและสารเคมีที่เป็นองค์ประกอบ ส่วนบริเวณทรานเอ็ชถึงพีเอสบีเอ และไอทีเอส1 สามารถใช้พิสูจน์เอกลักษณ์เพื่อแยกพืชสกุลสเตโมนาออกเป็นแต่ละชนิดได้ นอกจากนี้จากความแตกต่างระหว่างลำดับนิวคลีโอไทด์ของยีนแม็ทเค และไอทีเอส1 สามารถสร้างเครื่องหมายทางพันธุกรรมชนิดพีซีอาร์-อาร์เอฟแอลพีซึ่งให้รูปแบบการตัดด้วยเอนไซม์ตัดจำเพาะที่แสดงลักษณะลายพิมพ์ดีเอ็นเอที่แตกต่างกันของพืชสกุลสเตโมนาในแต่ละชนิดและสามารถประยุกต์ใช้ในการพิสูจน์เอกลักษณ์เครื่องยาได้

ผลการวิจัยแสดงให้เห็นว่าลำดับนิวคลีโอไทด์ดังกล่าวสามารถนำมาใช้พิสูจน์เอกลักษณ์พืชสกุลสเตโมนาทั้งหกชนิดที่มีในประเทศไทย เครื่องหมายทางพันธุกรรมพีซีอาร์-อาร์เอฟแอลพีที่พัฒนาขึ้นมานี้สามารถนำไปใช้เป็นวิธีการที่สะดวกในการพิสูจน์เอกลักษณ์พืชสกุลสเตโมนาและประยุกต์ใช้กับเครื่องยาที่ขายในท้องตลาดได้

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



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สาขาวิชา...........เภสัชเวท.........ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก..........................
ปีการศึกษา.........2550.............ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม..........................

// 4976575033 : MAJOR PHARMACOGNOSY
KEY WORD : *STEMONA* / DNA FINGERPRINT / PCR-RFLP / IDENTIFICATION

BOONYADIST VONGSAK : DNA FINGERPRINT ANALYSIS OF *STEMONA* IN THAILAND.
THESIS PRINCIPAL ADVISOR : ASST. PROF. SUCHADA SUKRONG, Ph.D., THESIS COADVISOR : ASSOC. PROF. ING-ON MONDRANONDRA, 127 pp.

In Thailand many species of *Stemona* were reported and biological activities such as insecticide, insect repellency and anti-tumor were different. Thus, accurate identification of *Stemona* is needed in order to ensure their efficacies. The identification based on morphological characters of each species alone is difficult because the morphology of *Stemona* is similar and they are often sold as crude drug which lose their original feature. The purpose of this study was to analyze DNA fingerprint of six *Stemona* in Thailand; *S. tuberosa* Lour., *S. collinsae* Craib, *S. phyllantha* Gagnep., *S. burkillii* Prain, *S. aphylla* Craib and *Stemona* sp., and developed PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) as a genetic marker in order to use as a convenient tool for identification.

The nucleotide sequences of three DNA regions; *mat*K, *trn*H-*psb*A and ITS1, were exploited to identify these six *Stemona* species in Thailand. A result of the comparison of partial *mat*K could be classified *Stemona* into two groups, *S. tuberosa* group and *S. collinsae* group, concerning with their morphology and chemical composition. The nucleotide sequences of *trn*H-*psb*A and ITS1 could be used to discriminate six *Stemona* species in Thailand. On the basis of difference among the partial *mat*K gene and ITS1 region, the PCR-RFLP analysis was performed. The restriction patterns showed distinct and polymorphic fingerprints among *Stemona* spp. and were able to apply for crude drug identification.

These results exhibited that the obtained nucleotide sequences could be used to identify *Stemona* in Thailand. PCR-RFLP genetic marker developed here could be used as a convenient tool for authentication of *Stemona* and also be applied to their commercial crude drugs.

Department : ......Pharmocognosy ....Student's signature : ......B. Vongsak......
Field of study : ...Pharmocognosy.....Principle advisor's signature : ......Suchada Sukrong......
Academic year : ....2007.............Co-advisor's signature : ....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to Assist. Prof. Dr. Suchada Sukrong, my thesis advisor, for her guidance, valuable suggestions, encouragement, patience, supports throughout my study. I thank her also for providing me an opportunity to grow as a good human.

My deepest gratitude and appreciation is also expressed to Assoc. Prof. Ing-On Mondranondra, my thesis co-advisor, for her great helps, guidance, valuable suggestions.

I would like to express heartfelt respect and sincere gratitude to Assoc. Prof. Dr. Srunya Vajrodaya, Assoc. Prof. Thatree Phadungcharoen and Assist. Prof Surapong Kengtong for kindness in collecting specimens and suggestions in my investigation.

Moreover, I would like to thank Assoc. Prof Dr. Kittisak Likhitwitayawuid and all of teachers in department of pharmacognosy for helpful discussion, and valuable suggestions for improvements.

My thanks are also extended to many people who have supported the specimens for their kindness, great helps in collecting specimens, and the staffs of phylogenetics laboratory in BIOTEC central research unit for providing valuable data in my thesis. I am especially thanks to Miss Woraluk Yodpetch for her great helps, advice, and good teaching in special techniques used in this study.

I thank everyone in the Department of Pharmacognosy and other persons whose names have not been mentioned here for helping me get through the difficult times, and for all the emotional support, entertainment, and friendships. In addition, I also thank the financial supports from the Graduate School of Chulalongkorn University (the 90th Anniversary of Chulalongkorn University-the Golden Jubilee Fund).

Finally, and most important, I take deepest pleasure in thanking my parents, my grand-mothers, grand-fathers, and my brother, who always love, care understanding and support me. This thesis and this degree are dedicated to them.

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LIST OF ABBREVIATIONS

18S rDNA	18S ribosomal RNA gene
A, T, G, C	Nucleotides containing the base adenine, thymine, guanine and cytosine, respectively
bp	Base pairs
°C	Degree Celcius
CI	Consistency index
cm	Centimeter
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
ddNTPs	Dideoxynucleotide triphosphates (ddATP, ddTTP, ddGTP, ddCTP)
EDTA	Ethylenediamine tetra acetic acid
ETS	External transcribed spacer
HCl	Hydrochloric acid
IGS	Intergenic spacer
ITS	Internal transcribed spacer
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
kb	Kilobase
*mat*K gene	Gene encoding maturase K
ME	Minimum evolution
$MgCl_2$	Magnesium chloride
ML	Maximum likelihood
ml	Milliliter
mM	Millimolar

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MP	Maximum parsimony
mtDNA	Mitochondrial DNA
NA	Not available
*ndh*F gene	Gene encoding NADH dehydrogenase F
nDNA	Nuclear DNA
ng	Nanogram
NJ	Neighbor joining
PAUP	Phylogenetic analysis using parsimony
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
RFLP	Restriction fragment length polymorphism
RI	Retention index
RNA	Ribonucleic acid
$tRNA^{Lys}$	Transfer RNA of Lysine
*trn*K gene	Gene encoding $tRNA^{Lys}$
UPGMA	Unweighted pair group method with arithmetic averages
μg	Microgram
μl	Microliter
μM	Micromolar
UV	Ultraviolet
V	Volt

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CHAPTER I

INTRODUCTION

Stemona contains approximately 20 species and represents the largest genus of the small monocotyledonous family Stemonaceae (Duyfjes, 1993). Many species prefer a seasonal climate and occur as perennial climbers or subshrubs with tufted tuberous roots in rather dry vegetation ranging from continental Asia and Japan through Southeast Asia to tropical Australia (Gagnepain, 1934; Duyfjes, 1993; Tsi *et al.*, 2000). In Thailand, many species of *Stemona*; *S. aphylla* Craib (เครือปุง), *S. burkillii* Prain (โป่งมดง่าม), *S. collinsae* Craib (หนอนตายหยาก), *S. phyllantha* Gagnep. (สามสิบกีบ), *S. tuberosa* Lour.(หนอนตายหยาก), *S. hutanguriana* W. Chuakul (สามสิบกีบน้อย), S. *cochinchinensis* Gagnep., *S. kerrii* Prain, *S. curtisii* Hook.f., were reported (Smitinand, 2001; Schinnerl *et al.*, 2007).

The roots of *Stemona* have been recommended in Thai, Chinese, Japanese, and Vietnamese traditional medicine for relieving cough and asthma and as anthelmintics, insect repellants and anti-cancer agents for a longtime (Chung *et al.*, 2003; Greger, 2006) and some are officially listed in Chinese Pharmacopoeia (2005) as antitussive traditional herbs. *Stemona* alkaloids represent a typical chemical character and so far are not detected in any other plant family (Greger, 2006). They are characterized by a pyrrolo[1,2-*a*]azepine core usually linked with two carbon chains mostly forming terminal lactone rings. To date, phytochemical investigations have led to isolation and structure elucidation of about 100 *Stemona* alkaloids from the tuberous roots of various species of *Stemona* (Greger, 2006). The underground parts are widely sold on local markets and herb-shops. However, because of the similar shapes of their tuberous roots, the same vernacular name such as "Non Tai Yak" is often used for different species of *Stemona* (Greger, 2006). Moreover, the same vernacular name is often used even for representatives from other plant families. (Kaltenegger *et al.*, 2003). This lack of proper methods for identifying plant material has already led to wide-spread confusions in the

chemical and pharmaceutical reports (Brem *et al.*, 2002; Kaltenegger *et al.*, 2003; Greger, 2006).

There were reports that *Clitoria macrophylla* Wall and *Asparagus* spp. were mistaken as *Stemona* (Brem *et al.*, 2002; Greger, 2006). The rotenoids stemonacetals were reported for *S. collinsae,* although, the likely compounds were originated from roots of the legume *Clitoria macrophylla*. The subsequent investigations found that neither rotenoids nor any other isoflavonoid derivatives have been detected in *S. collinsae* collected from different habitats (Brem *et al.*, 2002; Pacher *et al.*, 2002). The confusion could occur because *Clitoria macrophylla* were also sold under the name "Non Tai Yak" as well as *S. collinsae* (Brem *et al.*, 2002). In addition, the alkaloid asparagamine A was reported from the tuberous roots of *Asparagus racemosus* Willd. but asparagamine A was later repeatedly isolated as major compound from *S. collinsae* and named didehydrostemofoline (Seger *et al.*, 2004; Greger, 2006). The presumption that *Asparagus* has been mistaken as *Stemona* was supported by a colorimetric comparison of nine *Asparagus* collections from different provinces of China, where no alkaloids could be detected (Chong *et al.*, 1992; Greger, 2006). Furthermore, in the same genus, striking chemical differences were already observed between *S. collinsae* Craib and *S. tuberosa* Lour. leading to different biological activities, i.e. anti-tumor and insecticidal activities (Brem *et al.*, 2002; Rinner *et al.*, 2004). Thus, the usage of *Stemona* roots without proper identification passes serious problems for practical applications both in medicine and agriculture.

Therefore, accurate identification of the plant material is a prerequisite to ensure the efficacy of the tuberous roots of *Stemona*. Traditional 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 (Sahin *et al.,* 2007) and many extrinsic factors such as methods of cultivation, harvesting, drying and storing may affect the ultimate chemical profile of the herb (Schinnerl *et al.*, 2007). Moreover, medicinal plants are processed for use as crude drugs which often lose their original feature. Hence, it is difficult to determine the botanical origin of the crude drug through anatomical and chemotaxonomical studies.

DNA fingerprinting has become one of the most utilized approaches for inferring taxonomic and phylogenetic relationships and can be applied for identification of plants such as *Adenophora* (Zhao *et al.*, 2003), *Curcuma* (Xia *et al.*, 2005), *Derris* (Sukrong *et al.*, 2005), *Ephedra* (Changfeng *et al.*, 2005), *Panax* (Shim *et al.*, 2005), *Mitragyna* (Sukrong *et al.*, 2007), *Plantago* (Sahin *et al.*, 2007), etc. The chloroplast and nuclear ribosomal DNA are considered to be suitable genetic markers for analyzing the phylogenetic relationships among the species. Hence, three attractive targets, maturase gene (*mat*K), *trn*H-*psb*A region and nuclear internal transcribed spacers (ITS), were selected for molecular analysis. The *mat*K gene, a chloroplast genome encoded locus located within the intron of the chloroplast gene *trn*K, has high rates of substitution as compared to other chloroplast genes and its DNA sequence is one of the least conserved plastid genes (Fuse and Tamura, 2000; Ince *et al.*, 2005). For the plastid *trn*H-*psb*A region, there was the most variable plastid region in angiosperms and potentially usable DNA region for distinguishing plant species because of the high percentage of PCR success and ability to discrimination (Kress *et al.*, 2005). For nuclear ribosomal DNA, the regions encoding 18S-, 5.8S-, and 26S rRNA are highly conserved, whereas two internal transcribed spacers (ITS1 and ITS2) between the ribosomal RNA gene are variable and useful as possible sources of polymorphisms for plant identification (Sahin *et al.*, 2007).

To date, there have been a few nucleotide sequences of *Stemona* deposited in GenBank. Also, there are no studies which determine *mat*K, *trn*H-*psb*A and ITS sequences of *Stemona* in Thailand. In this study, the sequences of six *Stemona*; *S. tuberosa* Lour., *S. collinsae* Craib, *S. phyllantha* Gagnep, *S. burkillii* Prain, *S. aphylla* Craib and *Stemona* sp. were examined and the results would be used for their identification. Based on the differences among the sequences, the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) analysis was performed in order to develop a more convenient and efficient identification of *Stemona* species.

CHAPTER II

LITERATURE REVIEW

1. Plant Material

1.1 *Stemona* species

Stemona represents the largest genus of the small monocotyledonous family Stemonaceae (Duyfjes, 1993). They comprise about 40 species as follows: *Stemona acuta* C.H.Wright, *S. affinis* J.J.Sm., *S. angusta* I.Telford, *S. aphylla* Craib, *S. argyi* H.L., *S. asperula* J.J.Sm., *S. australiana* C.H.Wright, *S. burkillii* Prain, *S. cochinchinensis* Gagnep., *S. collinsae* Craib, *S. curtisii* Hook.f., *S. erecta* C.H.Wright, *S. filifolia* Schltr., *S. gloriosa* J.J.Sm., *S. gloriosoides* Voigt, *S. griffithiana* Kurz, *S. hutanguriana* W.Chuakul, *S. japonica* Franch. & Sav., *S. javanica* C.H.Wright, *S. kerrii* Craib, *S. kurzii* Prain, *S. lucida* Duyfjes, *S. mairei* K.Krause, *S. minor* Hook.f., *S. moluccana* Prain, *S. ovata* Nakai, *S. papuana* Schltr., *S. parviflora* C.H.Wright, *S. philippinensis* Merr., *S. phyllantha* Gagnep., *S. pierrei* Gagnep., *S. prostrata* I.R.H.Telford, *S. saxorum* Gagnep., *S. sessilifolia* Franch. & Sav., *S. shandongensis* D.K.Zang, *S. squamigera* Gagnep., *S. stenophylla* Diels, *S. sulensis* J.J.Sm., *S. tuberosa* Lour., *S. vagula* W.W.Sm., *S. versteegii* Schltr., and *S. wardii* W.W.Sm. (The International Plant Names Index, 2008).

They are distributed from continental Asia and Japan through Southeast Asia to tropical Australia (Gagnepain, 1934; Duyfjes, 1993; Tsi and Duyfjes, 2000). In Thailand, there are a number of *Stemona* species; *S. aphylla* Craib, *S. burkillii* Prain, *S. collinsae* Craib, *S. hutanguriana* W.Chuakul, *S. phyllantha* Gagnep., and *S. tuberosa* Lour., S. *cochinchinensis* Gagnep., *S. kerrii* Prain, *S. curtisii* Hook.f. (Smitinand, 2001; Schinnerl *et al.*, 2007). Many species prefer a seasonal climate and occur as perennial climbers or subshrubs with tufted tuberous roots. Stems are erect or climbing. Leaves are whorled, opposite, or alternate (Duyfjes, 1993; Tsi and Duyfjes, 2000). Macfarlane *et al.* (2002) reported reproductive form of *Stemona* as follows:

"...Inflorescence and flower features. Flowers solitary, or aggregated in 'inflorescences'; when solitary axillary. Inflorescence few-flowered. Flowers in cymes, or in racemes. The terminal inflorescence unit cymose, or racemose. Inflorescences axillary (in axils of leaves or scale leaves on apparently leafless stems). Flowers pedicellate. Pedicels articulated, the 'pedicel' above the articulation representing the slender, elongate base of the flower receptacle. *Flowers* bracteate; small, or medium-sized; regular; 2 merous; cyclic; pentacyclic. Perigone tube present, or absent. Perianth of 'tepals'; 4; 2 -whorled; isomerous (2+2); petaloid; similar in the two whorls; purple, or red to black. Androecium 4. Androecial members adnate (to the perianth); coherent (the broad stamens basally connate, with an internal extension that contacts the stigma); 1 -adelphous; 2 -whorled. Androecium exclusively of fertile stamens. *Stamens* 4; diplostemonous; more or less petaloid (due to the enlarged connectives). Filaments appendiculate (connectives much elongated above the anther cells, broad, somewhat petal-like). Anthers basifixed; dehiscing via longitudinal slits; introrse; tetrasporangiate; appendaged. The anther appendages apical (filiform, horn-like). *Pollen* shed as single grains. Gynoecium 2 carpelled. The pistil 1 celled. Carpels reduced in number relative to the perianth. Gynoecium syncarpous; synstylovarious; superior. Ovary unilocular; 1 locular. Gynoecium non-stylate, or stylate (sessile to subsessile). Styles apical. Placentation basal. Ovules in the single cavity 3–5 (i.e. 'few'); ascending; arillate; orthotropous.

Fruit and seed features. Fruit non-fleshy; dehiscent; a capsule (ovoid, somewhat compressed). Capsules two valvular. Seeds ovoid, with appendages to the long funicle; endospermic. Endosperm not ruminate; oily. *Seedling*. Hypocotyl internode absent. Mesocotyl absent. Seedling collar not conspicuous. Cotyledon hyperphyll compact; non-assimilatory. Coleoptile absent. Seedling cataphylls present. First leaf dorsiventral. Primary root persistent..."

The morphological comparisons of these species are illustrated in Table 1 (Gagnepain, 1934; Duyfjes, 1993; Tsi and Duyfjes, 2000; Wongsatit, 2000).

Table 1 Comparative morphology of *Stemona* spp. in Thailand

Characteristics	*S. tuberosa*	*S. phyllantha*	*S. collinsae*	*S. burkillii*	*S. hutangurina*	*S. aphylla*
Height (m)	Up to 4	NA	Up to 2	NA	Up to 0.3	NA
Leaves;						
Phyllotaxy	opposite	opposite and alternate	alternate	alternate	alternate	NA
shape	ovate or broadly ovate	ovate or triangular	ovate	ovate	ovate to ovate-oblong	NA
length (cm)	9-19.5	10-13	5-14	5-11	5-7.5	NA
width (cm)	3-14	NA	4-12	6.25-7.5	1.5-3.5	NA
apex	acuminate	acuminate	acuminate	acuminate	acute	NA
base	cordate	cordate	cordate	cordate	attenuate	NA
number of nerves	9-13	9	9-15	11-13	5	NA
petioles (cm)	1.5-7	1-1.2	1-6	10-15	1-3.8	NA
Inflorescence;						
bract						
shape	NA	linear-acuminate	NA	NA	linear-acuminate	linear-acuminate
length (mm)	5-15	5	10	10	1.5-2	4.5
pedicels (mm)	5-15	NA	NA	40	20-24	1.5
tepal						
color	outside green, purple or brown-red inside	NA	purple-green	outside pale green, purple or red inside	pink	NA
length (mm)	25-50	55	NA	10	7-13	25
width (mm)	4-10	7-11	NA	NA	2-2.5	55

NA = data not available

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Table 1 .Comparative morphology of *Stemona* spp. in Thailand (cont)

Characteristics	*S. tuberosa*	*S. phyllantha*	*S. collinsae*	*S. burkillii*	*S. hutangurina*	*S. aphylla*
stamen						
color	purple	NA	NA	pale green	white	NA
length (mm)	25-40	35	NA	NA	25-50	NA
anther length (mm)	8-15	NA	NA	NA	8-15	21
Seed						
number	10-20	NA	2-7	NA	2-3	NA
length (mm)	9-17	NA	NA	10	5.5-6.5	NA
width (mm)	NA	NA	NA	3	3-3.5	NA

NA = data not available

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1.2. Biological activities

The tuberous roots of *S. japonica, S. sessilifolia*, and *S. tuberosa*, known as "Radix Stemonae", have long been recommended in Chinese, Japanese, and Vietnamese traditional medicine for relieving asthma, chronic or acute cough, pulmonary tuberculosis, whooping cough and have also been used against enteric helminths and ectoparasites on humans and cattle (Pilli *et al.*, 2000; Pharmacopoeia Commission of People's Republic of China, 2005; Greger, 2006). In southeast Thailand, roots and leaves of "Non Tai Yak", are most probably derived from *S. collinsae* and traditional medical practitioners recommend them as scabicide, pediculocide, and against helmith worms (Prucksunand *et al.*, 1985; Akanitapichat *et al.*, 2005). Moreover, in leaf disk choice tests against fifth instar larvae of *Spodoptera*, strong antifeedant activity was observed for the crude extract of *S. collinsae*, whereas *S. tuberosa* clearly differed by its low toxicity but remarkable repellence (Brem *et al.*, 2002). In preliminary anti-tumor tests crude extracts of *S. tuberosa* and *S. collinsae* were compared for their effects on medullary thyroid carcinoma cells. Both extracts altered the phenotype of the cells from originally aggregating cells towards single-cell suspensions. However, the extract of *S. tuberosa* considerably enhanced apoptosis, whereas *S. collinsae* only moderately increased the apoptotic effect (Rinner *et al.,* 2004).

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2. Molecular Technique

2.1 The DNA sequencing

The DNA sequencing is one of the most informative techniques for the molecular systematic studies because nucleotide sequences directly reflect genetic information alteration. The rates and patterns of changes affect the evolution of genes and the organisms. Moreover, DNA sequences can be used for constructing the molecular phylogenetics of related organisms. 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 (Weising *et al.*, 2005).

Unlike animals, plants have three kinds of genomes, the chloroplast genomes (cpDNA) in addition to the nuclear (nDNA) and mitochondrial (mtDNA) genomes. The mtDNA is rarely used in molecular markers of plants due to its structure, size, and gene order are various depending on plant species (Kress *et al.*, 2005). The nDNA and cpDNA are commonly used to investigate in the molecular systematics and taxonomy of plants. The nDNA has more complexity and repetitive properties. On the other hand, the cpDNA is well suitable for evolutionary and phylogenetic studies because it is a relative abundant component of total DNA. In addition, it contains primarily single copy genes, and has a conservative rate of nucleotide substitution. The most common targets in cpDNA are *rbc*L, *ndh*F, *trn*H-*psb*A, *trn*K, and *mat*K, and the most common genes in nDNA is nuclear ribosomal gene; 18S rDNA, an internal transcribed spacer (ITS1), the 5.8S rDNA, a second internal transcribed spacer (ITS2), and finally the 26S rDNA (Kress *et al.*, 2005; Weising *et al.*, 2005).

2.2 Polymerase Chain Reaction (PCR)

PCR has been ingenious tool for research in molecular biology. PCR is so sensitive that only a single DNA molecule is needed and used for amplification, and single-copy genes can be routinely extracted out of complex mixtures of genomic sequences and visualized as distinct bands on agarose gels (Mullis *et al.*, 1994; and Bartlett and Sterling, 2003). In a typical PCR assay, three temperature-controlled steps can be discerned, which are repeated in a series of 25 to 50 cycles. A reaction mix consists of:

1. A buffer, usually containing Tris-HCl, KCl, and $MgCl_2$
2. A thermostable DNA-polymerase, which adds nucleotides to the 3′-end of a primer annealed to single-stranded DNA (ssDNA)
3. Four deoxyribonucleotide triphosphates [dNTPs]: dATP, dCTP, dGTP, dTTP
4. Two oligonucleotide primers
5. Template DNA

The principle of the cycling reaction is outlined in Figure 2.1. In the first step of the first cycle, the original template DNA is made single-stranded by raising the temperature to about 94 ◦C (denaturing step). In the second step, lowering the temperature to about 35 to 65 ◦C (depending on primer sequence and experimental strategy) results in primers annealing to their target sequences on the template DNA (annealing step). The primers will preferably hybridize to binding sites that are identical or highly homologous to their nucleotide sequence, although some mismatches (especially at the 5′-end) are allowed. For the third step, a temperature is chosen at which the activity of the thermostable polymerase is optimal; i.e., usually 65 to 72 ◦C (elongation step). The polymerase now extends the 3′-ends of the DNA-primer hybrids toward the other primer binding site. Because this happens at both primer-annealing sites on both DNA strands, the target fragment is completely replicated (cycle 1).

In the second cycle, the two resulting double-stranded DNAs are again denatured, and both the original strand and the product strand now act as templates. Repeating these three-step cycles 25 to 50 times results in the exponential amplification of the target amplicon between the 5′-ends of the two primer binding sites (Figure 2.1) (Weising *et al.*, 2005).

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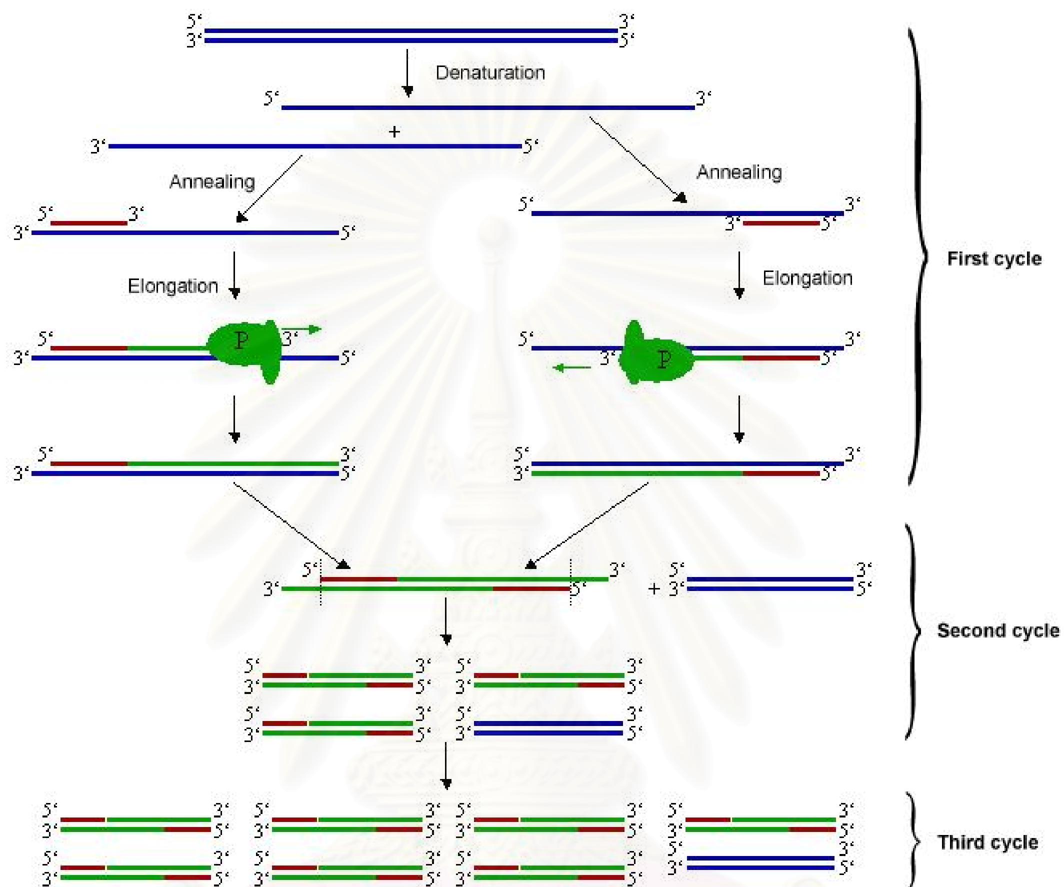

Figure 2.1 Principle of the polymerase chain reaction. A target DNA sequence is exponentially amplified with the help of flanking primers and a thermostable DNA polymerase. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing, and an elongation step. Primers are represented by red lines. In the initial stage of the reaction, both shorter and longer products are generated. Only the shortest possible fragments are amplified exponentially, and therefore the final products almost exclusively (Modified from http://www.juliantrubin.com/encyclopedia/biochemistry/pcr.html, 2008).

2.3 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) is one of the molecular markers. PCR-RFLP markers are generated in two steps. In the first step, a defined DNA sequence is amplified using a sequence-specific primer pair. This may result in differently size and hence informative PCR fragments. In the second step, the PCR product is digested with a restriction enzyme (Weising *et al.*, 2005). 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. 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.

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA. The enzyme makes two incisions, one through each of the sugar-phosphate backbones of the double helix without damaging the nitrogenous bases. The chemical bonds that the enzymes cleave can be reformed by other enzymes known as ligases, so that restriction fragments carved from different genes can be spliced together, provided their ends are complementary (Avise, 2004).

Several studies used PCR-RFLP analysis for investigations of many plants. For instance, Parducci and Szmidt (1999) used PCR-RFLP analysis of the chloroplast DNA of the genus *Abies* (family Pinaceae), to detect inter-specific variation in this genus. Xu *et al.* (2001) used PCR-RFLP for identification of wild and cultivated soybeans. Yang *et al.* (2004) developed PCR-RFLP analysis for correct identification of herbal drugs and plants of *Rheum* species. Wang *et al.* (2007) used PCR-RFLP analysis to differentiate the bulb of *Fritillaria cirrhosa* from other species of *Fritillaria* antitussive herb in China. Liu *et al.* (2007) developed a rapid and reliable PCR-RFLP method to accurately identify hybrids of *Leucadendron.*

3. DNA Regions

3.1 The *mat*K Gene

The *mat*K gene is approximately 1,500 base pairs (bp) in length (Figure 2.2) and encodes a maturase involved in splicing type II introns from RNA transcripts, located in the Large Single-Copy region (LSC) of the chloroplast genome (Figure 2.3) (Neuhaus and Link, 1987 and Wolfe *et al*., 1992). In all photosynthetic land plants so far examined, *mat*K is located within an intron of approximately 2,600 bp positioned between the 5′ and 3′ exons of the transfer RNA gene for lysine, *trn*K (Soltis *et al*., 1995).

The gene *mat*K is easily amplified using the highly conserved flanking coding regions that include the *trn*K exons. The rate of evolution of *mat*K makes this gene appropriate for resolving intergeneric or interspecific relationships in seed plants. Based on data for Saxifragaceae (Johnson and Soltis, 1995), Cornaceae (Xiang *et al*., 1998) and Taxodiaceae/Cupressaceae (Johnson and Soltis, 1995), the average numbers of nucleotide differences per site in pairwise comparisons for *mat*K are 3.2, 2.4, and 3.4 times higher, respectively, than for *rbc*L (the gene is able to encode the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase).

Figure 2.2 General map of *mat*K gene. Boxed areas represent coding regions and connecting lines represent spacer regions (Redrawn from Johnson and Soltis, 1995).

Figure 2.3 Gene map of *Dioscorea elephantipes* chloroplast genome illustrate location of many of the chloroplast regions. The arrows indicate the location of *mat*K, *psb*A and *trn*H (Hansen *et al.*, 2007).

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3.2 The *trn*H-*psb*A intergenic spacer region

The chloroplast *trn*H gene has been sequenced in different plant species, and was found to be well conserved during cpDNA evolution. This gene is usually found located near the LSC/IRA junction in higher plant chloroplast genomes, (Figures 2.3 and 2.4) such as in common bean, soybean, spinach and tobacco. It is, however, located within the inverted repeats of the rice cpDNA, and at the center of the LSC of the liverwort cpDNA. In pea and broad bean, the *trn*H gene is found downstream of the *psb*A gene (Careles *et al*, 1994). The length of the intergenic spacer between the *psb*A gene and the *trn*H gene varies from one plant to the other (Kress *et al.*, 2005).

The *trn*H-*psb*A intergeneric spacer, tested on 99 species in 80 genera from 53 plant families, was exhibited high divergence levels and easily amplified (Kress *et al.*, 2005; Rubinoff *et al.*, 2006). This spacer can also be used to test Ephedra in dietary supplements that sold in commercial markets (Techen *et al.*, 2005).

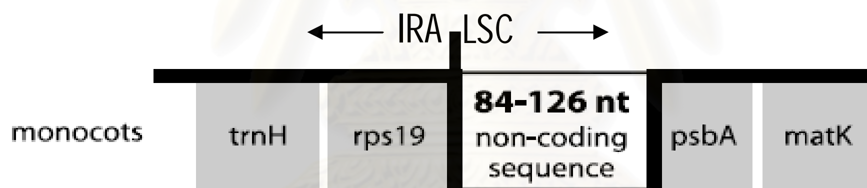

Figure 2.4 The map of *trn*H-*psb*A intergenic spacer region, rps19 could be observed in some genomes (Hansen *et al.*, 2007).

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3.3 Internal transcribed spacer (ITS)

The internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal DNA (Figure 2.5) has become an important gene locus for the molecular systematic investigation of angiosperms at the interspecific and intraspecific levels. The ITS region of rDNA, defined as the unit containing the ITS1 spacer, 5.8S rDNA gene and ITS2 spacer, has been proven to be a useful gene for screening different species of herbal medicine. For instance, ITS rDNA region is able to differentiate *Dendrobium* species (Zhang *et al.*, 2007), *Mitragyna* species (Sukrong *et al.*, 2007) and *Plantago* species (Sahin *et al.,* 2007).

The ITS region is an attractive target for molecular analysis, since 1) a large number of copies of these genes are present in the plant genome and 2) the regions encoding 18S-, 5.8S- and 26S rDNA are highly conserved, whereas two internal transcribed spacers (ITS1 and ITS2) between the ribosomal RNA genes are variable and useful as possible sources of polymorphisms for plant identification (Sahin *et al.,* 2007).

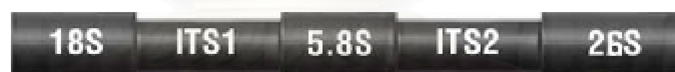

Figure 2.5 Schematic diagram of the nuclear rDNA internal transcribed spacer region. The three rDNA subunits: 18S, 5.8S and 26S are separated by internal transcribed spacers (ITS1 and ITS2) (Zhang *et al.*, 2007).

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CHAPTER III

MATERIALS AND METHODS

1. Materials

Fifteen specimens of *Stemona* species were collected from various localities in Thailand and preserved at the museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkon University, Thailand (Table 2). The pictures of herbarium specimens are shown in Appendix A. The specimens were examined by Assistance Professor Dr. Srunya Vajrodaya.

Four crude drugs of *Stemona* species were purchased at local retail sources and were sold as herbal medicines (Table 3 and Figure 3.1).

Table 2 *Stemona* samples were used in this study.

Samples	Date of collection	Locality	Voucher No.
Stemona tuberosa Lour.	20/10/2006	Lumpang	Vngb-060115
	20/10/2006	Phitsanulok	Vngb-060121
	21/5/2007	Kasetsart University, Nakornpathom	Vngb-070123
	22/12/2006	Chulalongkorn University, Bangkok	Vngb-070139
S. phyllantha Gagnep.	20/10/2006	Kanchanaburi	Vngb-060108
	20/10/2006	Kanchanaburi	Vngb-060120
	1/12/2006	Siriruckhachati Medicinal Plant Garden, Nakornpathom	Vngb-060131
	17/8/2007	Amphur BanChang, Rayong	Vngb-070147
S. collinsae Craib	20/10/2006	Saraburi	Vngb-060106
	20/10/2006	Nakhon Ratchasima	Vngb-060119
	5/12/2006	Kasetsart University, Nakornpathom	Vngb-060135
S. burkillii Prain	20/10/2006	Huay nai, Chiang Mai	Vngb-060110
	12/7/2007	Huay tueng tao, Chiang Mai	Vngb-070145
S. aphylla Craib	20/10/2006	Udon Thani	Vngb-060104
S. sp.	1/12/2006	Siriruckhachati Medicinal Plant Garden, Nakornpathom	Vngb-060130

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Table 3 Herbal drugs of *Stemona* species were used in this study.

Herbal drug names	Code no.	Localities of market	Date of collection
Non Tai Yak, tua mia (หนอนตายหยากตัวเมีย)	R1	Ratchaburi Province	12/2006
Non Tai Yak, tua phu (หนอนตายหยากตัวผู้)	R2	Ratchaburi Province	12/2006
Non Tai Yak (หนอนตายหยาก)	R3	Kanchanaburi Province	12/2006
Non Tai Yak (หนอนตายหยาก)	R4	Satun Province	4/2007

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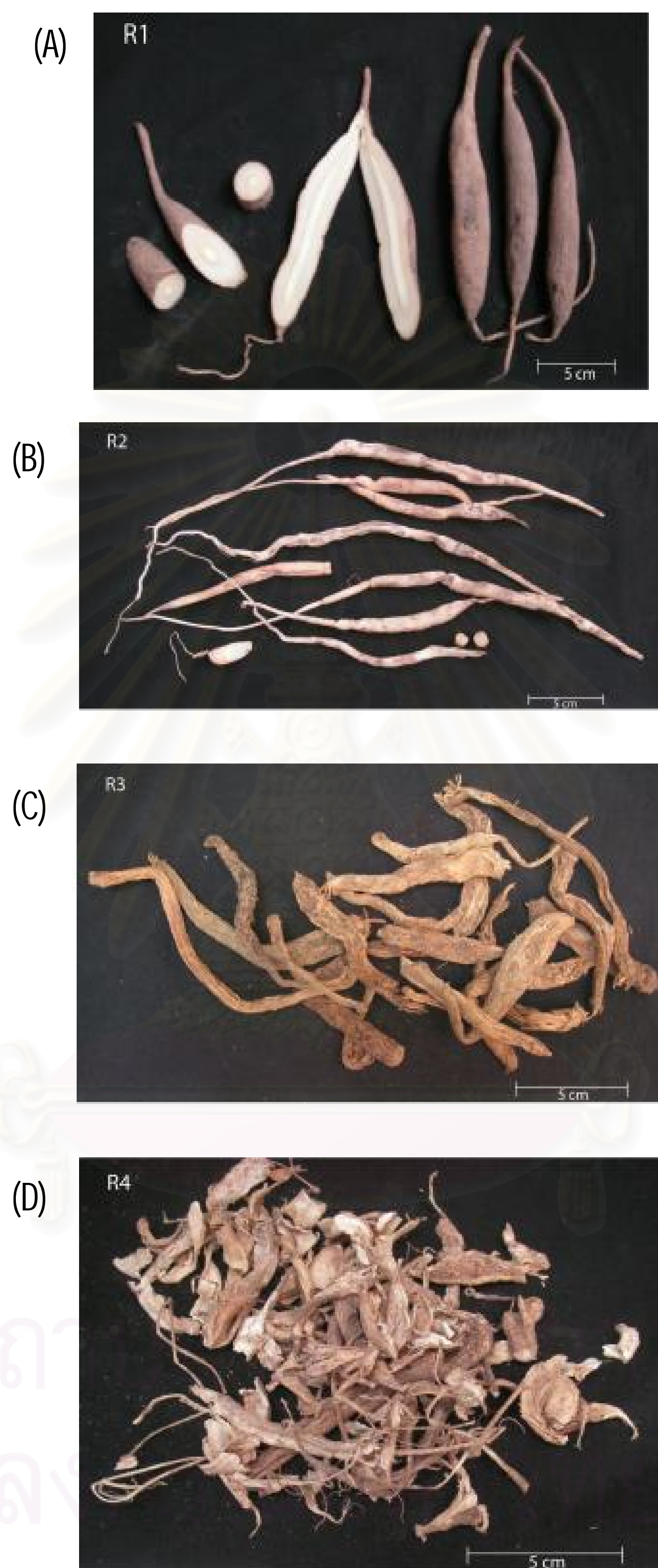

Figure 3.1 Herbal drugs of *Stemona* species used in this study. A, B, C, D, represent crude drugs R1, R2, R3, R4 respectively (see detail in Table 3)

2. Methods

2.1 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. Then the 50 µl of DNA solution was purified by a Geneclean® II Kit (QBiogene Inc., U.S.A.). For crude drugs, genomic DNA was extracted using MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE Biotechnologies, 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.

2.2 Primers Design for Different DNA Region

Partial *mat*K region

To amplify and sequence the *mat*K region, four primers were designed based on published complete *mat*K sequences of *Stemona japonica* database (NCBI GenBank, accession number AB040210). The designed primers were synthesized by Operon Biotechnologies (Germany). Details of these primers are presented in Table 4. The relative positions of the primers are shown in Figure 3.2.

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Table 4 PCR amplification primers and sequencing primers of partial *mat*K gene used in this study

Primer name	Primer sequence (5′ to 3′)	Direction
Stemoja-68F	TCC TTG AGG AGT ATA TTT ACG CAC T	forward
Stemoja-528F	GCA TTT ATT GCG ATT CTT TCT CC	forward
Stemoja-970R	TAC GAA TCC TGT GCG GTT GAG	reverse
Stemoja-1459R	CGT TCT CGA TGC GAC CTA TG	reverse

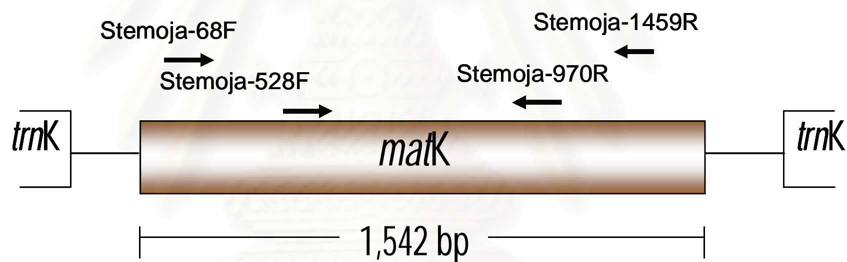

Figure 3.2 Relative positions of the PCR amplification primers and sequencing primers of partial *mat*K gene used in this study. Arrows (→) represent forward primers. Arrows (←) represent reverse primers.

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The *trn*H-*psb*A intergeneric spacer region

To amplify and sequence the *trn*H-*psb*A intergeneric spacer region, two primers were designed according to known genomic sequences from *Dioscorea elephantipes* (accession number EF38035), *Oryza sativa* (accession number X15901), *Nicotiana tabacum* (accession number Z00044) and *Atropa belladonna* (accession number NC004561). In addition, two inner forward primers, trnH-steF and trnH-steF774, were designed based on conserved regions of obtained *Stemona* sequences for confirmation of variable regions. The designed primers were synthesized by Operon Biotechnologies (Germany). Details of these primers are presented in Table 5. The relative positions of the primers are shown in Figure 3.3.

Table 5 PCR amplification primers and sequencing primers of *trn*H-*psb*A region used in this study

Primer name	Primer sequence (5¢to 3¢)	Direction
trnH-F	CGC ATG GTG GAT TCA CAA TCC	forward
trnH-steF	GCT CAA CAT ATA CGT ATG TCT G	forward
trnH-steF774	TCG AGG ACG TAG TTA TCC G	forward
psbA-diR	GTA ATG CAT GAA CGT AAT GCT C	reverse

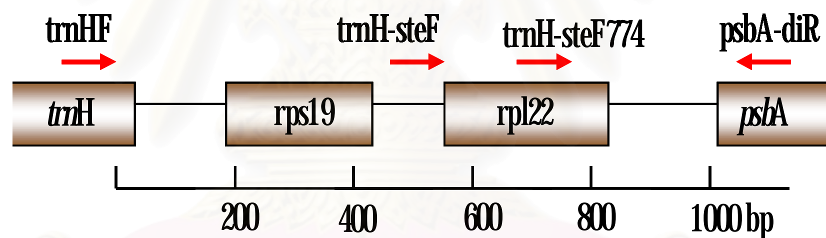

Figure 3.3 Relative positions of the PCR amplification primers and sequencing primers of *trn*H-*psb*A region used in this study. Arrows (→) represent forward primers. Arrows (←) represent reverse primers.

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The internal transcribed spacer (ITS) region

To amplify and sequence the internal transcribed spacer (ITS) region, Stmn-18S-F primer was designed based on published complete 18S rRNA sequences of *Stemona japonica* database (NCBI GenBank, accession number AF207028). Stmn-26S-68R primer and Stmn-26S-82R primer were designed from published 26S rRNA sequences of *Carludovica palmate* (accession number DQ008648), *Acorus gramineus* (accession number AF036490), *Tacca chantieri* (accession number AY095474,), *Dioscorea macrostachya* (accession number AF205123) and *Oryza sativa* (accession number M11585). Stmn-5S-R primer and Stmn-5S-nR primer were designed based on our obtained sequences of 5.8S rRNA regions of *Stemona.* The designed primers were synthesized by Operon Biotechnologies (Germany). Details of these primers are presented in Table 6. The relative positions of the primers are shown in Figure 3.4.

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Table 6 PCR amplification primers and sequencing primers of internal transcribed spacer used in this study

Primer name	Primer sequence (5¢to 3¢)	Direction
Stmn-18S-F	GAG AAG TCC ACT GAA CCT TAT C	forward
Stmn-26S-68R	CTA GGG GAA TCC TCG TAA G	reverse
Stmn-26S-82R	CCT AGT AAC GGC GAG CGA AC	reverse
Stmn-5S-R	GAG TTT TTG AAC GCA AGT TGC G	reverse
Stmn-5S-nR	GCA ATT CAC ACC AAG TAT C	reverse

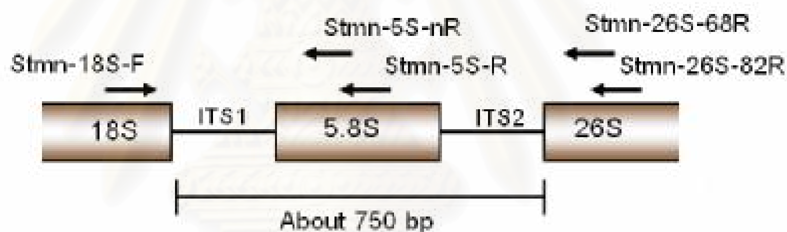

Figure 3.4 Relative positions of the PCR amplification primers and sequencing primers of ITS region used in this study. Arrows (⟶) represent forward primers. Arrows (⟵) represent reverse primers.

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2.3 PCR Amplification

Partial *mat*K Region

PCR amplification of partial *mat*K region was performed using 50 ng of DNA as a template in 50 µl of reaction mixture consisting of 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$ (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, Stemoja-68F and Stemoja-1459R.

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 94 ◦C for 5 minutes to ensure the complete seperatation of the DNA strands, followed by strand denaturation at 94 ◦C for 45 seconds, primer annealing at 52 ◦C for 45 seconds, and primer extension at 72 ◦C for 90 seconds for 29 cycles, and final extension step at 72 ◦C for 5 minutes to ensure that all amplicons are fully extended, then held at 4 ◦C.

The *trn*H-*psb*A intergeneric spacer region

PCR amplification of the *trn*H-*psb*A intergeneric spacer region was performed using 50 ng of total DNA as a template in 50 µl of reaction mixture consisting of 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$ (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, trnH-F and psbA-diR.

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 94 ◦C for 5 minutes to ensure the complete seperatation of the DNA strands, followed by strand denaturation at 94 ◦C for 45 seconds, primer annealing at 52 ◦C for 45 seconds, and primer extension at 72 ◦C for 90 seconds for 29 cycles, and final extension step at 72 ◦C for 5 minutes to ensure that all amplicons are fully extended, then held at 4 ◦C.

The internal transcribed spacer (ITS) region

PCR amplification of the ITS region was performed using 50 ng of total DNA as a template in 50 μl of reaction mixture consisting of 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 0.1 % Triton X-100, 3.5 mM $MgCl_2$ (Promega, U.S.A), 0.2 mM of each dNTPs, 2.0 U *Taq* DNA Polymerase (Promega, U.S.A), and 0.25 mM of each primer. Stmn-18S-F as forward primer and Stmn-26S-68R, Stmn-26S-82R, Stmn-5S-R and Stmn-5S-nR as reverse primers were used to amplify these regions.

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 94 ◦C for 5 minutes to ensure the complete seperatation of the DNA strands, followed by strand denaturation at 94 ◦C for 30 seconds, primer annealing at 50 ◦C to 52 ◦C for 30 seconds, and primer extension at 72 ◦C for 60 seconds for 29 cycles, and final extension step at 72 ◦C for 5 minutes to ensure that all amplicons are fully extended, then held at 4 ◦C.

2.4 Quantitation and Qualitation of DNA

Quantitation and qualitation of DNA based on the UV-induced fluorescence emitted by ethiduim bromide-DNA complexes were 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 (0.04M 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 ◦C before pouring gel into plastic gel form with the preset locations for the slots forming combs for casting the gel. In classification of *Stemona* using different size of *trn*H-*psb*A region, 2.5 % Metaphor® Agarose gel was prepared according to the manufacturer's protocol and used instead of normal agarose. After the gel was solid, the comb was carefully removed and the gel was inserted into an electrophoresis apparatus filled with 1XTAE buffer. One microliter of Nucleic Acid Sample Loading Buffer, 5X (Bio-Rad Laboratories Inc., U.S.A.) was added to the 4 μl of each DNA sample, and mixed before being loaded into each submerged slot. 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.

2.5 PCR Product Purification

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

2.6 Nucleotide Sequencing

Nucleotide sequences of purified PCR products were determined by Molecular Informatics Laboratory, Tech Dragon Ltd., Hong Kong.

2.7 Sequence Analysis and Phylogenetic Analysis

The consensus sequences were assembled and analyzed using the Multalin program (Corpet, 1998) and BioEdit program (Hall, 2004). The nucleotide sequence data of these regions were deposited in the DDBJ, EMBL, and GenBank. Nucleotide sequences with their accession numbers are shown in Table 10. Phylogenetic trees were generated using the computer program PAUP* (Version 4.0 beta 10a, Sinauer Assoc. Inc., U.S.A.). Parsimony analysis was performed using the Heuristic search method, with tree-bisection-reconnection (TBR) branch-swapping, MULTREES, a random addition sequence of 100 replicates. *Stichoneuron caudatum,* a member in family Stemonaceae, of these sequences was used as outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of topology of the consensus tree.

2.8 PCR-RFLP Analysis

PCR amplifications of partial *mat*K region and ITS1 region were performed using 50 ng of total DNA as a template in 50 µl of reaction mixture consisting of 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 0.1 % Triton X-100, 2.5 mM $MgCl_2$ for partial *mat*K region and 3.5 mM $MgCl_2$ for ITS1 region (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 partial *mat*K gene region of plant samples and crude drugs were Stemoja-68F and Stemoja-1459R. The ITS1 region was amplified by Stmn-18S-F primer and Stmn-5S-nR primer.

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 94 ◦C for 5 minutes, followed by strand denaturation at 94 ◦C for 30 seconds, primer annealing at 50 ◦C to 52 ◦C for 30 seconds, and primer extension at 72 ◦C for 45 seconds for 29 cycles, and final extension step at 72 ◦C for 5 minutes, then held at 4 ◦C.

The PCR products amplified by primers Stemoja-68F and Stemoja-1459R were digested with 5 units of restriction enzyme, *Mse*I and 10 units of *Bgl*I, separately (BioLabs, Inc., U.S.A.) at 37 ◦C for 3 hrs in PCR thermocycler. And the resulting PCR products amplified by primers Stmn-18S-F primer and Stmn-5S-nR primer, were digested with 5 units of each restriction enzyme, *Mse*I and *Dde*I singly (BioLabs, Inc., U.S.A.), at 37 ◦C for 3 hrs in PCR thermocycler.

The PCR product and the resulting restriction digests were detected by a 2.5 % agarose gel electrophoresis and stained with ethidium bromide, 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.). The size of fragments was estimated by comparison with a 1 kb plus DNA ladder (Invitrogen Corp., U. S. A.).

CHAPTER IV

RESULTS

1. Genomic DNA and PCR Amplification Products

Genomic DNA was isolated from the leaves of each specimen using the DNeasy® Plant Mini Kit (QIAGEN, Germany), and then purified by Geneclean® II Kit (QBiogene Inc., U.S.A.). Genomic DNA was examined on 0.8 % agarose gel electrophoresis (Figure 4.1). The purified DNA was stored at –20 °C until used.

Using the obtained DNA as templates, partial *mat*K region was amplified by PCR technique using primers, Stemoja-68F and Stemoja-1459R. The PCR products were about 1,300 bp in length (Figure 4.2).

The *trn*H-*psb*A intergeneric spacer was amplified using primers, trnH-F and psbA-diR. The PCR products were about 1,100 bp in length (Figure 4.3).

The internal transcribed spacer (ITS) of some *Stemona* spp. could be amplified by primers; Stmn-18S-F, Stmn-26S-68R, Stmn-26S-82R and Stmn-5S-R. Thus, newly designed primers Stmn-5S-nR with Stmn-18S-F primer were used to amplify all *Stemona* species (ITS1). The PCR products were about 365 bp in length (Figure 4.4).

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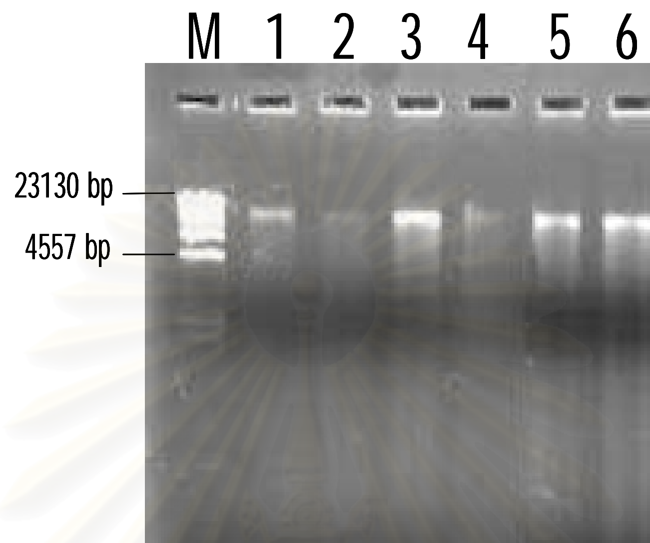

Figure 4.1 Agarose gel electrophoretogram of total DNA from *Stemona* species

Lane M : Lambda DNA-*Hind* III Digest

Lane 1 : *Stemona burkillii*

Lane 2 : *S. aphylla*

Lane 3 : *S. collinsae*

Lane 4 : *S.* sp.

Lane 5 : *S. phyllantha*

Lane 6 : *S. tuberosa*

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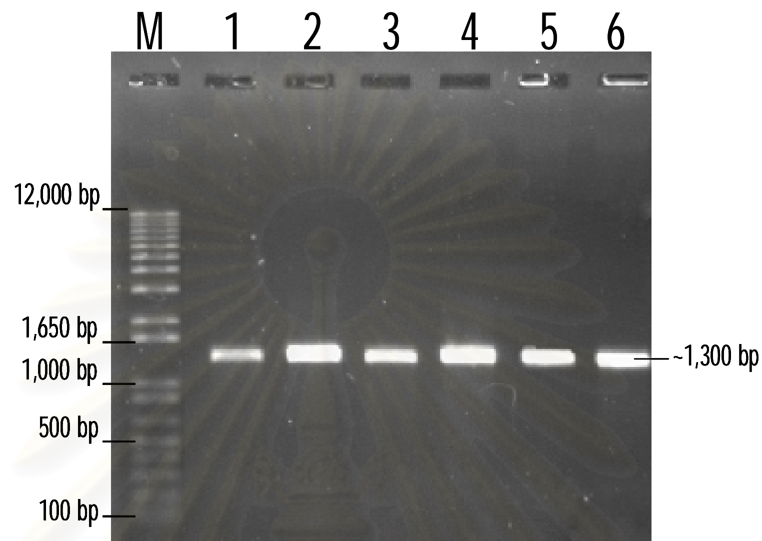

Figure 4.2 Agarose gel electrophoretogram of PCR products of partial *mat*K gene, by using primers; Stemoja-68F and Stemoja-1459R

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 : *Stemona burkillii*

Lane 2 : *S. aphylla*

Lane 3 : *S. collinsae*

Lane 4 : *S.* sp.

Lane 5 : *S. phyllantha*

Lane 6 : *S. tuberosa*

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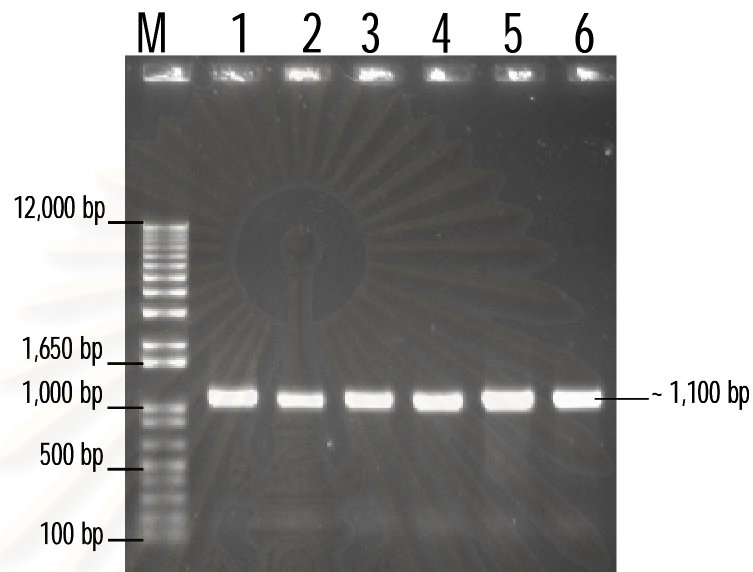

Figure 4.3 Agarose gel electrophoretogram of PCR products of the *trn*H-*psb*A intergeneric spacer (ITS), by using primers; trnH-F and psbA-diR

Lane M: 1 Kb plus DNA Ladder.

Lane 1 : *Stemona burkillii*

Lane 2 : *S. aphylla*

Lane 3 : *S. collinsae*

Lane 4 : *S.* sp.

Lane 5 : *S. phyllantha*

Lane 6 : *S. tuberosa*

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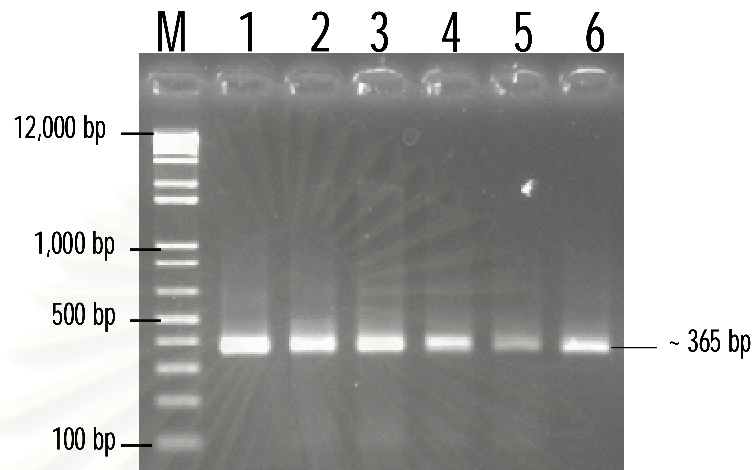

Figure 4.4 Agarose gel electrophoretogram of PCR products of the internal transcribed spacer (ITS) by using primers; Stmn-18S-F and Stmn-5S-nR

Lane M: 1 Kb plus DNA Ladder.

Lane 1 : *Stemona burkillii*

Lane 2 : *S. aphylla*

Lane 3 : *S. collinsae*

Lane 4 : *S.* sp.

Lane 5 : *S. phyllantha*

Lane 6 : *S. tuberosa*

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2. Sequence Analysis

2.1 The *mat*K gene sequences

The partial *mat*K sequences of all *Stemona* species amplified by primers, Stemoja-68F and Stemoja-1459R were found to be 1,306 bp in length. The specimens of the same species collected from different places have identical sequences of partial *mat*K gene. There were 9 sites of nucleotide substitutions (Table 7) among the six species and the position of each nucleotide was indicated based on complete *mat*K sequence of *S. japonica* (accession number AB040210). The nucleotide sequences of the partial *mat*K gene of *Stemona* spp. have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers in Table 9. The multiple sequence alignment of partial *mat*K sequences of all samples of *Stemona* was illustrated in Appendix B.

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Table 7 The partial *mat*K sequences with 9 variable sites recognized in *Stemona.* The position of each nucleotide was indicated based on complete *mat*K sequence of *S. japonica* (accession number AB040210).

*mat*K position / Species	465	608	780	861	910	940	1243	1390	1391
S. burkillii	C	C	G	A	T	C	T	T	T
S. aphylla	*	*	*	*	*	*	A	A	A
S. collinsae	*	*	*	*	*	*	A	G	G
S. sp.	*	*	*	C	*	*	A	*	*
S. phyllantha	T	C	A	*	C	A	A	*	*
S. tuberosa	T	C	A	*	C	A	A	*	*

An asterisk (*) indicates the nucleotide similar to *S. burkillii*

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2.2 The *trn*H-*psb*A intergeneric spacer region

The *trn*H-*psb*A intergeneric spacer region of *Stemona* spp. amplified by a pair of primers, trnH-F and psbA-diR, was determined to be 1061-1086 bp in length. The specimens of the same species collected from different places have identical sequences of *trn*H-*psb*A region, but in *S. collinsae*, the nucleotide sequences could be classified into 2 types; *S. collinsae* 1 *and S. collinsae* 2. Eighteen sites of nucleotide substitution and 28 sites of indel were observed (Table 8) and the first position of nucleotide sequence started form 59th of 5′ of *trn*H based on *Dioscorea elephantipes*, (accession number EF380353). Based on different size length of *trn*H-*psb*A region, primer trnH-F774 and primer psbA-diR were utilized to amplify short fragment this region. When the amplified products were compared, they could be clearly distinguished into two groups. The first group; *S. tuberosa* and *S. phyllantha*, had less than 300 bp and the second group; *S. burkillii, S. aphylla*, *S. collinsae* and *S.* sp., were more than 300 bp (Figure 4.5).

The nucleotide sequences of this region of *Stemona* spp. have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers shown in Table 9. The multiple sequence alignment of the *trn*H-*psb*A intergeneric spacer region of all samples of *Stemona* were illustrated in Appendix B.

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Table 8 The *trn*H-*psb*A region with 46 variable sites recognized in *Stemona*. The first position of nucleotide sequence started form 59th of 5′ of *trn*H based on *Dioscorea elephantipes*, (accession number EF380353).

Species \ Nucleotide position	22	102	188	826	828	829	830	832	833	836	837	839	840	841	843	875	889	890	891	892	893	909	911
S. burkillii	C	T	T	T	A	G	A	T	C	T	T	A	A	A	T	-	T	G	C	A	T	A	T
S. aphylla	*	*	G	A	T	T	T	A	A	G	A	T	C	T	A	A	*	*	*	*	*	*	-
S. collinsae1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	T	-
S. collinsae2	*	G	G	*	*	*	*	*	*	*	*	*	*	*	*	-	*	*	*	*	*	*	-
S. sp.	*	G	G	A	T	G	A	A	A	G	A	T	C	T	A	A	*	*	*	*	*	*	-
S. phyllantha	A	*	*	A	T	G	A	A	A	G	A	T	C	T	A	-	-	-	-	-	-	*	-
S. tuberosa	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	-	-	-	-	-	-	T	-

Species \ Nucleotide position	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934
S. burkillii	T	A	A	A	G	T	T	A	T	T	A	A	A	G	T	T	A	A	A	G	T	T	A
S. aphylla	-	-	-	*	*	*	*	*	A	A	G	T	T	A	*	*	*	*	*	*	*	*	*
S. collinsae1	-	-	-	-	-	-	-	-	-	A	C	T	T	T	A	A	T	*	*	C	*	*	T
S. collinsae2	-	-	-	-	-	-	-	-	-	A	G	T	T	A	*	*	*	*	*	*	*	*	*
S. sp.	-	-	-	-	-	-	-	-	-	A	G	T	T	A	*	*	*	*	*	*	*	*	*
S. phyllantha	-	-	-	-	-	-	-	-	-	A	G	T	T	A	-	-	-	-	-	-	-	-	-
S. tuberosa	-	-	-	-	-	-	-	-	-	A	C	T	T	T	-	-	-	-	-	-	-	-	-

A star indicates the nucleotide similar to *S. burkillii* and a hyphen reveals an indel.

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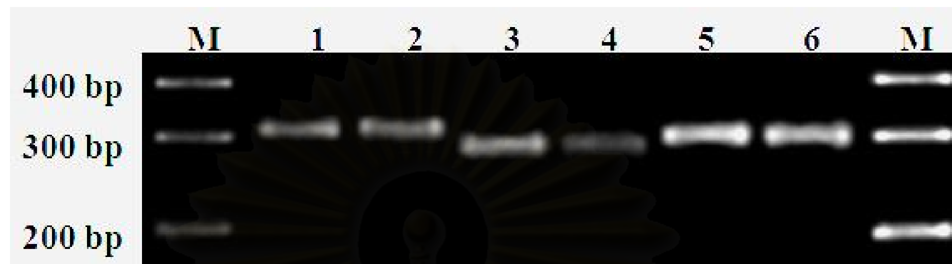

Figure 4.5 A 2.5 % MetaPhor® Agarose image of PCR products generated with a pair of primers, trnH-F774 and psbA-diR, flanking the *trn*H-*psb*A region using DNA from *Stemona* species.

Lane M: 1 Kb plus DNA Ladder.

Lane 1 : *Stemona burkillii*

Lane 2 : *S. aphylla*

Lane 3 : *S. collinsae*

Lane 4 : *S.* sp.

Lane 5 : *S. phyllantha*

Lane 6 : *S. tuberosa*

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2.3 The internal transcribed spacer (ITS) region

The internal transcribed spacer region amplified by a pair of primers, Stmn-18S-F and Stmn-5S-nR, was determined to be 363-368 bp in length. The specimens of the same species collected from different places have identical sequences of ITS region except for *S. collinsae*, of which the nucleotide sequences could be classified into 2 types; *S. collinsae* 1 *and S. collinsae* 2. One hundred and thirty-four sites of nucleotide substituion and 59 sites of indel were observed (Figure 4.6) and the first position of nucleotide sequence started from 1,711st of 5′ of 18S rDNA based on *S. japonica* (accession number AB207028). The nucleotide sequences of this region of *Stemona* spp. have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers in Table 9. The multiple sequence alignment of the ITS region of all samples of *Stemona* were illustrated in Appendix B.

Figure 4.6 Sequence alignment of 18S-ITS1-5.8S region of *Stemona* in Thailand. The position 1-38 are the partial 18S region which first position started form 1,711[st] of 5′ of 18S rDNA, ITS1 region corresponds to positions 39-306 (enclosed in box) and partial 5.8S region corresponds to positions 307-383. A dot indicates the nucleotides similar to *S. burkillii* and a hyphen reveals an indel

Table 9 The accession numbers of nucleotide sequence data obtained from this study and deposited in the GenBank database

Samples	Accession number of *mat*K	Accession number of *trn*H-*psb*A	Accession number of ITS
Stemona tuberosa Lour.	AB373230	AB373199	AB429262
Stemona phyllantha Gagnep.	AB373229	AB373198	AB429261
Stemona burkillii Prain	AB373225	AB373193	AB429268
Stemona collinsae Craib	AB373226	AB373195	AB429265
		AB373194	AB429266
Stemona aphylla Craib	AB373224	AB373192	AB429267
Stemona sp.	AB373228	AB373196	AB429264
Stichoneuron caudatum Ridley (out group)	AB373231	AB373200	AB429269

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3. Phylogenetic Analysis

Based on the partial *mat*K sequences of the six *Stemona* species determined, parsimony analysis was performed to produce parsimonious trees, with a consistency index (CI) of 1.000 and a retention index (RI) of 1.000. As shown in Figure 4.7, 50 % majority-rule consensus tree divided *Stemona* into two groups. *S. tuberosa* and *S. phyllantha* were in group 1 and separated from other species with a high bootstrap value (97%). The other, which are *S. collinsae, S. burkillii, S. aphylla* and *S.* sp. were in group 2 and separated from group 1 (*S. tuberosa* group) with a bootstrap value of 71%.

Based on the *trn*H-*psb*A region sequences of the six species determined, parsimony analysis was performed to produce parsimonious trees, with a consistency index (CI) of 0.9524 and a retention index (RI) of 0.9231. As shown in Figure 4.8, 50 % majority-rule consensus tree divided *Stemona* into two groups. *S.* sp., *S. aphylla* and *S. phyllantha* were in group 1 and separated from other species. The other, which are *S. burkillii*, *S. tuberosa*, *S. collinsae* 1 and *S. collinsae* 2 were in group 2 and separated from group 1 with a high bootstrap value of 98%.

Based on the ITS sequences of the six species determined, parsimony analysis was performed to produce parsimonious trees, with a consistency index (CI) of 0.8452 and a retention index (RI) of 0.6170. As shown in Figure 4.9, 50 % majority-rule consensus tree divided *Stemona* into two groups. *S. burkillii* and *S. collinsae* 1 were in group 1 and separated from other species with a bootstrap value of 68%. The other, which are *S. aphylla, S. phyllantha, S. collinsae* 2, *S.* sp. and *S. tuberosa* were in group 2 and separated from group 1 with a bootstrap value of 52%.

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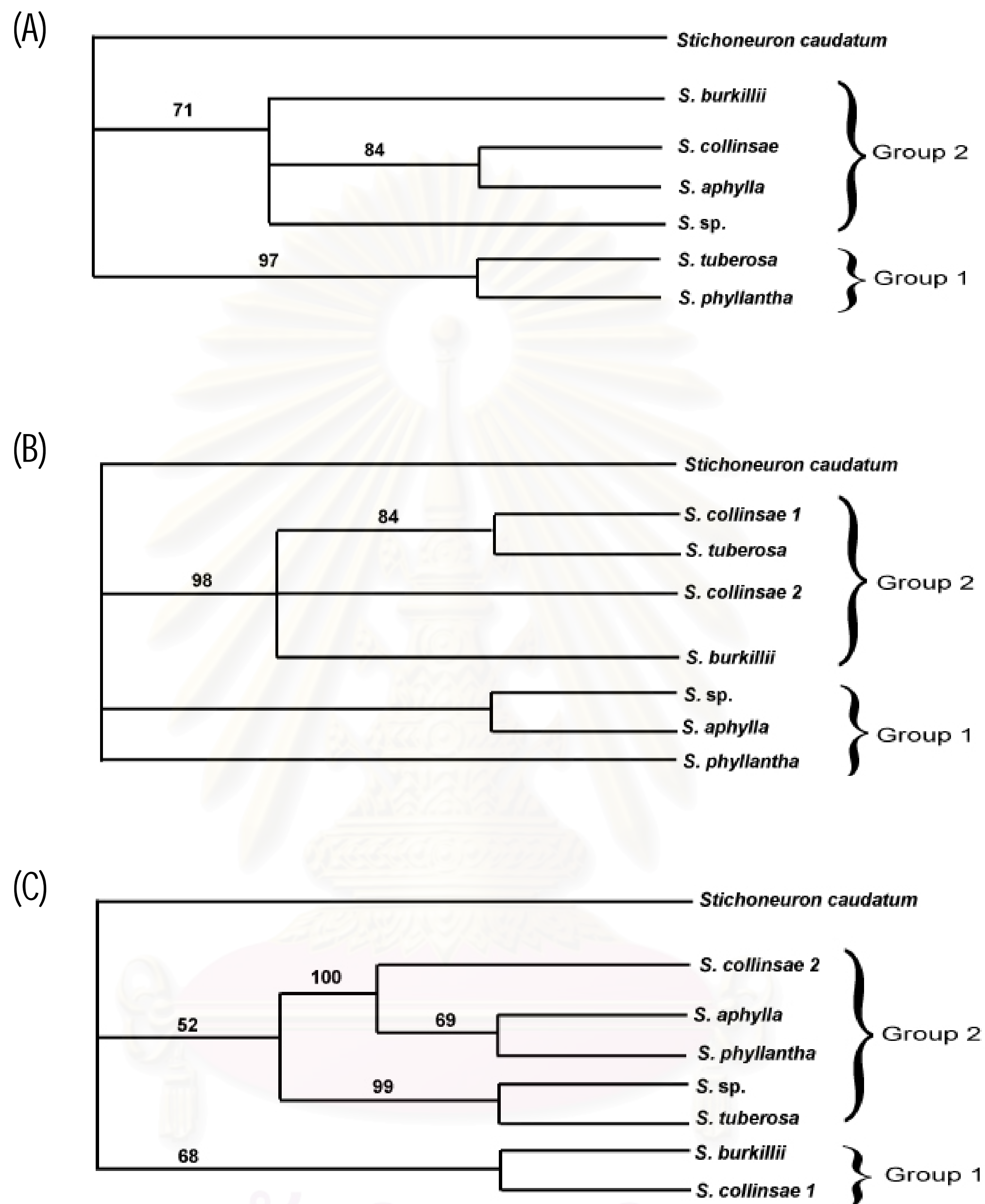

Figure 4.7 Comparison of 50%. majority-rule consensus tree of parsimony analysis produced on the basis of obtained sequences from this study. (A) represents Partial *mat*K sequences; Tree length = 83, CI= 1.0000, RI= 1.0000, RC= 1.0000. (B) represents the *trn*H-*psb*A region sequences. Tree length = 63, CI= 0.9524, RI = 0.9231, RC= 0.8791. (C) represents ITS region sequences. Tree length = 465, CI=0.8452, RI=0.6170, RC=0.5215. Number above line is the bootstrap value with 1000 replicates.

4. Identification of *Stemona* in Thailand by PCR-RFLP Analysis

*Bgl*I Digest

The PCR products of *Stemona* amplified with a pair of primers, Stemoja-528F and Stemoja-970R were 443 bp in length. The restriction enzyme *Bgl*I, which recognizes the sequence of 5′-GCCATTTTGGC-3′ was found to give diagnostic fragments among *Stemona* species. The partial *mat*K gene of *S.* sp. has a *Bgl*I restriction site at the nucleotide position 866 (Figure 4.8A). The resulting restriction digest showed two fragments of 105 and 338 bp (Figure 4.8B), while others species showed only one fragment of 443 bp (Figure 4.8B) because of no *Bgl*I restriction sites on the partial *mat*K gene (Figure 4.8A).

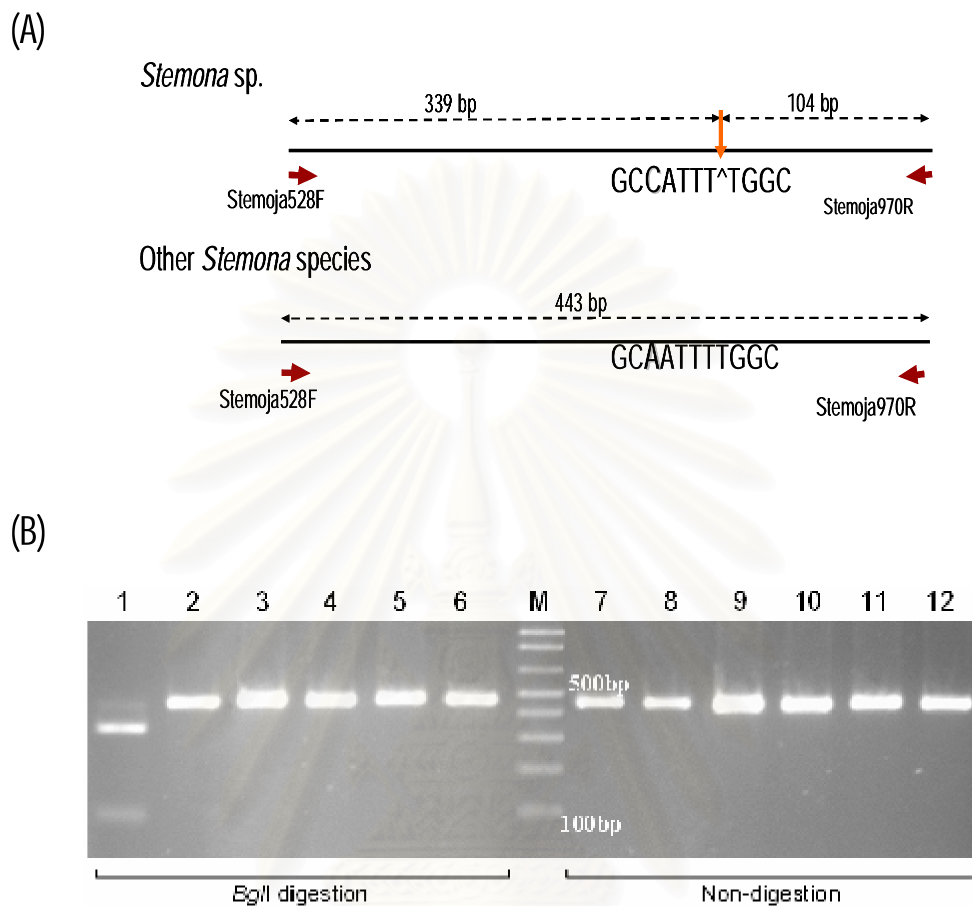

Figure 4.8 PCR-RFLP analysis of *Stemona* using the restriction enzyme *BglI* on partial *matK* gene

(A) *BglI* restriction sites in *Stemona* sp. Nucleotide with bold face indicates the defined marker nucleotide at position 862.

(B) Agarose gel electrophoretrogram of PCR products generated by primers Stemoja-528F and Stemoja-970R, digested with *BglI* (lanes 1–6) and non-digested (lanes 7-12). lane 1, 7: *S.* sp., lane 2, 8: *S. collinsae,* lane 3, 9: *S. aphylla*, lane 4, 10: *S. burkillii*, lane 5, 11: *S. phyllantha*, lane 6, 12: *S. tuberosa* and lane M: 1 kb plus DNA ladder.

*Mse*I Digest

The PCR products of *Stemona* amplified with a pair of primers, Stemoja-528F and Stemoja-970R were 443 bp in length. The restriction enzyme *Mse*I, which recognizes the sequence of 5′-TTAA-3′ was found to give diagnostic fragments of *S. tuberosa* and *S. phyllantha* from other species. The partial *mat*K gene of *S. tuberosa* and *S. phyllantha* has *Mse*I restriction sites at the nucleotide position 687 and 768 (Figure 4.9A). The resulting restriction digest showed three fragments of 160, 79, 203 bp, respectively (Figure 4.9B), while in the others had three *Mse*I restriction sites at the nucleotide position 608, 687 and 768 (Figure 4.9A). However, the resulting restriction digests showed two fragments of about 80 and 200 bp in electrophoretogram (Figure 4.9B) because closely fragment sizes are observed each fragment (79, 80, 81 bp).

By using another pair of primers, Stmn-18S-F and Stmn-5S-nR, the PCR products of *Stemona* were about 365 bp in length. The restriction enzyme *Mse*I was found to give diagnostic fragments between *S. tuberosa* and other species. The ITS region of *S. tuberosa* has a *Mse*I restriction site at the nucleotide position 229 (Figure 4.10A). The resulting restriction digest showed two fragments of 136 and 229 bp (Figure 4.10B), whereas others showed only one of non-digested fragment about 365 bp (Figure 4.10B).

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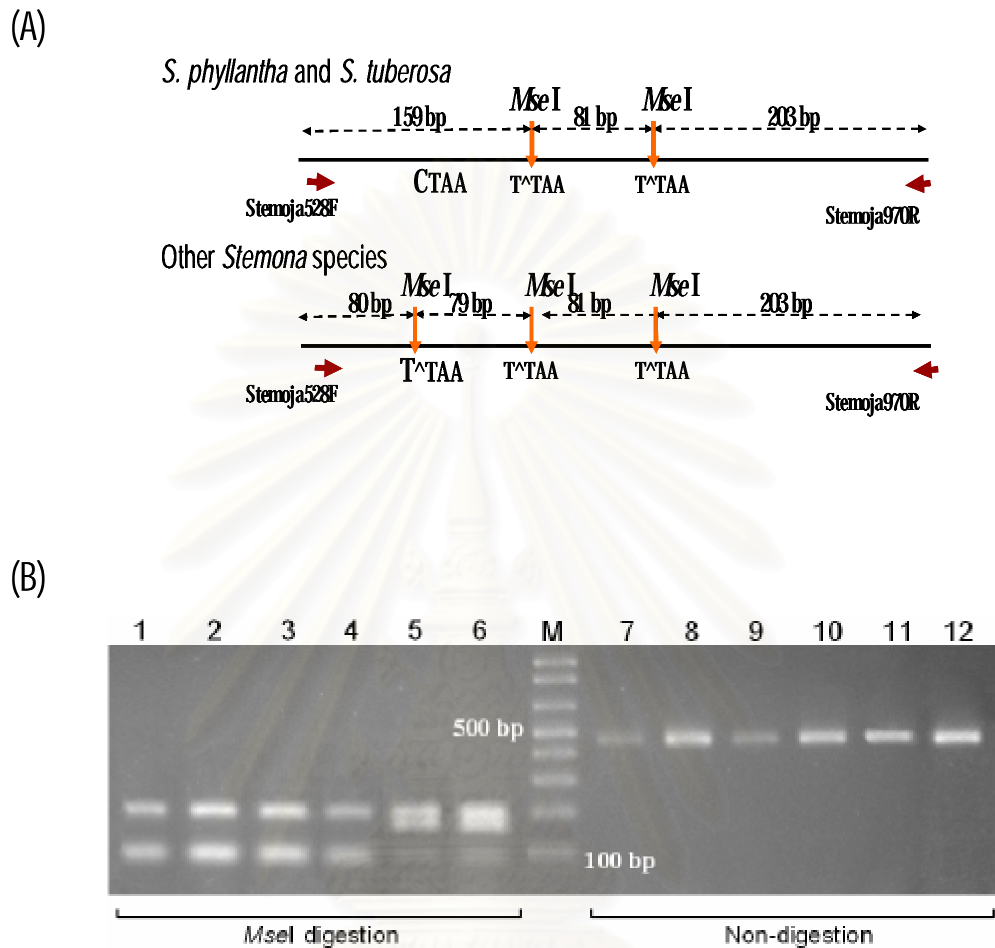


Figure 4.9 PCR-RFLP analysis of *Stemona* using the restriction enzyme *Mse*I on partial *mat*K gene

(A) *Mse*I restriction sites in *S. phyllantha* and *S. tuberosa*. Nucleotide with bold face indicates the defined marker nucleotide at position 608.

(B) Agarose gel electrophoretrogram of PCR product generated by primers Stemoja-528F and Stemoja-970R, digested with *Mse*I (lanes 1–6) and non-digested (lanes 7-12).

lane 1, 7: *S.* sp., lane 2, 8: *S. collinsae*, lane 3, 9: *S. aphylla*, lane 4, 10: *S. burkillii*, lane 5, 11: *S. phyllantha*, lane 6, 12: *S. tuberosa* and lane M: 1 kb plus DNA ladder.

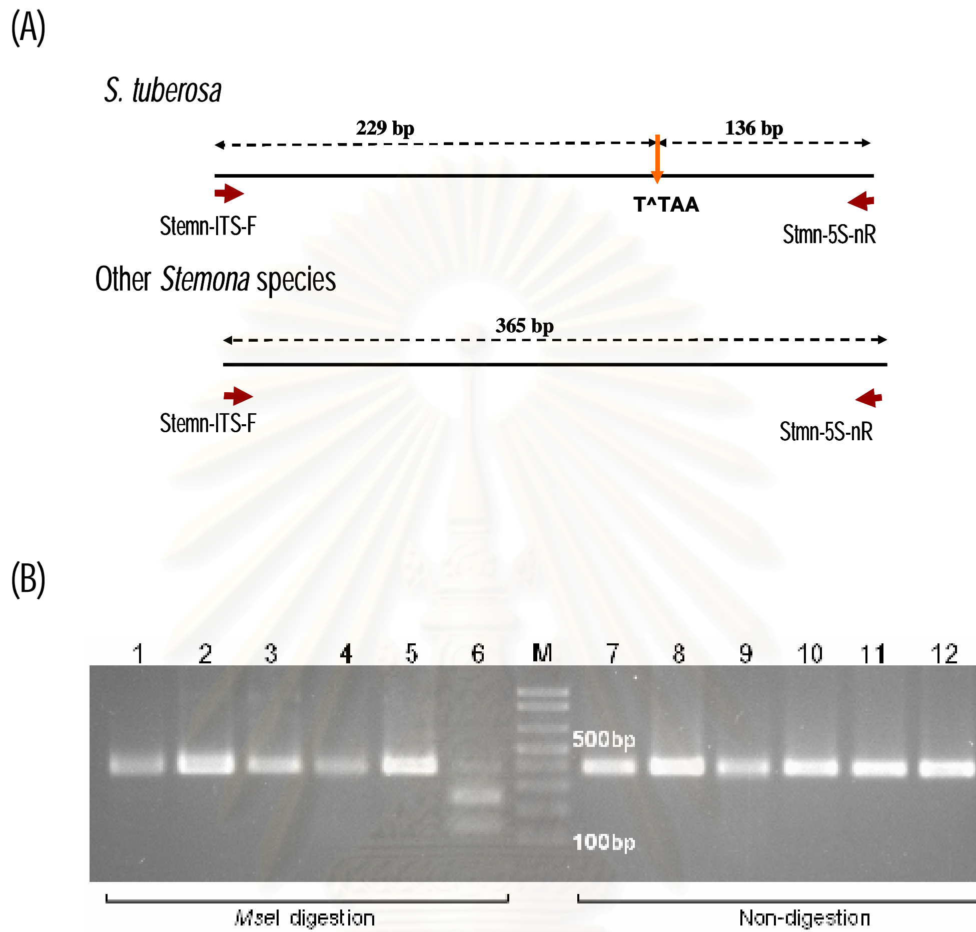


Figure 4.10 PCR-RFLP analysis of *Stemona* using the restriction enzyme *Mse*I on ITS region.

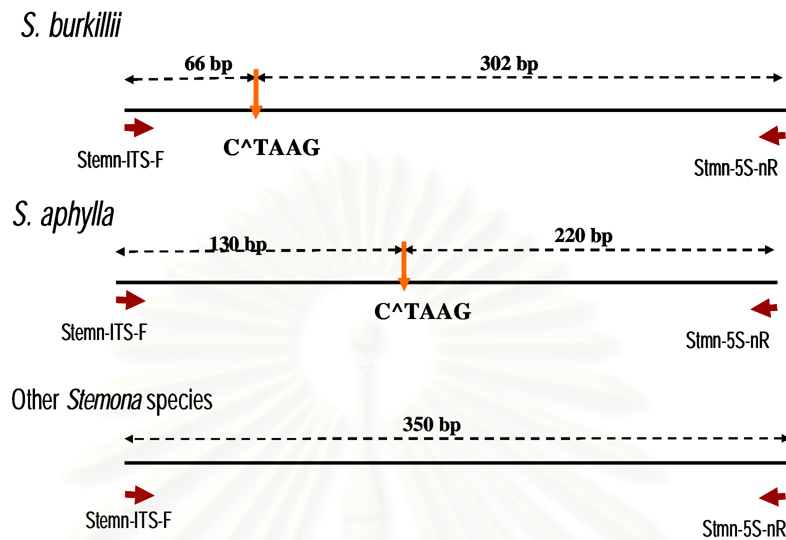
(A) *Mse*I restriction sites in *S. tuberosa*. The nucleotides indicate the defined marker nucleotide at position 228-231.

(B) Agarose gel electrophoretrogram of PCR product generated by primers Stmn-18S-F and Stmn-5S-nR, digested with *Mse*I (lanes 1–6) and non-digested (lanes 7-12). lane 1, 7: *S.* sp., lane 2, 8: *S. collinsae*, lane 3, 9: *S. aphylla*, lane 4, 10: *S. burkillii*, lane 5, 11: *S. phyllantha*, lane 6, 12: *S. tuberosa* and lane M: 1 kb plus DNA ladder.

*Dde*I Digest

The PCR products of *Stemona* amplified with a pair of primers, Stmn-18S-F and Stmn-5S-nR were about 365 bp in length. The restriction enzyme *Dde*I, which recognizes the sequence of 5′-CTNAG-3′ was found to give diagnostic fragments among *S. burkilii*, *S. aphylla* and other species. The ITS region of *S. burkilii* has a *Dde*I restriction site at the nucleotide position 67 (Figure 4.11A). The resulting restriction digest showed two fragments of 302 and 66 bp (Figure 4.11B). *S. aphylla* has a *Dde*I restriction site at the nucleotide position 122 (Figure 4.11A). The resulting restriction digest showed two fragments of 121 and 247 bp (Figure 4.11B) while others showed only one of non-digested fragment about 365 bp (Figure 4.11B) .

(A)



(B)

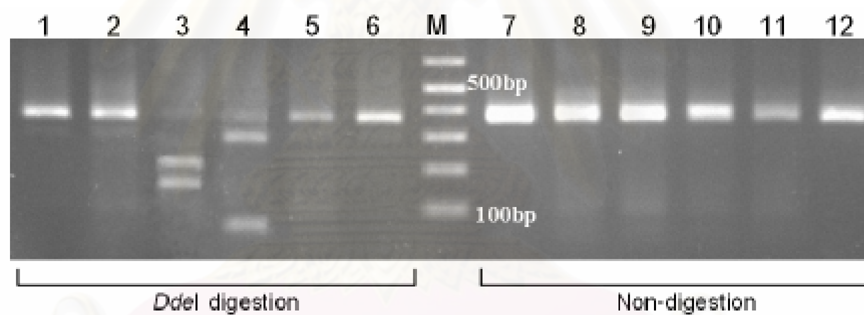


Figure 4.11 PCR-RFLP analysis of *Stemona* using the restriction enzyme *Dde*I on ITS region

(A) *Dde*I restriction sites in *S. burkillii* and *S. aphylla*. The nucleotides indicate the defined marker nucleotide at position 66-77 of *S. burkillii* and 121-126 of *S. aphylla* .

(B) Agarose gel electrophoretrogram of PCR product generated by primers Stmn-18S-F and Stmn-5S-nR, digested with *Dde*I (lanes 1–6) and non-digested lanes (7-12).

lane 1, 7: *S.* sp., lane 2, 8: *S. collinsae*, lane 3, 9: *S. aphylla*, lane 4, 10: *S. burkillii*, lane 5, 11: *S. phyllantha*, lane 6, 12: *S. tuberosa* and lane M: 1 kb plus DNA ladder.

The Figure 4.12 and Table 10 were generated for identification of each species by PCR-RFLP analysis. First, the PCR products of specimens amplified with a pair of primers, Stemoja-528F and Stemoja-970R were digested with *Bgl*I. Digestion of products with *Bgl*I resulted in two banding patterns with one cutting site in *S.* sp. while PCR products of other species; *S.burkillii*, *S. aphylla*, *S. collinsae*, *S. phyllantha*, and *S. tuberosa* showed only one of non-digested fragment. Second, for the other species, the PCR products were amplified by the similar primers as above and digested with *Mse*I. Digestion of products with *Mse*I divided into two patterns. *S. tuberosa* and *S. phyllantha* showed three fragments whereas the other species exhibited two fragments. Third, to separate *S. tuberosa* from the others, the PCR products were amplified with another pair of primers, Stmn-18S-F and Stmn-5S-nR and digested with *Mse*I. Digestion of products with *Mse*I exhibited two banding patterns with one cutting site in *S. tuberosa* while PCR products of other species showed only one of non-digested fragment. Finally, the PCR products amplified with primers Stmn-18S-F and Stmn-5S-nR were digested with *Dde*I. Digestion with *Dde*I showed three distinct types; two fragments of 130 and 220 bp in *S. aphylla*, two fragments of 66 and 302 bp in *S. burkillii* and non-restriction fragment in other species.

Crude drugs of *Stemona* in Table 3 were tested by PCR-RFLP analysis as the procedure in Figure 4.12. The results are shown in Figures 4.13-4.14. The crude drugs R1 and R3 had the restriction enzyme pattern similar to *S. phyllantha*. The pattern of crude drug R3 resembled *S. collinsae*. However, the restriction enzyme pattern of the crude drug R4 was not match to any species in this study.

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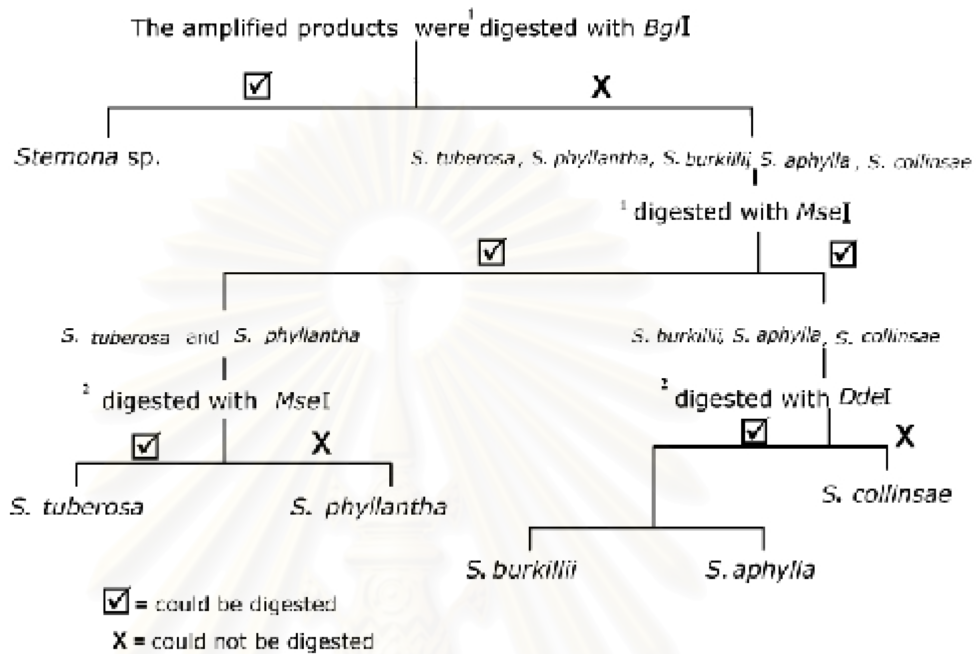

[1]PCR products were generated by primers Stemoja528F and Stemoja970R of partial *mat*K gene

[2]PCR products were generated by primers Stmn-ITS-F and Stmn-5S-nR of ITS region

Figure 4.12 Summary of restriction enzyme patterns of *Stemona* in Thailand

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Table 10 Summary of restriction fragment size in bp of *Stemona* in Thailand digested with *Bgl*I, *Mse*I, and *Dde*I

Restriction enzyme / Species	PCR products generated by primers; Stemoja-528F and Stemoja-970R		PCR products generated by primers; Stmn-18S-F and Stmn-5S-nR	
	*Bgl*I	*Mse*I	*Mse*I	*Dde*I
S. sp.	339, 104	80, 203	365	363
S. phyllantha	443	81, 159, 230	365	368
S. tuberosa	443	81, 159, 230	136, 229	365
S. aphylla	443	80, 203	365	130, 220
S. burkillii	443	80, 203	365	66, 302
S. collinsae	443	80, 203	365	365

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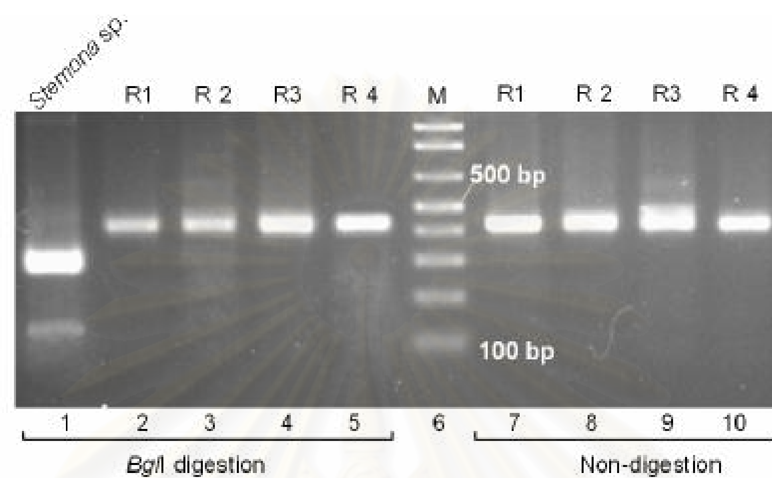
(A)



(B)

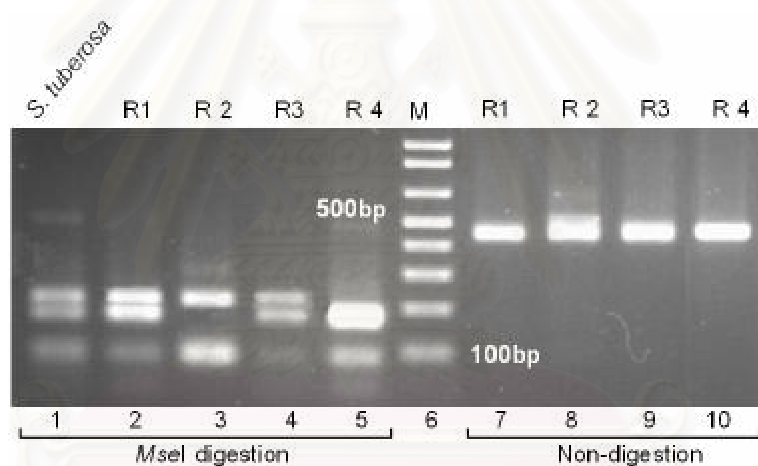
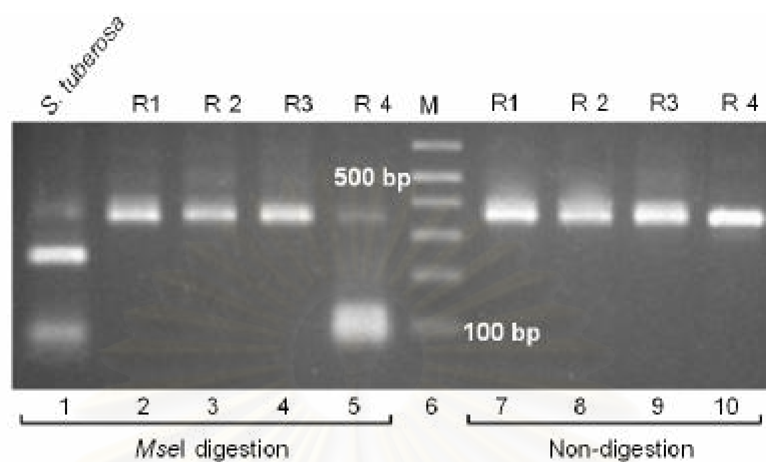


Figure 4.13 Agarose gel electrophoretogram of PCR products of four commercial crude drugs (R1-R4) generated by primers Stemoja-528F and Stemoja-970R.
(A) PCR products were digested with *BglI* (lanes 1-5) and non-digested (lanes 7-10).
lane 1: *S.* sp., lane 2,7: R1, lane 3, 8: R2, lane 4,9: R3, lane 5,10: R4, and
lane 6: 1 kb plus DNA ladder [M].
(B) PCR products were digested with *MseI* (lanes 1-5) and non-digested (lanes 7-10).
lane 1: *S. tuberosa*, lane 2,7: R1, lane 3, 8: R2, lane 4,9: R3, lane 5,10: R4, and
lane 6: 1 kb plus DNA ladder [M]

(A)



(B)

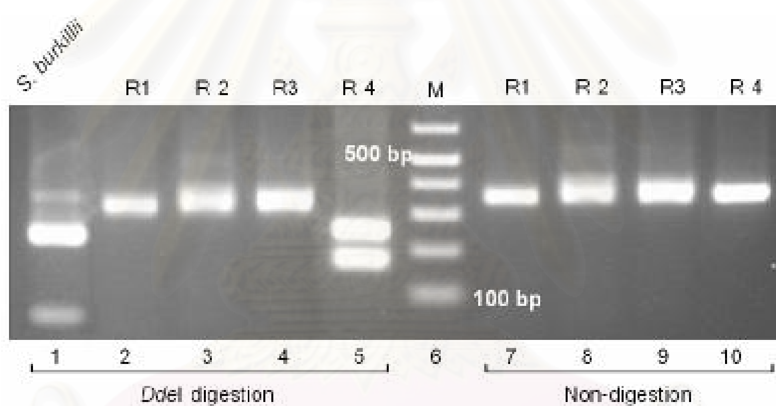


Figure 4.14 Agarose gel electrophoretogram of PCR products of four commercial crude drugs (R1-R4) generated by primers Stmn-18S-F and Stmn-5S-nR

(A) PCR products were digested with *Mse*I (lanes 1-5) and non-digested (lanes 7-10). lane 1: *S. tuberosa*, lane 2,7: R1, lane 3, 8: R2, lane 4,9: R3, lane 5,10: R4, and lane 6: 1 kb plus DNA ladder [M].

(B) PCR products were digested with *Dde*I (lanes 1-5) and non-digested (lanes 7-10). lane 1: *S. burkillii*, lane 2,7: R1, lane 3, 8: R2, lane 4,9: R3, lane 5,10: R4, and lane 6: 1 kb plus DNA ladder [M]

CHAPTER V

DISCUSSION

1.1 DNA sequence analysis

The present study was designed to differentiate *Stemona* species in Thailand. Partial *mat*K gene sequences of 15 specimens from six *Stemona* species distributed in Thailand showed completely identical sequence to the similar species despite the differences of their collections. Although the plastid *mat*K gene could be used to identify many plants (Zhu *et al.*, 2003 and Tamura *et al.*, 2004), the partial *mat*K sequences of *S. tuberosa* and *S. phyllantha* are identical, and the nucleotide sequence of *S. collinsae* are similar to that of *S. aphylla* as well. These results indicated that partial *mat*K sequences of *Stemona* in Thailand, having about 85 % in length from complete *mat*K gene based on *S. japonica* (accession number AB040210), were not enough to differentiate them. Thus, *trn*H-*psb*A and ITS region were used to increase discrimination power.

In addition, the specimens of the same species obtained from different locations have identical sequences of *trn*H-*psb*A region. In *S. collinsae,* nucleotide substitution was found variable while the nucleotide sequences of partial *mat*K gene resemble. The intraspecific variation probably occurred in *trn*H-*psb*A region of this species as in other plant families (Hamilton, 1999; Kondo *et al.*, 2007). Based on data combined from *trn*H-*psb*A sequences and morphological characteristics, *S. collinsae* could be classified into 2 types; *S. collinsae*-1, a short erect herb and *S. collinsae*-2, a climber. The length of *trn*H-*psb*A region in *Stemona* was determined to be 1061-1086 bp. The mostly variable sites of nucleotides were approximately located approximately at positions 840-860 and 920-950 which are informative for identification of *Stemona* in Thailand by DNA sequence technique. However, to develop a simple and efficient method such PCR-RFLP, this sequence may not be appropriate because the nucleotide substitutions were closely located. If PCR products were digested, the polymorphisms can not be detected easily. Hence, the ITS region was further examined to solve this problem since ITS was found to evolve faster than many plastid regions (Rubinoff *et al.*, 2006)

The ITS1 region of *Stemona* was determined to be 248-253 bp in length. The nucleotide sequences of ITS1 are highly variable and some nucleotide bases are not absolutely clear. The hybridization may occur in *Stemona* as reported in other plants (Shina *et al.*, 2006). Thus, larger sample sizes should be done to confirm this result in further study. However, in the present study, the ITS region could be used to identify *Stemona*. As a result of variable nucleotide sequences of ITS1 in *S. collinsae*, intraspecies nucleotide substitutions were observed as well as *trn*H-*psb*A region.

Based on the phylogenetic tree obtained from partial *mat*K gene sequences using parsimony analysis, *Stemona* in Thailand could be divided into two groups: 1) *S. tuberosa* and *S. phyllantha*, and 2) *S. collinsae*, *S. burkillii, S. aphylla* and *Stemona* sp., supported with high bootstrap value (Figure 4.7). This value indicated that the results are compiled to allow an estimate of the reliability of a particular grouping (Hall, 2004). These results correlated with former reports in morphological and chemotaxonomy studies (Gagnepain, 1934; Duyfjes, 1993; Schinnerl *et al.*, 2007). According to a morphological study, Gagnepain organized *S. tuberosa* and *S. phyllantha* into the same group because floral characters are almost similar except the fusion of peduncle with petiole and the size of perianth (Gagnepain, 1934; Duyfjes, 1993). In chemotaxonomy, stichoneurine- and croomine-type alkaloids were found in *S. tuberosa* and *S. phyllantha* while protostemonine- type alkaloids were found only in other species (Schinnerl *et al.*, 2007). Based on these alkaloids constituent, the biological activities are different. For instance, in *S. collinsae*, protostemonine-type alkaloids, didehydrostemofiline, showed the high insectoxicities while different provenances of *S. tuberosa* showed only very low activity or no activity. However, in *S. tuberosa*, stichoneurine-type alkaloids, tuberostemonine, showed outstanding repellency (Bem *et al.*, 2002). In a preliminary anti-tumor test crude extracts of *S. tuberosa* and *S. collinsae* were compared for their effects on medullary thyroid carcinoma cells. The extract of *S. tuberosa* considerably enhanced apoptosis, whereas that of *S. collinsae* only moderately increased the apoptotic effect (Rinner *et al.*, 2004). Based on the above-mentioned data, *Stemona* in Thailand could be classified into two groups: 1) *S. tuberosa* group: *S. tuberosa* and *S. phyllantha*, and 2) *S. collinsae* group: *S. collinsae*, *S. burkillii, S. aphylla* and *Stemona*

sp. Hence, the *mat*K sequence could support and confirm the relationship among *Stemona* spp. in Thailand and also consequently refer to biological activities.

However, based on the phylogenetic tree constructed from *mat*K gene, *trn*H-*psb*A region and ITS region, the results are dissimilar. These may be a consequence of different lineage sorting (Doyle and Davis, 1998). The plastid *mat*K gene are coding region (Komatsu *et al.*, 2001) whereas the two intergenic speacer of plastid *trn*H-*psb*A region and nuclear ITS region are non-coding region (Sang *et al.*, 1997). In addition, consistency index (CI) and retention index (RI) are often used as a measurement of accuracy of the topology (Hall, 2004). The phylogenetic tree obtained from *mat*K gene had higher value index than *trn*H-*psb*A region and ITS region. As the result of the comparison of *mat*K with *trn*H-*psb*A and *trn*L-*trn*F showed that *mat*K produced the best resolved phylogenetic tree (Sang *et al.*, 1997). This indicated that the phylogenetic tree obtained from *mat*K in this study had more high confidence than another two regions. However, to prove these phylogenetic hypotheses and produce the best phylogenetic reconstruction, the whole genomic sequencing is required.

1.2 PCR-RFLP analysis

DNA sequencing techniques have been used for classification of many medicinal plants and have revealed a variation among species, but the costs are relatively high (Yan *et al.*, 2007; Wang *et al.*, 2007). PCR-RFLP technique was employed to develop simple and efficient identification of *Stemona*. In this study, two pairs of primers: 1) Stemoja-528F and Stemoja-970R, and 2) Stmn-18S-F and Stmn-5S-nR, were used to amplify PCR products from genomic DNA in short fragments for application in crude drugs in subsequently study.

The PCR products amplified with a pair of primers, Stemoja-528F and Stemoja-970R, showed a band of 443 bp in all *Stemona.* Then, PCR products were digested with restriction enzymes *Bgl*I for separation of *Stemona* sp. from other species. For classification of *S. phyllantha* and *S. tuberosa* from other species, PCR products amplified with these primers were digested with restriction enzymes *Mse*I. Because of

the high similarity of the *mat*K sequences which amplified by these primers, they do not have the restriction site to differentiate all species. Another pair of primers, Stmn-18S-F and Stmn-5S-nR, was utilized for identification of the remaining species. Using these primers and digestion with restriction enzyme *Mse*I, *S. phyllantha* and *S. tuberosa* could be distinguished. Subsequently, PCR products were digested with enzyme *Dde*I can differentiate the remainder species: *S. burkillii*, *S. aphylla* and *S. collinsae.* (Table 10). Hence, the PCR-RFLP pattern of *Stemona* in this study indicated that the usage of tree restriction enzyme and two primer sets are enough for the identification of six *Stemona* in Thailand.

Some PCR products of the ITS1 regions amplified by a pair of primers, Stmn-18S-F and Stmn-5S-nR, could be used to differentiate *Stemona*. However, they could not be completely digested and consequently the fragment of 365 bp existed. Improper reaction conditions were not the reason for the under digestion because many reaction conditions were tested such as increasing the quantity of enzyme and prolonging reaction time. The partial digestion is probably due to hybridization between related species and these results are similar to a previous report on *Fritillariae cirrhosae* (Wang *et al.*, 2007). It is possible that not all copies of nrDNA ITS regions of *Stemona* have the same distinguishing base at restriction site because there are many copies of nrDNA in a set of chromatosome or hybridization between related species might occur (Wang *et al.*, 2007). However, partial digestion did not disturb the identification of *Stemona* as shown in Figure 4.10 and 4.11.

1.3 Crude drugs analysis

In the present study, a PCR-RFLP method was developed to identify *Stemona* in Thailand. In many crude drug samples, DNA degraded into small pieces due to the oxidative and hydrolytic processes during the preservation period or drug preparation. Consequently, long PCR products were difficult to be obtained (Yang *et al.*, 2004). Thus, short fragments of PCR products were used. The results showed that this

technique is a relatively simple method, and provides an effective and accurate tool for the identification of *Stemona,* even as crude drug samples.

In the case of crude drugs of *Stemona*, four specimens called as “Non Tai Yak” from herbal-shops were tested using procedure as mentioned in figure 4.12. Samples R1 and R3 showed PCR-RFLP pattern similar to *S. phyllantha* and R2 similar to *S. collinsae* (see detail of crude drugs in Table 3 and Figure 3.1). But the restriction enzyme pattern of sample R4 was distinct. The results proved that more than one species of *Stemona* in Thailand were sold on local markets under the same name as “Non Tai Yak” and not in the six species in this study. To know exactly the species of R3, increasing more authentic *Stemona* species should be studied.

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CHAPTER VI

CONCLUSION

In Thailand, the underground parts of *Stemona* have similar in shapes and are sold on local markets and in herbal-shops under the same vernacular name "Non Tai Yak (หนอนตายหยาก)". However, their alkaloid constituents are distinct. Consequently, the biological activities are different. The usage of *Stemona* roots without proper identification poses a risk for practical applications both in agriculture and medicine. Thus, accurate identification of these plants is needed to ensure their efficacies. The purpose of this study was to analyze the DNA fingerprints of six *Stemona* species in Thailand: *S. tuberosa* Lour., *S. collinsae* Craib, *S. phyllantha* Gagnep, *S. burkillii* Prain, *S. aphylla* Craib and *Stemona* sp., and to develop PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) for using as a convenient tool for identification.

The nucleotide sequences of three DNA regions; *mat*K, *trn*H-*psb*A, and ITS1 were examined for the identification of six *Stemona* species in Thailand. Based on parsimony analysis on obtained partial *mat*K sequences, *Stemona* could be divided into two groups, *S. tuberosa* group (*S. tuberosa* and *S. phyllantha*) and *S. collinsae* group (*S. collinsae*, *S. burkillii*, *S. aphylla*, and *S.* sp.), according to their morphology and chemical compositions. The nucleotide of the *trn*H-*psb*A and ITS1 region was determined to be 1,061-1,086 bp and 363-368 bp in length, respectively and could be used to differentiate *Stemona* in Thailand. Based on these sequences, *S. collinsae* could be divided into two subgroups with regard to their habit; a short erect herb or climber.

On the basis of the differences in nucleotide substitutions in *mat*K gene and ITS1 region, the PCR-RFLP analysis was demonstrated to be a convenient and effective tool for *Stemona* identification. Development of PCR-RFLP methods using singly two pairs of primers in amplification reactions; 1) Stemoja-528F and Stemoja-970R, and 2) Stmn-18S-F and Stmn-5S-nR, together with three restriction enzymes, *Bgl*I, *Mse*I and

*Dde*I, was achieved. Furthermore, developed PCR-RFLP technique can be applied for the authentication of *Stemona* crude drugs purchased from the markets.

In conclusion, this study exhibited an effective and accurate authentication of S*temona* in Thailand using nucleotide sequences of *mat*K, *trn*H-*psb*A and ITS1. A convenient and rapid PCR-RFLP analytical method established here can be used as a convenient tool for identification of *Stemona* and their crude drugs.

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APPENDICES

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APPENDIX A

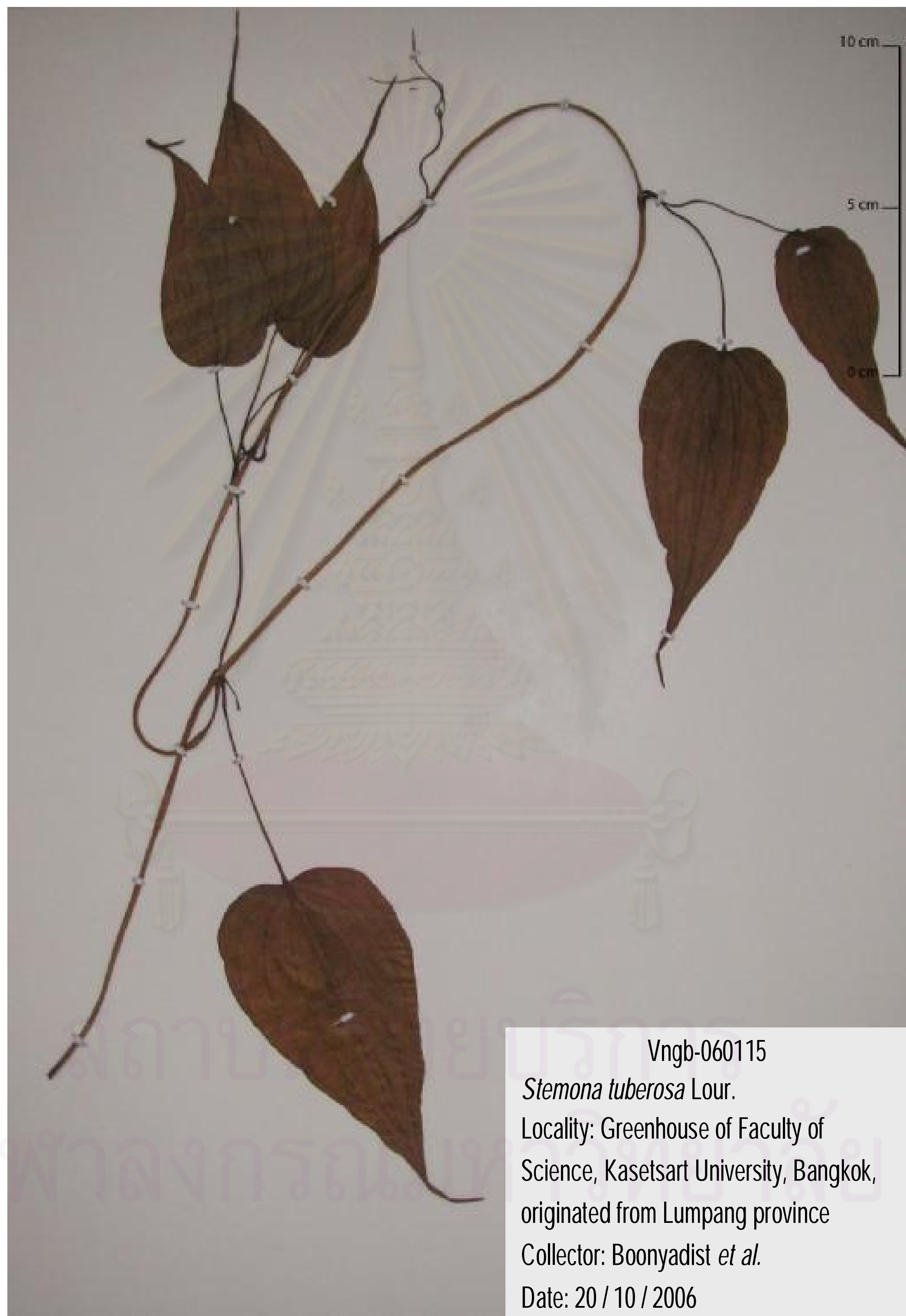

Figure A 1.1 *Stemona tuberosa* Lour.

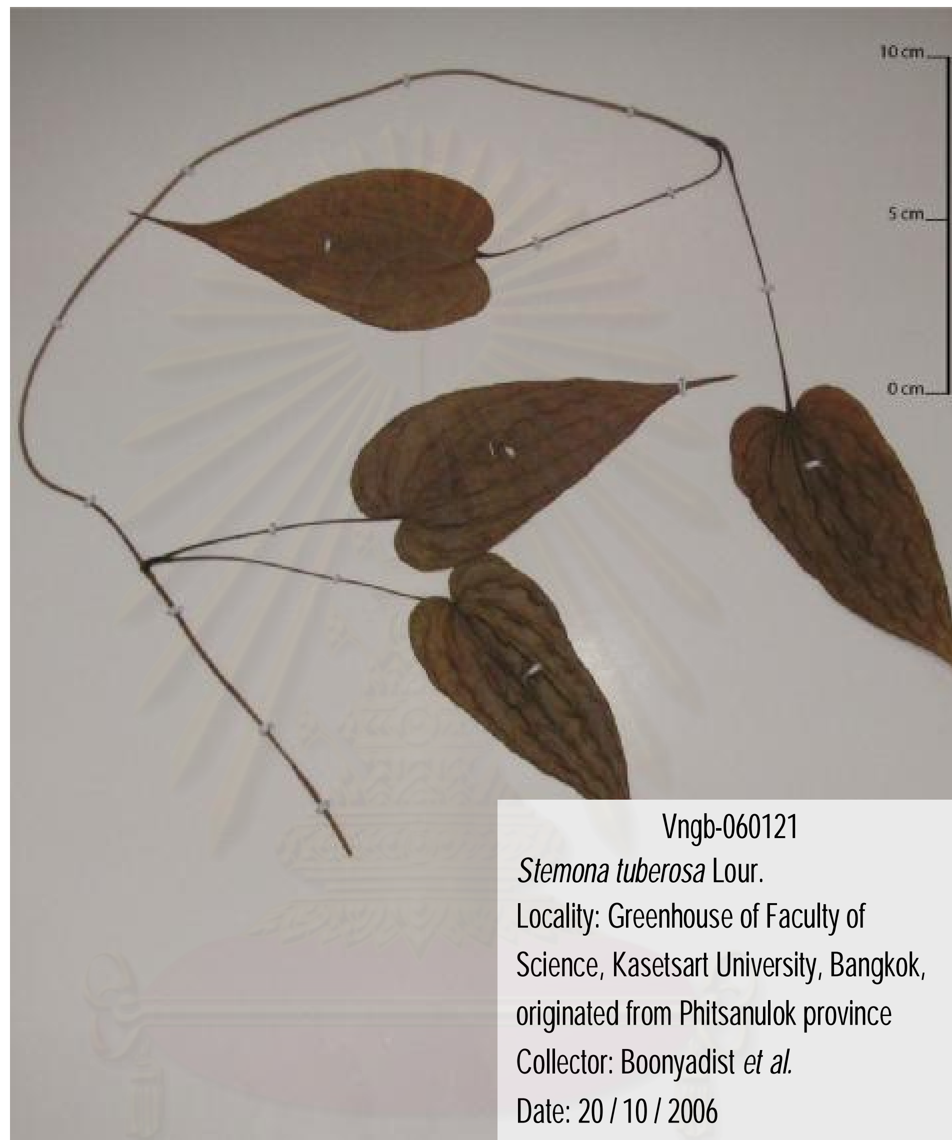

Figure A 1.2 *Stemona tuberosa* Lour.

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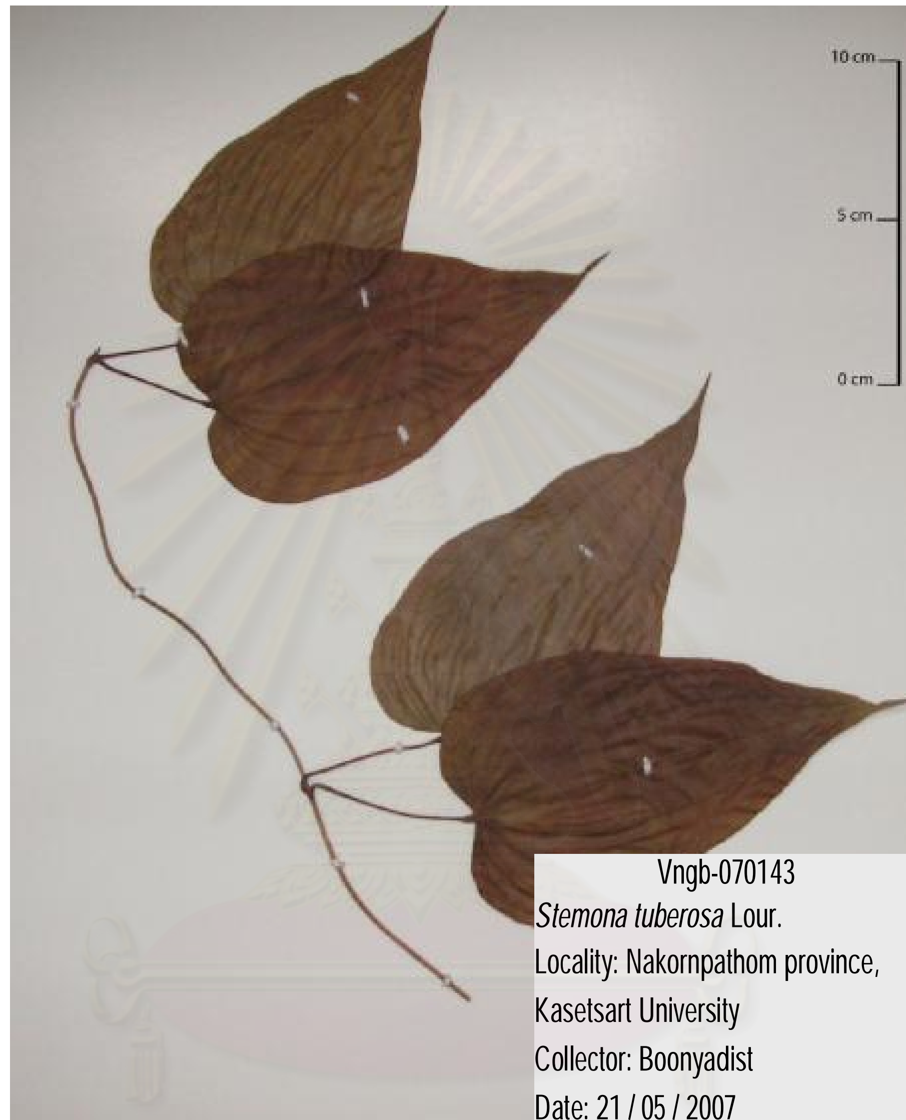

Figure A 1.3 *Stemona tuberosa* Lour.

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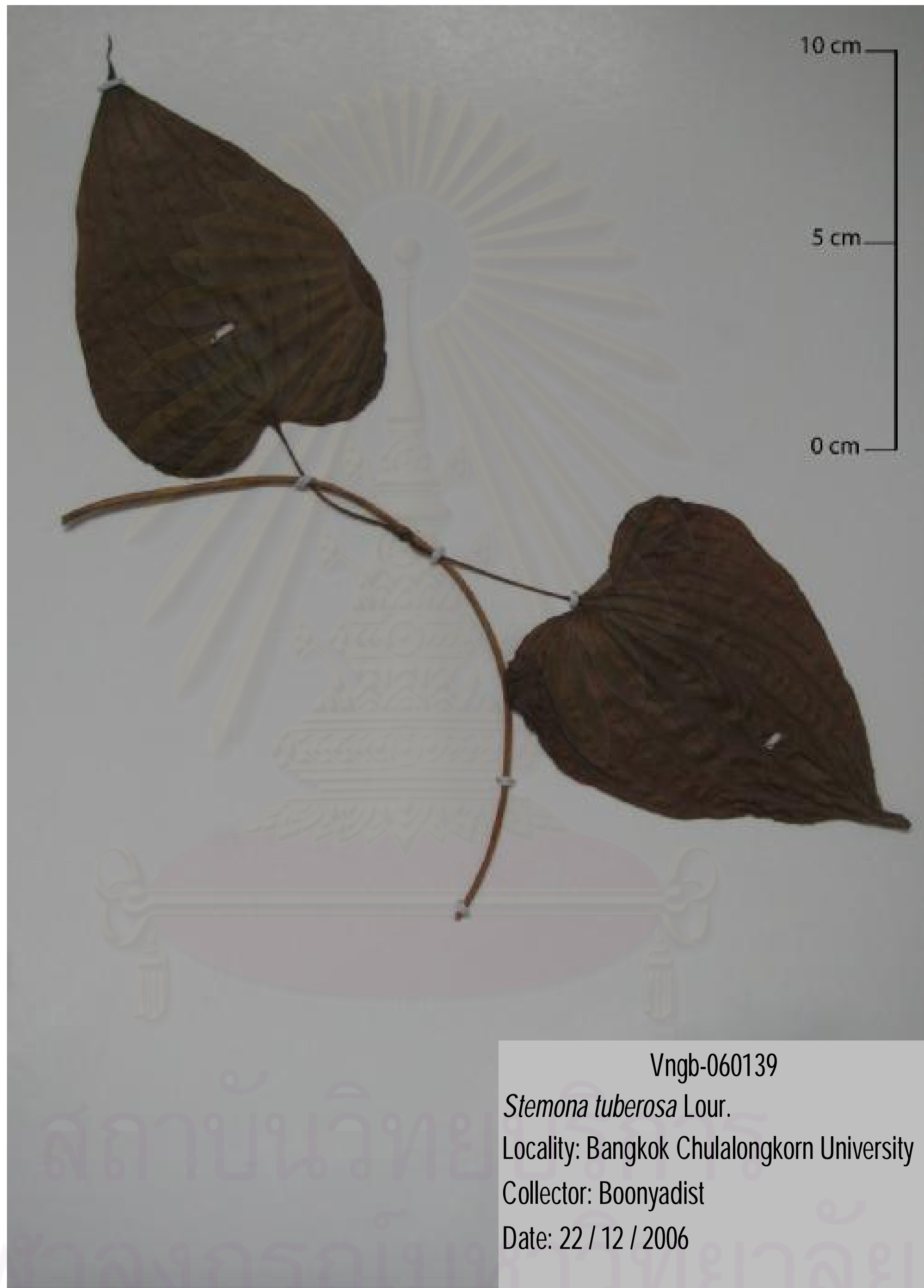

Figure A 1.4 *Stemona tuberosa* Lour.

Figure A 2.1 *Stemona phyllantha* Gagnep.

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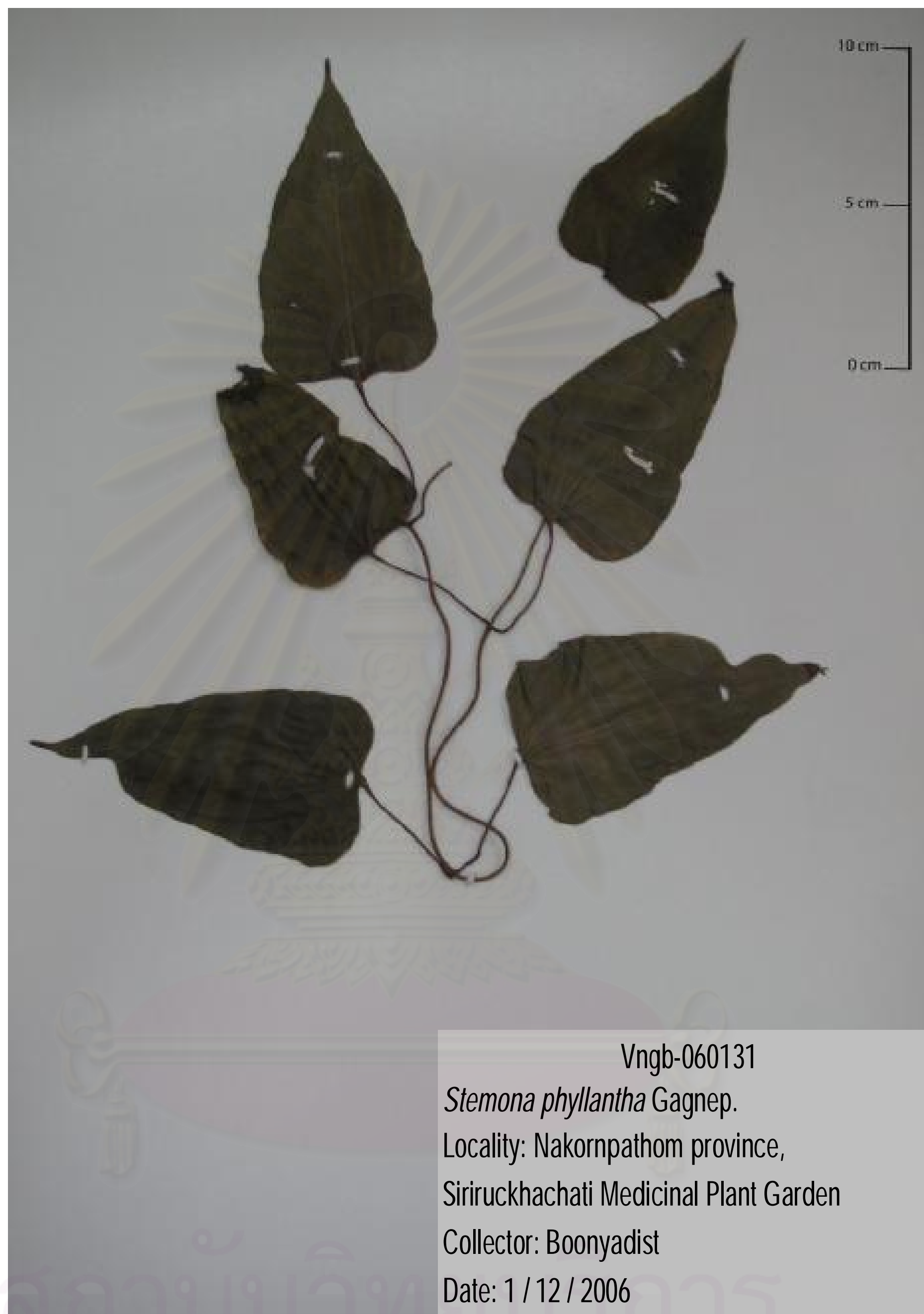
Figure A 2.2 *Stemona phyllantha* Gagnep.



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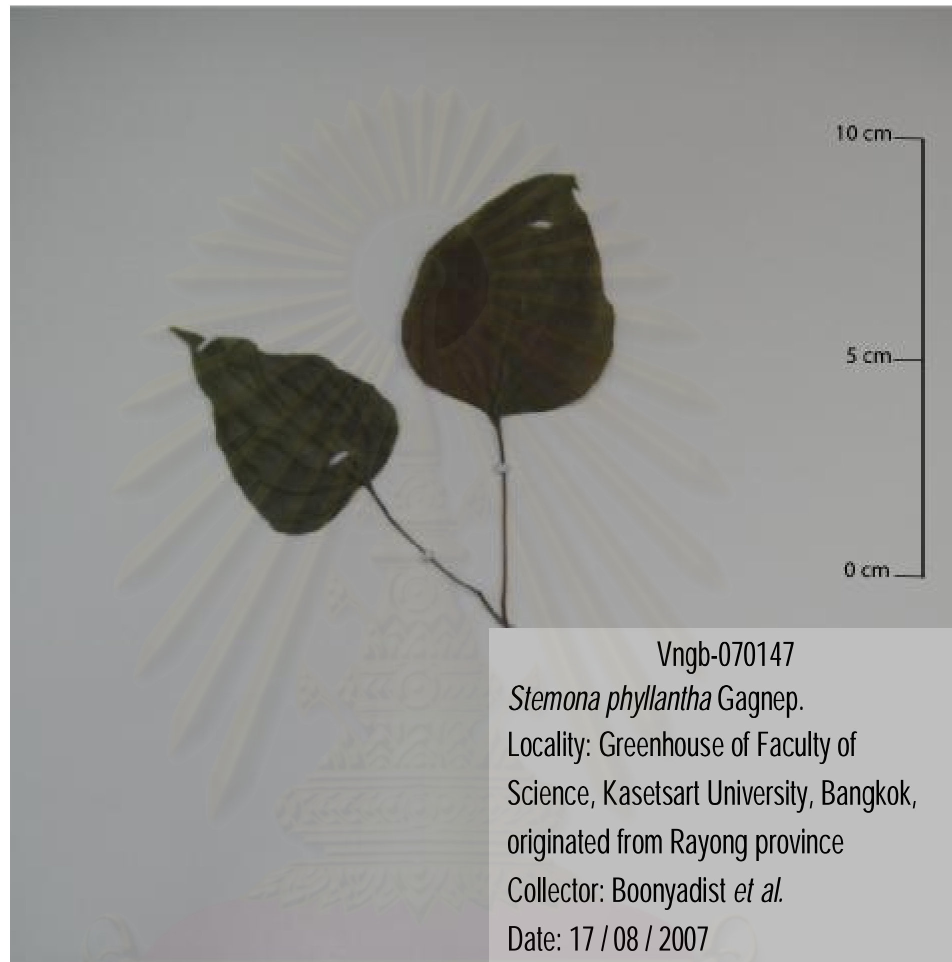

Figure A 2.4 *Stemona phyllantha* Gagnep.

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Figure A 3.1 *Stemona collinsae* Craib

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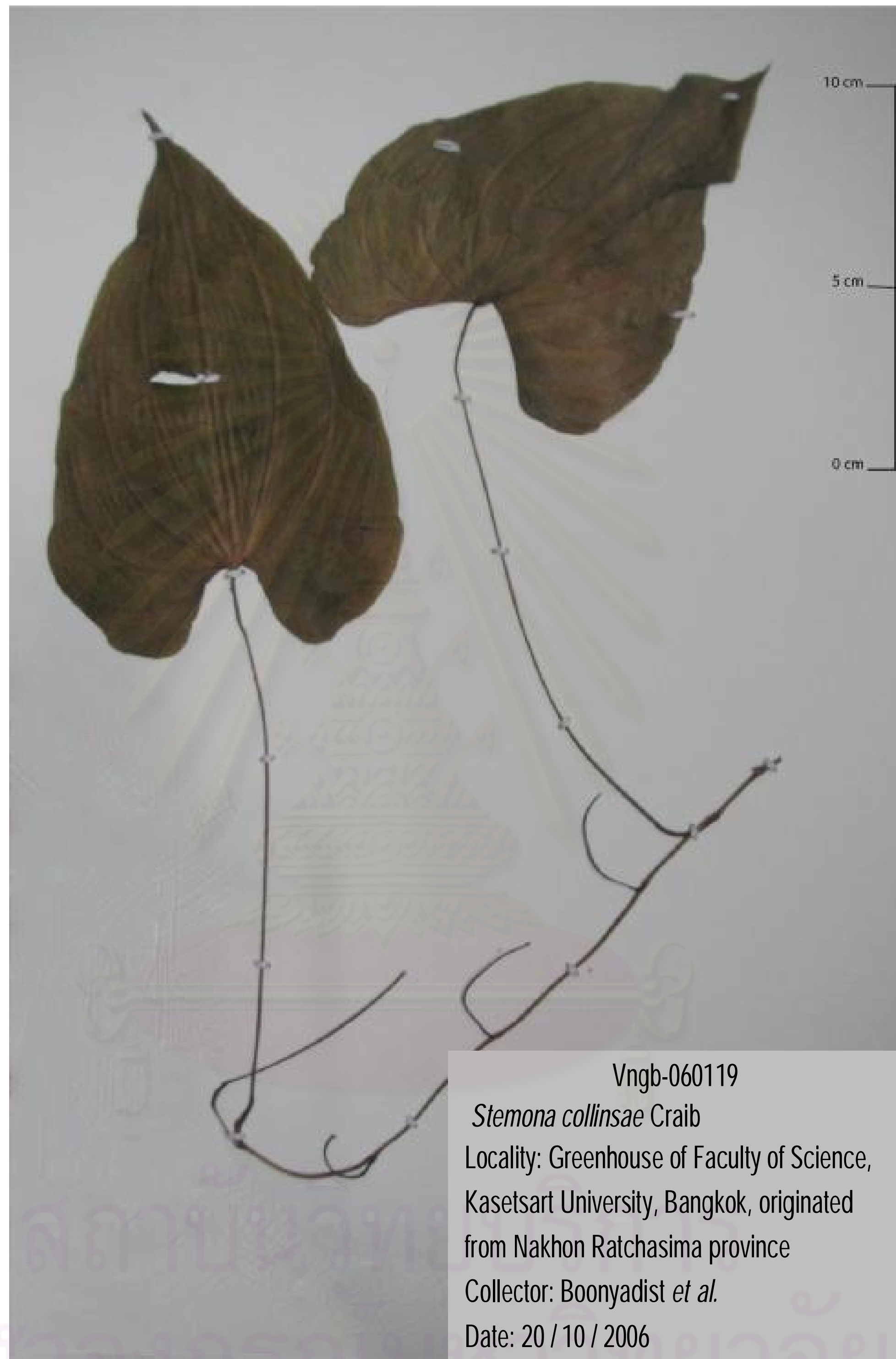

Figure A 3.2 *Stemona collinsae* Craib

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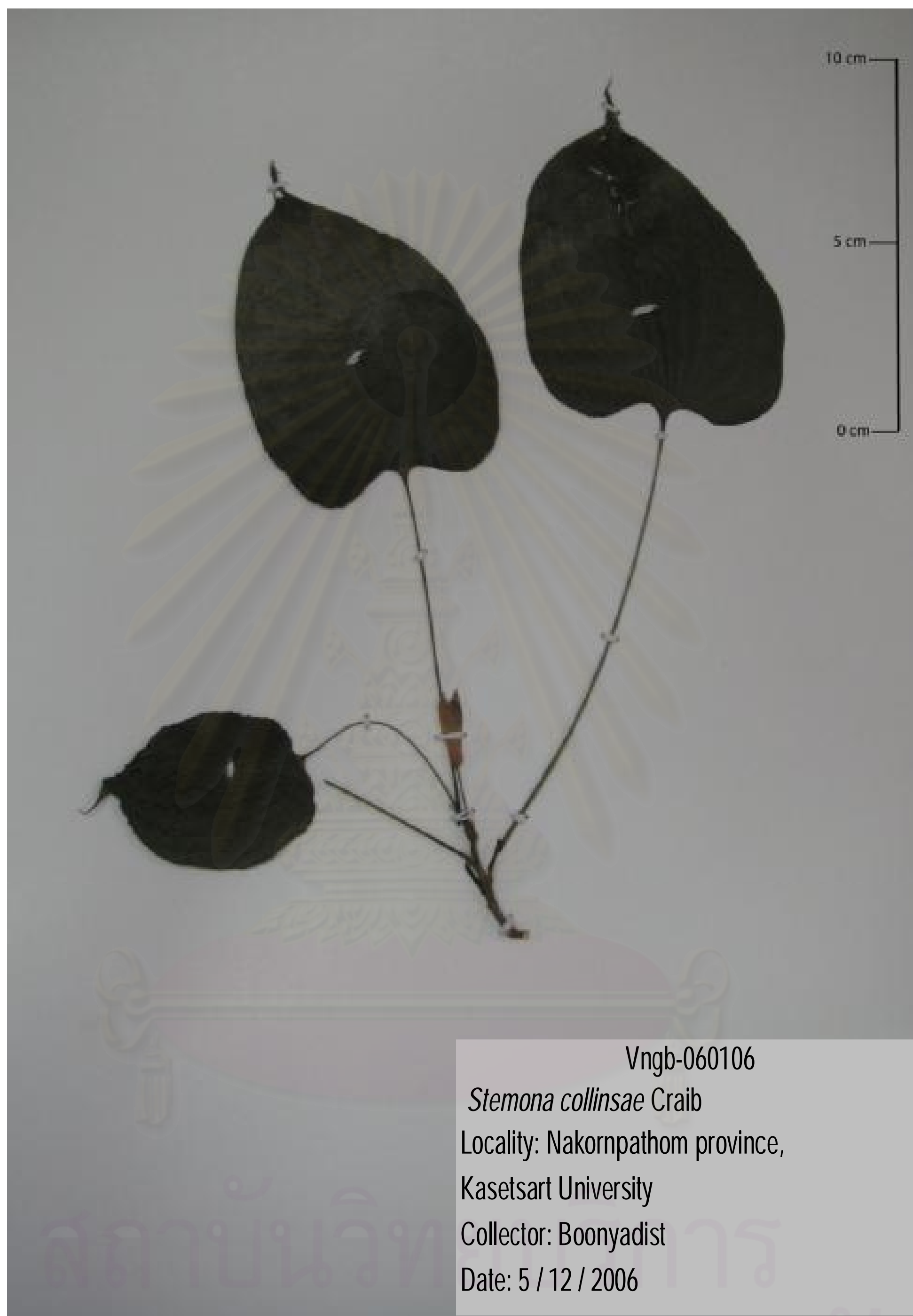

Figure A 3.3 *Stemona collinsae* Craib

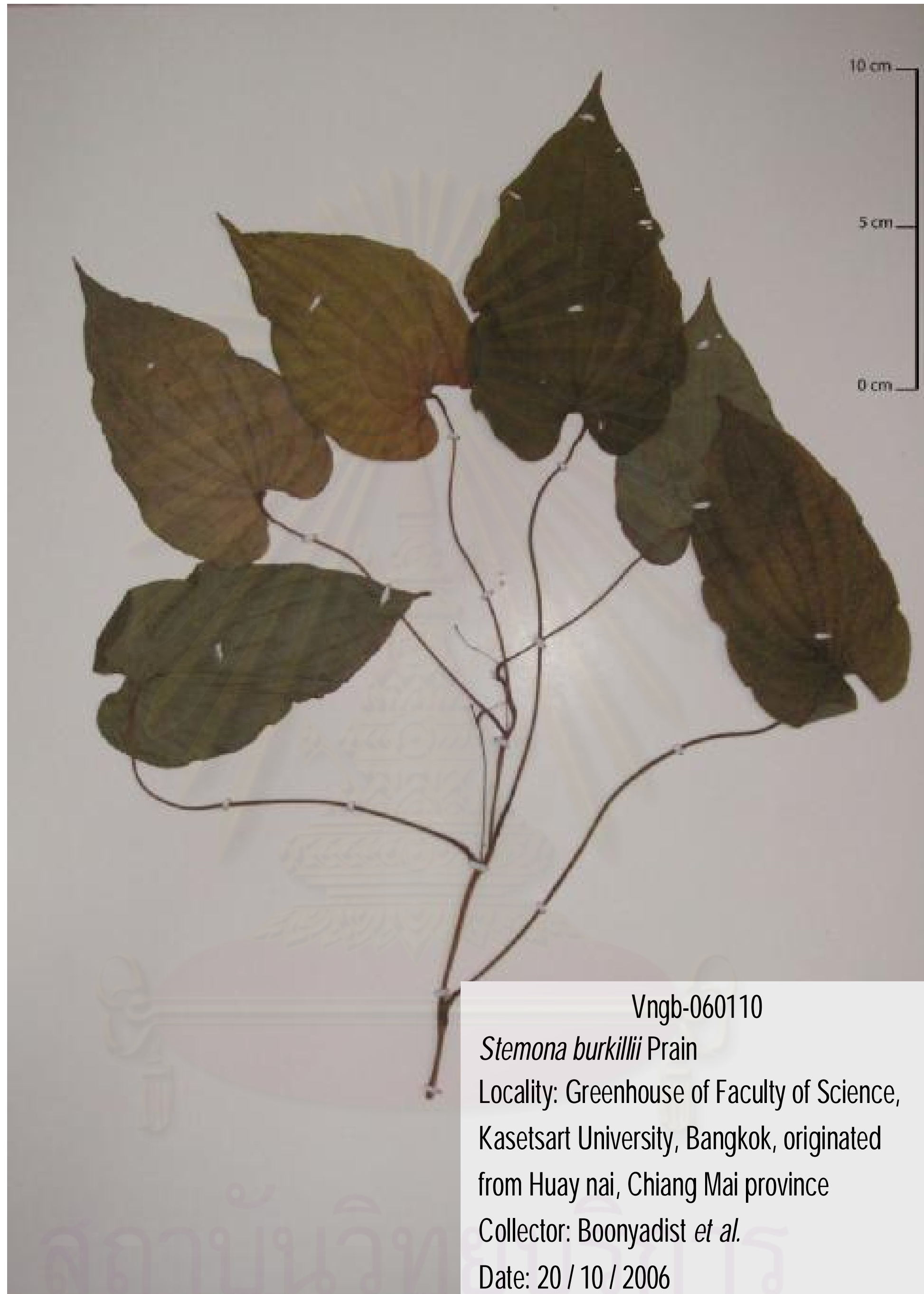

Figure A 4.1 *Stemona burkillii* Prain

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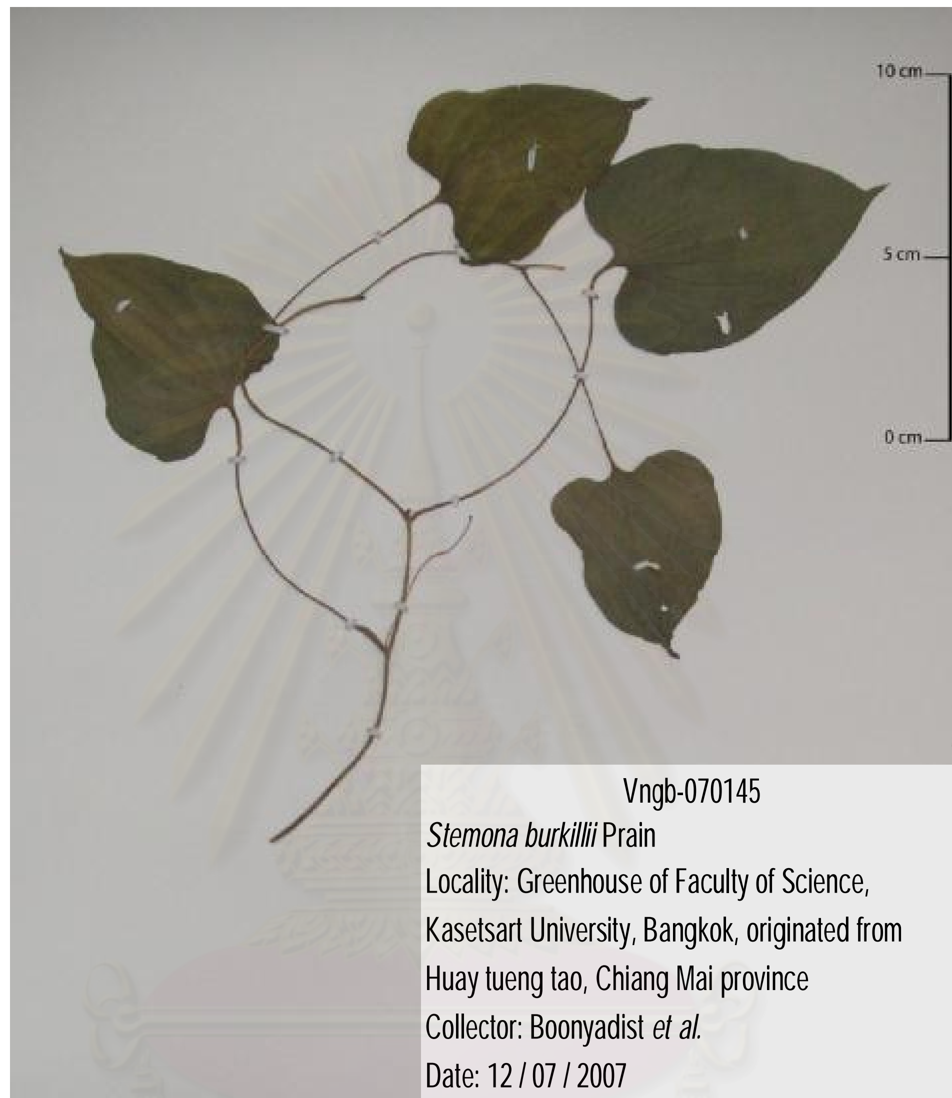

Figure A 4.2 *Stemona burkillii* Prain

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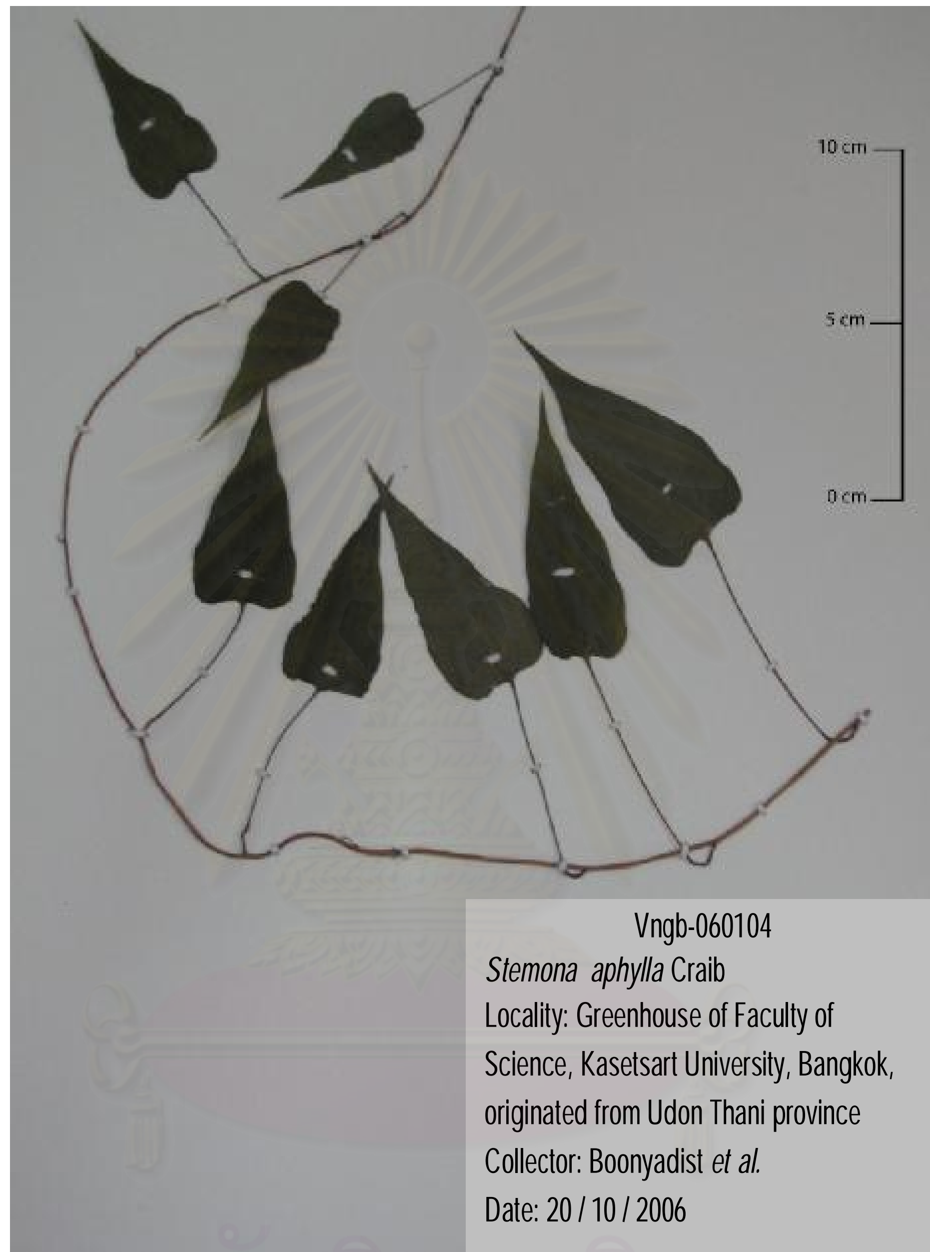

Figure A 5 *Stemona aphylla* Craib

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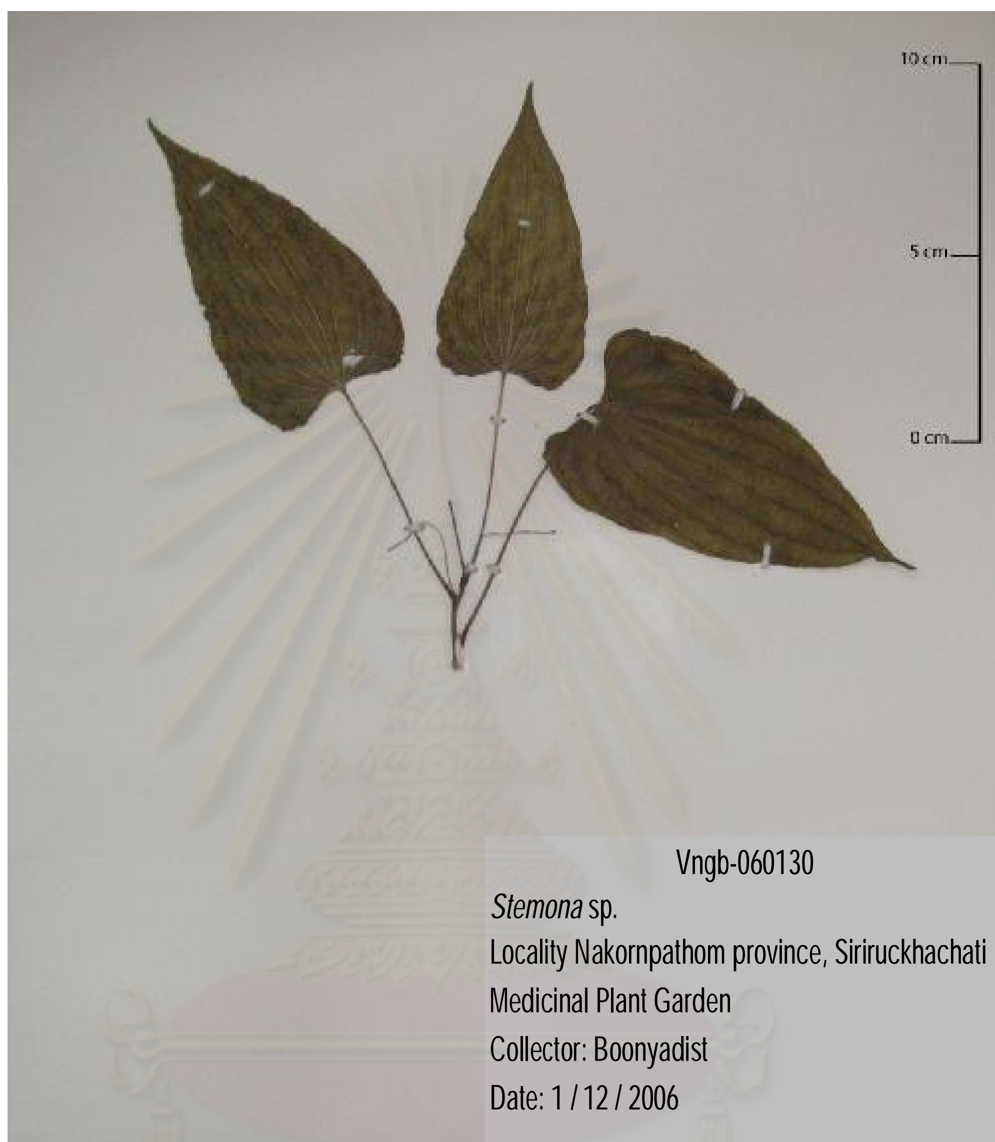

Figure A 6 *Stemona* sp.

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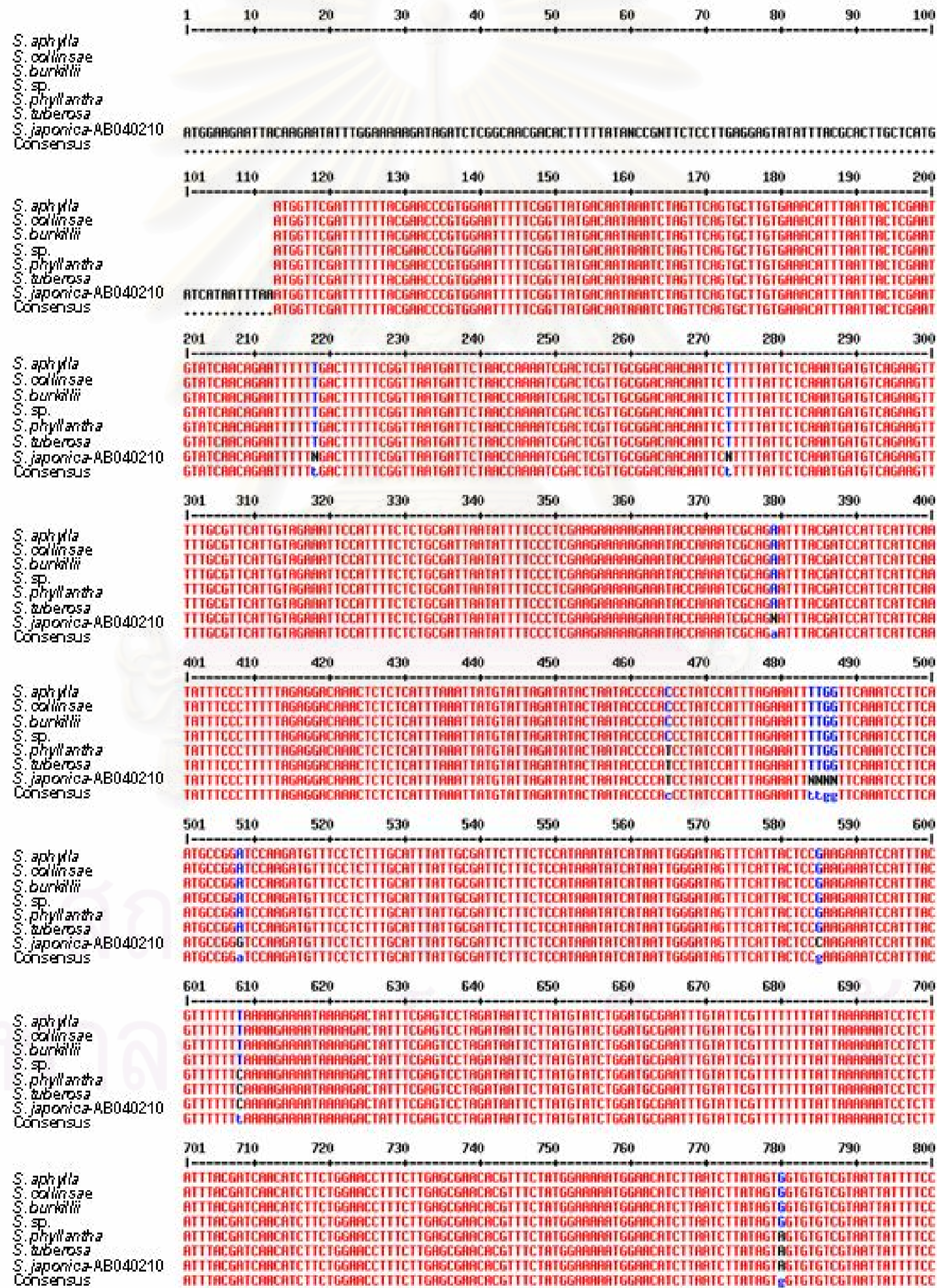
APPENDIX B

Figure B 1 The partial *mat*K gene sequence alignment of *Stemona* in Thailand compared with complete *mat*K sequence of *S. japonica* (accession number AB040210). The red and blue nucleotides are high and low consensus sequences, respectively.



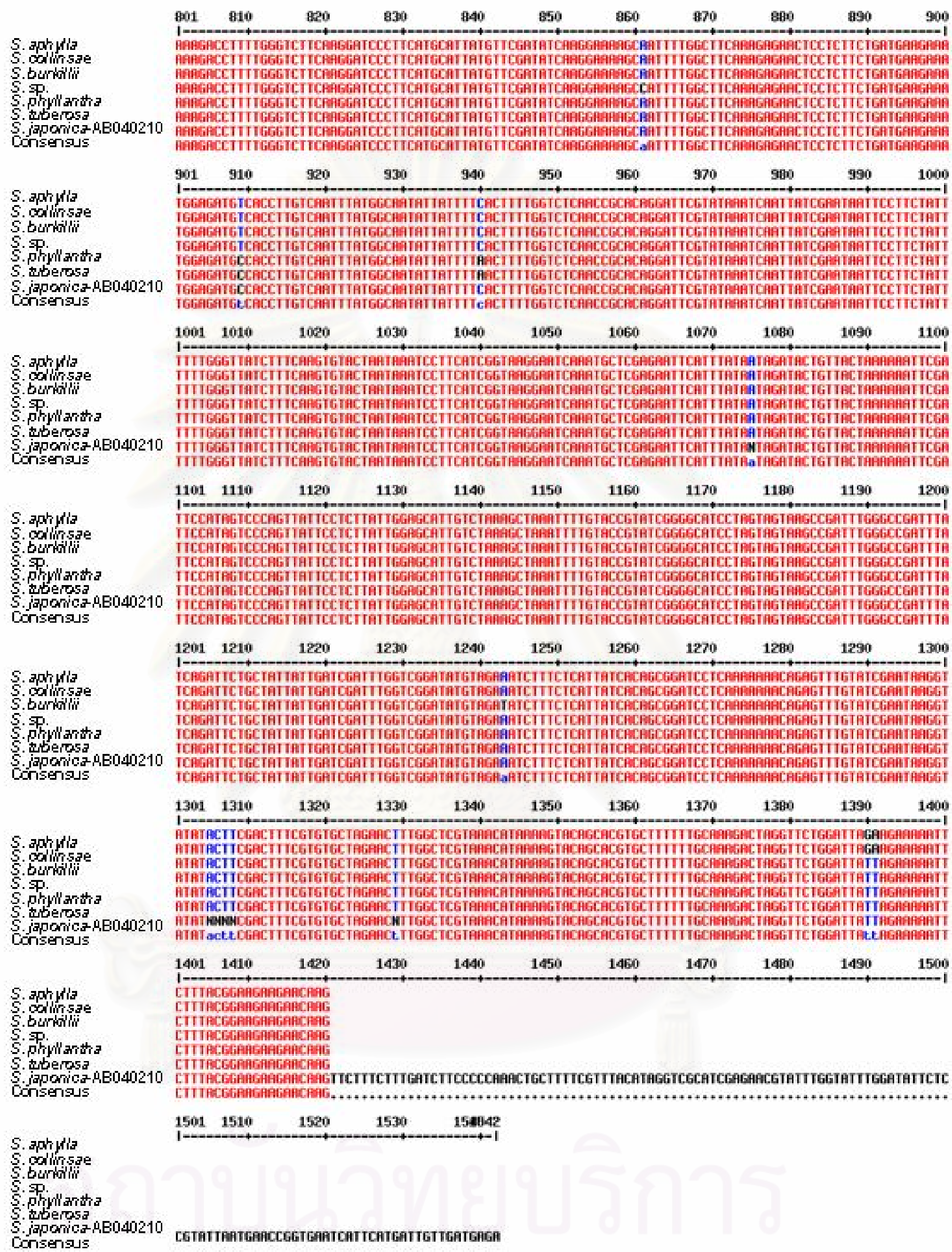


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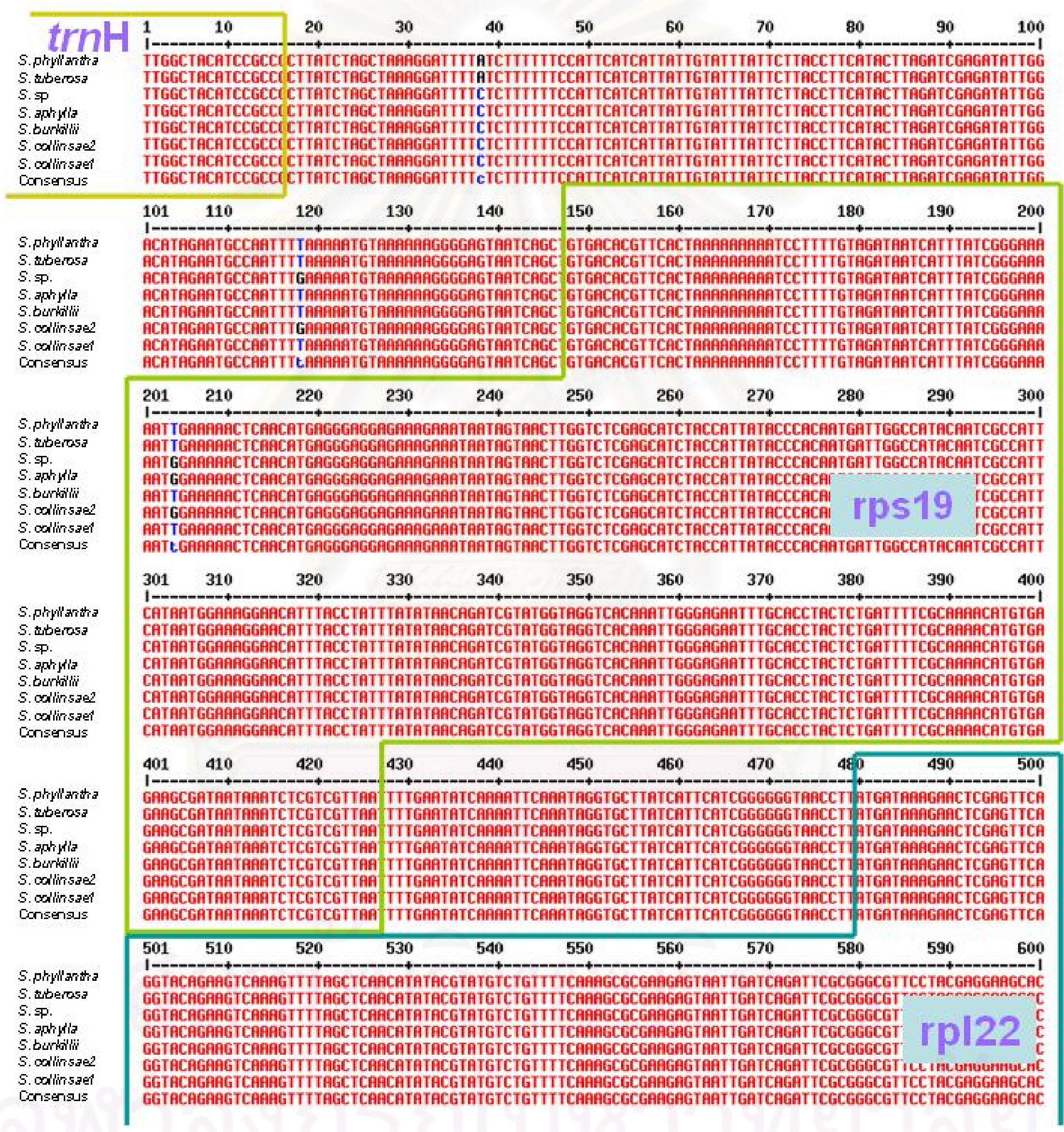
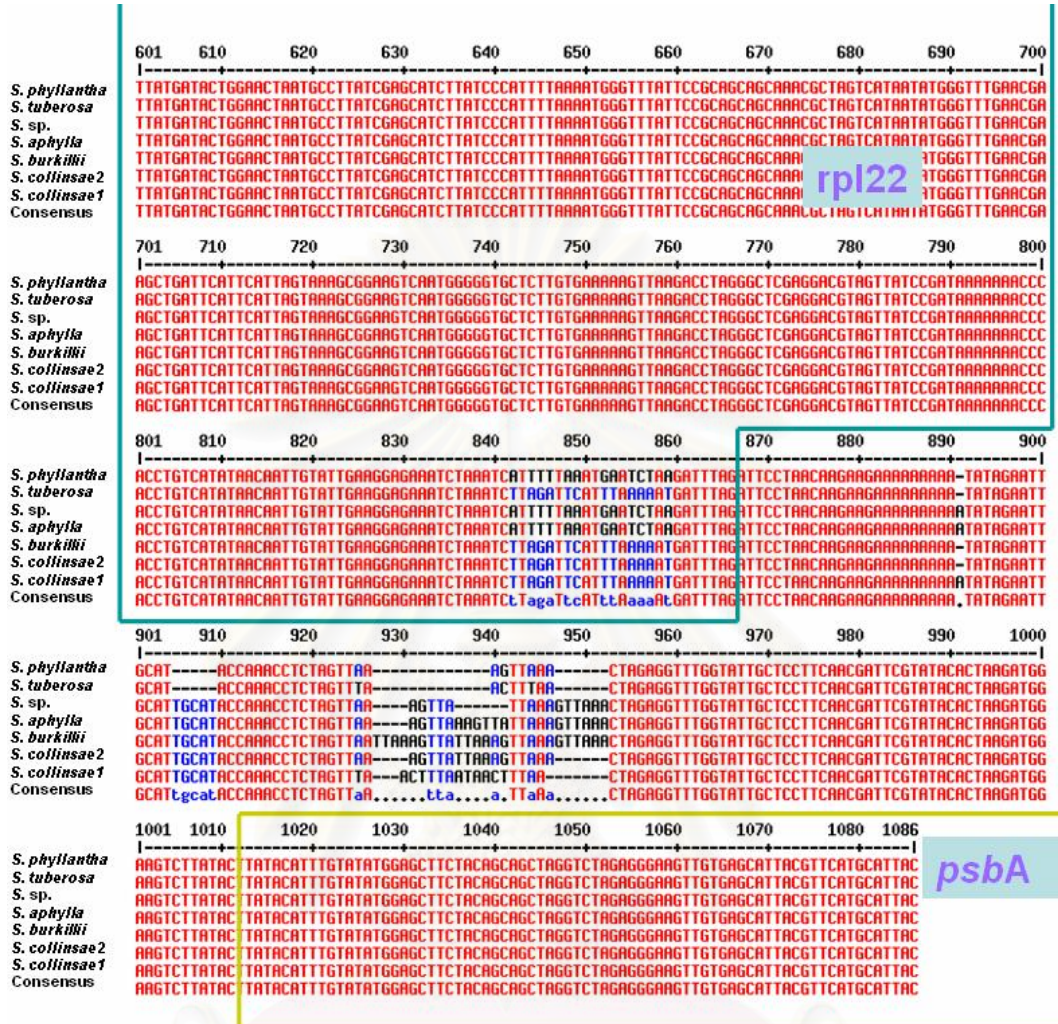
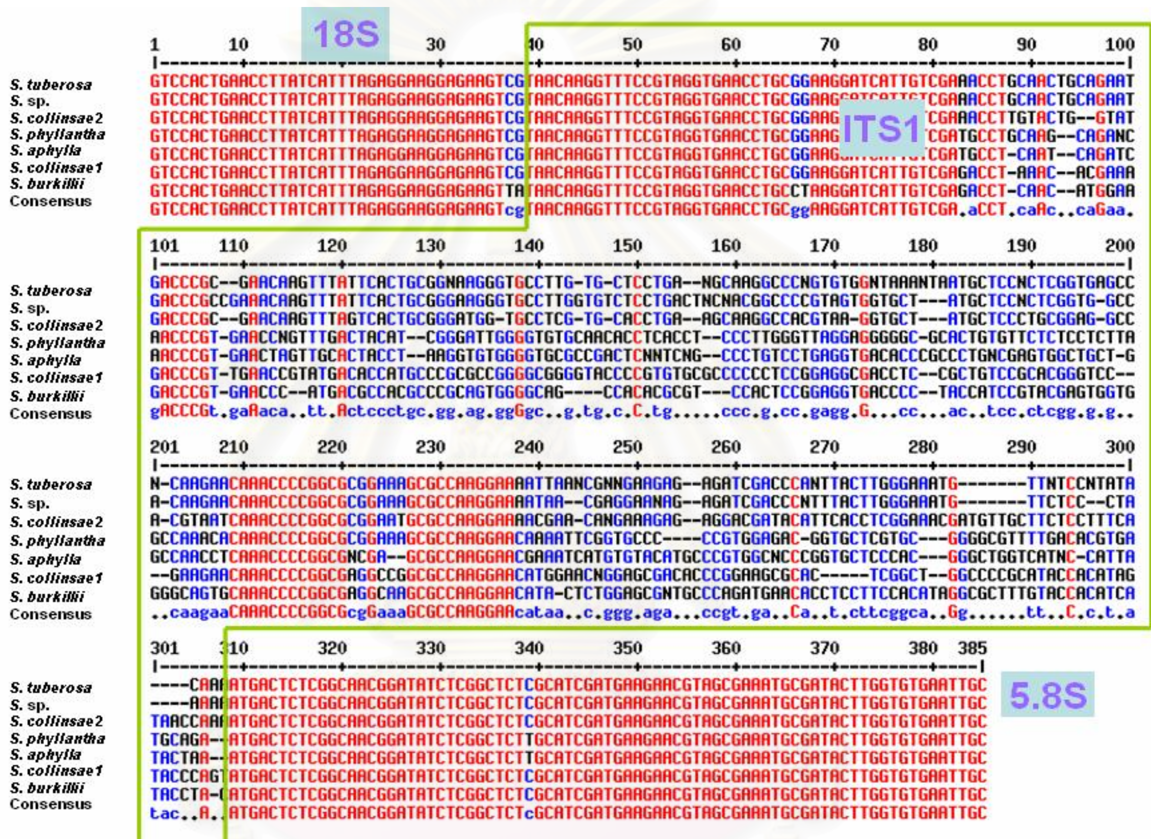
Figure B 2 Alignment of *trn*H-*psb*A region sequences from all six species of *Stemona* spp. in Thailand Hyphen (-) denotes alignment gap. The rps19 and rpl22 are boundaries between *trn*H-*psb*A region in positions 148-426 and 480-866, respectively. The red and blue nucleotides are high and low consensus sequences, respectively.





rpl22
psbA
S. phyllantha
S. tuberosa
S. sp.
S. aphylla
S. burkillii
S. collinsae2
S. collinsae1
Consensus

Figure B 3 The Sequence Alignments of 18S-ITS1-5.8S Region of *Stemona* in Thailand. The ITS 1 region corresponds to positions 39-308 (enclosed in box). Gaps (-) are introduced for the best alignment. The red and blue nucleotides are high and low consensus sequences, respectively.



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APPENDIX C

Data of DNA sequences obtained from this study and were submitted to GenBank database

1. DNA sequences of partial matK gene

1.1 *S. tuberosa* Lour.

ACCESSION AB373230

FEATURES Location/Qualifiers

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/identified_by="Srunya Vajrodaya"

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1.2 *S. phyllantha* Gangep.

ACCESSION AB373229

FEATURES Location/Qualifiers

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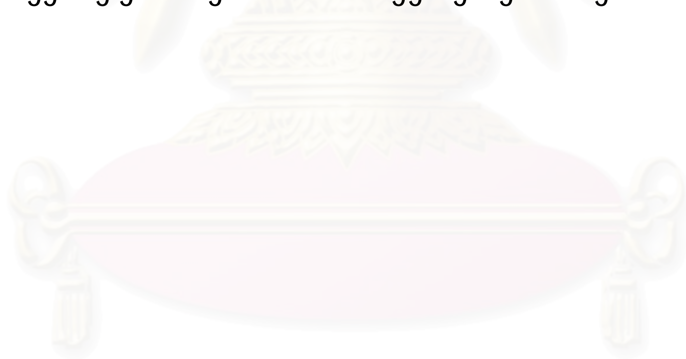

1.3 *S. collinsae* Craib

ACCESSION AB373226

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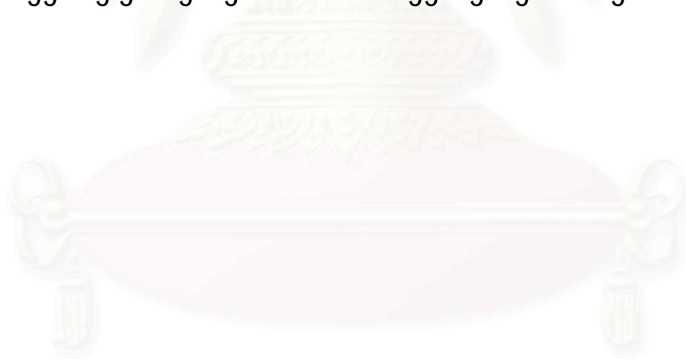

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1.4 *S. burkillii* Prain

ACCESSION AB373225

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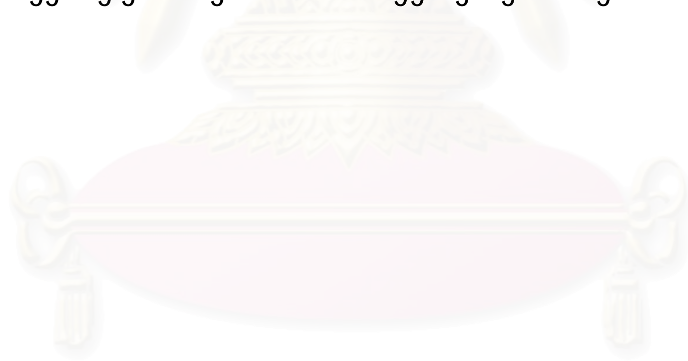

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1.5 *S. aphylla* Craib

ACCESSION AB373224

FEATURES Location/Qualifiers

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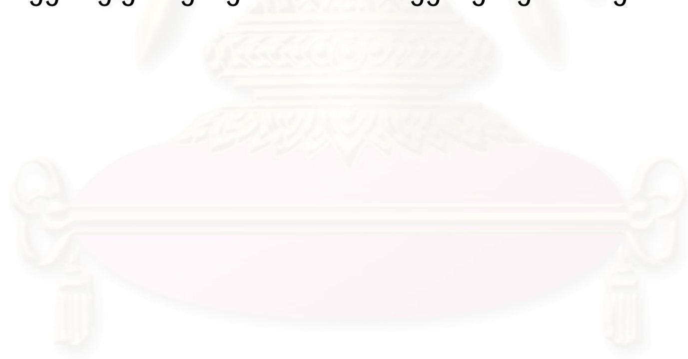

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1.6 *S.* sp.

ACCESSION AB373228

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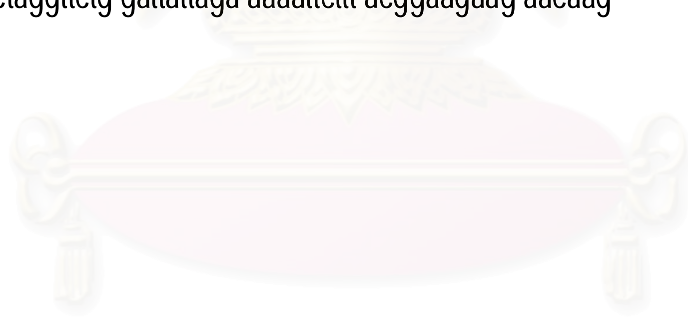

2. DNA sequences of *trn*H-*psb*A region

2.1 *S. tuberosa* Lour.

ACCESSION AB373199

FEATURES Location/Qualifiers

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/country="Thailand: Lumpang province"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organelle="plastid:chloroplast"

/organism="Stemona tuberosa"

tRNA complement(<1..16)

/gene="trnH"

/product="tRNA-His"

misc_feature 17..986

/note="psbA-trnH intergenic spacer"

CDS 148..426

/codon_start=1

/gene="rps19"

/product="30S ribosomal protein S19"

/transl_table=11

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IGHTIAIHNGKEHLPIYITDRMVGHKLGEFAPTLIFAKHVRSDNKSRR"

CDS 480..845

/codon_start=1

/gene="rpl22"

/product="50S ribosomal protein L22"

/transl_table=11

/translation="MIKNSSSGTEVKVLAQHIRMSVFKARRVIDQIRGRSYEEALMIL

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

2.2 *S. phyllantha* Gangep.

ACCESSION AB373198

FEATURES Location/Qualifiers

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/country="Thailand: Kanchanaburi province"
/identified_by="Srunya Vajrodaya"
/mol_type="genomic DNA"
/organelle="plastid:chloroplast"
/organism="Stemona phyllantha"

tRNA complement(<1..16)
/gene="trnH"
/product="tRNA-His"

misc_feature 17..986
/note="psbA-trnH intergenic spacer"

CDS 148..426
/codon_start=1
/gene="rps19"
/product="30S ribosomal protein S19"
/transl_table=11

/translation="MTRSLKKNPFVDNHLSGKIEKLNMREEKEIIVTWSRASTIIPTM
IGHTIAIHNGKEHLPIYITDRMVGHKLGEFAPTLIFAKHVRSDNKSRR"

CDS 480..866
/codon_start=1
/gene="rpl22"
/product="50S ribosomal protein L22"
/transl_table=11

/translation="MIKNSSSGTEVKVLAQHIRMSVFKARRVIDQIRGRSYEEALMIL

สถาบันวิทยบริการ
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ELMPYRASYPILKWVYSAAANASHNMGLNEADSFISKAEVNGGALVKKLRPRARGRSY
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CDS complement(987..>1061)
/codon_start=1
/gene="psbA"
/product="photosystem II D1 protein"
/transl_table=11
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2.3.1 *S. collinsae* Craib -1

ACCESSION AB373194

FEATURES Location/Qualifiers

source 1..1076

/country="Thailand: Saraburi"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organelle="plastid:chloroplast"

/organism="Stemona collinsae"

tRNA complement(<1..16)

/gene="trnH"

/product="tRNA-His"

misc_feature 17..1001

/note="psbA-trnH intergenic spacer"

CDS 148..426

/codon_start=1

/gene="rps19"

/product="30S ribosomal protein S19"

/transl_table=11

/translation="MTRSLKKNPFVDNHLSGKIEKLNMREEKEIIVTWSRASTIIPTM

IGHTIAIHNGKEHLPIYITDRMVGHKLGEFAPTLIFAKHVRSDNKSRR"

CDS 480..845

/codon_start=1

/gene="rpl22"

/product="50S ribosomal protein L22"

/transl_table=11

/translation="MIKNSSSGTEVKVLAQHIRMSVFKARRVIDQIRGRSYEEALMIL

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PIKKPTCHITIVLKEKSKS"

CDS complement(1002..>1076)

/codon_start=1

/gene="psbA"

/product="photosystem II D1 protein"

/transl_table=11

/translation="VMHERNAHNFPLDLAAVEAPYTNV"

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2.3.2 *S. collinsae* Craib -2

ACCESSION AB373195

FEATURES Location/Qualifiers

source 1..1075

/country="Thailand: Nakhon Ratchasima"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organelle="plastid:chloroplast"

/organism="Stemona collinsae"

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/gene="trnH"

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misc_feature 17..1000

/note="psbA-trnH intergenic spacer"

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/codon_start=1

/gene="rps19"

/product="30S ribosomal protein S19"

/transl_table=11

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CDS 480..845

/codon_start=1

/gene="rpl22"

/product="50S ribosomal protein L22"

/transl_table=11

/translation="MIKNSSSGTEVKVLAQHIRMSVFKARRVIDQIRGRSYEEALMIL

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/gene="psbA"
/product="photosystem II D1 protein"
/transl_table=11
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2.4 *S. burkillii* Prain

ACCESSION AB373193

FEATURES Location/Qualifiers

source 1..1085

/country="Thailand: Chiang Mai"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organelle="plastid:chloroplast"

/organism="Stemona burkillii"

tRNA complement(<1..16)

/gene="trnH"

/product="tRNA-His"

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/note="psbA-trnH intergenic spacer"

CDS 148..426

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/product="30S ribosomal protein S19"

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IGHTIAIHNGKEHLPIYITDRMVGHKLGEFAPTLIFAKHVRSDNKSRR"

CDS 480..845

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/gene="rpl22"

/product="50S ribosomal protein L22"

/transl_table=11

/translation="MIKNSSSGTEVKVLAQHIRMSVFKARRVIDQIRGRSYEEALMIL

สถาบันวิทยบริการ
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2.5 *S. aphylla* Craib

ACCESSION AB373192

FEATURES Location/Qualifiers

source 1..1082

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/organism="Stemona aphylla"

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/product="tRNA-His"

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/note="psbA-trnH intergenic spacer"

CDS 148..426

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/product="30S ribosomal protein S19"

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CDS 480..866

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/gene="rpl22"

/product="50S ribosomal protein L22"

/transl_table=11

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2.6 *S.* sp.

ACCESSION AB373196

FEATURES Location/Qualifiers

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/identified_by="Boonyadist Vongsak"

/mol_type="genomic DNA"

/organelle="plastid:chloroplast"

/organism="Stemona hutanguriana"

tRNA complement(<1..16)

/gene="trnH"

/product="tRNA-His"

misc_feature 17..1001

/note="psbA-trnH intergenic spacer"

CDS 148..426

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/gene="rps19"

/product="30S ribosomal protein S19"

/transl_table=11

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CDS 480..866

/codon_start=1

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/transl_table=11

/translation="MIKNSSSGTEVKVLAQHIRMSVFKARRVIDQIRGRSYEEALMIL

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ELMPYRASYPILKWVYSAAANASHNMGLNEADSFISKAEVNGGALVKKLRPRARGRSY
PIKKPTCHITIVLKEKSKSFLNESKI"

CDS complement(1002..>1076)
/codon_start=1
/gene="psbA"
/product="photosystem II D1 protein"
/transl_table=11
/translation="VMHERNAHNFPLDLAAVEAPYTNV"

ORIGIN

1 ttggctacat ccgcccctta tctagctaaa ggattttctc tttttccat tcatcattat
61 tgtatttatt cttaccttca tacttagatc gagatattgg acatagaatg ccaatttgaa
121 aaatgtaaaa aaggggagta atcagctgtg acacgttcac taaaaaaaaa tccttttgta
181 gataatcatt tatcgggaaa aatggaaaaa ctcaacatga gggaggagaa agaaataata
241 gtaacttggt ctcgagcatc taccattata cccacaatga ttggccatac aatcgccatt
301 cataatggaa aggaacattt acctatttat ataacagatc gtatggtagg tcacaaattg
361 ggagaatttg cacctactct gattttcgca aaacatgtga gaagcgataa taaatctcgt
421 cgttaatttt gaatatcaaa attcaaatag gtgcttatca ttcatcgggg ggtaacctta
481 tgataaagaa ctcgagttca ggtacagaag tcaaagtttt agctcaacat atacgtatgt
541 ctgttttcaa agcgcgaaga gtaattgatc agattcgcgg gcgttcctac gaggaagcac
601 ttatgatact ggaactaatg ccttatcgag catcttatcc cattttaaaa tgggtttatt
661 ccgcagcagc aaacgctagt cataatatgg gtttgaacga agctgattca ttcattagta
721 aagcggaagt caatgggggt gctcttgtga aaaagttaag acctagggct cgaggacgta
781 gttatccgat aaaaaaaccc acctgtcata taacaattgt attgaaggag aaatctaaat
841 catttttaaa tgaatctaag atttagattc ctaacaagaa gaaaaaaaaa atatagaatt
901 gcattgcata ccaaacctct agttaaagtt attaaagtta aactagaggt ttggtattgc
961 tccttcaacg attcgtatac actaagatgg aagtcttata cttatacatt tgtatatgga
1021 gcttctacag cagctaggtc tagagggaag ttgtgagcat tacgttcatg cattac

//

3. DNA sequences of ITS1

3.1 *S. tuberosa* Lour.

ACCESSION AB429262

FEATURES Location/Qualifiers

source 1..365

/country="Thailand: Lumpang province"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organism="Stemona tuberosa "

rRNA <1..38

/product="18S ribosomal RNA"

misc_RNA 39..288

/note="internal transcribed spacer 1"

rRNA 289..>365

/product="5.8S ribosomal RNA"

ORIGIN

1 GTCCACTGAA CCTTATCATT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA

61 CCTGCGGAAG GATCATTGTC GAAACCTGCA ACTGCAGAAT GACCCGCGAA CAAGTTTATT

121 CACTGCGGNA AGGGTGCCTT GTGCTCCTGA NGCAAGGCCC NGTGTGGNTA AANTAATGCT

181 CCNCTCGGTG AGCCNCAAGA ACAAACCCCG GCGCGGAAAGCGCCAAGGAAAATTAANCGN

241 NGAAGAGAGA TCGACCCANT TACTTGGGAA ATGTTNTCCN TATACAAAAt GACTCTCGGC

301 AACGGATATC TCGGCTCTCG CATCGATGAA GAACGTAGCG AAATGCGATA CTTGGTGTGA

361 ATTGC

//

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3.2 *S. phyllantha* Gangep.

ACCESSION AB429261

FEATURES Location/Qualifiers

source 1..367

/country="Thailand: Kanchanaburi province"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organism="Stemona phyllantha"

rRNA <1..38

/product="18S ribosomal RNA"

misc_RNA 39..290

/note="internal transcribed spacer 1"

rRNA 291..>367

/product="5.8S ribosomal RNA"

ORIGIN

1 GTCCACTGAA CCTTATCATT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA

61 CCTGCGGAAG GATCATTGTC GATGCCTGCA AGCAGANCAA CCCGTGAACC NGTTTGACTA

121 CATCGGGATT GGGGTGTGCA ACACCTCACC TCCCTTGGGT TAGGAGGGGG CGCACTGTGT

181 TCTCTCCTCT TAGCCAAACA CAAACCCCGG CGCGGAAAGC GCCAAGGAAC AAAATTCGGT

241 GCCCCCGTGG AGACGGTGCT CGTGCGGGGC GTTTTGACAC GTGATGCAGA ATGACTCTCG

301 GCAACGGATA TCTCGGCTCT TGCATCGATG AAGAACGTAG CGAAATGCGA TACTTGGTGT

361 GAATTGC

//

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3.3.1 *S. collinsae* Craib -1

ACCESSION AB429265

FEATURES Location/Qualifiers

source 1..368

/country="Thailand: Saraburi province"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organism="Stemona collinsae"

rRNA <1..38

/product="18S ribosomal RNA"

misc_RNA 39..291

/note="internal transcribed spacer 1"

rRNA 292..>368

/product="5.8S ribosomal RNA"

ORIGIN

1 GTCCACTGAA CCTTATCATT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA

61 CCTGCGGAAG GATCATTGTC GAGACCTAAA CACGAAAGAC CCGTTGAACC GTATGACACC

121 ATGCCCGCGC CGGGGCGGGGTACCCCGTGTGCGCCCCCCTCCGGAGGCGACCTCCGCTGT

181 CCGCACGGGTCCGAAGAACAAACCCCGGCGAGGCCGGCGCCAAGGAACATGGAACNGGAG

241 CGACACCCGG AAGCGCACTC GGCTGGCCCC GCATACCACA TAGTACCCAG TATGACTCTC

301 GGCAACGGAT ATCTCGGCTC TCGCATCGAT GAAGAACGTA GCGAAATGCG ATACTTGGTG

361 TGAATTGC

//

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3.3.2 *S. collinsae* Craib -2

ACCESSION AB429266

FEATURES Location/Qualifiers

source 1..367

/country="Thailand: Nakhon Ratchasima province"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organism="Stemona collinsae"

rRNA <1..38

/product="1 8S ribosomal RNA"

misc_RNA 39..290

/note="internal transcribed spacer 1"

rRNA 291..>367

/product="5.8S ribosomal RNA"

ORIGIN

1 GTCCACTGAA CCTTATCATT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA

61 CCTGCGGAAG GATCATTGTC GAAACCTTGT ACTGGTATGA CCCGCGAACA AGTTTAGTCA

121 CTGCGGGATG GTGCCTCGTG CACCTGAAGC AAGGCCACGT AAGGTGCTAT GCTCCCTGCG

181 GAGGCCACGT AATCAAACCC CGGCGCGGAATGCGCCAAGGAAAACGAACANGAAAGAGAG

241 GACGATACAT TCACCTCGGA AACGATGTTG CTTCTCCTTT CATAACCAAA AtGACTCTCG

301 GCAACGGATA TCTCGGCTCT CGCATCGATG AAGAACGTAG CGAAATGCGA TACTTGGTGT

361 GAATTGC

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3.4 *S. burkillii* Prain

ACCESSION AB429268

FEATURES Location/Qualifiers

Source 1..368

/country="Thailand: Chiang Mai province"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organism="Stemona burkillii"

rRNA <1..38

/product="18S ribosomal RNA"

misc_RNA 39..291

/note="internal transcribed spacer 1"

rRNA 292..>368

/product="5.8S ribosomal RNA"

ORIGIN

1 GTCCACTGAA CCTTATCATT TAGAGGAAGG AGAAGTTATA ACAAGGTTTC CGTAGGTGAA

61 CCTGCCTAAG GATCATTGTC GAGACCTCAA CATGGAAGAC CCGTGAACCC ATGACGCCAC

121 GCCCGCAGTG GGGCAGCCAC ACGCGTCCACTCCGGAGGTGACCCCTACCATCCGTACGAG

181 TGGTGGGGCA GTGCAAACCC CGGCGAGGCAAGCGCCAAGGAACATACTCTGGAGCGNTGC

241 CCAGATGAAC ACCTCCTTCC ACATAGGCGC TTTGTACCAC ATCATACCTA CATGACTCTC

301 GGCAACGGAT ATCTCGGCTC TCGCATCGAT GAAGAACGTA GCGAAATGCG ATACTTGGTG

361 TGAATTGC

//

3.5 *S. aphylla* Craib

ACCESSION AB429267

FEATURES Location/Qualifiers

source 1..368

country="Thailand: Udon Thani province"

/identified_by="Srunya Vajrodaya"

/mol_type="genomic DNA"

/organism="Stemona aphylla"

rRNA <1..38

/product="18S ribosomal RNA"

misc_RNA 39..291

/note="internal transcribed spacer 1"

rRNA 292..>368

/product="5.8S ribosomal RNA"

ORIGIN

1 GTCCACTGAA CCTTATCATT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA

61 CCTGCGGAAG GATCATTGTC GATGCCTCAA TCAGATCAAC CCGTGAACTA GTTGCACTAC

121 CTAAGGTGTG GGGTGCGCCG ACTCNNTCNG CCCTGTCCTGAGGTGACACCCGCCCTGNCG

181 AGTGGCTGCT GGCCAACCTC AAACCCCGGC GNCGAGCGCC AAGGAACGAA TCATGTGTA

241 CATGCCCGTG GCNCCCGGTG CTCCCACGGG CTGGTCATNC CATTATACTA AATGACTCTC

301 GGCAACGGAT ATCTCGGCTC TTGCATCGAT GAAGAACGTA GCGAAATGCG ATACTTGGTG

361 TGAATTGC

//

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3.6 *S.* sp.

ACCESSION AB429264

FEATURES Location/Qualifiers

source1.. 363

/country="Thailand: Nakornpathom, Siriruckhachati Medicinal Plant Garden"

/identified_by="Boonyadist Vongsak"

/mol_type="genomic DNA"

/organism="Stemona hutanguriana "

rRNA <1..38

/product="18S ribosomal RNA"

misc_RNA 39..286

/note="internal transcribed spacer 1"

rRNA 287..>363

/product="5.8S ribosomal RNA"

ORIGIN

1 GTCCACTGAA CCTTATCATT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA
61 CCTGCGGAAG GATCATTGTC GAAACCTGCA ACTGCAGAAT GACCCGCCGA AACAAGTTTA
121 TTCACTGCGG GAAGGGTGCC TTGGTGTCTC CTGACTNCNA CGGCCCCGTA GTGGTGCTAT
181 GCTCCNCTCG GTGGCCACAA GAACAAACCC CGGCGCGGAAAGCGCCAAGGAAAATAACGA
241 GGAANAGAGA TCGACCCNTT TACTTGGGAA ATGTTCTCCC TAAAAAATGA CTCTCGGCAA
301 CGGATATCTC GGCTCTCGCA TCGATGAAGA ACGTAGCGAA ATGCGATACT TGGTGTGAAT
361 TGC

//

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VITA

Mr. Boonyadist Vongsak was born on May, 1982 in Bangkok, Thailand. He graduated Bechelor's Degree of Sciences in Pharmacy in 2005 from the Faculty of Pharmacy, Silpakorn University.

Poster Presentation

Vongsak B., Pooltong N., Phisutthinusart S., Sittisombut C. (2005). Anti-oxidant activity of some food herbal extracts. The 2nd AASP Symposium & APEM Conference, 2005. The Bangkok Monthien Riverside Hotel, Thailand. 14-17 Nov., 2005.

Grant

He received the financial supports from the Graduate School of Chulalongkorn University (the 90th Anniversary of Chulalongkorn University-the Golden Jubilee Fund) in 2007.

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