การวิเคราะห์ลำดับนิวคลีโอไทด์ของยีนแม็ทเคของกวางเครือขาว และกวางเครือแดง และการประยุกต์ใช้เป็นเครื่องหมายพันธุกรรมโดยเทคนิคพีซีอาร์-อาร์เอฟแอลพีเพื่อการพิสูจน์เอกลักษณ์

นางสาว วรลักษณ์ ยอดเพชร

ศูนย์วิทยทรัพยากร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเวท ภาควิชาเภสัชเวท
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ปีการศึกษา 2550

จุฬาลงกรณ์มหาวิทยาลัย
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

SEQUENCE ANALYSIS OF *mat*K GENE OF *PUERARIA CANDOLLEI* AND *BUTEA SUPERBA* AND THE APPLICATION OF PCR-RFLP GENETIC MARKER FOR IDENTIFICATION

Miss Woraluk Yodpetch

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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จุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	SEQUENCE ANALYSIS OF *mat*K GENE OF *PUERARIA CANDOLLEI* AND *BUTEA SUPERBA* AND THE APPLICATION OF PCR-RFLP GENETIC MARKER FOR IDENTIFICATION
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วรลักษณ์ ยอดเพชร: การวิเคราะห์ลำดับนิวคลีโอไทด์ของยีนแม็ทเคของกวาวเครือขาว และกวาวเครือแดง และการประยุกต์ใช้เป็นเครื่องหมายพันธุกรรมโดยเทคนิคพีซีอาร์-อาร์เอฟแอลพีเพื่อการพิสูจน์เอกลักษณ์. (SEQUENCE ANALYSIS OF *mat*K GENE OF *PUERARIA CANDOLLEI* AND *BUTEA SUPERBA* AND THE APPLICATION OF PCR-RFLP GENETIC MARKER FOR IDENTIFICATION) อ.ที่ปรึกษา : ผศ. ร.ต.อ. หญิง ดร. สุชาดา สุขหร่อง, อ.ที่ปรึกษาร่วม : รศ. ธาตรี ผดุงเจริญ, 102 หน้า.

การพิสูจน์เอกลักษณ์และควบคุมคุณภาพพืชสมุนไพรมีความจำเป็นอย่างยิ่งที่จะต้องทำเป็นอันดับแรก เพื่อให้เกิดความมั่นใจในคุณภาพ ความปลอดภัยและประสิทธิผลของยาจากสมุนไพร วัตถุประสงค์ของงานวิจัยชิ้นนี้ คือ เพื่อศึกษาลำดับนิวคลีโอไทด์ของยีนแม็ทเคของกวาวเครือขาว [(*Pueraria candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham และ *Pueraria candollei* Graham ex Benth. var. *candollei*)] และกวาวเครือแดง Red Kwao Khruea (*Butea superba* Roxb.) และนำข้อมูลที่ได้ไปจัดความสัมพันธ์ทางวงศ์วานวิวัฒนาการ และประยุกต์เครื่องหมายทางพันธุกรรมพีซีอาร์-อาร์เอฟแอลพี (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) เพื่อใช้เป็นเครื่องมือที่สะดวกต่อการพิสูจน์เอกลักษณ์

ผลการวิจัยพบว่า ลำดับนิวคลีโอไทด์ของยีนแม็ทเคของกวาวเครือขาวมีความยาว 1,521 คู่เบส ในขณะที่กวาวเครือแดงมีความยาว 1,527 คู่เบส ซึ่งเป็นผลจากการที่มีการสอดแทรกหรือหายไปของเบส 6 คู่เบส จากการศึกษาตัวอย่างกวาวเครือขาว 10 ต้น พบว่ามีตำแหน่งเบสที่ถูกแทนที่ทั้งหมด 5 ตำแหน่ง และเมื่อเปรียบเทียบลำดับนิวคลีโอไทด์ระหว่างกวาวเครือขาวและกวาวเครือแดงทั้งหมด 14 ต้น พบว่ามีตำแหน่งเบสที่ถูกแทนที่ทั้งหมด 83 ตำแหน่ง จากการวิเคราะห์ความสัมพันธ์ทางวงศ์วานวิวัฒนาการที่สร้างโดยวิธี parsimony analysis พบว่ากวาวเครือขาวถูกแบ่งเป็นสองกลุ่มและถูกแบ่งแยกออกมาจากกลุ่มของกวาวเครือแดง

จากข้อมูลความแตกต่างกันระหว่างลำดับนิวคลีโอไทด์ของกวาวเครือทั้งสองชนิด สามารถนำมาสร้างเครื่องหมายทางพันธุกรรมพีซีอาร์-อาร์เอฟแอลพีและพบว่าผลจากการตัดด้วยเอนไซม์ตัดจำเพาะ *Eco*RI และ *Dde*I สามารถใช้ในการแยกความแตกต่างระหว่างกวาวเครือขาวและกวาวเครือแดงได้

ผลการวิจัยแสดงให้เห็นว่าข้อมูลลำดับนิวคลีโอไทด์ของยีนแม็ทเคของกวาวเครือขาวและกวาวเครือแดง สามารถนำมาใช้หาความสัมพันธ์ทางวงศ์วานวิวัฒนาการระหว่างพืชทั้งสองชนิดได้ และเครื่องหมายทางพันธุกรรมพีซีอาร์-อาร์เอฟแอลพีที่พัฒนาขึ้นมานี้สามารถนำไปใช้ในการพิสูจน์เอกลักษณ์กวาวเครือทั้งสองชนิดและยังสามารถประยุกต์ใช้กับผลิตภัณฑ์กวาวเครือที่วางขายตามท้องตลาดได้อีกด้วย

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จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา.....เภสัชเวท.....ลายมือชื่อนิสิต.....วรลักษณ์ ยอดเพชร.....

สาขาวิชา.....เภสัชเวท.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4876601633 : MAJOR PHARMACOGNOSY
KEY WORD : *PUERARIA CANDOLLEI* / *BUTEA SUPERBA* / *mat*K GENE / GENETIC MARKER / PCR-RFLP / IDENTIFICATION

WORALUK YODPETCH: SEQUENCE ANALYSIS OF *mat*K GENE OF *PUERARIA CANDOLLEI* AND *BUTEA SUPERBA* AND THE APPLICATION OF PCR-RFLP GENETIC MARKER FOR IDENTIFICATION
THESIS ADVISOR : ASSIST. PROF. SUCHADA SUKRONG, Ph.D.,
THESIS COADVISOR : ASSOC. PROF. THATREE PADUNGCHAROEN, 102pp.

The accurate identification and quality control of plant material is, therefore, an essential prerequisite for ensuring the quality, safety, and efficacy of herbal medicines. The purpose of this study was to examine the *mat*K gene sequences of White Kwao Khruea [(*Pueraria candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham and *Pueraria candollei* Graham ex Benth. var. *candollei*)] and Red Kwao Khruea (*Butea superba* Roxb.) and used the results for their classification and phylogenetic studies, as well as developed PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) method in order to use as a convenient tool for identification.

The complete *mat*K gene of White Kwao Khruea was 1,521 bp in length, whereas that in Red Kwao Khruea was found to be 1,527 bp due to a 6-bp indels (insertions or deletions). Five sites of nucleotide substitutions were detected in 10 specimens of White Kwao Khruea. A total of 83 sites of substitutions were observed in 14 specimens of Both Kwao Khruea. Their phylogenetic analysis using parsimony analysis showed that the specimens of White Kwao Khruea were divided into two clades and were separated from Red Kwao Khruea.

Based on the differences among the sequences, the PCR-RFLP analysis was performed. The restriction patterns of DNA amplified from partial *mat*K gene with two restriction enzymes, *Eco*RI and *Dde*I showed distinct and polymorphic fingerprints between White Kwao Khruea and Red Kwao Khruea.

These results suggest that the *mat*K gene sequences of White Kwao Khruea and Red Kwao Khruea can be used to study of phylogenetic relationships. Moreover, PCR-RFLP genetic markers developed here can be used as a convenient tool for identification of both types of Kwao Khruea and can be applied to their commercial products.

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Department.....Pharmacognosy..... Student's signature.....
Field of study.....Pharmacognosy.....Advisor's signature.....
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 student.

My deepest gratitude and appreciation is also expressed to Assoc. Prof. Thatree Padungcharoen, my thesis co-advisor, for her great helps, guidance, valuable suggestions, and kindness in collecting specimens.

I would like to express heartfelt respect and sincere gratitude to Assoc. Prof. Dr. Kittisak Likhitwitayawuid, Assoc. Prof. Dr. Chaiyo Chaichantipyuth, and Dr. Piyarat Chareonsap, for serving on my thesis committee, helpful discussion, and valuable suggestions for improvements.

I would like to special thanks the many people who have supported the specimens: Assoc. Prof. Dr. Chaiyo Chaichantipyuth for his kindness in collecting specimens. My special thanks are also extended to Dr. Charan Ditchaiwong for his kindness, great helps in collecting specimens, and providing valuable data for my thesis.

I am especially thanks to Miss Juthatip Manissorn 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. Especially, I am obliged to my best friend, Miss Jiranuch Mingmuang, who was always available for help, chats, fun, and everything.

Finally, and most important, I take deepest pleasure in thanking my parents, my grand-fathers, grand-mothers, my sisters and 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
AFLP	Amplified fragment length polymorphism
AP-PCR	Arbitrarily primed PCR
A, T, G, C	Nucleotides containing the base adenine, thymine, guanine and cytosine, respectively
bp	Base pairs
BSU	BioService Unit
°C	Degree Celcius
CI	Consistency index
cm	Centimeter
cpDNA	Chloroplast DNA
DAF	DNA amplification fingerprinting
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
ITS-1	Internal transcribed spacer 1
ITS-2	Internal transcribed spacer 2
kb	Kilobase
KCl	Potassium chloride
LS	Least squares
*mat*K gene	Gene encoding maturase K
ME	Minimum evolution
$MgCl_2$	Magnesium chloride
ML	Maximum likelihood

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ml	Milliliter
mM	Millimolar
MP	Maximum parsimony
mtDNA	Mitochondial DNA
*ndh*F gene	Gene encoding NADH dehydrogenase F
nDNA	Nuclear DNA
ng	Nanogram
NJ	Neighbor joining
PAA	Polyacrylamide
PAUP	Phylogenetic analysis using parsimony
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction- Restriction fragment length polymorphism
RAPD	Random amplified polymorphic DNA
*rbc*L gene	Gene encoding the large subunit of the ribulose-bisphosphate carboxylase
RFLP	Restriction fragment length polymorphism
RI	Retention index
RNA	Ribonucleic acid
TAE	Tris acetate EDTA
Tris	Tris (hydroxymethyl) aminomethane
$tRNA^{Lys}$	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

The name "Kwao Khruea (กวาวเครือ)" is commonly applied to more than ten different plants in different genera. At least three types of Kwao Khruea are commonly known. They are White Kwao Khruea (กวาวเครือขาว), Red Kwao Khruea (กวาวเครือแดง), and Black Kwao Khruea (กวาวเครือดำ), all of which were recorded in Luang Anusan's pamphlet (Suntara, 1931). The tuberous roots of them have long been used in traditional Thai herbal medicine for rejuvenation, promotion of human physical appearance such as breasts enhancing, regrowing hair on the bald and turning the hair from white to black as well as improving complexion in both women and men (Suntara, 1931). In the northern part of Thailand, the powder derived from their tuberous roots was consumed for rejuvenating and aphrodisiac purposes (Wanandorn, 1933). When Kwao Khruea is mixed with honey, it could be used as a general tonic or nourishment (Nilanidhi *et al.*, 1957). Recently, White and Red Kwao Khruea are becoming of great interest for commercialization development.

White Kwao Khruea or *Pueraria candollei* Graham ex Benth. belongs to the family Leguminosae, subfamily Papilionoideae, tribe Phaseoleae (Ridley, 1967; Suvatti, 1978). It was first discovered and identified by Vatna in 1939 as *Butea superba* Roxb. because of their superficial resemblance (Bounds and Pope, 1960). There are two varieties of *P. candollei* found in Thailand, *P. candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham and *P. candollei* Graham ex Benth. var. *candollei* (Smitinand, 2001; Srijugawan and Ditchaiwong, 2005). Prathanturarug *et al.* (2000) found that *P. candollei* Graham ex Benth. var. *candollei* was also used as a source of White Kwao Khruea by Thai herbalists. Vegetative botanical characters of both varieties were almost the same, only the pods were different. Other names of White Kwao Khruea are Kwao Khruea (กวาวเครือ), Khruea khao pu (เครือขาวปู), and Talan Khruea (ตาลานเครือ) (Smitinand, 2001). There are some differences in chemical components between the two varieties of *P. candollei* as indicated by TLC fingerprinting (Prathanturarug *et al.*, 2000). The tuberous roots of White Kwao Khruea have been analyzed by chromatography technique and found to

produce many chemical substances with estrogenic activities such as miroestrol, deoxymiroestrol, daidezein, genistein, coumestran, puerarin, kwakhurin, mirificin, isoflavonoid, *etc*. (Bounds and Pope, 1960; Cain, 1960; Kashemsanta *et al*., 1963; Ingham *et al*., 1986a, 1986b, 1988, 1989; Tahara *et al*., 1987; Chansakaow *et al*., 2000a, 2000b).

Red Kwao Khruea or *Butea superba* Roxb. also belongs to the family Leguminosae, subfamily Papilionoideae, tribe Phaseoleae (Ridley, 1967; Suvatti, 1978). Several local names are used in various parts of Thailand such as Kwao Khruea (กวาวเครือ), Chan Khruea (จานเครือ), Tan Chom Thong (ตานจอมทอง), Thong Khruea (ทองเครือ), Pho-ta-ku (โพ้ะตุ), and Pho-mue (โพมือ) (Smitinand, 2001). The tuber and stem of plant have been used in traditional medicine and believed to promote strength and male potency (Suntara, 1931). The plant preparation appears to improve an erectile function in patients with erectile dysfunction (Cherdshewasart and Nimsakul, 2003). The tuberous roots of Red Kwao Khruea contained five groups of chemical constituents namely; carboxylic acids, steroids, steroid glycosides, flavonoids, and flavonoid glycosides (Roengsamran *et al*., 2000).

In recent years, White and Red Kwao Khruea draw a lot of interest from the public. Many products of them in the forms of cream, capsule, tablet, and solution have been developed for both domestic consumption and exportation, and widely used as an age rejuvenation drug as well as cosmetics. Examples are breast enlargement creams, skin moisturizers, shampoo, hair tonic, mask, and eye gels (Cherdshewasart, 2005).

The accurate identification and quality control of the plant material is, therefore, an essential prerequisite for ensuring the quality, safety, and efficacy of the tuberous roots of White and Red Kwao Khruea and other herbal medicines. In addition, authentication of herbal plants is also an extremely important prerequisite for chemical and pharmacological investigations of herbal medicines. General approaches to herbal identification depend on morphological, anatomical, and chemical analyses, but these characteristics are often affected by environmental and/or developmental factors during plant growth (Li *et al*., 1994). Nevertheless, the use of chromatographic techniques and marker compounds to standardize botanical

preparations has limitations because of the varied sources and chemical complexity of such preparations. In particular, many extrinsic factors such as methods of cultivation, harvesting, drying and storing may affect the ultimate chemical profile of a given herb. Moreover since many associated agents and bonding agents are mixed with herbal extracts at 90-100ºC for 2-4 hours, it is difficult to identify the plants involved in the final products (extracts, capsules, liquids) by TLC (Shim *et al.*, 2005). On the other hand, medicinal plants are processed for use as crude drugs. Through these processes, some morphological and anatomical characteristics and some chemical constituents are changed. Therefore, it is difficult to determine the botanical origins of crude drugs through anatomical and chemotaxonomical studies.

DNA based polymorphism assay may offer an alternative method to identify herbal medicine. By using polymerase chain reaction (PCR) technology, nanogram quantities of DNA are required to amplify and yield sufficient amounts of template DNA for molecular genetic analysis (Cai *et al.*, 1999). The analysis of DNA has the advantages of being applicable to all parts of plants and not being affected by conditions of culture (Shim *et al.*, 2003). Furthermore, a number of recent studies have indicated that DNA markers are ideal tools for elucidating the molecular evolution and phylogeny of the species concerned, as well as for identifying crude herbal materials (Xue *et al.*, 2006). Medicinal products whose origins are often controversial, such as *Angelica gigas* root, *Saposhnikovia* root, *Coix* seed, *Citrus unshiu* peel, *Angelica decursiva* radix, *Magnolia* bark, *Polygonatum* rhizome, *Bupleurum* root, *Mitragyna* leaf, *Derris* root, *etc.* have been intensively studied and other herbal medicines have also been analyzed by DNA markers (Shim *et al.*, 2005; Sukrong *et al.*, 2005, 2007).

DNA sequencing has become one of the most utilized approaches for inferring taxonomic and phylogenetic relationships of animals and plants, and can be applied for identification of organisms. In plants, chloroplast DNA is considered to be suitable for analyzing the phylogenetic relationships among the species. The *mat*K gene, a chloroplast genome encoded locus located within the intron of the chloroplast gene *trn*K, encodes a maturase on the large single-copy section adjacent to the inverted repeat of every plant families, has high rates of substitution compared to other chloroplast genes and its DNA sequence is one of the least conserved plastid

genes; therefore, has been effectively used in plant evolution and address the phylogenetic questions in various taxonomic levels (Ito *et al.*, 1999; Fuse and Tamura, 2000; Ince *et al.*, 2005).

To date, there is no study which determines the *mat*K gene sequences of *P. candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham, *P. candollei* Graham ex Benth. var. *candollei*, and *B. superba* Roxb. In this study, the *mat*K gene sequences of these plants were examined and the results would be used for their classification and phylogenetic studies, as well as characterization for medical authentication. 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 method. In PCR-RFLP analysis, the restriction patterns of PCR product amplified from partial *mat*K gene with two restriction enzymes, *Eco*RI and *Dde*I showed distinct and polymorphic fingerprints between White and Red Kwao Khruea. Therefore, genetic markers developed here should aid the quality control and authentication the presence of White and Red Kwao Khruea DNA in crude drugs or even capsule preparations sold on the market.

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CHAPTER II

LITERATURE REVIEW

1. White Kwao Khruea

White Kwao Khruea (กวาวเครือขาว) belongs to the family Leguminosae, subfamily Papilionoideae, tribe Phaseoleae (Ridley, 1967; Suvatti, 1978). It was first discovered and identified by Vatna in 1939 as *Butea superba* Roxb. because of their superficial resemblance (Bounds and Pope, 1960). Later, in 1952, it was recognized as a new species and reidentified as *Pueraria mirifica* by Airy Shaw and Kasin Suvatabandhu (Kashemsanta *et al*., 1952). In 1990, Niyomdham reduced this species to a variety of *Pueraria candollei* Graham ex Benth., and reidentified as *Pueraria candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham (Niyomdham, 1992).

There are two varieties of *P. candollei* found in Thailand, i.e. var. *mirifica* and var. *candollei* (Smitinand, 2001; Srijugawan and Ditchaiwong, 2005). Prathanturarug *et al*. (2000) found that *P. candollei* Graham ex Benth. var. *candollei* was also used as a source of White Kwao Khruea by Thai herbalists. Vegetative botanical characters of both varieties were almost the same but pods were different. Other names of White Kwao Khruea are Kwao Khruea (กวาวเครือ), Khruea khao pu (เครือเขาปู), and Talan Khruea (ตาลานเครือ) (Smitinand, 2001). The plant is a long-living twinning wood. The leaves are pinnately trifoliate stipulate; terminal leaflet. The tuberous roots are varied in sizes and shapes depending on the environment in which they exist. The flowers are bluish-purple legume shaped. Flowering occurs during late January to early April. The length of the inflorescences of certain flowers is approximately 20-150 cm. The flower contains five sepals and the petals are one standard with two keels. The pods are slender typically short or elongate, with hairs (var. *mirifica*) or no hair (var. *candollei*), including 1-10 single seeds when fully mature and dry which turn into brown color (Smitasiri and Wungjai, 1986; Prathanturarug *et al*., 2000; Srijugawan and Ditchaiwong, 2005).

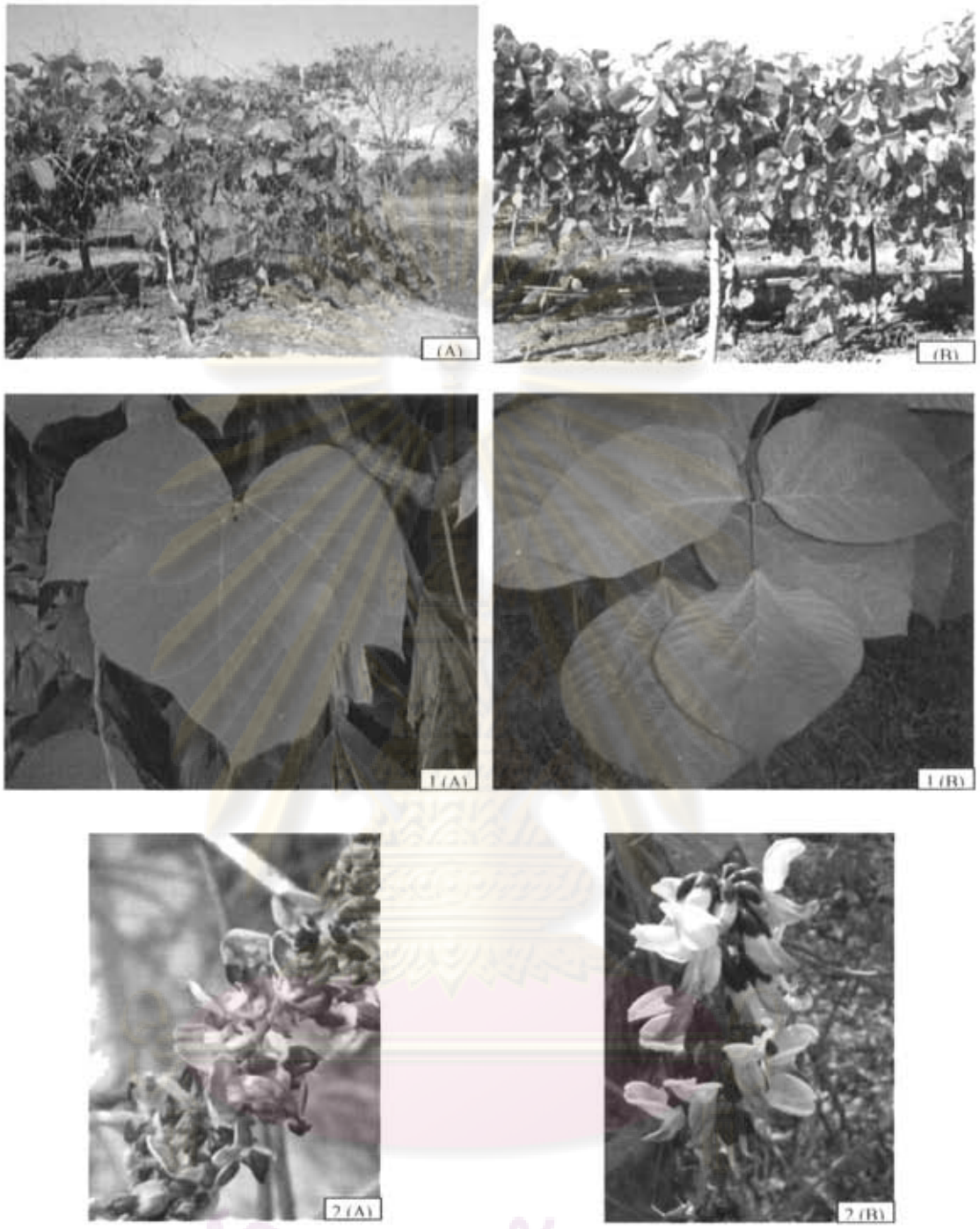

Figure 2.1 *Pueraria candollei* var. *mirifica* (A) and *P. candollei* var. *candollei* (B)
(1) Leaves (2) Flowers

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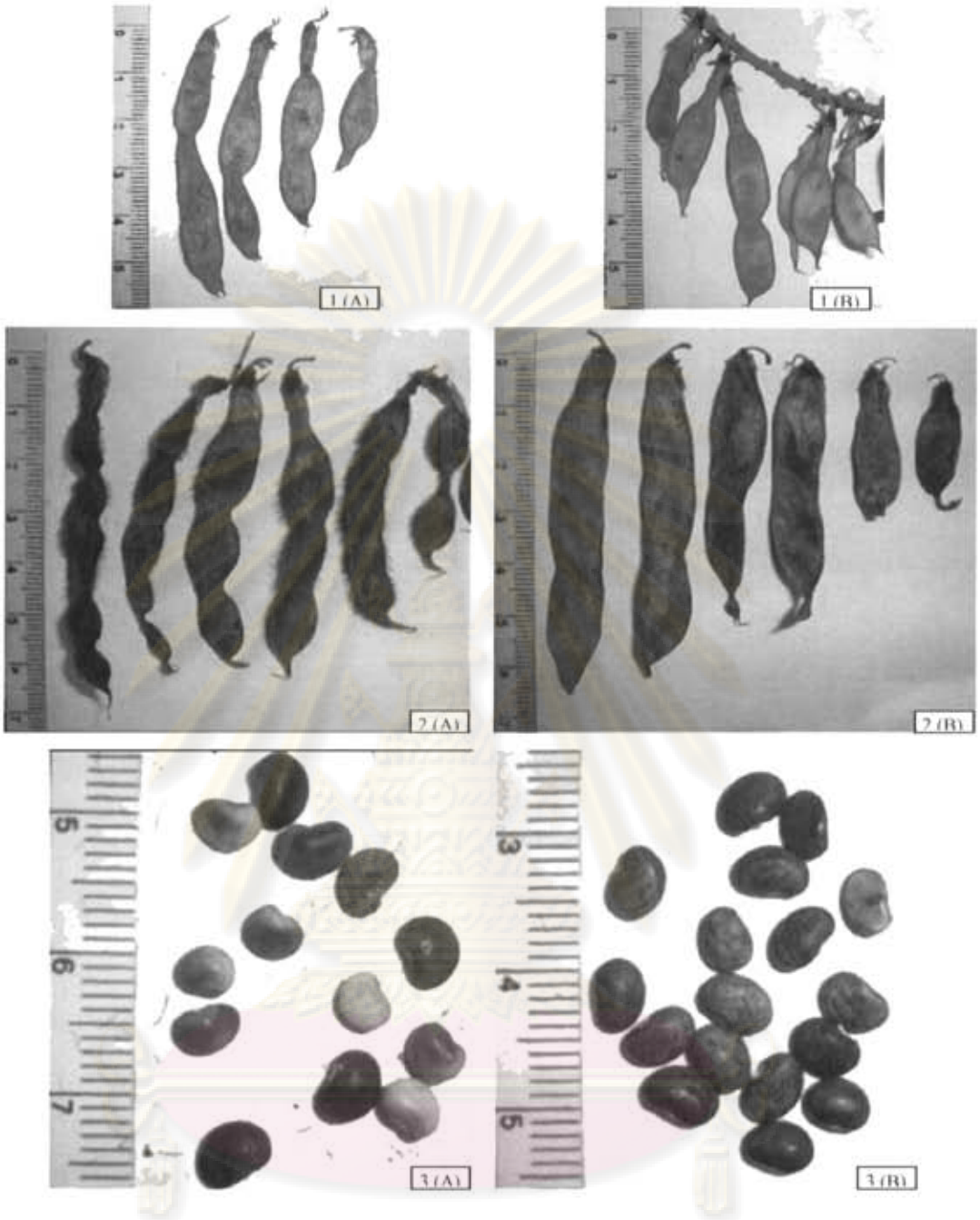

Figure 2.2 *Pueraria candollei* var. *mirifica* (A) and *P. candollei* var. *candollei* (B)
(1) Pods (2) Dried pods (3) Seeds

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Figure 2.3 Tuberous roots of *Pueraria candollei*

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2. Red Kwao Khruea

Red Kwao Khruea (กวาวเครือแดง) or *Butea superba* Roxb. belongs to the family Leguminosae, subfamily Papilionoideae, tribe Phaseoleae (Ridley, 1967; Suvatti, 1978). The local names are different in various parts of Thailand such as Kwao Khruea (กวาวเครือ), Chan Khruea (จานเครือ), Tan Chom Thong (ตานจอมทอง), Thong Khruea (ทองเครือ), Pho-ta-ku (โพ้ตะกู), and Pho-mue (โพมือ) (Smitinand, 2001). *B. superba* is a large size climber. The tuberous root is elongated with red sap released once cut. The leaves are pinnately trifoliate, acuminate leaflet and long leafstalk. The flowers are large with yellowish-orange color. The petals are three times longer than the calyx. The pods are 3-4 inches long, oblong shaped with silvery silky short hair (Kruz, 1877; Brandis, 1900; Aubréville and Leroy, 1979).

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Figure 2.4 *Butea superba* Roxb.

(1) Leaves (2) Flower (3) Tuberous roots (4) Pod

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3. Molecular Markers

Molecular markers have proved their utility in plant science such as taxonomy, physiology, forensic botany, and genetics. As the science of plant genetic progressed, researchers have tried to explore these molecular techniques for their application in commercially important plants such as food crops, horticultural plants and recently in pharmaceutical sciences for the characterization of herbal medicine. The DNA-based molecular markers can be categorized into 3 major techniques (Joshi *et al.*, 2004).

'Hybridization-based methods'

'PCR-based methods'

'Sequencing-based markers'

3.1 Hybridization-Based Methods

Hybridization-based methods including Restriction Fragment Length Polymorphisms (RFLP) are unequal lengths of DNA fragments obtained by cutting genomic DNA with restriction enzymes at specific sites. On an agarose gel, RFLP can be visualized using radiolabeled complementary DNA sequences. Polymorphisms are analyzed after hybridization by observing present or absent bands. There is no need for PCR amplification of DNA in this method. A routine southern blot experiment is used instead. Normally, RFLP is used to identify the origins of a particular plant species, setting the stage for mapping its evolution. There are some problems with the RFLP method of DNA fingerprinting. Firstly, the results do not specifically indicate the chance of a match between two organisms. Secondly, the process involves a lot of money and labor, which not many laboratories can afford. Finally, unlike the microsatellites, a few loci in the assay must suffice (Vasudevan, 2007).

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3.2 PCR-Based Markers

PCR-based methods are the amplification of DNA fragments *in vitro* using thermostable DNA polymerase and either random or specific primers. For example, Random Amplified Polymorphic DNA (RAPD), Arbitrarily Primed PCR (AP-PCR),

DNA Amplification Fingerprinting (DAF), Amplified Fragment Length Polymorphism (AFLP), and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) are well known methods.

(1) Random Amplified Polymorphic DNA (RAPD)

It is a type of PCR reaction using oligoprimers (8-12 nucleotides). The prior knowledge of the DNA sequences for the targeted gene is not required. The primers bind somewhere in the sequences as random amplification. The polymorphic bands are performed by agarose gel electrophoresis. RAPD technique was used as the species-specific markers. For instance, Sukrong *et al.* (2005) used this technique to distinguish five *Derris* species. Echeverrigaray *et al.* (2001) were successful to classify the thyme cultivars, Burpee, Blumen, Battle, SEM, Tropical and Isla by RAPD analysis and their essential oil composition. In addition, the chemical content and genomics of Italian garlic and rice were analyzed. The results of Italian garlic revealed the correlation between its chemicals and genetic materials (Brandolini *et al.*, 2005).

RAPD marker is widely used by many researchers, because the method is rapid and inexpensive, and does not require much genetic information. However, the disadvantage of this marker is reproducibility. The specimens should be replicated in the same and suitable condition to ensure the reproducible pattern (Atienzar and Jha, 2006).

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

PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) is one of the molecular markers. Another acronym of this marker technique is CAPS (Cleaved Amplified Polymorphic Sequences). 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 already result in differently sized 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 (Figure 2.3). 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. For example, *Eco*RI (named after the bacterium *Escherichia coli* from which it was isolated) acts like precise scalpel that cuts double-stranded DNA wherever the non-methylated sequence 5′-GAATTC-3′ occurs. *Dde*I (isolated from *E. coli* strain that carries the *Dde*I gene from *Desulfovibrio desulfuricans*) cuts double-stranded DNA wherever the non-methylated sequence 5′-CTNAG-3′ occurs. Several hundred such enzymes, most with different recognition sequences, have been isolated and characterized from various bacterial strains and are commercially available (Avise, 2004).

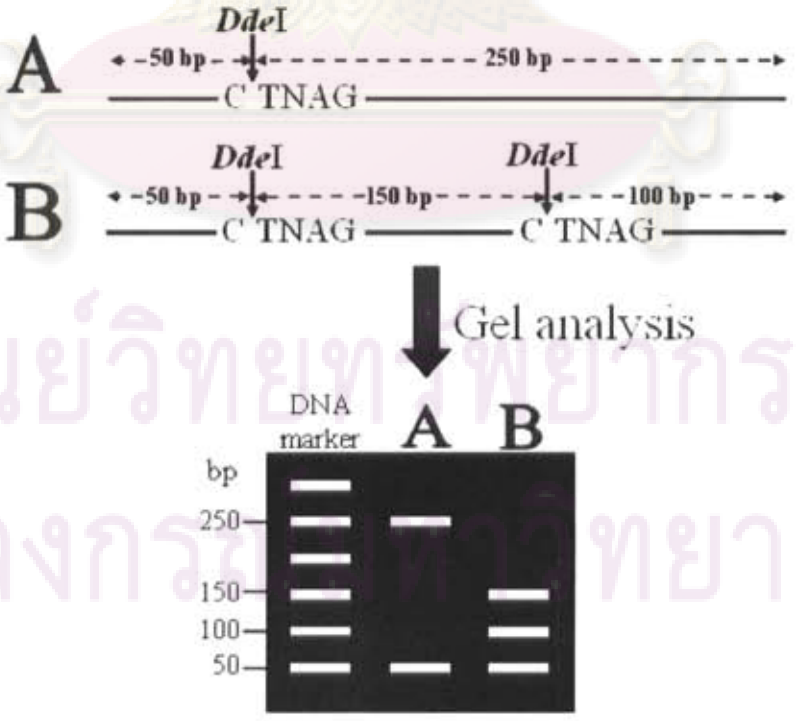

Figure 2.5 Principle of PCR-RFLP

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Several studies used PCR-RFLP analysis for investigation 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 *Bulbus Fritillariae cirrhosae* from other species of *Bulbus Fritillariae* antitussive herb in China. Liu *et al.* (2007) developed PCR-RFLP a rapid and reliable method to accurately identify hybrids of *Leucadendron*. Not only plants but in another organisms, PCR-RFLP analysis was used. For instance, Everett and Andersen (1999) used PCR-RFLP method to distinguish the nine bacteria *Chlamydiaceae* species for taxonomic, epidemiological and pathological studies and for improved diagnostic capability. Ratcliffe *et al.* (2003) used this method to identify the morphologically similar Diptera (flies) larvae that are important in forensic entomology for estimating the time and location of death. Ferreira *et al.* (2005) used PCR-RFLP as an identification tool for two closely related species of bats of genus *Platyrrhinus*. Zhang *et al.* (2007) used this method to distinguish species of red snappers (fishes of the genus *Lutjanus*) among commercial salted fish products. Abdel-Rahman and Ahmed (2007) used PCR-RFLP technique for the rapid, specific and sensitive identification of buffalo's, cattle's, and sheep's milk. Sasazaki *et al.* (2007) described the development of PCR-RFLP DNA markers to discriminate between Japanese and Australian beef.

(3) Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) technology was introduced by Zabeau, Vos, and coworkers (Zabeau and Vos, 1993; Vos *et al.*, 1995) and represents an ingenious combination of RFLP analysis and PCR. AFLP technology is applicable to all organisms without previous sequence information, and generally results in highly informative fingerprints. It rapidly became one of the most popular and powerful approaches to detect DNA polymorphisms. The AFLP reaction comprises two principal steps (Figure 1.4). In the first step, genomic DNA is digested with two different restriction enzymes producing sticky ends, and double-stranded synthetic adapters of a defined sequence are ligated to both ends of all restriction

fragments. Adapter and restriction site sequences then provide universal primer binding sites for subsequent PCR reactions that comprise the second step (Debener and Mattiesch, 1999).

Typically, two successive PCRs are performed on the restricted template, using specifically designed primers that allow only a subset of the restriction fragments to be amplified. To achieve this, the 5′-portions of the primers are made complementary to the adapters, whereas the 3′-ends extend by a few, arbitrarily chosen nucleotides (so-called selective bases or selective nucleotides) into the restriction fragment (Figure 2.4). Exact matching of the 3′-ends of a primer is essential for amplification. Therefore, only those restriction fragments are amplified in which the 3′-primer extensions match the sequences flanking the restriction sites. Statistically, each selective base added to one of the primers reduces the complexity of banding patterns fourfold. Thus, only 1/16 of the total set of fragments are amplified if there is one selective base on each side (+1 primers), 1/256 in case of two (+2 primers), and 1/4096 in case of three (+3 primers). A touchdown PCR program is used to maximize specificity. In the standard procedure described by Vos *et al.* (1995), one of the selective primers is radioactively labeled, the amplification products are separated on highly resolving sequencing gels, and banding patterns are visualized by autoradiography (Weising *et al*, 2005).

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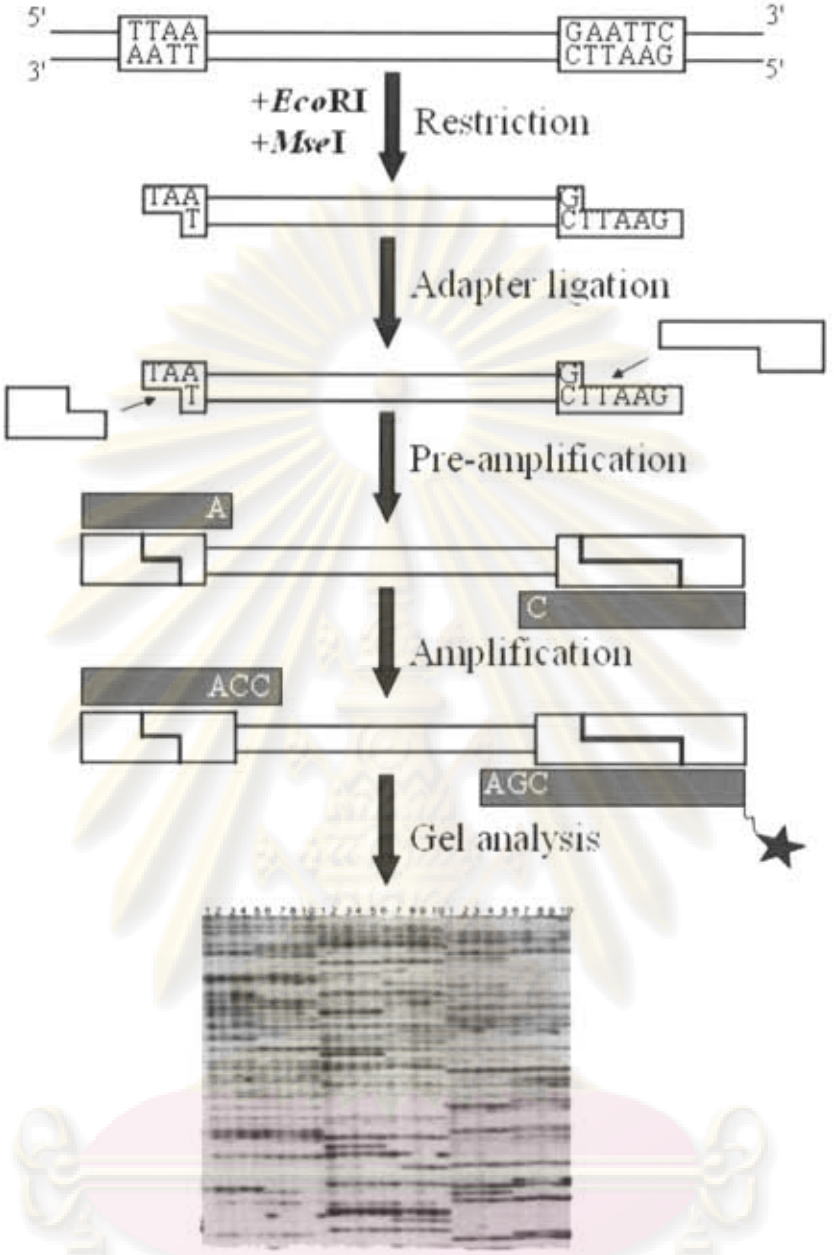

Figure 2.6 Principle of the AFLP strategy. Genomic DNA is digested with two restriction enzymes (here *Eco*RI and *Mse*I) and specific adapters are ligated to both ends of all resulting fragments. Two successive PCRs are then performed using specific primer pairs, of which the 5′-portions are complementary to the adapters and the restriction site, and the 3′-ends extend by one or a few selective bases into the interior of the restriction fragment. Usually one of the primers is labeled by a radioisotope or a fluorochrome (indicate by a star). Amplification products obtained by the second, selective PCR are separated on sequencing gels. (Redrew from Weising *et al*, 2005)

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3.3 Sequencing-Based Markers

The nucleotide sequencing is one of the most techniques to utilize the phylogenetic history. DNA sequence data are the power of informative tool for molecular systematics, and comparative analysis of DNA sequences is becoming increasingly important in plant systematics. There are two major reasons why nucleotide sequencing is useful in systematics of plants. First, the nucleotides are the basic units of information encoded in organisms. Second, the potential sizes of data sets are immense. 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. On the negative side, DNA sequencing can be prohibitively tedious and expensive when very large numbers of individuals have to be assayed (e.g., in population genetics, phylogeography, and marker-assisted plant breeding program) (Weising *et al*, 2005). Furthermore, different genes or parts of the genome might evolve at different rates. The selection of genes or any parts of genome depends on the taxonomic levels.

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. The nDNA and cpDNA are commonly able to investigate in the molecular systematics and taxonomy of plants. The nDNA is more complexity and repetitive properties. On the other hand, the cpDNA is well suitable for evolutionary and phylogenetic studies because cpDNA; 1) is a relative abundant component of total DNA, 2) contains primarily single copy genes, 3) has a conservative rate of nucleotide substitution. The most common genes in cpDNA are *rbc*L, *ndh*F, *trn*K/*mat*K gene, chloroplast ribosomal gene, etc. The most common genes in nDNA is nuclear ribosomal gene consisting of a transcribed region that comprises an external transcribed spacer (ETS), followed by 18s rDNA, an internal transcribed spacer (ITS-1), the 5.8s rDNA, a second internal transcribed spacer (ITS-2), and finally the 26s rDNA. Each repeat is separated from the next repeat by an intergenic spacer (IGS) (Soltis *et al*., 1995).

For instance, Zhao *et al.* (2003) and Xia *et al.* (2005) studied the sequences of 5s-rRNA spacer domain and assessed the chemicals of traditional Chinese medicine, *Angelica* (Danggui) and *Curcuma*, respectively. The *ndh*F gene and ITS sequences were used in durian identification for the investigation of phylogeny of core Durineae and related family (Nyffeler and Baum, 2000; Nyffeler and Baum, 2001). The sequences of ITS regions, ITS-1 and ITS-2 were used as DNA markers to characterize *Ceratinia siliqua*, *Cyamopsis tetragonoloba* (Urdiain *et al.*, 2004; Urdiain *et al.*, 2005), alfalfa and red clover (Lum *et al.*, 2005).

4. The *mat*K Gene

The *mat*K gene, formerly known as ORF-K (Open Reading Frame K), is emerging as yet another gene with potential contributions to plant molecular systematics and evolution (Johnson and Soltis, 1994, 1995; Hilu and Liang, 1997). The *mat*K gene is located in the Large Single-Copy region (LSC) of the chloroplast genome (Figure 2.5); it is approximately 1,500 base pairs (bp) in length and encodes a maturase involved in splicing type II introns from RNA transcripts (Neuhaus and Link, 1987; 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).

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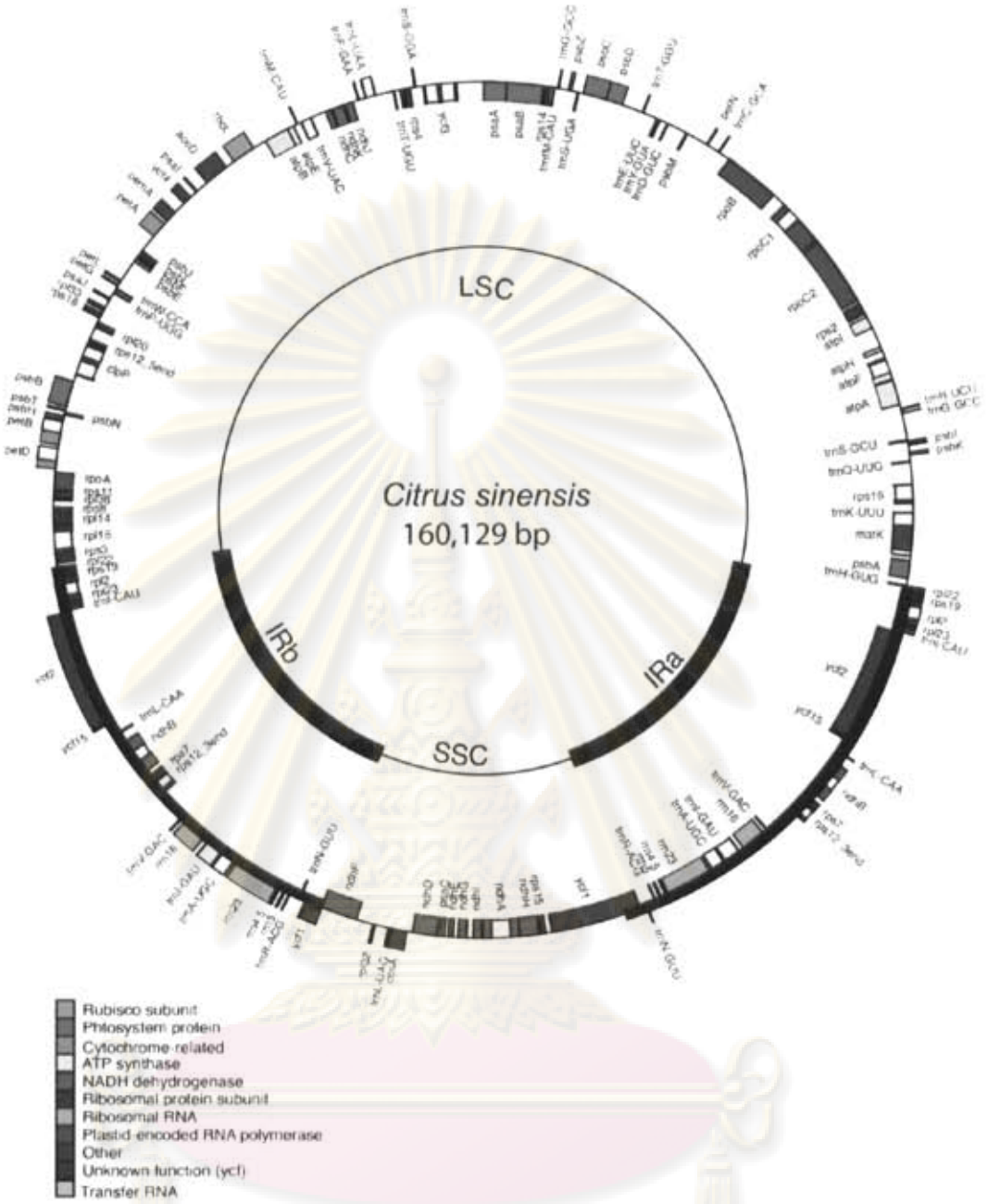

Figure 2.7 Gene map of *Citrus sinensis* (L.) chloroplast genome, illustrating location of many of the chloroplast regions. Genes located on the inside of the map are transcribed counterclockwise, and genes on the outside are transcribed clockwise. The inner circle shows where the Small Single-Copy region (SSC), Large Single-Copy region (LSC), and Inverted Repeats (IR) are located. The thick lines on the actual map are the IRs. (Available from http://www.biomedcentral.com/1471-2229/6/21)

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The gene *mat*K, as well as the noncoding regions that flank it, is easily amplified using the highly conserved flanking coding regions that include the *trn*K exons and the gene *rps*16 and *psb*A (Figure 2.6). 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. In Polemoniaceae, the number of nucleotide differences is on average 1.9 times greater for *mat*K than ITS. Sequences of *mat*K may therefore be informative at the generic and species levels (Johnson and Soltis, 1995) that can be seen clearly in Figure 2.7. In Polemoniaceae, Saxifragaceae and Cornaceae, the numbers of transitions and transversions are essentially identical; in contrast, *rbc*L is biased toward transitions (Johnson and Soltis, 1995; Xiang *et al.*, 1998).

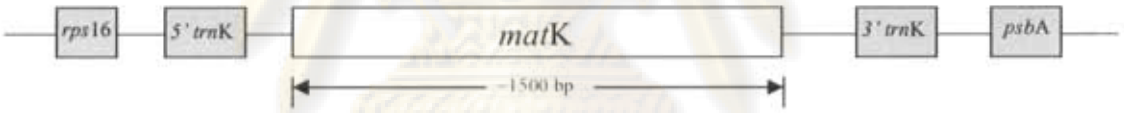

Figure 2.8 General map of *mat*K gene. Boxed areas represent coding regions and connecting lines represent spacer regions. (Redrawn from Johnson and Soltis, 1995)

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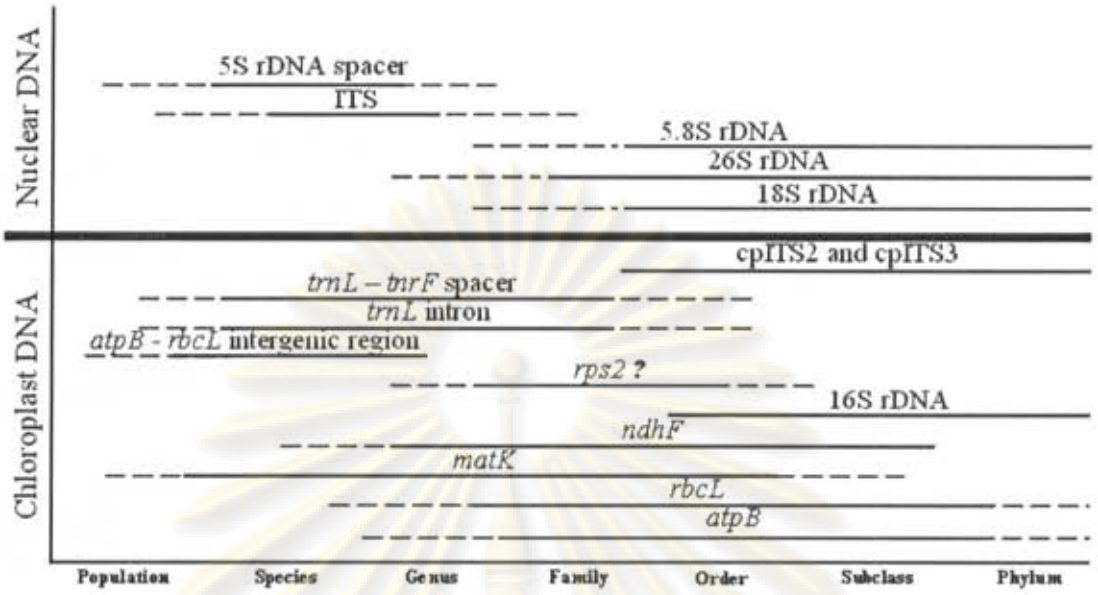

Figure 2.9 Approximate taxonomic level of utility of various chloroplast, nuclear, and mitochondrial genes and DNA regions used in phylogenetic reconstruction based on angiosperms. Question mark (?) refers to genes that have been rarely used; dashes (- - - -) designate the approximate upper or lower limits of applicability (i.e., the region may work at this level in some groups). (Modified from Johnson and Soltis, 1995)

Indels (insertions or deletions) are likely to be present in a *mat*K data matrix of any taxonomic breadth. All of the indels in the families noted are small, either 3, 6, or 9 bp. Hence, alignment of *mat*K sequences is easily accomplished by eyes. Although for any DNA region, the homology of indels may be difficult to ascertain, a posterior mapping of *mat*K indels onto trees based on nucleotide substitutions reveals that these indels are often parsimony-informative, providing additional support for monophyletic groups identified by base substitutions (Johnson and Soltis, 1995).

Well-resolved generic and specific-level phylogenies have been obtained using *mat*K sequences in many plant families such as Polemoniaceae (Johnson and Soltis, 1995; Johnson *et al.*, 1996), Saxifragaceae (Johnson and Soltis, 1995; Johnson *et al.*, 1996), Orchidaceae (Jarrell and Clegg, 1995), Poaceae (Liang and Hilu, 1996), Apiales (Plunkett *et al.*, 1997a, 1997b), Cornaceae (Xiang *et al.*, 1998), Leguminosae

(Hu *et al.*, 2000), Zingiberaceae (Zhu *et al.*, 2003), *etc.* In several angiosperm families, *rbc*L and *mat*K data have been combined, providing enhanced resolution and internal support compared to either gene alone (e.g., Soltis *et al.*, 1996; Plunkett *et al.*, 1997b; Xiang *et al.*, 1998; Tamura *et al.*, 2004).

5. The PCR Generation

Polymerase Chain Reaction (PCR)

PCR is based on the enzymatic *in vitro* amplification of DNA. Since the introduction of thermostable DNA polymerases in 1988 (Saiki *et al.*, 1998), the use of PCR in research and clinical laboratories has increased tremendously, and tens of thousands of publications as well as numerous books document the success of the technique (e.g., Innis *et al.*, 1990; Mullis *et al.*, 1994; 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 selectivity of the reaction is determined by the choice of the primer(s). Primers are single-stranded pieces of DNA (oligonucleotides) with sequence complementarity to template sequences flanking the targeted region. To allow for exponential amplification, the primers must anneal in opposite directions, so that their 3′-ends face the target amplicon. Amplification is most efficient when the two primer binding sites are not further apart than approximately 4 kb. However, amplification products of more than 10 kb can be obtained under optimal conditions (Cardle *et al.*, 2000).

The principle of the cycling reaction is outlined in Figure 2.8. 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 a template. 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 (short products in Figure 2.8). Other, longer fragments are also generated, but these are only linearly amplified and their proportion in the final product is negligible (Weising *et al.*, 2005).

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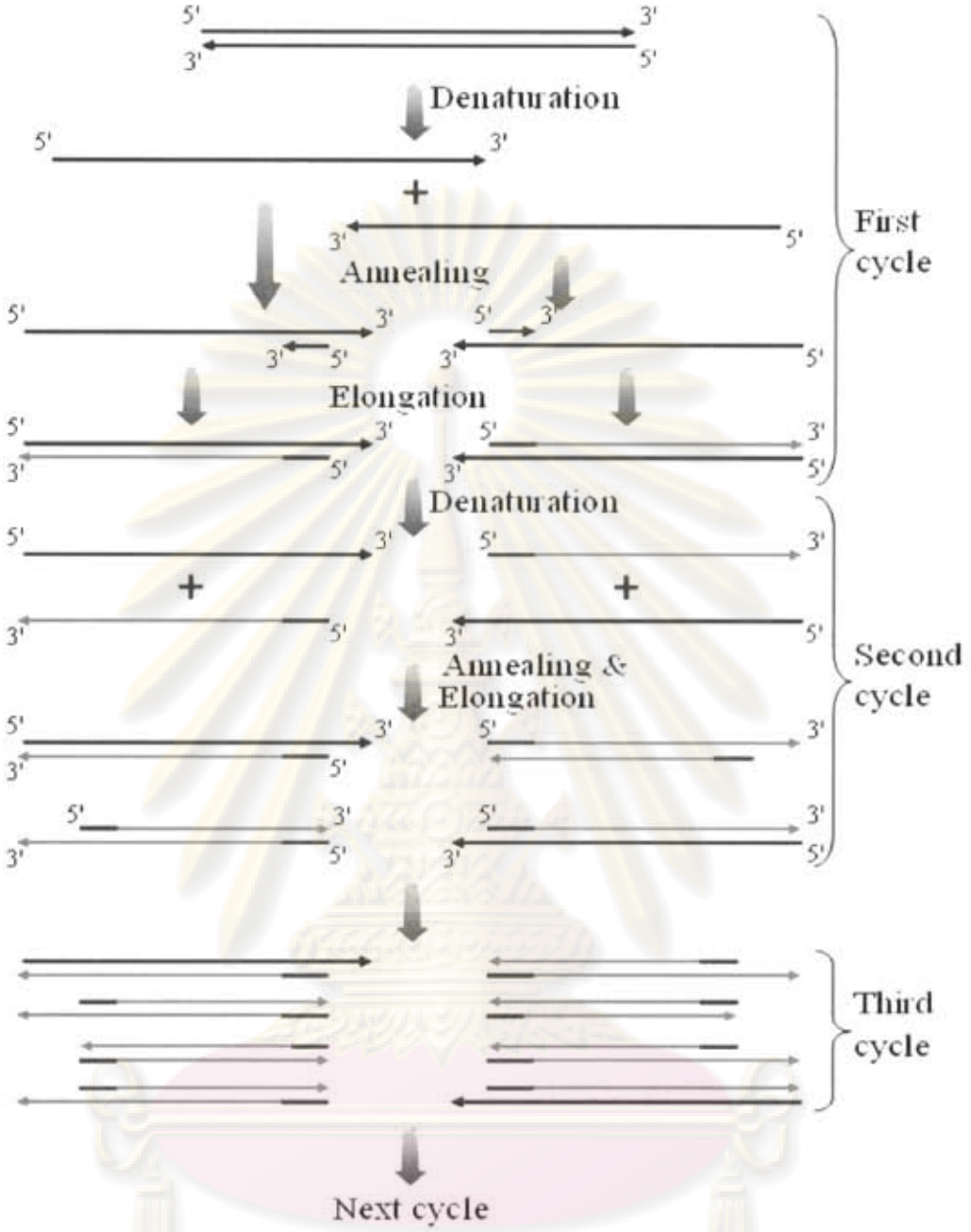

Figure 2.10 Principle of the polymerase chain reaction. A target DNA sequence is exponentially amplified with the help of flanking primers and a thermostable DNA polymerase. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing, and an elongation step. Primers are 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 predominate the final product almost exclusively.

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Primer Design

The selection of primers for given PCR can determine the efficiency and specificity of the PCR. Several variables must be taken into consideration when designing PCR primers. Ideally, specific primers should be 15 to 25 bases long, contain 40 to 60% GC, anneal to the template at about 55°C, slightly below the melting temperature (T_m). The primer sequence must be chosen such that there is no polyG or polyC that can promote non-specific annealing. PolyA and polyT stretches are also to be avoided these will breath and open-up stretches of the primer template complex. This can lower the efficiency of amplification. Both polypyrimidine (T, C) and polypurine (A, G) stretches should also be avoided. Moreover, a primer should not contain sequences that allow hairpin formation and primer-dimer formation (Weising *et al.*, 2005).

A hairpin is a structure formed by a single DNA molecule in which a portion on one part of the DNA hybridizes to a complementary portion within the same DNA strand, forming a structure resembling a hairpin (Figure 2.9A). When a PCR primer forms a hairpin, it adversely affects the primer's ability to bind and extend at the target site. In the worst case, the hairpin includes a base pair of the 3′-end and an overhang of the 5′-end (Figure 2.9B). Such a structure allows the extension by DNA polymerase along the primer and will result in the formation of a primer that will not be complementary to the template and will not be extended if hybridized (Figure 2.9C) (Bartlett and Sterling, 2003).

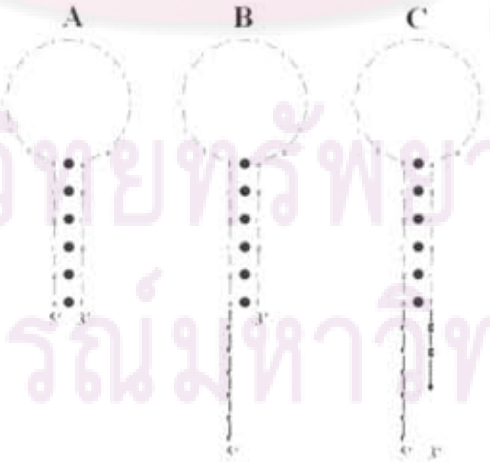

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Figure 2.11 Hairpin structures (Modified from Bartlett and Sterling, 2003)

A primer-dimer is the hybridization of two primers together (Figure 2.10A). There are two possibilities for these, homodimers and heterodimers. Homodimers are formed from the hybridization of the same species of primer together. Heterodimers are the duplex of two different primer sequences hybridizing together. The result of either of these is that the primers will not be as efficient in hybridizing to the target. As with hairpins, the worst case is that in which the 3′-end of one of the primers is base paired and there is a 5′ overhang (Figure 2.10B). In this case, the primer will extend, using the other primer as a template, rendering the extended primer unable to prime the desire template (Figure 2.10C). Even worse than with hairpins, this situation leads to amplification of the primer dimers and rapid depletion of usable primers (Bartlett and Sterling, 2003).

Figure 2.12 Dimer structures (Redrawn from Bartlett and Sterling, 2003)

It is well established that the 3′ terminal position in PCR primers is essential for the control of mis-priming. A G or C residue at the 3′ end is *'GC clamp'*, helps to ensure correct binding at the 3′ end due to the stronger hydrogen bonding of G/C residue. It also helps to improve the efficacy of the reaction by minimizing any breathing that might occur. At present, various computer programs are available that assist in primer design (e.g., Fast PCR, Primers3, Oligo, and Primo Pro 3.4). These programs can calculate many parameters as mentioned above so primer design is so easily.

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6. DNA Sequencing and Sequence Alignment

DNA Sequencing

Two basic strategies of DNA sequencing were devised in the mid-1970s. The so-called chemical degradation method of Maxam and Gilbert (1997) employs chemicals that cleave behind specific bases in an end-labeled DNA molecule. This treatment generates four nested sets of labeled cleavage products, each terminating at a specific base. After separation on highly resolving, denaturing polyacrylamide gels, these fragment sets are visualized by autoradiography. The resulting sequence ladder can be read directly from the autoradiogram.

The chain termination method described by Sanger *et al.* (1997) exploits the 5′- to 3′-strand extension activity of a DNA polymerase in the presence of a base-specific chain terminator. A typical Sanger sequencing reaction is set up to contain the denatured template DNA, a target-specific sequencing primer, the DNA polymerase, and the four dNTPs in an appropriate buffer system. Reactions are aliquoted into four microtubes, each containing a specific 2′-3′-dideoxynucleotide (ddATP, ddTTP, ddCTP, ddGTP). These nucleotide analogs are properly recognized by the polymerase and incorporated into the growing chain. However, because ddNTPs lack a 3′-OH group, they are not extended, and the chain is terminated at the precise position where the ddNTP is added. The concentration ratios of dNTPs and ddNTPs need to be carefully balanced, so that all position termination points along the DNA chain are represented in the set of reaction products. Labeling is achieved by incorporating either labeled primers or labeled ddNTPs in the reaction. As in the case of the chemical method, a ladder of bands is produced after high-resolution, denaturing polyacrylamide gel electrophoresis.

The Sanger method has become the most widely used technique for sequencing DNA, and several variants have been developed. Whereas a genetically engineered form of phage T7 DNA polymerase is routinely used in the standard protocol, the so-called cycle sequencing variant (Murray, 1989) employs a thermostable *Taq* DNA polymerase and a thermal profile to generate single-stranded

sequence template by asymmetric PCR. Cycle sequencing works equally well with double-stranded and single-stranded templates, and requires less template DNA than the standard methodology.

Although based on the same principle, currently used sequencing protocols differ slightly from each other, depending on the type and conformation of the template (plasmid vs. PCR product; single- vs. double-stranded DNA), type of label employed (radioisotopes vs. fluorescence), target of the labeling reaction (primers vs. terminators), and the type of polymerase used (thermostable vs. thermosensitive) (Weising *et al.*, 2005).

Sequence Alignment

In bioinformatics, a sequence alignment is a way of arranging the primary sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences. Aligned sequences of nucleotide or amino acid residues are typically represented as rows within a matrix. Alignments are commonly represented both graphically and in text format. In almost all sequence alignment representations, sequences are written in rows arranged so that aligned residues appear in successive columns. In text formats, aligned columns containing identical or similar characters are indicated with a system of conservation symbols. Many sequence visualization programs also use color to display information about the properties of the individual sequence elements; in DNA and RNA sequences, this equates to assigning each nucleotide its own color. Sequence alignments can be stored in a wide variety of text-based file formats, many of which were originally developed in conjunction with a specific alignment program or implementation. Most web-based tools allow a number of input and output formats, such as FASTA format and GenBank format (Corpet, 1988).

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7. Phylogenetic Analysis

Phylogenetic analysis of DNA sequences has become an important tool for studying the evolutionary history of organisms. Since the rate of sequence evolution varies extensively with gene or DNA segment, one can study the evolutionary relationships of virtually all levels of classification of organisms (e.g. kingdom, phyla, families, genera, species, and intraspecific populations) by using different genes or DNA segments. Phylogenetic analysis is also important for clarifying the evolutionary pattern of multigene families as well as for understanding the process of adaptive evolution at the molecular level.

There are many statistical methods that can be used for reconstructing phylogenetic trees from molecular data. Commonly used methods are classified into three major groups: (1) distance methods, (2) likelihood method, and (3) parsimony methods (Nei and Kumar, 2000).

7.1 Distance Methods

In distance methods or distance matrix methods, evolutionary distances are computed for all pairs of taxa, and a phylogenetic tree is constructed by considering the relationships among these distance values. There are many different methods of constructing trees from distance data. The methods that have proved to be useful for actual data analysis i.e. Unweighted Pair-Group Method Using Arithmetic Averages (UPGMA), Least Squares (LS) Method, Minimum Evolution (ME) Method, and Neighbor Joining (NJ) Method (Nei and Kumar, 2000).

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7.2 Maximum Likelihood Methods

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In maximum likelihood (ML) methods, the likelihood of observing a given set of sequence data for a specific substitution model is maximized for each topology, and the topology that gives the highest maximum likelihood is chosen as the final tree. The parameters to be considered are not the topologies but the branch lengths for each topology, and the likelihood is maximized to estimate branch lengths (Nei and Kumar, 2000).

7.3 Maximum Parsimony Methods

In these maximum parsimony (MP) methods, four or more aligned nucleotide sequences are considered, and the nucleotide of ancestral taxa are inferred separately at each site for a given topology under the assumption that mutational changes occur in all directions among the four nucleotides. The smallest number of nucleotide substitutions that explain the entire evolutionary process for the topology is then computed. This computation is done for all potentially correct topologies, and the topology that requires the smallest number of substitutions is chosen to be the best tree. The theoretical basis of this method is William of Ockham's philosophical idea that the best hypothesis to explain a process is the one that requires the smallest number of assumptions.

In the search for MP trees, nucleotide sites that have the same nucleotide for all taxa (invariable sites) are eliminated from the analysis, and only variable sites are used. However, not all variable sites are useful for finding an MP tree topology. A nucleotide site to be informative for constructing an MP tree, there must be at a least two different kinds of nucleotides, each represented at least two times. These sites are called informative sites. Because only sites contribute to finding MP trees, it is important to have many informative sites to obtain reliable MP trees. However, when the extent of homoplasy (backward and parallel substitutions) is high, MP trees would not be reliable even if there many informative sites available. For this reason, the consistency index is used to measure the extent of homoplasy. This index for a single nucleotide site is given $c_i = m_i/s_i$, where m_i is the minimum possible number of substitutions at the site for any conceivable topology, and s_i is the minimum number of substitutions required for the topology under consideration. However, the lower bound of the consistency index is not 0, and c_i varies with topology. For this reason, the retention index is used for quantity. The retention index is given by $r_i = (g_i - s_i)/(g_i - m_i)$, where g_i is the maximum possible number of substitutions at the single nucleotide site for any conceivable tree under the parsimony principle and is equal to the number of substitutions required for a star topology when the most frequent nucleotide is placed at the central node. The retention index becomes 0 when the site is least informative for MP tree construction, that is, $g_i = s_i$. The c_i and r_i are considered for one site. In practice, these values are computed for all informative

sites, and the ensemble or overall consistency index (CI) and overall retention index (RI) for all sites are considered. These indices are defined as $CI = \Sigma_i m_i / \Sigma_i s_i$ and $RI = (\Sigma_i g_i - \Sigma_i s_i) / (\Sigma_i g_i - \Sigma_i m_i)$, where i refers to the single nucleotide site. These indices are often used as measure of accuracy of the topology obtained. When there are no backward and no parallel substitutions, CI = 1 and RI = 1. In this case, the topology is uniquely determined.

One of the effective ways of testing the reliability of an MP tree is to use the bootstrap test. In this test, the reliability of an inferred tree is examined. A set of nucleotide sites is randomly sampled with replacement from the original set, and this random set that has the same number of nucleotide sites as that of the original set used for constructing a new tree. This process is repeated many times (over 100 times), and the reliability of the inferred tree is evaluated by the percentage of times in which each branching pattern is found among all the replicate bootstrap trees (Nei and Kumar, 2000).

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CHAPTER III

SEQUENCE ANALYSIS OF *mat*K GENE OF *PUERARIA CANDOLLEI* AND *BUTEA SUPERBA*

1. Materials

Plant materials were collected from either forests or plantations in Thailand. All of the collected plant materials, ten samples of White Kwao Khruea and four samples of Red Kwao Khruea and their localities are listed in Table 1. Locations of plant materials are shown in Figure 3.1. Herbarium specimens have been deposited in the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The pictures of herbarium specimens are shown in Appendix A. The morphological characters of plant specimens were observed and are shown in Table 2.

The White Kwao Khruea specimens were identified and compared with the voucher specimens TH. Wongpasert no. 043-75 (BKF no. 141022) and J. F. Maxwell, no. 09-297 (BKF no. 091885), the Red Kwao Khruea specimens were identified and compared with the voucher specimens R. Pooma no. 796 (BKF no. 101288) stored in the herbarium of the Office of Forest and Plant Conservation Research National Park, Wildlife and Plant Conservation Department, Thailand.

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Table 1 Plant specimens used in this study and their accession numbers of *mat*K gene sequences

Plant names	Species	Code no.	Locality of voucher	Date of collection	Voucher no.	Habitus	GenBank accession no.
White Kwao Khruea (กวาวเครือขาว)	*Pueraria candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham or *P. candollei* Graham ex Benth. var. *candollei*	W01	Nonthaburi	2005.5.9	TH090505	cultivated	EU106108
		W02	Bangkok	2005.9.9	WY090905	cultivated	EU106106
		W03	Bangkok	2005.9.29	WY290905	cultivated	EU106106
		W05	Kanchanaburi	2005.12.28	SS281205	cultivated	EU106108
		W07	Kanchanaburi	2005.12.28	WY281205	wild	EU106109
		W16	Nakhon Pathom	2006.1.27	WY270106	cultivated	EU106110
		W20	Khon Kaen	2006.2.15	TH150206	cultivated	EU106107
		W21	Kanchanaburi	2005.9.29	CC290905	wild	EU106106
		W22	Chiang Mai	2007.4.21	WY210407	cultivated	EU106108
		W23	Prachuap Khiri Khan	2007.4.21	WY210407	cultivated	EU106108
Red Kwao Khruea (กวาวเครือแดง)	*Butea superba* Roxb.	R08	Kanchanaburi	2006.3.23	TH230306	wild	EU106111
		R09	Chachoengsao	2006.2.13	TH130206	wild	EU106111
		R12	Bangkok	2006.7.5	TH050706	cultivated	EU106111
		R15	Kalasin	2007.8.1	BR010807	cultivated	EU106111

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Table 2 Morphological characteristics of plant specimens used in this study

Plant names	Species	Code no.	Terminal Leaflet			Pods		Seeds
			Shape	Base	Apex	hairs	No hairs	
White Kwao Khruea (กวาวเครือขาว)	*Pueraria candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham	W01	elliptic	obtuse	acuminate	nd	nd	nd
		W02	elliptic	obtuse	acuminate	nd	nd	nd
		W03	elliptic	obtuse	acuminate	nd	nd	nd
		W05	elliptic	obtuse	acuminate	nd	nd	nd
		W07	elliptic	acute	acuminate	nd	nd	nd
	or	W16	elliptic	obtuse	acuminate	nd	nd	nd
		W20	elliptic	obtuse	acuminate	nd	✓	striation
	P. candollei Graham ex Benth. var. *candollei*	W21	elliptic	obtuse	acuminate	nd	nd	nd
		W22	elliptic	obtuse	acuminate	✓	nd	plain
		W23	elliptic	obtuse	acuminate	✓	nd	plain
Red Kwao Khruea (กวาวเครือแดง)	*Butea superba* Roxb.	R08	nd	nd	nd	nd	nd	nd
		R09	nd	nd	nd	nd	nd	nd
		R12	rhombic	obtuse	cuspidate	nd	nd	nd
		R15	rhombic	obtuse	cuspidate	nd	nd	nd

nd means no data

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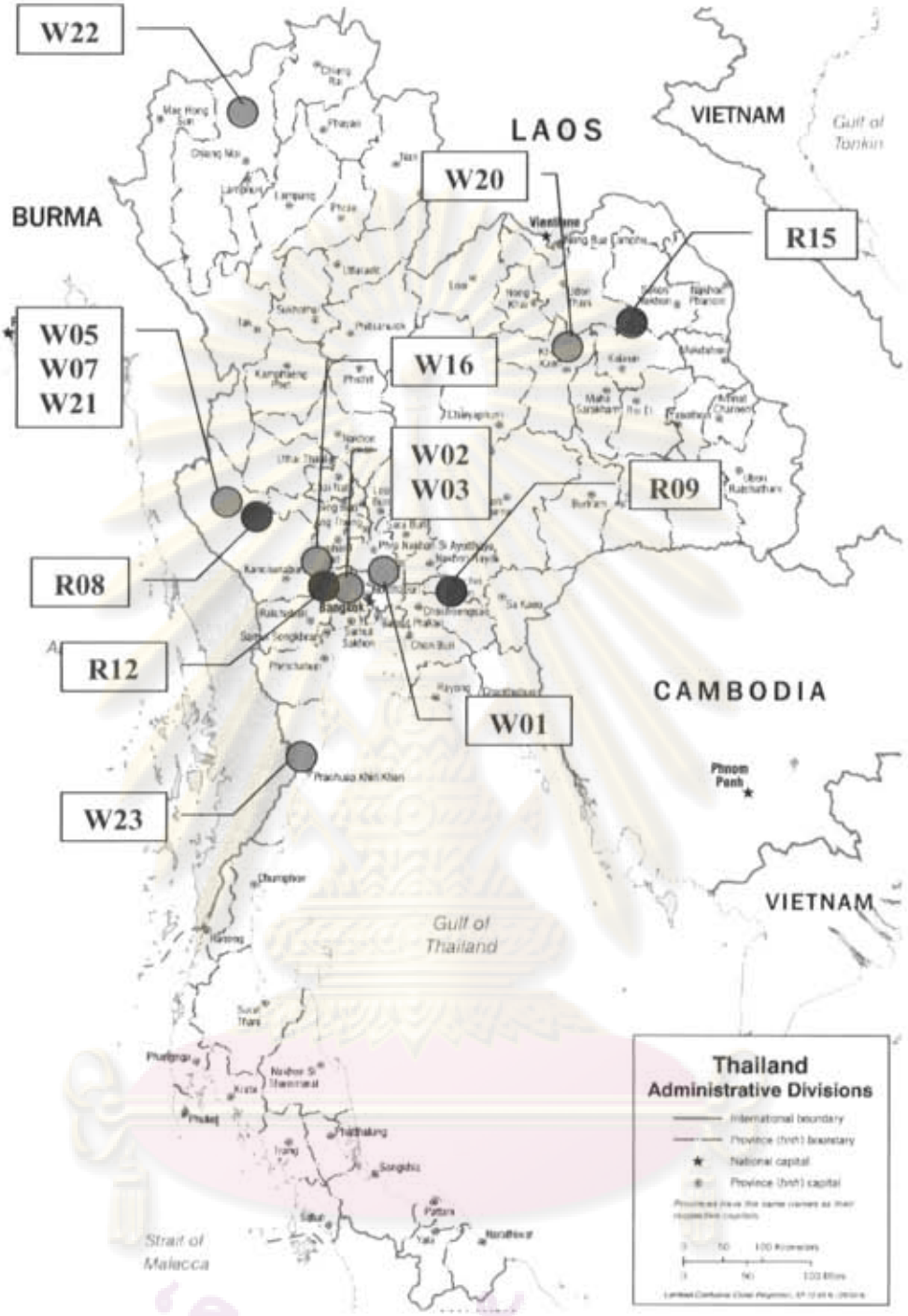

Figure 3.1 Locations for collecting plant specimens of White Kwao Khruea and Red Kwao Khruea

Represents White Kwao Khruea

Represents Red Kwao Khruea

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2. Methods

Total DNA Extraction

Fresh or dried leaves of each sample were ground under liquid nitrogen to a fine powder using a mortar and pestle. Total DNA was extracted using a DNeasy® Plant Mini Kit (QIAGEN, Germany), following the manufacturer's protocol. Then the 50 µl of DNA solution was purified by a Geneclean® II Kit (QBiogene Inc., U.S.A.). Total genomic DNA was performed on 0.8 % agarose gel electrophoresis stained by ethidium bromide and visualized under UV light to determine quality and quantity. A Lambda DNA-*Hind* III Digest (New England BioLabs Inc., U.S.A.) was used as standard molecular size. The extracted DNA was kept at -20°C for further use as template in PCR amplification.

Design Oligonucleotide Primers

To amplify and sequence the *mat*K region, seven primer sets were designed from published genomic DNA sequences database (NCBI GenBank) (http://www.ncbi.nlm.nih.gov), including the sequences of *trn*K-*mat*K regions of *Nicotiana tabacum* (L.) (accession number Z00044), *Arabidopsis thaliana* (L.) Heynh. (accession number NC000932) and *Pueraria Montana* (Lour.) Merr. (accession number AY582972). Initially, *trn*K-*mat*K regions of *Nicotiana tabacum* (accession number Z00044), *Arabidopsis thaliana* (accession number NC000932) and *Pueraria montana* (accession number AY582972) were compared, then other species were aligned and flanking conserved regions were selected. The designed primers were synthesized by Operon Biotechnologies (Germany).

Details of these primers are presented in Table 3. The relative positions of the primers are shown in Figure 3.2. The locations of amplification primers and sequencing primers on *trn*K-*mat*K region based on *Pueraria montana* (Lour.) Merr. retrived from GenBank (accession number AY582972) are shown in Figure 3.3.

Table 3 PCR amplification primers and sequencing primers of *mat*K gene used in this study

Primer name	Primer sequence (5′ to 3′)	Direction
Pue-F00	CCG TTG ATG GAT TTG ACT TGT	forward
Pue-F03	TAC CCT ATC CTA TCC ATC TG	forward
Pue-F04	TCT CCC AGC ATT CAT TTC ACT	forward
Pue-R02	GAA CCC CGA ATC TTG ACT CTT	reverse
Pue-R05	ACC GGC TTA CTA ATG GGA TGA	reverse
*mat*K-BMP-F-20	CTG TTT TGA TCA TAT CGC ACT	forward
*mat*K-BMP-R-21	GCA CAC GGC TTT CCC TAT	reverse

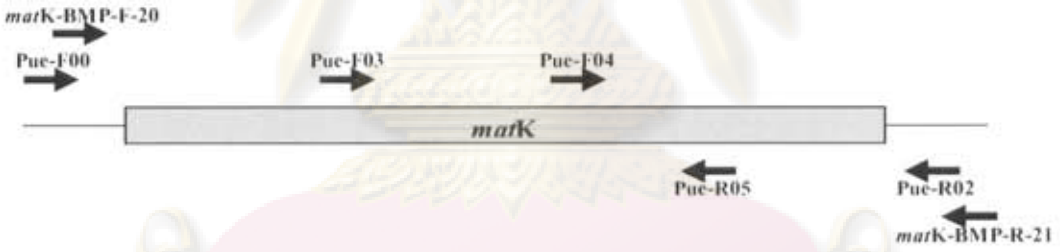

Figure 3.2 Relative positions of the PCR amplification primers and sequencing primers used in this study. Arrows (➡) represent forward primers. Arrows (⬅) represent reverse primers.

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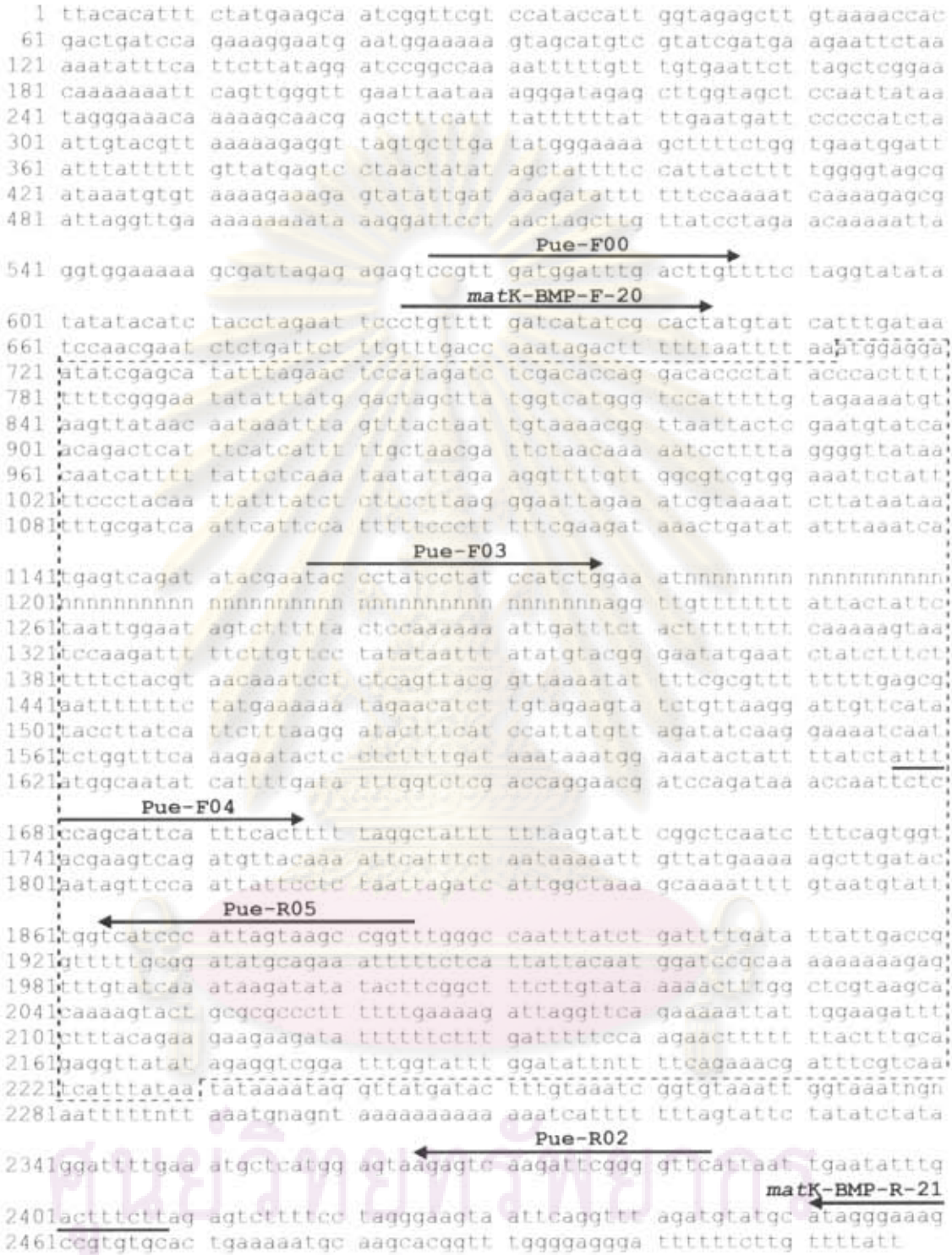

Figure 3.3 Locations of PCR amplification primers and sequencing primers on *trn*K-*mat*K region based on *Pueraria montana* (Lour.) Merr. retrieved from GenBank (accession number AY582972). The red nucleotides in dash box represents *mat*K gene.

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PCR Amplification of *mat*K Region

PCR amplification of *mat*K region was performed using 50 ng to 100 ng of total DNA as a template in 50 µl of reaction mixture consisting of 10 mM Tris-HCL (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ (Promega, U.S.A), 0.2 mM of each dNTPs, 1.5 U *Taq* DNA Polymerase (Promega, U.S.A), and 0.25 mM of each primer. Two pairs of amplification primers were used to amplify *mat*K gene region of *Pueraria* spp. and *Butea* spp. as follows: Pue-F00 and Pue-R02 for *Pueraria* spp.; *mat*K-BMP-F-20 and *mat*K-BMP-R-21 for *Butea* spp.

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 40 seconds, primer annealing at 50°C to 58°C for 40 seconds, and primer extension at 72°C for 90 seconds for 30 cycles, and final extension step at 72°C for 4 minutes to ensure that all amplicons are fully extended, then held at 4°C.

Quantitation and Qualitation of DNA

Quantitation and qualitation of DNA based on the UV-induced fluorescence emitted by ethiduim bromide-DNA complexes was used in this study. The PCR products were running 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. After the gel was solid, carefully removed the comb and inserted the gel into an electrophoresis apparatus filled with 1XTAE buffer. Added 1 µl of Nucleic Acid Sample Loading Buffer, 5X (Bio-Rad Laboratories Inc., U.S.A.) to the 4 µl of each DNA sample, mixed before loading 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.

PCR Product Purification

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

Nucleotide Sequencing

Nucleotide sequences of purified PCR products were determined by BioService Unit: BSU, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. Primers for sequencing in the forward direction were Pue-F00, Pue-F03, Pue-F04, and *mat*K-BMP-F-20. Primers Pue-R02, Pue-R05, and *mat*K-BMP-R-21 were used for the complementary strand.

Sequence Analysis and Phylogenetic Analysis

Five or more overlapping 700-900 bp sequences per sample were usually obtained, and the consensus sequences were assembled and analyzed using the SeqMan program (DNAstar, U.S.A.) and BioEdit (Hall, 2004). The nucleotide sequence data of *mat*K region were deposited in the DDBJ, EMBL, GenBank nucleotide sequence database with the accession numbers shown in Table 1. Multiple sequence alignments were evaluated with the freeware program CLUSTAL_X (Thompson *et al.*, 1997) and Multalin (Corpet, 1998). 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. *Glycine max* (L.) Merr. (accession number AF142700) belonging to the same family was used as outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of topology of the consensus tree.

3. Results

Total DNA and PCR Products

Total genomic DNA was isolated from leaves of each specimen by using the DNeasy® Plant Mini Kit (QIAGEN, Germany), and then DNA in solution was purified by Geneclean® II Kit (QBiogene Inc., U.S.A.). Genomic DNA was examined on 0.8% agarose gel electrophoresis. The size of isolated total DNA in each specimen was varied as shown in the Figure 3.4. The purified DNA was stored at –20°C until used.

Using the obtained total DNA as templates, complete *mat*K gene were amplified by PCR technique using primers, Pue-F00 and Pue-R02 for *Pueraria* spp.; *mat*K-BMP-F-20 and *mat*K-BMP-R-21 for *Butea* spp.

The resulting of both PCR products was about 1,800 bp in length (Figure 3.5).

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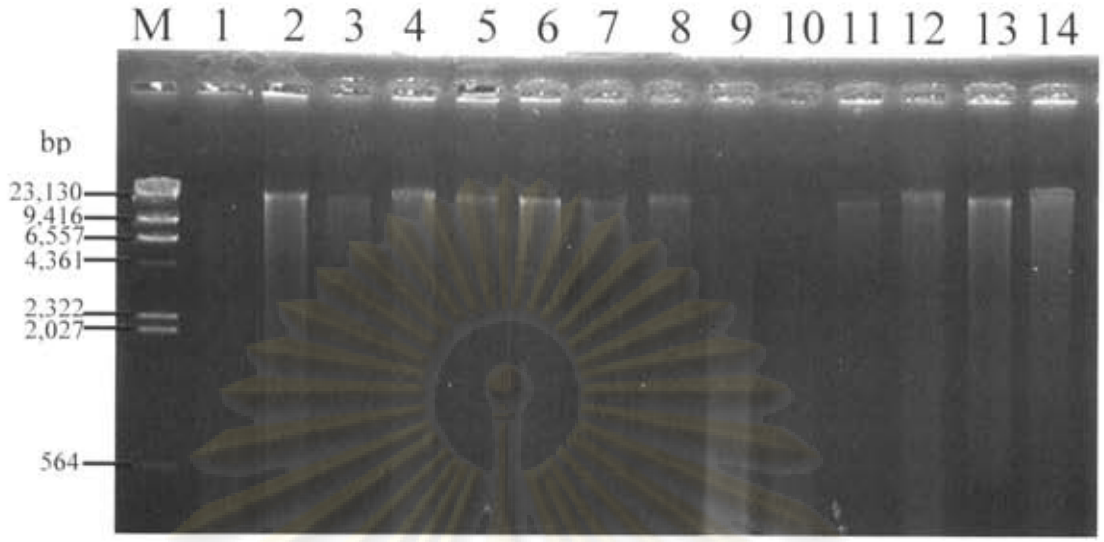

Figure 3.4 Agarose gel electrophoretogram of total DNA from White Kwao Khruea and Red Kwao Khruea

Lane M: Lambda DNA-*Hind* III Digest (The sizes are 564, 2027, 2322, 4361, 6557, 9416, and 23130 bp, respectively.)
Lane 1: White Kwao Khruea (W01, Nonthaburi)
Lane 2: White Kwao Khruea (W02, Bangkok)
Lane 3: White Kwao Khruea (W03, Bangkok)
Lane 4: White Kwao Khruea (W05, Kanchanaburi)
Lane 5: White Kwao Khruea (W07, Kanchanaburi)
Lane 6: White Kwao Khruea (W16, Nakhon Pathom)
Lane 7: White Kwao Khruea (W20, Khon Kaen)
Lane 8: White Kwao Khruea (W21, Kanchanaburi)
Lane 9: White Kwao Khruea (W22, Chiang Mai)
Lane 10: White Kwao Khruea (W23, Prachuap Kiri Khan)
Lane 11: Red Kwao Khruea (R08, Kanchanaburi)
Lane 12: Red Kwao Khruea (R09, Chachoengsao)
Lane 13: Red Kwao Khruea (R12, Bangkok)
Lane 14: Red Kwao Khruea (R15, Kalasin)

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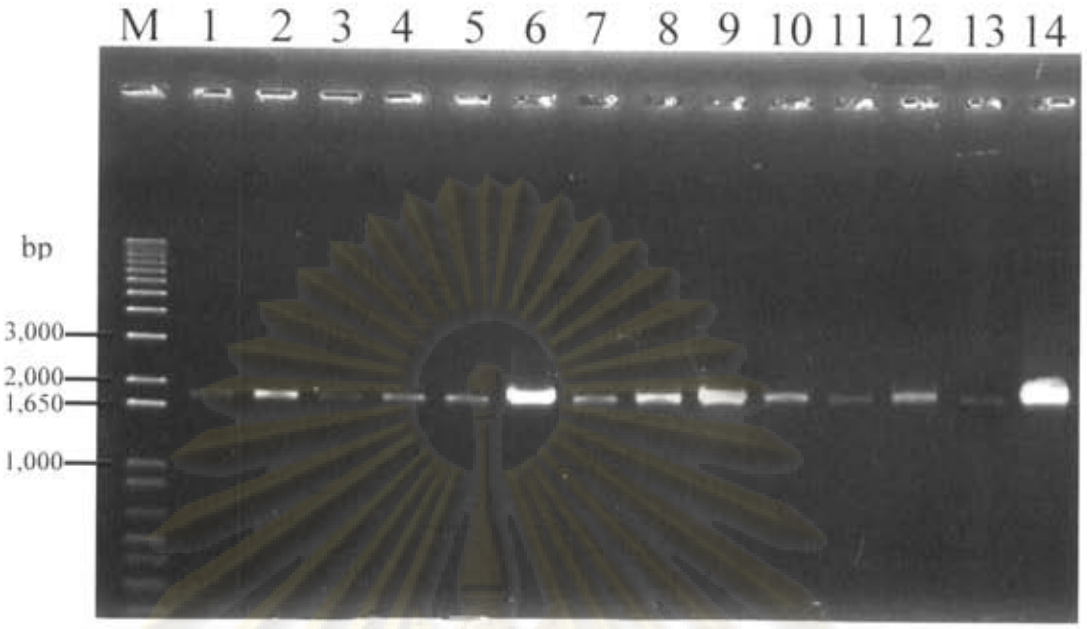

Figure 3.5 Agarose gel electrophoretogram of PCR products of complete *mat*K gene
Lane M: 1 Kb plus DNA Ladder. (The sizes are 100, 200, 300, 400, 500, 650, 850, 1000, 1650, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000 and 12000 bp, respectively.)
Lane 1-10: White Kwao Khruea (W01, W02, W03, W05, W07, W16, W20, W21, W22, and W23, respectively), using primers, Pue-F00 and Pue-R02.
Lane11-14: Red Kwao Khruea (R08, R09, R12 and R15, respectively), using primers, *mat*K-BMP-F-20 and *mat*K-BMP-R-21.

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The *mat*K Gene Sequence

The complete *mat*K gene of *P. candollei* (White Kwao Khruea) was found to be 1,521 bp in length whereas that in *B. superba* (Red Kwao Khruea) was found to be 1,527 bp in length due to a 6-bp indels at the nucleotide positions 627-632. White Kwao Khruea showed sequence variation intraspecies. Five sites of nucleotide substitutions were detected in 10 specimens of White Kwao Khruea. A total of 83 sites of substitutions were observed in 14 specimens of Both Kwao Khruea (Table 4). The multiple sequence alignment of *mat*K sequences of all samples of White Kwao Khruea and Red Kwao Khruea were illustrated in Appendix C. At the nucleotide position 1393, White Kwao Khruea were separated into two groups. The thymine substitutions were found at this position of the first group which are White Kwao Khruea code no. W02 (from Bangkok), W03 (from Bangkok), W21 (from Kanchanaburi), and W20 (from Khon Kaen). While, the guanine substitutions were found at the same position of the second group which are White Kwao Khruea code no. W01 (from Nonthaburi), W05 (from Kanchanaburi), W07 (from Kanchanaburi), W16 (from Nakhon Pathom), W22 (from Chiang Mai), and W23 (from Prachuap Khiri Khan).

The nucleotide sequences of the *mat*K gene of White Kwao Khruea and Red Kwao Khruea have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers EU106106, EU106107, EU106108, EU106109, EU106110, and EU106111 (see Appendix B).

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Table 4 Comparison of *mat*K gene sequences among ten specimens of White Kwao Khruea and four specimens of Red Kwao Khruea

Plant names	Code no.	Locality of voucher	16	17	42	53	55	63	93	95	144	170	171	204	214	219	241	252	256	279	289	293	331	341	345	415	417	420	433	493	510	531	551	590	609	627	628	629	630	631	632	637	707	708	724	759	766	779	782	791	795	813	820
White Kwao Khruea	W02	Bangkok	G	C	A	C	C	A	A	C	T	G	G	C	A	C	A	C	C	A	G	G	T	G	A	C	G	A	G	T	C	G	A	C	T	–	–	–	–	–	–	C	G	G	A	C	C	C	T	G	A	T	A
(ขาวเขียว)	W03	Bangkok	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W21	Kanchanaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W20	Khon Kaen	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W01	Nonthaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W05	Kanchanaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W22	Chiang Mai	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W23	Prachuap Khiri Khan	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W07	Kanchanaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
	W16	Nakhon Pathom	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	–	–	–	–	–	*	*	*	*	*	*	*	*	*	*	*	*
Red Kwao Khruea	R08	Kanchanaburi	A	T	C	T	T	G	C	T	A	*	T	G	G	T	T	T	A	C	*	T	C	A	T	T	T	*	A	C	T	A	G	*	G	G	T	T	C	C	T	T	T	A	G	A	A	T	C	T	C	C	C
(ขาวเขียวแดง)	R09	Chachoengsao	A	T	C	T	T	G	C	T	A	*	T	G	G	T	T	T	A	C	*	T	C	A	T	T	T	*	A	C	T	A	G	*	G	G	T	T	C	C	T	T	T	A	G	A	A	T	C	T	C	C	C
	R12	Bangkok	A	T	C	T	T	G	C	T	A	*	T	G	G	T	T	T	A	C	*	T	C	A	T	T	T	*	A	C	T	A	G	*	G	G	T	T	C	C	T	T	T	A	G	A	A	T	C	T	C	C	C
	R15	Kalasin	A	T	C	T	T	G	C	T	A	*	T	G	G	T	T	T	A	C	*	T	C	A	T	T	T	*	A	C	T	A	G	*	G	G	T	T	C	C	T	T	T	A	G	A	A	T	C	T	C	C	C

Plant names	Code no.	Locality of voucher	912	947	963	965	975	977	999	1023	1036	1047	1053	1119	1125	1155	1158	1215	1218	1265	1275	1276	1286	1290	1296	1305	1349	1393	1395	1428	1469	1512	1513	1523
White Kwao Khruea	W02	Bangkok	C	G	G	T	C	C	A	C	C	G	A	A	A	A	T	C	G	C	G	A	A	A	T	G	C	T	T	G	G	C	G	T
(ขาวเขียว)	W03	Bangkok	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	W21	Kanchanaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	W20	Khon Kaen	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	W01	Nonthaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*
	W05	Kanchanaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*
	W22	Chiang Mai	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*
	W23	Prachuap Khiri Khan	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*
	W07	Kanchanaburi	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*
	W16	Nakhon Pathom	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*
Red Kwao Khruea	R08	Kanchanaburi	T	A	T	G	A	A	G	A	G	A	G	G	T	G	G	A	A	A	A	C	G	C	C	A	T	G	A	T	T	G	A	C
(ขาวเขียวแดง)	R09	Chachoengsao	T	A	T	G	A	A	G	A	G	A	G	G	T	G	G	A	A	A	A	C	G	C	C	A	T	G	A	T	T	G	A	C
	R12	Bangkok	T	A	T	G	A	A	G	A	G	A	G	G	T	G	G	A	A	A	A	C	G	C	C	A	T	G	A	T	T	G	A	C
	R15	Kalasin	T	A	T	G	A	A	G	A	G	A	G	G	T	G	G	A	A	A	A	C	G	C	C	A	T	G	A	T	T	G	A	C

The numbers above sequence are aligned nucleotide positions. Asterisks (*) indicate the identical nucleotides with those of White Kwao Khruea (W02) in the first line. Dashes (--) denote alignment gaps.

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Phylogenetic Analysis

The phylogenetic tree was constructed on the basis of complete *mat*K gene sequences of 14 specimens of White Kwao Khruea and Red Kwao Khruea. Parsimony analysis showed that six most parsimonious trees fully resolved tree with a length of 1,444, a consistency index (CI) of 1.000 and a retention index (RI) of 1.000. As shown in Figure 3.6, strict consensus tree divided the White Kwao Khruea into two clades (clade I and clade II). The four specimens of Red Kwao Khruea were in the same clade (clade III) and separated from White Kwao Khruea with a high bootstrap value (100%). White Kwao Khruea (clade II) which are W01 (from Nonthaburi), W05 (from Kanchanaburi), W07 (from Kanchanaburi), W16 (from Nakhon Pathom), W22 (from Chiang Mai), and W23 (from Prachuap Khiri Khan) was separated from White Kwao Khruea (clade I) which are W02 (from Bangkok), W03 (from Bangkok), W21 (from Kanchanaburi), and W20 (from Khon Kaen) with a bootstrap value of 64%.

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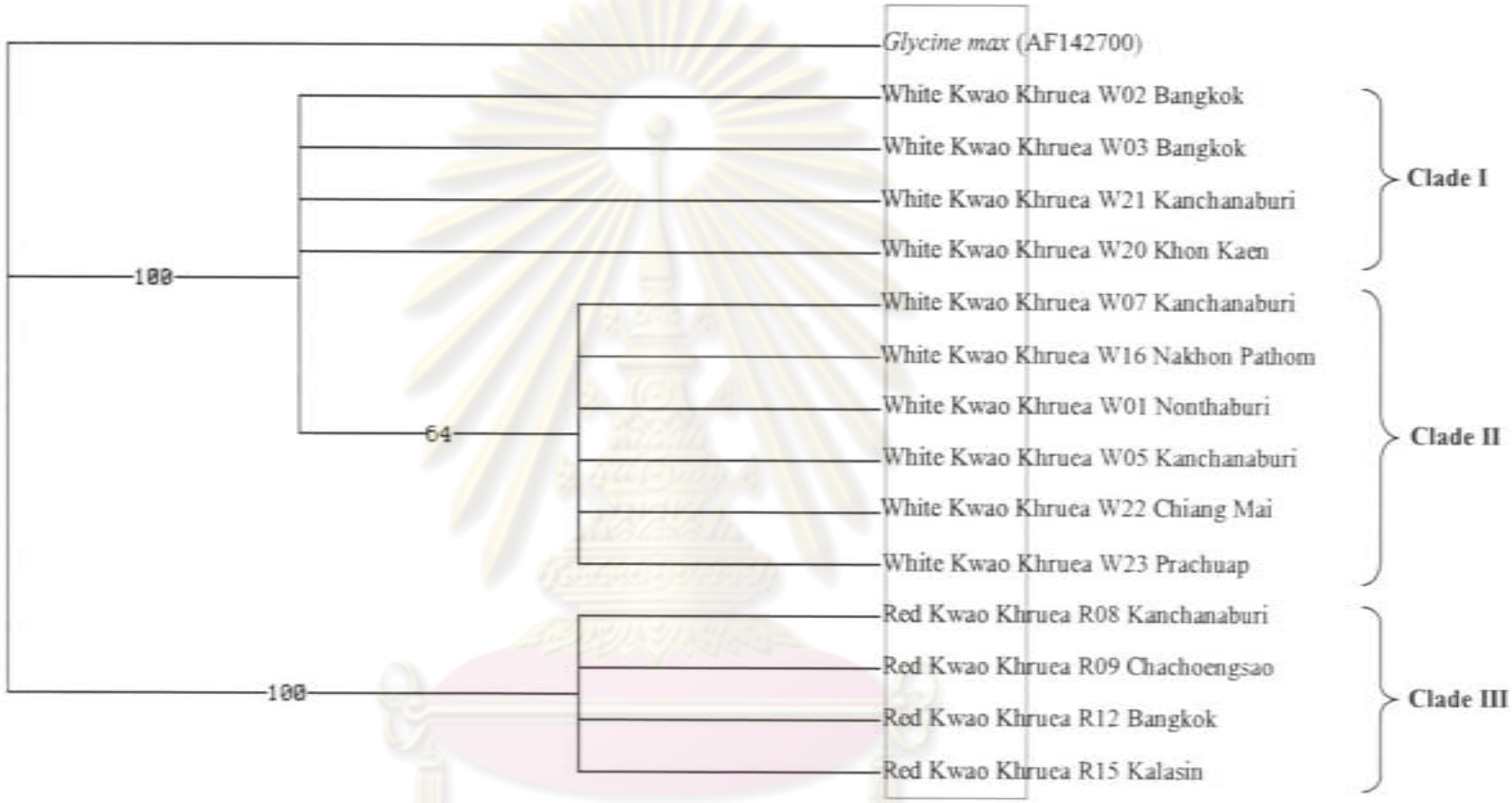

Figure 3.6 Strict consensus tree of six most parsimonious tree
The six most parsimonious tree constructed on the basis of maximum parsimonious analysis. Tree length=1,444, CI=1.000, RI=1.000. Numbers on the lines indicate bootstrap values with 1,000 replicates.

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4. Discussion

The present study was designed to determine the identification markers of White Kwao Khruea and Red Kwao Khruea and to clarify their phylogenetic relationships. Complete *mat*K gene sequences of 10 specimens of White Kwao Khruea (1521 bp) and 4 specimens of Red Kwao Khruea (1527 bp) were elucidated. In the White Kwao Khruea, not only interspecies but also intraspecies nucleotide substitutions were observed. This result agrees with previous research (Srijugawan and Ditchaiwong, 2005) in that there was genetic variation among White Kwao Khruea specimens, although these specimens were collected from the same source. The result of sequence comparison of the *mat*K gene revealed that 83 sites of nucleotide substitutions had been detected. Based on phylogenetic tree obtained from complete *mat*K gene sequences, the specimens of White Kwao Khruea were divided into two clades (clade I and clade II). This result is consistent with a previous study in that *P. candollei* have two varieties which are *P. candollei* var. *candollei* (pods without hair) and *P. candollei* var. *mirifica* (pods with hairs) (Smitinand, 2001; Srijugawan and Ditchaiwong, 2005). Data in Table 2 shown that pods of W20 (from Khon Kaen) in clade I are without hair (*P. candollei* var. *candollei*). While, the pods of W22 (from Chiang Mai), and W23 (from Prachuap Khiri Khan) in clade II are with hairs (*P. candollei* var. *mirifica*). The result of sequence comparison of the *mat*K gene revealed that key nucleotide differentiating *P. candollei* var. *candollei* from *P. candollei* var. *mirifica* existed at position 1393. From this finding, this position could be used for identifying these two varieties of *P. candollei*. However, the present study dose not has enough data of the characteristic of White Kwao Khruea pods. It is recommended that further studies, including larger sample sizes and investigating the characteristic of pods, should be done to confirm this result.

All four specimens of Red Kwao Khruea were in the same clade (clade III) and were separated from White Kwao Khruea. There are 83 different nucleotide sites among both Kwao Khruea. These 83 sites can be used to identify and clarify the phylogenetic relationships of them.

In this study, the phylogenetic tree was constructed on the basis of complete *mat*K gene sequences of 10 specimens of White Kwao Khruea and 4 specimens of Red Kwao Khruea. Furthermore, 83 sites nucleotides substitution can be used as identification markers of White Kwao Khruea and Red Kwao Khruea.

CHAPTER IV

POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) OF *PUERARIA CANDOLLEI* AND *BUTEA SUPERBA* AND THEIR APPLICATION FOR IDENTIFICATION

1. Materials

. The leaves of White Kwao Khruea (code no. W03 and W22) and Red Kwao Khruea (code no. R08 and R09) were used in this study. The tuberous roots of White Kwao Khruea (code no. W21) and Red Kwao Khruea (code no. R12) were also investigated. All of the collected plant materials and their localities are listed in Table 1 in Chapter III. Seven commercial products of White Kwao Khruea and Red Kwao Khruea were purchased at a local retail source and were in the form of dried ground plant material enclosed in capsules and sold as herbal medicines (Table 4). Both herbarium specimens and commercial products have been deposited in the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The pictures of herbarium specimens are shown in appendix A, and the pictures of commercial products are shown in Figure 4.1.

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Table 5 Commercial products used in this study and their botanical origins

Herbal drug names	Botanical origin	Code no.	Market	Date of collection
White Kwao Khruea capsules (ยาแคปซูลกวาวเครือขาว)	*Pueraria candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham or *P. candollei* Graham ex Benth. var. *candollei*	WP1	Tha Phra Chan, Bangkok	2006.10.17
		WP2	Tha Chang, Bangkok	2006.102.17
		WP3	Tha Maharat, Bangkok	2006.10.17
Red Kwao Khruea capsules (ยาแคปซูลกวาวเครือแดง)	*Butea superba* Roxb.	RP1	Tha Phra Chan, Bangkok	2006.10.17
		RP2	Tha Chang, Bangkok	2006.10.17
		RP3	Tha Maharat, Bangkok	2006.10.17
		RP4	Sai Yok Noi, Kanchanaburi	2005.12.28

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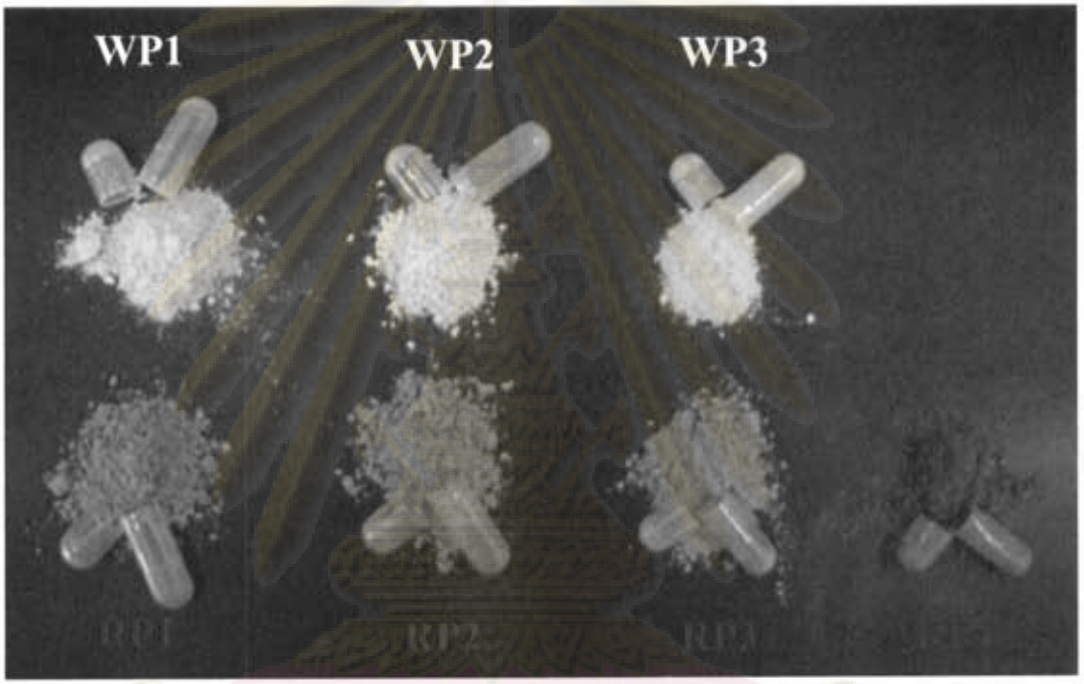

Figure 4.1 Commercial products of White Kwao Khruea and Red Kwao Khruea

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2. Methods

Total DNA Extraction

Fresh leaves, silica gel dried leaves, or tuberous roots of samples were ground under liquid nitrogen to a fine powder using a mortar and pestle. Fifty milligrams of plant tissue or 100 mg of dried material from the capsule were used for total DNA extraction using the 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.). Total genomic DNA was performed on 0.8 % agarose gel electrophoresis stained by ethidium bromide and visualized under UV light to determine quality and quantity. A Lambda DNA-*Hind* III Digest (New England BioLabs Inc., U.S.A.) was used as standard DNA size. The extracted total DNA was kept at -20°C for further use as template in PCR amplification.

Design Oligonucleotide Primers

Two pairs of primer were designed based on our nucleotide sequence of the *mat*K region of White Kwao Khruea and Red Kwao Khruea (accession number EU106106, EU106107, EU106108, EU106109, EU106110, and EU106111). The 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 4.2. The locations of amplification primers on partial *mat*K region based on our sequence of *Butea superba* Roxb. (accession number EU106111) are shown in Figure 4.3.

Table 6 PCR amplification primers of partial *mat*K gene used in PCR-RFLP method

Primer name	Primer sequence (5′ to 3′)	Direction
461F	TCC ATC TGG AAA TCT TGG TTC	forward
960R	GAT CGT TCC TGG TTG AGA CC	reverse
1179F	GGT TTG GGC CAA TTT ATC TG	forward
1489R	CCA AAT ACC AAA TCC GAC CT	reverse

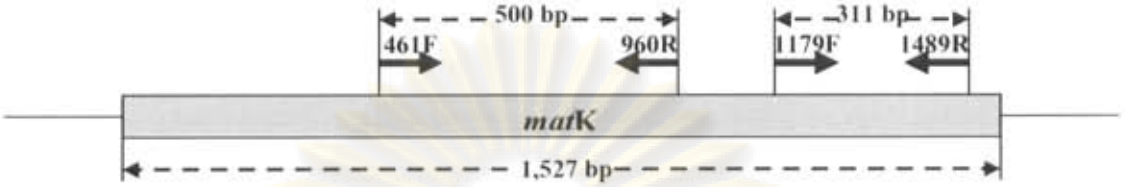

Figure 4.2 Relative positions of the PCR amplification primers used in PCR-RFLP method. Arrows (➞) represent forward primers. Arrows (⬅) represent reverse primers.

```
   1 atggaggaat atcgaatata tttagaactc catagatctc gccaccagga catcttatac
  61 ccgctttttt ttcgggaata tatttatgga ctcgtttatg gtcatgggtc catttttgta
 121 gaaaaaaatg taggttataa caaaaaattt agtttactaa ttgtaaaacg tttaattact
 181 cgaatgtatc aacagactca tttgatcatt tttgctaatg attctaacaa aaatcctttt
 241 tggggttata ataataattt ttattctcaa ataatattcg aaggttttgt tgtcgtcgtg
 301 gagattctat tttcctaca attatttatc ccttccttaa aggatttaga aatcgtaaaa
 361 tcttataata atttgcgatc aattcattcc atttttccct ttttcgaaga taaatttata
                                                        461F
 421 tatttaaatc ataagtcaga tatacgaata ccctatccta tccatctgga aatcttggtt
 481 caaatccttc gacattggat aaaagatgtt tctttctttc atttattaag attgtttttt
 541 tattactatt gtaattggaa tagtcttttt actccaaaaa aatggatttc tacttttttt
 601 tcaaaaagga atccaagatt tttcttgttc ctattcttat ataatttata tgtacgggaa
 661 tatgaatcta tctttctttt tctacgtaac aaatcctctc agttactatt aaaatatttt
 721 cgcgtttttt ttgagcgaat ttttttctat gaaaaaatag aacatattgt agaagtattt
 781 gctaaggatt tttcctatac cttatcattc ttcaaggatc ctttcatcca ttatgttaga
 841 tatcaaggaa aatcaattct ggtttcaaag aatactcctc ttttgataaa taaatggaaa
                                                        960R
 901 tactatttta tttatttatg gcaatgtcat tttgatattt ggtctcaacc aggaacgatc
 961 catagaaacc aattatacca gcattcattt cacttttgg gctatttttt aagtattcgg
1021ctaaatcttt cagtggtacg aagtcaaatg ttgcaaaatt catttctaat aaaaattgtt
1081atgaaaaagc ttgatacaat agttccaatt attcctctga ttagttcatt ggctaaagca
                                                    1179F
1141aaattttgta atgtgttggg tcatcccatt agtaagccgg tttgggccaa tttatctgat
1201tttgatatta ttgaacgatt tttgcggata tgcagaaatt tttctcatta ttacaatgga
1261tccgaaaaaa aaaaacgttt gtatcgaatc agatacatac ttcgactttc ttgtataaaa
1321actttggctc gtaagcacaa aagtactgtg cgcacttttt tgaaaagatt aggttcagaa
1381aaattattgg aagaattctt tacagaagaa gaagatattt tttcttttat ttttccaaga
                                                1489R
1441acttctttta ctttgcagag gttatatata ggtcggattt ggtatttgga tattcttttc
1501agaaacgatt tgatcaatca ttcataa
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Figure 4.3 Locations of PCR amplification primers on *mat*K gene of *Butea superba* Roxb. (accession number EU106111)

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PCR-RFLP Analysis

PCR amplification of partial *mat*K region was performed using 50 ng to 100 ng of total DNA as a template in 50 µl of reaction mixture consisting of 10 mM Tris-HCL (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ (Promega, U.S.A), 0.2 mM of each dNTPs, 1.5 U *Taq* DNA Polymerase (Promega, U.S.A), and 0.25 mM of each primer. Two pairs of amplification primers were used to amplify partial *mat*K gene region of plant samples and commercial products as follows: 1) 461F forward primer and 960R reverse primer, and 2) 1179F forward primer and 1489R reverse 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 40 seconds, primer annealing at 48°C to 56°C for 40 seconds, and primer extension at 72°C for 40 seconds for 30 cycles, and final extension step at 72°C for 4 minutes, then held at 4°C. In the case of commercial products of Red Kwao Khruea, nested PCR method was applied: the first PCR product was used as a template of the second PCR amplification then the second PCR product was used as a template of the third PCR amplification.

The PCR products which were amplified using primers 461F and 960R, were digested with 2.5 units of restriction enzyme, *Dde*I (BioLabs, Inc., U.S.A.) at 37°C for 4 hrs. And the resulting PCR products which were amplified using primers 1179F and 1489R, were digested with 2.5 units of restriction enzyme, *Eco*RI (BioLabs, Inc., U.S.A.) at 37°C for 4 hrs.

The PCR product and the resulting restriction digests were detected by a 2.0% 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.).

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3. Results

Total DNA

The total DNA isolated from leaves of White Kwao Khruea (code no. W03 and W22) and Red Kwao Khruea (code no. R08 and R09) were obtained (Figure 3.4 in Chapter III), and those from tuberous roots of White Kwao Khruea (code no. W21), Red Kwao Khruea (code no. R12), and drug samples (WP1, WP2, WP3, RP1, RP2, RP3, and RP4) were obtained (Figure 4.4).

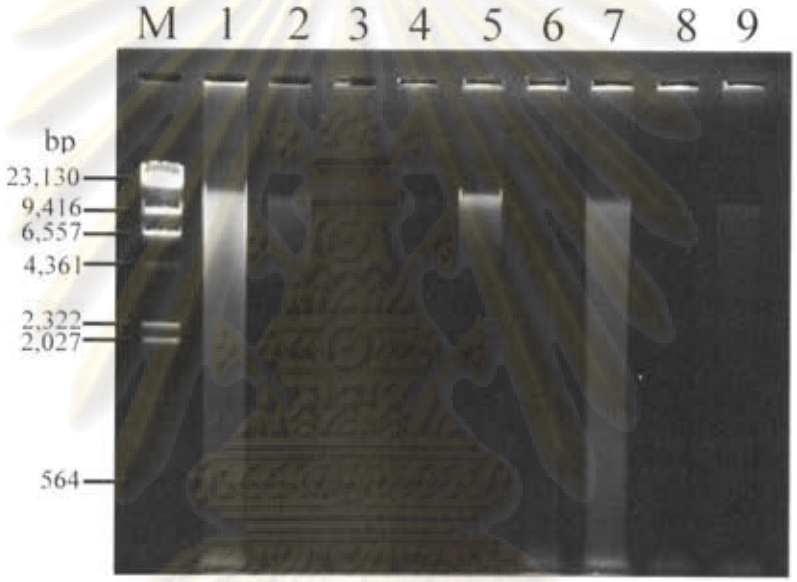

Figure 4.4 Agarose gel electrophoretogram of total DNA from tuberous roots and drug samples of White Kwao Khruea and Red Kwao Khruea

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

Lane 1: Tuberous root of White Kwao Khruea (W21, Kanchanaburi)

Lane 2: White Kwao Khruea capsules (WP1)

Lane 3: White Kwao Khruea capsules (WP2)

Lane 4: White Kwao Khruea capsules (WP3)

Lane 5: Tuberous root of Red Kwao Khruea (R12, Bangkok)

Lane 6: Red Kwao Khruea capsules (RP1)

Lane 7: Red Kwao Khruea capsules (RP2)

Lane 8: Red Kwao Khruea capsules (RP3)

Lane 9: Red Kwao Khruea capsules (RP4)

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Identification of White Kwao Khruea and Red Kwao Khruea by PCR-RFLP Analysis

***Dde*I Digest**

The PCR products of both Kwao Khruea amplified with a pair of primers, 461F and 960R were 500 bp in length. The restriction enzyme *Dde*I, which recognizes the sequence of 5′-CTNAG-3′ was found to give diagnostic fragments among the two species. The *mat*K gene of White Kwao Khruea had a *Dde*I restriction site at the nucleotide position 698-702 (Figure 4.5A). The resulting restriction digest showed two fragments of 238 and 264 bp (Figure 4.5B), while in the Red Kwao Khruea, they had two *Dde*I restriction sites at the nucleotide position 698-702 and 782-786 (Figure 4.5A). The resulting restriction digest showed three fragments of 84, 178 and 238 bp in electrophoretogram (Figure 4.5B).

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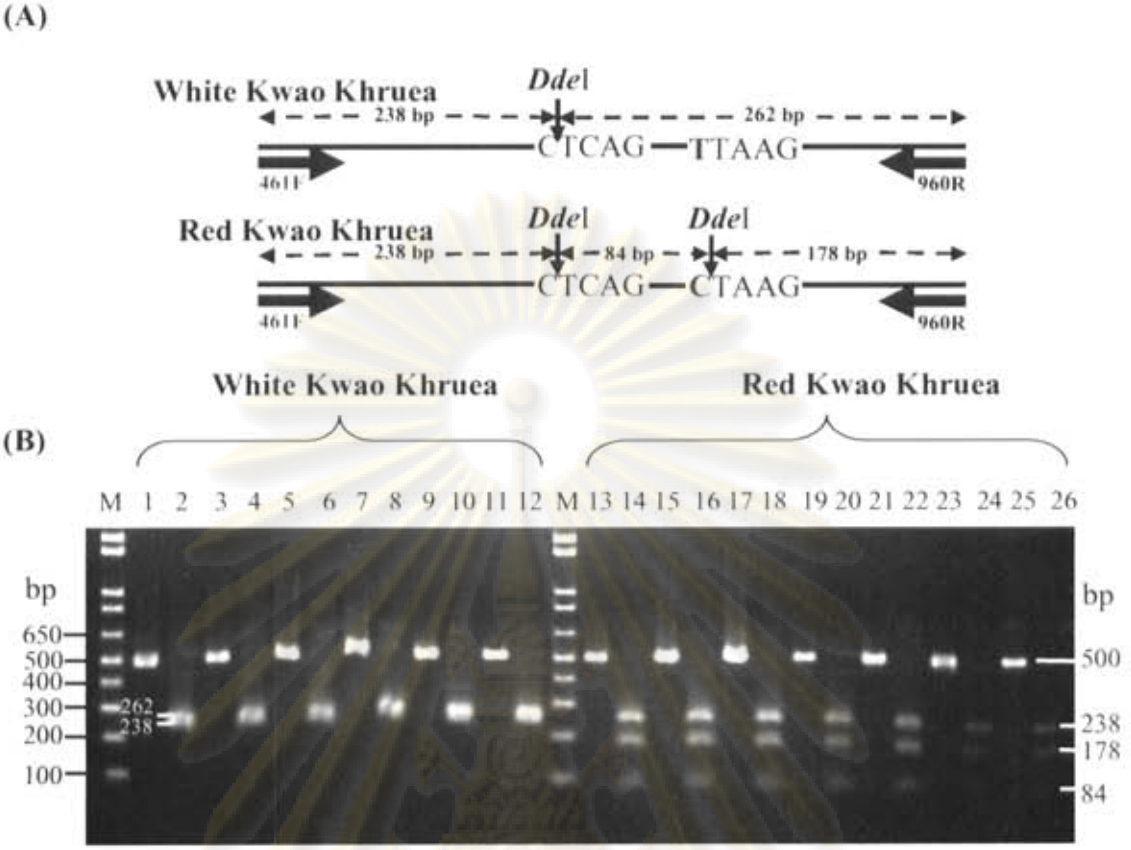

Figure 4.5 PCR-RFLP analysis of White Kwao Khruea and Red Kwao Khruea using the restriction enzyme *Dde*I on partial *mat*K gene

(A) *Dde*I restriction sites in White Kwao Khruea and Red Kwao Khruea. Red nucleotide indicates the defined marker nucleotide at position 782.

(B) Agarose gel electrophoretrogram of PCR product generated by primers 461F and 960R (odd-numbered lanes), and then digested with *Dde*I (even-numbered lanes);

lane M: 1 Kb plus DNA Ladder

lane 1, 2: leaves of White Kwao Khruea (W03, Bangkok)

lane 3, 4: leaves of White Kwao Khruea (W22, Chiang Mai)

lane 5, 6: tuberous root of White Kwao Khruea (W21, Kanchanaburi)

lane 7, 8: commercial product (WP1)

lane 9, 10: commercial product (WP2)

lane 11, 12: commercial product (WP3)

lane 13, 14: leaves of Red Kwao Khruea (R08, Kanchanaburi)

lane 15, 16: leaves of Red Kwao Khruea (R09, Chachoengsao)

lane 17, 18: tuberous root of Red Kwao Khruea (R12, Bangkok)

lane 19, 20: commercial product (RP1)

lane 21, 22: commercial product (RP2)

lane 23, 24: commercial product (RP3)

lane 25, 26: commercial product (RP4)

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***Eco*RI Digest**

The PCR products of both Kwao Khruea amplified with a pair of primers, 1179F and 1489R were 311 bp in length. The restriction enzyme *Eco*RI, which recognizes the sequence of 5′-GAATTC-3′ was found to give diagnostic fragments among the two species. The *mat*K gene of Red Kwao Khruea had a *Eco*RI restriction site at the nucleotide position 1393-1398 (Figure 4.6A). The resulting restriction digest showed two fragments of 96 and 215 bp (Figure 4.6B), whereas that of White Kwao Khruea showed only one fragment of 311 bp (Figure 4.6B) because they do not have the *Eco*RI restriction site on the *mat*K gene (Figure 4.6A).

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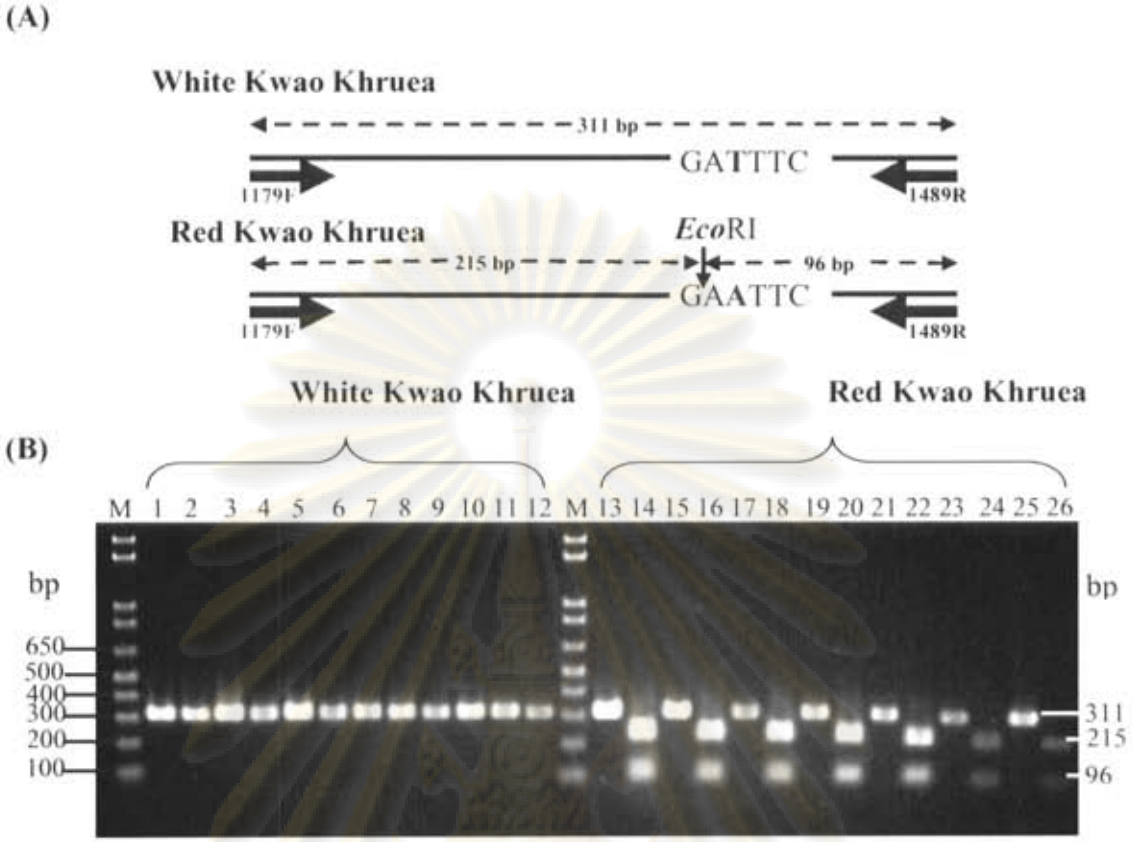

Figure 4.6 PCR-RFLP analysis of White Kwao Khruea and Red Kwao Khruea using the restriction enzyme *Eco*RI on partial *mat*K gene

(A) *Eco*RI restriction site in White Kwao Khruea and Red Kwao Khruea. Red nucleotide indicates the defined marker nucleotide at position 1395.

(B) Agarose gel electrophoretrogram of PCR product generated by primers 1179F and 1489R (odd-numbered lanes), and then digested with *Eco*RI (even-numbered lanes);

lane M: 1 Kb plus DNA Ladder
lane 1, 2: leaves of White Kwao Khruea (W03, Bangkok)
lane 3, 4: leaves of White Kwao Khruea (W22, Chiang Mai)
lane 5, 6: tuberous root of White Kwao Khruea (W21, Kanchanaburi)
lane 7, 8: commercial product (WP1)
lane 9, 10: commercial product (WP2)
lane 11, 12: commercial product (WP3)
lane 13, 14: leaves of Red Kwao Khruea (R08, Kanchanaburi)
lane 15, 16: leaves of Red Kwao Khruea (R09, Chachoengsao)
lane 17, 18: tuberous root of Red Kwao Khruea (R12, Bangkok)
lane 19, 20: commercial product (RP1)
lane 21, 22: commercial product (RP2)
lane 23, 24: commercial product (RP3)
lane 25, 26: commercial product (RP4)

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4. Discussion

In the present study, on the basis of two marker nucleotides at positions 782 and 1395 of the *mat*K gene sequence of White Kwao Khruea and Red Kwao Khruea in Chapter III, PCR-RFLP method using restriction enzyme *Dde*I and *Eco*RI was established as a convenient and efficient method for identification. Since in many drug samples, DNA was degraded into small pieces due to the oxidative and hydrolytic process during preservation period or drug preparation, longer PCR products were difficult to be obtained. Therefore, two pairs of primer sets; 1) 461F and 960R, and 2) 1179F and 1489R were designed, which produced short fragments, to allow differentiation of drug samples. The plant specimens and commercial prodructs were amplified with these primers, giving a band of 500 bp and 311 bp, respectively. Subsequently, the 500 bp and 311 bp PCR products were digested with restriction enzymes *Dde*I and *Eco*RI, respectively. 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 PCR products revealed polymorphisms after separation on agarose gel. For the plant specimens and commercial products identified as White Kwao Khruea, two fragments (238 bp and 262 bp) were observed in the *Dde*I digestion of the PCR products, while those identified as Red Kwao Khruea, showed three fragments (84 bp, 178 bp, and 238 bp). In the *EcoR*I digestion, the plant specimens and commercial products identified as Red Kwao Khruea showed two fragments (96 bp and 215 bp), whereas those of White Kwao Khruea showed a single band of 311 bp.

In the case of commercial products of Red Kwao Khruea, PCR amplification failed at first, PCR products of partial *mat*K gene were not detected, since DNA isolation from the crude drugs or commercial products of herbal medicine was difficult because of the high amounts of polysaccharides and secondary metabolites that formed insoluble complexes with nucleic acids during extraction (Cai *et al.*, 1996 and Pandey *et al.*, 1999). So, nested PCR method was required. The first PCR products were used as a template of the second PCR amplification then the second PCR product were used as a template of the third PCR amplification. From this method, PCR products were obtained.

PCR-RFLP method was also applied for combined herbal drugs such as samples WP3 and RP3 which combined with *Piper nigrum* Linn. (พริกไทย), *Cyperus rotundus* Linn. (หัวแห้วหมู), *Tinospora crispa* (L.) Miers ex Hook.f. *et* Thoms. (บอระเพ็ด), *Elephantopus scaber* Linn. (ไคไม่รู้ล้ม), and *Diospyros rhodocalyx* Kuarz. (ตะโกนา). The designed primers were specific to White Kwao Khruea and Red Kwao Khruea, thus DNA of the other species were not amplified and were not affected the PCR-RFLP analysis.

In this study, a newly developed PCR-RFLP method was established as a convenient tool for the differentiation of the White Kwao Khruea from the Red Kwao Khruea. It could be used to identify both Kwao Khruea in drug samples sold as a single drug or in combination with other herbal drugs. PCR-RFLP method is relatively simple, reliable, repeatable, and provides effective and accurate identification of both types of Kwao Khruea.

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CHAPTER V

CONCLUSION

1. The *mat*K gene sequences of ten specimens of *Pueraria candollei* Graham ex Benth. var. *mirifica* (Airy Shaw *et* Suvatabandhu) Niyomdham and *P. candollei* Graham ex Benth. var. *candollei* were determined. As a result, intraspecies nucleotide substitutions were observed. Phylogenetic tree constructed using parsimony analysis suggested that *P. candollei* can be divided into two groups which are related to the two varieties of *P. candollei*.
2. The *mat*K gene sequences of four specimens of *Butea superba* Roxb. were determined and shown to have identical sequence despite of differences in collection places.
3. Marker nucleotides for identification of *P. candollei* and *B. superba* were determined by the sequence comparison of *mat*K gene region.
4. On the basis of the defined marker nucleotides at positions 782 and 1395, PCR-RFLP analysis was designed as a convenient and efficient method for identification. PCR-RFLP methods using two restriction enzymes *Dde*I and *Eco*RI were developed for identifying White Kwao Khruea and Red Kwao Khruea and can be applied to not only all parts of plants but also to their commercial products.
5. Nested PCR method was required for some drug samples in PCR-RFLP analysis.
6. PCR-RFLP analysis can be applied to commercial products which are in combination with other herbal drugs.

In conclusion, development of a reliable marker for authentication of herbal plants is important since their pharmacological effects are known to be different according to the composition of their chemical constituents. In addition, using misidentified plant products can cause serious side-effects or toxicity. Therefore, the reliable *mat*K gene sequence and PCR-RFLP markers established in this study will provide an important tool for quality control and standardization of both types of Kwao Khruea and their products.

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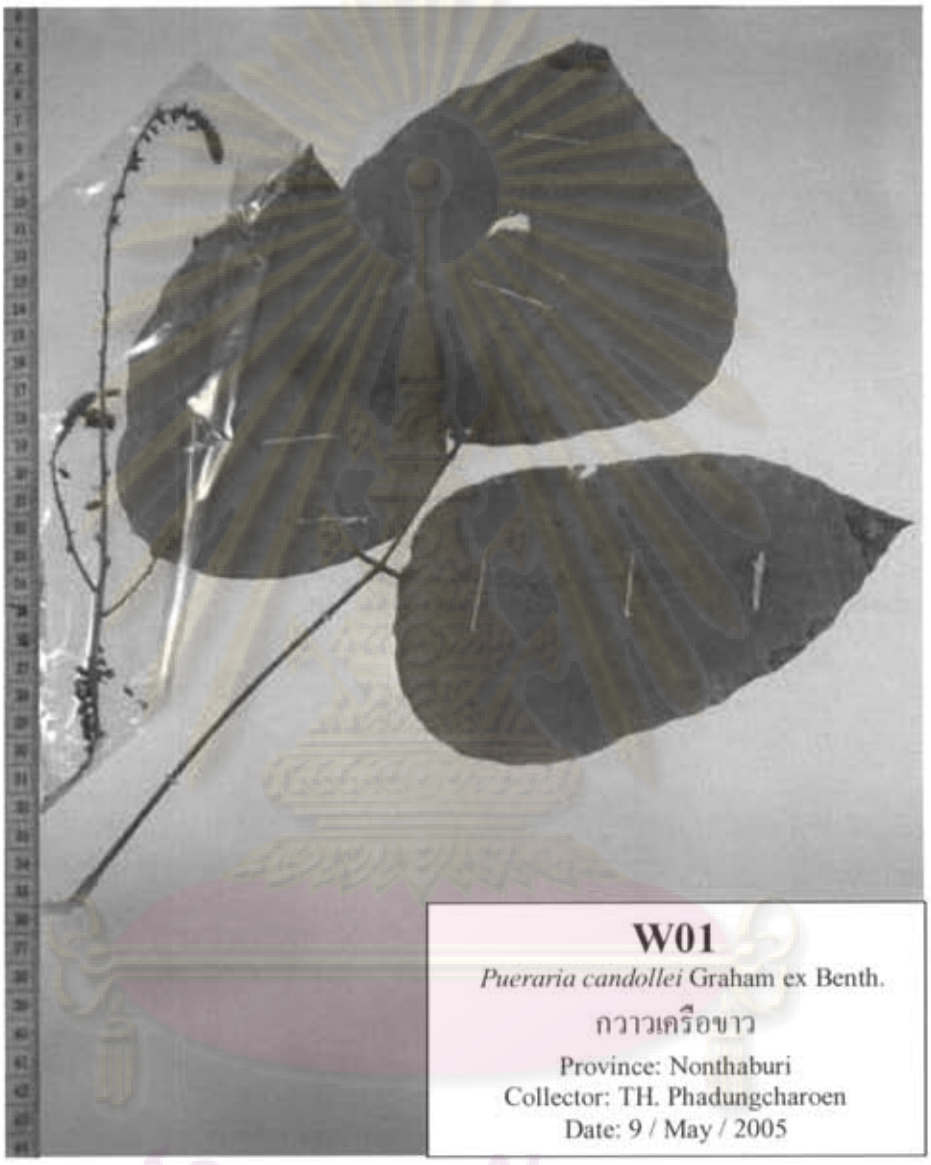
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

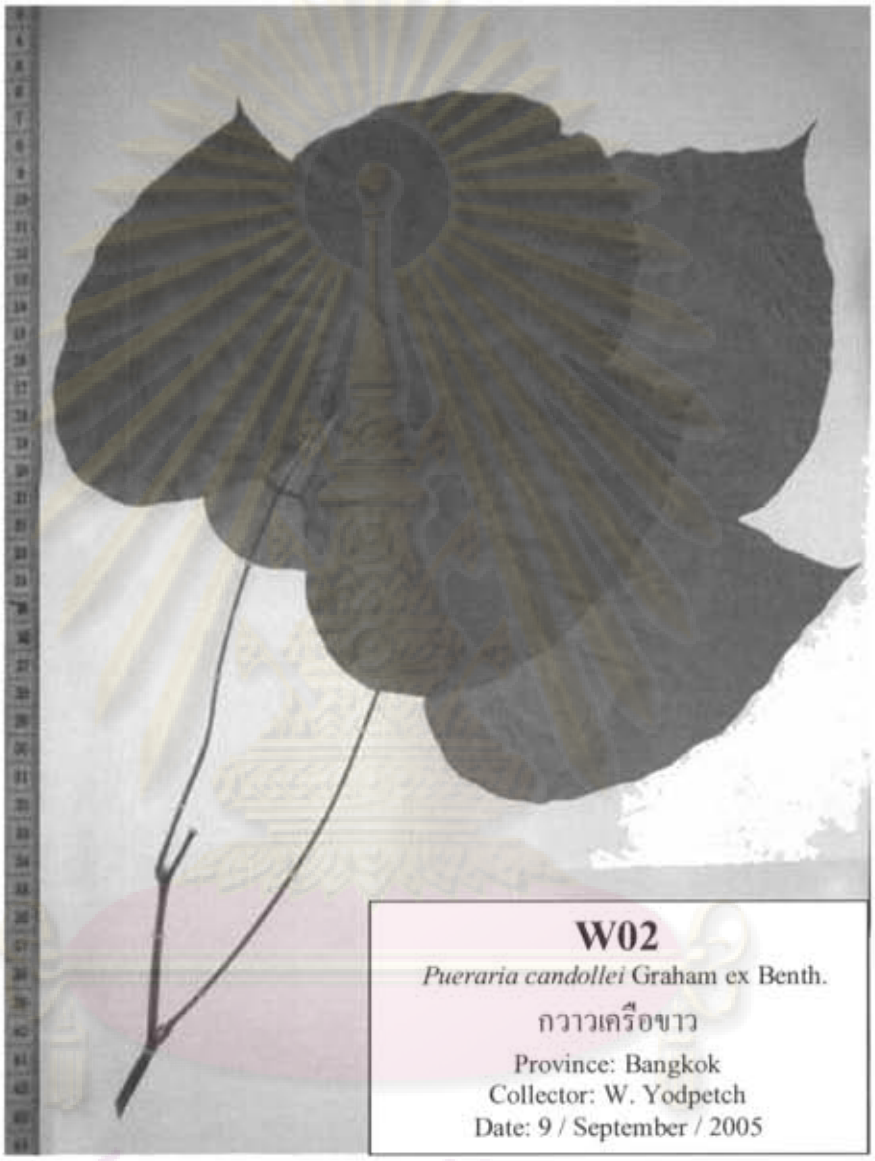
ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX A



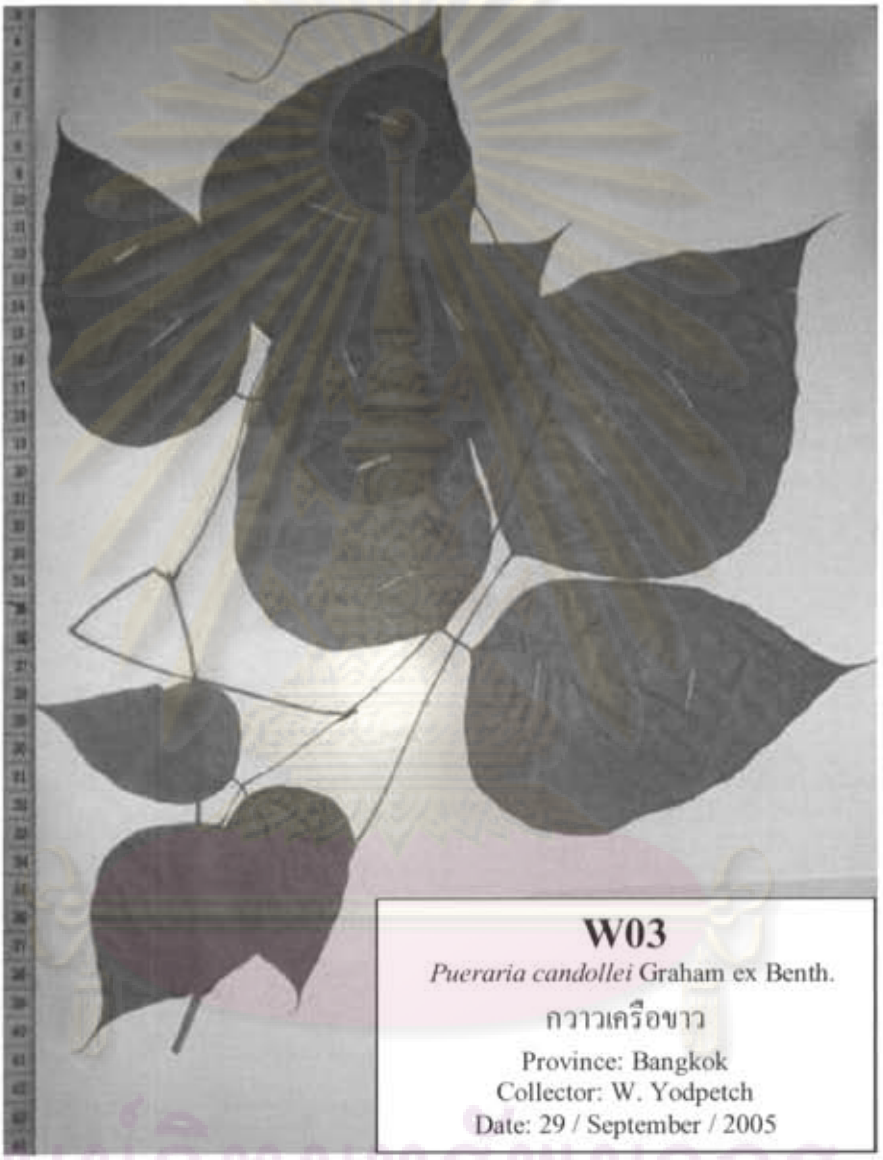
Figture A 1 *Pueraria candollei* Graham ex Benth. (W01)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



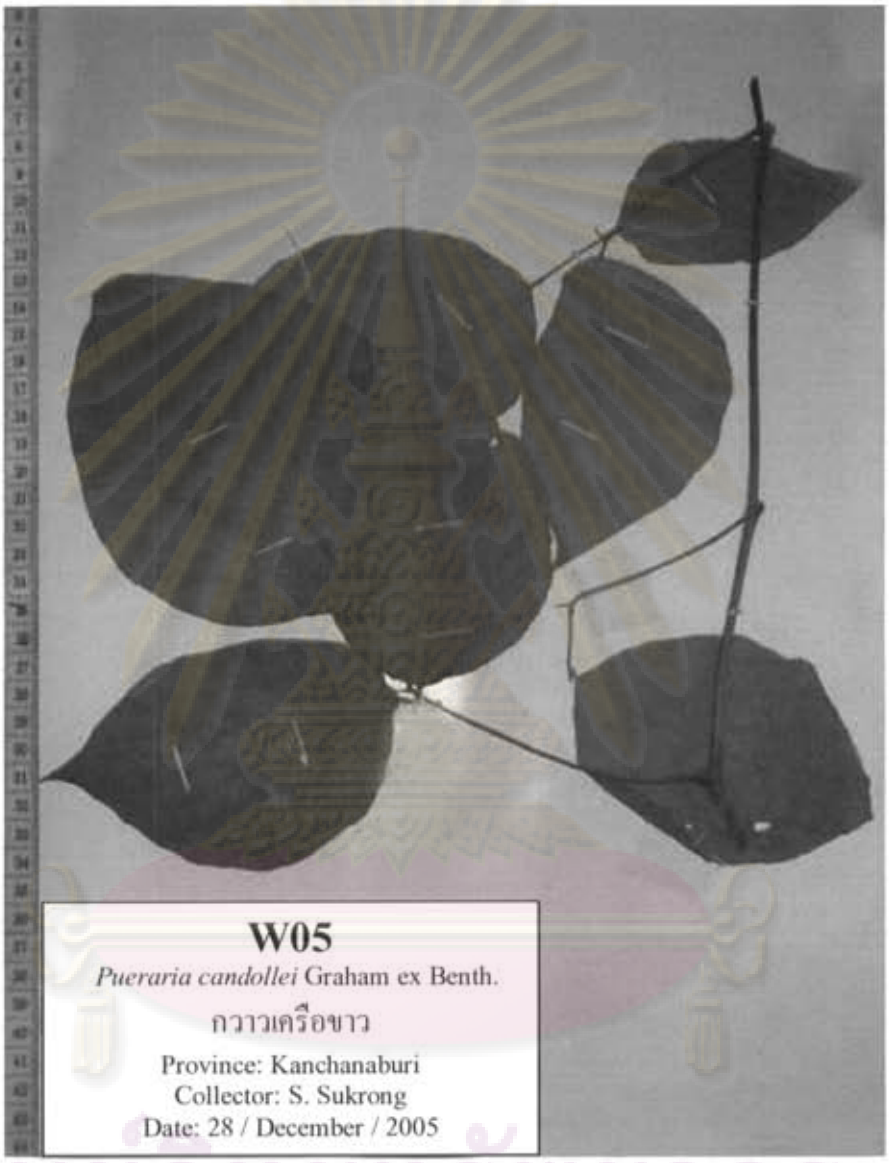
Figture A 2 *Pueraria candollei* Graham ex Benth. (W02)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



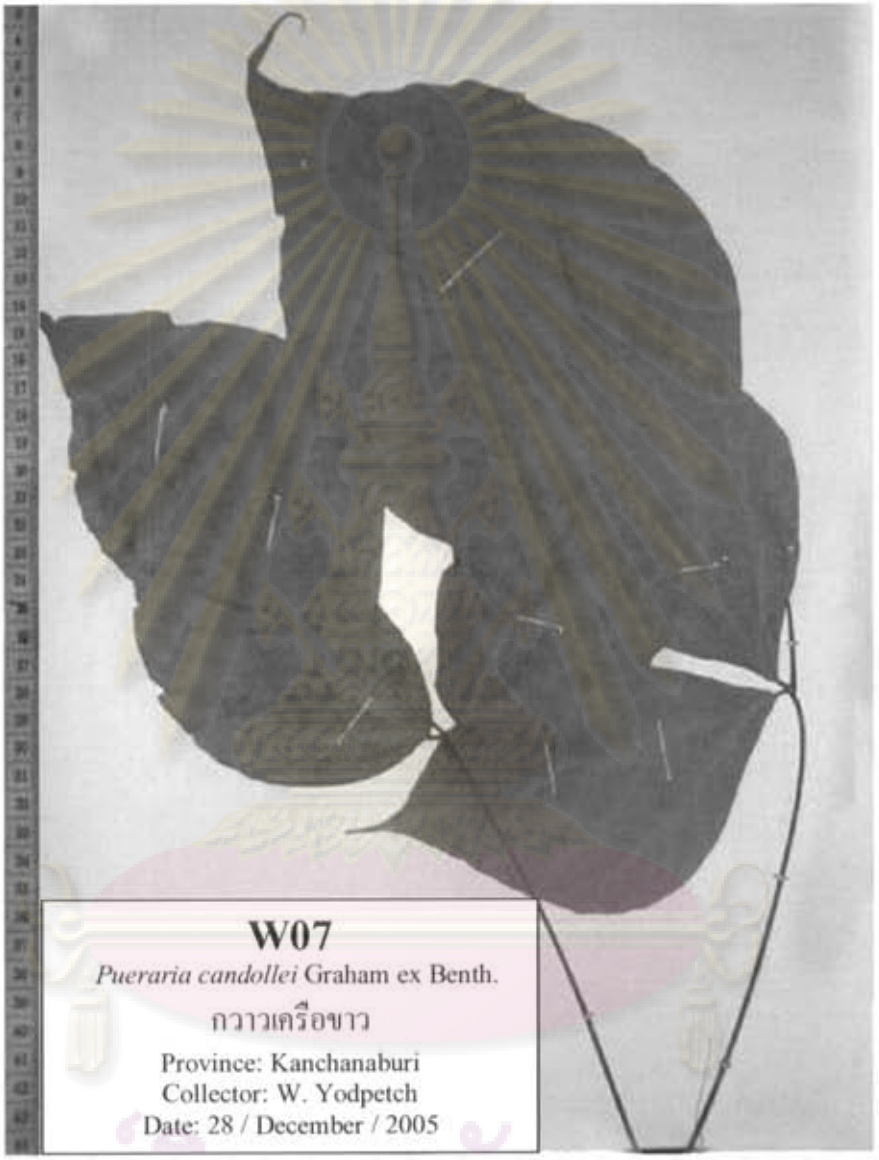
Figture A 3 *Pueraria candollei* Graham ex Benth. (W03)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



Figture A 4 *Pueraria candollei* Graham ex Benth. (W05)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



Figture A 5 *Pueraria candollei* Graham ex Benth. (W07)

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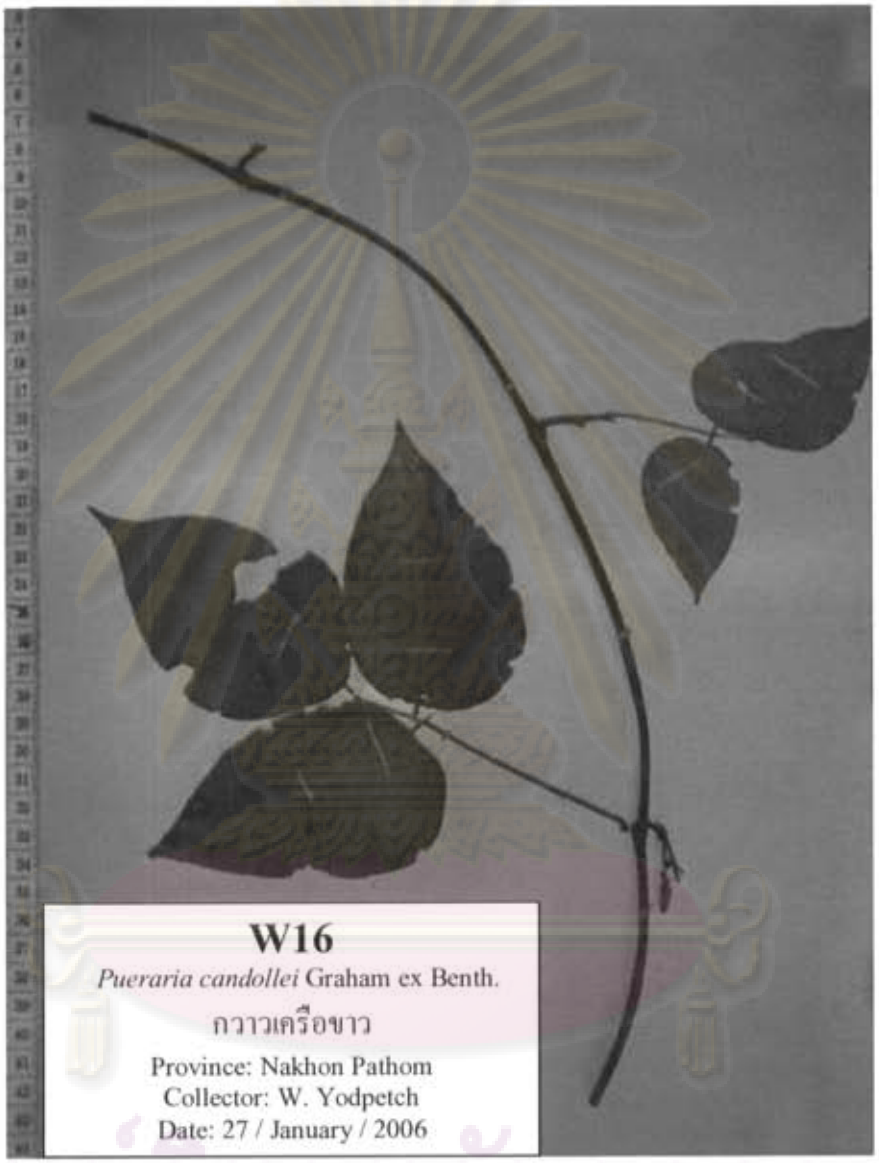

Figture A 6 *Pueraria candollei* Graham ex Benth. (W16)

ศูนย์วิทยทรัพยากร
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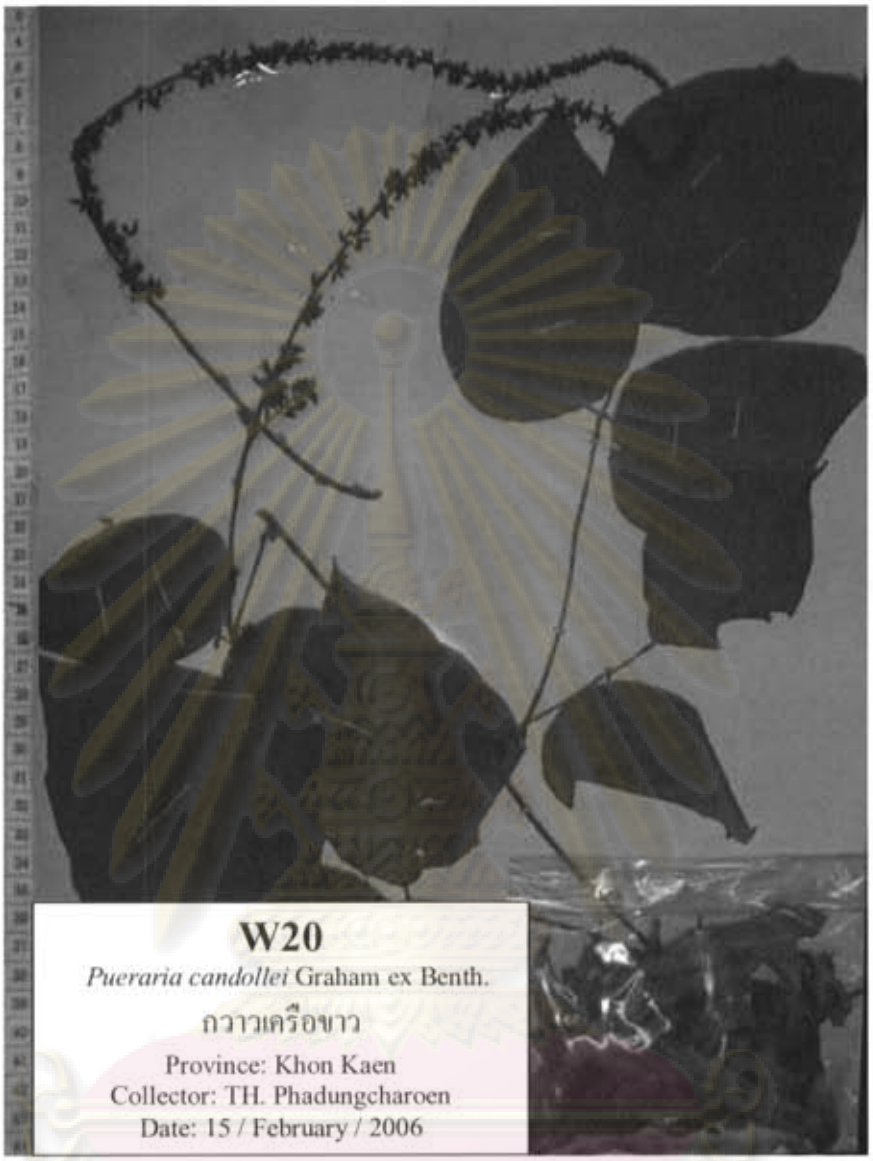
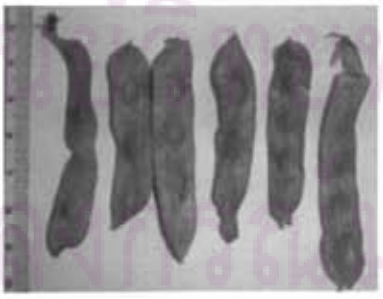


Figture A 7 *Pueraria candollei* Graham ex Benth. (W20)



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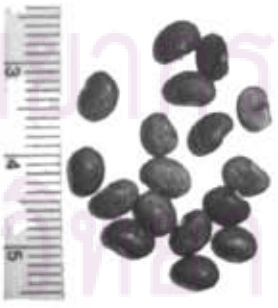

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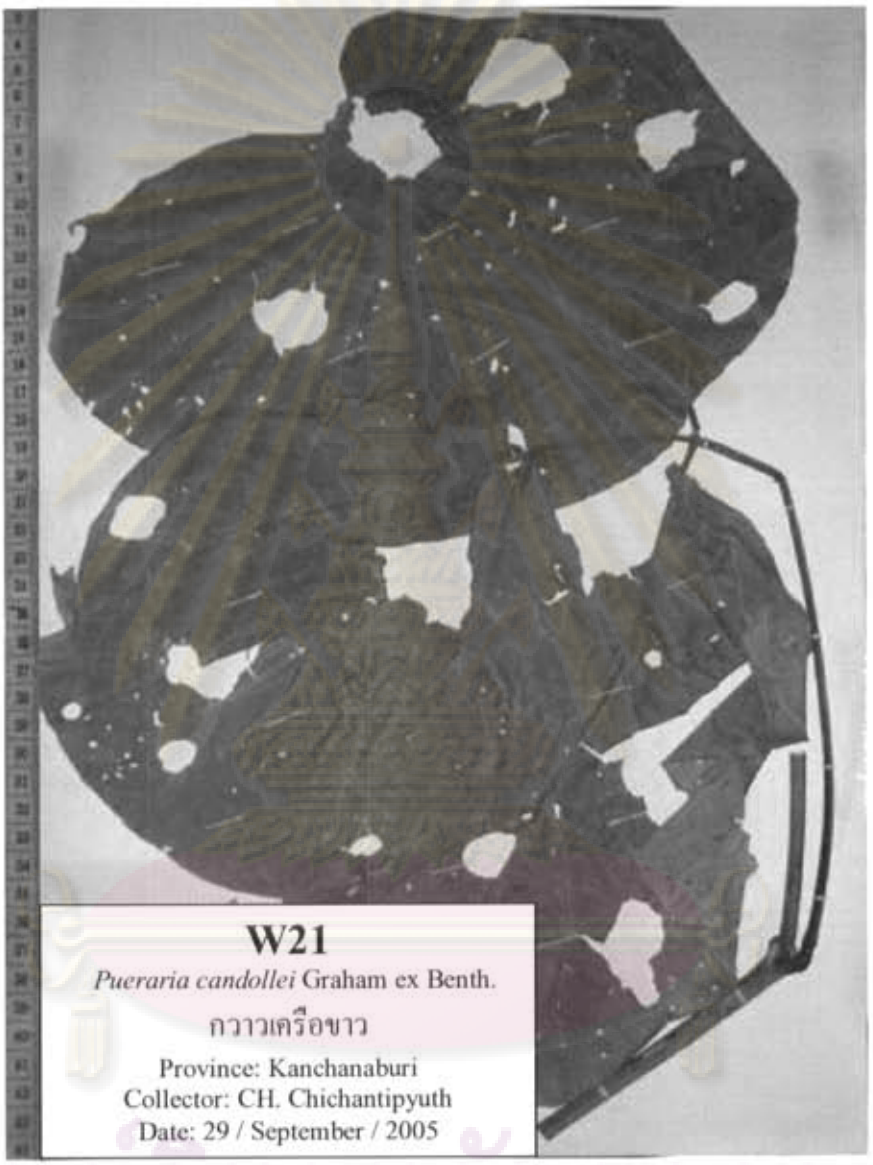

Figture A 8 *Pueraria candollei* Graham ex Benth. (W21)

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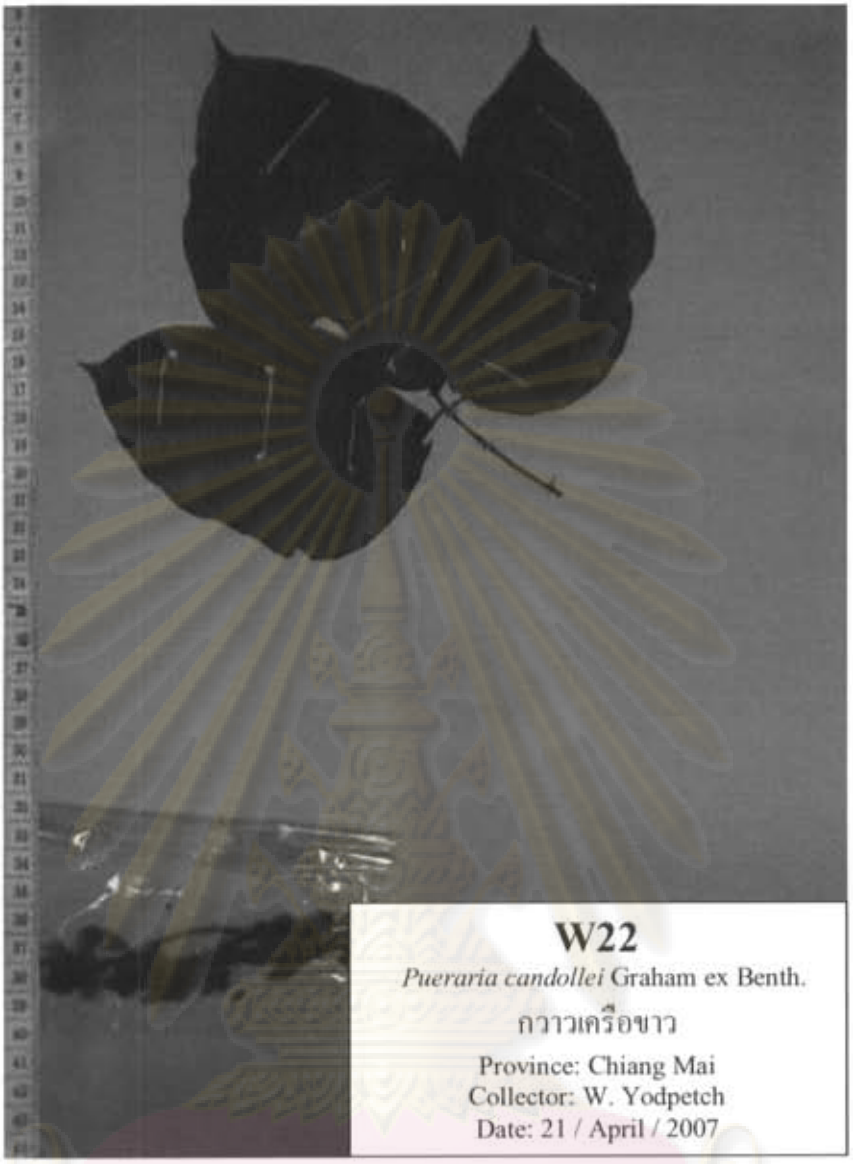

Figure A 9 *Pueraria candollei* Graham ex Benth. (W22)

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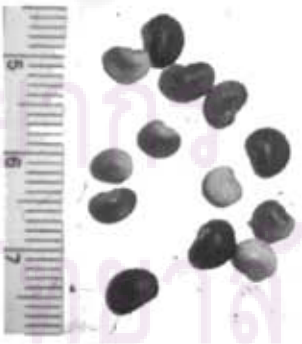

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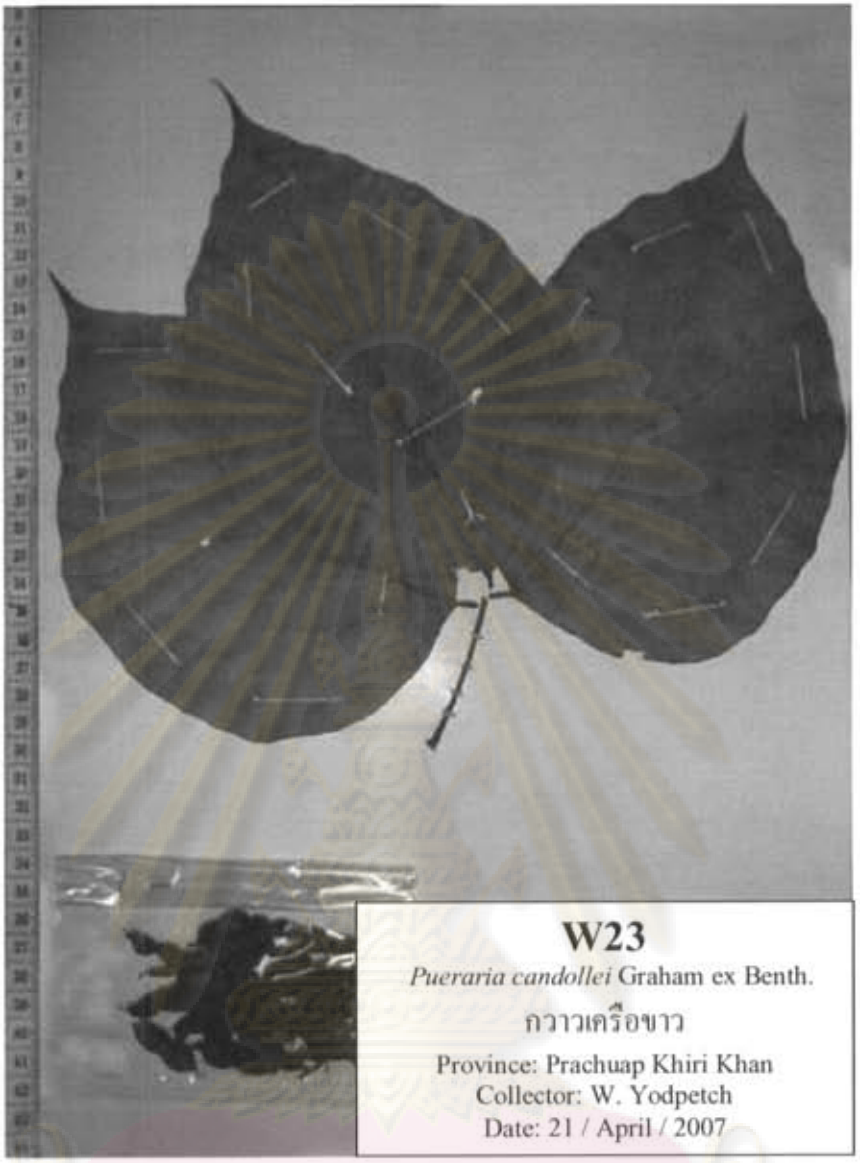

Figure A 10 *Pueraria candollei* Graham ex Benth. (W23)

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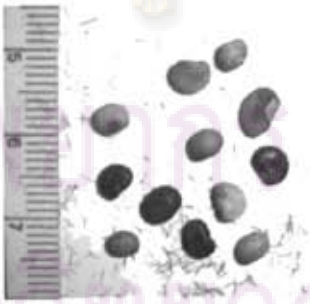

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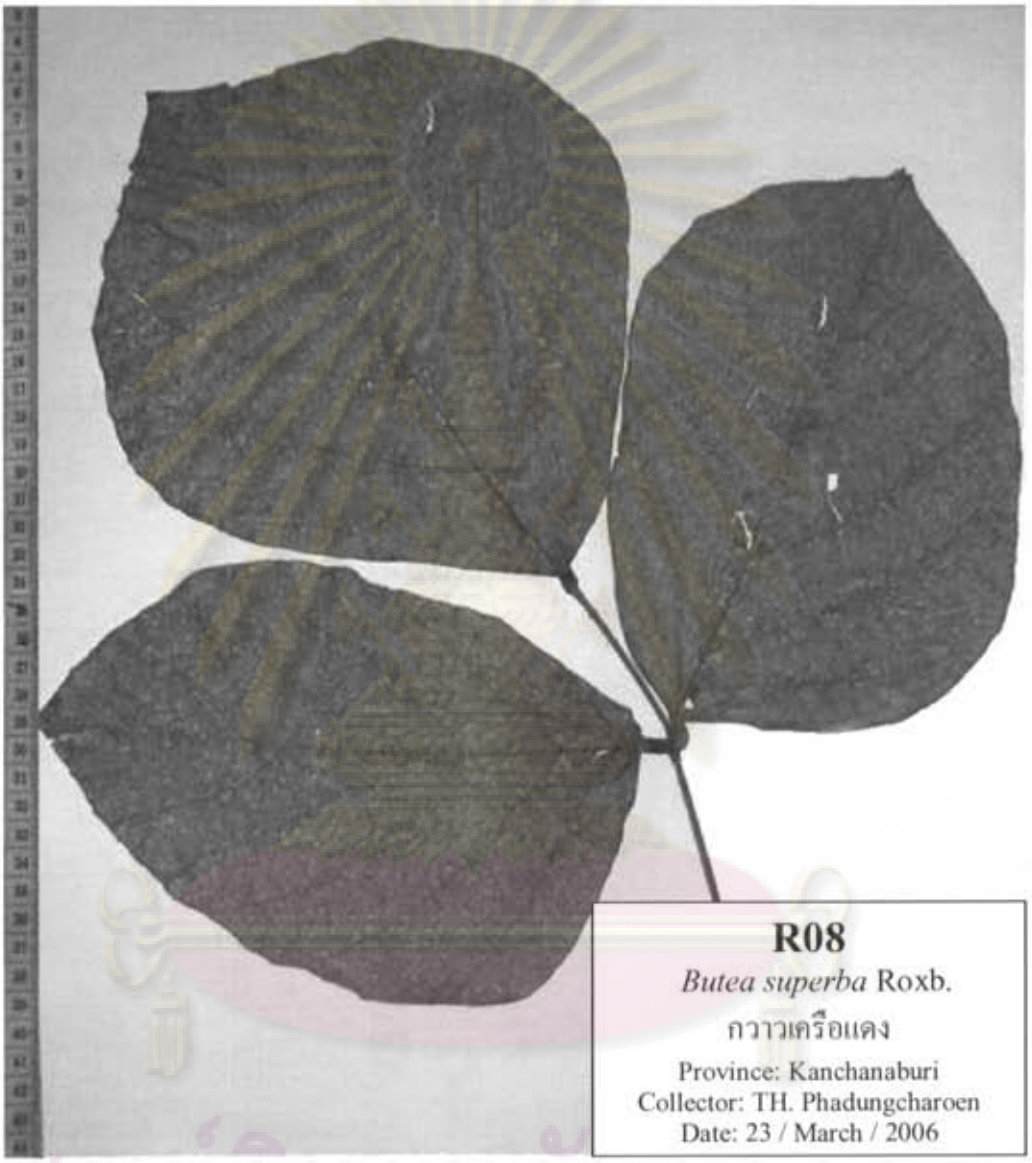

Figture A 11 *Butea superba* Roxb. (R08)

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Figture A 12 *Butea superba* Roxb. (R09)

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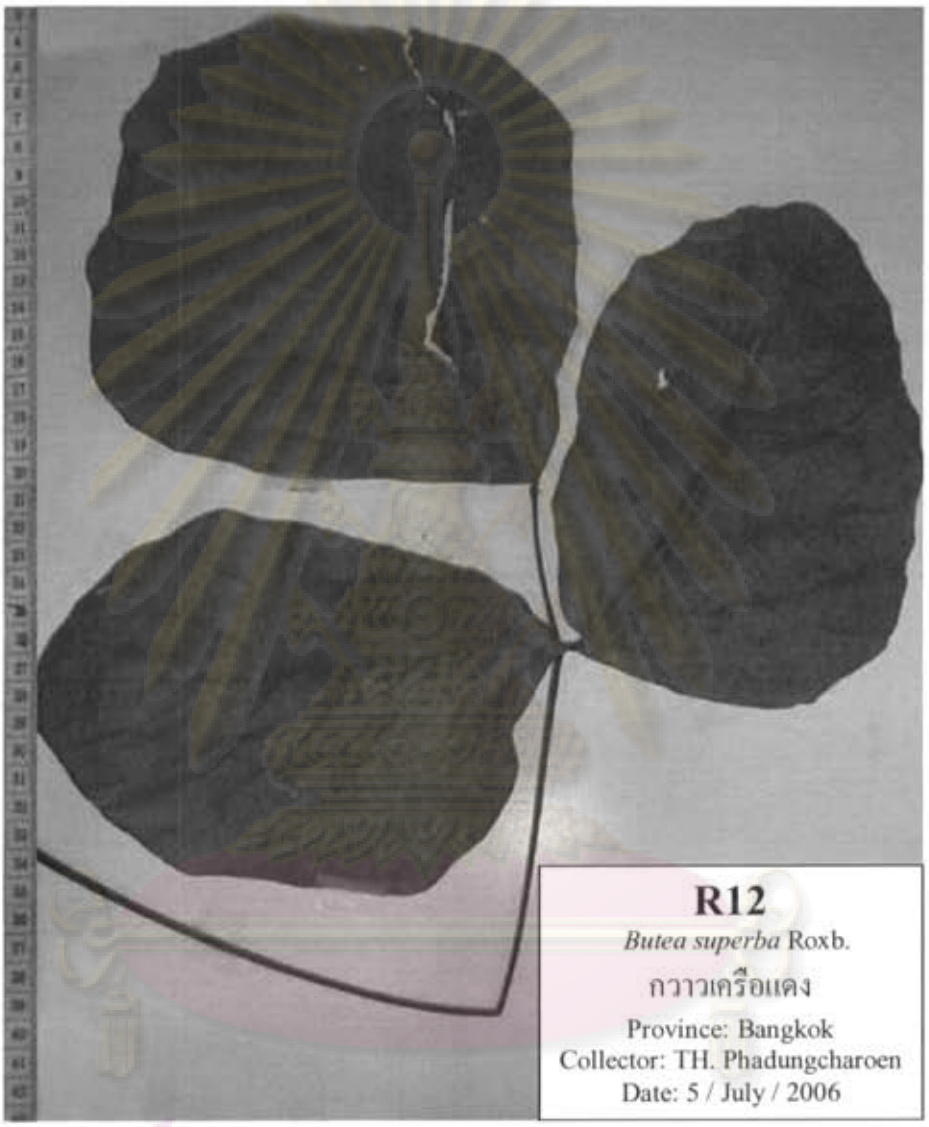

Figture A 13 *Butea superba* Roxb. (R12)

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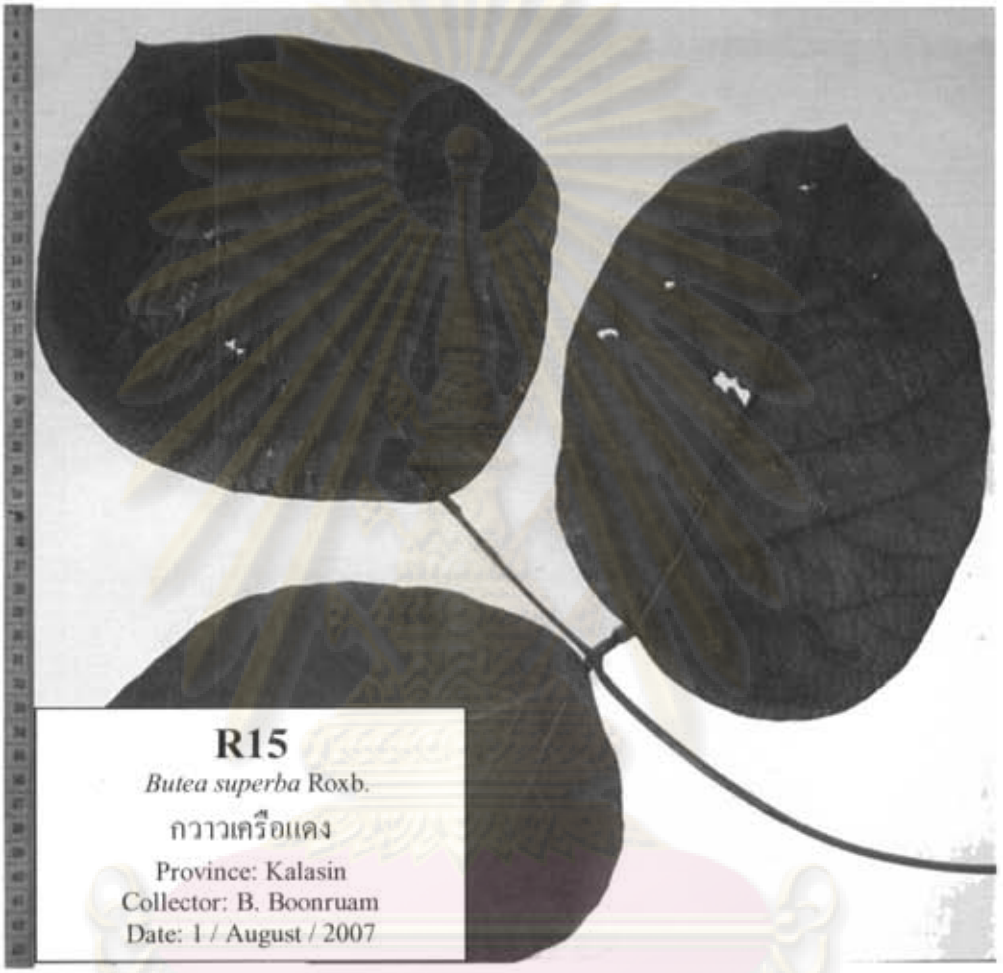

Figture A 14 *Butea superba* Roxb. (R15)

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APPENDIX B

Data of DNA sequences which were submitted to GenBank database

1. White Kwao Khruea (code no. W02, W03, and W21)

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ACCESSION   EU106106
VERSION
KEYWORDS    .
SOURCE      chloroplast Pueraria candollei
  ORGANISM  Pueraria candollei
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REFERENCE   1  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
TITLE       Sequence Analysis of matK Gene of Pueraria candollei and
            Butea superba and the Application of PCR-RFLP Genetic
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-AUG-2007) Pharmacognosy, Chulalongkorn
            University, Phayathai, Bangkok 10330, Thailand
COMMENT     Pueraria candollei was classified into Eukaryota;
            Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
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ศูนย์วิทยทรัพยากร
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ศูนย์วิทยทรัพยากร
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2. White Kwao Khruea (code no. W20)

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DEFINITION  Pueraria candollei maturase (matK) gene, complete cds;
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VERSION
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SOURCE      chloroplast Pueraria candollei
  ORGANISM  Pueraria candollei
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REFERENCE   1  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
TITLE       Sequence Analysis of matK Gene of Pueraria candollei and
            Butea superba and the Application of PCR-RFLP Genetic
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
  TITLE     Direct Submission
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            University, Phayathai, Bangkok 10330, Thailand
COMMENT     Pueraria candollei was classified into Eukaryota;
            Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
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121 gaaaaaaatg taggttataa caataaattt agtttactaa ttgtaaaacg gttaattact
181 cgaatgtatc aacagactca tttcatcatt tttactaacg attctaacaa aaatccttt
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241 aggggttata acaatcattt ttattctcaa ataatattag aaggttttgt tggcgtcgtg
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361 tcttataata atttgcgatc aattcattcc atttttccct ttttcgaaga taaactgatc
421 tatttaaatc atgagtcaga tatacgaata ccctatccta tccatctgga aatcttggtt
481 caaatccttc gatattggat aaaagatgtc tctttctttc atttattaag gttgttttt
541 tattactatt ataattggaa tagtcttttt actccaaaaa aatggatttc tactttttt
601 tcaaaaagta atccaagatt tttcttattc ctatataatt tatatgtacg ggaatatgaa
661 tctatctttc tttttctacg taacaaatcc tctcagttac ggttaaaata ttttcgcatt
721 ttttttgagc gaatttttt ctatgaaaaa atcgaacatc ttgtagaagt atctgttaag
781 gattgttcat ataccttatc attctttaag gatactttca tccattatgt tagatatcaa
841 ggaaaatcaa ttctggtttc aaagaatact cctcttttga taaataaatg gaaatactat
901 tttatctatt tatggcaatg tcattttgat atttggtctc gaccaggaac gatccagata
961 aaccaattct cccagcattc atttcacttt ttaggctatt ttttaagtat tcggctcaat
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1081aagcttgata caatagttcc aattattcct ctaattagat cattggctaa agcaaaattt
1141tgtaatgtat ttggtcatcc cattagtaag ccggtttggg ccaatttatc tgattttgat
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1261aaaaaaaaga gtttgtatca aataagatat atacttcggc tttcttgtat aaaaactttg
1321gctcgtaagc acaaaagtac tgcgcgcact tttttgaaaa gattaggttc agaaaaatta
1381ttggaatatt tctttacaga agaagaagat atttttttctt tgatttttcc aagaacttct
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1501gatttcgtca atcatttata a
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ศูนย์วิทยทรัพยากร
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3. White Kwao Khruea (code no. W01, W05, W22, and W23)

```
LOCUS       EU106108          1521 bp    DNA     linear  31-AUG-2007
DEFINITION  Pueraria candollei maturase (matK) gene, complete cds;
            plastid.
ACCESSION   EU106108
VERSION
KEYWORDS    .
SOURCE      chloroplast Pueraria candollei
  ORGANISM  Pueraria candollei
            Unclassified.
REFERENCE   1  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
TITLE       Sequence Analysis of matK Gene of Pueraria candollei and
            Butea superba and the Application of PCR-RFLP Genetic
            Marker for Identification
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-AUG-2007) Pharmacognosy, Chulalongkorn
            University, Phayathai, Bangkok 10330, Thailand
COMMENT     Pueraria candollei was classified into Eukaryota;
            Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
            eudicotyledons; rosids; eurosids I; Fabales; Fabaceae;
            Papilionoideae; Phaseoleae; Pueraria.

            woranui24@gmail.com.
FEATURES             Location/Qualifiers
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                     /organism="Pueraria candollei"
                     /organelle="plastid:chloroplast"
                     /mol_type="genomic DNA"
                     /country="Thailand"
                     /note="PCR_primers=fwd_name: Pue-F00,
                       rev_name: Pue-R02"
     gene            1..1521
                     /gene="matK"
     CDS             1..1521
                     /gene="matK"
                     /codon_start=1
                     /transl_table=11
                     /protein_id="PROT_1_ EU106108"

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QILRYWIKDVSFFHLLRLFFYYYYNWNSLFTPKKWISTFFSKSNPRFFLFLYNLYVRE
YESIFLFLRNKSSQLRLKYFRIFFERIFFYEKIEHLVEVSVKDCSYTLSFFKDTFIHY
VRYQGKSILVSKNTPLLINKWKYYFIYLWQCHFDIWSRPGTIQINQFSQHSFHFLGYF
LSIRLNLSVLRSQMLQNSFLIKIVMKKLDTIVPIIPLIRSLAKAKFCNVFGHPISKPV
WANLSDFDIIDRFLRICRNFSHYYNGSAKKKSLYQIRYILRLSCIKTLARKHKSTART
FLKRLGSEKLLEDFFTEEEDIFSLIFPRTSFTLQRLYRGRIWYLDILFRNDFVNHL"
BASE COUNT 480 a 232 c 218 g 591 t
ORIGIN
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 61 ccacttttt ttcgggaata tatttatgga ctagcttatg gtcatgggtc catttttgta
121 gaaaaaaatg taggttataa caataaattt agtttactaa ttgtaaaacg gttaattact
181 cgaatgtatc aacagactca tttcatcatt tttactaacg attctaacaa aaatcctttt
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241 aggggttata acaatcattt ttattctcaa ataatattag aaggttttgt tggcgtcgtg
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361 tcttataata atttgcgatc aattcattcc atttttccct ttttcgaaga taaactgata
421 tatttaaatc atgagtcaga tatacgaata ccctatccta tccatctgga aatcttggtt
481 caaatccttc gatattggat aaaagatgtc tctttctttc atttattaag gttgtttttt
541 tattactatt ataattggaa tagtcttttt actccaaaaa aatggatttc tactttttt
601 tcaaaaagta atccaagatt tttcttattc ctatataatt tatatgtacg ggaatatgaa
661 tctatctttc tttttctacg taacaaatcc tctcagttac ggttaaaata ttttcgcatt
721 ttttttgagc gaatttttt ctatgaaaaa atcgaacatc ttgtagaagt atctgttaag
781 gattgttcat ataccttatc attctttaag gatactttca tccattatgt tagatatcaa
841 ggaaaatcaa ttctggtttc aaagaatact cctcttttga taaataaatg gaaatactat
901 tttatctatt tatggcaatg tcattttgat atttggtctc gaccaggaac gatccagata
961 aaccaattct cccagcattc atttcacttt ttaggctatt ttttaagtat tcggctcaat
1021ctttcagtgc tacgaagtca gatgttacaa aattcatttc taataaaaat tgttatgaaa
1081aagcttgata caatagttcc aattattcct ctaattagat cattggctaa agcaaaattt
1141tgtaatgtat ttggtcatcc cattagtaag ccggtttggg ccaatttatc tgattttgat
1201attattgacc ggtttttgcg gatatgcaga aatttttctc attattacaa tggatccgca
1261aaaaaaaaga gtttgtatca aataagatat atacttcggc tttcttgtat aaaaactttg
1321gctcgtaagc acaaaagtac tgcgcgcact tttttgaaaa gattaggttc agaaaaatta
1381ttggaagatt tctttacaga agaagaagat atttttctt tgatttttcc aagaacttct
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1501gatttcgtca atcatttata a
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4. White Kwao Khruea (code no. W07)

```
LOCUS       EU106109          1521 bp    DNA     linear  31-AUG-2007
DEFINITION  Pueraria candollei maturase (matK) gene, complete cds;
            plastid.
ACCESSION   EU106109
VERSION
KEYWORDS    .
SOURCE      chloroplast Pueraria candollei
  ORGANISM  Pueraria candollei
            Unclassified.
REFERENCE   1  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
TITLE       Sequence Analysis of matK Gene of Pueraria candollei and
            Butea superba and the Application of PCR-RFLP Genetic
            Marker for Identification
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-AUG-2007) Pharmacognosy, Chulalongkorn
            University, Phayathai, Bangkok 10330, Thailand
COMMENT     Pueraria candollei was classified into Eukaryota;
            Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
            eudicotyledons; rosids; eurosids I; Fabales; Fabaceae;
            Papilionoideae; Phaseoleae; Pueraria.

            woranui24@gmail.com.
FEATURES             Location/Qualifiers
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                     /organelle="plastid:chloroplast"
                     /mol_type="genomic DNA"
                     /country="Thailand"
                     /note="PCR_primers=fwd_name: Pue-F00,
                       rev_name: Pue-R02"
     gene            1..1521
                     /gene="matK"
     CDS             1..1521
                     /gene="matK"
                     /codon_start=1
                     /transl_table=11
                     /protein_id="PROT_1_ EU106109"

/translation="MEEYRAYLELHRSRHQDTLYPLFFREYIYGLAYGHGSIFVEKNV
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LFSLQLFISSLRELEIVKSYNNLRSIHSIFPFFEDKLIYLNHESDIRIPYPIHLEILV
QILRYWIKDVSFFHLLRLFFYYYYNWNSLFTPKKWIYTFFSKSNPRFFLFLYNLYVRE
YESIFLFLRNKSSQLRLKYFRIFFERIFFYEKIEHLVEVSVKDCSYTLSFFKDTFIHY
VRYQGKSILVSKNTPLLINKWKYYFIYLWQCHFDIWSRPGTIQINQFSQHSFHFLGYF
LSIRLNLSVLRSQMLQNSFLIKIVMKKLDTIVPIIPLIRSLAKAKFCNVFGHPISKPV
WANLSDFDIIDRFLRICRNFSHYYNGSAKKKSLYQIRYILRLSCIKTLARKHKSTART
FLKRLGSEKLLEDFFTEEEDIFSLIFPRTSFTLQRLYRGRIWYLDILFRNDFVNHL"
BASE COUNT 481 a 231 c 218 g 591 t
ORIGIN
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 61 ccacttttt ttcgggaata tatttatgga ctagcttatg gtcatgggtc cattttgta
121 gaaaaaaatg taggttataa caataaattt agtttactaa ttgtaaaacg gttaattact
181 cgaatgtatc aacagactca tttcatcatt tttactaacg attctaacaa aaatccttt
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241 aggggttata acaatcattt ttattctcaa ataatattag aaggttttgt tggcgtcgtg
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361 tcttataata atttgcgatc aattcattcc atttttccct ttttcgaaga taaactgata
421 tatttaaatc atgagtcaga tatacgaata ccctatccta tccatctgga aatcttggtt
481 caaatccttc gatattggat aaaagatgtc tctttctttc atttattaag gttgtttttt
541 tattactatt ataattggaa tagtcttttt actccaaaaa aatggattta tactttttt
601 tcaaaaagta atccaagatt tttcttattc ctatataatt tatatgtacg ggaatatgaa
661 tctatctttc tttttctacg taacaaatcc tctcagttac ggttaaaata ttttcgcatt
721 ttttttgagc gaatttttt ctatgaaaaa atcgaacatc ttgtagaagt atctgttaag
781 gattgttcat ataccttatc attctttaag gatactttca tccattatgt tagatatcaa
841 ggaaaatcaa ttctggtttc aaagaatact cctcttttga taaataaatg gaaatactat
901 tttatctatt tatggcaatg tcattttgat atttggtctc gaccaggaac gatccagata
961 aaccaattct cccagcattc atttcacttt ttaggctatt ttttaagtat tcggctcaat
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1321gctcgtaagc acaaaagtac tgcgcgcact tttttgaaaa gattaggttc agaaaaatta
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ศูนย์วิทยทรัพยากร
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5. White Kwao Khruea (code no. W16)

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LOCUS       EU106110          1521 bp    DNA     linear  31-AUG-2007
DEFINITION  Pueraria candollei maturase (matK) gene, complete cds;
            plastid.
ACCESSION   EU106110
VERSION
KEYWORDS    .
SOURCE      chloroplast Pueraria candollei
  ORGANISM  Pueraria candollei
            Unclassified.
REFERENCE   1  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
TITLE       Sequence Analysis of matK Gene of Pueraria candollei and
            Butea superba and the Application of PCR-RFLP Genetic
            Marker for Identification
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-AUG-2007) Pharmacognosy, Chulalongkorn
            University, Phayathai, Bangkok 10330, Thailand
COMMENT     Pueraria candollei was classified into Eukaryota;
            Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
            eudicotyledons; rosids; eurosids I; Fabales; Fabaceae;
            Papilionoideae; Phaseoleae; Pueraria.

            woranui24@gmail.com.
FEATURES             Location/Qualifiers
     source          1..1521
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                     /organelle="plastid:chloroplast"
                     /mol_type="genomic DNA"
                     /country="Thailand"
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                       rev_name: Pue-R02"
     gene            1..1521
                     /gene="matK"
     CDS             1..1521
                     /gene="matK"
                     /codon_start=1
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                     /protein_id="PROT_1_ EU106110"
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QILRYWIKDVSFFHLLRLFFYYYYNWNSLFTPKKWISTFFSKSNPRFFLFLYNLYVRE
YESIFLFLRNKSSQLRLKYFRIFFERIFFYEKIEHLVEVSVKDCSYTLSFFKDTFIHY
VRYQGKSILVSKNTPLLINKWKYYFIYLWQCHFDIWSRPGTIQINQFSQHSFHFLGYF
LSIRLNLSVLRSQMLQNSFLIKIVMKKLDTIVPIIPLIRSLAKAKFCNVFGHPISKPV
WANLSDFDIIDRFLRICRNFSHYYNGSAKKKSLYQIRYILRLSCIKTLARKHKSTART
FLKRLGSEKLLEDFFTEEEDIFSLIFPRTSFTLQRLYRGRIWYLDILFRNDFVNHL"
BASE COUNT 481 a 233 c 216 g 591 t
ORIGIN
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121 gaaaaaaatg taggttataa caataaattt agtttactaa ttgtaaaaca gttaattact
181 cgaatgtatc aacagactca tttcatcatt tttactaacg attctaacaa aaatcctttt
241 aggggttata acaatcattt ttattctcaa ataatattag aaggttttct tggcgtcgtg
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421 tatttaaatc atgagtcaga tatacgaata ccctatccta tccatctgga aatcttggtt
481 caaatccttc gatattggat aaaagatgtc tctttctttc atttattaag gttgtttttt
541 tattactatt ataattggaa tagtcttttt actccaaaaa aatggatttc tactttttt
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ศูนย์วิทยทรัพยากร
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6. Red Kwao Khruea (code no. R08, R09, R12, and R15)

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LOCUS       EU106111          1527 bp    DNA     linear  31-AUG-2007
DEFINITION  Butea superba maturase (matK) gene, complete cds;
            plastid.
ACCESSION   EU106111
VERSION
KEYWORDS    .
SOURCE      chloroplast Butea superba
  ORGANISM  Butea superba
            Unclassified.
REFERENCE   1  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
TITLE       Sequence Analysis of matK Gene of Pueraria candollei and
            Butea superba and the Application of PCR-RFLP Genetic
            Marker for Identification
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1521)
  AUTHORS   Sukrong,S., Yodpetch,W. and Phadungcharoen,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-AUG-2007) Pharmacognosy, Chulalongkorn
            University, Phayathai, Bangkok 10330, Thailand
COMMENT     Pueraria candollei was classified into Eukaryota;
            Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
            eudicotyledons; rosids; eurosids I; Fabales; Fabaceae;
            Papilionoideae; Phaseoleae; Butea.

            woranui24@gmail.com.
FEATURES             Location/Qualifiers
     source          1..1527
                     /organism=" Butea superba "
                     /organelle="plastid:chloroplast"
                     /mol_type="genomic DNA"
                     /country="Thailand"
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                       F20,rev_name: matK-BMP-R-21"
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     CDS             1..1527
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QILRHWIKDVSFFHLLRLFFYYYCNWNSLFTPKKWISTFFSKRNPRFFLFLFLYNLYV
REYESIFLFLRNKSSQLLLKYFRVFFERIFFYEKIEHIVEVFAKDFSYTLSFFKDPFI
HYVRYQGKSILVSKNTPLLINKWKYYFIYLWQCHFDIWSQPGTIHRNQLYQHSFHFLG
YFLSIRLNLSVVRSQMLQNSFLIKIVMKKLDTIVPIIPLISSLAKAKFCNVLGHPISK
PVWANLSDFDIIERFLRICRNFSHYYNGSEKKKRLYRIRYILRLSCIKTLARKHKSTV
RTFLKRLGSEKLLEEFFTEEEDIFSFIFPRTSFTLQRLYIGRIWYLDILFRNDLINHS"
BASE COUNT 482 a 224 c 215 g 606 t
ORIGIN
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121 gaaaaaaatg taggttataa caaaaaattt agtttactaa ttgtaaaacg tttaattact
181 cgaatgtatc aacagactca tttgatcatt tttgctaatg attctaacaa aaatcctttt
241 tggggttata ataataattt ttattctcaa ataatattcg aaggttttgt tgtcgtcgtg
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481 caaatccttc gacattggat aaaagatgtt tctttctttc atttattaag attgtttttt
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721 cgcgtttttt ttgagcgaat ttttttctat gaaaaaatag aacatattgt agaagtattt
781 gctaaggatt tttcctatac cttatcattc ttcaaggatc ctttcatcca ttatgttaga
841 tatcaaggaa aatcaattct ggtttcaaag aatactcctc ttttgataaa taaatggaaa
901 tactatttta tttatttatg gcaatgtcat tttgatattt ggtctcaacc aggaacgatc
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1441acttctttta ctttgcagag gttatatata ggtcggattt ggtatttgga tattcttttc
1501agaaacgatt tgatcaatca ttcataa
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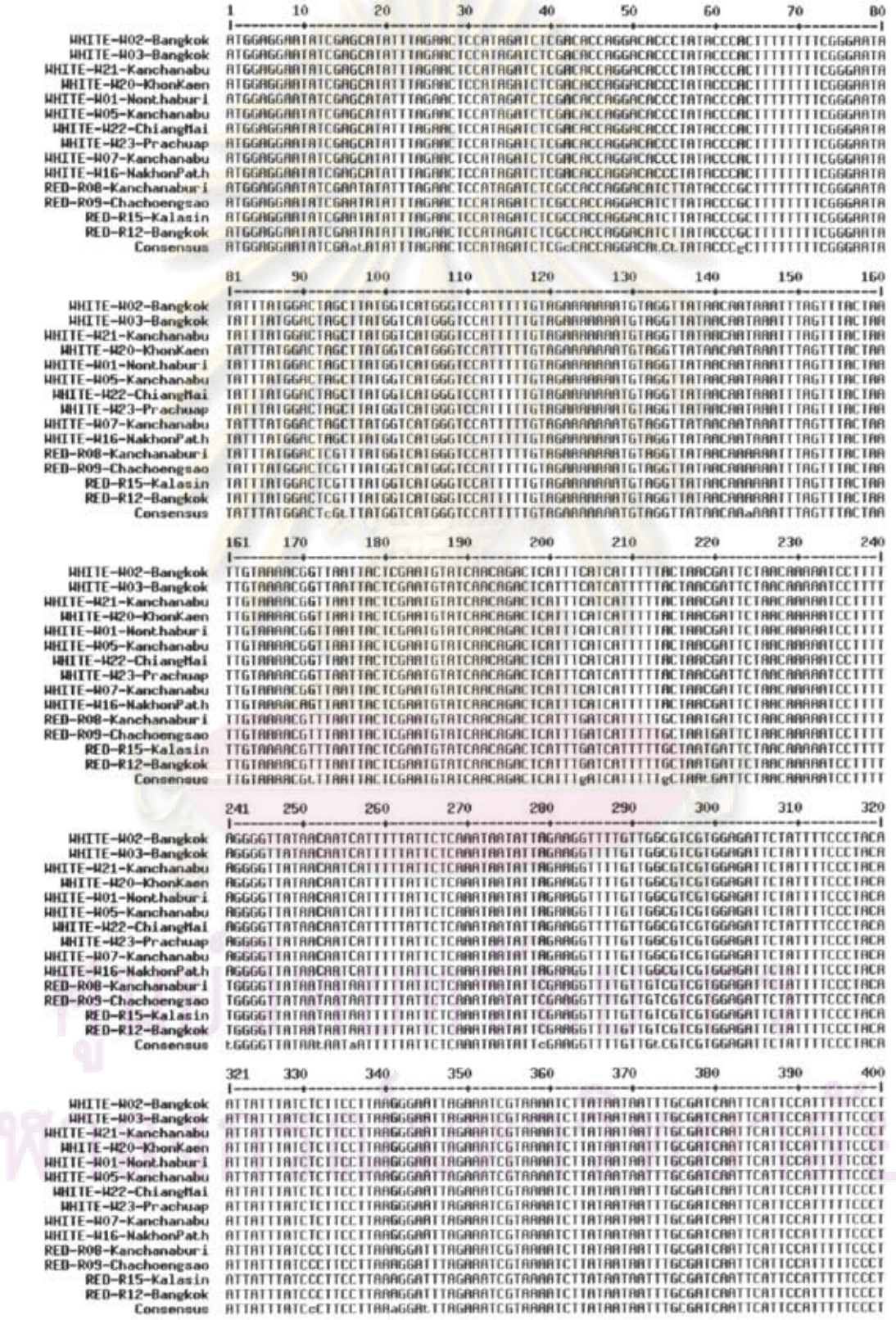
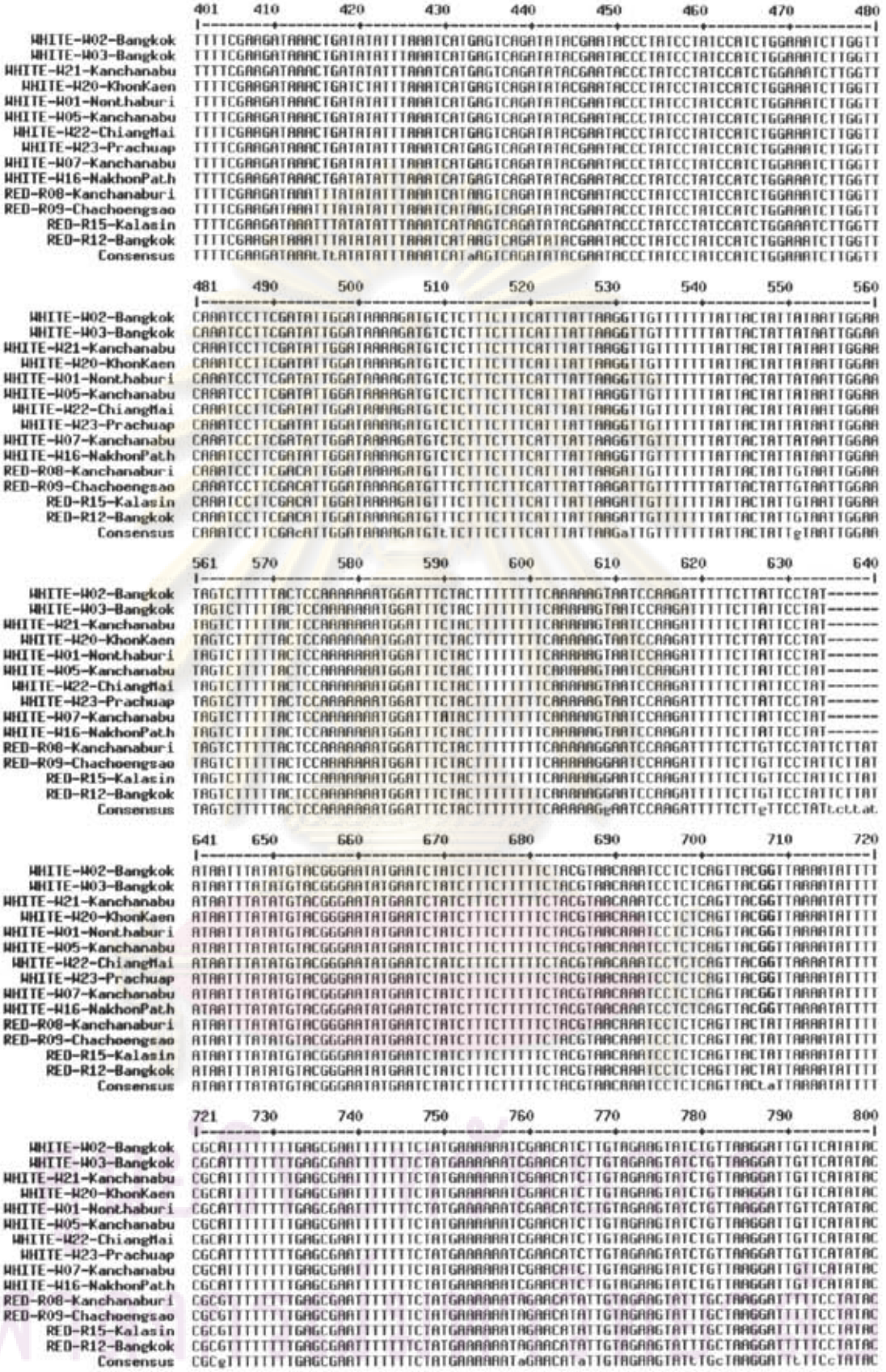
ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX C

*mat*K Gene Sequence Alignment of White Kwao Khruea and Red Kwao Khruea

The red and blue nucleotides are high and low consensus sequences, respectively.

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WHITE-W03-Bangkok TTTTCGAAGATAAACTGATATATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
WHITE-W21-Kanchanabu TTTTCGAAGATAAACTGATATATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
WHITE-W20-KhonKaen TTTTCGAAGATAAACTGATCTATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
WHITE-W01-Nonthaburi TTTTCGAAGATAAACTGATATATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
WHITE-W05-Kanchanabu TTTTCGAAGATAAACTGATATATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
WHITE-W22-ChiangMai TTTTCGAAGATAAACTGATATATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
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WHITE-W07-Kanchanabu TTTTCGAAGATAAACTGATATATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
WHITE-W16-NakhonPath TTTTCGAAGATAAACTGATATATTTAAATCATGAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
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RED-R09-Chachoengsao TTTTCGAAGATAAATTTATATATTTAAATCATAAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
RED-R15-Kalasin TTTTCGAAGATAAATTTATATATTTAAATCATAAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
RED-R12-Bangkok TTTTCGAAGATAAATTTATATATTTAAATCATAAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
Consensus TTTTCGAAGATAAAKTtATATATTTAAATCATaAGTCAGATATACGAATACCCTATCCTATCCATCTGGAAATCTTGGTT
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WHITE-W16-NakhonPath CAAATCCTTCGATATTGGATAAAAGATGTCTCTTTCTTTCATTTATTAAGGTTGTTTTTTTATTACTATTATAATTGGAA
RED-R08-Kanchanaburi CAAATCCTTCGACATTGGATAAAAGATGTTTCTTTCTTTCATTTATTAAGATTGTTTTTTTATTACTATTGTAATTGGAA
RED-R09-Chachoengsao CAAATCCTTCGACATTGGATAAAAGATGTTTCTTTCTTTCATTTATTAAGATTGTTTTTTTATTACTATTGTAATTGGAA
RED-R15-Kalasin CAAATCCTTCGACATTGGATAAAAGATGTTTCTTTCTTTCATTTATTAAGATTGTTTTTTTATTACTATTGTAATTGGAA
RED-R12-Bangkok CAAATCCTTCGACATTGGATAAAAGATGTTTCTTTCTTTCATTTATTAAGATTGTTTTTTTATTACTATTGTAATTGGAA
Consensus CAAATCCTTCGAcATTGGATAAAAGATGTcTCTTTCTTTCATTTATTAAGaTTGTTTTTTTATTACTATTgTAATTGGAA
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WHITE-W03-Bangkok TAGTCTTTTTACTCCAAAAAAATGGATTTCTACTTTTTTTTCAAAAAGTAATCCAAGATTTTTCTTATTCCTAT------
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WHITE-W01-Nonthaburi TAGTCTTTTTACTCCAAAAAAATGGATTTCTACTTTTTTTTCAAAAAGTAATCCAAGATTTTTCTTATTCCTAT------
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RED-R12-Bangkok CGCGTTTTTTTTGAGCGAATTTTTTTCTATGAAAAAATAGAACATATTGTAGAAGTATTTGCTAAGGATTTTTCCTATAC
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RED-R12-Bangkok     CTTATCATTCTTCAAGGATCCTTTCATCCATTATGTTAGATATCAAGGAAAATCAATTCTGGTTTCAAAGAATACTCCTC
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WHITE-W07-Kanchanabu TTTTGATAAATAAATGGAAATACTATTTTATCTATTTATGGCAATGTCATTTTGATATTTGGTCTCGACCAGGAACGATC
WHITE-W16-NakhonPath TTTTGATAAATAAATGGAAATACTATTTTATCTATTTATGGCAATGTCATTTTGATATTTGGTCTCGACCAGGAACGATC
RED-R08-Kanchanaburi TTTTGATAAATAAATGGAAATACTATTTTATTTATTTATGGCAATGTCATTTTGATATTTGGTCTCAACCAGGAACGATC
RED-R09-Chachoengsao TTTTGATAAATAAATGGAAATACTATTTTATTTATTTATGGCAATGTCATTTTGATATTTGGTCTCAACCAGGAACGATC
RED-R15-Kalasin     TTTTGATAAATAAATGGAAATACTATTTTATTTATTTATGGCAATGTCATTTTGATATTTGGTCTCAACCAGGAACGATC
RED-R12-Bangkok     TTTTGATAAATAAATGGAAATACTATTTTATTTATTTATGGCAATGTCATTTTGATATTTGGTCTCAACCAGGAACGATC
Consensus           TTTTGATAAATAAATGGAAATACTATTTTATtTATTTATGGCAATGTCATTTTGATATTTGGTCTCaACCAGGAACGATC

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WHITE-W21-Kanchanabu CAGATAAACCAATTCTCCCAGCATTCATTTCACTTTTTAGGCTATTTTTTAAGTATTCGGCTCAATCTTTCAGTGCTACG
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WHITE-W22-ChiangMai CAGATAAACCAATTCTCCCAGCATTCATTTCACTTTTTAGGCTATTTTTTAAGTATTCGGCTCAATCTTTCAGTGCTACG
WHITE-W23-Prachuap  CAGATAAACCAATTCTCCCAGCATTCATTTCACTTTTTAGGCTATTTTTTAAGTATTCGGCTCAATCTTTCAGTGCTACG
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WHITE-W16-NakhonPath CAGATAAACCAATTCTCCCAGCATTCATTTCACTTTTTAGGCTATTTTTTAAGTATTCGGCTCAATCTTTCAGTGCTACG
RED-R08-Kanchanaburi CATAGAAACCAATTATACCAGCATTCATTTCACTTTTTGGGCTATTTTTTAAGTATTCGGCTAAATCTTTCAGTGGTACG
RED-R09-Chachoengsao CATAGAAACCAATTATACCAGCATTCATTTCACTTTTTGGGCTATTTTTTAAGTATTCGGCTAAATCTTTCAGTGGTACG
RED-R15-Kalasin     CATAGAAACCAATTATACCAGCATTCATTTCACTTTTTGGGCTATTTTTTAAGTATTCGGCTAAATCTTTCAGTGGTACG
RED-R12-Bangkok     CATAGAAACCAATTATACCAGCATTCATTTCACTTTTTGGGCTATTTTTTAAGTATTCGGCTAAATCTTTCAGTGGTACG
Consensus           CATAgAAACCAATTaTaCCAGCATTCATTTCACTTTTTgGGCTATTTTTTAAGTATTCGGCTaAATCTTTCAGTGgTACG

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WHITE-W03-Bangkok   AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
WHITE-W21-Kanchanabu AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
WHITE-W20-KhonKaen  AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
WHITE-W01-Nonthaburi AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
WHITE-W05-Kanchanabu AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
WHITE-W22-ChiangMai AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
WHITE-W23-Prachuap  AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
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WHITE-W16-NakhonPath AAGTCAGATGTTACAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
RED-R08-Kanchanaburi AAGTCAAATGTTGCAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTGA
RED-R09-Chachoengsao AAGTCAAATGTTGCAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTGA
RED-R15-Kalasin     AAGTCAAATGTTGCAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTGA
RED-R12-Bangkok     AAGTCAAATGTTGCAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTGA
Consensus           AAGTCAaATGTTgCAAAATTCATTTCTAATAAAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTgA

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WHITE-W20-KhonKaen  TTAGATCATTGGCTAAAGCAAAATTTTGTAATGTATTTGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
WHITE-W01-Nonthaburi TTAGATCATTGGCTAAAGCAAAATTTTGTAATGTATTTGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
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WHITE-W22-ChiangMai TTAGATCATTGGCTAAAGCAAAATTTTGTAATGTATTTGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
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WHITE-W07-Kanchanabu TTAGATCATTGGCTAAAGCAAAATTTTGTAATGTATTTGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
WHITE-W16-NakhonPath TTAGATCATTGGCTAAAGCAAAATTTTGTAATGTATTTGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
RED-R08-Kanchanaburi TTAGTTCATTGGCTAAAGCAAAATTTTGTAATGTGTTGGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
RED-R09-Chachoengsao TTAGTTCATTGGCTAAAGCAAAATTTTGTAATGTGTTGGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
RED-R15-Kalasin     TTAGTTCATTGGCTAAAGCAAAATTTTGTAATGTGTTGGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
RED-R12-Bangkok     TTAGTTCATTGGCTAAAGCAAAATTTTGTAATGTGTTGGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
Consensus           TTAGtTCATTGGCTAAAGCAAAATTTTGTAATGTgTTgGGTCATCCCATTAGTAAGCCGGTTTGGGCCAATTTATCTGAT
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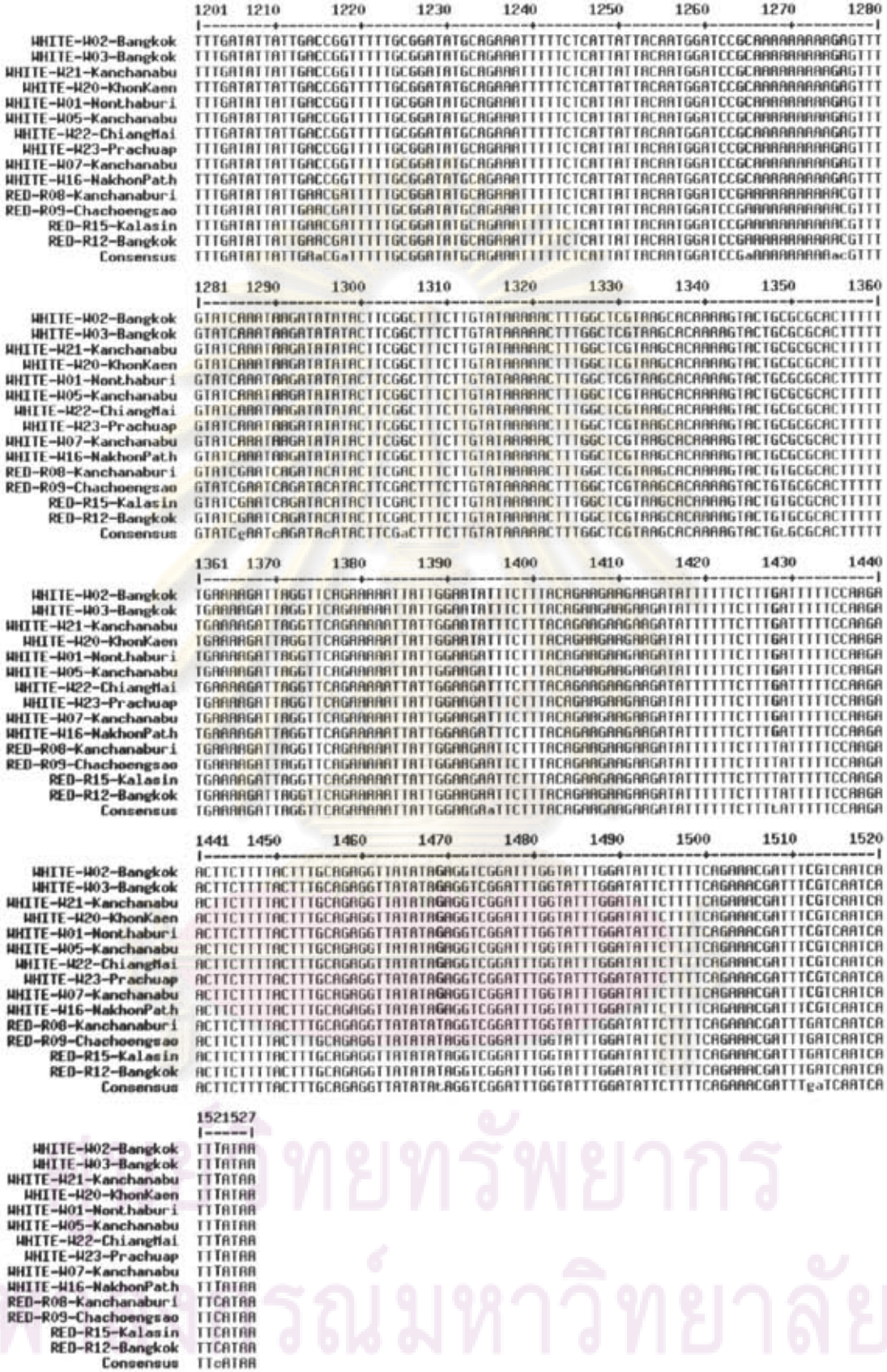

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Poster Presentation

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