การศึกษาอนุกรมวิธานของ *Hoya siamica* ชนิดเชิงซ้อนในประเทศไทย

นางสาวดวงใจ ตั้งมั่นในธรรม

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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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TAXONOMIC STUDY OF THE *Hoya siamica* COMPLEX IN THAILAND

Miss Duangjai Tungmunnithum

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By	Miss Duangjai Tungmunnithum
Field of Study	Botany
Thesis Advisor	Assistant Professor Manit Kidyoo, Ph.D.
Thesis Co-advisor	Assistant Professor Chumpol Khunwasi, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

..... Chairman

(Professor Thaweesakdi Boonkerd, Ph.D.)

..... Thesis Advisor

(Assistant Professor Manit Kidyoo, Ph.D.)

..... Thesis Co-advisor

(Assistant Professor Chumpol Khunwasi, Ph.D.)

..... Examiner

(Sroynapa Yannawat, Ph.D.)

..... External Examiner

(Somran Suddee, Ph.D.)

ดวงใจ ตั้งมั่นในธรรม: การศึกษาอนุกรมวิธานของ *Hoya siamica* ชนิดเชิงซ้อนในประเทศไทย. (TAXONOMIC STUDY OF THE *Hoya siamica* COMPLEX IN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. มานิต คิดอยู่, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ชุมพล คุณวาสี, 79 หน้า.

ในอดีตสถานะทางอนุกรมวิธานของ *Hoya siamica* ชนิดเชิงซ้อนยังไม่ชัดเจน เนื่องจากมีความแปรผันของลักษณะทางสัณฐานวิทยาสูงมาก วัตถุประสงค์ของวิทยานิพนธ์นี้คือการตรวจสอบลักษณะทางสัณฐานวิทยา กายวิภาค เรณูวิทยาและข้อมูลเชิงชีวโมเลกุลของ *H. siamica* ชนิดเชิงซ้อนในประเทศไทยเพื่อระบุสถานะทางอนุกรมวิธานที่ชัดเจน ความหลากหลายของรูปร่างใบ ลักษณะของเส้นแขนงใบ รูปร่างของกระบังรอบและคอร์พัสคูลัมเป็นเกณฑ์สำคัญในการแบ่งพืชที่ศึกษาออกเป็น 5 แบบ เรียกว่าแบบ I-V ข้อมูลสัณฐานวิทยาเชิงคุณภาพนี้แสดงให้เห็นว่าแบบ I แตกต่างจากแบบอื่นๆอย่างเห็นได้ชัดโดยมีเส้นแขนงใบที่เห็นไม่ชัดเจนและคอร์พัสคูลัมรูปไข่กว้าง ส่วนแบบ II-V มีเส้นแขนงใบที่เห็นชัดเจนและเป็นร่องและคอร์พัสคูลัมรูปหัวลูกศร แบบ II และ III มีกะบังรอบรูปไข่กลับและมุมด้านนอกป้าน แต่แบบ IV และ V มีกะบังรอบรูปรีถึงรูปไข่กลับและมุมด้านนอกแหลม โดยแบบ II-V มีรูปร่างใบที่แตกต่างกัน นอกจากนี้ลักษณะเชิงปริมาณยังแสดงให้เห็นว่า *Hoya siamica* ชนิดเชิงซ้อนในประเทศไทยสามารถจำแนกได้เป็นสองกลุ่ม กลุ่มแรกประกอบด้วย แบบ I และกลุ่มที่สองประกอบด้วยแบบ II-V สอดคล้องกับลักษณะกายวิภาคของแผ่นใบที่แบ่งได้ 2 กลุ่ม กลุ่มแรก (แบบ I) มีชั้นมีโซฟิลล์ที่สามารถแบ่งออกเป็นสองชั้นได้ชัดเจน ในขณะที่กลุ่มที่สอง (แบบ II-V)ไม่สามารถแบ่งได้ นอกจากนี้การศึกษา 5 บริเวณของคลอโรพลาสต์ดีเอ็นเอของพืชชนิดเชิงซ้อนนี้ด้วยเทคนิค PCR-RFLP พบว่าไม่มีความแตกต่างกัน ดังนั้นแบบ I ของ *Hoya siamica* ชนิดเชิงซ้อนควรจัดอยู่ในระดับ variety เนื่องจากมีความหลากหลายของลักษณะทางสัณฐานวิทยา เรณูวิทยาและกายวิภาคที่ปรากฏในการศึกษาครั้งนี้สูงมาก นอกจากนี้ แบบ II-V ของ *H. siamica* ชนิดเชิงซ้อนดังที่ได้นำเสนอในการศึกษาครั้งนี้เป็นรูปแบบความแปรผันที่เกิดขึ้นภายในชนิดเดียวกันของ *H. siamica* ผลการศึกษาทั้งหมดชี้ให้เห็นว่า *H. siamica* ชนิดเชิงซ้อนในประเทศไทย ประกอบด้วย *H. siamica* Craib และ variety

ภาควิชา .....พฤกษศาสตร์..... ลายมือชื่อนิสิต.....

สาขาวิชา ...พฤกษศาสตร์..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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DUANGJAI TUNGMUNNITHUM : TAXONOMIC STUDY OF THE *Hoya siamica* COMPLEX IN THAILAND. ADVISOR: ASST. PROF. MANIT KIDYOO, Ph.D., CO-ADVISOR: ASST. PROF. CHUMPOL KHUNWASI, Ph.D., 79 pp.

Previously, taxonomic status of *Hoya siamica* complex was still dubious due to extremely variable in morphological characters. The aim of this thesis is to investigate morphological, anatomical, palynological and molecular biological characters of the *H. siamica* complex in Thailand so as to define its taxonomic status. The variation in shape of leaf, characters of lateral veins, corona and corpusculum provide important criteria for dividing the studied plants into 5 forms i.e. form I-V. These qualitative morphological data showed that form I is obviously different from the others by obscure lateral veins and broadly ovate corpusculum. Form II-V have conspicuous lateral veins, grooved on upper surface and arrow head corpusculum. Form II and III have obovate corona with obtuse outer angle but form IV and V have elliptic-obovate corona with acute outer angle. From II-V are different in leaf shapes. The quantitative characters showed that *H. siamica* complex in Thailand can be divided into two groups, the first group composed of form I and the second group composed of form II-V. Likewise, the anatomical studies showed that this complex can be classified into 2 groups. The first group (form I) have mesophyll which can be obviously divided into two layers, whereas the second group (form II-IV) cannot be divided. The PCR-RFLP technique was subsequently used to detect diversity of 5 chloroplast DNA regions of this complex which reveals no difference. Therefore, Form I of the *H. siamica* complex is treated as a variety, due to their great variation in morphological, palynological and anatomical characters as present in this study. In additional, forms II-V of the *H. siamica* complex as presented in this study indicate that they are variable form of a single species of *H. siamica*. In all, it is proposed here that *H. siamica* complex in Thailand composed of *H. siamica* Craib and a variety.

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List of Abbreviations

A, T, C, G	=	nucleotide containing the base Adenine, Thymine, Cytosine and Guanine, respectively
BCU	=	Professor Kasin Suvatabhundbu Herbarium, Department of Botany, Chulalongkorn University
bp	=	base pair
°C	=	degree celsius
cm	=	centimeter
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates
EDTA	=	ethylenediamine tetraacetic acid
g	=	gram
K	=	Kew Herbarium, Royal Botanic Gardens, England
Kb	=	kilobase pair
M	=	molar
$MgCl_2$	=	magnesium chloride
mg	=	milligram
mm	=	millimeter
mM	=	millimolar
ml	=	milliliter
NaCl	=	sodium chloride
ng	=	nanogram
PCR	=	polymerase chain reaction
RFLP	=	restriction fragment length polymorphism
Tris	=	tris(hydroxyl methyl) aminomethane
UV	=	ultraviolet
µg	=	microgram
µl	=	microlitre
µm	=	micrometer
V	=	volt

CHAPTER I

INTRODUCTION

The genus *Hoya* R. Br., belongs to the family Asclepiadaceae, published in 1810 by Robert Brown in honor of his friend, Thomas Hoy, the gardener. It mainly occurs from Asian region to the Pacific Islands and Northern Australia (Mabbery, 1997; Wanntorp *et al*., 2006). Like many Asclepiadaceaus plants, *Hoya* is milky weed with succulent leaves that varies in shape and thickness. Its inflorescence is umbelliform, positively or negatively geotropic, flower with star-shaped corona, and also have pollinarium between corona lobe, each pollinarium compose of two groups of pollen mass called pollinium, a corpusculum and each pollinium have their own translator. (Forster *et al.* 1998; Rintz, 1978; Mabbery, 1997; Wanntorp *et al*., 2006).

Figure1. Geographical distribution of *Hoya* (Wanntorp *et al*., 2006)

In Thailand, most of the *Hoya* specimens were collected during the 1920s by Arthur Francis George Kerr (Kerr, 1951). The taxonomic report of the genus *Hoya* was in Florae Siamensis Enumeratio 60 years ago (Kerr, 1951). Since then, they have been

no further intensive research on this genus. Inasmuch as they have the great variation in their morphological characters, which provide the confusion in both identification and classification processes. At present, the genus *Hoya* is being revised for the Flora of Thailand Project. At least 36 species in Thailand were described (Thaithong, 1995). Nevertheless, many species in the genus *Hoya* have confused taxonomic status because of the great morphological variations. *Hoya siamica* Craib is one of those problematic species.

H. siamica Craib published in 1911 by W. G. Craib. It has been discovered by A. F. G. Kerr at Doi Sutep, Chiang Mai Province, Thailand 100 years ago (Craib, 1911). This species occurs in hill evergreen forest at about 1,000 m elevation (Craib, 1911; Thaithong, 1995). *H. siamica* is an extremely variable species, with a great variation in texture, shape, size and venation of leaves, petiole length, number of flowers per inflorescence, pedicel length and size and color of flowers (Craib, 1911; Kerr, 1951; Ping-tao, *et al*., 1995). Consequently, identification and classification of this species is still uncertain. Previously, anatomical, palynological and molecular data together with its description that covered variations of this plant group in Thailand is not available.

Aim of the thesis

Aim of this thesis is to investigate morphological, anatomical, palynological and molecular data of the *H. siamica* complex in Thailand. These data will be used to determine the taxonomic status of this complex species in Thailand.

CHAPTER II

LITERATURE REVIEW

The complex species is a group of closely related taxa at and/or just below the species level (Stage, 1984) has great variation in morphological characters. Hence, the taxonomic status of the complex species is rather difficult to define and give rise to the question that there exists one or more taxa. To clarify this problem, morphological, anatomical, palynological and molecular biological methods are used.

Morphological and anatomical studies were often used for determining taxonomic status of complex species in several groups of flowering plants. In the study of complex species in the genus *Malmea* (Annonaceae), 53 morphological characters were used to determine their taxonomic status. The researcher reported that 238 herbarium specimens could be divided into 12 groups. (Chatrou,1997). Likewise, morphological data, together with anatomical data were an effective instrument for determining the taxonomic status of two closely related species, *Bouteloua aristidoides* and *Bouteloua eriopoda*, which differ from each other in number of branches per inflorescence, branch length, number of spikelets per branch, and branches deciduous versus persisten (Columbus, 1999). In the study of *Carex backii* complex (Saarela and Ford, 2001), (*Phyllostachyae*: Cyperaceae) the researchers hypothesize that the *C. backii* complex is composed of three distinct species: *C. backii*, *C. saximontana* and an undescribed species (Currently named as *C. cordillerana*). And also they presented the results of a morphological and anatomical studies are effectively used to determine the taxonomic status of *C. backii* complex.

For taxonomic studies of the genus *Hoya*, Forster and Liddle (1991) reported that not only vegetative but also reproductive structures were very useful for describing the taxonomic status of *H. australis* complex. Both qualitative and quantitative morphological characters were useful for recognizing five subspecies within *H. australis* complex. Furthermore, many reproductive structures, such as corona and gynostegia were widely used for identification and classification in Asclepiadaceae (Liede and Kunze, 1993). Similarly, Wanntorp' s study concluded that the combination of leaf, inflorescence and pollinium characters were useful for distinguishing *Hoya* species rather than corona morphology alone (Wanntorp, 2007). Kunze and Wanntorp (2008) reported that characters of corona were the best tools for describing the differences between species of the genus *Hoya* and *Dischidia.* In addition in 2009, the research about the genus *Clemensiella* (Asclepiadoideae: Asclepiadaceae) both vegetative and reproductive character were used to prove the taxonomic status of *Clemensiella mariae* Livish. & Meve and *Clemensiella omlorii* Schltr. These researchers found that the shape of pollinarium, corolla lobes and corona were efficient instruments (Meve, *et al.* 2009). Many researches reported that discriminant analyses was a useful technique to determine the importance characters for classification of many plants. The obvious examples are the study of *H. parasitica* (Asclepiadaceae) complex in Thailand (Kidyoo *et al*, 2005), *Cascabela-Thevetia* complex species (Alvarado-Cárdenas and Ochoterena, 2007), *Cassia* sensu lato (Boonkerd, Pechsri and Baum, 2005) and *Bauhinia pottsii* G. Don (Boonkerd, Saengmanee and Baum, 2002). Besides these studies, the palynological data was widely used as powerful instrument for determining the taxonomic status of several complex species, especially in the species level (Erdtman, 1952). In addition, Rintz (1978), who is the expert in Asclepiadaceae suggested that the shape of pollinium can be used to distinguish most of the species in the genus *Hoya.* In the same way, Fishbein's research (2001) reported that the pollinarium was probably the most generally used for taxonomic study in many species of the family Asclepiadaceae. For instance, many researches confirmed that pollinarium characters, such as shape and size of pollinium, translator length and shape and size of

corpusculum were potentially used for classify *Hoya* species (Rintz, 1978; Kleijn and van Donkelaar, 2001; Wanntorp, 2007).

In the era of molecular's blooming, molecular data has become an important approach for systematic study (Doyle, 1992). Weising and Gardner (1999) generated a set of 10 consensus primer pairs based on multiple alignment of mononucleotide repeat-flanking regions in conserved chloroplast DNA (cpDNA) from a large number of plants both monocot and dicot. They also suggested that universal primers targeted to mononucleotide repeats might serve as general tools for studying chloroplast variation in angiosperm. In addition, Wang and Szmidt (1994) studied the variation of cpDNA of complex species in the genus *Pinus* from Asia. The authors analyzed cpDNA variation in multiple individuals and populations of *P. tabulaeformis* Carriere, *P. yunnanensis* Franch. and *P. massoniana* Lamb. They suggested that the study of variation of cpDNA was an effective method to examine the taxonomic status of this complex. Moreover, in the studied of cpDNA variation in *P. parviflora* complex (Watano, Imazu and Shimizu, 1995), the researchers reported that this method was useful to determine the complex species. Furthermore, 10 cpDNA microsatellite primer pairs, which designed by Weising & Gardner (Weising and Gardner, 1999) were tested in this experiment. They amplified overlapping fragments spanning the large single copy (LSC) region from eudicots. These primer pairs were tested on 20 plant species, belonging to 13 families. These researchers found that many primer pairs were robust with all species. They recommended that conserved cpDNA primer pairs were useful in plant molecular genetics studies.

Afterwards, the PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) method has become one of the common tools for solving taxonomic problems. Miller and Spooner (1999) used PCR-RFLP method to find the relationships in the wild potato *Solanum brevicaule* complex, and also define their taxonomic status. Likewise, in the study of Wang, Szmidt and Nguyen (2000) the taxonomic status of *Pinus krempfii* Lecomte was examined by restriction fragment length polymorphism method. Their results indicated that *P. krempfii* Lecomte clearly

belonged to the genus *Pinus*. As for the study of *Hoya*, there was a large number of taxonomic works indicating that the PCR-RFLP was useful for answering many taxonomic problems. For example, the study of *H. parasitica* complex (Kidyoo, *et al*, 2005) and many species in the genus *Hoya* (Wanntorp, 2007; 2009; Wanntorp, *et al.,* 2006)

To conclude, it is obvious that the morphological, anatomical, palynological and molecular studies were useful for determining the taxonomic status of many complex species. These effectively processes can be applied for study of the "*H. siamica* complex" in Thailand.

CHAPTER III

MORPHOLOGICAL, ANATOMICAL AND PALYNOLOGICAL STUDY

Introduction

At present, taxonomic status of the *H. siamica* complex is still uncertain due to its great morphological variation. Nevertheless, the original description (Craib, 1911) does not cover many characters of *H. siamica* complex in Thailand. Moreover, anatomical, palynological data together with its description that covered variations of this plant group in Thailand is not available. These variations make confusion in its identification and classification. Pollinarium morphology are observed in this study, focusing on translator length, shape and size of corpusculum and pollinium that can solve the taxonomic problems in many plant species, such as *Hoya australis* complex (Forster and Liddle, 1991) and *Hoya parasitica* complex (Kidyoo *et al*, 2005). Moreover, the pollinarium and/or corpusculum shape have been reported as effective characters for classification of species in the genus *Hoya* (Wanntorp, 2007; 2009; Kunze and Wanntorp, 2008). The obvious example is *Hoya balaensis* Kidyoo & Thaithong (Kidyoo and Thaithong, 2007).

The hypothesis of this research is that *H. siamica* complex in Thailand may contain a cryptic species or variety. To prove this, the investigation of all the morphological, palynological and anatomical variations are needed to determine the taxonomic status of this species. In addition, the key and description were constructed.

Materials and methods

1. Plant materials

1.1 Specimen collection

The living specimens of *H. siamica* from the previous recorded location in Thailand during March 2009 – May 2010. Moreover, detailed descriptions of all specimens collected together with habitats were thoroughly noted and photographs were taken. The mature stem and leaves of the living plants were fixed in FAA solution (Formaline Acetic acid Alcohol) for anatomical study. The remainder specimens, including branches, leaves and flowers were preserved in 70% ethanol for further morphological studies. Moreover, Herbarium specimens were prepared using standard herbarium techniques (ทวีศักดิ์ บุญเกิด และคณะ, 2530) and kept at the Professor Kasin Suvatabhundbu Herbarium, Department of Botany, Chulalongkorn University (BCU).

1.2 Specimens determinations

Specimens were determined using the previous available taxonomic keys in Flora of Indo-China (Constantin, 1912), Flora of China (Ping-tao *et al*., 1995), *Kerr* 724 (holo K) and the original description (Craib, 1911).

2. Morphological and palynological study

2.1 A total of 47 specimens were qualitatively examined from both vegetative and reproductive organs, such as leaf shape, leaf venation, color and shape of sepal, corolla and corona, etc. by using a stereo microscope and a compound light microscope.

2.2 Flowers of each specimen were collected, and preserved in 70% ethanol for further palynological study. These preserved flowers were treated in 95% and 100% ethanol respectively at least 1 hour for each step. The solution was drained,

and then the pollinaria were detected from the corona of each flowers under the stereo microscope and put on a glass slide. Then, pollinaria were mounted in permount. Their dimension were measured by means of imaging analysis software of Nikon digital sight DS-L2. The pollinarium characters were observed and describes based on the corpusculum, translator and pollinium both shape and size. Permanent slides of the whole samples were deposited at BCU.

2.3 Total of 45 quantitative characters of both vegetative and reproductive characters (Table 1) of 47 specimens were studied by taking photographs and measuring these characters, using electronic measurements tool of Adobe Photoshop CS4. And then, discriminant analysis was used to determine the important characters for classification the *H. siamica* complex. Procedure CLASSIFY in SPSS version 16 (IBM, 2011).

Table1. The 45 quantitative characters of both vegetative and reproductive characters of the *H. siamica* complex

Abbreviation	Characters
C1	leaf length in cm
C2	leaf width in cm
C3	distance from base to the widest point of leaf in cm
C4	leaf shape (calculated by C3/C1)
C5	petiole length in cm
C6	diameter of petiole in cm
C7	pedicel length in mm
C8	diameter of pedicel in mm
C9	ratio of leaf length and petiole length (calculated by C1/C5)
C10	ratio of peduncle length and pedicle length (calculated by C11/C7)
C11	peduncle length in cm
C12	sepal length in mm
C13	sepal width in mm
C14	diameter of corolla in mm
C15	corolla length in mm (calculated by C20+C18+C19)
C16	corolla lobe length in mm (calculated by C18+C19)
C17	diameter of corolla tube in mm
C18	distance from base to the widest point of corolla lobe in mm
C19	corolla lobe apex length in mm
C20	corolla tube length in mm
C21	corolla lobe width in mm
C22	corolla lobe base width in mm
C23	diameter of corona in mm
C24	diameter of coronal receptacle in mm

Abbreviation	Characters
C25	thickness of corona lobes in mm
C26	distance between corona lobes in mm
C27	corona lobe length in mm
C28	distance from base to the widest point of corona lobe in mm
C29	corona lobe shape (calculated by C28/C27)
C30	corona lobe width in mm
C31	ratio of corona diameter and coronal receptacle diameter (calculated by C23/C24)
C32	ratio of corona diameter and corolla tube diameter (calculated by C23/C17)
C33	pollinium length in mm
C34	pollinium width in mm
C35	corpusculum length in mm (calculated by C36+C37)
C36	upper apex of corpusculum length in mm
C37	lower apex of corpusculum length in mm
C38	corpusculum width in mm
C39	translator length in mm
C40	ratio of pollinium length and pollinium width in mm (calculated by C33/C34)
C41	ratio of corpusculum length and corpusculum width in mm (C35/C38)
C42	number of guard cells per 0.5 mm^2
C43	stoma length in micron
C44	stoma width in micron
C45	guard cell length in micron

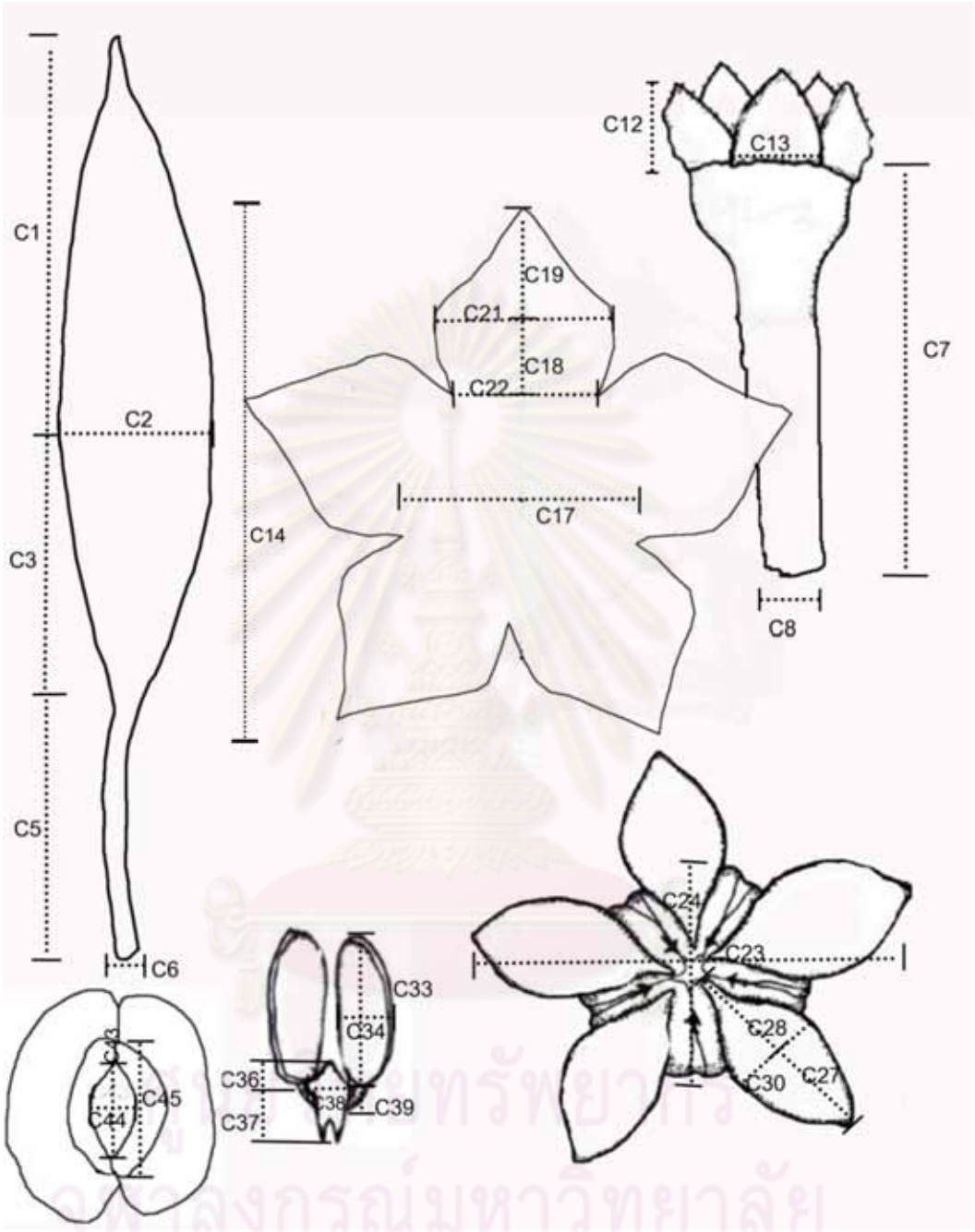

Figure 2 Measurements of leaf and floral parts. A: leaf, B: calyx and pedicel, C: corolla, D: corona, E: pollinarium, F: stoma and guard cell.

3. Anatomical study

Anatomical characters of the mature stems and leaves were studied from cross section through mature lamina and internodes of all specimens of the *H. siamica* complex. This cross section techniques were modified from Bradbury (1973), have been used throughout. Moreover, epidermal characters of both adaxial and abaxial surface of epidermis were observed by using the techniques modified from Ruzin (1999).

3.1 Anatomy of stem and leaf in cross section

All sections were cut using a rotary microtome (Plant Microtome Automatic MT-3) at 50 -80 μm thickness without embedding. Then the sections were stained by safranin O-fast green mixture in 2-ethoxy ethanol, then they were differentiated in 2-ethoxy ethanol. After this stage the sections were cleared in xylene and mounted on slides in permount. All of the permanent slides of the whole samples were deposited at BCU.

3.2 Epidermal characters

The preserved mature leaf samples were transferred to potassium hydroxide 10% and then were warm in a water-bath for 30 minutes. Next, the samples were washed with distilled water. After this step, abaxial and adaxial surfaces of epidermis were peeled off and stained with safranin O, destained by 70% ethanol, dehydrated in 70, 95 and 100% ethanol respectively. Then, abaxial and adaxial surfaces of epidermis were cleared in xylene and mounted on slides in permount. All of the permanent slides of the whole samples were deposited at BCU.

4. Taxonomic treatment based on morphological, palynological and anatomical data

Taxonomic treatment of the "*H. siamica* complex" was carried out based on morphological, palynological and anatomical data. Full descriptions including ecological and distribution of each group were prepared. Furthermore, a comprehensive key to the "*H. siamica* complex" were constructed.

Results

1. Habitat and distribution

The *H. siamica* complex can be found throughout floristic regions in Thailand on a specific elevation, estimated at about 940-2295 m elevation at Huai Yang Waterfall National Park (Prachuapkhirikhan Province), Phu Luang (Loei Province), Doi Sutep and Kio Mae Pan (Chiang Mai Province), Cha Pan Pee Forest Park (Chiang Rai Province) and Khao Yai (Prachinburi Province) (Figure 4). These plants are long climbing epiphytes in hill evergreen forest. *H. siamica* complex from Huai Yang Waterfall National Park (Southwestern Thailand) usually grow on rocks in open areas (Figure 3E and 3F) at 940-1100 m elevations. *H. siamica* complex from Phu Luang (Northeastern Thailand) grow on tree's branches or stems in shade areas (Figure 3D) at 1473-1520 m elevations. *H. siamica* complex from Khao Yai (Southeastern Thailand) grow on tree's branches or stems in shade areas (Figure 3C) at 1150-1470 m elevations. *H. siamica* complex from Doi Sutep, Cha Pan Pee Forest Park and Kio Mae Pan (Northern Thailand) grow on tree's branches in shade areas (Figure 3A and 3B) at 1463-1500 m, 1100-1200 m elevations and 2280-2295 m elevations, respectively.

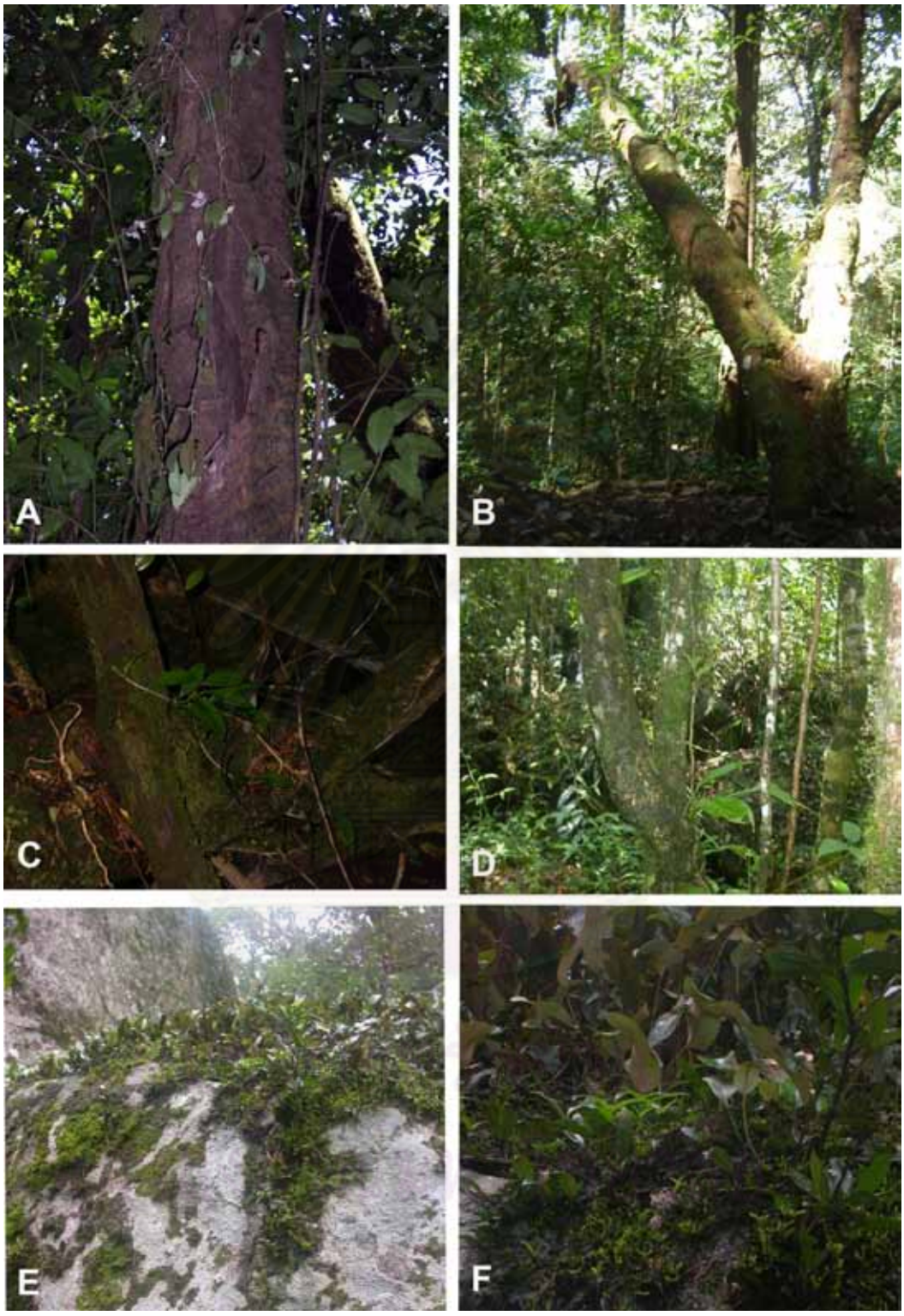

Figure 3. Habitats of the *H. siamica* complex from difference floristic regions of Thailand A: Kio Mae Pan (Chiang Mai Province), B: Doi Sutep and Kio Mae Pan (Chiang Mai Province), C: Khao Yai (Prachinburi Province), D: Phu Luang (Loei Province), E-F: Huai Yang Waterfall National Park (Prachuapkhirikhan Province).

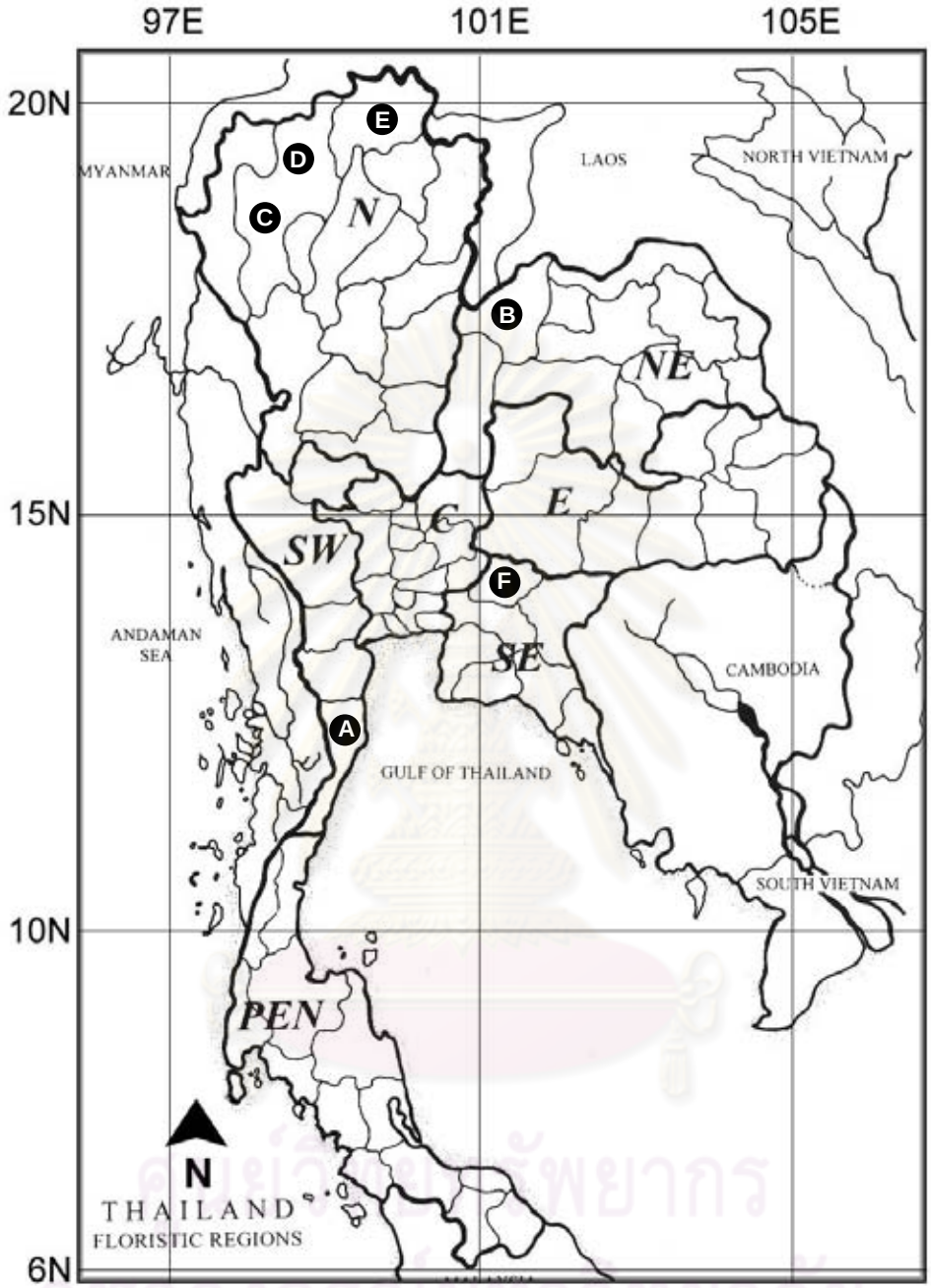

Figure 4. Distribution of *H. siamica* complex in Thailand A: Huai Yang Waterfall National Park (Prachuapkhirikhan Province), B: Phu Luang (Loei Province), C: Doi Sutep (Chiang Mai Province), D: Kio Mae Pan (Chiang Mai Province), E: Cha Pan Pee Forest Park (Chiang Rai Province) and F: Khao Yai (Prachinburi Province).

2. Morphology and palynology of the *H. siamica* complex

2.1 Variation of the *H. siamica* complex

Leaf shape: oblong, elliptic, elliptic-oblong or oblanceolate, leaf apex acute or acuminate, base cuneate or acute; leaves texture: coriaceous to succulently coriaceous; midrib grooved on upper surface; lateral veins: conspicuous and grooved or obscure; petioles: slender or stout, glabrous. Color of corolla: light pink on inner surface and dark violet spots on pink outer surface; white inner surface and white outer surface; white inner surface and pink spots on white outer surface or pink spots on light pink outer surface; green inner surface and light green outer surface or white pearl inner surface and pink spots on white outer surface. Corona shape; ovate, obovate or elliptic-obovate; inner angle of corona: acute or acuminate; outer angle of corona: obtuse or acute. Color of corona: white, creamy white, light red, pink or light violet. Pollinium shape: broadly oblong-obovate or narrowly oblong-obovate. Corpusculum shape: broadly ovate or arrow head.

2.2 Comparative morphology and palynology of the *H. siamica* complex

Despite the great observed variations (i.e., leaf shape, lateral veins conspicuous and grooved or obscure, corona shape, outer angle of corona, pollinium shape and corpusculum shape). The variations of this complex are tentatively classified into five morphological forms by a comparison of morphological and palynological characters. These morphological forms are named as form I-V. A key to forms and descriptions of each form are presented below.

Key to forms of the *Hoya siamica* complex in Thailand

1A. Main lateral veins obscure, broadly ovate corpusculum …….…….……. **form I**

1B. Main lateral veins conspicuous, grooved, arrow head corpusculum

2A. Obovate corona, outer angle obtuse

3A. Oblong leaves …… ……..……...…….....….. **form II**

3B. Elliptic leaves ……..…..……..……..…….. **form III**

2B. Elliptic-obovate corona, outer angle acute

4A. Oblanceolate leaves…….. ……..…..... **form IV**

4B. Elliptic leaves ……..……..……. **form V**

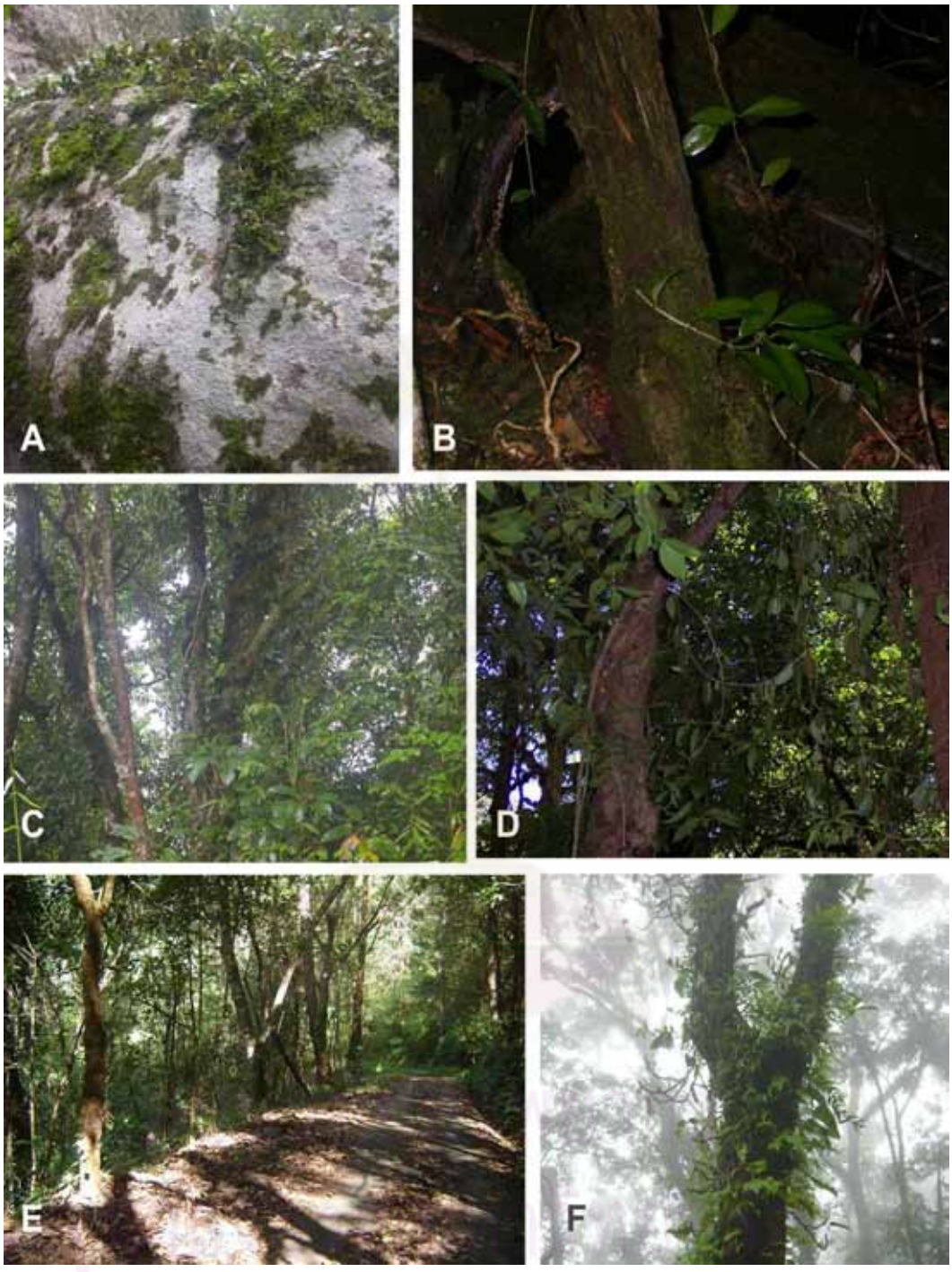

Figure 5. Habitats of the five forms of *H. siamica* complex in Thailand A: form I, Huai Yang Waterfall National Park; B: form II, Khao Yai; C: form III, Phu Luang; D: form IV, Kio Mae Pan; E: form V, Doi Sutep; F: Cha Pan Pee Forest Park

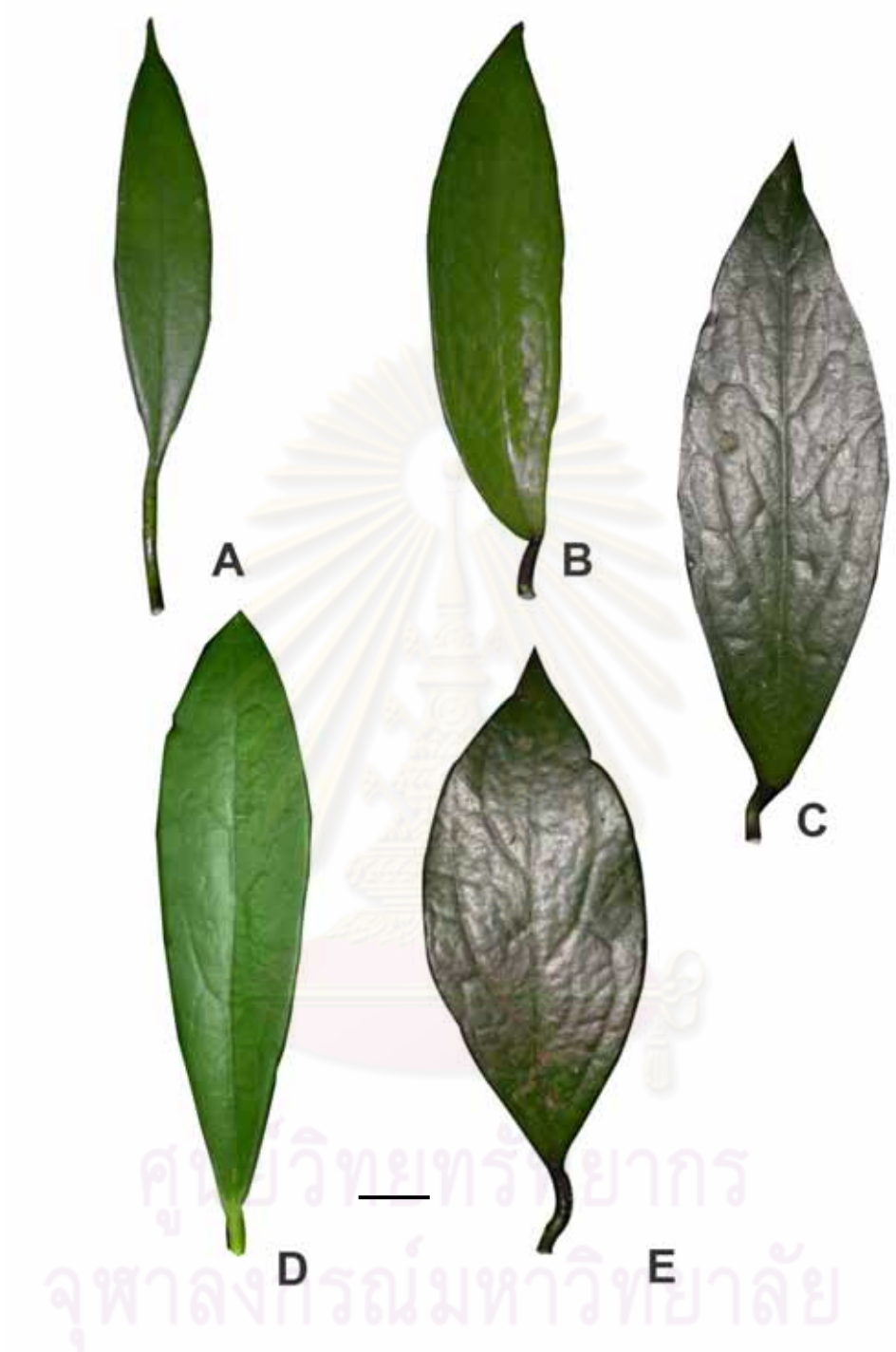

Figure 6. Leaves of *H. siamica* complex in Thailand. A: form I, Huai Yang Waterfall National Park, *D. Tungmunnithum* 67; B: form II, Khao Yai, *D. Tungmunnithum* 3; C: form III, Phu Luang, *D. Tungmunnithum* 39; D: form IV, Kio Mae Pan, *D. Tungmunnithum* 48; E: form V, Kio Mae Pan, *D. Tungmunnithum* 41. Bar= 1.5 cm

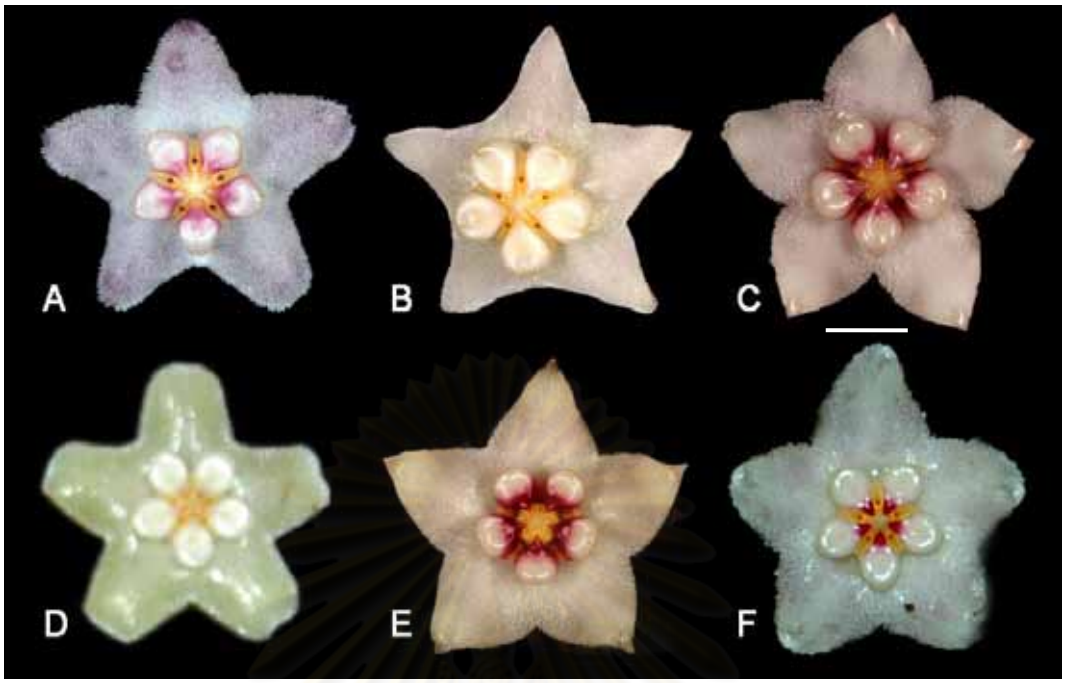

Figure 7. Variations of flowers and flower parts of *H. siamica* complex. A: form I, Huai Yang Waterfall National Park, *D. Tungmunnithum* 67; B: form II, Khao Yai, *D. Tungmunnithum* 2; C: form II, Phu Luang: *D. Tungmunnithum* 11; D: form IV, Kio Mae Pan, *D. Tungmunnithum* 46; E: form III, Phu Luang, *D. Tungmunnithum* 19; F: form V, Kio Mae Pan, *D. Tungmunnithum* 43. Bar = 5 mm

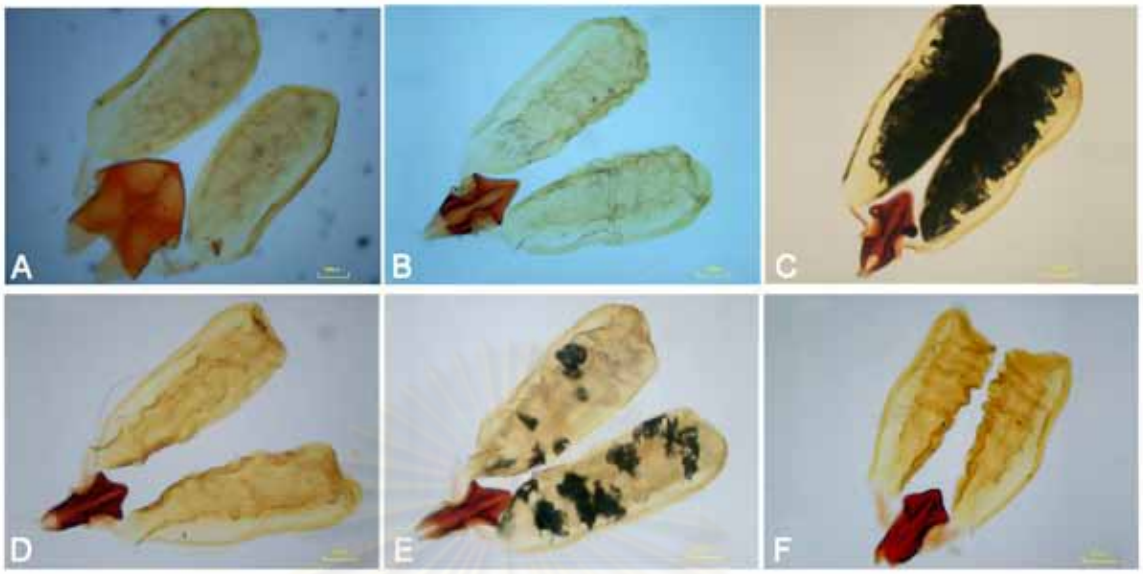

Figure 8. Variations of the pollinaria of *H. siamica* complex. A: form I, Huai Yang Waterfall National Park, *D. Tungmunnithum* 67; B: form II, Khao Yai, *D. Tungmunnithum* 2; C: form II, Phu Luang: *D. Tungmunnithum* 11; D: form IV, Kio Mae Pan, *D. Tungmunnithum* 46; E: form III, Phu Luang, *D. Tungmunnithum* 19; F: form V, Kio Mae Pan, *D. Tungmunnithum* 43. Bar = 100 µm

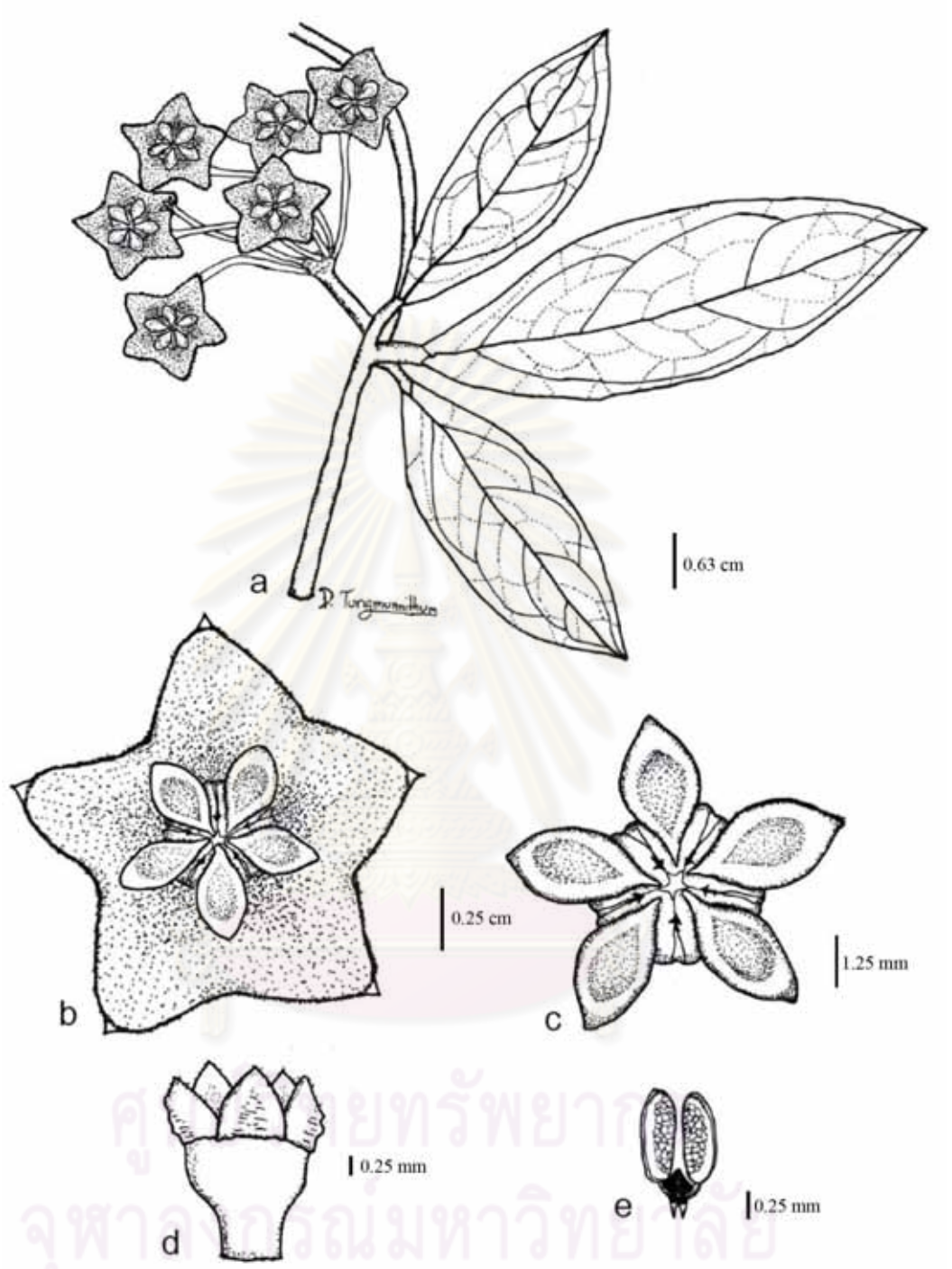

Figure 9. *H. siamica* Craib drawn by D. Tungmunnithum from *Kerr* 724 (holo K)

a. branch; b–d. flower; e. pollinarium

Descriptions of each form

Form I (Figure 5A, 6A 7A and 8A)

Leaves succulently and thickly coriaceous, glabrous, narrowly elliptic or narrowly elliptic-oblong, apex narrowly acute, base cuneate, midrib grooved on the upper surface, lateral veins obscure; slender petiole, 1.44-2.93 cm long. Umbel 4-9-flowered, peduncle 2.40-3.00 cm long; glabrous pedicel, 18.86-23.5 mm long. Sepal broadly ovate, 0.97-1.59 mm long, 0.77-1.32 mm wide. Light pink corolla, 5.19-8.27 mm diam., inner surface puberulent except the angle of corolla lobe, dark violet spots on pink outer surface, obovate corona, 1.62-2.86 mm long, 1.51-2.48 mm wide, broadly acute and white outer angle, acuminate and violet inner angle. Broadly oblong-obovate pollinium, 0.63-0.70 mm long, 0.29-0.33 mm wide; translator stout 0.11-0.15 mm long; broadly ovate corpusculum, 0.44-0.55 mm long, 0.28-0.39 mm wide. Fruit not seen.

Ecology and distribution: on rocks in open areas, hill evergreen forest; Southwestern Thailand at 940-1150 m elevation.

Specimens examined: Huai Yang Waterfall National Park, Prachuapkhirikhan Province: *D. Tungmunnithum* 66, 67, 68, 69 & 70, Khao Yai, Kanchanaburi Province: *C. F. van Beusekom and C. Phengkhlai* 199 (BKF).

Form II (Figure 5B, 6B, 7B, 7C, 8B and 8C)

Leaves coriaceous, glabrous, oblong, apex and base acute, midrib grooved on the upper surface, lateral veins conspicuous; stout petiole, 1.00-2.03 cm long. Umbel 7-13-flowered; peduncle, 5.00-7.30 cm long; glabrous pedicel, 20.68-22.22 mm long. Ovate to narrowly ovate sepal 1.43-1.87 mm long, 1.08-1.53 mm wide. White corolla,

8.28-9.380 mm diam., puberulent inner surface except the angle of corolla lobe, glabrous with white outer surface, obovate corona, 2.30-3.28 mm long, 1.42-2.46 mm wide, obtuse and white outer angle, acute and white or light red inner angle. Narrowly oblong-obovate pollinium, 0.42-0.75 mm long, 0.21-0.23 mm wide; translator 0.09- 0.11 mm long; arrow head corpusculum, 0.31-0.45 mm long, 0.10-0.17 mm wide. Fruit not seen.

Ecology and distribution: On trees or on rocks in shade areas, hill evergreen forest, Southeastern Thailand at 1100-1470 m elevation and Northeastern Thailand at 1200-1520 m elevation

Specimens examined: Khao Yai, Prachinburi Province: *D. Tungmunnithum* 2, 3 & 7; *B. Hansen, G. Seidenfaden and T. Smitinand* 11366 (BKF); Phu Luang, Loei Province: *D. Tungmunnithum* 10, 11 & 18, *C. Chermsiri-vathana*, 877 (BK) and *O. Thaithong* 997 (BCU)

Form III (Figure 5C, 6C, 7E and 8E)

Leaves coriaceous, glabrous, elliptic, apex and base acute, midrib grooved on the upper surface, lateral veins conspicuous; stout petiole, 1.00-2.00 cm long. Umbel 7-11-flowered; peduncle, 3.40-7.30 cm long; glabrous pedicel, 13.44-24.00 mm long. ovate sepal, 1.05-2.40 mm long, 0.89-1.57 mm wide. White corolla, 6.08-7.73 mm diam., inner surface puberulent except the angle of corolla lobe, glabrous with white, pink spots on white or pink spots on light pink and outer surface, Corona broadly obovate, 1.86-3.04 mm long, 1.52-2.64 mm wide, obtuse and white outer angle, acute and red inner angle. Pollinium narrowly oblong-obovate, 0.64-0.75 mm long, 0.20-0.24 mm wide;

translator 0.65-0.74 mm long; narrowly arrow head corpusculum, 0.31-0.40 mm long, 0.13-0.15 mm wide. Fruit not seen.

Ecology and distribution: On tree or on rocks in shade areas, hill evergreen forest, Northeastern Thailand at 1463-1470 m elevation and Northern Thailand at 1500 m elevation

Specimens examined: Phu Luang, Loei Province: *D. Tungmunnithum* 12, 16, 17, 19 & 39; Doi Chiengdao, Chiang Mai Province: *A. F. G. Kerr* 5610 (BK)

Form IV (Figure 5D, 6D, 7D and 8D)

Leaves coriaceous, glabrous, narrowly oblanceolate or oblanceolate, apex and base acute; midrib grooved on the upper surface, onspicuous lateral veins c; stout petiole, 1.10-1.94 cm long. Umbel 8-14-flowered, peduncle, 2.00-5.50 cm long; glabrous pedicel, 16.24-20.46 mm long. Ovate sepal, 1.20-2.12 mm long, 0.66-1.32 mm wide. Green corolla, 7.18-8.90 mm diam., inner surface puberulent except the angle of corolla lobe, glabrous with light green outer surface; elliptic-obovate corona ,1.65-2.40 mm long, 1.32-2.24 mm wide, acute and white outer angle, acute-acuminate and light violet inner angle; oblong-obovate pollinium, 0.42-0.75 mm long, 0.21-0.24 mm wide; translator 0.90-0.11 mm long; narrowly arrow head corpusculum, 0.31-0.45 mm long, 0.10-0.12 mm wide. Fruit not seen.

Ecology and distribution: On trees or on rocks in shade areas, hill evergreen forest, Northern Thailand at 2283-2295 m elevation

Specimens examined: Kio Mae Pan, Chiang Mai Province: *D. Tungmunnithum* 46, 47, 48, 52 & 53

Form V (Figure 5E-F, 6E, 7F and 8F)

Leaves coriaceous, glabrous, elliptic, apex and base acute; midrib grooved on the upper surface, conspicuous lateral veins; stout petiole, 1.00-1.22 cm long. Umbel 7-12-flowered, peduncle, 1.20-5.30 cm long; pedicel glabrous, 14.02-25.24 mm long. Ovate sepal 1.10-2.10 mm long, 0.76-1.57 mm wide. White pearl corolla, 6.29-9.54 mm diam., inner surface puberulent except the angle of corolla lobe, glabrous with pink spots on white outer surface, elliptic-obovate corona 1.08-3.33 mm long, 1.42-2.52 mm wide, acute and white outer angle, acute and pink inner angle. Narrowly oblong-obovate pollinium, 0.24-0.74 mm long, 0.19-0.25 mm wide; translator, 0.70-0.11 mm long; narrowly arrow head corpusculum, 0.32-0.44 mm long, 0.14-0.16 mm wide. Fruits, a follicle, pod-like with brown spots on green surface, straight glabrous and numerous seeds.

Ecology and distribution: On trees or on rocks in shade areas, hill evergreen forest, Northern Thailand at 1500-2288 m elevation

Specimens examined: Kio Mae Pan, Chiang Mai Province: *D. Tungmunnithum* 41, 42, 43, 44, 45, 49, 50, 51, 55, 85, 86, 87, 88 & 89; Cha Pan Pee Forest Park, Chiang Rai Province: *S. Wongpakam* s. n.; Doi Sutep, Chiang Mai Province: *Kerr* 724 (holo K), *D. Tungmunnithum* 90, 91 & 92, *T. Shimizu and M. Hutoh* 10561 (BKF) and *J.F. Maxwell* 1159 (BKF)

Table 2. Comparison of the morphological character of the five Forms of the *H. siamica* complex found in Thailand.

Characters	Form I (Huai Yang)	Form II (Khao Yai & Phu Luang)	Form III (Phu Luang)	Form IV (Kio Mae Pan)	Form V (Cha Pan Pee, Doi Sutep & Kio Mae Pan)	Holotype (Doi Sutep)
Leaf shape	Narrowly elliptic	Oblong	Elliptic	Obanceolate	Elliptic	Elliptic
Leaf base	Cuneate	Acute	Acute	Acute	Acute	Acute
Leaf apex	Acute -acuminate	Acute	Acute	Acute	Acute	Acute
Lateral veins	Obscure	conspicuous and grooved	conspicuous and grooved	conspicuous and grooved	conspicuous and grooved	conspicuous and grooved
Peduncle length	2.40 - 3.00 cm	5.00 - 7.30 cm	3.40-7.30 cm	2.00 - 5.50 cm	1.20 - 5.30 cm	1.90-2.40 cm
Corona scale shape	Obovate	Obovate	Obovate	Elliptic-obovate	Elliptic-obovate	Elliptic-obovate
Inner angle of corona	Acuminate	Acute	Acute	Acute-acuminate	Acute	Acute
Outer angle of corona	Acute	Obtuse	Obtuse	Acute	Acute	Acute
Pollinium shape	Broadly oblong-obovate	Narrowly oblong-obovate	Narrowly oblong-obovate	Narrowly oblong-obovate	Narrowly oblong-obovate	Narrowly oblong-obovate
Corpusculum shape	Broadly ovate	Arrow head	Narrow arrow head	Narrow arrow head	Narrow arrow head	Arrow head
Petiole	Slender	Stout	Stout	Stout	Stout	Stout

2.3 Discriminant analysis of the *H. siamica* complex

The ordination plot on the canonical axes 1 and 2 (Figure 10) shows that the five morphological forms can be separated into 2 groups in canonical axis 1. Group 1 consists of form I of *H. siamica* complex. Group 2 is the largest group, which consists of form II-V of *H. siamica* complex.

The percentage of the total variance (Table 3) shows that canonical discriminant function 1 should be used to determine the important of the quantitative characters of the *H. siamica* complex. Canonical variable 1 was 95.65 % correlated with the 45 characters and explained 68.4% of the total variance (Table 3). Accordingly, this axis was highly associated with corpusculum width (C38) (Table 4 and Figure 11).

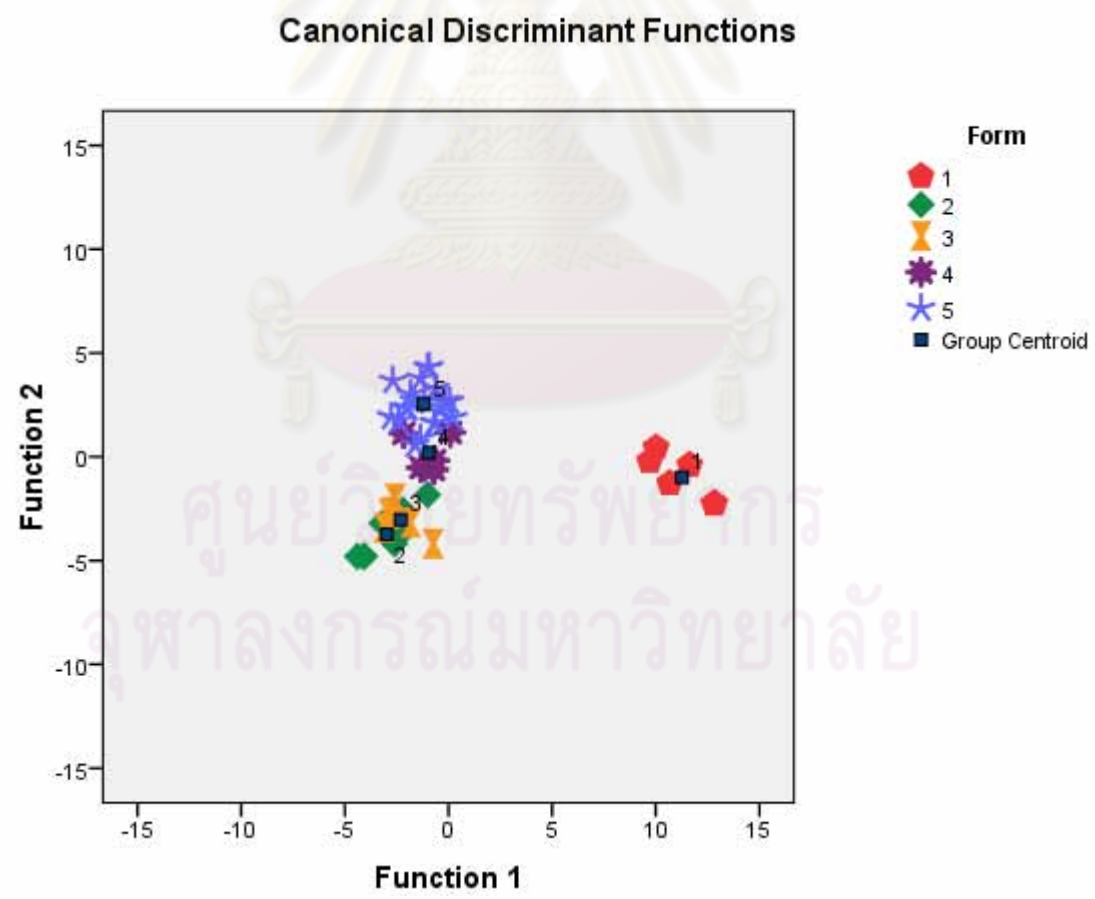

Figure 10. The ordination plot on the canonical axes 1 and 2 of the five morphological forms of the *H. siamica* complex.

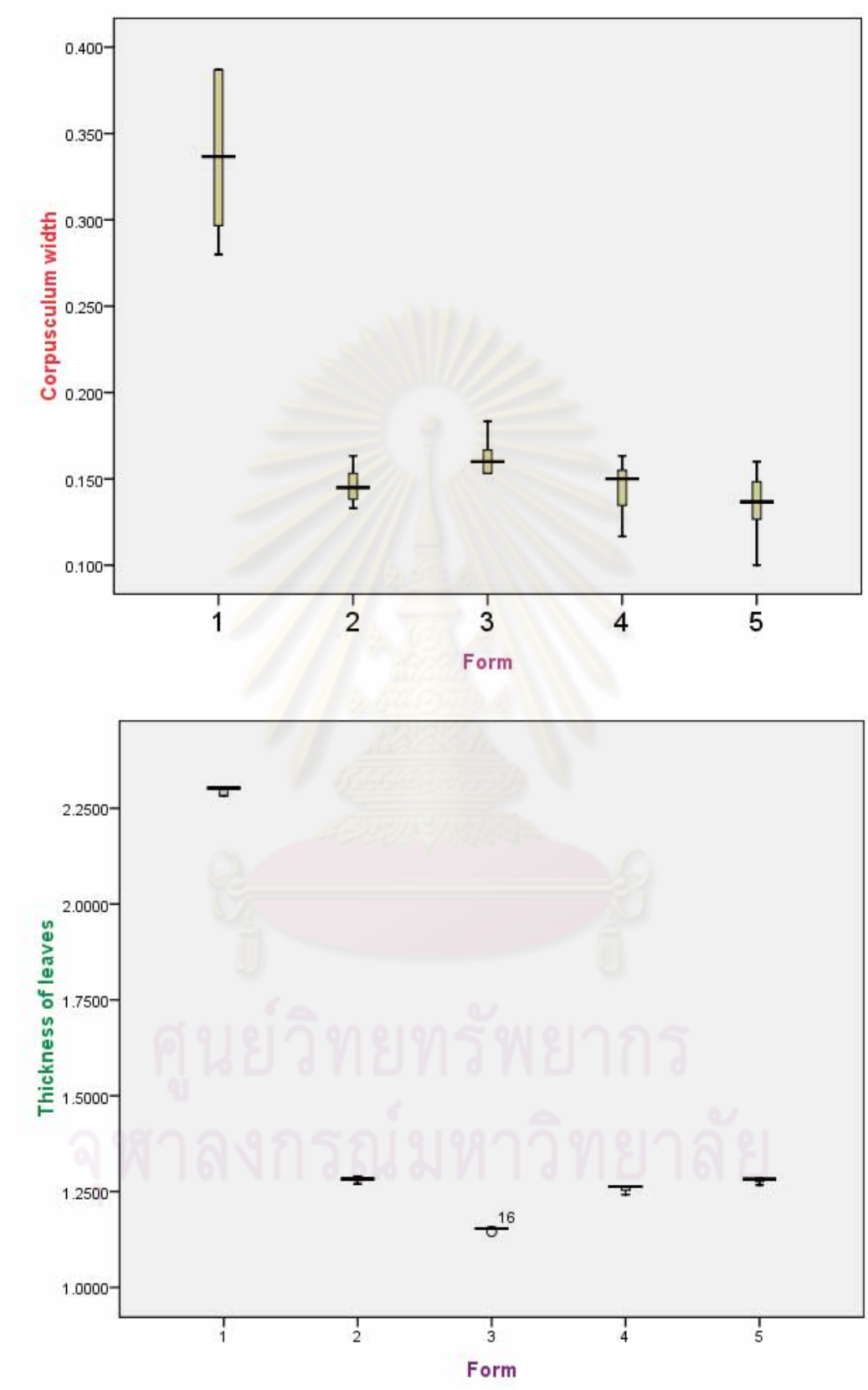

Figure 11. Boxplots of important quantitative characters of *H. siamica* complex.

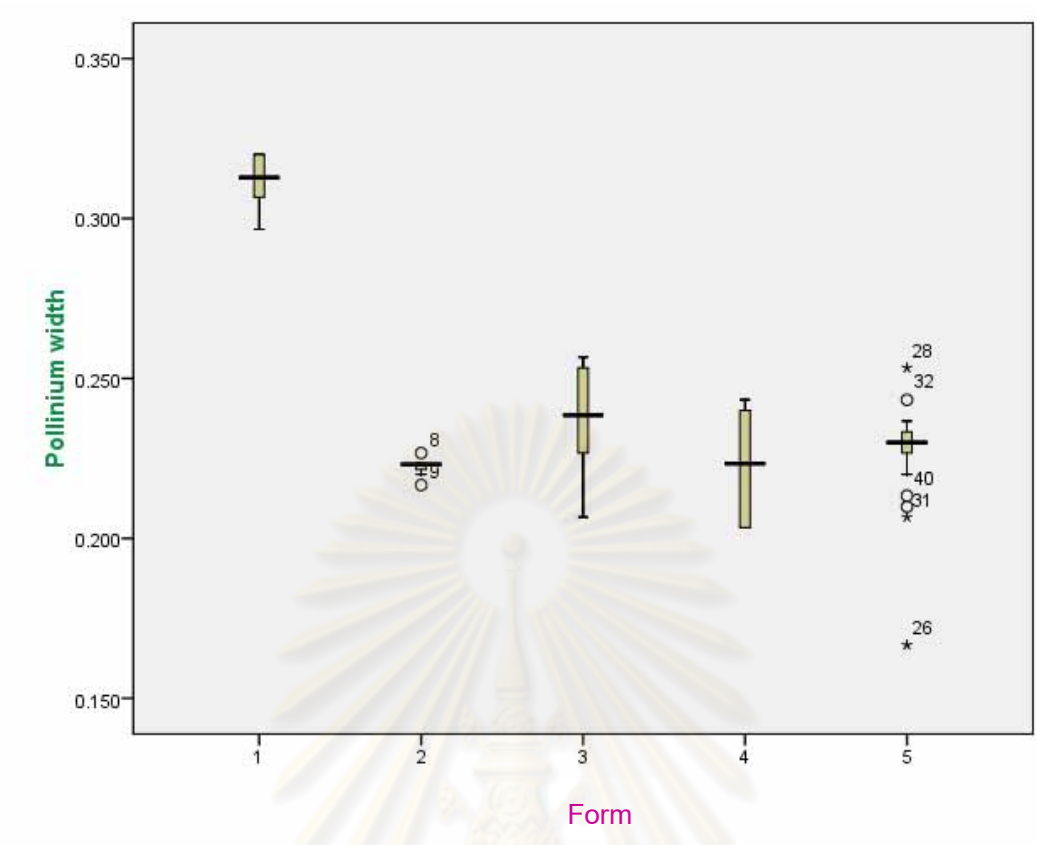

Figure11. (continue)

3. Anatomy of the *H. siamica* complex

Stems Anatomy

The stems of *H. siamica* complex are round in cross section (Figure 12A-16A). Anatomical characters of the mature stem in all specimens are rather uniform in cross-section. The epidermis have a thick cuticle, lenticels and bark, epidermal cells are square in shapes. Moreover, the periderm appears in the sub-epidermal layers, which are composed of thin-walled cells (Figure 12C-16C). The cortex layer consists of all most polyhedral parenchymal cells. Furthermore, a layer of long-polyhedral sclereid appears between the cortex (Figure 12B and 12D, 13B and 13D, 14B and 14, 15B and 15D, 16B and 16D). Prismatic crystals are also found in the cortex and pith. The secondary phloem tissue is on outer side of the secondary xylem tissues (Figure 12B-16B). Its pith cells are homogeneous, composed of polyhedral parenchyma cells (Figure 12B-16B).

Leaves Anatomy

Anatomical characters of the mature leaf of the *H. siamica* complex both abaxial and adaxial, epidermis consist of small epidermal cells, and also the outer walls are covered with cuticle, which is a thin waterproof layer. Its vascular bundle of midrib is a simple arc-shape (Figure 12E-F, 13E-F, 14E-F, 15E-F, 16E-F), and is composed of xylem and phloem tissue. The stomatal type of all specimens of the *H. siamica* complex is “Paracyctic” (Figure 12G-16G), the stoma accompanied on either side by one or more subsidiary cells parallel to the long axis of the pore and guard cell. However, each specimen was different in number per area, 10-31 cells per 0.5 mm^2. Its epidermal cells were triangular to polyhedral in shape (Figure 12G and 12H, 13G and 13H, 14G and 14H, 15G and 15H, 16G and 16H). The mesophyll of *H. siamica* complex can be obviously divided into two groups. Group 1 composed of the specimens from Huai Yang Waterfall National Park (morphological form I) can be obviously divided into two layers, dense and sparse parenchyma cells (Figure 17A). Moreover, their leaves are 2.28-2.32 mm, which are thicker than the others, 1.15-1.28 mm (Figure 17). Whereas group 2 composed of the specimens from Khao Yai, Phu Luang, Doi Sutep, Cha Pan Pee Forest Park, and Kio Mae Pan (morphological form II-IV) cannot be divided (Figure 17B-E).

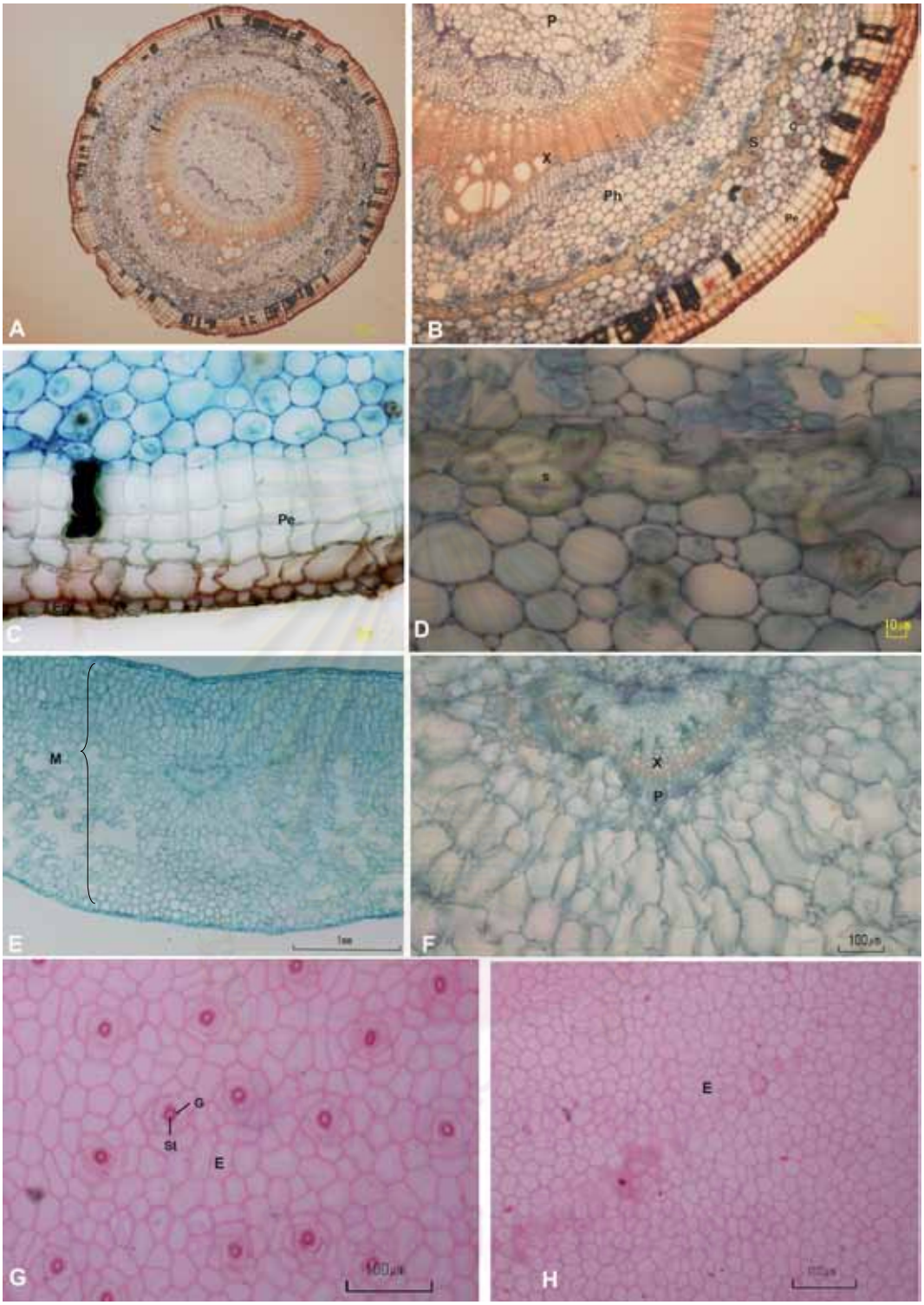

Figure 12. The stem and leaves anatomy of form I. A-D: x-section of stem (P = pith, Ep = epidermis, C = cortex, Ph = phloem, X = xylem, Pe = periderm, S = sclereid) E-F: x-section of leaves, G: abaxial surface, H: adaxial surface (M = mesophyll, P = phloem, X = xylem, G = guard cell, St = stoma, E = epidermal cells) Specimen numbers (A-D, F: *D. Tungmunnithum* 69, E: *D. Tungmunnithum* 67, G-H: *D. Tungmunnithum* 70). Bar (A= 50 µm, G-H, B = 100 µm, C-D = 10 µm, E = 1 mm)

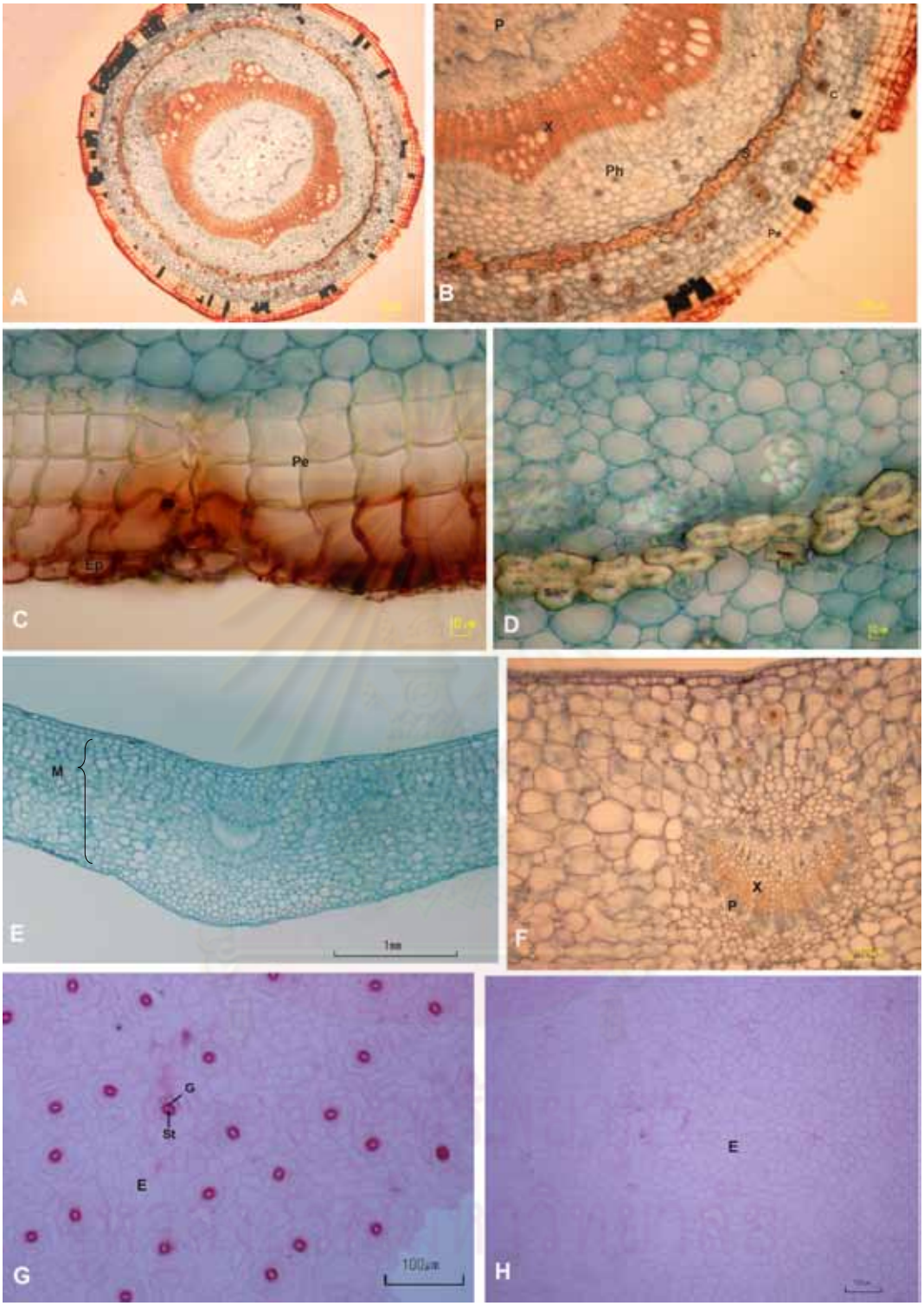

Figure 13. The stem and leaves anatomy of form II. A-D: x-section of stem (P = pith, Ep = epidermis, C = cortex, Ph = phloem, X = xylem, Pe = periderm, S = sclereid) E-F: x-section of leaves, G: abaxial surface, H: adaxial surface (M = mesophyll, P = phloem, X = xylem, G = guard cell, St = stoma, E = epidermal cells) Specimen numbers (A-D, F: *D. Tungmunnithum* 2, E: *D. Tungmunnithum* 3, G-H: *D. Tungmunnithum* 18). Bar (A= 50 μm, G-H, B = 100 μm, C-D = 10 μm, E = 1 mm)

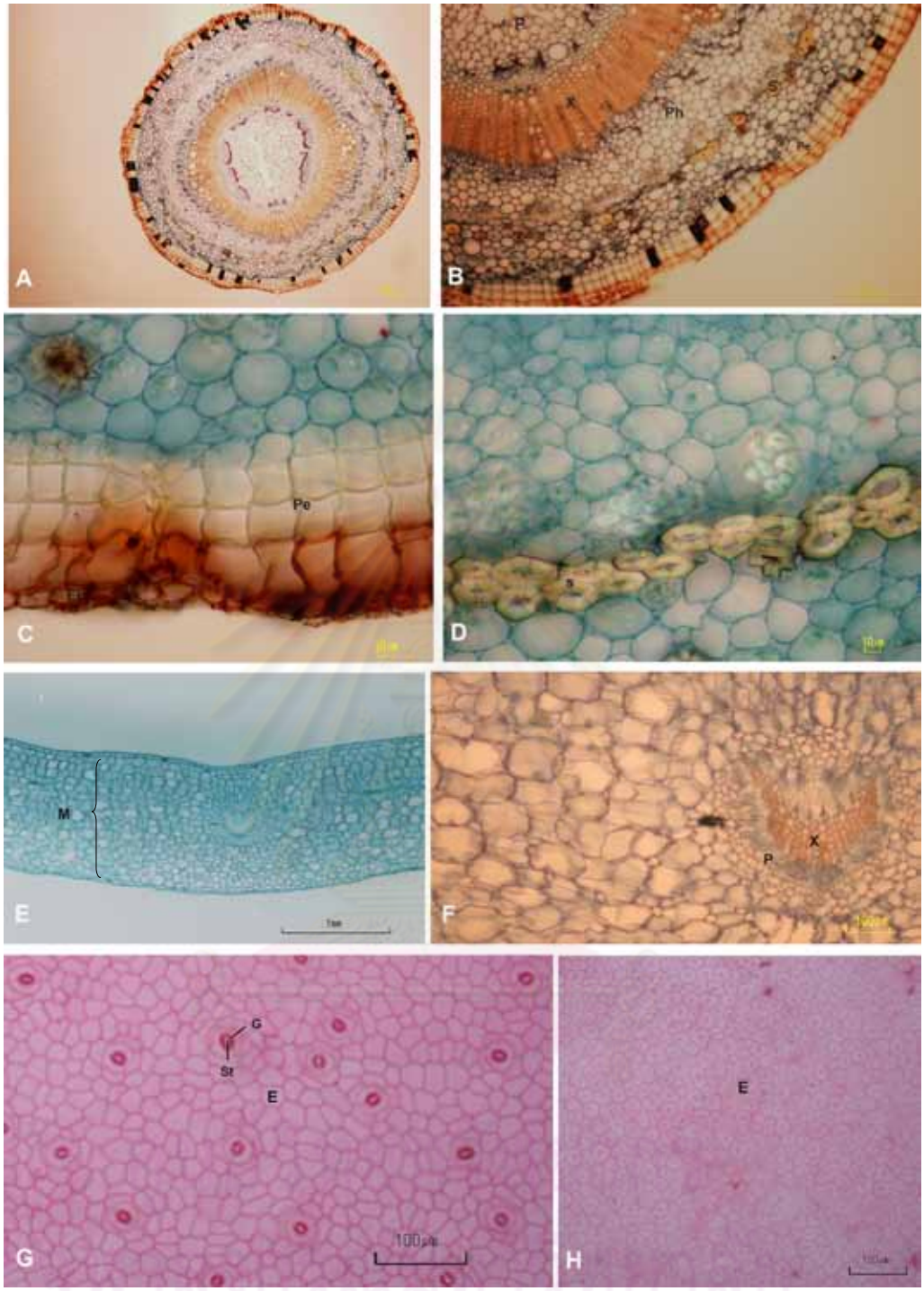

Figure 14. The stem and leaves anatomy of form III. A-D: x-section of stem (P = pith, Ep = epidermis, C = cortex, Ph = phloem, X = xylem, Pe = periderm, S = sclereid) E-F: x-section of leaves, G: abaxial surface, H: adaxial surface (M = mesophyll, P = phloem, X = xylem, G = guard cell, St = stoma, E = epidermal cells) Specimen numbers (A-D, F: *D. Tungmunnithum* 12, E: *D. Tungmunnithum* 17, G: *D. Tungmunnithum* 19, H: *D. Tungmunnithum* 12) Bar (A= 50 µm, G-H, B = 100 µm, C-D = 10 µm, E = 1 mm)

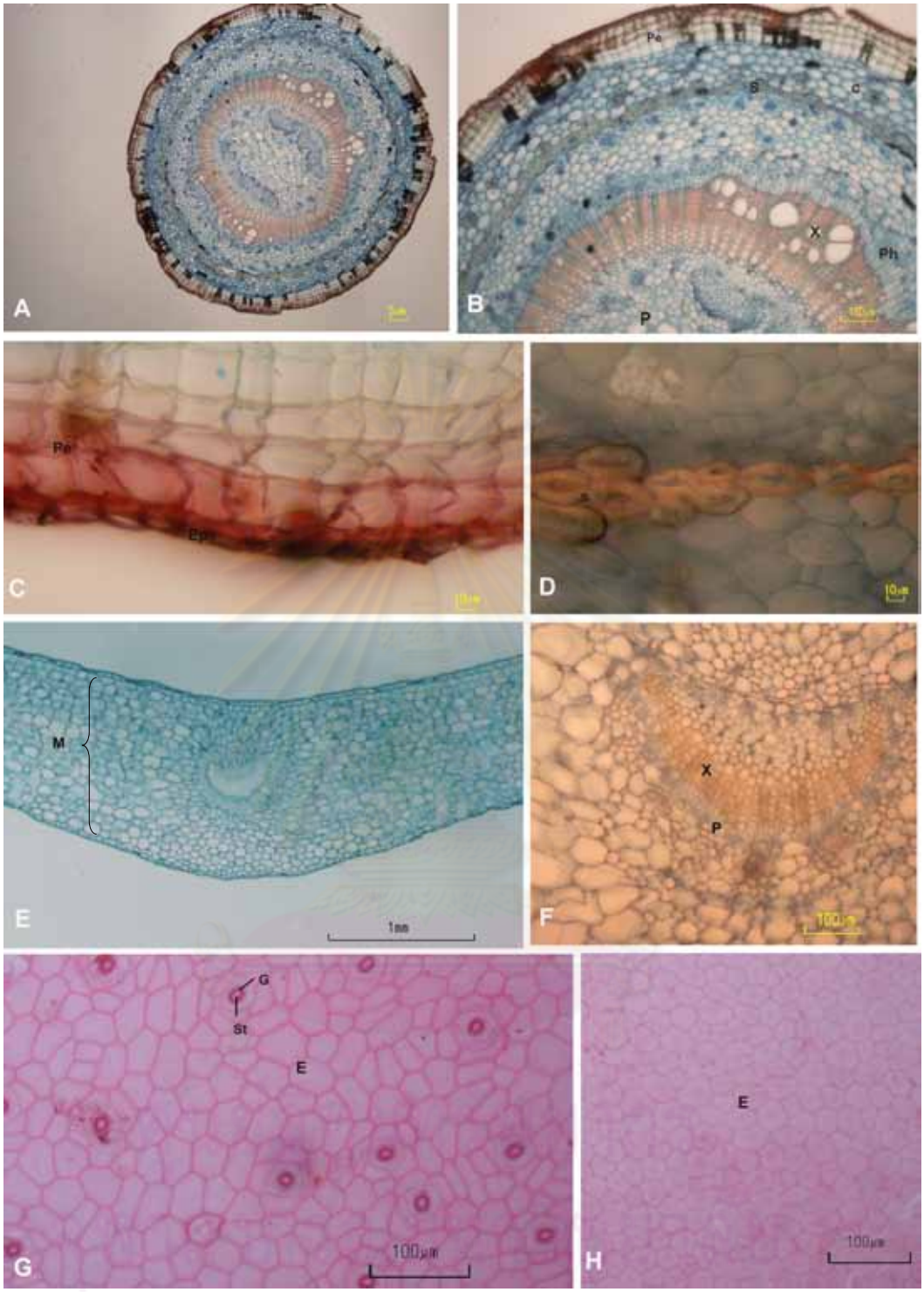

Figure 15. The stem and leaves anatomy of form IV. A-D: x-section of stem (P = pith, Ep = epidermis, C = cortex, Ph = phloem, X = xylem, Pe = periderm, S = sclereid) E-F: x-section of leaves, G: abaxial surface, H: adaxial surface (M = mesophyll, P = phloem, X = xylem, G = guard cell, St = stoma, E = epidermal cells) Specimen numbers (A-D, F: *D. Tungmunnithum* 46, E: *D. Tungmunnithum* 47, G: *D. Tungmunnithum* 48, H: *D. Tungmunnithum* 47). Bar (A= 50 µm, G-H, B = 100 µm, C-D = 10 µm, E = 1 mm)

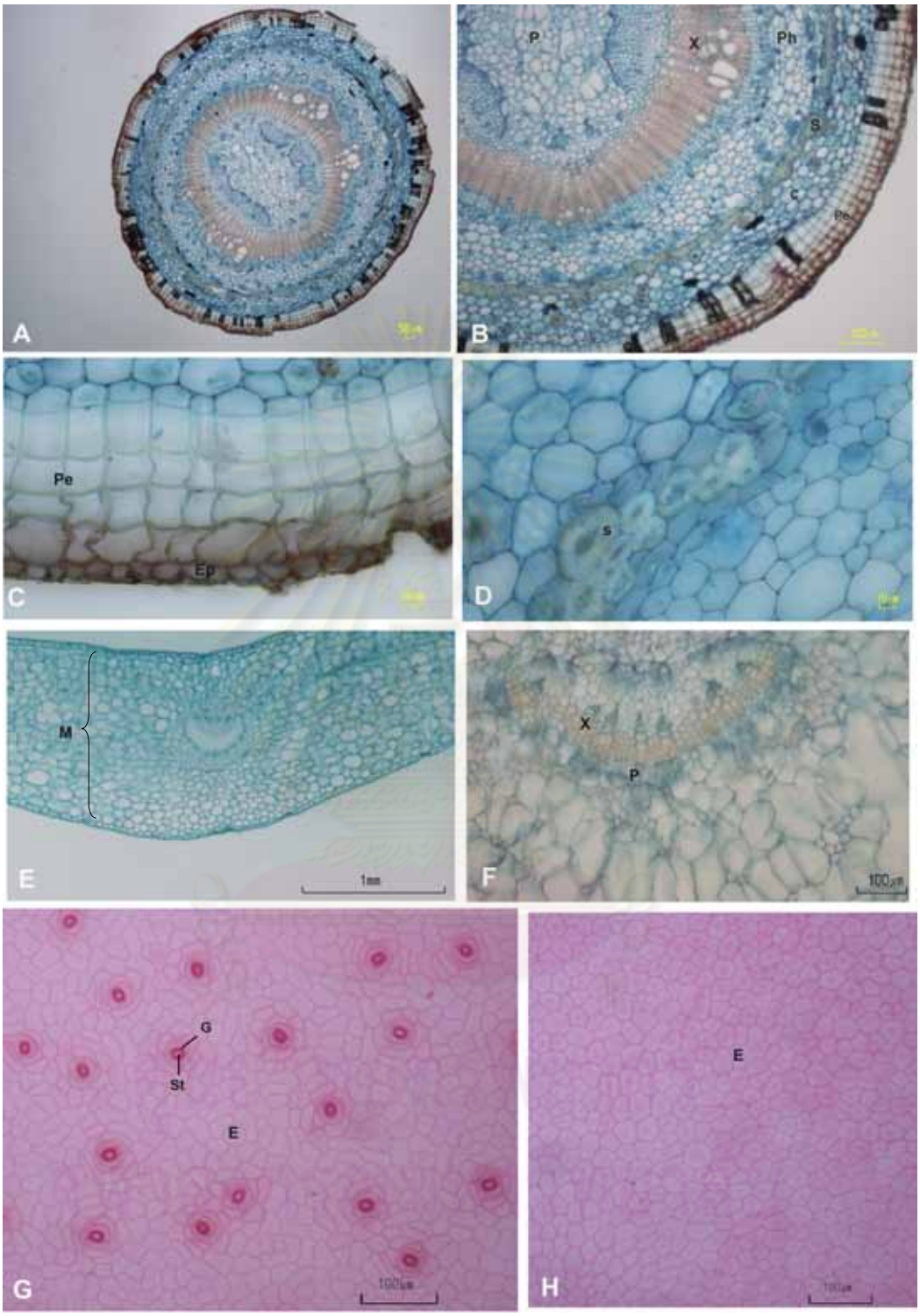

Figure 16. The stem and leaves anatomy of form V. A-D: x-section of stem (P = pith, Ep = epidermis, C = cortex, Ph = phloem, X = xylem, Pe = periderm, S = sclereid) E-F: x-section of leaves, G: abaxial surface, H: adaxial surface (M = mesophyll, P = phloem, X = xylem, G = guard cell, St = stoma, E = epidermal cells) Specimen number (A-D, F: *D. Tungmunnithum* 85, E: *D. Tungmunnithum* 90, G-H: *D. Tungmunnithum* 43). Bar (A= 50 μm, G-H, B = 100 μm, C-D = 10 μm, E = 1 mm)

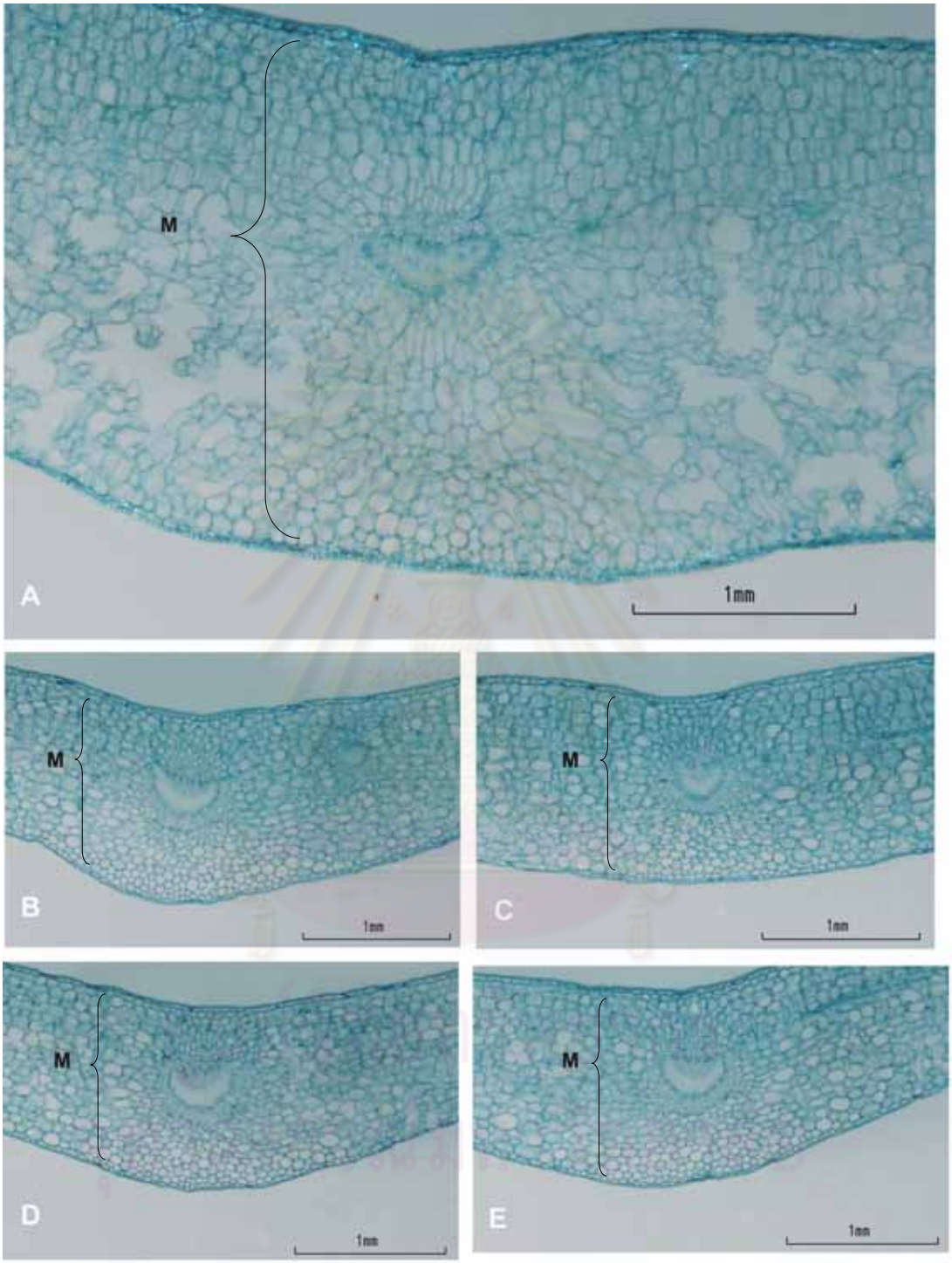

Figure 17. Comparative mesophyll of the five forms (M = mesophyll). A: cross-section of leaves in form I, B: cross-section of leaves in form II, C: cross-section of leaves in form III, D: cross-section of leaves in form IV, E: cross-section of leaves in form V. Specimen number (A: *D. Tungmunnithum* 69, B: *D. Tungmunnithum* 3, C: *D. Tungmunnithum* 17, D: *D. Tungmunnithum* 47 and E: *D. Tungmunnithum* 90. Bars (A - E = 1 mm)

Discussion

In this research, samples of *H. siamica* complex were collected from the floristic regions throughout Thailand. The results from this study indicate that morphological characters of *H. siamica* complex are more diverse than those described from holotype by Craib (Craib, 1911). These plants are various in leaf shape (Figure 6): elliptic, elliptic-oblong, oblanceolate, and oblong. Petiole is slender (Figure 6A) or stout (Figure 6B-E). Lateral veins are obscure (Figure 6A) or conspicuous (Figure 6B-E). The holotype specimen has elliptic leaves, stout petiole and conspicuous lateral veins (Figure 9A). *H. siamica* complex in Thailand have a variation in shape of corona scale that can be obovate or elliptic-obovate. Outer angle of corona is acute or obtuse (Figure 7). Corpusculum shape is broadly obovate or arrow head (Figure 7). As for holotype (Figure 9C, 9E), corona scale is elliptic-obovate. Outer angle of corona is acute. Corpusculum is arrow head shape. Moreover, ranges of leaf length, leaf width, petiole length, sepal size, corolla size, pedicel length, peduncle length and pollinium length and width are wider than holotype.

The original description did not include detail of lateral veins, pollinium and corpusculum shape. However, these characters can be used to classify these plants into morphological forms. Furthermore, many researches report that pollinarium characters are the important characters for classifying the genus *Hoya* into the species leval. (Forster and Liddle, 1991; Kleijn and van Donkelaar, 2001; Wanntorp, 2007; 2009; Kunze and Wanntorp, 2008).

This study found that *H. siamica* complex could be divided into five forms based on qualitative morphological and palynological characters: leaves shape, leaves venation, corona shape, pollinium and corpusculum shape (Table 2). Form I is different from holotype and other forms in both vegetative and reproductive structures. It has elliptic-narrowly lanceolate leaf with cuneate base, obscure lateral veins, slender petiole, broadly ovate corpusculum and broardly oblong-obovate pollinium. Holotype and form II-V have various leaf shapes, such as oblong, oblanceolate, elliptic and lanceolate, with acute base, conspicuous lateral veins, stout petiole, arrow head

corpusculum and narrowly oblong-obovate pollinium. Form II and form III have obovate corona shape and obtuse outer angle, but these characters are different when compare with holotype and other forms. Form II differs from form III only in leaf shape. Form II has oblong leaf while form III has elliptic leaf. Form IV is different from holotype and form V in leaf shape. Form IV has oblanceolate leaf while form V has elliptic leaf. It is clear that corpusculum and pollinium shape are the important reproductive characters for classifying the *H. siamica* complex in Thailand.

The results from discriminant analysis, which analyzed all specimens of the *H. siamica* complex and 45 quantitative characters of both vegetative and reproductive structures of each specimen indicates that they can be obviously divided in to 2 groups: group 1 consists of morphological form I and group 2 consists of morphological form II-V of *H. siamica* complex. Furthermore, the ordination plot (Figure 10) shows that Group 1 (morphological form I) can be obviously separated from the others. Group 2 (morphological form II-V) should be classified to the same taxon. Moreover, it shows that three characters, i.e. corpusculum width, thickness of leaves and pollinium width are the effective characters for classifying this complex. For this reason, it is clear now that the five morphological forms should not be the variations of a single species of *H. siamica* complex. "*H. siamica* complex" in Thailand comprise at least two species or varieties.

Moreover, some keys used the number of flowers per inflorescence, leaf width and pedicel length to classify this plant (Ping-tao, Gilbert and Stevens, 1995), but this study indicates that leaf length, leaf width, petiole length, sepal size, number of flowers per inflorescence, pedicel length, color of corolla and corona are not effective characters for classifying this plant species because these characters are highly variable even within the same population. In addition, the five morphological forms of the complex occur in different habitats throughout Thailand. Form I occurs in southwestern Thailand at 940-1150 m elevation, on rock in open areas. Form II occurs in southeastern and northeastern Thailand at 1100-1520 m elevation. From III occurs in northern and

northeastern Thailand at 1463-1500 m elevation. Form VI and V occurs in northern Thailand at 2283-2295 m and 1500-2288 m elevation respectively. Likewise, many researches indicated that width (Davis, 1983) and thickness (Feild, *et al.* 2001) of leaves and color of flower parts (Forster and Liddle, 1991) were not useful characters for classification, these characters were found to differ under various growth conditions in various habitats.

The results from anatomical study shows that mesophyll of *H. siamica* complex can be classified into 2 groups. The first group composed of specimens from Huai Yang Waterfall National Park (morphological form I) can obviously be divided into two layers, dense and sparse parenchyma cells. The second group composed of the other specimens (morphological form II-IV), which cannot be divided. Hence, this anatomical data support the morphological and palynological data that form I is obviously difference from the others. Moreover, the leaf thickness of the first group is higher than the second group. However, the thickness of leaf should not be used to classify this complex because it may be related to environmental effects such as, light, humid and elevation. An obvious example from the study of acclimation of leaf anatomy in *Amborella trichopoda* indicated that plants of the same species which growth in different environment, such as sun and shade have different leaf thickness (Feild, *et al.* 2001) like the study of Dickison (Dickison and Weitzman, 1996), which reported that plants of the same species, but grow in difference habitat can have different leaf thickness. Additionally, this study suggests that the future study should be carried out on these plants by cultivating them in a green house and examine their mesophyll. This will help to determine whether an environment affects on the character of mesophyll or not.

To conclude, the results from morphological, anatomical and palynological data of the *H. siamica* complex in Thailand indicated that this complex should be consisted of two species or varieties. The specimen from Huai Yang Waterfall National Park (morphological form I, Group 1 of ordinary plot in discriminant analysis and anatomy) is possibly a new species or varieties of the *H. siamica* complex in Thailand.

CHAPTER IV

PCR-RFLP STUDY

Introduction

At present, many molecular techniques have been widely used to study both structure and evolution of genes. Polymerase chain reaction (PCR)- Restriction Fragment Length Polymorphisms (RFLPs) is one of those techniques, which uses a set of primer pairs to amplify the regions of genome via polymerase chain reaction and the PCR-products are digested by the a set of restriction endonucleases to detect polymorphisms. Size of these fragments are separated by using gel electrophoresis. The presence and absence of fragments resulted from changes in recognition sites are used to identify species or populations. Moreover, this method is easy and rapid (Gielly and Taberlet, 1994).

Similarly, the study of variations in chloroplast DNA (cpDNA) has become a common tool in taxonomic studies (Doyle, 1992). Many researches indicate that cpDNA is an extremely valuable molecule for studying phylogenetic relationships between closely related species (Palmer 1987; Palmer *et al.* 1988). Furthermore, PCR-RFLP analysis of chloroplast DNA by using universal primers is one of the effective methods to clearify taxonomic status in several plant taxa. An obvious example is the studies of *Solanum brevicaule* complex (Miller and Spooner, 1999), *Pinus krempfii* (Wang, Szmidt and Nguyen, 2000), *Hoya parasitica* complex (Kidyoo *et al*, 2005) and many species in the genus *Hoya* (Wanntorp, *et al.,* 2006, Wanntorp, 2007; 2009)

Due to the highly variation in many characters of "*H. siamica* complex" in Thailand, the molecular investigations are needed to clarify their taxonomic status. Aim of this study is to investigate cpDNA variations by using the PCR-RFLP technique in order to determine taxonomic status of the *H. siamica* complex in Thailand. Moreover, this study may be the first report of molecular data, especially the PCR-RFLP analysis of this complex.

Materials and methods

1. Plant materials

Total 17 living specimens collected from various parts of Thailand, which are cover the population and variation of the *H. siamica* complex were used for PCR-RFLP analyses (Table 5).

Table5. Sampling specimen numbers of the *H. siamica* complex in Thailand.

N0.	Locality	Specimen number	Morphological form
1	Huai Yang Waterfall National Park, (Prachuapkhirikhan Province)	66	I
2	Huai Yang Waterfall National Park, (Prachuapkhirikhan Province)	67	I
3	Huai Yang Waterfall National Park, (Prachuapkhirikhan Province)	69	I
4	Khao Yai (Prachinburi Province)	2	II
5	Khao Yai (Prachinburi Province)	3	II
6	Phu Luang (Loei Province)	10	II
7	Phu Luang (Loei Province)	11	II
8	Phu Luang (Loei Province)	12	III
9	Phu Luang (Loei Province)	17	III
10	Phu Luang (Loei Province)	19	III
11	Kio Mae Pan (Chiang Mai Province)	46	IV
12	Kio Mae Pan (Chiang Mai Province)	47	IV
13	Kio Mae Pan (Chiang Mai Province)	48	IV
14	Kio Mae Pan (Chiang Mai Province)	87	V
15	Kio Mae Pan (Chiang Mai Province)	89	V
16	Doi Sutep (Chiang Mai Province)	90	V
17	Doi Sutep (Chiang Mai Province)	91	V

2. DNA extraction and PCR amplification

The total genomic DNA was extracted from fresh leaf by using the NucleoSpin Plant II Kit (MACHEREY-NAGEL). All DNA was kept at -20 °C for the PCR amplification. And then, five different regions of cpDNA were amplified by using the universal primers (Table 6, Figure 18). Amplifications were used 50 µl of reaction mixture containing 250 µM dNTPs (iNtRON Biotechnology), 1.5 mM MgCl2, 1 µM each primers,1x Taq DNA polymerase buffer, 2.5 unit of Taq DNA polymerase (iNtRON Biotechnology) and 20-50 ng of total genomic DNA as the template. The PCR amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc.), with a heated lid, using an initial cycle at 94 °C for 4 minutes, followed by 40 cycles of 45 second at 94 °C; 45 second at 52 °C to 57.5°C; 3 minutes to 4 minutes at 72 °C (annealing temperature and extension time depend on the length of the fragment to be amplified; Table 6), and a final extension for 10 minutes at 72 °C.

Table 6. PCR regions of amplification by using five cpDNA universal primer pairs (Grivet *et al.*, 2001).

No.	Regions	Primers
1	C_1C	rpoC1: 5'-GCACAAATTCCRCTTTTTATRGG-3' trnC: 5'-CGACACCCRGATTTGAACTGG-3'
2	HK	trnH: 5'-ACGGGAATTGAACCCGCGCA-3' trnK: 5'- CCGACTAGTTCCGGGTTCGA-3'
3	K_1K_2	rnK: 5'–GGGTTGCCCGGGACTCGAAC-3' trnK: 5'–CAACGGTAGAGTACTCGGCTTTTA-3'
4	TC	trnT: 5'-GCCCTTTTAACTCAGTGGTA-3' psbC: 5'-GAGCTTGAGAAGCTTCTGGT-3'
5	VL	trnV: 5'- CGAACCGTAGACCTTCTCGG-3' rbcl: 5'-GCTTTAGTCTCTGTTTGTGG-3'

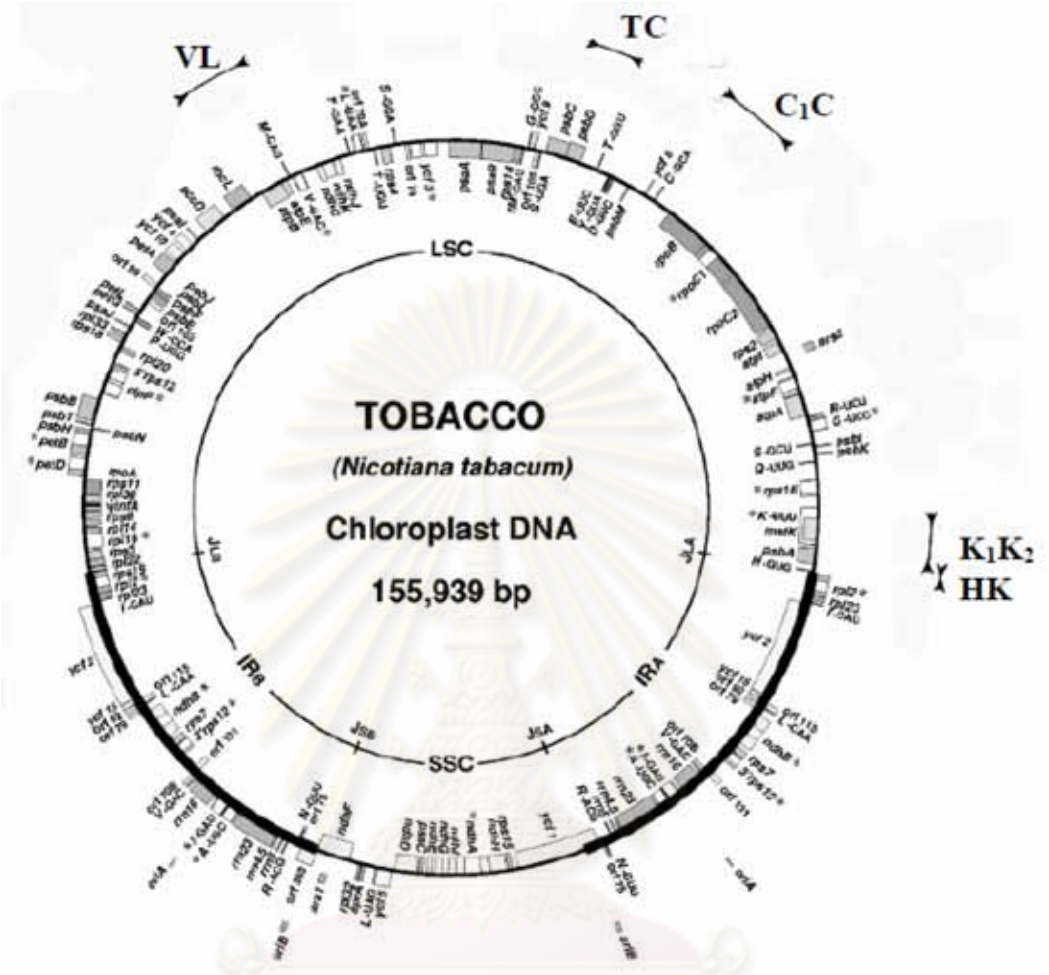

Figure 18. Location of the 5 regions in this study base on the tobacco cpDNA genome (Grivet *et al.*, 2001).

3. Agarose gel electrophoresis

Agarose gel electrophoresis was performed so as to check the results of DNA extraction and PCR amplification by using 1% in TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA pH 8.0, respectively). Two µl of the loading dye (0.25% bromphenol blue, 40% ficoll 400 and 0.25% xylene cyanol) was mixed with 5 µl of each DNA sample, and loaded into each well. The 1 kb DNA ladder (Sib Enzyme)

was used as the standard molecular size. Electrophoresis was carried out until bromphenol blue, which is the lowest dye migrated to approximately 3-5 cm from the bottom of gel. The gel was stained with a 15 µl of ethidium bromide solution about 30 minutes, and then destained by distilled water about 15 minutes. Finally, visualized under UV light and photographed.

4. Restriction enzyme digestion

The five restriction enzymes in the Table 7 (Fermentas) were used to digest the PCR products. The digestion was carry out in a 10 µl volume containing approximately 30 ng of the PCR products. The reaction mixture was incubated at 37 °C overnight with 3-5 units of enzyme in 1X enzyme buffers. The 1 kb DNA ladder and 100 bp DNA ladder (Sib Enzyme) were used as the standard molecular size. The fragments were separated on 2 % agarose gel at 80 V for about 4.5-5 hours, stained with ethidium bromide, visualized under UV light and photographed.

Table 7. List of restriction enzymes used for analyses of cpDNA variation of *H. siamica* complex in Thailand.

No.	Restriction enzymes	Recognition site	Incubated Temp. °C
1	*Eco*R I	G/AATTC	37
2	*Msp*I (*Hpa*II)	CC/GG	37
3	*Rsa*I	GT/AC	37
4	*Hae*III (*Bsu*RI)	GG/CC	37
5	*Ase*I (*Vsp*I)	AT/TAAT	37

Results

PCR amplification

The selected regions of chloroplast DNA, which are the five intergenic-spacers (HK, K_1K_2, C_1C, TC, and VL) were amplify by using the PCR technique. The approximated size of PCR products of these regions and degree of amplification are shown in Table 8, and Figures 19-23.

Table 8. PCR conditions size of the amplified fragments and degree of amplification using 5 cpDNA universal primer pairs in *Hoya siamica* complex in Thailand.

Abberv. Of cpDNA primer[a]	PCR conditions		Size (bp)	Degree of amplification [b]
	Annealing Temperature (°C)	Extension time (min)		
C_1C	52	4	4490	+
HK	55	4	1560	++
K_1K_2	57.5	3	2480	++
TC	52.5	4	3030	+
VL	55	4	3730	+

[a] Abbreviations are in Grivet *et al.* (2001)

[b] +, faint amplification; ++, good amplification

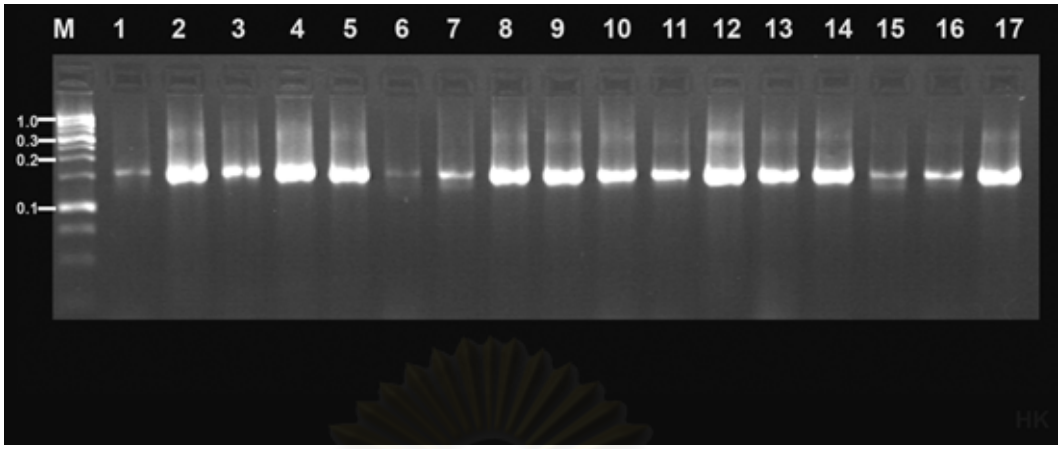

Figure 19. PCR amplified of HK region of cpDNA of "*Hoya siamica* complex". Lane M: 1 kb ladder, Lane 1-17: PCR amplified products of 17 *H. siamica* complex individuals (Table 5).

Figure 20. PCR amplified of K_1K_2 region of cpDNA of "*Hoya siamica* complex". Lane M: 1 kb ladder, Lane 1-17: PCR amplified products of 17 *H. siamica* complex individuals (Table 5).

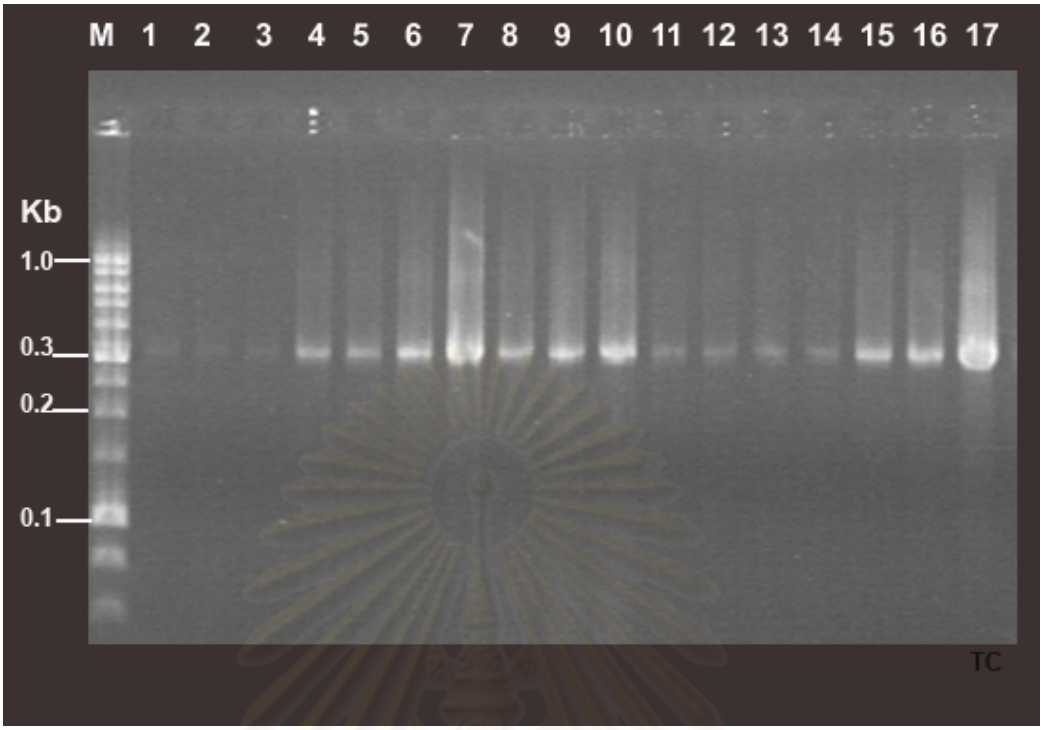

Figure 21. PCR amplified of TC region of cpDNA of "*Hoya siamica* complex". Lane M: 1 kb ladder, Lane 1-17: PCR amplified products of 17 *H. siamica* complex individuals (Table 5).

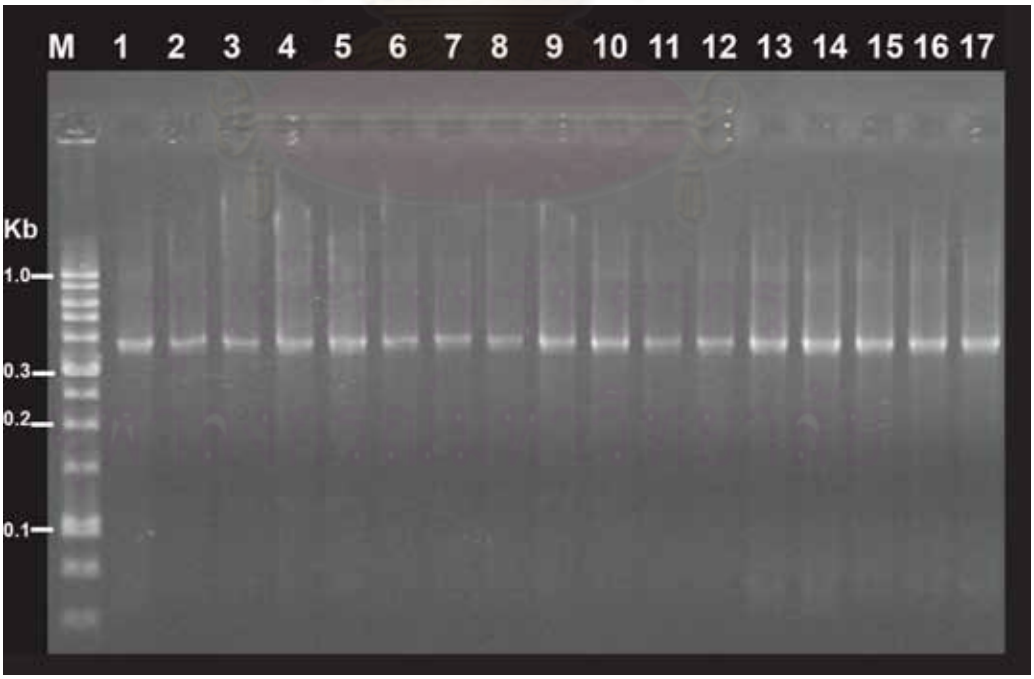

Figure 22. PCR amplified of VL region of cpDNA of "*Hoya siamica* complex". Lane M: 1 kb ladder, Lane 1-17: PCR amplified products of 17 *H. siamica* complex individuals (Table 5).

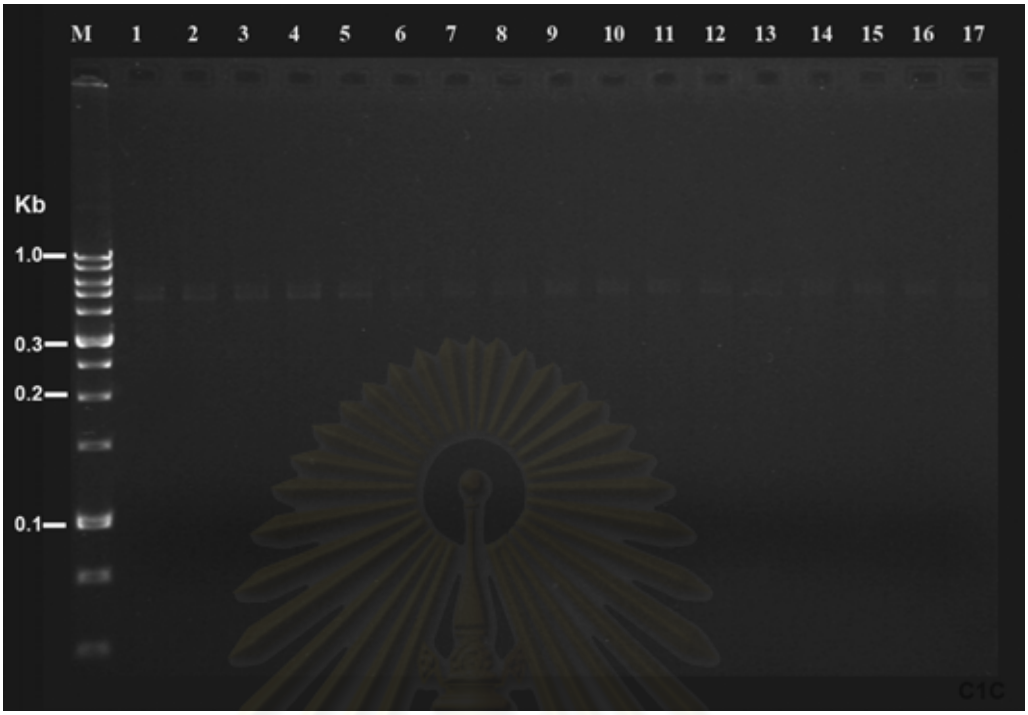

Figure 23. PCR amplified of C_1C region of cpDNA of "*Hoya siamica* complex". Lane M: 1 kb ladder, Lane 1-17: PCR amplified products of 17 *H. siamica* complex individuals (Table 5).

PCR-RFLP analysis

Each of the five amplified fragments (HK, K_1 K_2, C_1C, TC, and VL) was digested by five restriction enzymes (*Bsu*I, *Eco*RI, *Msp*I, *Rsa*I and *Vsp*I) generating 25 primer-enzyme combinations (Table 9). All fragment amplified by five primer pairs showed the monomorphic patterns with the five restriction enzymes (Table 10 and Figures 24-48).

Table 9. The results of digestions of five amplified cpDNA regions with five restriction enzymes

	Amplified cpDNA				
Enzymes	C1C	HK	K1K2	TC	VL
*Bsu*I	3	2	4	4	5
*Eco*RI	4	1	2	4	3
*Msp*I	4	1	3	4	5
*Rsa*I	4	4	4	6	8
*Vsp*I	4	2	3	4	2

Table 10. Numbers and appoximated size of restriction fragments and number of monomorphic patterns recognized by PCR-RFLP analysis.

Regions	Restriction enzymes	No. of pattern	No. of bands	Approximated fragment size (bp)	Specimens
C_1C	*Bsu*I	1	3	2550, 1110,630, 200	all 17 specimens
	*Eco*RI	1	4	1440, 1400, 850, 650, none visually detected band	all 17 specimens
	*Msp*I	1	4	2240, 1300, 700, 250	all 17 specimens
	*Rsa*I	1	4	2100, 1300, 550, 450, none visually detected band	all 17 specimens
	*Vsp*I	1	4	1800, 1200, 920, 570	all 17 specimens
HK	*Bsu*I	1	2	1200, 360	all 17 specimens
	*Eco*RI	1	1	1560	all 17 specimens
	*Msp*I	1	1	1560	all 17 specimens
	*Rsa*I	1	4	775, 310, 265, 210	all 17 specimens
	*Vsp*I	1	2	1220, 340	all 17 specimens
K_1K_2	*Bsu*I	1	4	1400, 550, 300, 230	all 17 specimens
	*Eco*RI	1	2	2000, 480	all 17 specimens
	*Msp*I	1	3	1170, 700, 610	all 17 specimens
	*Rsa*I	1	4	1450, 500, 330, 200	all 17 specimens
	*Vsp*I	1	3	1000, 730, 750	all 17 specimens

Table10. (Continued)

Regions	Restriction enzymes	No. of pattern	No. of bands	Approximated fragment size (bp)	Specimens
TC	*Bsu*I	1	4	1500, 700, 630, 200	all 17 specimens
	*Eco*RI	1	4	895, 725, 705, 460, none visually detected band	all 17 specimens
	*Msp*I	1	4	1490, 520, 390, 380, none visually detected band	all 17 specimens
	*Rsa*I	1	6	1400, 500,400, 360, 220, 150	all 17 specimens
	*Vsp*I	1	4	870, 750, 740, 670	all 17 specimens
VL	*Bsu*I	1	5	1300, 950, 610, 385, 290, none visually detected band	all 17 specimens
	*Eco*RI	1	3	3100, 310, 210, none visually detected band	all 17 specimens
	*Msp*I	1	5	1380, 900, 600, 450, 400	all 17 specimens
	*Rsa*I	1	8	860, 675, 520, 430, 360, 300, 260, 200, none visually detected band	all 17 specimens
	*Vsp*I	1	2	2200, 1500, none visually detected band	all 17 specimens

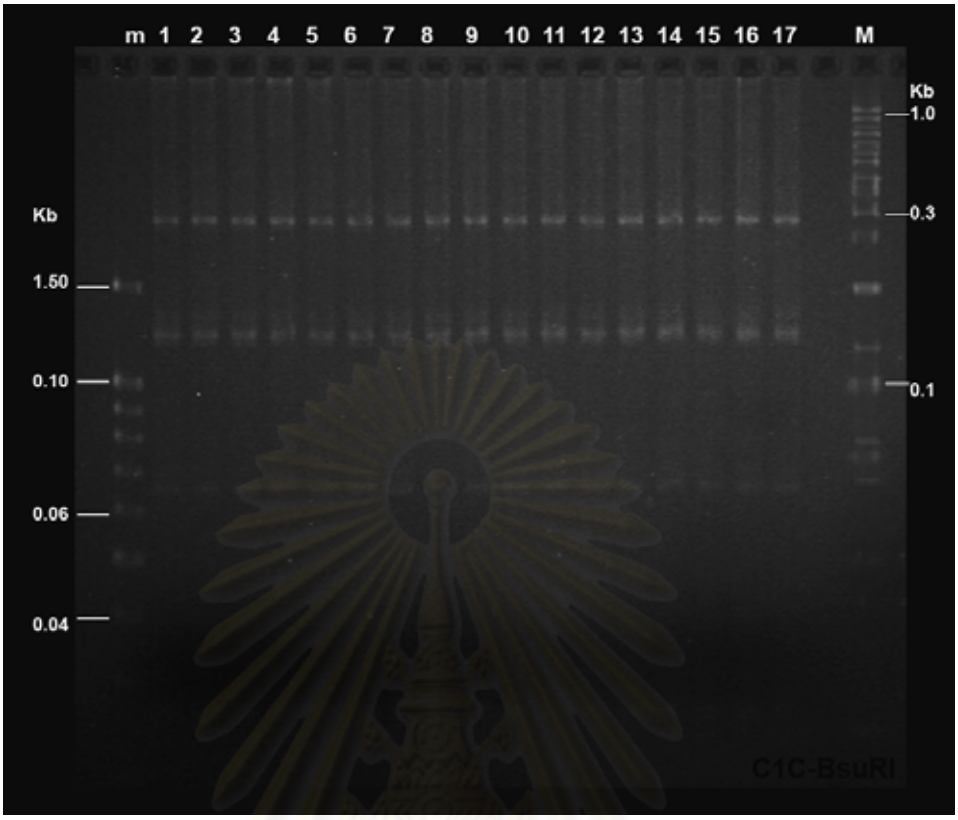

Figure 24. PCR-RFLP in C_1C region of cpDNA of "*Hoya siamica* complex" digested with *Bsu*RI. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

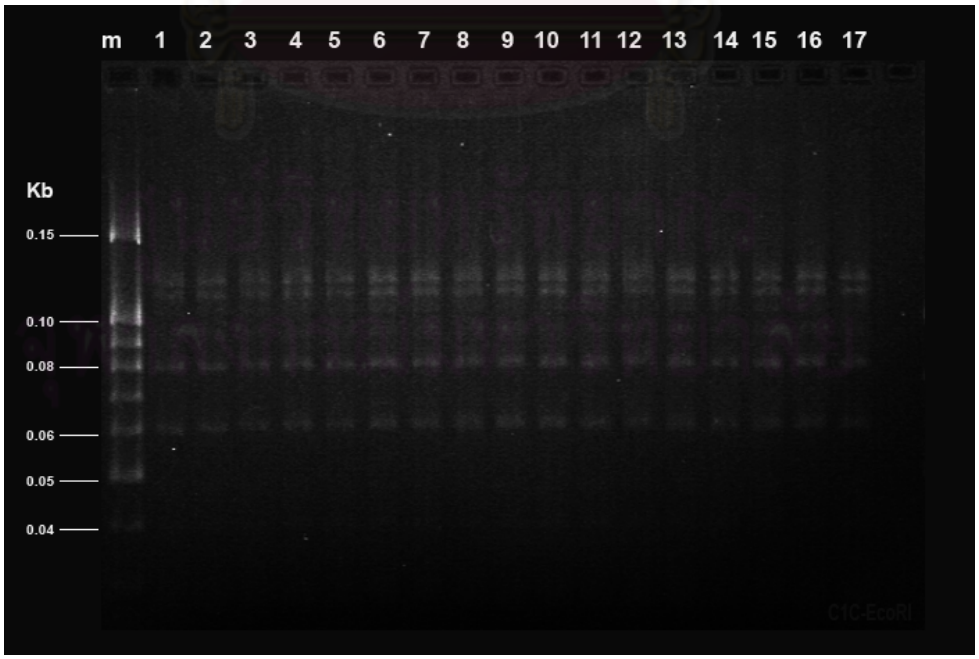

Figure 25. PCR-RFLP in C_1C region of cpDNA of "*Hoya siamica* complex" digested with *Eco*RI. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

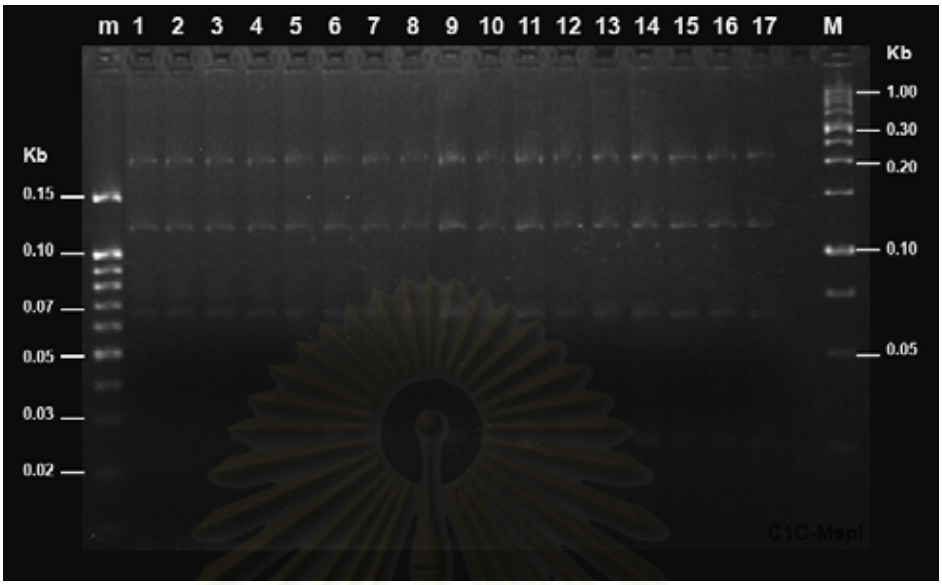

Figure 26. PCR-RFLP in C_1C region of cpDNA of "*Hoya siamica* complex" digested with *Msp*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

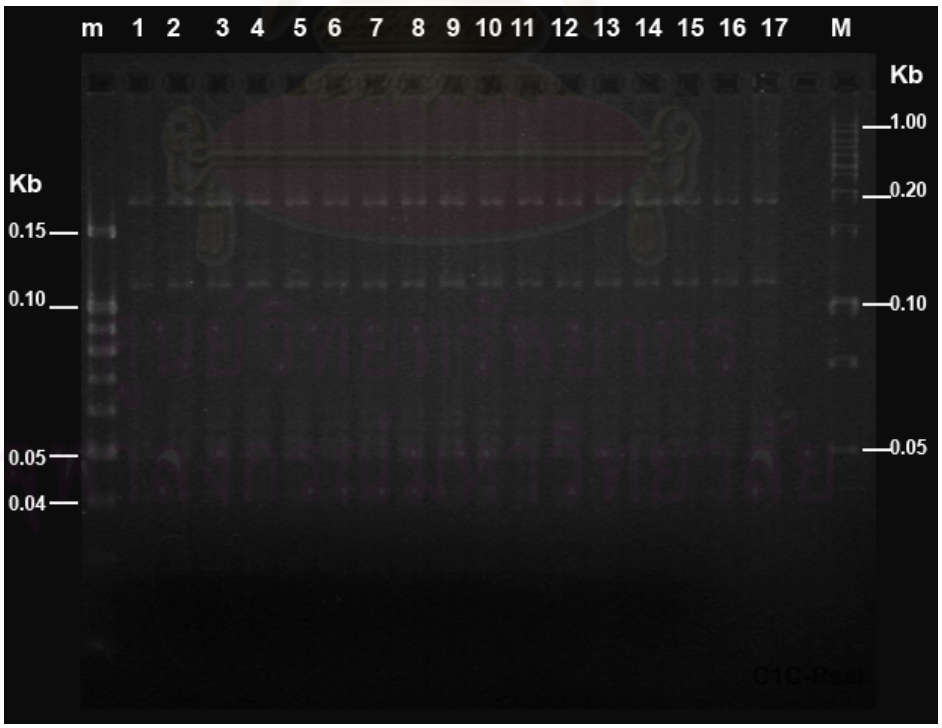

Figure 27. PCR-RFLP in C_1C region of cpDNA of "*Hoya siamica* complex" digested with *Rsa*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

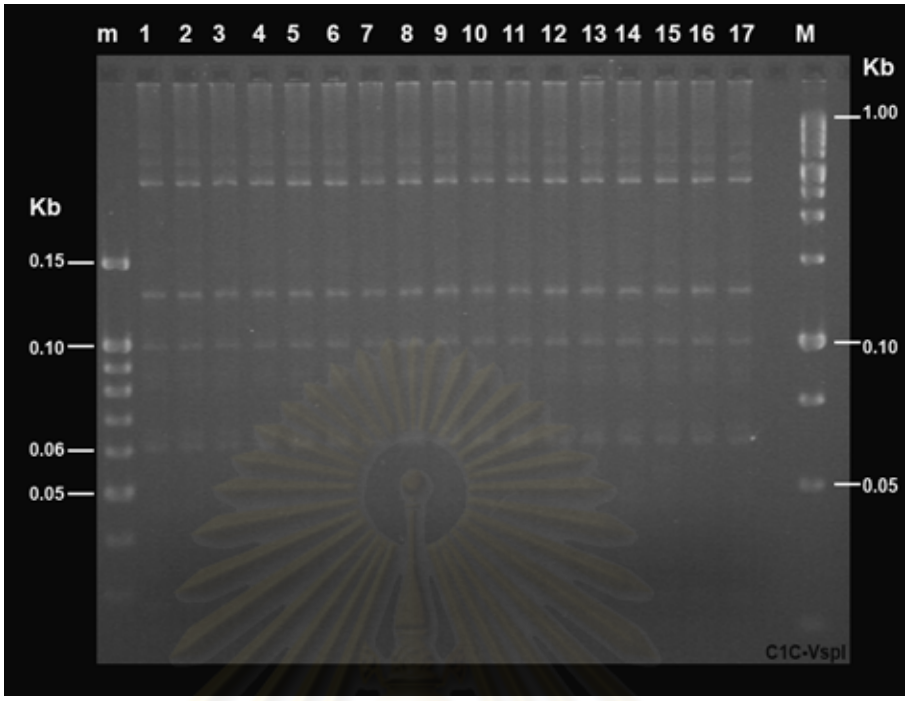

Figure 28. PCR-RFLP in C_1C region of cpDNA of "*Hoya siamica* complex" digested with *Vsp*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

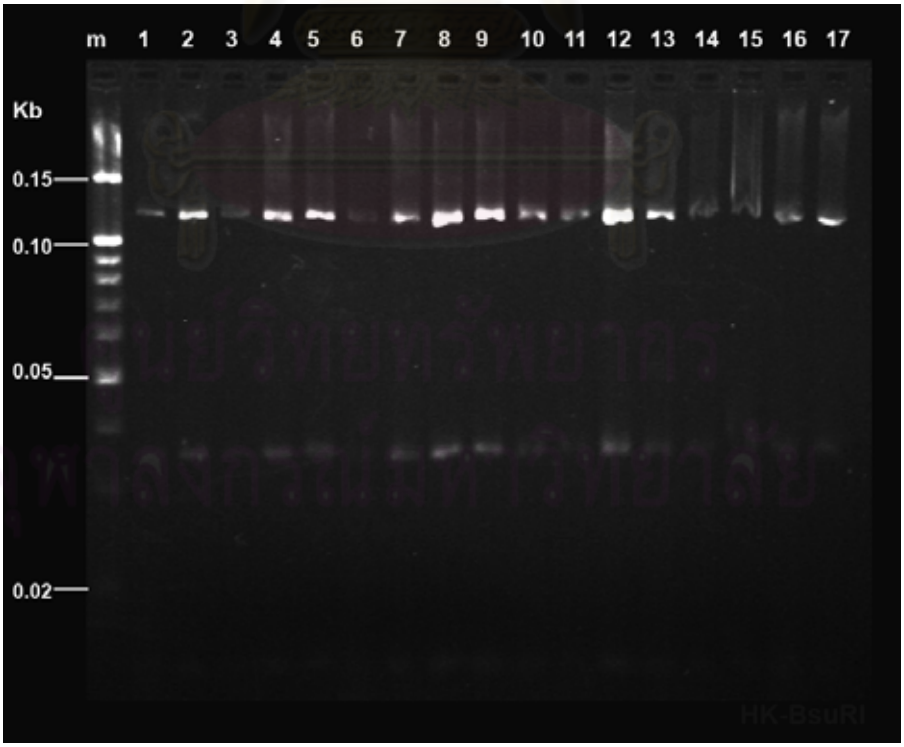

Figure 29. PCR-RFLP in HK region of cpDNA of "*Hoya siamica* complex" digested with *Bsu*RI. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

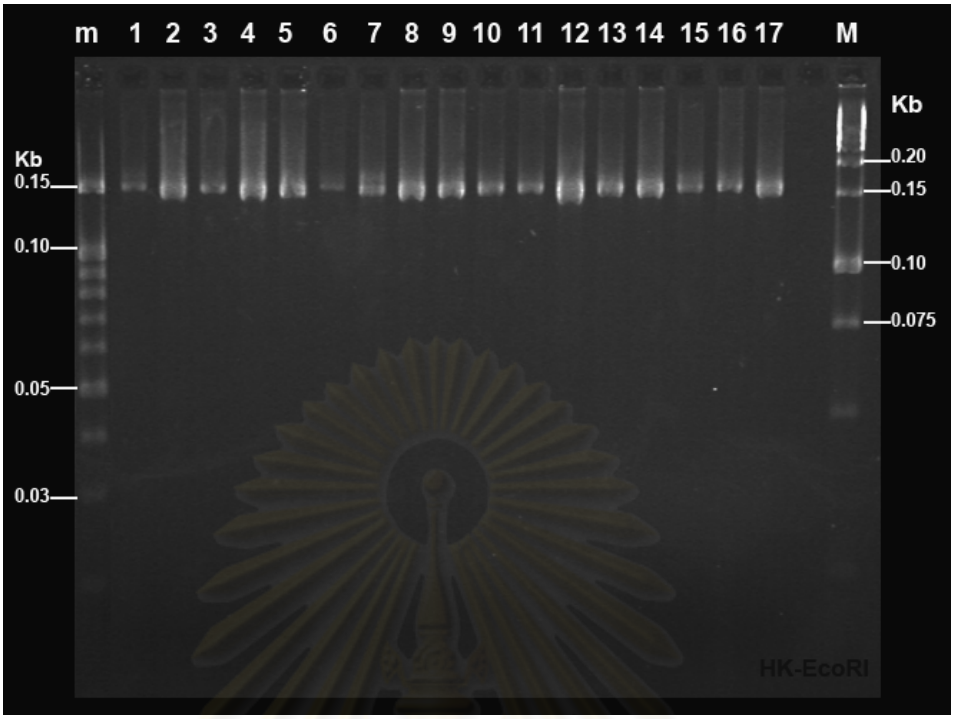

Figure 30. PCR-RFLP in HK region of cpDNA of "*Hoya siamica* complex" digested with *Eco*RI. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

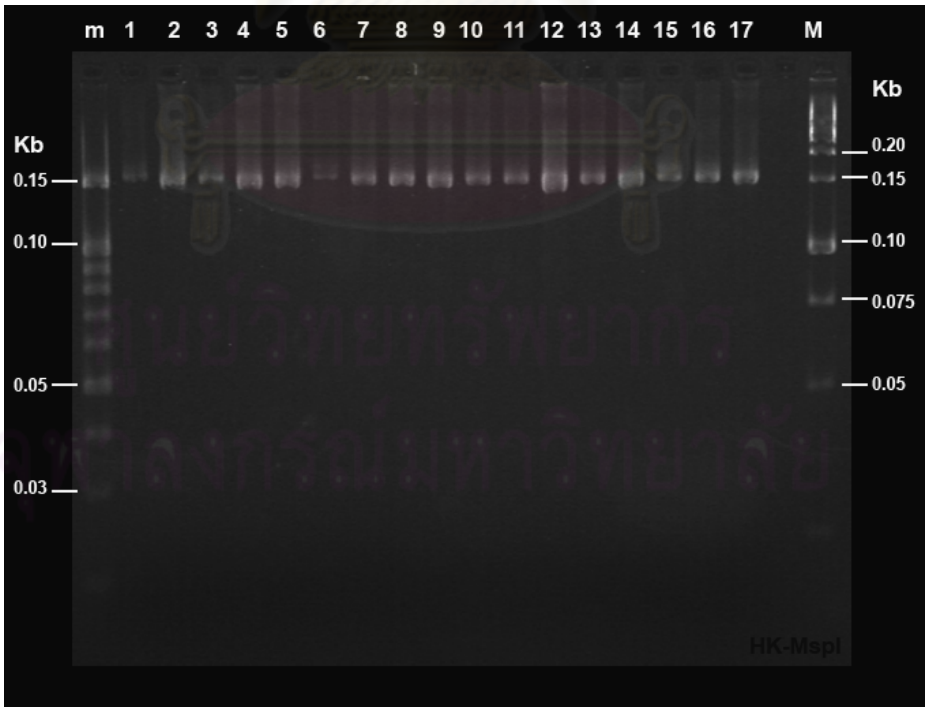

Figure 31. PCR-RFLP in HK region of cpDNA of "*Hoya siamica* complex" digested with *Msp*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

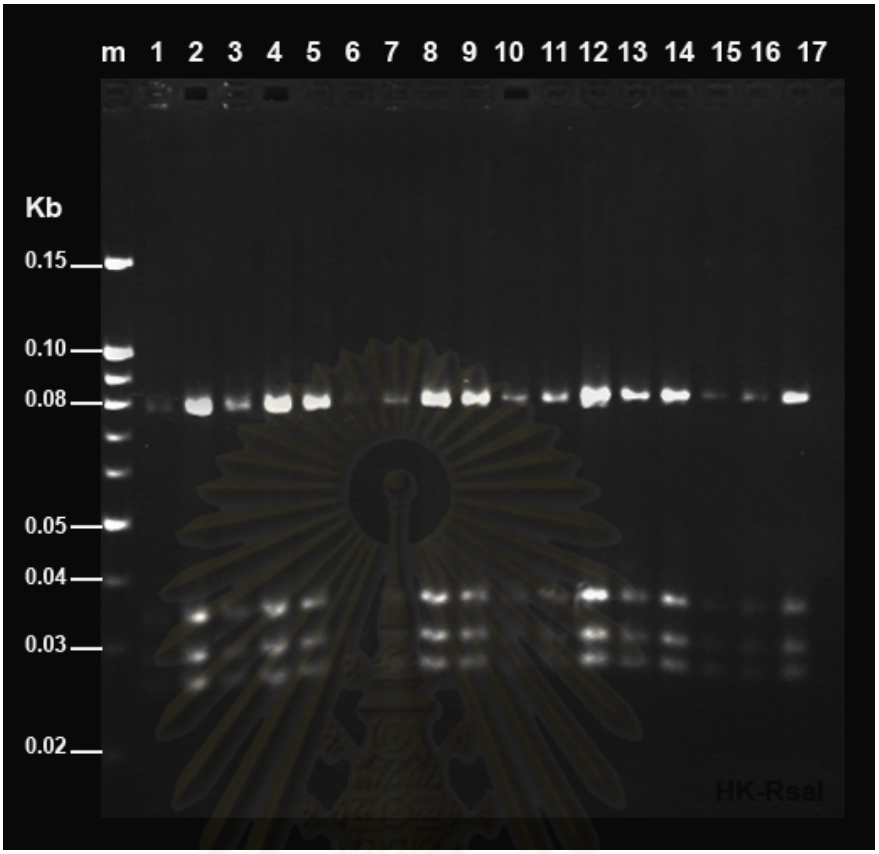

Figure 32. PCR-RFLP in HK region of cpDNA of "*Hoya siamica* complex" digested with *Rsa*I. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

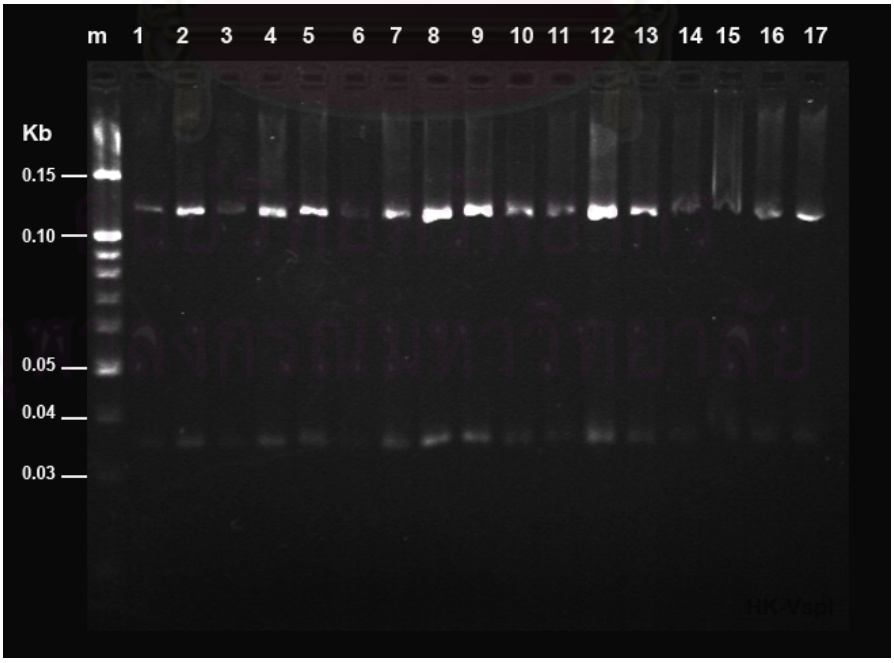

Figure 33. PCR-RFLP in HK region of cpDNA of "*Hoya siamica* complex" digested with *Vsp*I. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

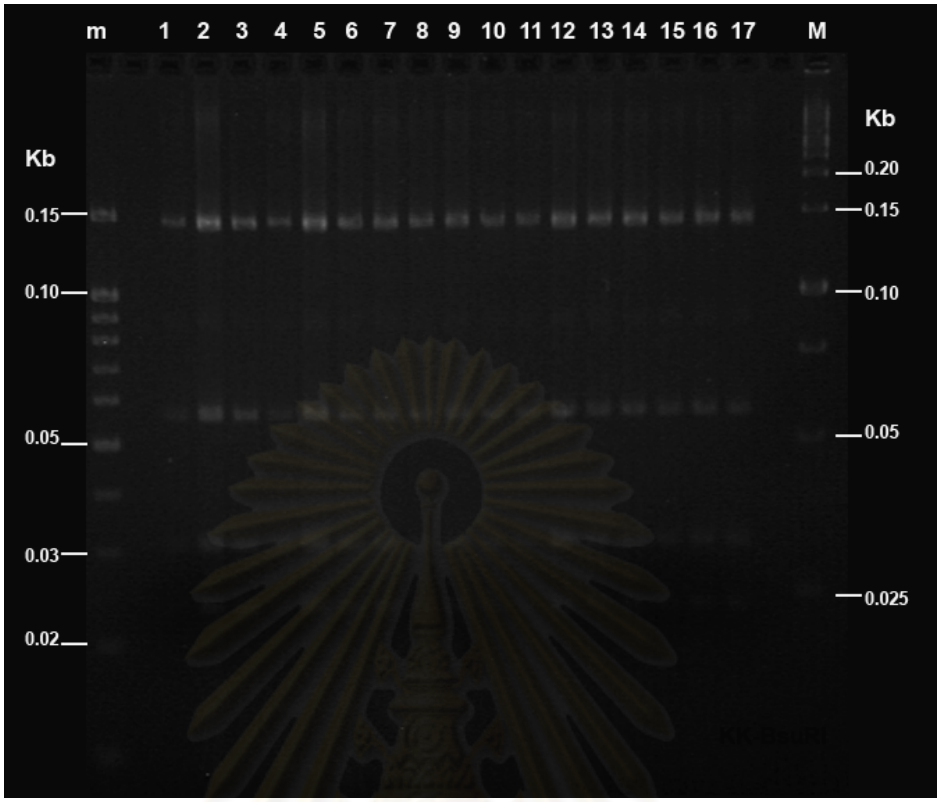

Figure 34. PCR-RFLP in K_1K_2 region of cpDNA of "*Hoya siamica* complex" digested with *Bsu*RI. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

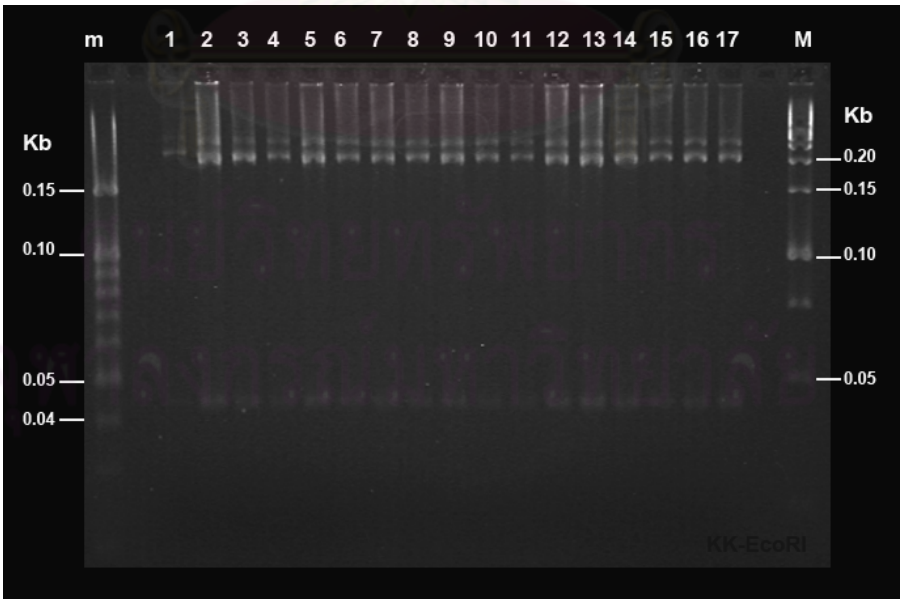

Figure 35. PCR-RFLP in K_1K_2 region of cpDNA of "*Hoya siamica* complex" digested with *Eco*RI. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

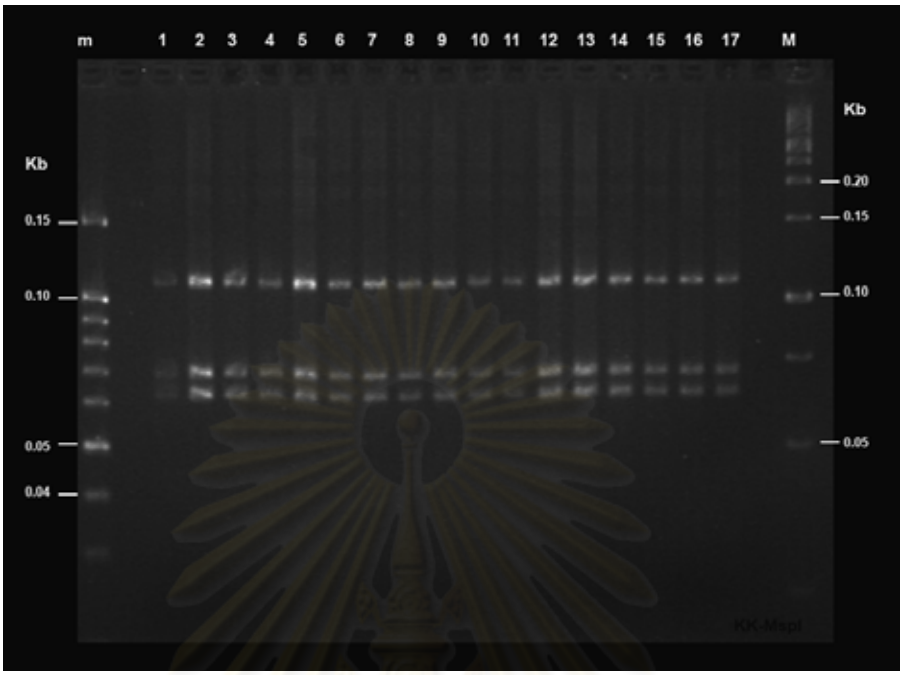

Figure 36. PCR-RFLP in K_1K_2 region of cpDNA of "*Hoya siamica* complex" digested with *Msp*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

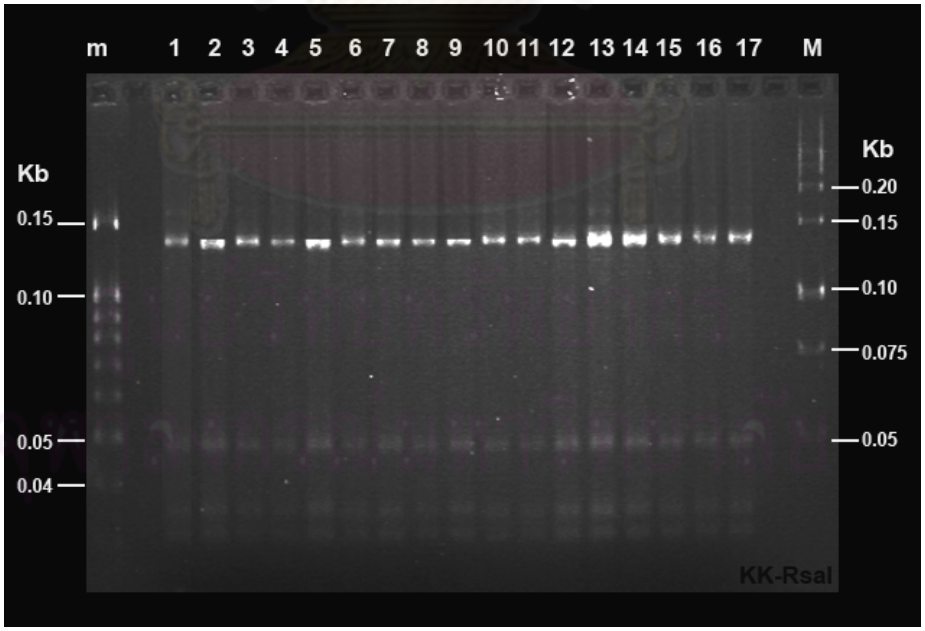

Figure 37. PCR-RFLP in K_1K_2 region of cpDNA of "*Hoya siamica* complex" digested with *Rsa*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

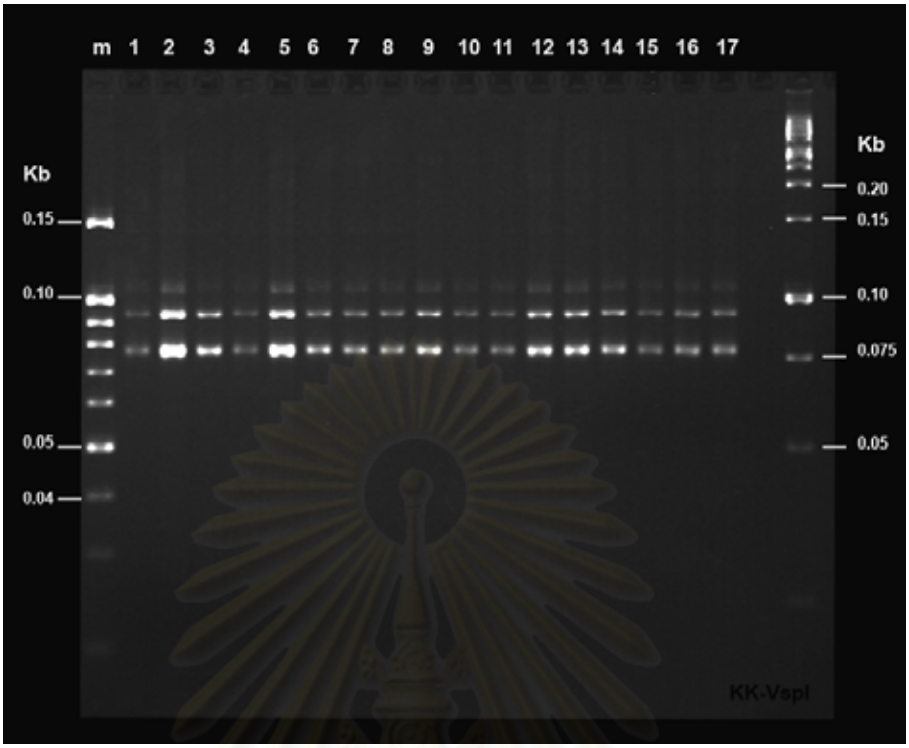

Figure 38. PCR-RFLP in K_1K_2 region of cpDNA of "*Hoya siamica* complex" digested with *Vsp*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

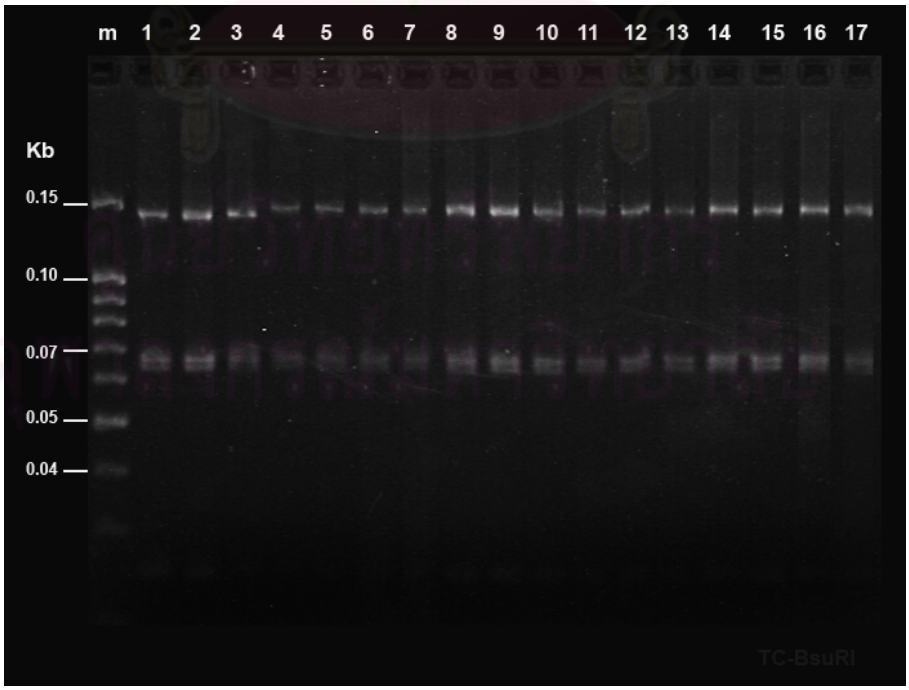

Figure 39. PCR-RFLP in TC region of cpDNA of "*Hoya siamica* complex" digested with *Bsu*RI. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

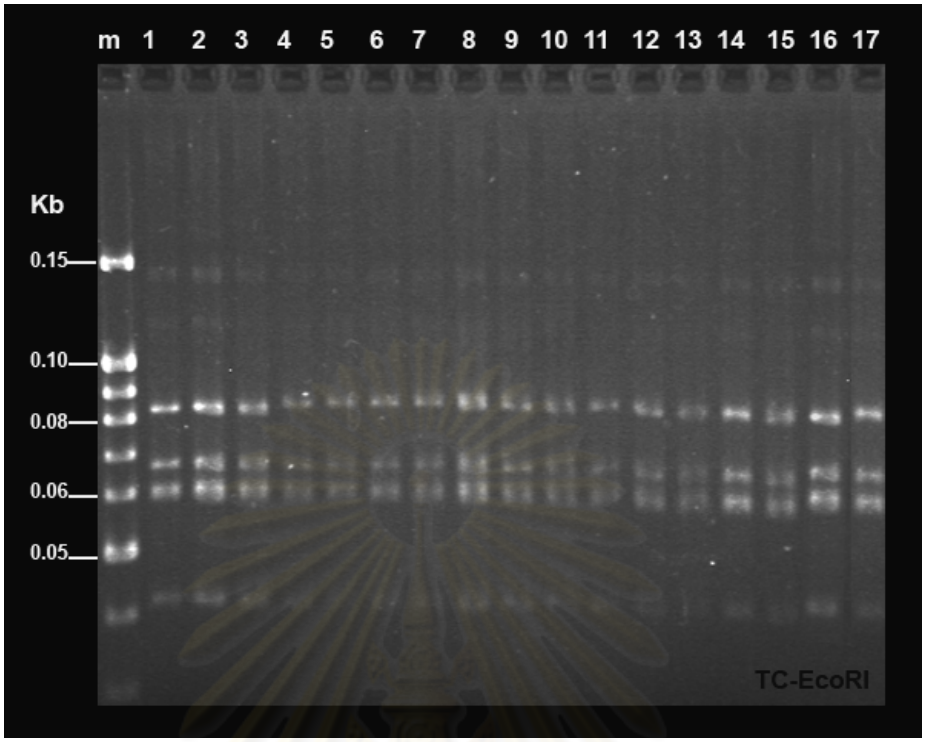

Figure 40. PCR-RFLP in TC region of cpDNA of "*Hoya siamica* complex" digested with *Eco*RI. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

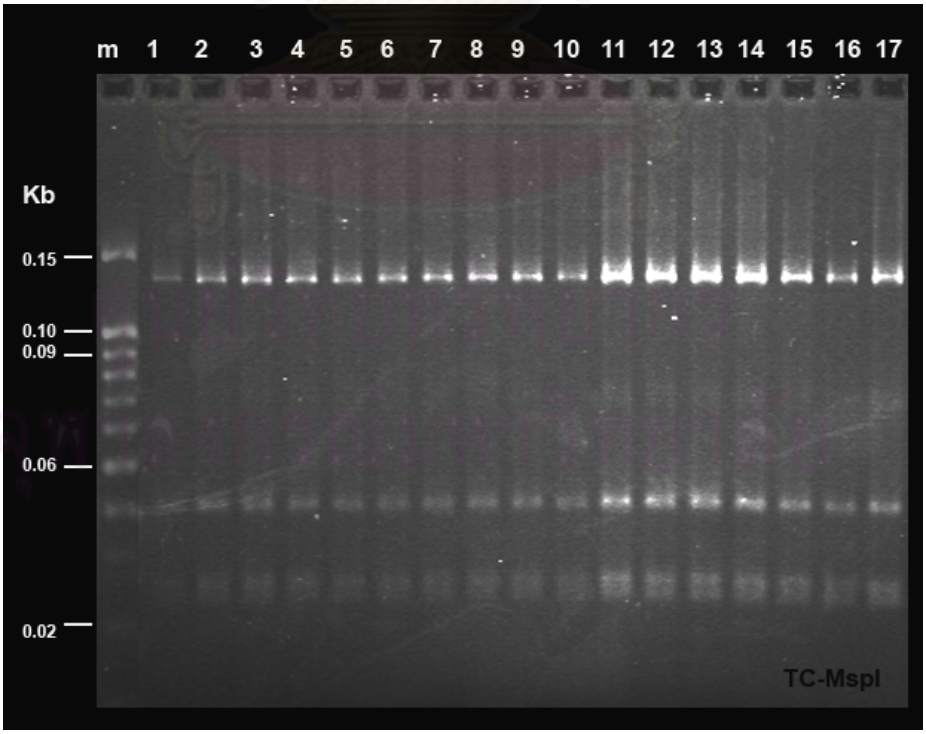

Figure 41. PCR-RFLP in TC region of cpDNA of "*Hoya siamica* complex" digested with *Msp*I. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

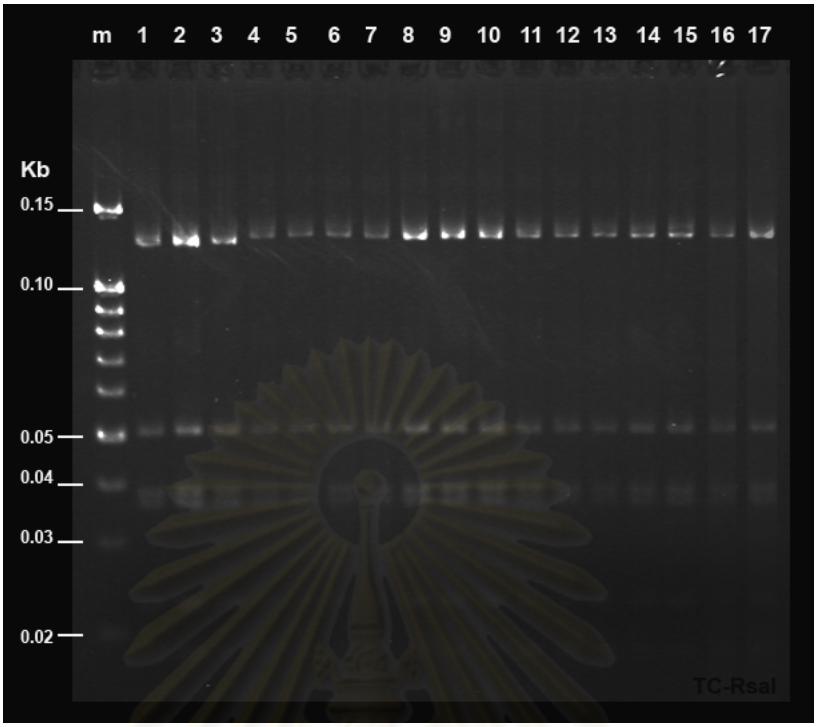

Figure 42. PCR-RFLP in TC region of cpDNA of "*Hoya siamica* complex" digested with *Rsa*I. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5)

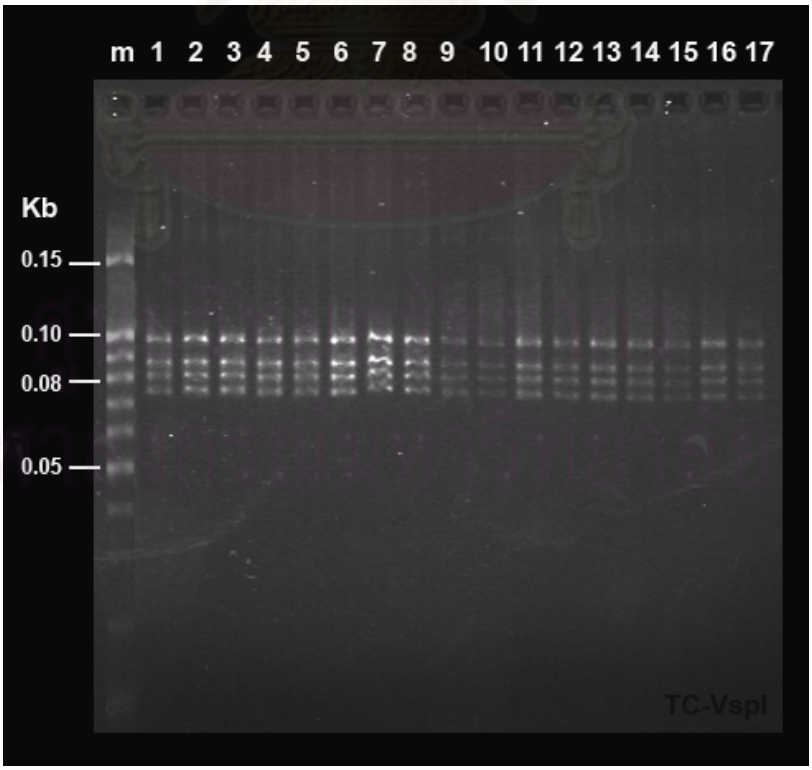

Figure 43. PCR-RFLP in TC region of cpDNA of "*Hoya siamica* complex" digested with *Vsp*I. Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

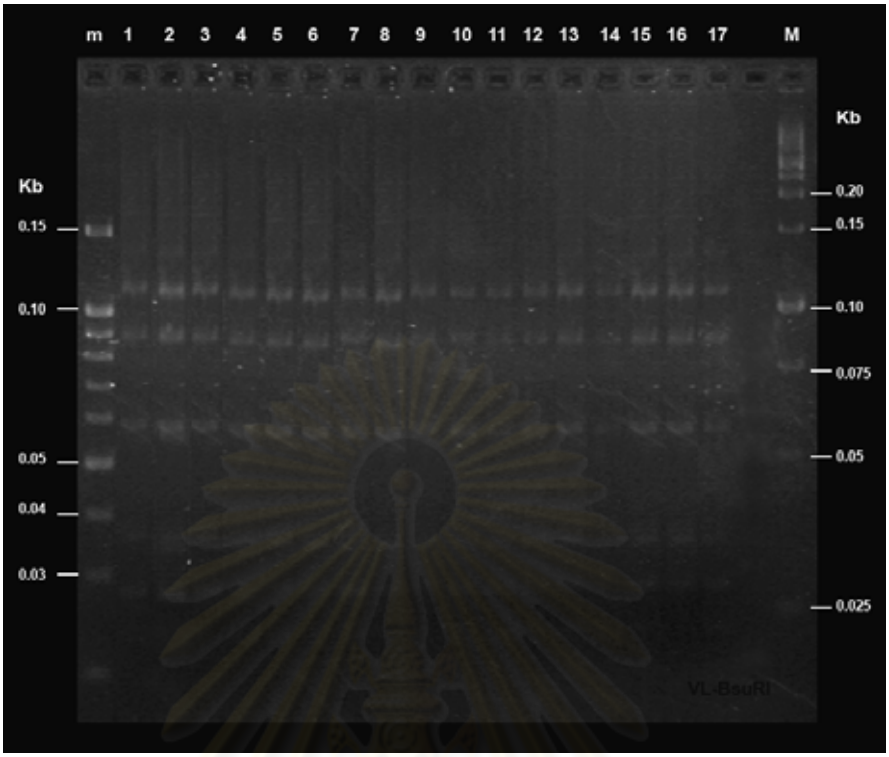

Figure 44. PCR-RFLP in VL region of cpDNA of "*Hoya siamica* complex" digested with *Bsu*RI. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

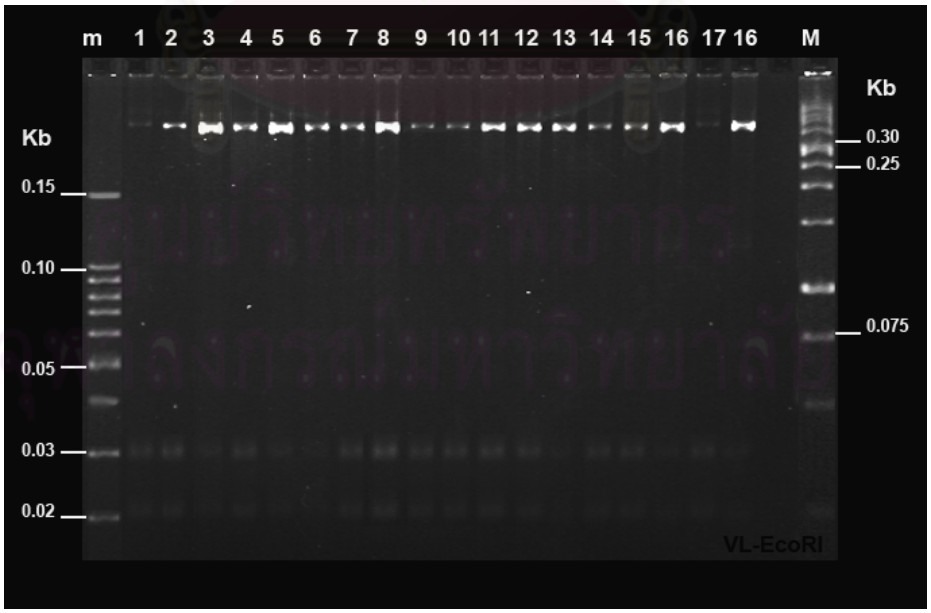

Figure 45. PCR-RFLP in VL region of cpDNA of "*Hoya siamica* complex" digested with *Eco*RI. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

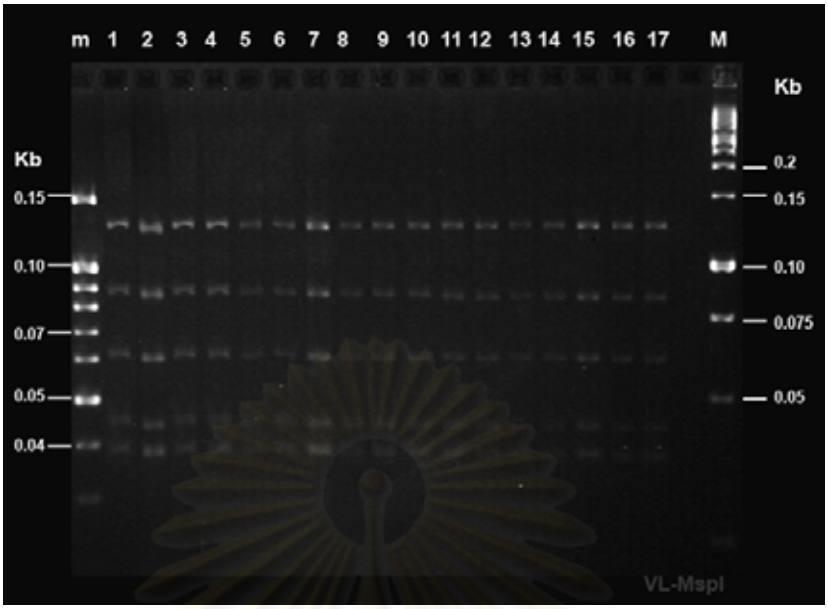

Figure 46. PCR-RFLP in VL region of cpDNA of "*Hoya siamica* complex" digested with *Msp*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

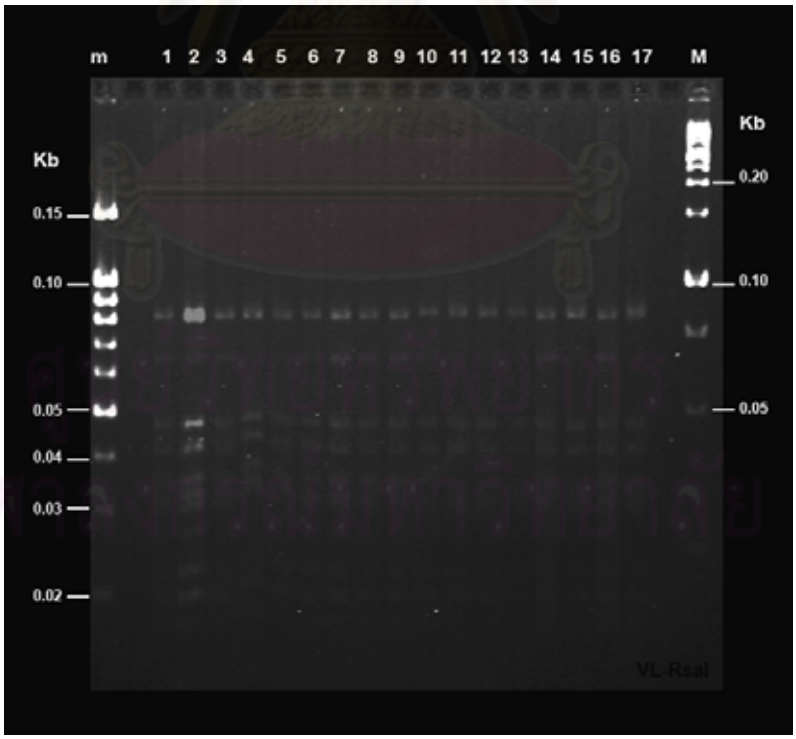

Figure 47. PCR-RFLP in VL region of cpDNA of "*Hoya siamica* complex" digested with *Rsa*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

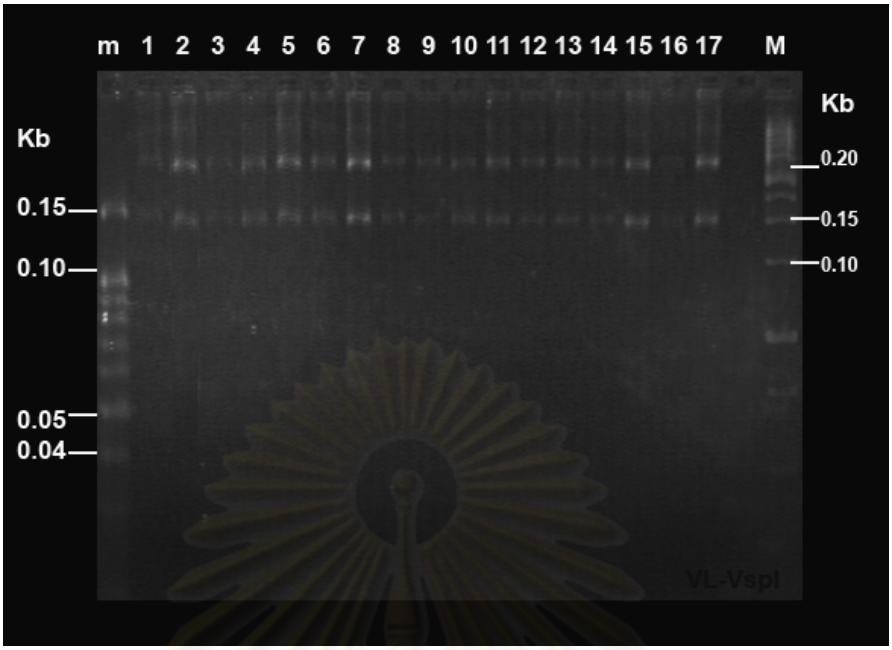

Figure 48. PCR-RFLP in VL region of cpDNA of "*Hoya siamica* complex" digested with *Vsp*I. Lane M: 1 kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-17: Samples of *H. siamica* complex individuals (Table5).

Discussion

In this study, the PCR products from five different chloroplast DNA regions were digested by five restriction enzymes. The result showed that there were no different between specimens of *H. siamica* complex in Thailand. All PCR products-enzyme combinations showed the monomorphic patterns (Table 10 and Figures 24-48). Consequently, the five intergenic-spacers (HK, K_1K_2, C_1C, TC, and VL) of chloroplast DNA of *H. siamica* complex in Thailand are low variation, when study by RFLP technique using five restriction enzymes (*Bsu*I, *Eco*RI, *Msp*I, *Rsa*I and *Vsp*I).

Nevertheless, I suggest that further study should be carried out by using more primer pairs of cpDNA regions and more restriction endonuclease so as to increase probability of cleavage and restriction site. Moreover, other powerful techniques should be used such as, DNA sequencing technique, which can present all sequence of the specimens, or acrylamide gel technique, which can detect a small size DNA fragments (smaller size than 100 bp) and separate the bands of fragments better than agarose gel.

CHEPTER V

GENERAL CONCLUSION

The *Hoya siamica* complex is highly variable species not only in vegetative but also reproductive characters (Craib, 1911; Kerr, 1951; Ping-tao, *et al*., 1995) and has never been intensively studied for a long time. Accordingly, the original description (Craib, 1911) does not covered several characters of the recent *H. siamica* complex in Thailand. Hence, the taxonomic status of this complex is still uncertain.

This study shows that *H. siamica* complex in Thailand is an extremely variable species, which can be divided into five morphological forms called form I, II, III, IV and V based on qualitative morphological and palynological characters, i.e. leaves shape, conspicuous or obscure lateral veins, corona shape, outer angle of corona, pollinium and corpusculum shape. Moreover, the members of the complex occur in different habitats, form I grow in open area on rock or tree, in evergreen forest about 940-1150 m. elevations. From II-V grow in shady area, on rock or on tree's branches or stem in evergreen forest about 1100-2295 m. elevations. These qualitative morphological data shows that form I is obviously different from the others by its obscure lateral veins and broadly ovate corpusculum shape. Furthermore, the results from the quantitative morphological, palynological and anatomical characters supports that *H. siamica* complex in Thailand can be divided in to two groups, the first group composed of form I and the second group composed of form II-V. The important characters, which are used to separate the two groups are pollinium width and corpusculum width h. In addition, the anatomical studies support both qualitative and quantitative morphological and palynological studies that morphological form I differ from the other forms by their mesophyll can be obviously divided into two layers, dense and sparse parenchyma cells, whereas morphological form II-IV cannot be divided. The result from PCR-RFLP technique of 5 different chloroplast DNA regions reveals no difference between this complex species.

The results of morphological, palynological and anatomical studies indicate that *H. siamica* complex in Thailand have high morphological, palynological and anatomical variations. These variations should not be the results of infraspecific variations of a single species, *H. siamica*. Hence, the complex should be segregated into species, or variety. However, there is no universal definition of the species concept. Generally, a species should be separated by obvious morphological differences from the other closely related species and this difference related to adaptation to different geographical areas or environment and usually distinct ecological habitats, for the varieties, they are recognizable by morphological variations within species (Davis and Gilmartin, 1985). In this study, vegetative and reproductive characters were used to define a species. Therefore, the five form of *H. siamica* complex are best treated as *H. siamica* together with a variety. Form I of the *H. siamica* complex is ranked in variety level, due to their great variation in morphological, palynological and anatomical characters. In additional, the close relationships among forms II-V of the *H. siamica* complex, as presented in this study, indicates that they are a variable form of a single species of *H. siamica.*

In conclusion, the investigation of morphological, palynological, anatomical and molecular data of the *H. siamica* complex in Thailand shows that this complex composed of *H. siamica* and its variety.

Last but not least, this study shows the great variations of *H. siamica* complex in Thailand. However, this study is the first report of the intensive taxonomic study, which covers the morphological, anatomical, palynological and molecular data of this complex. The results of this research served as a basis knowledge for the further applied studies.

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APPENDIX

Table 3. Canonical discriminant functions of 5 morphological forms based on 45 vegetative and reproductive characters.

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	21.965[a]	68.4	68.4	.978
2	7.501[a]	23.4	91.8	.939
3	2.343[a]	7.3	99.1	.837
4	.305[a]	.9	100.0	.483

a. First 4 canonical discriminant functions were used in the analysis.

Table 4. Poolled within canonical structure of 5 morphological forms based on 45 vegetative and reproductive characters

Structure Matrix

	Function			
	1	2	3	4
SQRTC38	.620*	-.317	-.054	.411
SQRTC35[a]	.380*	-.066	.182	-.177
SQRTC37[a]	.342*	-.044	.019	.148
SQRTC27[a]	-.335*	.083	-.042	-.088
SQRTC6[a]	.282*	.219	.085	.201
SQRTC29[a]	.262*	.048	.212	.120
SQRTC11[a]	-.260*	-.080	.087	.010
SQRTC30[a]	-.255*	.037	-.117	.020
SQRTC10[a]	-.197*	-.077	.042	.028
SQRTC22[a]	-.179*	.099	-.105	-.125
SQRTC32[a]	.160*	-.082	.034	-.134
SQRTC17[a]	-.130*	.039	.042	.054
SQRTC7[a]	-.111*	.015	.045	-.009
SQRTC1	-.449	-.620*	.423	-.064
SQRTC21	-.332	.418*	-.331	.327
SQRTC4[a]	.018	.294*	.251	.187
SQRTC8[a]	.233	.277*	.187	.109
SQRTC14[a]	.225	-.261*	.136	.168
SQRTC39[a]	.042	.240*	.163	-.006
SQRTC24[a]	.042	-.213*	.082	-.182
SQRTC42[a]	.002	-.191*	-.155	-.144
SQRTC28	-.116	.019	.440*	-.223
SQRTC3[a]	-.099	.137	.376*	.183
SQRTC2[a]	-.246	-.190	.246*	-.142
SQRTC26	-.035	.333	.165	.593*
SQRTC40[a]	-.030	.021	.001	-.433*
SQRTC25[a]	-.070	-.070	-.117	-.385*
SQRTC31[a]	.022	-.115	.034	-.362*
SQRTC41	-.075	.120	.187	-.355*

	Function			
	1	2	3	4
SQRTC23[a]	.060	-.206	.085	-.347*
SQRTC44[a]	-.038	.257	.032	.331*
SQRTC20[a]	-.096	-.025	-.065	.325*
SQRTC36[a]	.194	.062	.070	.303*
SQRTC5[a]	.092	.142	-.004	.288*
SQRTC9[a]	-.169	-.254	.090	-.275*
SQRTC43[a]	.017	.261	-.032	.274*
SQRTC34[a]	-.019	-.078	.017	.259*
SQRTC15[a]	-.066	.079	-.070	.229*
SQRTC33[a]	-.174	.160	.006	-.208*
SQRTC19[a]	-.034	.092	-.012	.190*
SQRTC13[a]	-.014	-.060	-.156	-.183*
SQRTC12[a]	-.068	-.014	-.036	-.164*
SQRTC45[a]	.133	-.101	-.036	-.161*
SQRTC18[a]	.023	.091	-.105	-.137*
SQRTC16[a]	-.025	.112	-.043	.115*

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions
Variables ordered by absolute size of correlation within function.

*. Largest absolute correlation between each variable and any discriminant function

a. This variable not used in the analysis.

BIOGRAPHY

Miss Duangjai Tungmunnithum was born on October 24, 1985 at Suphanburi province. Since 2006–present, she has got the scholarships from Development and Promotion of Science and Technology Talent Project (DPST) of the Institute for the Promotion of Teaching Science and Technology. She graduated in Bachelor of Science with Second Class Honors from the Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand in 2009. Then, she continued her study in Master of Science in Botany Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand from 2009-2011.