มอร์โฟเมตรีของใบ ความแปรผันทางพันธุกรรม และวงศ์วานทางวิวัฒนาการของกวาวเครือแดง *Butea superba* ในประเทศไทย

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LEAF MORPHOMETRY, GENETIC VARIATION, AND PHYLOGENY OF *Butea superba* IN THAILAND

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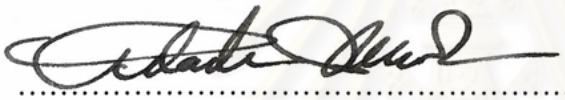

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จิรัฏติกาล แก้วเมืองมูล: มอร์โฟเมตรีของใบ ความแปรผันทางพันธุกรรม และวงศ์วานทางวิวัฒนาการของกวาวเครือแดง *Butea superba* ในประเทศไทย (LEAF MORPHOMETRY, GENETIC VARIATION, AND PHYLOGENY OF RED KWAO KRUA *Butea superba* IN THAILAND) อ.ที่ปรึกษา: ผศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, อ.ที่ปรึกษาร่วม: รศ. ดร. วิชัย เชิดชีวศาสตร์, 125 หน้า

กวาวเครือแดง *Butea superba* Roxb. เป็นพืชสมุนไพรไทยในกลุ่มพืชตระกูลถั่ว รากมีลักษณะเป็นหัวมีประโยชน์ในการใช้เสริมสมรรถภาพทางเพศของเพศชายได้ ในงานวิจัยนี้ได้ทำการวิเคราะห์ทางมอร์โฟเมตรีและทางพันธุกรรมของกวาวเครือแดงจากแหล่งต่างๆ ทำการเก็บตัวอย่างใบที่โตเต็มที่จาก 29 แหล่ง เพื่อการวิเคราะห์ทางด้านมอร์โฟเมตรีของใบ และเก็บใบจาก 34 แหล่ง เพื่อการวิเคราะห์ทางด้านพันธุกรรม ในส่วนของการวิเคราะห์ทางมอร์โฟเมตรีได้นำค่าพารามิเตอร์มา 9 ค่าเพื่อใช้ในการวัด ซึ่งประกอบไปด้วย ความยาวก้านใบ (PL), เส้นผ่านศูนย์กลางของก้านใบ (PD), ความยาวก้านใบประกอบ (RL), ความยาวข้อใบ (PLL), ความยาวของใบปลาย (TLL), ความกว้างของใบปลาย (TLB), ความยาวหูใบ (SPL), มุมองศาที่ฐานของใบปลาย (AB°) และจำนวนคู่ของเส้นใบที่ใบปลาย (NPV) นำผลของการวัดมาวิเคราะห์ด้วยวิธี factor analysis และจัดกลุ่มด้วยวิธี cluster analysis จากการวิเคราะห์ด้วย factor analysis ได้ค่าพารามิเตอร์ที่เหมาะสมต่อการทดลองเพียง 7 ค่าจากทั้งหมด 9 ค่า และนำมาจัดกลุ่มได้ 3 factor เมื่อพิจารณาจากการจัดกลุ่มด้วยการพลอตกราฟของ factor score ไม่สามารถจัดกลุ่มสายพันธุ์กวาวเครือแดงในประเทศไทยได้อย่างชัดเจน โดยใช้ค่าพารามิเตอร์ดังกล่าว นอกจากนี้พบว่าผลของเดนโดรแกรมที่ได้จาก cluster analysis ให้ผลที่สอดคล้องกับกราฟของ factor score โดยไม่สามารถแยกสายพันธุ์กวาวเครือแดงได้อย่างชัดเจน อย่างไรก็ตามพบว่าผลของ correlation ของ factor score กับละติจูดและลองทิจูดแสดงให้เห็นถึง clinal pattern ของความยาวของใบกวาวเครือแดงในประเทศไทย กล่าวคือขนาดของใบจะมีความยาวเพิ่มขึ้นจากทิศเหนือไปทิศใต้ใน factor ที่ 1 และมีความยาวลดลงจากทิศเหนือไปทิศใต้ใน factor ที่ 2

ในส่วนของการวิเคราะห์ทางพันธุกรรม ทำการศึกษาความแปรผันที่บริเวณ *rbc*L, *trn*LF-cd และ *trn*LF-cf โดยทำการเพิ่มปริมาณลำดับเบสในบริเวณดังกล่าวโดยเทคนิคพีซีอาร์ ได้ขนาดผลิตภัณฑ์ที่ความยาว 300, 550 และ 1,000 bp ตามลำดับ หลังจากทำการหาลำดับเบสของผลิตภัณฑ์ จึงทำการจัดกลุ่มโดยใช้ maximum parsimony (MP) และวิธี neighbor-joining วงศ์วานทางวิวัฒนาการของ *rbc*L แสดงให้เห็นถึงความแปรผันทางวิวัฒนาการของสายพันธุ์ของกวาวเครือแดงในประเทศไทยต่ำ ส่วนวงศ์วานทางวิวัฒนาการของ *trn*LF-cd และ *trn*LF-cf แสดงให้เห็นถึงความแปรผันทางวิวัฒนาการของสายพันธุ์ของกวาวเครือแดงในประเทศไทยสูง นอกจากนั้นยังได้ทำการวิเคราะห์ความแปรผันทางพันธุกรรมโดยใช้เทคนิคอาร์เอพีดี ซึ่งพบว่าผลการทดลองที่ได้สอดคล้องกับผลของการวิเคราะห์โดยการใช้ลำดับเบสของบริเวณ *trn*LF-cd และ *trn*LF-cf สามารถสรุปได้ว่าสายพันธุ์ของกวาวเครือแดงในประเทศไทยมีความหลากหลายทางพันธุกรรมสูง

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JIRATTIKARN KAEWMUANGMOON: LEAF MORPHOMETRY, GENETIC VARIATION, AND PHYLOGENY OF RED KWAO KRUA *Butea superba* IN THAILAND. THESIS ADVISOR: ASST. PROF. CHANPEN CHANCHAO, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. WICHAI CHERDSHEWASART, D.Sc., 125 pp.

Butea superba (Red Kwao Krua) are Thai herbal leguminous plants. Its tuberous roots are widely used for estrogen replacement therapy. It reported that Kwao Krua from different localities performed different estrogenic activity. Morphometric and genetic analyses were used in this research. Leaves *of B. superba* were collected from 29 localities throughout Thailand for morphometric analysis and from 34 localities for genetic analysis. In morphometric analysis, 9 parameters [petiole length (PL), petiole diameter (PD), rachis length (RL), petiolet length (PLL), terminal leaflet length (TLL), terminal leaflet breadth (TLB), stipule length (SPL), angle of first leaf border (AB^{o}), and number of pairs of primary veins (NPV)] were used for factor and cluster analyses. For factor analysis, 7 out of 9 morphometric characters were selected as new variable and could be grouped into 3 new factors. Due to graph plotting of factor scores, no cultivars could be separated from each others. Moreover, a dendrogram generated by cluster analysis supported the graph of factor score that *B. superba* cultivars could not separate into groups. However, result on correlation analysis of factor scores against latitude and longitude shows clinal patterns in morphometric characters of *B. superba* leaf in Thailand. From the North to the South, leaf length increase in size in factor 1 but decreases in size in factor 2.

In genetic analysis, variation of *rbcL*, *trnL*F-cd, and *trnL*F-cf regions was determined. Amplified PCR products of 300, 550, and 1,000 bp by PCR were obtained, respectively. After direct sequencing, nucleotide base were obtained and clustered by using maximum parsimony (MP) and neighbor-joining (NJ) method. Considering a phylogenetic tree of *rbc*L, low genetic variation was obtained but not 2 phylogenetic trees of *trn*LF-cd and *trn*LF-cf. Furthermore, RAPD was used to investigate genetic variation. The obtained result also supported the result by direct sequencing method in both *trn*LF-cd and *trn*LF-cf region. It can summarize that *B. superba* cultivars had high genetic variation.

Field of study	Biotechnology	Student's signature Jirattikarn K.
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ABBREVIATIONS

A, T, G, C	deoxy nucleotide triphosphate (dNTP) containing Adenine, Thymine, Cytosine, and Guanine, respectively
bp	base pair
°C	degree Celcius
DNA	deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
HCl	hydrochloric acid
kb	kilobase
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mtDNA	mitochondrial DNA
ng	nanogram
NJ	Neighbor Joining
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
Rbcl	RuBisCo large subunit
rpm	revolution per minute
sec	second
TEMED	N, N, N, N'-tetra methyl ethylene diamine
Tris	tris (hydroxyl methyl) aminomethane
trnLF	RNA-Leucine-Phenylalanine
UPGMA	Unweighted Pair Group Method using Arithmetic averages
UV	ultraviolet
V	volt
µg	microgram
µl	microlitre
µM	micromolar

CHAPTER I

INTRODUCTION

Butea superba Roxb. is an herb in family Leguminosae with the common Thai name of “red Kwao Krua”. It is a crawler plant, wraps itself around a large tree. The leaves are compound with alternate 3-leaflets. Flower color is yellowish orange. It grows well in an outdoor area (Roengsumran *et al*., 2000). *B. superba* is abundantly distributed in deciduous forest in many parts of Thailand. This indigenous herb has been traditionally consumed among Thai males for the purpose of rejuvenation as well as maintenance of sexual performance or prevention of erectile dysfunction (Suntara, 1931).

Crude extract of *B. superba* tuberous root contained 2 groups of chemical constituents, carboxylic acid, steroid, steroid glycoside, flavonoid, and flavonoid glycoside (Ruksil, 1995). Recently 5 chemical including A carpin (Medicarpin) and four isoflavone were isolated from the tuber roots of *B. superba* Roxb. (Ngamrojanavanich *et al*., 2007). Flavonoid and flavonoid glycoside could inhibit cAMP phosphodiesterase which stimulates the function of the central nervous system and leads to the increase in male sexual performance (Roengsumran *et al* ., 2000). It had an effect on anti-proliferation of MCF-7 and Hela cells (Cherdshewasart *et al*., 2004a,b). Moreover, chemicals in *B. superba* can exhibit significant inhibitory activity on acetylcholinesterase and can increase levels of acetylcholine in a body. This is helpful in clinical trial on erectile dysfunction in Thai- male and in treatment of Alzheimer’s disease (Cherdshewasart and Nimsakul, 2003; Ingkaninan *et al*., 2003).

According to a large-scale survey on the distribution and diversity of Kwao Krua since 1998, *P. mirifica* can be widely distributed throughout different locations or habitats (Cherdshewasat, Subtang, and Dahlan, 2006). *P. mirifica* was found in 28 provinces while *B. superba* was found in 24 provinces (Pulcharoen, 2005). It is reported that *P. mirifica* from different locations performs different bioactivity and contains various isoflavone contents (Parnruensan, 2000; Cherdshewasart, Kitsamai and Malaivijitnond, 2006).

Morphometric and genetic variation of *B. superba* in Thailand has not been reported although many cultivars have been found throughout the country.

In other plants of leguminosae, morphometric and genetic variation was reported by using morphometric method, DNA sequencing, and Random Amplified Polymorphic DNA (RAPD) technique. Moreno-Sánchez (2004) studied graphic approach of *Archaeopteris* leaves by using morphometric analysis. Mienie, Smith and Pretorius (1995) used RAPD technique to identify South African soybean cultivars. Kass and Wink (1996) investigated the molecular evolution of the leguminosae and determined a phylogeny of legumes in three subfamilies based on *rbc*L-sequences. Weder (2002) used RAPD-polymerase chain reaction (RAPD-PCR) technique to identify legume species in food. Zhang, Yang, and Rao (2005) discovered genetic variation of amphicarpic species, *Amphicarpaea edgeworthii* Benth (Leguminosae) based on RAPD markers. They used 13 RAPD primers to investigate the variation and found that genetic variation was high among populations and was similar within populations. Martins *et al*. (2006) studied genetic variation among and within Portuguese landraces of common white bean (*Phaseolus vulgaris* L.) by RAPD analysis.

Morphometric and genetic variation of red Kwao Krua in Thailand is scared. In *B. superba*, morphometric and genetic variation has never been reported. Therefore, we aim to determine both variations of *B. superba* cultivars in Thailand. Samples were collected from various provinces. Nine morphometric characters in mature leaf was analyzed. In addition, variation in partial sequences of RuBisCO large subunit (*rbc*L) and transfer RNA-Leucine-Phenylalanine in the chloroplast DNA (*trn*L-F) would be studied by using DNA sequencing and RAPD analysis. Molecular phylogenetic relationship among *B. superba* cultivars in Thailand would be analysed. The obtained result will provide information on basic biology, biodiversity, geographic variation, and genetic relationship among *B. superba* cultivars in Thailand. In addition, it may apply to conservation biology of *B. superba* and agricultural knowledge of herbal plants in Thailand. Therefore, our study on *B. superba* will provide a first survey on the population genetic structure by using molecular markers. Also, it may help to select the best cultivar of *B. superba* for pharmaceutical application in the future.

CHAPTER II

LITERATURE REVIEW

Butea superba Roxb. is one of well-known Kwao Krua plants in Thailand. Its common name is “red Kwao Krua”. As traditional medicine, its tuberous root and stem is always used in male rejuvenation. It could provide strength and power and could increase sexual performance in male. People believe that it is one of a miracle herb (Sutjit, 2003; Tanasugarm, 2001).

2.1 Botanical characteristics of *Butea superba* Roxb.

Butea superba Roxb. is a plant in family Leguminosae and subfamily Papilionoideae. Its local names are Kwao Krua, Jan Krua, Tan Jom Thong and Thong Krua (Samitinun, 1980). *B. superba* Roxb. is a climber plant growing independently, and wrapping itself around trees. Leaves are pinately-tree foliates and acuminate leaflet. It leaf stalk is long. A flower is large in size with yellowish orange color (Figure 1) and blooms during winter to summer. Petals are three times longer than calyx. A pod is 3-4 inches long and oblong in shape with silvery silky short hair [Kurz, 1877; Brandis, 1990; Cherdshewasart (unpublished)]. Roots of mature plant are 8 to 9 inches long and later turn into tubers. If it is cuts, the tuberous roots will release red sap. It can reproduce through seeds (sexual reproduction) and stem cutting (asexual reproduction).

B. superba is a large twining wood found in a deciduous forest in the northern, the central, the western, and the northeastern regions of Thailand. It was found in the same habitat as *Pueraria mirifica* and also found in a mountainous area.

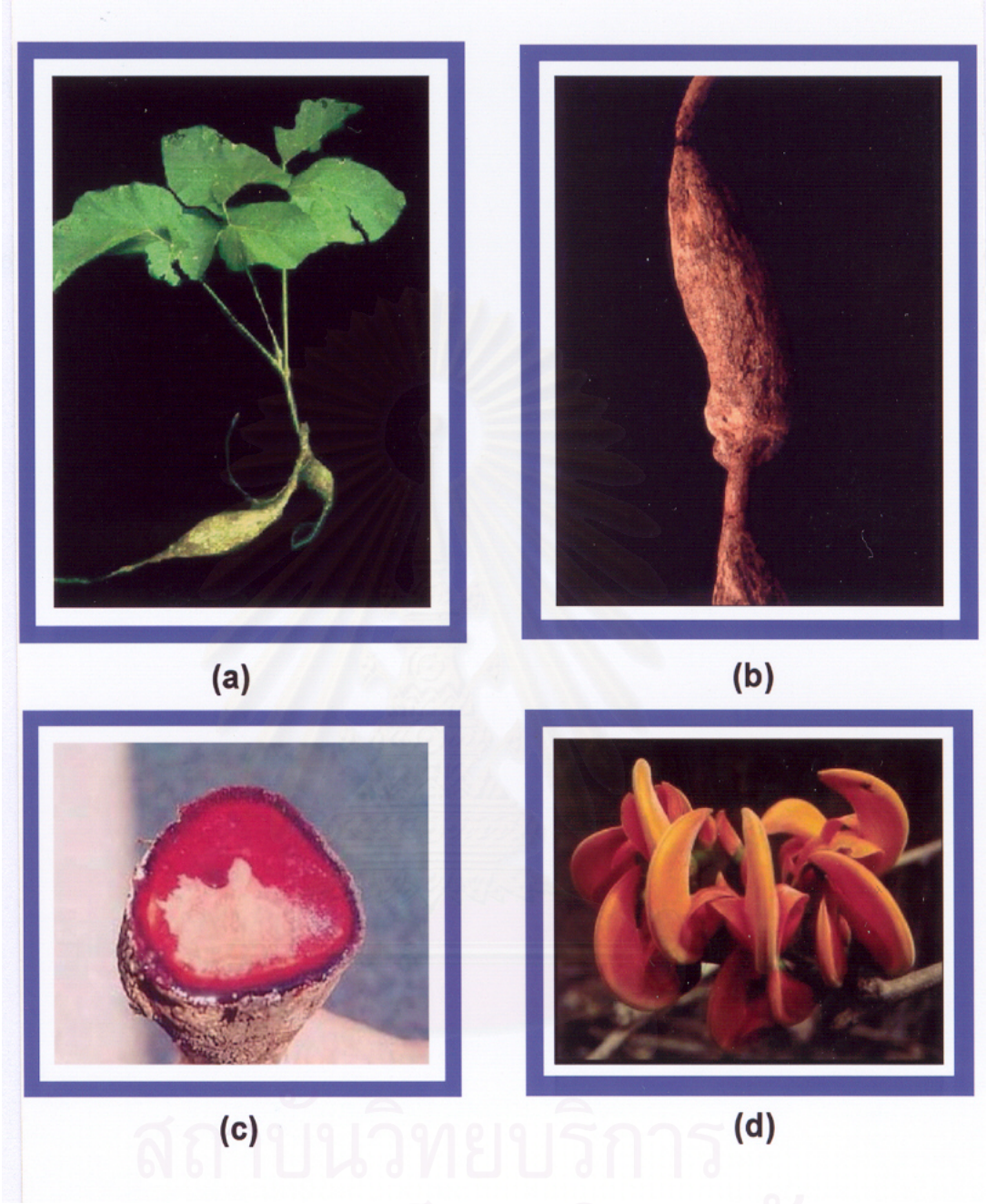


Figure 1. *Butea superba* Roxb. Its feature (a), tuberous root (b), stem (c), and flowers (d) were presented. (Photo by W. Cherdshewasart)

2.2 Taxonomy of the genus Butea (Honton, 2005)

Regnum ***Plantae***
Common name: Plant kingdom

Division ***Magnoliophyta*** Cronquist
Common name: *Angiosperms*

Subdivision ***Magnoliophytina*** Frohne & U. Jensen ex Reveal

Classis ***Rosopsida*** Batsch

Subclassis ***Rosidae*** Takht.

SuperOrdo ***Fabanae*** R. Dahlgren ex Reveal

Ordo ***Fabales*** Bromhead
Common name: *Legumes*

Familia ***Leguminosae*** Juss.

Genus ***Butea* Roxb**. ex Will.
Common name: *Butea*

Specie ***Butea superba*** Roxb.
Common name: *red Kwao Krua*

2.3 Previous work of the genus Butea

Razdan *et al*. (1969) investigated anti-fertility effects and pharmacological actions of *Butea frondosa* seed by using an alcoholic extract, chloroform extract, and aqueous extract. They found that only the alcohol extract is active. It has a distinct anti-fertility effect on rats without a clear-cut of dose-response relationship. Estrous cycle was unaffected by the extracts. Differences between control and treated groups were insignificant regarding to anti-estrogenic activity and androgenic activity. Pharmacological and toxic effects are probably unrelated to the anti-fertility action of the extract.

Mehta *et al*. (1983) studied isolation and *in vitro* antimicrobial efficiency of *Butea monosperma* seed oil to human pathogenic bacteria and phytopathogenic fungi. The *in vitro* antimicrobial efficiency was studied by filter paper disk method against several human pathogenic bacteria and fungi. Result showed that the isolated gave a significant bactericidal and fungicidal effect.

Bhargava (1986) isolated butin from seeds of *Butea monosperma* (Figure 2) and administered orally to adult female rats at the doses of 5, 10, and 20 mg/kg BW from day 1 to day 5 of pregnancy. It showed anti-implantation activity at 40%, 70%, and 90% of treated animals, respectively. Besides, butin is known as weak estrogen.

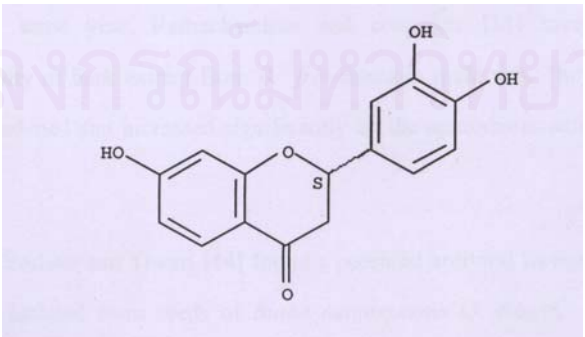


Figure 2. Structure of butin from seeds of *Butea monosperma.*

Bandara *et al.* (1989) isolated petroleum and ethyl acetate crude extract of *Butea monosperma* stem barks (Figure 3). They found medicarpin compound that it showed significant antifungal activity against *Cladosporium cladosporioides.*

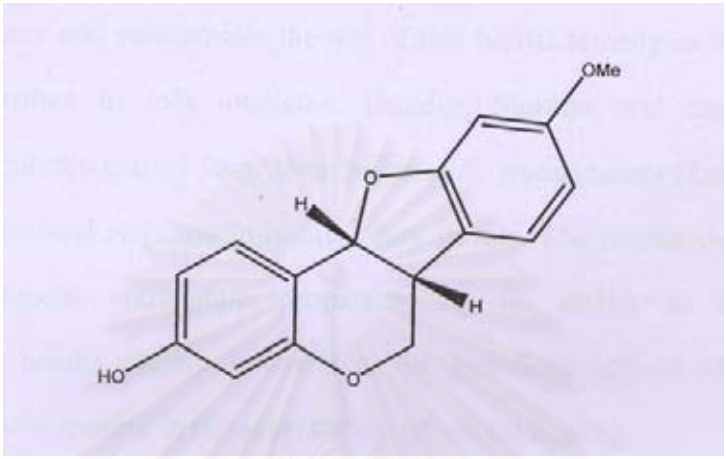


Figure 3. Structure of medicarpin of *Butea monosperma* stem barks.

Prashanth et al. (2001) investigated methanol extract of Butea monosperma seeds in India. Due to in vitro test, it showed significantly anthelmintic activity.

Soman et al. (2004) studied an effect of Butea fromdosa leave extract on stress, anxiety, and cognition on rats. The results showed that both aqueous and alcoholic extracts possess anti-stress activity. In addition, Ramachandran et al. (2004) investigated the aphrodisiac activity of B. frondosa bark extract on male rats. They reported that the extract could reduce and increase aphrodisiac activity significantly on male rats.

Yavada et al. (2005) found a potentially anti-viral flavone glycoside isolated from B. monosperma O. Kuntz seed. Its structure was determined by various spectral analysis and chemical degradations as 5, 2'-dihydroxy-3, 6, 7-trimethoxy-flavone -5-O-β-D-xylopyranosyl-(1→4)-O-β-D-glucopyranoside (Figure 4). Furtheremore, Gunakkunru et al. (2005) investigated the anti-diarrhea potential by the ethanol

extract of B. monosperma (Lam) Kuntz stem barks. Their experiments were on Wistar albino rats. The obtained results establish the efficacy and substantiation folk medicine for a non-specific treatment for diarrhea. Besides, Sumitra *et al.* (2006) investigated alcoholic extract of *B. monosperma* (Lam) Kuntz stem barks on healing tissue injury in rats. The results also showed that *B. monosperma* extract possesses antioxidant properties which are able to reduce lipid peroxidation.

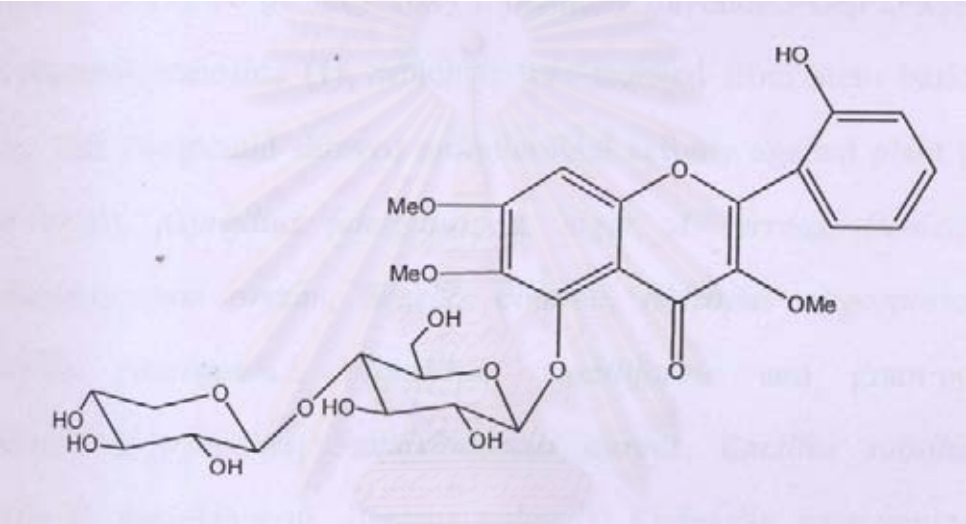


Figure 4. Structure of 5, 2'-dihydroxy-3, 6, 7-trimethoxyflavone-5-*O*-*β*-D-xylopyranosyl-(1→4)-*O*-*β*-D-glucopyranoside of *Butea monosperma* stem barks.

2.4 Previous work of *Butea superba* Roxb.

Ruksilp (1995) investigated a tuberous root of *B. superba* Roxb. collected from Lampang province. It was finely crushed and extracted by hexane, chloroform, methanol, and water. A fraction of crude extract by column chromatography led to isolation of 5 compounds. These compounds were a mixture of carboxylic acids (C_{22}-C_{26}), a mixture of steroids (campesterol, stigmasterol, and *β*-sitosterol), a mixture of

steroid glycosides (*β*-sitosterol-3-*O*-*β*-D-glycopyranoside and stigmateryl-3- *O*-*β*-D-glycopyranoside), 3, 7, 3'-trihydroxy-4'-methoxyflavone, and 3, 3'-dihydroxy-4'-methoxyflavone-7-*O*-*β*-D-glycopyranoside (Figure 5 and 6).

In 1998, Yavada and Reddy discovered new bioactive compounds. There are flavonol glycoside, 3, 5, 7, 3', 4'-pentahydroxy-8-methoxy-flavonol-3-*O*-*β*-D-xylopyranosyl (1→2) -α-L-rhamnopyranoside. All of them were isolated from stem barks of *B. superba* Roxb. They showed antimicrobial activity against 1) plant pathogenic fungi which are *Trich viride, Aspergillus fumigatus, A. niger, A. terrus, Penicillium expansum, Helmtnthosporium oryzae, Botxitis cinerea, Rhizopus oligosporus, R. chinensis, Klebsiella pneumoniae, Fusarium moniliforme,* 2) gram-positive bacteria which are *Streptococcus phyogenus, Staphylococcus aureus, Bacillus subtilis,* and gram-negative bacteria which are *Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Pseudomonas aeruginosa.* The maximum inhibitory effect was to *H. oryzae, A. niger, B. cinerea* and gram-positive bacteria.

Roengsumran *et al*. (2000) found 2 compounds of 3, 7, 3'-trihydroxy-4'-methoxyflavone and 3, 5'-dihydroxy-4'-ethoxyflavone-7-*O*-*β*-D-glycopyranoside. Both of them showed inhibition of cAMP phosphodiesterase activity which was capable to stimulate the function of the central nervous system at IC_{50} value of 190 and 58 μg/ml, respectively.

Manosroi *et al.* (2001) reported the preliminary chronic toxicity of *B. superba* on rats at the doses of 5 and 100 mg/kg BW. There was no adverse effect on essential organs except the sperm count was increased. Abnormal sperms were found at the treatment of 5 mg/kg BW.

Pongpanparadon *et al.* (2002) determined the primary toxicological effects of *B. superba* Roxb. Dried powder by micronucleus and dominant lethal tests were

undertaken. The results showed that 1,000 mg/kg BW/day of aqueous solution was more significantly effective in inducing the formation of micronuclei in polychromatic erythrocytes than control ($p<0.01$). *B. superba* Roxb. extract had no effect on body weight of treated rats. In addition, Boongapim (2002) assessed the vasodilating effect of isolated human umbilical vein with mode of action of ethanol extract of *B. superba* Roxb. The results showed inhibitory effect of *B. superba* Roxb. extract on histamine-induced vascular contraction. Moreover, the inhibitory effect depended on time and KCl concentration. It was related to endothelium function.

Ingkaninan *et al*. (2003) searched new acetylcholinesterase (AChE) inhibitor from 32 plants used in Thai traditional rejuvenating and neurotonic remedies such as a drug for the symptomatic treatment of Alzheimer's disease. They reported that the methanol extract from stem barks of *B.superba* Roxb. showed 50-65% inhibitory activity on AChE. In addition, Cherdshewasart and Nimsakul (2003) studied the effect of *B. superba* Roxb. on erectile dysfunction (ED) on Thai males aged 30-70 years. Three month randomized double-blind clinical trial was carried out in volunteers with ED. The result showed that 82.4% of patients exhibited noticeable improvement without apparent toxicity.

Cherdshewasart, Cheewasopit and Picha (2004) investigated ethanol extract of *B. superba* Roxb. with proliferation and anti-proliferation effect on the growth of MCF-7 cells at 10, 100 and 1,000 μg/ml with and ED_{50} value of 370.91 μg/ml. The data was evaluated after 4 days of incubation. Also, the results presented the relation of a possible anti-estrogen mechanism and a potent cytotoxic effect.

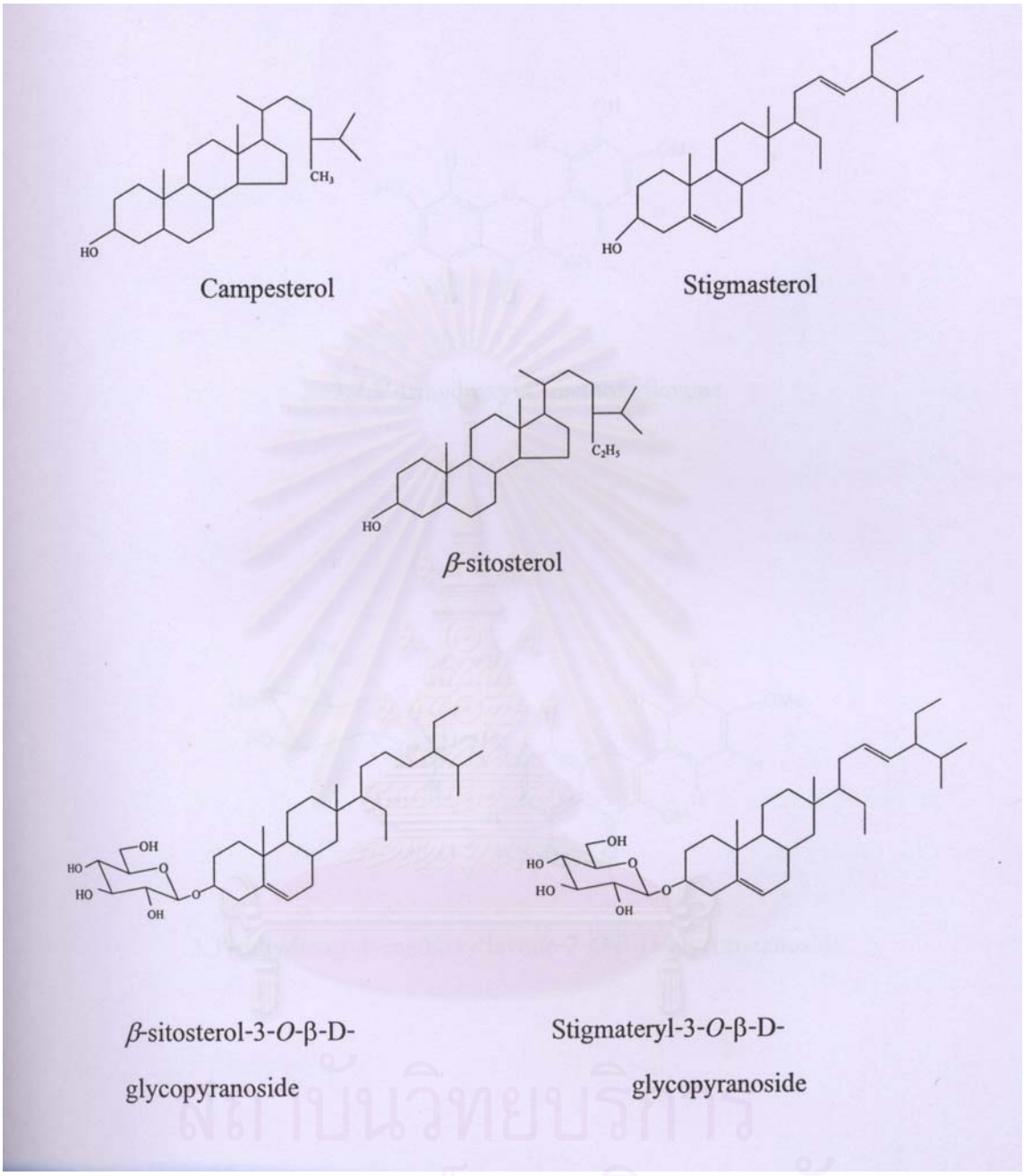
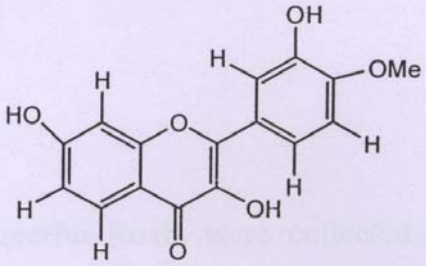
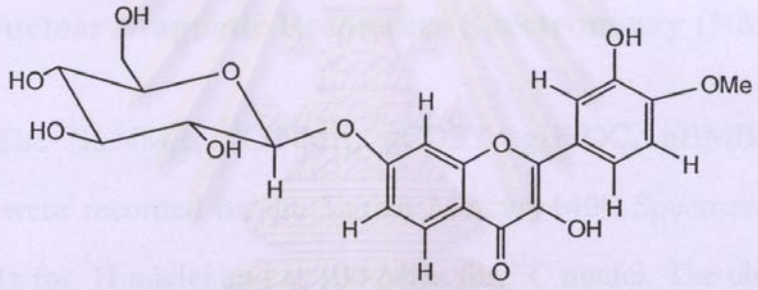


Figure 5. Structure of the chemical constituents of *Butea superba* Roxb. (Ruksilp, 1995).



3,7,3′-trihydroxy-4′-methoxyflavone



3,3′-dihydroxy-4′-methoxyflavone-7-*O*-β-D-glycopyranoside

Figure 6. Structure of the chemical constituents of *Butea superba* Roxb. (Ruksilp, 1995).

2.5 Chemical constituents of *Butea superba* Roxb.

A plant tuber exhibited some chemicals closely related to that of *P. mirifica* but some chemicals are different. *B. superba* tuberous root contained 5 groups of chemical constituents. They are carboxylic acid, steroid, steroid glycoside, flavonoid, and flavonoid glycoside (Table 1, Raksilp, 1995; Loontaisong, 2005). Macromolecules (protein, lipid, and starch) were also found. Flavonoid glycoside (3, 7-dihydroxy-8-methoxyflavone 7-O-α-L-rhamnopyranoside) was found in stem of Indian *B. superba* (Yavada and Reddy, 1998).

Table 1. Chemical constituents of *B. superba* tuber.

Category	Chemical	References
Carboxylic acid	Straight chain carboxylic acid (C_{22}-C_{26})	Ruksilp, 1995
	3-hexacosanoyloxy-propane-1, 2-diol	Loontaisong, 2005
Steroid	Campesterol, stigmasterol, β-sitosterol.	
Steroid glycoside	β-sitosteryl 1-3-o-β-D-glucopyranoside, stigmasteryl 1-3-o-β-D-glucopyranoside	
Flavonoid	3, 7, 3-trihydro-4'-methoxyflavone	Ruksilp,1995
Flavonoid glycoside	3- 3'-dihydroxy-4'-methoxyflavone-7-o-β-D-glucopyranoside	Ruksilp, 1995
	3, 7-dihydroxy-8-methoxyflavone-7-O-α-L-rhamnopyranoside	Yavada and Reddy, 1998
	5, 4'-dihydroxy-7-methoxy-isoflavone	Loontaisong, 2005

	(Prunetin)	
	3-hydroxy-9-methoxypterocarpan (Medicarpin)	Loontaisong, 2005
	7-hydroxy-4'-methoxy-isoflavone (Pormononetin)	Loontaisong, 2005
	7-hydroxy-6-4'-dimethoxyisoflavone	Loontaisong, 2005
	7, 4'-dimethoxyisoflavone (Butein and Butin)	Subba and Seshadri, 1949

2.6 Morphometric analysis

Morphometry is the measurement of particular structures of organisms and analysed by statistics. Morphometric methods are usually the best substitutes of complicating qualitative descriptions of shape variables (Moreno-Sánchez, 2004). To identify species, morphology characters are the most evident features. They are the basis for the description and identification of cultivars (Perries, 1998). Many researches used several organs of plants to study morphometry such as leaf, seed, fruits, flower, etc. For example, Ridder-Numan *et al*. (1997) used pollen morphology to identify plants in 4 genera of *Butea, Kunstleria, Meizotropis,* and *Spatholobus.* Moreno-Sánchez (2004) used 3 parameters of leaf shape for comparison of plants in genus *Archaeopteris*. Andrés-Agustín *et al.* (2006) studied morphometry of organs of cherimoya (*Annona cherimola* Mill.) and analysed fruit parameters in order to characterize cultivars. The data was applied to germplasm selection. The multivariate analyses were performed by Principal Component Analysis method (PCA) of Factor analysis and then statistically clustered by the unweighted pair group method with

arithmetic average (UPGMA). A dendrogram could be used to separate the interesting plants into groups.

2.7 Plant molecular phylogenetic

2.7.1 Phylogenetic systematics

Phylogenetic systematics or simply ‘phylogenetics’ is a methodology described by Willi Hennig, a German entomologist in 1950. Phylogeny indicates the origin and evolution of a set of organisms, usually a set of species. A major task of systematics is to determine the ancestral relationship among groups of organisms.

There are several types of characters which can be used for phylogenetic analyses such as morphological characters and molecular characters. Morphological characters are the simple source of characters in most groups of organisms. External characters are more predominant than internal characters because they are easy to observe. Some of them can be quantified as a reference. For example, color patterns may be measured by wavelength. Molecular character is an alternative to use for phylogenetic analysis. For example, in 1972, Kohne *et al.* introduced DNA sequencing to analyse phylogeny of primate. After that, most phylogeneticists have been interested in finding nucleotide sequences, especially at non-coding regions to determine the relationship among organisms.

There are 2 different major methods to construct phylogenetic trees. The first method is a ‘distance method’ which converts aligned sequences into a pairwise distance matrix and inputs that matrix into a tree constructing method. The second method is a ‘discrete method’ which considers an individual nucleotide site directly. The distance method should be used when the original data are in the form of genetic distances. However, if we have the nucleotide or protein sequences, we should analyse them with a discrete method in order to avoid the loss of information that

occurs when sequences are converted into distances. The discrete methods are different from the distance methods as they operate directly on the sequences rather than pair wise distances.

Neighbor-joining is a popular distance method. In a tree, sum of branch lengths is minimal. The major discrete method is maximum parsimony which chooses the tree (or trees) that require fewest evolutionary changes.

2.7.2 Technique for phylogenetic analysis in plants

2.7.2.1 PCR amplification on gene target

For phylogenetic studies in plants, a target gene must be selected. Different genes have different mutation rates. Analysis of non-coding regions permits assessment of phylogenetic relationships at lower taxonomic levels because they evolve more rapidly than coding regions.

For example, target genes used in phylogenetic analysis are chloroplast genes (Clegg *et al*., 1994; Kelchner, 2000; Olmstead *et al*., 1998). The modes of chloroplast DNA evolution (cpDNA) in plants are usually conserved in terms of genome sizes, structures, gene contents, and linear orders of genes among lineages of land plants. This conservative mode suggests that any change in structure and content of chloroplast genome may provide significant phylogenetic implications and therefore be useful for the study of phylogenetic relationships. The chloroplast genome evolves at a slower rate than the nuclear genome. However, some regions change either more rapidly or more slowly than the average. Nucleotide substitution rates vary among plant lineages. Therefore, cpDNA is useful in determining the relationship of organisms at the inter-generic, the inter-specific, and the intra-specific levels (Plamer,

1998). Later, specific primers will be designed for Polymerase Chain Reaction (PCR) to amplify the gene target.

The basic protocol of PCR (Taberlet *et al*., 1991) is simple as below: (1) Double stranded DNA is denatured at high temperature to form single strands (templates); (2) Short oligonucleotide primers bind complementary to the single strand templates at the flanking ends at lower annealing temperature; (3) The temperature is raised for primers extension in order to synthesize new strands; and (4) The newly synthesized double stranded DNA are denatured at high temperature. The cycles have been repeated many times in order that the amplification of target DNA can perform continuously. The amount of increased target DNA will be exponential. PCR is a powerful technique in plant molecular systematics because it can provide reproducible targeted DNA from herbarium specimens. The ability of amplification can determine the quality of DNA whether it is already degraded or is still good (Doyle *et al.* 1995).

Savolainen *et al.* (1995) found that amplification of DNA from some herbarium samples might be difficult sometimes because there was some oxidized material co-precipitated with DNA. Also, they found that the addition of some certain additives could overcome the inhibiting activities of some herbarium extracts.

2.7.2.2 RAPD analysis

Randomly Amplified Polymorphic DNA (RAPD) is a Polymerase Chain Reaction (PCR)-based technique. Arbitrary primers are used to detect changes in the DNA sequence at random sites in the genome (Yoke-Kqueen, 2006). Accordding to Martinez *et al.* (1998), fingerprint of genomic DNA was able to detect hybrids and races of species. There are 2 steps (amplification and electrophoresis) involving in RAPD.

RAPD method described by Williams *et al.* (1990) is faster and less expensive than RFLP analysis. This technique has been used in cultivar identification of many crops including *Stylosanthes* (Kazan *et al*., 1993), papaya (Stiles *et al*., 1993), celery (Yang and Quiros, 1993), and apple (Koller et al., 1993). There are few reports on the use of RAPD in characterization of soybean (Lark *et al*., 1992; Caetanoanolles *et al*., 1993; Paiva *et al*., 1994; Prabhu and Gresshoff, 1994). Additionally, Williams *et al.* (1990) could differentiate 2 soybean species by RAPD analysis. Lark *et al*. (1992) used RAPD analysis on soy bean species and found that they consisted of 11 domesticated cultivars, 9 wild cultivars, and 5 perennial cultivars.

2.7.3 Plant chloroplast DNA

The ribulose 1, 5 bisphosphate carboxylase/ oxygenase (*rbc*L) is the most widely used gene in plant phylogenetic construction (Palmer *et al*., 1998; Chase and Ablert, 1998; Soltis and Soltis, 1998). It was chosen to generate large molecular dataset of angiosperms (Chase *et al*., 1993) and was used in large-scale analyses of green plants (Lewis *et al*., 1997; Källersjö *et al.*, 1998; Cuenoud *et al*., 2000; Savolainen *et al*., 2000; Albach *et al*., 2001).

Recent molecular phylogenetic studies using nucleotide sequences of the gene encoding the large subunit of *rbc*L successfully revealed the phylogenetic relationship. Phylogenetic studies using chloroplast DNA could confirm the evolutionary distinctiveness of evolutionary lineages of species (Downie *et al*., 2000; Wu *et al*., 2006).

Nowadays, chloroplast DNA transfer RNA-Leucine and phenylalanine region (*trn*L-F) is also widely used in studying molecular phylogenetic. Due to Figure 7, these regions are non-coding sites which their sequences are conserved and highly varied.

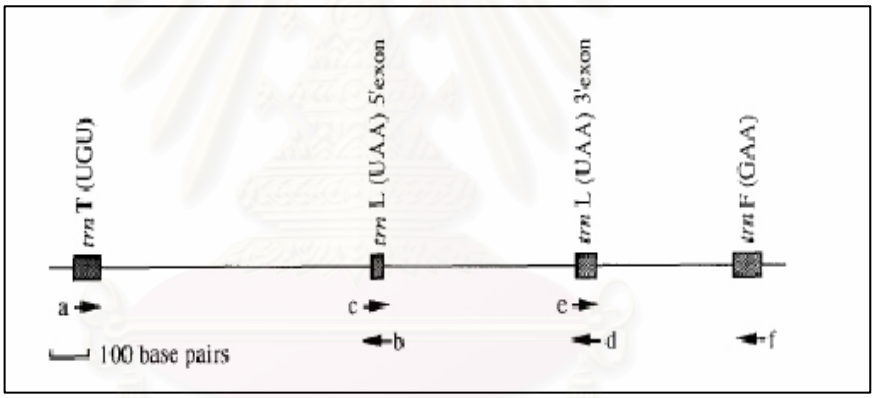


Figure 7. Map of chloroplast DNA *trn*L-F region in plants. It illustrates many universal primers (a-f) used in phylogenetic study (Taberlet *et al.*, 1991).

Many researches used partial sequences of *rbc*L and *trn*L-F to study molecular evolution in plant to study molecular phylogenetics (Käss and Wink, 1996; Negrisolo *et al*., 2004; Kathriarachchi *et al*., 2005; Huang, 2005; Müller *et al*., 2006; Chung *et al*., 2007).

2.7.4 Program for phylogenetic tree construction

Before construction, nucleotide sequences would be analyzed by using these following computer programs:

BioEdit: It is a program for biological sequence editing. It runs on Windows 95/ 98/ NT/ 2000/ XP or on general PC computer. It provides the basic functions for protein and nucleic sequence editing, alignment, and analysis.

Chromas**:** It is also a PC program that is used to check and compare the DNA sequence data that are received by sequencing process. Then, these data are modified into Fasta formatted file before they would be aligned by using Clustal program.

Clustal X (PC program) and Clustal W (http://www.ebi.ac.uk/clustalw): There are 2 computer tools in generating DNA or protein data matrix. All DNA sequences would be aligned all together (multiple sequence alignment). Also, the best matches for selected sequences would be calculated. The Clustal programs can compare all sequences from left to right. Then, similarity of DNA data matrix can be seen.

PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b10: It is a Macintosh computer program to construct a phylogenetic tree. This program works only on Macintosh Power PC and mainly uses maximum parsimony searching approaches to analyze the completely aligned data matrix. All data must be converted

and saved as Nexus formatted file before phylogenetic tree construction will be conducted. Phylogenetic analyses are performed by using neighbor-joining (NJ) and unweighted pair group method with arithmetic mean (UPGMA) methods in this program. Then, bootstrap analysis with 1,000 replicates is applied by the PAUP in order to evaluate supporting for nodes estimated in a parsimony tree. Lastly, the trees will present the relationship.

2.7.5 Maximum parsimony and neighbor-joining

Data matrix is used to calculate the relationships among taxa. There are several methods (either discrete or distance) to construct a phylogenetic tree (or trees). One most popular method among discrete methods is ‘maximum parsimony’ which chooses the tree (or trees) that requires the fewest evolutionary changes (Page and Holmes, 1998).

Neighbor-joining is distance methods and is widely used for phylogenetic tree construction. Neighbor-joining technique is a clustering method but can not optimize a fitting criterion between tree and data. However, it is a good method to estimate a minimum evolution tree.

CHAPTER III

MATERIALS AND METHODS

3.1 Morphometric analysis

3.1.1 Morphometric study equipments

- Vernia caliper
- Ruler

3.1.2 Collection of leaves sample

Leave of *Butea superba* Roxb. were collected from several locations in Thailand (except the South) since Febuary 2005 to October 2006 (Figure 11). Twenty-five mature leaves at about 2 meters from its shoots were picked for morphometry. Moreover, leave of *B. monosperma* (synonym: *B. frondosa*) were classified as an outgroup.

3.1.3 Measurement

Nine characters were measured and analyzed statistically (Figure 8-10).The used characters were petiole length (PL), petiole diameter (PD), rachis length (RL), petiolet length (PLL), terminal leaflet length (TLL), terminal leaflet breadth (TLB), stipule length (SPL), angle of first leaf border (AB^{o}), and number of pairs of primary veins (NPV).

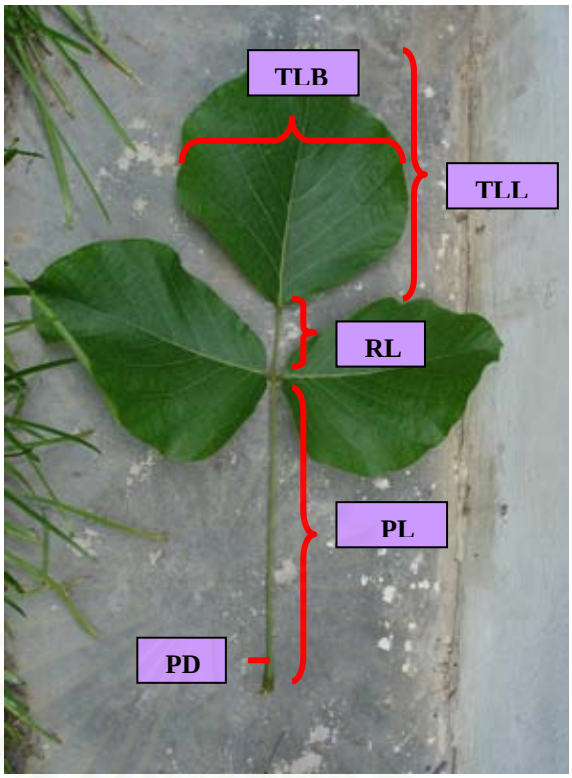


Figure 8. Parameters of terminal leaflet breadth (TLB), terminal leaflet length (TLL), rachis length (RL), petiole length (PL), and petiole diameter (PD).

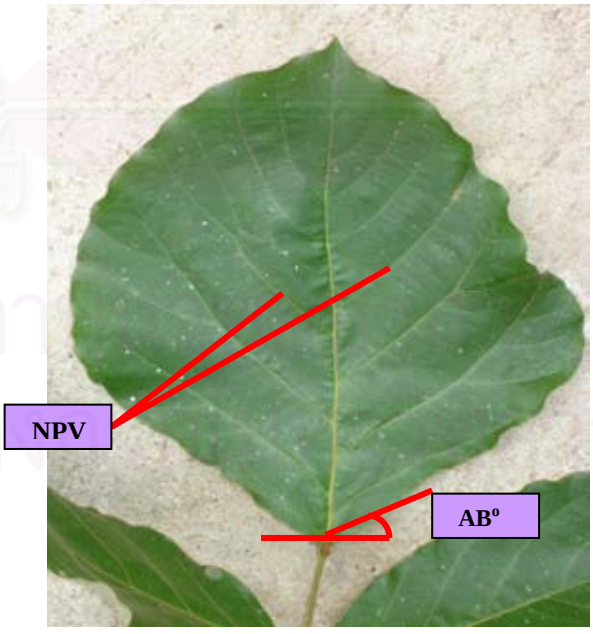


Figure 9. Parameters of number of pairs of primary veins (NPV) and angle of first leaf border (AB^{o}).

.

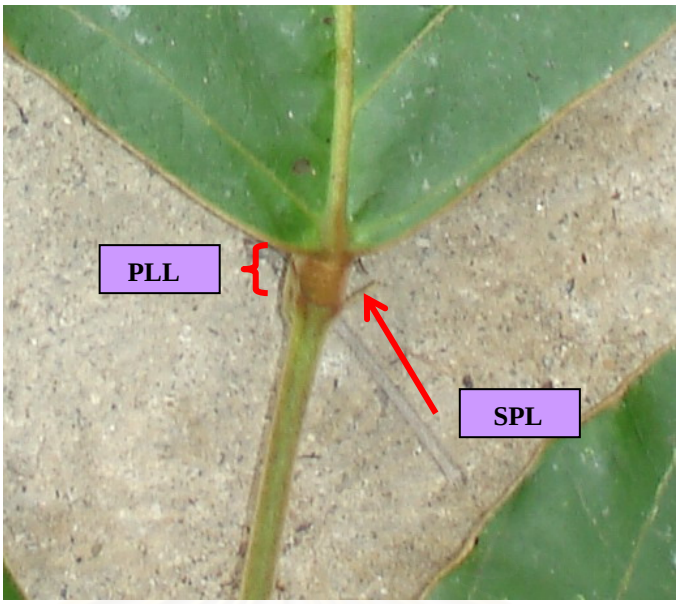


Figure 10. Parameters of petiolet length (PLL) and stipule length (SPL).

3.1.4 Data analysis

A statistic was used to perform a factor analysis on the leave length of 9 characters. This method provides characters those have larger loadings in various factors and allows the parsimonious reduction in the number of characters needed for further analysis. After that, cluster analysis (SPSS for windows 14.0) was used to investigate the relationship among cultivars. Finally, correlation was used to explore clinal patterns in the characteristics of *B. superba* leaves in Thailand.

No.	Province	Code name
1	Kanchanaburi	KC 1-3
2	Khon Kaen	KK
3	Chantaburi	CT
4	Chachoengsao	CC
5	Chonburi	CB
6	Chaiyaphum	CHY
7	Chiangrai	CR 1-2
8	Tak	TK
9	Nakorn ratchasima	Nak
10	Nakhon sawan	NS 1-3
11	Buriram	BR
12	Prachenburi	PB
13	Phitsanulok	PS 1-3
14	Phetchaboon	PC
15	Ratchaburi	Rat 1-4
16	Lopburi	LB
17	Lampang 1	LP1
18	Lampang 2	LP2
19	Loei	LY
20	Sakhonnakorn	SK
21	Saraburi	SR
22	Sukhothai	SU
23	Nongbualamphu	NB
24	Uttaradit	UTT

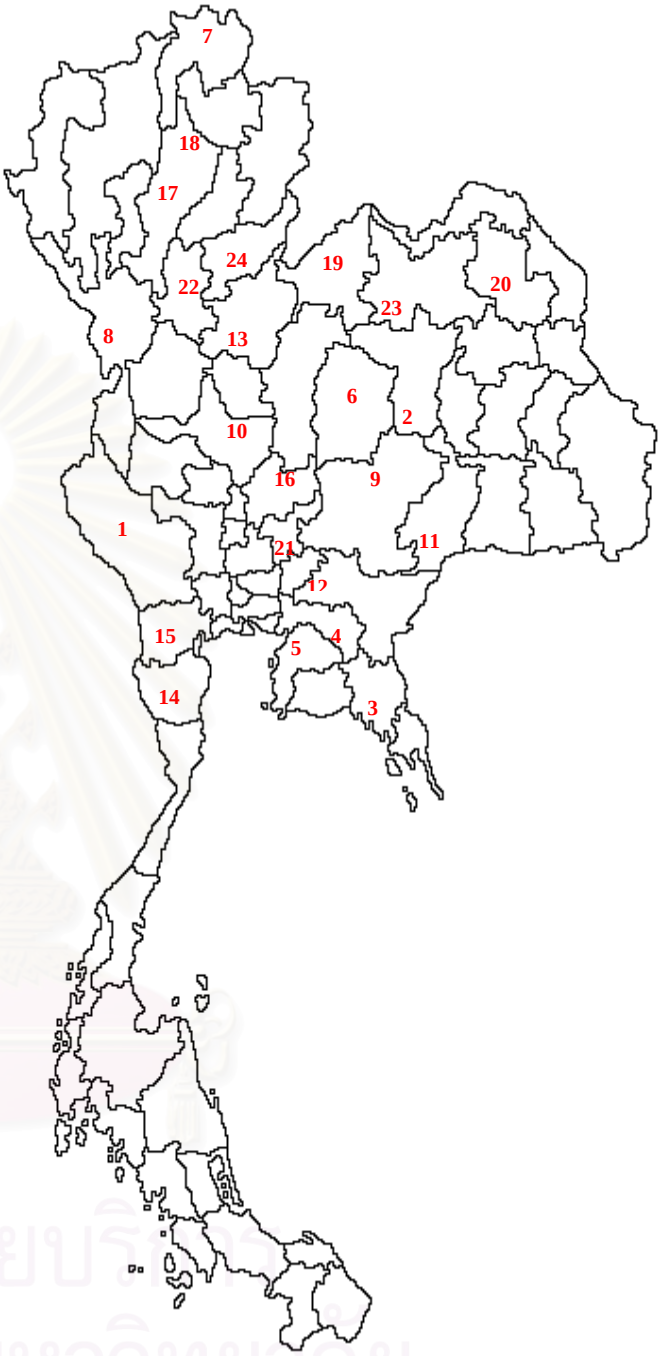


Figure 11. Map of leaf collection. Thirty four cultivars of *B. superba* were collected from 24 provinces in Thailand for both morphometric and genetic analyses.

3.2 Genetic analysis

3.2.1 Instruments

- Autoclave, model: Conbraco, Conbraco Ind. Inc., USA
- Automatic micropipette P10, P20, P100, P200, and P1000 (Gilson-medical electronics, S.A., France)
- Freezer (-20°C)
- Horizontal gel electrophoresis apparatus, model: Mupid, Advance Co., Ltd., Japan
- High speed microcentrifuge, model: Centrifuge 5410 (Eppendorf, Germany)
- Magnetic stirrer, model: PC-320 (Corning, USA)
- Polaroid camera, model: direct screen instant camera DS 34 H-34 (Peca products, UK)
- Microincubator, model: M-36, Taitec, Japan
- Incubator, model: Memmert, Germany
- Microwave oven, model: Sharp carousel R7456 (Sharp, Thailand)
- PCR machine, model: GeneAmp® PCR system 9700 (Applied Biosystem, Singapore)
- Electronic UV transilluminator (Ultra ium Inc., USA)
- Vortex, model: MS I minishaker (IKA-works, Inc., USA)

3.2.2 Inventory supplies

- Polaroid film
- Filter paper Whatman 3 mm (Whatman international Ltd., England)
- Microcentrifuge tubes (0.5 and 1.5 ml)
- Pipette tips (10, 200, and 1000 μl)

- Thin-wall microcentrifuge tube (0.2 ml)

- Whatman laboratory sealing film (Whatman international Ltd., England)

3.2.3 Chemicals

- Absolute ethanol, CH_3CH_2OH, M. W. = 46.07 (Merck, Germany)

- Agarose (Research organics, USA)

- Boric acid (Research organics, USA)

- Ethedium bromide

- DNA ladder marker 100 bp (catalog # SM0321), Fermentas Life Science

- DNA λ *Hin*dIII marker (catalog # SM0101), Fermentas Life Science

- Ethylene diamine tetra-acetic acid (EDTA), $C_{10}H_{16}N_2O_8$, M. W. = 292.2 (Serve feinbiochemica GmbH & Co., USA)

- 95% Ethyl alcohol, CH_3CH_2OH, M.W. = 46, Thailand

- 2x PCR Master mix solution (*i*-Taq) (catalog # 25027), iNtRON BIOTECHNOLOGY

- QIAquick® PCR purification kit (catalog # 28104), Qiagen, Germany

- *AccuPrep*® PCR purification kit (catalog # K-3034), BIONEER, Korea

- QIAamp® DNA mini kit (catalog # 51304), Qiagen, Germany

- Nucleospin® Plant mini kits (catalog # 740570.50), MACHEREY-NAGEL, Germany

- Tris-(Hydroxymrtyl)-aminomethane, $NH_2C(CH_2OH)_3$, M.W. = 121.14, Pharmacia Biotech, USA

3.2.4 PCR primers

All oligonucleotides (Table 2 and 3) were synthesized by Bioservice unit of National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

Table 2. Lists of 5 primers for PCR amplification.

Primer name	**Direction**	**Sequence (5´ to 3´)**	**Reference**
*rbc*L (BuL3)	forward	AGGTTCTGTTACTAACATGT	-
*rbc*L (BuR3)	reverse	GGTCTCTCCAACGCATAAAT	-
*trn*L (UAA) 5' exon primer_c	forward	CGAAATCGGTAGACGCTACG	Taberlet *et al.*, 1991
*trn*L (UAA) 3' exon primer_d	reverse	GGGGATAGAGGGACTTGAAC	Taberlet *et al.*, 1991
*trn*F (GAA) primer_f	reverse	ATTTGAACTGGTGACACGAG	Taberlet *et al.*, 1991

3.2.5 RAPD primers

Table 3. Lists of 5 arbitrary primers for RAPD reaction

Primer	**Sequence (5' to 3')**	**Reference**
OPA-07	GAAACGGGTG	Mienie *et al.* (1995)
OPA-12	TCGGCGATAG	Mienie *et al.* (1995)
OPA-19	CAAACGTCGG	Mienie *et al.* (1995)
OPC-15	GACGGATCAG	Mienie *et al.* (1995)
OPD-2	GGACCCAACC	Mienie *et al.* (1995)

All 5 RAPD primers were designed and selected due to Mienie *et al.* (1995).

3.2.6 Collection of young leaves

Fresh young leaves of *B. superba* were collected from several part of Thailand. Furthermore, *B. monosperma* (synonym *B. frondosa*) was used as control. Localities of sample collections of 34 cultivars are shown in Table 4. Fresh young leaves were stored at -20^{0}C until DNA extraction was performed.

Table 4. Thirty five cultivars of *B. superba* were collected in Thailand and their code name.

Number	Cultivars	Code
1	Kanchanaburi	KC 1
2	Kanchanaburi	KC 2
3	Kanchanaburi	KC 3
4	Khon Kaen	KK
5	Chantaburi	CT
6	Chachoengsao	CC
7	Chonburi	CB
8	Chaiyaphum	CHY
9	Chiangrai	CR 1
10	Chiangrai	CR 2
11	Tak	TK
12	Nakhon ratchasima	NAK
13	Nakhon sawan	NS 1
14	Nakhon sawan	NS 2
15	Nakhon sawan	NS 3
16	Buriram	BR

Number	Cultivars	Code
17	Prachenburi	PB
18	Phitsanulok	PS 1
19	Phitsanulok	PS 2
20	Phitsanulok	PS 3
21	Phetchaboon	PC
22	Ratchaburi	RAT 1
23	Ratchaburi	RAT 2
24	Ratchaburi	RAT 3
25	Ratchaburi	RAT 4
26	Lopburi	LB
27	Lampang1	LP 1
28	Lampang2	LP 2
29	Loei	LY
30	Sakhonnakorn	SK
31	Saraburi	SR
32	Sukhothai	SU
33	Nongbualamphu	NB
34	Uttaradit	UTT

3.2.7 DNA extraction

Genomic DNA was extracted from an individual cultivar of *B. superba* by either of 2 kits as below.

- Genomic DNA extraction by DNeasy plant mini kit (Qiagen, catalog # 69103)

A leaf powder ground in liquid nitrogen was mixed by 400 µl of AP1 buffer and 4 µl of 100 mg/ml RNase A stock solution. It was vortexed, incubated at 65°C for 10 min, and mixed by inverting during incubation. Later, 130 µl of buffer AP2 was added, mixed, and incubated for 5 min on ice. The lysate was applied to a QIAshredder spin-column set and centrifuged for 2 min. A flow-through fraction was transferred to a new tube and mixed by 1.5 volumes of AP3. The mixture (650 µl) was applied to the DNeasy mini spin-column set and centrifuged for one min. After that flow-through was discarded. The remaining sample was added to the spin column and was centrifuged for another min. The column was placed in a new tube and 500 µl of AW buffer was added. It was then centrifuged for another min. More 500 µl of AW buffer added, and centrifuged for 2 min. The spin column was transferred to a new tube and 50 µl of 65°C preheated buffer AE was transferred onto the DNAeasy membrane. The column was incubated for 5 min at RT and then centrifuged for 1 min. More 50 µl of preheated buffer was added to elute DNA. DNA was finally stored at -20 °C freezer.

- Genomic DNA extraction by NucleoSpin® Plant mini kits (MACHEREY-NAGEL, catalog # 740570.50)

Dry tissue (20 mg) was ground by a pestle and motar, mixed by 400 µl of buffer CO, and homogenized. Ten µl of RNase A solution (1 mg/ml) was added. The

mixture was then incubated at 60°C for 30 min. Centrifugation of the mixture was done at maximum speed for 5 min and 300 µl of the clear lysate was transferred to a new microcentrifuge tube. Three hundred µl of buffer C4 and 200 µl of absolute ethanol were added into the tube (C4 buffer and absolute ethanol must be premixed before used). The mixture was loaded into a provided 2 ml Nucleospin® plant column, centrifuged for 1 min. Later, the flow-through was discarded. Buffer CW (400 µl) was added to the membrane of the column. About 700 µl and 200 µl of buffer C5 were then used to wash a silica membrane for the second and third times, respectively. To dry the silica membrane completely, the column was centrifuged at maximum speed for another 2 min. Finally, a highly pure genomic DNA was eluted from the membrane by 50 µl heated buffer CE twice. The eluted DNA solution was kept at -20°C before used.

3.2.8 Agarose gel electrophoresis

In order to determine the quality of genomic DNA, 0.8% (w/v) agarose gel was prepared. The loading sample was mixed between 5 µl of genomic DNA and 1x loading dye (5x loading dye: 25 mM Tris-HCl at pH 7.0, 0.05% bromophenol blue, 150 mM EDTA, and 25% glycerol). Also, λ *Hin*d III marker (200 ng) was used as a standard marker. Electrophoresis was performed by using 1x TBE buffer (0.05 M Tris-HCl at pH 8.0, 0.05 M Boric acid, and 0.65 M EDTA) as running buffer at 100 V for 50 min. After that, the gel was stained by 10 µg/ml Ethedium bromide (EtBr) for 5 min and destained by d-H_2O for 30 min. Genomic DNA was visible under UV light and photographed.

3.2.9 Polymerase Chain Reaction (PCR)

Primers were designed by using Primer 3 program (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Forward primers (BuL3: 5'- AGGTT CTGTT ACTAA CATGT -3' and primer_c: 5'- CGAAA TCGGT AGACG CTACG -3') and reverse primers (BuR3: 5'- GGTCT CTCCA ACGCA TAAAT -3', primer_d: 5'- GGGGA TAGAG GGACT TGAAC -3', and primer_f: 5'- ATTTG AACTG GTGAC ACGAG -3') were synthesized. PCR reaction was carried out in 2x PCR Master mix solution (*i*-Taq) (catalog # 25027), iNtRON BIOTECHNOLOGY, 2 μM of each FW and RW primer, and genomic DNA (200 ng). PCR condition by *rbc*L *and trn*LF amplification were as followed: 94°C for 2 min, 30 sec, followed by 35 cycles of 94°C for 1 min; 59°C for 1 min; and 72°C for 3 min, and a final extension step at 72°C for 10 min. The PCR product was electrophoresed on 1.2% agarose gel at 100 V for 1 h.

3.2.10 PCR product purification

Any contaminants in PCR mixture must be removed by purification before sequencing. In this research, 2 kits were used to purify PCR product.

- Purification by QIAquick® PCR purification kit (Qiagen, catalog # 28104**)**

Five times volume of buffer PB were mixed with 1x volume of PCR product. The mixture was then transferred to a QIAquick® spin column which would be centrifuged at 13,000 rpm for 1 min. Flow through (FT) was discarded. Buffer PE of 750 μl was added to the column which would be centrifuged at 13,000 rpm for 1 min. After that, FT was discarded again. The column was centrifuged additionally at

13,000 rpm for 1 min. The column was removed to a new 1.5 ml microcentrifuge tube. Buffer EB (30 μl) was added to the center of the column. It was incubated at RT for 2 min and was centrifuged at 8,000 rpm for 1 min.

- Purification by *AccuPrep*® PCR purification kit (BIONEER, catalog # K-3034)

Five times volume of buffer PB was added to 1x volume of the PCR reaction. A binding column was placed in a 2 ml tube. Later, sample was applied to the column. It was centrifuged at maximum speed for 30-60 sec. The flow-through was discarded. Buffer WB (500 μl) was added to the column tube. It was centrifuged at maximum speed for 30-60 sec. Again, the flow-through was discarded. More buffer WB (500 μl) was added. Additional centrifugation was performed to confirm that the membrane was completely dry. The binding column was placed in a clean 1.5 ml tube. Buffer EL (30 μl) was added to the center of the binding column filter. It was incubated at RT for 1 min and centrifuged at maximum speed for 1 min. The eluted DNA was kept at -20^{o}C.

3.2.11 DNA sequencing and phylogenetic analysis

Purified PCR products were sequenced by Bioservice unit (BSU) and Research center, Ramathibodi hospital. Then, partial DNA sequences were aligned initially by using the multiple sequence alignment program CLUSTAL X. The data were saved to NEXUS file formatted for further phylogenetic tree construction. Phylogenetic analyses were performed by using neighbor-joining (NJ) and UPGMA (PAUP*4.0b10) (Swofford, 2000). In order to investigate the support for nodes estimated in a parsimony tree, bootstrap analysis with 1000 replicates were undertaken by PAUP*4.0b10.

3.2.12 Random Amplified Polymorphic DNA (RAPD)

Due to Mienie *et al.* (1995), 15 RAPD primers were tried. Considering amplification ability, 5 primers were selected (Table 3). A reaction was carried out in a volume of 20 µl. It contained 10 µl of PCR master mix, 4 µl of primer, 4 µl of sterile water, and 2 µl of DNA.

An amplification reaction was performed by the 2400 thermal controller with the following cycles: 94°C for 2 min, 30 sec, followed by 45 cycles of 94°C for 1 min; 36°C for 1.5 min; and 72°C for 3 min, and a final extension step at 72°C for 10 min. Each reraction of 35 DNA samples was amplified twice to ensure that the PCR profiles were reproducible. Amplified products were electrophoresed on 2.0% agarose gel at 80 V for 1 h 30 min, stained by Ethedium bromide, and photographed by Polaroid camera under UV light.

3.2.13 RAPD data analysis

Amplified bands in a size range from 0.1 to 1.5 kb were scored. Neighbour-joining cluster analysis was performed to demonstrate the relationships among populations by considering Nei-Li genetic distance (PAUP*4.0b10).

CHAPTER IV

RESULTS

4.1 Morphometry

4.1.1 Collection of leaves

Butea superba leaves were collected from 5 parts (the northern, the northeastern, the central, the western, and the eastern parts) of Thailand. In term of morphometric analysis, we could collect mature leaves from only 29 of total 34 cultivars (in 24 provinces). Moreover, 34 cultivars could be collected for genetic analysis. The detail was in Table 5.

Table 5. Collection of leave in Thailand. Non-analysis (N/A) indicated unavailable cultivars of collections.

No.	Province	Code name	Morphometric analysis	Genetic analysis
1	Kanchanaburi	KC 1 KC 2 KC 3	+ + N/A	+ + +
2	Khon Kaen	KK	+	+
3	Chantaburi	CT	+	+
4	Chachoengsao	CC	+	+
5	Chonburi	CB	+	+
6	Chaiyaphum	CHY	N/A	+
7	Chiangrai	CR 1 CR 2	+ N/A	+ +
8	Tak	TK	+	+
9	Nakorn ratchasima	Nak	+	+
10	Nakhon sawan	NS 1 NS 2 NS 3	+ + +	+ + +

No.	Province	Code name	Morphometric analysis	Genetic analysis
11	Buriram	BR	+	+
12	Prachenburi	PB	+	+
13	Phitsanulok	PS 1 PS 2 PS 3	+ + +	+ + +
14	Phetchaboon	PC	N/A	+
15	Ratchaburi	Rat 1 Rat 2 Rat 3 Rat 4	+ + + +	+ + + +
16	Lopburi	LB	+	+
17	Lampang 1	LP1	+	+
18	Lampang 2	LP2	+	+
19	Loei	LY	+	+
20	Sakhonnakorn	SK	+	+
21	Saraburi	SR	+	+
22	Sukhothai	SU	+	+
23	Nongbualamphu	NB	+	+
24	Uttaradit	UTT	N/A	+

4.1.2 Factor analysis

In each population, Principal Component Analysis (PCA) method of factor analyses was performed by using raw data of each of 9 morphometric characters. After that, factor loadings would be obtained. Since only factor loading greater than 0.6 would be selected for further analysis, there are only 7 qualified morphometric characters as indicated below (Figure 8-10):

1. petiole diameter – PD
2. number of pairs of primary veins – NPV
3. stipule length – SPL

4. petiole length – PL
5. rachis length – RL
6. terminal leaflet breadth – TLB
7. angle of first leaf border – AB^{o}

The factor analysis of leave length of 7 selected morphometric characters can divide them into 3 groups. First factor was accounted for 34.24% of total variation and was mainly associated with petiole diameter (PD), number of pairs of primary veins (NPV), rachis length (RL), and terminal leaflet breadth (TLB). The 2nd factor was accounted for 17.17% and was mainly associated with stipule length (SPL) and petiolet length (PLL). The 3rd factor was mainly associated with angle of first leaf border (AB^{o}). This factor was accounted for 11.48% of total variation.

Interactive graph was based on factor 1, factor 2, and factor 3. Figure 12-14 showed the distribution of *B. superba* populations.

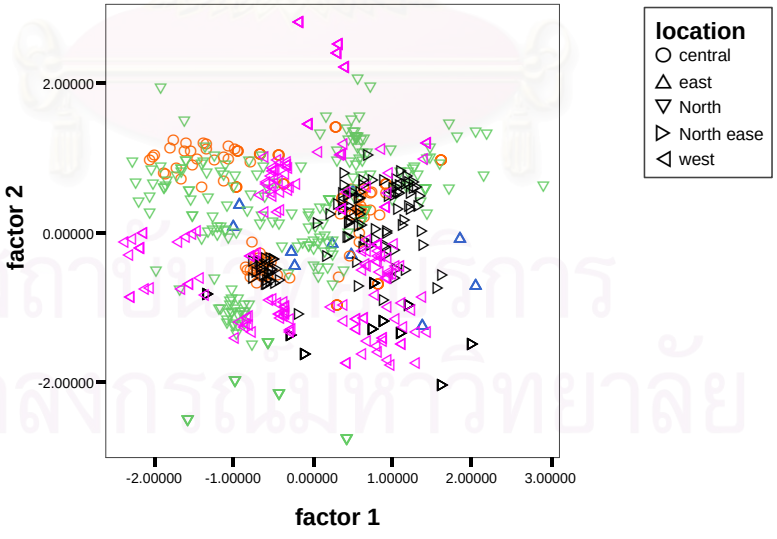


Figure 12. Plots of factor score 1 and factor scores 2 generated by Principal Component Analysis (PCA). *B. superba* were coded by collecting locations.

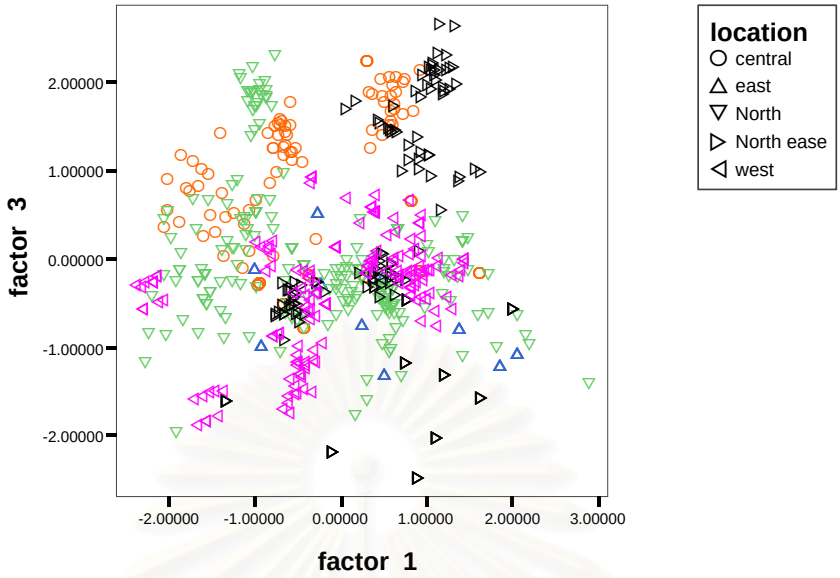


Figure 13. Plots of factor scores 1 and factor score 3 generated by Principal Component Analysis (PCA). *B. superba* were coded by collecting locations.

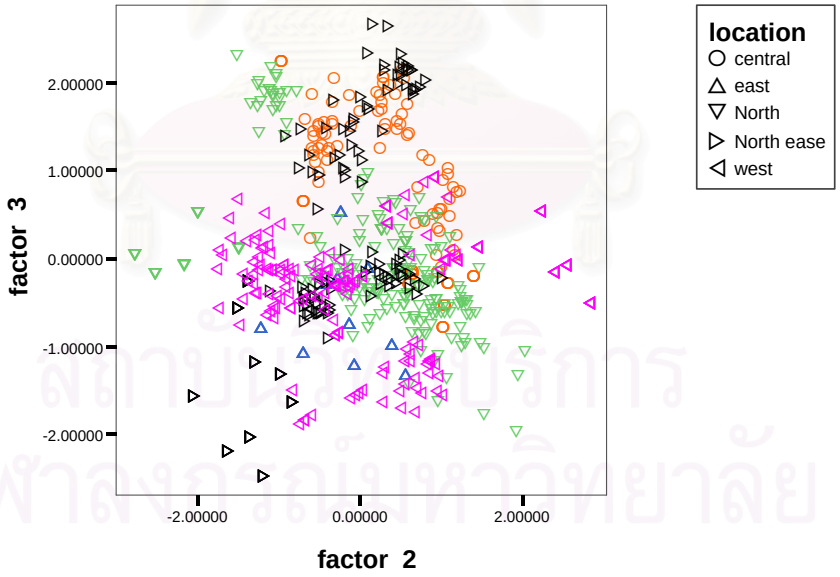


Figure 14. Plots of factor scores 2 and factor score 3 generated by Principal Component Analysis (PCA). *B. superba* were coded by collecting locations.

4.1.3 Cluster analysis

A dendrogram was constructed by a cluster analysis of the squeared euclidian distances between means of factor scores (Figure 15). Dendrograms revealed that collected *B. superba* could not separate into groups.

4.1.4 Clinal patterns in the characteristic of *B. superba* in Thailand

To explore clinal patterns in the characteristics of *B. superba*, factor scores were plotted against latitude and longitude. Gradual transitions of characters from the South to the North and the West to the East are indicated (Figure 16-21). Result of correlation analyses of factor scores against latitude and longitude are summarized in Table 6. A distinct and highly significant slope ($P \leq 0.05$) is observed in latitude and longitude. In conclusion, from the North to the South, leaf length increase in size in factor 1 but decrease in size in factor 2.

Table 6. Correlation of geographic trends in morphometric characters of *B. superba* from Thailand. ** Correlation is significant at the 0.01 level (2-tailed).

Predictor	Dependent variable	R value	P significant
Latitude	Factor 1 (PD,RL,NPV,TLB)	0.212**	0.00**
	Factor 2 (SPL, PLL)	-0.241**	0.00**
	Factor 3 (AB^0)	-0.68	0.068
Longtitude	Factor 1 (PD,RL, NPV,TLB)	0.020	0.595
	Factor 2 (SPL, PLL)	0.058	0.116
	Factor 3 (AB)	0.046	0.218

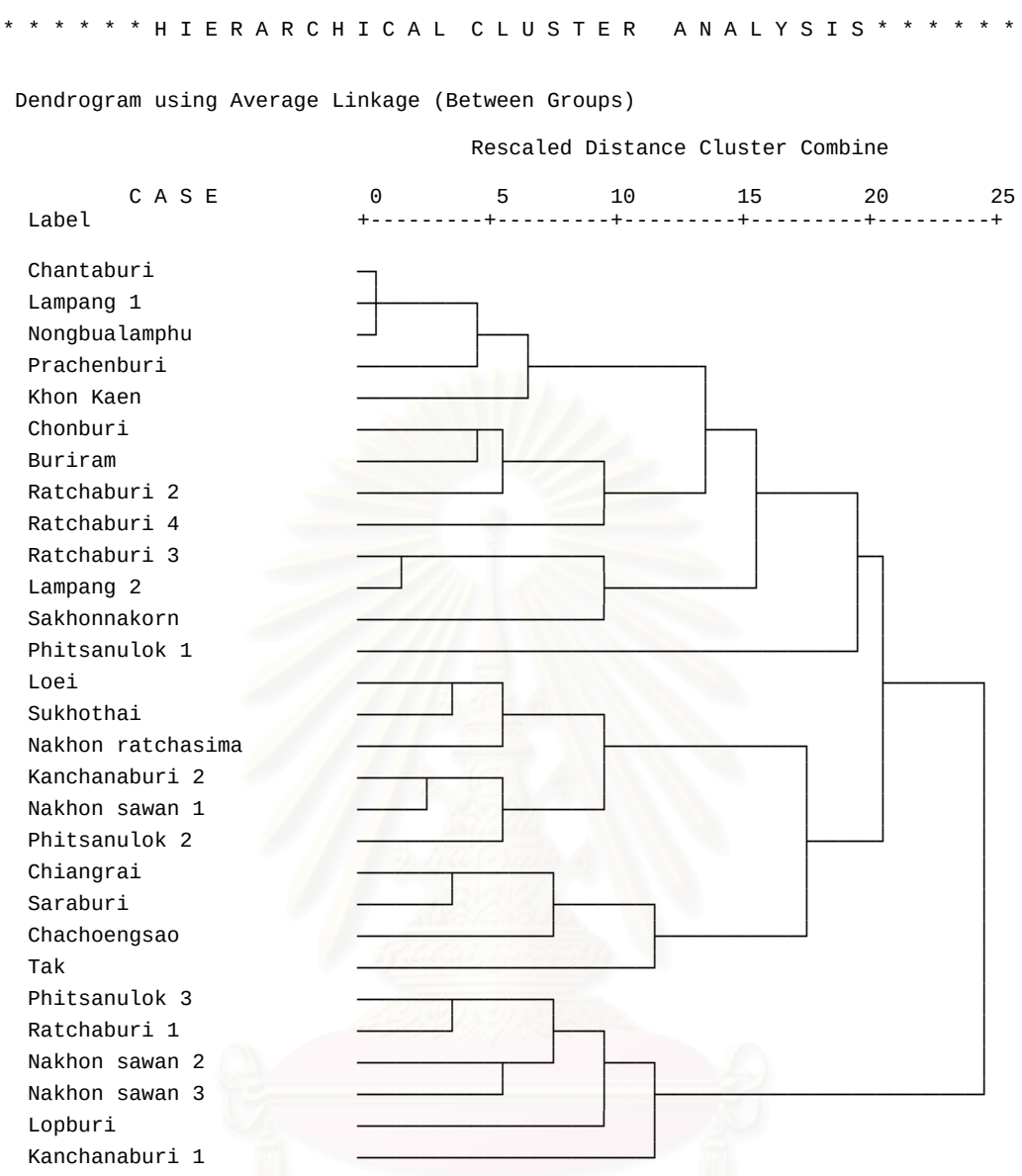


Figure 15. A dendrogram constructed by a cluster analysis. *B. superba* is classified by collecting locations.

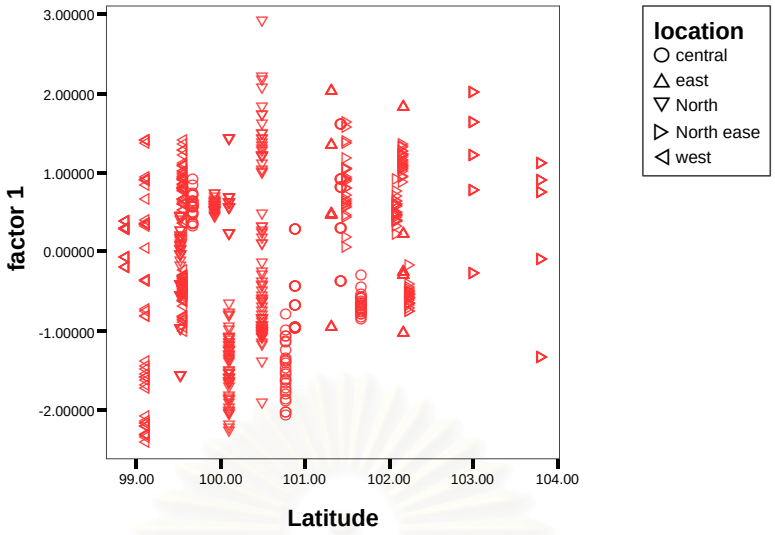


Figure 16. Geographic trends in morphometric characters of *B. superba* in Thailand, latitude and ordinate; factor score 1 as derived from PCA. Value labels refer to major sampling locations.

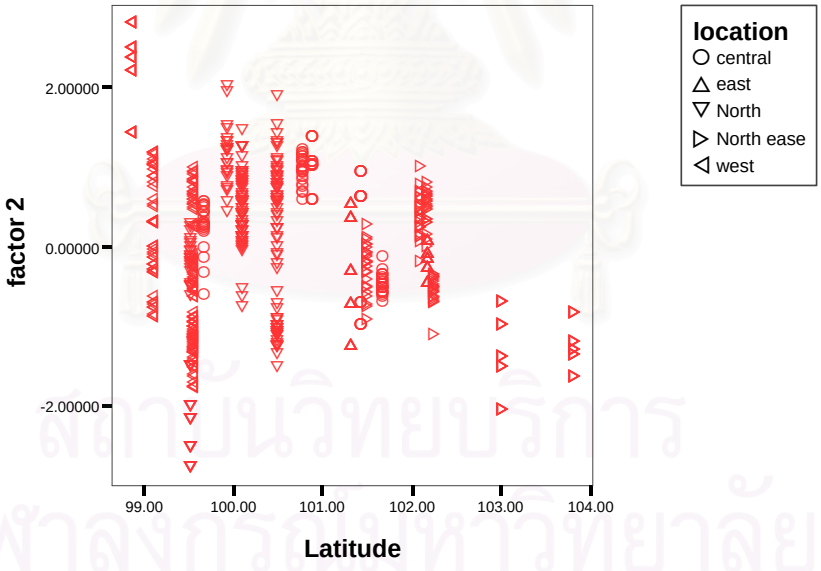


Figure 17. Geographic trends in morphometric characters of *B. superba* in Thailand, latitude and ordinate; factor score 2 as derived from PCA. Value labels refer to major sampling locations.

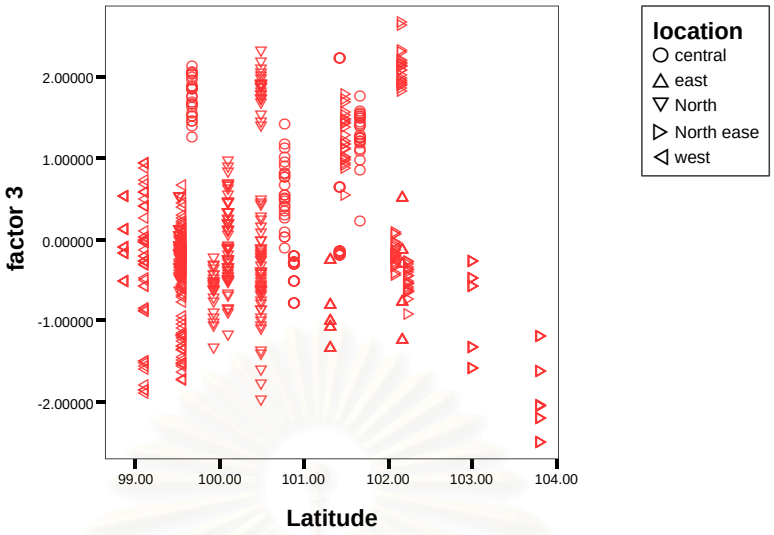


Figure 18. Geographic trends in morphometric characters of *B. superba* in Thailand, latitude and ordinate; factor score 3 as derived from PCA. Value labels refer to major sampling locations.

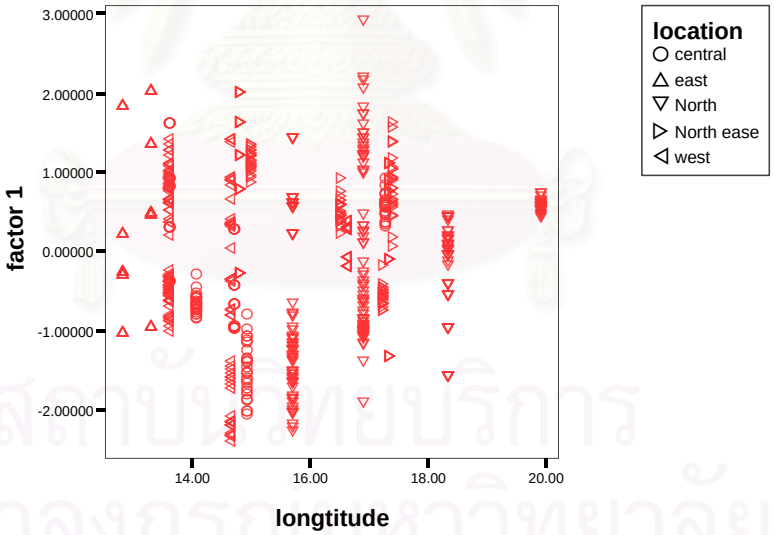


Figure 19. Geographic trends in morphometric characters of *B. superba* in Thailand, longitude and ordinate; factor score 1 as derived from PCA. Value labels refer to major sampling locations.

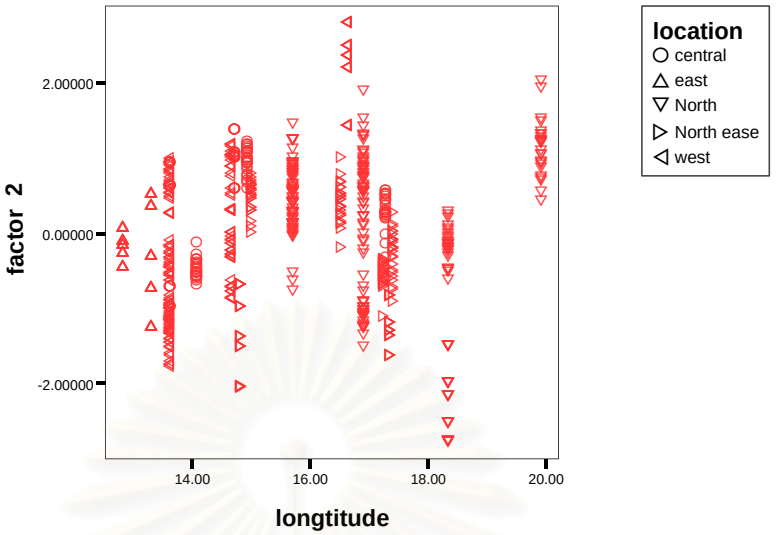


Figure 20. Geographic trends in morphometric characters of *B. superba* in Thailand, longitude and ordinate; factor score 2 as derived from PCA. Value labels refer to major sampling locations.

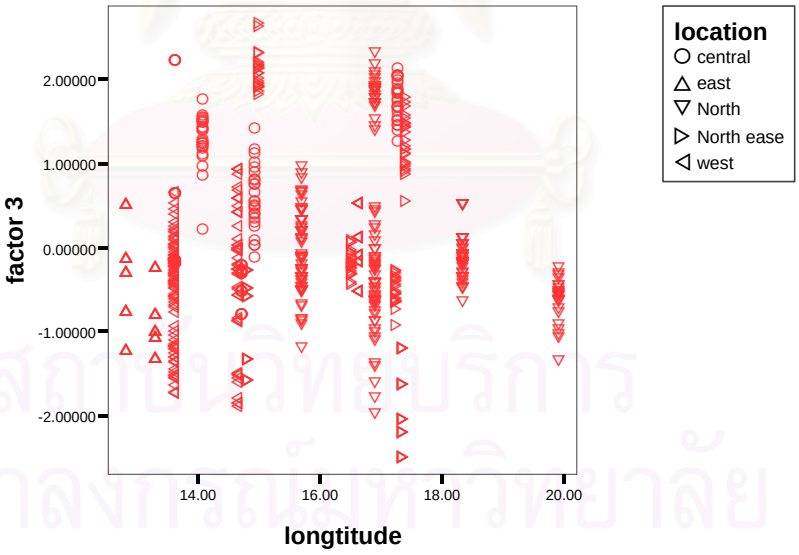


Figure 21. Geographic trends in morphometric characters of *B. superba* in Thailand, longitude and ordinate; factor score 3 as derived from PCA. Value labels refer to major sampling locations

4.2 Genetic variation analysis

4.2.1 DNA extraction

Genomic DNA of fresh young leaves of *B. superba* were extracted by QIAamp® DNA mini kit and Nucleospin® DNA mini kit. Good quality of genomic DNA is determined by sharp and high molecular weight (MW) band on agarose gel. High MW of genomic DNA (about 23 kb in length) is presented (Figure 22).

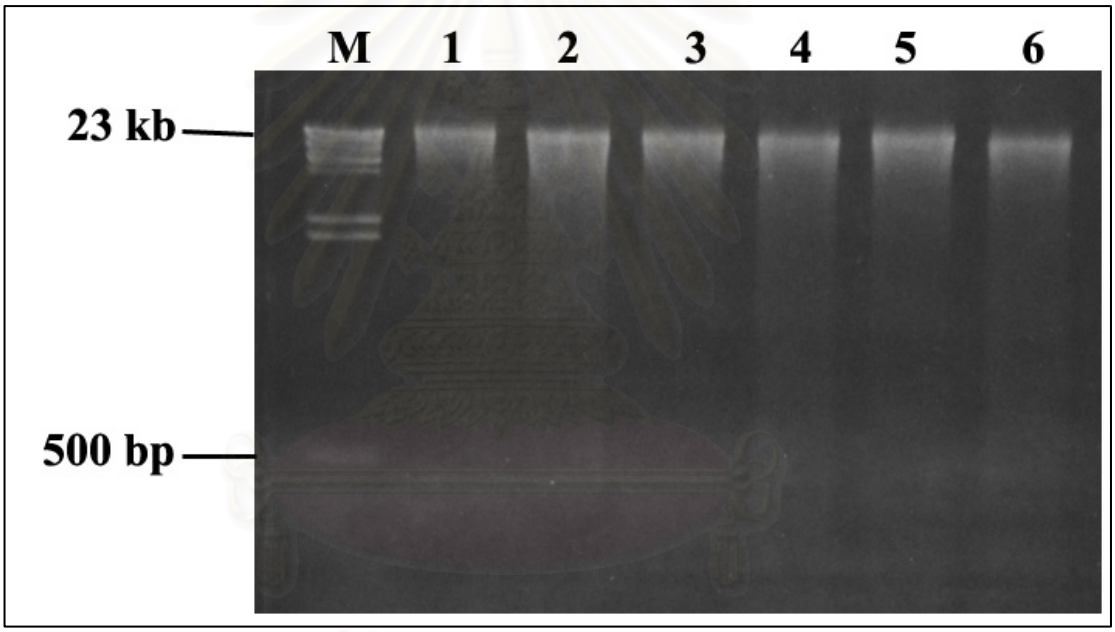


Figure 22. High MW DNA of *B. superba* on 0.8% agarose gel. Lane 1-6 indicate individual genomic DNA while lane M represents λ *Hin*d III as standard DNA marker.

4.2.2 PCR amplification

PCR is a technique for *in vitro* DNA amplification of specific sequence by simultaneous primer extension of complementary strand of DNA. After electrophoresis on 1.2% agarose gel and EtBr staining, PCR product was visible under UV light. Size of the product was estimated by comparing to 100 bp DNA ladder. Due to primer design, expected PCR products amplified by *rbc*L, *trn*LF-cd and *trn*LF-cf primers were 300 bp, 550 bp, and 1,000 bp, respectively (Figure 23-25 and Table 7).

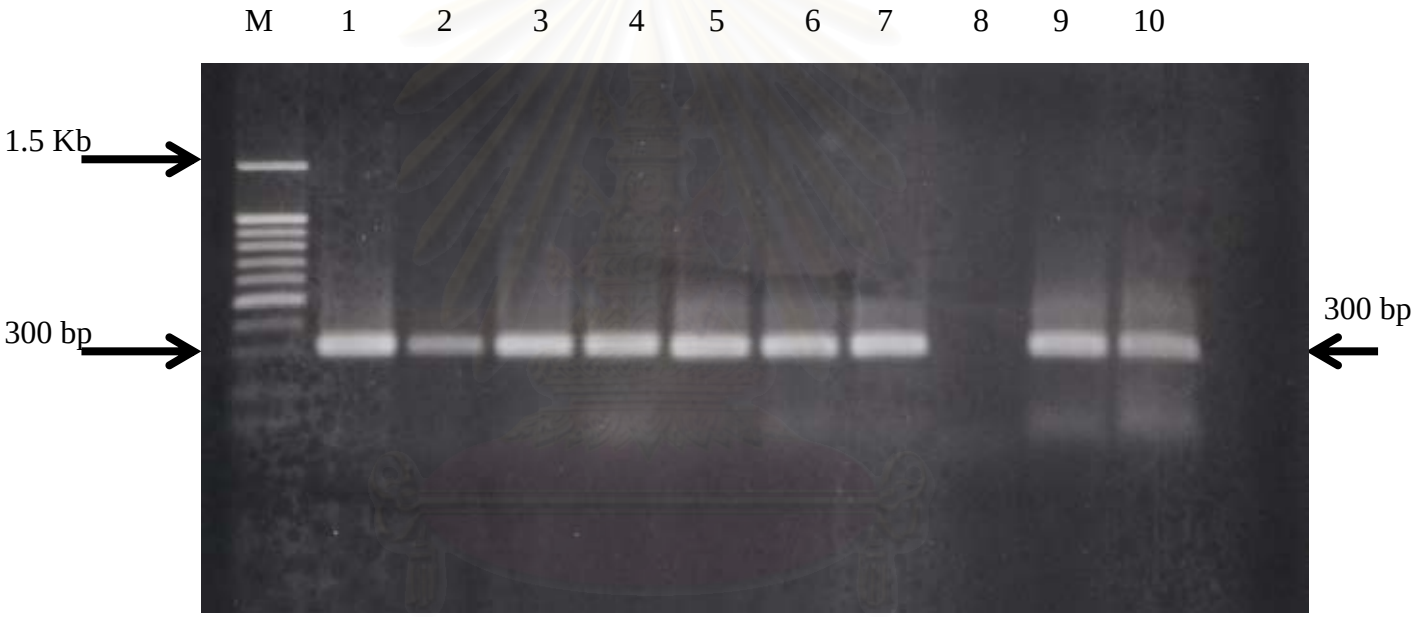


Figure 23. PCR products of *rbc*L on 1.2% agarose gel. Lane 1-7 and 9-10 contain the PCR product of *B. superba*. In addition, lane 8 contains no PCR product of *Butea sp.* (outgroup control). Lane M represents 100 bp ladder as DNA marker.

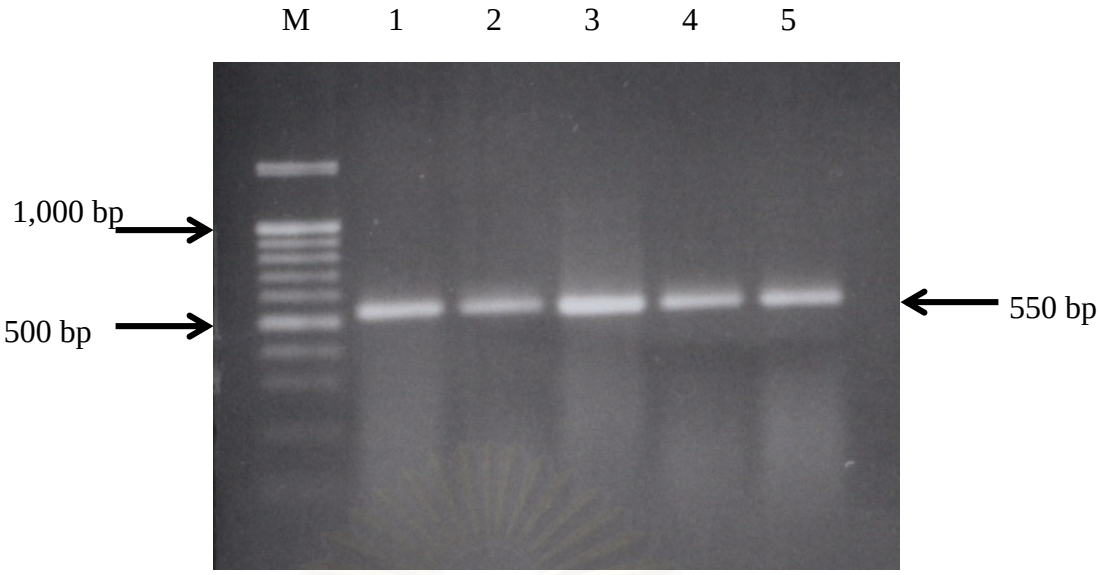


Figure 24. PCR products of *trn*LF-cd on 1.2% agarose gel. Lane 1-5 contains the PCR product of *B. superba*. Lane M represents 100 bp ladder as DNA marker.

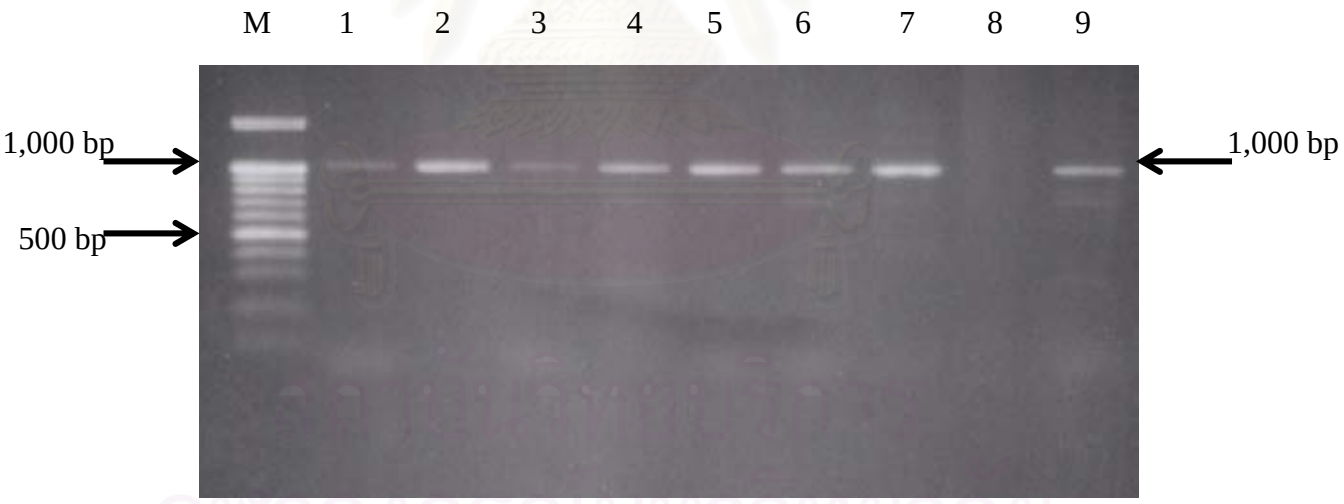


Figure 25. PCR products of *trn*LF-cf on 1.2% agarose gel. Lane 1-7 and 9 contains the PCR product of *B. superba*. In addition, lane 8 contains no PCR product of outgroup control (*Butea sp.*). Lane M represents 100 bp ladder as DNA marker.

Table 7. The results of PCR amplification by 3 pairs of primers.

+ indicated a visible PCR band while the – indicated an invisible PCR band.

No.	**Cultivars**	**Code**	***rbc*L (BuL 3 and BuR 3)**	***trn*LF-cd**	***trn*LF-cf**
1	Kanchanaburi 1	KC 1	+	+	+
2	Kanchanaburi 2	KC 2	+	+	+
3	Kanchanaburi 3	KC 3	+	+	+
4	Khon Kaen	KK	+	+	+
5	Chantaburi	CT	+	+	+
6	Chachoengsao	CC	+	+	+
7	Chonburi	CB	+	+	+
8	Chaiyaphum	CHY	+	+	+
9	Chiangrai 1	CR 1	+	+	+
10	Chiangrai 2	CR 2	+	+	+
11	Tak	TK	+	+	+
12	Nakhon ratchasima	NAK	+	+	+
13	Nakhon sawan 1	NS 1	+	+	+
14	Nakhon sawan 2	NS 2	+	+	+
15	Nakhon sawan 3	NS 3	+	+	+
16	Buriram	BR	+	+	+
17	Prachenburi	PB	+	+	+
18	Phitsanulok 1	PS 1	+	+	+
19	Phitsanulok 2	PS 2	+	–	–
20	Phitsanulok 3	PS 3	+	+	+
21	Phetchaboon	PC	+	+	+
22	Ratchaburi 1	RAT 1	+	+	+
23	Ratchaburi 2	RAT 2	+	+	+
24	Ratchaburi 3	RAT 3	+	+	+
25	Ratchaburi 4	RAT 4	+	+	+

No.	Species	Code	*rbc*L BuL 3 and BuR 3	*trn*LF-cd	*trn*LF-cf
26	Lopburi	LB	+	+	+
27	Lampang 1	LP 1	+	+	+
28	Lampang 2	LP 2	+	+	+
29	Loei	LY	+	+	–
30	Sakhonnakorn	SK	+	+	+
31	Saraburi	SR	+	+	+
32	Sukhothai	SU	+	+	+
33	Nongbualamphu	NB	+	+	+
34	Uttaradit	UTT	+	+	+

Outgroup controls

No.	Species	Code	*rbc*L BuL 3 and BuR 3	*trn*LF-cd	*trn*LF-cf
1	*B. monosperma* (syn. *B. frondosa)*	Bm	+	–	–
2	*Butea sp.*	Con	–	–	–
3	*P. mirifica*	Pm	+	+	+
4	*P. lobata*	Pl	+	+	+

+ indicated a visible PCR band while the – indicated an invisible PCR band

According to Table 7, *rbc*L primer could amplify DNA of all 34 cultivars of *B. superba* and DNA of *B. monosperma*, *P. mirifica*, and *P. lobata* (outgroup control). The *trn*LF-cd primer could amplify DNA of 33 cultivars of *B. superba* except DNA of Phitsanulok 2 cultivar. Moreover, *trn*LF-cf primer could amplify DNA of 32 cultivars of *B. superba* except DNA of Phitsanulok 2 and Loei cultivars.

Table 8. Results of sequencing of PCR product recorded in Table 7. P indicates positive/ obtained nucleotide sequence. F indicates negative/ failed to obtained nucleotide sequence. In addition, N/A indicates no analysis/ no PCR band.

No.	**Cultivars**	**Code**	***rbc*L (BuL 3 and BuR 3)**	***trn*LF-cd**	***trn*LF-cf**
1	Kanchanaburi 1	KC 1	P	P	F
2	Kanchanaburi 2	KC 2	P	F	F
3	Kanchanaburi 3	KC 3	P	P	P
4	Khon kaen	KK	P	P	P
5	Chantaburi	CT	P	P	P
6	Chachoengsao	CC	P	P	P
7	Chonburi	CB	P	P	P
8	Chaiyaphum	CHY	P	P	P
9	Chiangrai 1	CR 1	P	P	P
10	Chiangrai 2	CR 2	P	P	P
11	Tak	TK	P	F	F
12	Nakhon ratchasima	NAK	P	P	P
13	Nakhon sawan 1	NS 1	F	P	P
14	Nakhon sawan 2	NS 2	P	P	P
15	Nakhon sawan 3	NS 3	P	P	P
16	Buriram	BR	P	P	P
17	Prachenburi	PB	P	P	P
18	Phitsanulok 1	PS 1	P	F	F
19	Phitsanulok 2	PS 2	P	F	N/A
20	Phitsanulok 3	PS 3	P	P	P
21	Phetchaboon	PC	P	P	P
22	Ratchaburi 1	RAT 1	P	P	P
23	Ratchaburi 2	RAT 2	P	P	P

No.	Cultivars	Code	*rbc*L (BuL 3 and BuR 3)	*trn*LF-cd	*trn*LF-cf
24	Ratchaburi 3	RAT 3	P	P	P
25	Ratchaburi 4	RAT 4	P	P	P
26	Lopburi	LB	P	P	F
27	Lampang 1	LP 1	F	P	P
28	Lampang 2	LP 2	P	P	P
29	Loei	LY	P	P	N/A
30	Sakhonnakorn	SK	P	P	P
31	Saraburi	SR	P	P	P
32	Sukhothai	SU	P	P	P
33	Nongbualamphu	NB	P	F	F
34	Uttaradit	UTT	P	P	P

Table 8. (continued)

Outgroup control

No.	Species	Code	*rbc*L BuL 3 and BuR 3	*trn*LF-cd	*trn*LF-cf
1	*B. monosperma* (syn. *B. frondosa)*	Bm	P	N/A	N/A
2	*Butea sp.*	Con	N/A	N/A	N/A
3	*P. mirifica*	Pm	P	P	P
4	*P. lobata*	Pl	P	P	P

4.2.3 Sequence analysis

After PCR amplification, PCR products of *rbc*L of *B. superba* from all collecting localities in Thailand were purified and sequenced. In Table 8, nucleotide sequences of chloroplast gene, *rbc*L, were obtained from 32 cultivars of *B. superba*, 29 cultivars of *trn*LF-cd regions, and 25 sequences of *trn*LF-cf regions, respectively.

The obtained sequence length of *rbc*L, *trn*LF-cd, and *trn*LF-cf regions were 247, 229, and 410 bp, respectively. They contain high A+T content with the average of 58.58% on *rbc*L region, 67.46% in *trn*LF-cd region, and 63.87% in *trn*LF-cf region (Table 9-11). In addition, multiple alignment sequence by clustal X comparisons revealed nucleotide variation in the form of single base pair substitution (Figure 22-24). Then, the pairwise distance in pair of *rbc*L, *trn*LF-cd, and *trn*LF-cf sequences by PAUP*4.0b10 were 0-1.2%, 0 - 51.22%, and 0 - 98.23 %, respectively (Table 12, 13 and 14).

Alignment: BuL3 and BuR3 primers of *rbc*L region

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
PS2             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
Nak             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
CHY             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
Rat4            TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
KC3             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
Rat3            TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
BR              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
Rat2            TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
Rat1            TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
KC1             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
NS3             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
KK              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
BM(outgroup)    TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
NS2             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
PS3             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
SU              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
NB              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
Utt             TGCGCGCTCT ACGTCTGGGG GATTTGCGAA TCCCTATTTC TTATATTAAA
Tak             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
PC              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
KC2             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
SR              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
CT              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
LY              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
PB              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
CR1             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
LB              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
SK              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
CB              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
PM(outgroup)    TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
PL(outgroup)    TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
LP2             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
PS1             TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
CC              TGCGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATATTAAA
CR2             TACGCGCTCT ACGTCTGGAG GATTTGCGAA TCCCTATTTC TTATGTTAAA
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
PS2             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Nak             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
CHY             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Rat4            ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
KC3             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Rat3            ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
BR              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Rat2            ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Rat1            ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
KC1             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
NS3             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
KK              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
BM              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
NS2             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
PS3             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
SU              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
NB              ACTTTACAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Utt             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Tak             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
PC              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
KC2             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
SR              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
CT              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
LY              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
PB              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
CR1             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
LB              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
SK              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
CB              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
PM              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
PL              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
LP2             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
PS1             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
CC              ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
CR2             ACTTTCCAAG GTCCGCCTCA TGGCATCCAA GTTGAGAGAG ATAAATTGAA
Clustal Co      ***** **** ********** ********** ********** **********
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                ....|....| ....|....| ....|....| ....|....| ....|....|
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Nak             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
CHY             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
Rat4            CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
KC3             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
Rat3            CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
BR              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
Rat2            CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
Rat1            CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
KC1             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
NS3             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
KK              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
BM              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
NS2             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
PS3             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
SU              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
NB              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
Utt             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
Tak             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
PC              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
KC2             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
SR              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
CT              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
LY              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
PB              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
CR1             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
LB              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
SK              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
CB              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
PM              CAAGTATGGT CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
PL              CAAGTATGGT CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
LP2             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
PS1             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
CC              CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
CR2             CAAGTATGGC CGTCCCCTAT TAGGATGTAC TATTAAACCT AAATTGGGGT
Clustal Co      *********  ********** ********** ********** **********
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
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Nak             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
CHY             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
Rat4            TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
KC3             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
Rat3            TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
BR              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
Rat2            TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
Rat1            TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
KC1             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
NS3             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
KK              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
BM              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
NS2             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
PS3             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
SU              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
NB              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
Utt             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
Tak             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
PC              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
KC2             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
SR              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
CT              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
LY              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
PB              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
CR1             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
LB              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
SK              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
CB              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
PM              TATCCGCTAA GAATTATGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
PL              TATCCGCTAA GAATTATGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
LP2             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
PS1             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
CC              TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
CR2             TATCCGCTAA GAATTACGGT AGAGCAGTTT ATGAATGTCT TCGTGGGGGA
Clustal Co      ********** ****** *** ********** ********** **********
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                ....|....| ....|....| ....|....| ....|....| ....|..
                        210        220        230        240
PS2             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Nak             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
CHY             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Rat4            CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
KC3             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Rat3            CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
BR              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Rat2            CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Rat1            CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
KC1             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
NS3             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
KK              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
BM              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
NS2             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
PS3             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
SU              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
NB              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Utt             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Tak             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
PC              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
KC2             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
SR              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
CT              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
LY              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
PB              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
CR1             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
LB              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
SK              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
CB              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
PM              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
PL              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
LP2             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
PS1             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
CC              CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
CR2             CTTGATTTTA CCAAAGATGA TGAAAATGTG AATTCCCAAC CATTTAT
Clustal Co      ********** ********** ********** ********** *******
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Figure 26. A 247 bp character matrix based on partial *rbc*L of chloroplast DNA sequences of 32 cultivars of *B. superba*. Asterisks * indicate that all samples provide nucleotide identity.

Alignment: *trn*LF-c and *trn*LF-d primers of *trn*LF-cd region

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
CR2             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTATAAAG TGATAATAAA
LP1             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
KC3             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
Rat4            CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
CB              CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
Utt             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
KK              CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
CT              CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
PC              CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
NS3             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
NS2             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
Rat3            CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
Rat2            CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
Rat1            CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
LP2             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
CR1             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
CHY             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
PS3             CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAT
CC              CCAAATCCTG TTTCCTGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
Pm              CCAAATCCCG TTTTCCGAAA ACAAAGAAAA GTTCGGAAAG TGATAATAAA
Pl              CCAAATCCCG TTTTCCGAAA ACAAAGAAAA GTTCGGAAAG TGATAATAAA
SK              CCAAATCCTG TTGGCCGAGA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
NS1             CCAAATCCTG TCCCCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
LB              CCAAATCGTG TCCCCCGAAA ACAAGGGAAA GTTTGGAAAG TGATAATAAA
LY              CCAAATCCTG TCCCCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
SR              CCAAATCCTG TCCTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATAAA
Nak             CCAAATCCTG TCTTCCGAAA ACGAAGAAAA GTTTAGAAAG TGATAATAAA
SU              CCAAATCCTG TTTTCCGAAA ACAAAGAAAA GTTTAGAAAG TGATAATATG
KC1             CCAAATCCTG TTTTCCGAAA ACGAAGAAAA GTTTAGAAAG TGATAATAAA
BR              CCAAATCCTG TCCCCCGAAA ACGGGAAAAG GTTTAGAAAG TGATAATAAA
PB              CCAAATCCTG TTTTCCGAAA ACGACGAAAA GTTTAGAAAG TGATAATAAT
Clustal Co      *******  * *   * ** * **     **  ***   **** ********
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
CR2             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
LP1             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
KC3             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
Rat4            AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
CB              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
Utt             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
KK              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
CT              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
PC              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
NS3             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
NS2             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
Rat3            AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
Rat2            AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
Rat1            AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
LP2             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
CR1             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
CHY             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
PS3             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
CC              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
Pm              AAAGGGATAG GTGCAGAGAC TCAATGGAAG CTGTTCTAAC AAACGGAGTT
Pl              AAAGGGATAG GTGCAGAGAC TCAATGGAAG CTGTTCTAAC AAACGGAGTT
SK              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAAGGGAGTT
NS1             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGACCT
LB              AAAGGGATGG GTGCAGAGAC TCGGTGGAAG CTGTTCTAAC AAATGGAGCT
LY              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
SR              AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC AAATGGAGTT
Nak             AAAGGGATAG GCGCAGAGAC TCGATGGAAG CTGTTCTAAC ACATGGAGTT
SU              AAAGGGATAG GCGCAGAGAC TCGATGGAAG CTGTTCTAAC ACATGGAGTT
KC1             AAAGGGATAG GTGCAGAGAC TCGATGGAAG CTGTTCTAAC ACATGGAGTT
BR              AAAGGGATAG GTGCAGAGAC TCGATGGAAG GTGTTGTAAC ACATGGAGAT
PB              TAAGGGAGTG GCGCTGAGAC TCGTTGGTTG GTGTTGTAAC ACATGGAGTT
Clustal Co       ******  * * ** ***** **  ***  *  **** **** * * ***  *
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
CR2             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
LP1             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
KC3             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
Rat4            GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
CB              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
Utt             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
KK              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
CT              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
PC              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
NS3             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
NS2             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
Rat3            GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
Rat2            GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
Rat1            GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
LP2             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
CR1             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
CHY             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
PS3             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
CC              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
Pm              GACGATTTTT CCTTTTTGCA TTAGGAAAAG AATCCTTCCA TCAAAATTCC
Pl              GACGATTTTT CCTTTTTGCA TTAGGAAAAG AATCCGTCCA TCAAAATTCC
SK              GAATA----- -CTTTTTGCG TTATGAAAGG GATCATTCCA TCAAAATT--
NS1             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
LB              GACTA----- -CTTTTTGCG TAGGAAAGGA AATCATTCCC TCAAAATT--
LY              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
SR              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
Nak             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAAAATT--
SU              GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCT TCACGATT--
KC1             GACTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCACGATT--
BR              GAGTA----- -CTCCTTGCA CTAGGAAAGG AATCCTTCCA TCACGATT--
PB              GAGTA----- -CTTTTTGCA TTAGGAAAGG AATCATTCCA TCAACGTT--
Clustal Co      **  *       **  ****       **     ***  ***  ***   **
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
CR2             ---------- ---------- ---------- ---------- --TTCAATTG
LP1             ---------- ---------- ---------- ---------- --TTCAATTG
KC3             ---------- ---------- ---------- ---------- --TTCAATTG
Rat4            ---------- ---------- ---------- ---------- --TTCAATTG
CB              ---------- ---------- ---------- ---------- --TTCAATTG
Utt             ---------- ---------- ---------- ---------- --TTCAATTG
KK              ---------- ---------- ---------- ---------- --TTCAATTG
CT              ---------- ---------- ---------- ---------- --TTCAATTG
PC              ---------- ---------- ---------- ---------- --TTCAATTG
NS3             ---------- ---------- ---------- ---------- --TTCAATTG
NS2             ---------- ---------- ---------- ---------- --TTCAATTG
Rat3            ---------- ---------- ---------- ---------- --TTCAATTG
Rat2            ---------- ---------- ---------- ---------- --TTCAATTG
Rat1            ---------- ---------- ---------- ---------- --TTCAATTG
LP2             ---------- ---------- ---------- ---------- --TTCAATTG
CR1             ---------- ---------- ---------- ---------- --TTCAATTG
CHY             ---------- ---------- ---------- ---------- --TTCAATTG
PS3             ---------- ---------- ---------- ---------- --TTCAATTG
CC              ---------- ---------- ---------- ---------- --TTCAATTG
Pm              AGGAATGGAT CAAAGATAAA CATATATATA CTGAAATACT ATTTCAATTG
Pl              AGGAATGGAT CAAAGATAAA CATATATATA CTGAAATACT ATTTCAATTG
SK              ---------- ---------- ---------- ---------- --TTCAATTG
NS1             ---------- ---------- ---------- ---------- --TTCAATTG
LB              ---------- ---------- ---------- ---------- --TTGAATTG
LY              ---------- ---------- ---------- ---------- --TTCAATTG
SR              ---------- ---------- ---------- ---------- --TTCAATTG
Nak             ---------- ---------- ---------- ---------- --TTCAATTG
SU              ---------- ---------- ---------- ---------- --TTCAATTG
KC1             ---------- ---------- ---------- ---------- --TTCAATTG
BR              ---------- ---------- ---------- ---------- --TTCAATTG
PB              ---------- ---------- ---------- ---------- --TTCAATTG
Clustal Co                                                     ** *****
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250
CR2              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
LP1              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
KC3              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
Rat4             ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
CB               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
Utt              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
KK               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
CT               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
PC               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
NS3              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
NS2              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
Rat3             ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
Rat2             ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
Rat1             ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
LP2              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
CR1              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
CHY              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
PS3              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
CC               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
Pm               ATTAATGA-- -AGATCCATT TGTGATCAAA ATATTCACAA ATGAAAGATG
Pl               ATTAATGA-- -AGATCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
SK               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
NS1              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
LB               ATTAATGAGG GAGCTCCGTT TGT---AAAA ATATTCACAA ATGAAAGATG
LY               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAGGATG
SR               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
Nak              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCCCAA ATGAAAGATG
SU               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAGGGATG
KC1              ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCTCAA ATGAGGGATG
BR               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAAAGATG
PB               ATTAATGA-- -AGCTCCATT TGTGATAAAA ATATTCACAA ATGAGGGATG
Clustal Co       ********    ** *** ** ***    *** ****** *** ****  ****
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                ....|....| ....|....| ....|....| ...
                        260        270        280
CR2             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
LP1             TGAATCAAAT CACTGTCCAA GTTGAAGAAA AGA
KC3             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Rat4            TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
CB              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Utt             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
KK              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
CT              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
PC              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
NS3             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
NS2             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Rat3            TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Rat2            TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Rat1            TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
LP2             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
CR1             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
CHY             TGAATCAAAT CAAT-TCCGA GTTGAAGAAA AGA
PS3             TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
CC              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Pm              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Pl              TGAATCAA-- ---T-TCCAA GTTGAAGAAA AGA
SK              TGGATGAAAT CCAT-TCCAA GTTGAAGAAA AGA
NS1             TGAATCAAAT CAAT-TCTAA GTTGAAGAAA AGA
LB              AGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
LY              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
SR              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Nak             TGAATCACAT CAAT-TCCAA GTTGAAGAAA AGA
SU              TGAATCACAT CAAT-TGCTC GTTGATGAAA AGA
KC1             TGAATCACAT CCAT-TCTTC GTTGAAGAAA AGA
BR              TGGATGAAAT CCAT-TCCAA GTTGAAAAAA GGA
PB              TGAATCAAAT CAAT-TCCAA GTTGAAGAAA AGA
Clustal Co       * ** *       * *     *****  ***  **
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Figure 27. A 283 bp character matrix based on partial *trn*LF-cd of chloroplast DNA sequences of 29 cultivars of *B. superba*. Asterisks * indicate that all samples provide nucleotide identity.

Alignment: *trn*LF-cf gene

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
PS3             CCAAATCCTG CGGGGGGGGG GAAGAAAGCT TAGTTTAGAA AGTGATAATA
SK              CCAAATCCTG TTGGCCGAG- -AACAAAGAA AAGTTTAGAA AGTGATAATA
Pm              CCAAATCCCG TTTTCCGAA- -AACAAAGAA AAGTTCGGAA AGTGATAATA
Pl              CCAAATCCCG TTTTCCGAA- -AACAAAGAA AAGTTCGGAA AGTGATAATA
CR2             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTATAA ACTGATAATA
NS1             CCAAATCCTG T--CCCCCGA AAACAAAGAA AAGTTTAGAA AGTGATAATA
CC              CCAAATCCTG TTTCCTCCGA AAACAAAGAA AAGTTTAGAA AGTGATAATA
Rat4            CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
CR1             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
Rat3            CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
NS2             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
LP1             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
NS3             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
PC              CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
CB              CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
Rat2            CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
LP2             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
KC3             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
Rat1            CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
CHY             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
CT              CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
KK              CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
Utt             CCAAATCCTG TTTTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
SR              CCAAATCCTG TCCTCCGAA- -AACAAAGAA AAGTTTAGAA AGTGATAATA
Nak             CCAAATCCTG TCTTCCGAA- -AACGAAGAA AAGTTTAGAA AGTGATAATA
SU              CCAAATCCTG TTTTCCGAA- -AACGAAGAA AAGGTTAGAA AGTGATAATA
PB              CCAAAGCCTG TTTGCCGAC- -AACGTCGAA AAGGTGGGAA AGTGATAATT
BR              CCAAAGCCTG TCCCCCGAA- -AACGG-GAA AAGGTGGGAA AGTGATAATT
Clustal Co      ***** ** *            **    *    ** *   ** * *******

                ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
PS3             ATAAAGGGAT AGGTGCAGAG ACTCGATGGG AAGCTGTTCT AACAAATGGA
SK              AAAAAGGGAT AGGTGCAGAG ACTCCCTGG- ATGCTGTTCT AACAAAGGGA
Pm              AAAAAGGGAT AGGTGCAGAG ACTCAATGG- AAGCTGTTCT AACAAACGGA
Pl              AAAAAGGGAT AGGTGCAGAG ACTCAATGG- AAGCTGTTCT AACAAACGGA
CR2             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
NS1             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
CC              AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
Rat4            AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
CR1             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
Rat3            AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
NS2             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
LP1             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
NS3             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
PC              AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
CB              AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
Rat2            AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
LP2             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
KC3             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
Rat1            AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
CHY             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
CT              AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
KK              AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
Utt             AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
SR              AAAAAGGGAT AGGTGCAGAG ACTCGATGG- AAGCTGTTCT AACAAATGGA
Nak             AGAAAGGGAT AGGCGCAGAG ACTCGATGG- AAGCTGTTCT AACACATGGA
SU              TGAAAGGGAT AGGCGCAGAG ACTCGATGG- AAGCTGTTCT AACACATGGA
PB              TGTAAGGGAG TGGCGCTGAG ACTCGTTGG- TTGGTGTTGT TACTCACGGA
BR              TGAAAGGGAG AGGCGCAGAG ACTCGATGG- AAGGTGTTGT AACACATGGA
Clustal Co        ******   ** ** *** ****  ***    * **** *  **  * ***
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
PS3             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
SK              GTTGAATA-- ----CTTTTT GCGTTATGAA AGGGATCATT CTCTCAAAA-
Pm              GTTGACGATT TTTCCTTTTT GCATTAGGAA AAGAATCCTT CCATCAAAAT
Pl              GTTGACGATT TTTCCTTTTT GCATTAGGAA AAGAATCCGT CCATCAAAAT
CR2             GTTGACTACT CTT-CCTTTT GCATTGGGAA AGGAATCATT CCATCAAAA-
NS1             CCTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
CC              GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
Rat4            GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
CR1             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
Rat3            GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
NS2             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
LP1             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
NS3             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
PC              GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
CB              GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
Rat2            GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
LP2             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
KC3             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
Rat1            GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
CHY             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
CT              GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
KK              GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
Utt             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
SR              GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCATCAAAA-
Nak             GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCCTT CCAGCACAA-
SU              GTTGACTA-- ----CTTTTT GCATTAGGAA AGGAATCATT CCTTCACGA-
PB              GTTGAGTA-- ----CTCCTT GCTCTAGGAA AGGAATCCTT CCCGCACGT-
BR              GATGAGTA-- ----CTCCTT GCACTAGGAA AGGAATCCTT CCATCACGA-
Clustal Co        ***  *       *   ** **  *  *** * * ***  * *   **


                  ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
PS3             ---------- ---------- ---------- ---------- ---TTTTCAA
SK              ---------- ---------- ---------- ---------- ---GAATAAA
Pm              TCCAGGAATG GATCAAAGAT AAACATATAT ATACTGAAAT ACTATTTCAA
Pl              TCCAGGAATG GATCAAAGAT AAACATATAT ATACTGAAAT ACTATTTCAA
CR2             ---------- ---------- ---------- ---------- ---TTTTCAA
NS1             ---------- ---------- ---------- ---------- ---TTTTCAA
CC              ---------- ---------- ---------- ---------- ---TTTTCAA
Rat4            ---------- ---------- ---------- ---------- ---TTTTCAA
CR1             ---------- ---------- ---------- ---------- ---TTTTCAA
Rat3            ---------- ---------- ---------- ---------- ---TTTTCAA
NS2             ---------- ---------- ---------- ---------- ---TTTTCAA
LP1             ---------- ---------- ---------- ---------- ---TTTTCAA
NS3             ---------- ---------- ---------- ---------- ---TTTTCAA
PC              ---------- ---------- ---------- ---------- ---TTTTCAA
CB              ---------- ---------- ---------- ---------- ---TTTTCAA
Rat2            ---------- ---------- ---------- ---------- ---TTTTCAA
LP2             ---------- ---------- ---------- ---------- ---TTTTCAA
KC3             ---------- ---------- ---------- ---------- ---TTTTCAA
Rat1            ---------- ---------- ---------- ---------- ---TTTTCAA
CHY             ---------- ---------- ---------- ---------- ---TTTTCAA
CT              ---------- ---------- ---------- ---------- ---TTTTCAA
KK              ---------- ---------- ---------- ---------- ---TTTTCAA
Utt             ---------- ---------- ---------- ---------- ---TTTTCAA
SR              ---------- ---------- ---------- ---------- ---TTTTCAA
Nak             ---------- ---------- ---------- ---------- ---TTTTCAA
SU              ---------- ---------- ---------- ---------- ---TTTTCAA
PB              ---------- ---------- ---------- ---------- ---TTTTCAT
BR              ---------- ---------- ---------- ---------- ---TTTTCAA
Clustal Co                                                      * *
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
PS3              TTGATTAATG AAGGCTCCAT TTGT-GATAA AAATATTCTA AATGA--AAG
SK               TTGATAAATG AAG-CTCCAT TTGT-GGGAA AAAAATTCCC CCCTGCTGGG
Pm               TTGATTAATG AAG-ATCCAT TTGT-GATCA AAATATTCAC AAATGA-AAG
Pl               TTGATTAATG AAG-ATCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
CR2              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
NS1              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
CC               TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
Rat4             TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
CR1              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
Rat3             TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
NS2              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
LP1              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
NS3              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
PC               TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
CB               TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
Rat2             TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
LP2              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
KC3              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
Rat1             TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
CHY              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
CT               TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
KK               TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
Utt              TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
SR               TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-AAG
Nak              TTGATTAATG AAGCTCCCTC TTGT-GATAA AAATATTCCC AAATGA-GAG
SU               TTGATTAATG AAG-CTCCAT TTGT-GATAA AAATATTCAC AAATGA-GGG
PB               TTGAGGGGTG GTGCTCCCTC TTGTCGATTA TAATATTC-- ---TGAGGGG
BR               TTGAGGAATA GAAGCTCCAC TTGT-GAAAA AAATATTCAC AAATGGGGGG
Clustal Co       ****    *       **   **** *   *  ** ****            *


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
PS3              ATGTGAATCA AATCAAT--T CCAAGGT-TG AAGAAAAGAT GGAATAT-TC
SK               AGGTAGGTGA AATCCAC--C CCCCGTTGTA GAAAAAAGGC GAATAAA-TT
Pm               ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
Pl               ATGTGAATC- ----AAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
CR2              ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
NS1              ATGTGAATCA AATCAAT--T CTAAGTT--G AAGAAAAGAT GGAATAT-TC
CC               ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
Rat4             ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
CR1              ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
Rat3             ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
NS2              ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
LP1              ATGTGAATCA AATCACTG-T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
NS3              ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
PC               ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
CB               ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
Rat2             ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
LP2              ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
KC3              ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
Rat1             ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
CHY              ATGTGAATCA AATCAAT--T CCGAGTT--G AAGAAAAGAT GGAATAT-TC
CT               ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAG-TC
KK               ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
Utt              ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAT-TC
SR               ATGTGAATCA AATCAAT--T CCAAGTT--G AAGAAAAGAT GGAATAG-TC
Nak              ATGTGAATCA CATCAAT--T CCGGGTT--G ATGAAAAGAA GGAGTGTGTC
SU               ATGTGAATCA CATCAAT--T GCTCGTT--G ATGAAAAGAT GGAGTGTGTC
PB               AGGATGTGT- CTCCGACGTC CCCTGTT--G CCGCCCAGAG GGGGGGGTCT
BR               AGAGGACCCA CTCCAGAGAC ACTCGAT--G A-AAACAGGA GGGGGGGTGT
Clustal Co       *                       * *          **   *
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
PS3              CTTGGATCAA ATCATTCACG TTCCGTCATA ATCTGATAGA TCCCTTTGAA
SK               ATGGGATCAA GTCTTTTTC- -TCCATAAAC AGATAGAAAC TTTTTTGGAG
Pm               ATTG-ATCAA ATTATTCAC- -TCCATCATA ATCTGATAGA TCCCTTGAAG
Pl               ATTG-ATCAA ATTATTCAC- -TCCATCATA ATCTGATAGA TCCCTTGAAG
CR2              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
NS1              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
CC               ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
Rat4             ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
CR1              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
Rat3             ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
NS2              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
LP1              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
NS3              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
PC               ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
CB               ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
Rat2             ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
LP2              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
KC3              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
Rat1             ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
CHY              ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
CT               ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
KK               ATTG-ATCAA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
Utt              GTTG-ATCGA ATCATTCAC- -TCCATCATA ATCTGATAGA TCCTTTGAAC
SR               ATTG-GTCAA ATCATTCAC- -TCCATCATA GTCTGATAGA TCCTTTGAAC
Nak              GTTG-AGCAA GTCAGTCAC- -TCCATCATA GTCTGATAGA TCCTTTGTAC
SU               GGTG-GTCAA GTCAGTCAC- -TCCATCATA GTCTGATGGA TCCTTTGTCC
PB               GTGT--TCTG GCAAGCTCT- --CCTTGGTA AGCGGAGGGA TCCTCTGTTT
BR               GCAA--GTCG GTCACGCCA- --TCATGGCA AGAGGAGACA TCGTCCACCT
Clustal Co                           * *                    *

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
PS3              CAACTG--AA CAATCAGACG AGAATAA-GG ATAGAGTCCT ---ATTCTAC
SK               ACACGGGAAA AAATAGGACG GGGACAA-AA GATGCCCCCT TATGTTCTAC
Pm               A--------T TAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
Pl               AACTGA---T TAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
CR2              A-ACGG--AT CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
NS1              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
CC               AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
Rat4             AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
CR1              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
Rat3             AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
NS2              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
LP1              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
NS3              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
PC               AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
CB               AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
Rat2             AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
LP2              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
KC3              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
Rat1             AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
CHY              AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
CT               AACTGA---T CAATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
KK               AACTGAT--C GAGTCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
Utt              AACTGA---T CGATCAGACG AGAATAA-AG ATAGAGTCCT ---ATTCTAC
SR               AACTGA---T CAGTCAGACG AGAATAA-GG ATAGAGTCCT ---ATTCTAC
Nak              AACTGTG-TC GAGCGAGACG AGAATAATGG ATAGAGTCCT ---ATTCTAC
SU               ATCTGTT--C GGTCGAGACG AGAGTGT-GG ATAGAGTCCT ---ATTCTAC
PB               -TGTGG---G GGATCGGCCG AGGGGAA-CG CTCGCGTCCG ---GTCCGGC
BR               GTGTGG---G GGA---GACG AGAGCGG--G ATAGAGTCCT ---ATTCGAC
Clustal Co                   * **  *            *   **      * *  *
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                ...|....| ....|....| ....|....| ....|....| ....|....|
                      410        420        430        440        450
PS3             ATGTCAATAC CGACAACAAT GAAATTTATA GCGGGAGGAA -AATCCGTCG
SK              AGGGCATTCC CCAAAAAAAG GGAATTTTTA GGGGGGAGAA -AATCCCCCC
Pm              ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
Pl              ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
CR2             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
NS1             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
CC              ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
Rat4            ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
CR1             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
Rat3            ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
NS2             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
LP1             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
NS3             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
PC              ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
CB              ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
Rat2            ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
LP2             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
KC3             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
Rat1            ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
CHY             ATGTCAATAC CGACAACAAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
CT              ATGTCAATAC CGACACCAAT GATATTTATA GTAAGAGGAA -AATCCGTCG
KK              ATGTCAATAC CGACGACGAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
Utt             ATGTCAATAC CGACAACGAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
SR              ATGTCAATAC CGACAACGAT GAAATTTATA GTAAGAGGAA -AATCCGTCG
Nak             GTGTCTATAC CGAGGGCGGT GGAAGTTATA GTAAGAGGAA -AATCCGGCG
SU              GGGTCGATAC CGA-GGCGGT GATAGGTATA GTAAGAGGAA -AGTCCGGCG
PB              CTGTCGGTGG GGGGGGCGTT GGGTTTTTTT TTTGGAGGGG CAACCCGGCT
BR              GGGCCGATAG GGAGGGCGGA GAGAGGAAAA GTAAGAGGAA -AGTCCGGCC
Clustal Co        * *  *              *           *  *    *  **  *

                ....|....| ....|....| ....|....| ....|....| ..
                      460        470        480        490
PS3             --ACTTTAGA AAGTCGTGAG GGT
SK              CCACTTTAAA AATCGGGGGG GGT
Pm              --ACTTAAGA AATCGTGAGG GTT
Pl              --ACTTAAGA AATCGTGAGG GTT
CR2             --ACTTTAGA AATCGTGAGG GTT
NS1             --ACTTTAGA AATCGTGAGG GTT
CC              --ACTTTAGA AATCGTGAGG GTT
Rat4            --ACTTTAGA AATCGTGGGG GTT
CR1             --ACTTTAGA AATCGTGAGG GTT
Rat3            --ACTTTAGA AATCGTGAGG GTT
NS2             --ACTTTAGA AATCGTGAGG GTT
LP1             --ACTTTAGA AATCGTGAGG GTT
NS3             --ACTTTAGA AATCGTGAGG GTT
PC              --ACTTTAGA AATCGTGAGG GTT
CB              --ACTTTAGA AATCGTGAGG GTT
Rat2            --ACTTTAGA AATCGTGAGG GTT
LP2             --ACTTTAGA AATCGTGAGG GTT
KC3             --ACTTTAGA AATCGTGAGG GTT
Rat1            --ACTTTAGA AATCGTGAGG GTT
CHY             --ACTTTAGA AATCGTGAGG GTT
CT              --ACTTTAGA AATCGTGAGG GTT
KK              --ACTTTAGA AATCGTGAGG GTT
Utt             --ACTTTAGA AATCGTGAGG GTT
SR              --ACTTTAGA AATCGTGAGG GTT
Nak             --GCTTTTGT GGGCGTGGGG GGG
SU              --TGTTTTGT GGGCGTGAGG GGG
PB              --TCTTGGGG GAAAGGGGGG GGT
BR              GGCCTTGGGG GGGAGGGGGG GGC
Clustal Co          **             * *
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Figure 28. A 473 bp character matrix based on partial *trn*LF-cf of chloroplast DNA sequences of 26 cultivars of *B. superba*. Asterisks * indicate that all samples provide nucleotide identity.

Table 9. Percentages of base composition of *rbc*L sequences of *B. superba*.

Taxon	A	C	G	T	# sites
CC	0.28455	0.19919	0.21545	0.30081	246
LP2	0.28455	0.19919	0.21545	0.30081	246
PS1	0.28455	0.19919	0.21545	0.30081	246
CT	0.28455	0.19919	0.21545	0.30081	246
LY	0.28455	0.19919	0.21545	0.30081	246
PB	0.28455	0.19919	0.21545	0.30081	246
CR1	0.28455	0.19919	0.21545	0.30081	246
LB	0.28455	0.19919	0.21545	0.30081	246
SK	0.28455	0.19919	0.21545	0.30081	246
CB	0.28455	0.19919	0.21545	0.30081	246
SR	0.28455	0.19919	0.21545	0.30081	246
KC2	0.28455	0.19919	0.21545	0.30081	246
NB	0.28862	0.19512	0.21545	0.30081	246
Tak	0.28455	0.19919	0.21545	0.30081	246
PC	0.28455	0.19919	0.21545	0.30081	246
PS3	0.28455	0.19919	0.21545	0.30081	246
SU	0.28455	0.19919	0.21545	0.30081	246
NS2	0.28455	0.19919	0.21545	0.30081	246
NS3	0.28455	0.19919	0.21545	0.30081	246
KK	0.28455	0.19919	0.21545	0.30081	246
Rat1	0.28455	0.19919	0.21545	0.30081	246
KC1	0.28455	0.19919	0.21545	0.30081	246
BR	0.28455	0.19919	0.21545	0.30081	246
Rat2	0.28455	0.19919	0.21545	0.30081	246
KC3	0.28455	0.19919	0.21545	0.30081	246
CR2	0.28455	0.19919	0.21545	0.30081	246
Rat3	0.28455	0.19919	0.21545	0.30081	246
CHY	0.28455	0.19919	0.21545	0.30081	246
Rat4	0.28455	0.19919	0.21545	0.30081	246
PS2	0.28455	0.19919	0.21545	0.30081	246
Nak	0.28455	0.19919	0.21545	0.30081	246
Utt	0.28049	0.19919	0.21951	0.30081	246
BM	0.28455	0.19919	0.21545	0.30081	246
PM	0.28455	0.19106	0.21545	0.30894	246
PL	0.28455	0.19106	0.21545	0.30894	246
Mean	0.28455	0.19861	0.21556	0.30128	246.00
Mean %	28.46%	19.86%	21.56%	30.13%	

Table 10. Percentages of base composition of *trn*LF-cd sequences of *B. superba.*

Taxon	A	C	G	T	# sites
CR1	0.41485	0.13100	0.18341	0.27074	229
KC3	0.41485	0.13100	0.18341	0.27074	229
LP2	0.41485	0.13100	0.18341	0.27074	229
Nak	0.39738	0.15284	0.18777	0.26201	229
PB	0.35371	0.13537	0.21834	0.29258	229
NS2	0.41485	0.13100	0.18341	0.27074	229
NS3	0.41485	0.13100	0.18341	0.27074	229
PC	0.41485	0.13100	0.18341	0.27074	229
PS3	0.41048	0.13100	0.18341	0.27511	229
Rat1	0.41485	0.13100	0.18341	0.27074	229
Rat2	0.41485	0.13100	0.18341	0.27074	229
Rat3	0.41485	0.13100	0.18341	0.27074	229
Rat4	0.41485	0.13100	0.18341	0.27074	229
SR	0.41485	0.13974	0.18341	0.26201	229
SU	0.36245	0.14847	0.20524	0.28384	229
CR2	0.41485	0.13100	0.17904	0.27511	229
CT	0.41485	0.13100	0.18341	0.27074	229
KK	0.41485	0.13100	0.18341	0.27074	229
NS1	0.41485	0.14847	0.17904	0.25764	229
Utt	0.41485	0.13100	0.18341	0.27074	229
SK	0.39738	0.12664	0.21397	0.26201	229
BR	0.37991	0.15721	0.22271	0.24017	229
CB	0.41485	0.13100	0.18341	0.27074	229
CC	0.41485	0.13100	0.18341	0.27074	229
CHY	0.41048	0.13100	0.18777	0.27074	229
KC1	0.36681	0.14847	0.20087	0.28384	229
LB	0.38428	0.14410	0.23144	0.24017	229
LY	0.41048	0.14410	0.18777	0.25764	229
LP1	0.41048	0.13537	0.18341	0.27074	229
Pm	0.39738	0.15284	0.17467	0.27511	229
Pl	0.40175	0.14847	0.17904	0.27074	229
Mean	0.40485	0.13678	0.18932	0.26905	229
Mean%	40.49%	13.68%	18.93%	26.91%	

Table 11. Percentages of base composition of *trn*LF-cf sequences of *B. superba.*

Taxon	A	C	G	T	# sites
CR1	0.39268	0.15854	0.17317	0.27561	410
KC3	0.39268	0.15854	0.17317	0.27561	410
LP2	0.39268	0.15854	0.17317	0.27561	410
Nak	0.31220	0.17073	0.25854	0.25854	410
PB	0.15122	0.21707	0.36098	0.27073	410
NS2	0.39268	0.15854	0.17317	0.27561	410
NS3	0.39268	0.15854	0.17317	0.27561	410
PC	0.39268	0.15854	0.17317	0.27561	410
PS3	0.36341	0.15122	0.21951	0.26585	410
Rat1	0.39268	0.15854	0.17317	0.27561	410
Rat2	0.39268	0.15854	0.17317	0.27561	410
Rat3	0.39268	0.15854	0.17317	0.27561	410
Rat4	0.39024	0.15854	0.17561	0.27561	410
SR	0.38049	0.16341	0.18780	0.26829	410
SU	0.28780	0.16829	0.27073	0.27317	410
CR2	0.39024	0.16098	0.17317	0.27561	410
CT	0.38780	0.16098	0.17561	0.27561	410
KK	0.38537	0.15854	0.18293	0.27317	410
NS1	0.39268	0.16829	0.17073	0.26829	410
Utt	0.38293	0.15854	0.18293	0.27561	410
SK	0.35122	0.17805	0.23902	0.23171	410
BR	0.29268	0.20244	0.34878	0.15610	410
CB	0.39268	0.15854	0.17317	0.27561	410
CC	0.39268	0.16098	0.17317	0.27317	410
CHY	0.39024	0.15854	0.17561	0.27561	410
LP1	0.39024	0.16098	0.17561	0.27317	410
Pm	0.40976	0.15366	0.16098	0.27561	410
Pl	0.40976	0.15122	0.16585	0.27317	410
Mean	0.37099	0.16385	0.19747	0.26768	410.00
Mean (%)	37.1%	16.39%	19.75%	26.77%	

4.2.4 Phylogenetic analysis

Partial *rbc*L, *trn*LF-cd, and *trn*LF-cf sequences of *B. superba* collected in Thailand (the northern, the northeastern, the western, the eastern, and the central parts) were analyzed to gain insights into the inter- and intra- genetic relationships. The phylogenetic relationships were inferred by using the neighbor-joining (NJ) and Maximum pasimony Method (MP).

Phylogenetic trees of *rbc*L showed 32 cultivars of *B. superba* and 3 species of outgroup (*B. monosperma, P. mirifica, and P. lobata*) as in Figure 29. Considering the tree, *B. superba* cultivars could not separate into groups. They had high similarity sequence but low genetic distance (Table 12).

A phylogenetic tree of *trn*LF-cd (Figure 30) showed 2 major groups (I and II). Group I had 3 minor groups (IA, IB, and IC). The IA minor group had high similarity sequence and low genetic distance (Table 13). According to the IB minor group, a sequence of Sukhothai cultivar was closely similar to Kanchanaburi 1 cultivar with 86 bootstrap values. In addition, the IC minor group had high variation but could not show bootstrap value which is less than 50%. Moreover, Group II had only Sakhonnakorn cultivar that separates from the others.

A phylogenetics tree of *trn*LF-cf (Figure 31) showed 2 major groups. Group I is composed of 2 minor groups. Group II is composed of only Sakhonnakorn cultivar of *B. superba* (same results with *trn*LF-cd). The IA minor group had low genetic distance and had high similarity sequence within groups (Table 14). The IB minor group had high variation within groups. Prachenburi and Buriram cultivars were separated into the IB minor group and had high bootstrap value with 99%.

According to 3 phylogenetic trees, *B. superba* cultivars had high variation in *trn*LF-cd and *trn*LF-cf regions but not in *rbc*L gene. In addition, Sukhothai cultivar was closely similar to Kanchanaburi 1 cultivar with 86 bootstrap values in *trn*LF-cd region. Prachenburi and Buriram cultivars were closely similar in sequences with 99% bootstrap value in *trn*LF-cf region.

Moreover, Sakhonnakorn cultivar was separated from the others due to compared sequences of *trn*LF-cd and *trn*LF-cf regions.

Figure 29. A rooted phylogenetic tree of *rbc*L sequence inferred by neighbor-joining method. Confidence probabilities are shown on the branches.

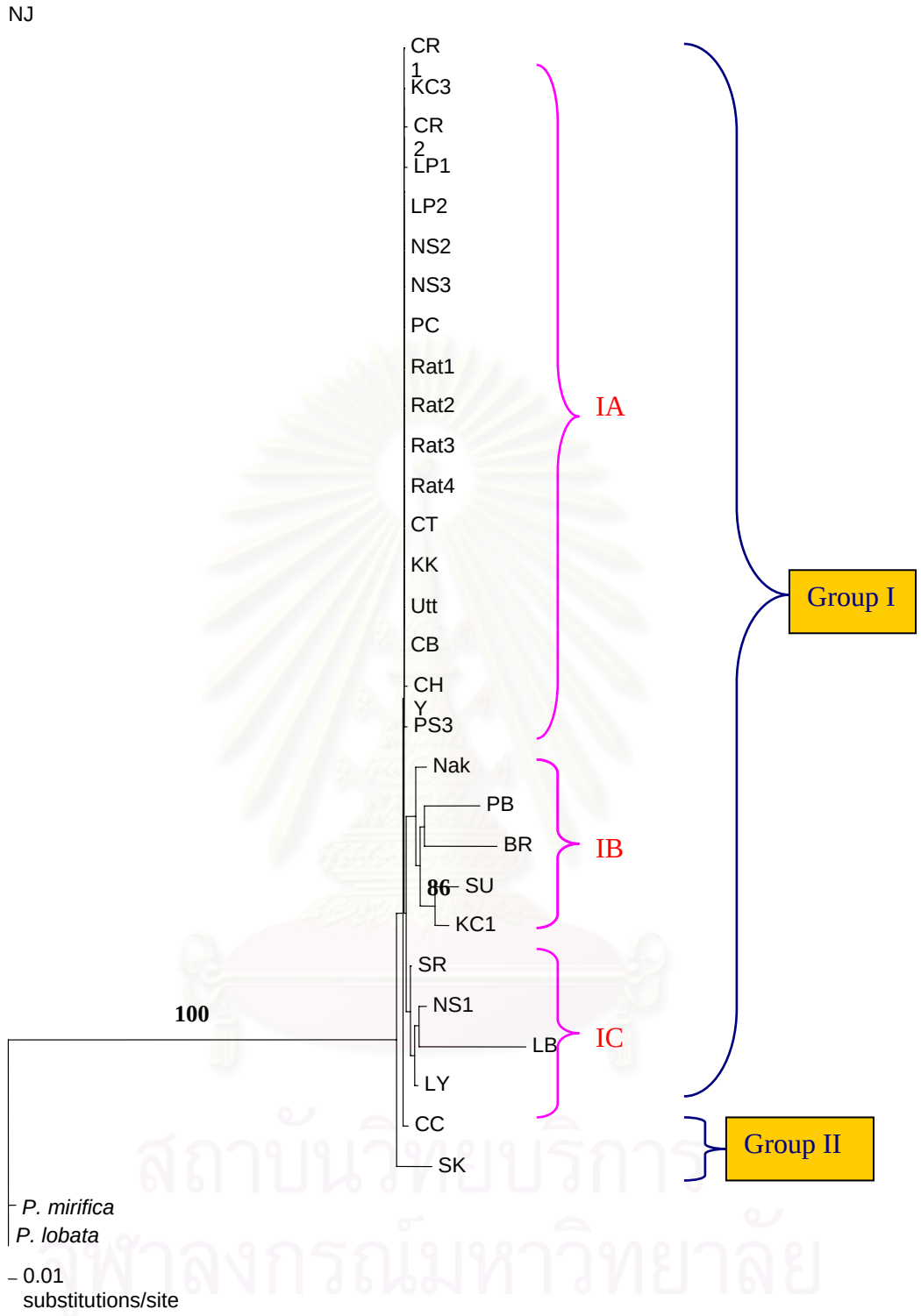


Figure 30. A rooted phylogenetic tree of *trn*LF-cd sequence inferred by neighbor-joining method. Confidence probabilities are shown on the branches.

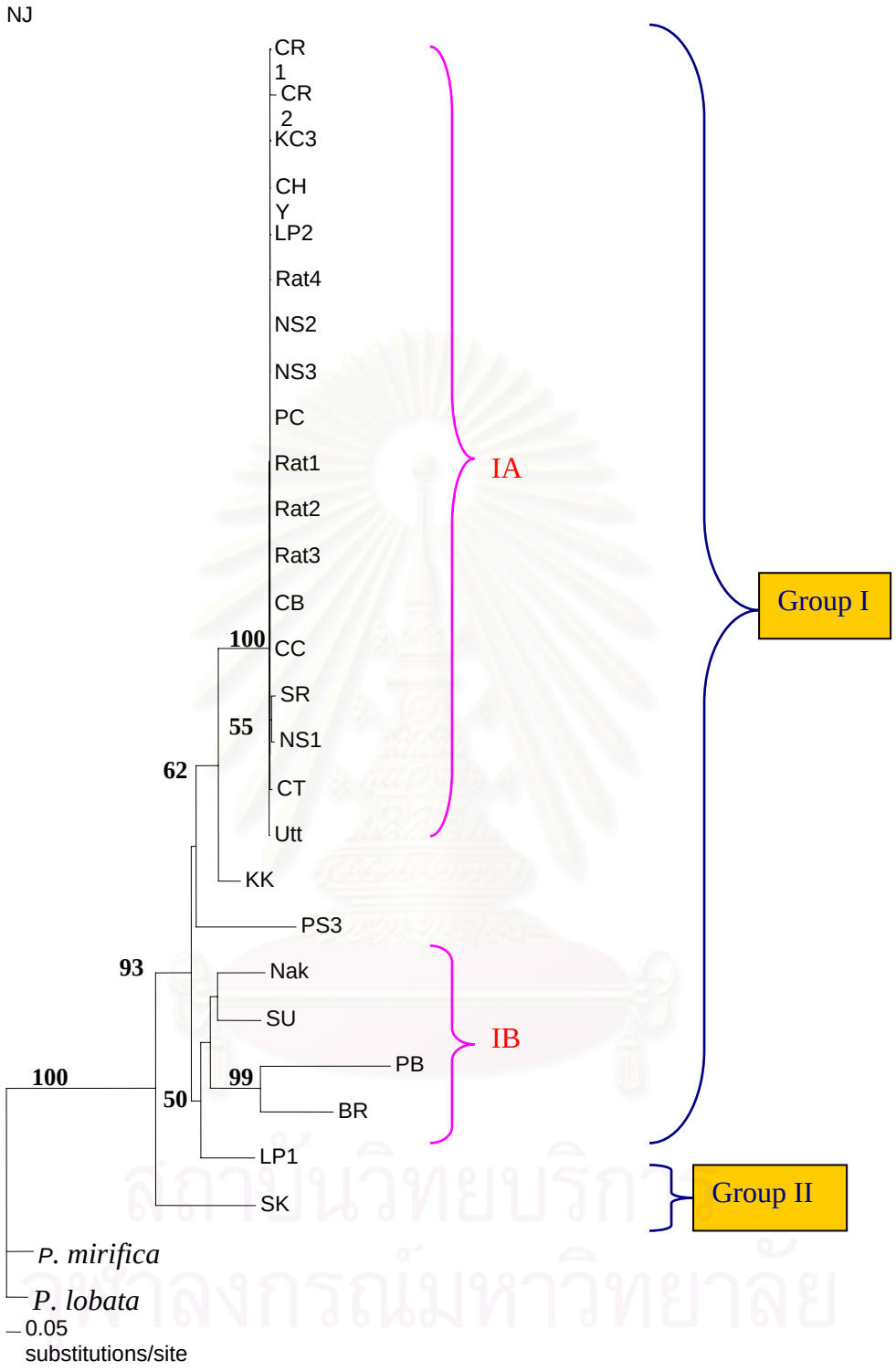


Figure 31. A rooted phylogenetic tree of *trn*LF-cf sequence inferred by neighbor-joining method. Confidence probabilities are shown on the branches.

Table 12. The *rbc*L sequence divergence (%) based on pairwise comparisons among *B. superba* samples in Thailand (see Table 4 for abbreviated names).

Kimura 2-parameter distance matrix

	1	2	3	4	5	6	7	8	9
1 CC	-								
2 LP2	0.00000	-							
3 PS1	0.00000	0.00000	-						
4 CT	0.00000	0.00000	0.00000	-					
5 LY	0.00000	0.00000	0.00000	0.00000	-				
6 PB	0.00000	0.00000	0.00000	0.00000	0.00000	-			
7 CR1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	-		
8 LB	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	-	
9 SK	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	-
10 CB	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
11 SR	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
12 KC2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
13 NB	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408
14 Tak	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
15 PC	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
16 PS3	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
17 SU	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
18 NS2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
19 NS3	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20 KK	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
21 Rat1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
22 KC1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
23 BR	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
24 Rat2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
25 KC3	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
26 CR2	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820
27 Rat3	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
28 CHY	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
29 Rat4	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
30 PS2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
31 Nak	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
32 Utt	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408
33 BM	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
34 PM	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820
35 PL	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820

Kimura 2-parameter distance matrix (continued)

	10	11	12	13	14	15	16	17	18
10 CB	-								
11 SR	0.00000	-							
12 KC2	0.00000	0.00000	-						
13 NB	0.00408	0.00408	0.00408	-					
14 Tak	0.00000	0.00000	0.00000	0.00408	-				
15 PC	0.00000	0.00000	0.00000	0.00408	0.00000	-			
16 PS3	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	-		
17 SU	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	-	
18 NS2	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	-
19 NS3	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
20 KK	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
21 Rat1	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
22 KC1	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
23 BR	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
24 Rat2	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
25 KC3	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
26 CR2	0.00820	0.00820	0.00820	0.01231	0.00820	0.00820	0.00820	0.00820	0.00820
27 Rat3	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
28 CHY	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
29 Rat4	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
30 PS2	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
31 Nak	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
32 Utt	0.00408	0.00408	0.00408	0.00818	0.00408	0.00408	0.00408	0.00408	0.00408
33 BM	0.00000	0.00000	0.00000	0.00408	0.00000	0.00000	0.00000	0.00000	0.00000
34 PM	0.00820	0.00820	0.00820	0.01231	0.00820	0.00820	0.00820	0.00820	0.00820
35 PL	0.00820	0.00820	0.00820	0.01231	0.00820	0.00820	0.00820	0.00820	0.00820

Kimura 2-parameter distance matrix (continued)

	19	20	21	22	23	24	25	26	27
19 NS3	-								
20 KK	0.00000	-							
21 Rat1	0.00000	0.00000	-						
22 KC1	0.00000	0.00000	0.00000	-					
23 BR	0.00000	0.00000	0.00000	0.00000	-				
24 Rat2	0.00000	0.00000	0.00000	0.00000	0.00000	-			
25 KC3	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	-		
26 CR2	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	-	
27 Rat3	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00820	-
28 CHY	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00820	0.00000
29 Rat4	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00820	0.00000
30 PS2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00820	0.00000
31 Nak	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00820	0.00000
32 Utt	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.00408	0.01235	0.00408
33 BM	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00820	0.00000
34 PM	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.01653	0.00820
35 PL	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.00820	0.01653	0.00820

Kimura 2-parameter distance matrix (continued)

	28	29	30	31	32	33	34	35
28 CHY	-							
29 Rat4	0.00000	-						
30 PS2	0.00000	0.00000	-					
31 Nak	0.00000	0.00000	0.00000	-				
32 Utt	0.00408	0.00408	0.00408	0.00408	-			
33 BM	0.00000	0.00000	0.00000	0.00000	0.00408	-		
34 PM	0.00820	0.00820	0.00820	0.00820	0.01235	0.00820	-	
35 PL	0.00820	0.00820	0.00820	0.00820	0.01235	0.00820	0.00000	-

Table 13. The *trn*LF-cd sequence divergence based on pairwise comparisons among the *B. superba* samples in Thailand (see Table 4 for abbreviated names).

Kimura 2-parameter distance matrix

	1	2	3	4	5	6	7	8	9
1 CR1	-								
2 KC3	0.00000	-							
3 LP2	0.00000	0.00000	-						
4 Nak	0.02668	0.02668	0.02668	-					
5 PB	0.08796	0.08796	0.08796	0.08803	-				
6 NS2	0.00000	0.00000	0.00000	0.02668	0.08796	-			
7 NS3	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	-		
8 PC	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	-	
9 PS3	0.00438	0.00438	0.00438	0.03121	0.08305	0.00438	0.00438	0.00438	-
10 Rat1	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
11 Rat2	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
12 Rat3	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
13 Rat4	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
14 SR	0.00881	0.00881	0.00881	0.02668	0.09781	0.00881	0.00881	0.00881	0.01323
15 SU	0.06377	0.06377	0.06377	0.06380	0.10845	0.06377	0.06377	0.06377	0.06378
16 CR2	0.00438	0.00438	0.00438	0.03121	0.09291	0.00438	0.00438	0.00438	0.00879
17 CT	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
18 KK	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
19 NS1	0.02681	0.02681	0.02681	0.04510	0.11804	0.02681	0.02681	0.02681	0.03132
20 Utt	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
21 SK	0.04964	0.04964	0.04964	0.07820	0.13871	0.04964	0.04964	0.04964	0.05432
22 BR	0.11381	0.11381	0.11381	0.11357	0.14996	0.11381	0.11381	0.11381	0.11883
23 CB	0.00000	0.00000	0.00000	0.02668	0.08796	0.00000	0.00000	0.00000	0.00438
24 CC	0.00881	0.00881	0.00881	0.03586	0.09781	0.00881	0.00881	0.00881	0.01323
25 CHY	0.00439	0.00439	0.00439	0.03125	0.09286	0.00439	0.00439	0.00439	0.00879
26 KC1	0.05434	0.05434	0.05434	0.04982	0.10316	0.05434	0.05434	0.05434	0.05904
27 LB	0.14529	0.14529	0.14529	0.16672	0.23438	0.14529	0.14529	0.14529	0.15051
28 LY	0.01778	0.01778	0.01778	0.03586	0.09781	0.01778	0.01778	0.01778	0.02224
29 LP1	0.00438	0.00438	0.00438	0.03121	0.09291	0.00438	0.00438	0.00438	0.00879
30 Pm	0.47025	0.47025	0.47025	0.52096	0.60463	0.47025	0.47025	0.47025	0.47850
31 Pl	0.46219	0.46219	0.46219	0.51226	0.59520	0.46219	0.46219	0.46219	0.47037

Kimura 2-parameter distance matrix (continued)

	10	11	12	13	14	15	16	17	18
10 Rat1	-								
11 Rat2	0.00000	-							
12 Rat3	0.00000	0.00000	-						
13 Rat4	0.00000	0.00000	0.00000	-					
14 SR	0.00881	0.00881	0.00881	0.00881	-				
15 SU	0.06377	0.06377	0.06377	0.06377	0.07339	-			
16 CR2	0.00438	0.00438	0.00438	0.00438	0.01323	0.06854	-		
17 CT	0.00000	0.00000	0.00000	0.00000	0.00881	0.06377	0.00438	-	
18 KK	0.00000	0.00000	0.00000	0.00000	0.00881	0.06377	0.00438	0.00000	-
19 NS1	0.02681	0.02681	0.02681	0.02681	0.01772	0.09306	0.03132	0.02681	0.02681
20 Utt	0.00000	0.00000	0.00000	0.00000	0.00881	0.06377	0.00438	0.00000	0.00000
21 SK	0.04964	0.04964	0.04964	0.04964	0.05434	0.11799	0.05432	0.04964	0.04964
22 BR	0.11381	0.11381	0.11381	0.11381	0.10340	0.15577	0.11883	0.11381	0.11381
23 CB	0.00000	0.00000	0.00000	0.00000	0.00881	0.06377	0.00438	0.00000	0.00000
24 CC	0.00881	0.00881	0.00881	0.00881	0.01778	0.07339	0.01323	0.00881	0.00881
25 CHY	0.00439	0.00439	0.00439	0.00439	0.01328	0.06377	0.00879	0.00439	0.00439
26 KC1	0.05434	0.05434	0.05434	0.05434	0.06386	0.04500	0.05904	0.05434	0.05434
27 LB	0.14529	0.14529	0.14529	0.14529	0.13436	0.21842	0.15051	0.14529	0.14529
28 LY	0.01778	0.01778	0.01778	0.01778	0.00881	0.07339	0.02224	0.01778	0.01778
29 LP1	0.00438	0.00438	0.00438	0.00438	0.01323	0.06854	0.00879	0.00438	0.00438
30 Pm	0.47025	0.47025	0.47025	0.47025	0.48678	0.52981	0.47850	0.47025	0.47025
31 Pl	0.46219	0.46219	0.46219	0.46219	0.47845	0.52096	0.47037	0.46219	0.46219

Kimura 2-parameter distance matrix (continued)

	19	20	21	22	23	24	25	26	27
19 NS1	-								
20 Utt	0.02681	-							
21 SK	0.06861	0.04964	-						
22 BR	0.10836	0.11381	0.12415	-					
23 CB	0.02681	0.00000	0.04964	0.11381	-				
24 CC	0.02681	0.00881	0.05434	0.11381	0.00881	-			
25 CHY	0.03141	0.00439	0.05434	0.11911	0.00439	0.01328	-		
26 KC1	0.07346	0.05434	0.09789	0.12415	0.05434	0.06386	0.05434	-	
27 LB	0.13412	0.14529	0.18270	0.22993	0.14529	0.14529	0.15085	0.21229	-
28 LY	0.01772	0.01778	0.05908	0.10340	0.01778	0.01778	0.02233	0.06386	0.13436
29 LP1	0.03132	0.00438	0.05432	0.11883	0.00438	0.01323	0.00879	0.05904	0.15051
30 Pm	0.52126	0.47025	0.50507	0.62491	0.47025	0.48678	0.47845	0.52096	0.58899
31 Pl	0.51241	0.46219	0.49694	0.61460	0.46219	0.47845	0.47025	0.51226	0.57878

Kimura 2-parameter distance matrix (continued)

	28	29	30	31
28 LY	-			
29 LP1	0.02224	-		
30 Pm	0.49526	0.47037	-	
31 Pl	0.48678	0.46237	0.00879	-

Table 14. The *trn*LF-cf sequence divergence based on pairwise comparisons among the *B. superba* samples in Thailand (see Table 4 for abbreviated names).

Kimura 2-parameter distance matrix

	1	2	3	4	5	6	7	8	9
1 CR1	-								
2 KC3	0.00000	-							
3 LP2	0.00000	0.00000	-						
4 Nak	0.52649	0.52649	0.52649	-					
5 PB	0.94144	0.94144	0.94144	0.79525	-				
6 NS2	0.00000	0.00000	0.00000	0.52649	0.94144	-			
7 NS3	0.00000	0.00000	0.00000	0.52649	0.94144	0.00000	-		
8 PC	0.00000	0.00000	0.00000	0.52649	0.94144	0.00000	0.00000	-	
9 PS3	0.58785	0.58785	0.58785	0.45311	0.95934	0.58785	0.58785	0.58785	-
10 Rat1	0.00000	0.00000	0.00000	0.52649	0.94144	0.00000	0.00000	0.00000	0.58785
11 Rat2	0.00000	0.00000	0.00000	0.52649	0.94144	0.00000	0.00000	0.00000	0.58785
12 Rat3	0.00000	0.00000	0.00000	0.52649	0.94144	0.00000	0.00000	0.00000	0.58785
13 Rat4	0.00244	0.00244	0.00244	0.52649	0.93210	0.00244	0.00244	0.00244	0.58785
14 SR	0.01986	0.01986	0.01986	0.52632	0.94029	0.01986	0.01986	0.01986	0.61563
15 SU	0.51255	0.51255	0.51255	0.31356	0.80233	0.51255	0.51255	0.51255	0.65375
16 CR2	0.01980	0.01980	0.01980	0.56218	0.97915	0.01980	0.01980	0.01980	0.62661
17 CT	0.00736	0.00736	0.00736	0.53108	0.94029	0.00736	0.00736	0.00736	0.59836
18 KK	0.25400	0.25400	0.25400	0.51255	0.91490	0.25400	0.25400	0.25400	0.58926
19 NS1	0.01482	0.01482	0.01482	0.54683	0.97915	0.01482	0.01482	0.01482	0.61563
20 Utt	0.00985	0.00985	0.00985	0.52147	0.91391	0.00985	0.00985	0.00985	0.58241
21 SK	0.73440	0.73440	0.73440	0.71704	1.04451	0.73440	0.73440	0.73440	0.80947
22 BR	0.75144	0.75144	0.75144	0.61715	0.69653	0.75144	0.75144	0.75144	0.94576
23 CB	0.00000	0.00000	0.00000	0.52649	0.94144	0.00000	0.00000	0.00000	0.58785
24 CC	0.00000	0.00000	0.00000	0.52649	0.94144	0.00000	0.00000	0.00000	0.58785
25 CHY	0.00244	0.00244	0.00244	0.52147	0.94144	0.00244	0.00244	0.00244	0.59334
26 LP1	0.50083	0.50083	0.50083	0.41805	0.95811	0.50083	0.50083	0.50083	0.62110
27 Pm	0.98232	0.98232	0.98232	1.05742	1.62518	0.98232	0.98232	0.98232	1.09660
28 Pl	0.95574	0.95574	0.95574	1.01056	1.68612	0.95574	0.95574	0.95574	1.07926

Kimura 2-parameter distance matrix (continued)

	10	11	12	13	14	15	16	17	18
10 Rat1	-								
11 Rat2	0.00000	-							
12 Rat3	0.00000	0.00000	-						
13 Rat4	0.00244	0.00244	0.00244	-					
14 SR	0.01986	0.01986	0.01986	0.02240	-				
15 SU	0.51255	0.51255	0.51255	0.50755	0.51732	-			
16 CR2	0.01980	0.01980	0.01980	0.02232	0.04029	0.52694	-		
17 CT	0.00736	0.00736	0.00736	0.00982	0.02235	0.52186	0.02733	-	
18 KK	0.25400	0.25400	0.25400	0.25046	0.27928	0.38060	0.26777	0.26058	-
19 NS1	0.01482	0.01482	0.01482	0.01733	0.02490	0.54323	0.03509	0.02230	0.27556
20 Utt	0.00985	0.00985	0.00985	0.01235	0.02495	0.51255	0.02997	0.01728	0.25758
21 SK	0.73440	0.73440	0.73440	0.73440	0.74093	0.70353	0.76093	0.75417	0.69712
22 BR	0.75144	0.75144	0.75144	0.74403	0.72336	0.58404	0.75037	0.74302	0.72551
23 CB	0.00000	0.00000	0.00000	0.00244	0.01986	0.51255	0.01980	0.00736	0.25400
24 CC	0.00000	0.00000	0.00000	0.00244	0.01986	0.51255	0.01980	0.00736	0.25400
25 CHY	0.00244	0.00244	0.00244	0.00490	0.02240	0.51255	0.02232	0.00982	0.25758
26 LP1	0.50083	0.50083	0.50083	0.49575	0.52227	0.31499	0.51481	0.51061	0.18474
27 Pm	0.98232	0.98232	0.98232	0.98232	0.98839	1.08078	1.00030	0.99230	0.88808
28 Pl	0.95574	0.95574	0.95574	0.95574	0.97974	1.01448	0.98378	0.96494	0.92583

Kimura 2-parameter distance matrix (continued)

	19	20	21	22	23	24	25	26	27
19 NS1	-								
20 Utt	0.02495	-							
21 SK	0.76093	0.72795	-						
22 BR	0.73577	0.72954	0.96546	-					
23 CB	0.01482	0.00985	0.73440	0.75144	-				
24 CC	0.01482	0.00985	0.73440	0.75144	0.00000	-			
25 CHY	0.01733	0.01235	0.73440	0.75144	0.00244	0.00244	-		
26 LP1	0.53178	0.50083	0.62019	0.72245	0.50083	0.50083	0.50083	-	
27 Pm	1.03364	0.96681	0.93873	1.24867	0.98232	0.98232	0.99026	0.86721	-
28 Pl	1.00734	0.93986	0.92686	1.24612	0.95574	0.95574	0.96387	0.90104	0.16585

4.2.5 **RAPD analysis**

Five primers of RAPD were selected and analyzed by neighbour-joining cluster to demonstrate the relationships among cultivars using Nei-Li genetic distance (PAPU*4.0, Swofford, 1998). Only a reproducible band was score as 1 (presence). Non reproducible band was score as 0 (absence). Total of 48 RAPD fragments by 5 primers (OPA19, OPA12, OPA7, OPD2, and OPC15) were visible. Polymorphic bands by each primer were 10, 10, 11, 7, and 10, respectively (Table 18). Primer OPA19 gave RAPD bands ranging from 200 bp - 1200 bp. Primer OPA12 gave RAPD bands ranging from 200 bp – 1500 bp. Primer OPA7 gave RAPD bands ranging from 200 bp – 1500 bp. Primer OPD2 gave RAPD band ranging from 200 bp – 1000 bp. Finally, primer OPC15 gave RAPD band ranging from 200 bp – 1500 bp.

Table 15. Total number of bands, monomorphic, and polymorphic band within 34 cultivars of *B. superba* and *B. monosperma* revealed by RAPD analysis using primer OPA19, OPA12, OPA7, OPD2, and OPC15.

Primer name	No. of total bands	No. of monomorphic bands	No. of polymorphic bands
OPA19	10	0	10
OPA12	10	0	10
OPA7	11	0	11
OPD2	7	0	7
OPC15	10	0	10
Total	48	0	48 (100%)

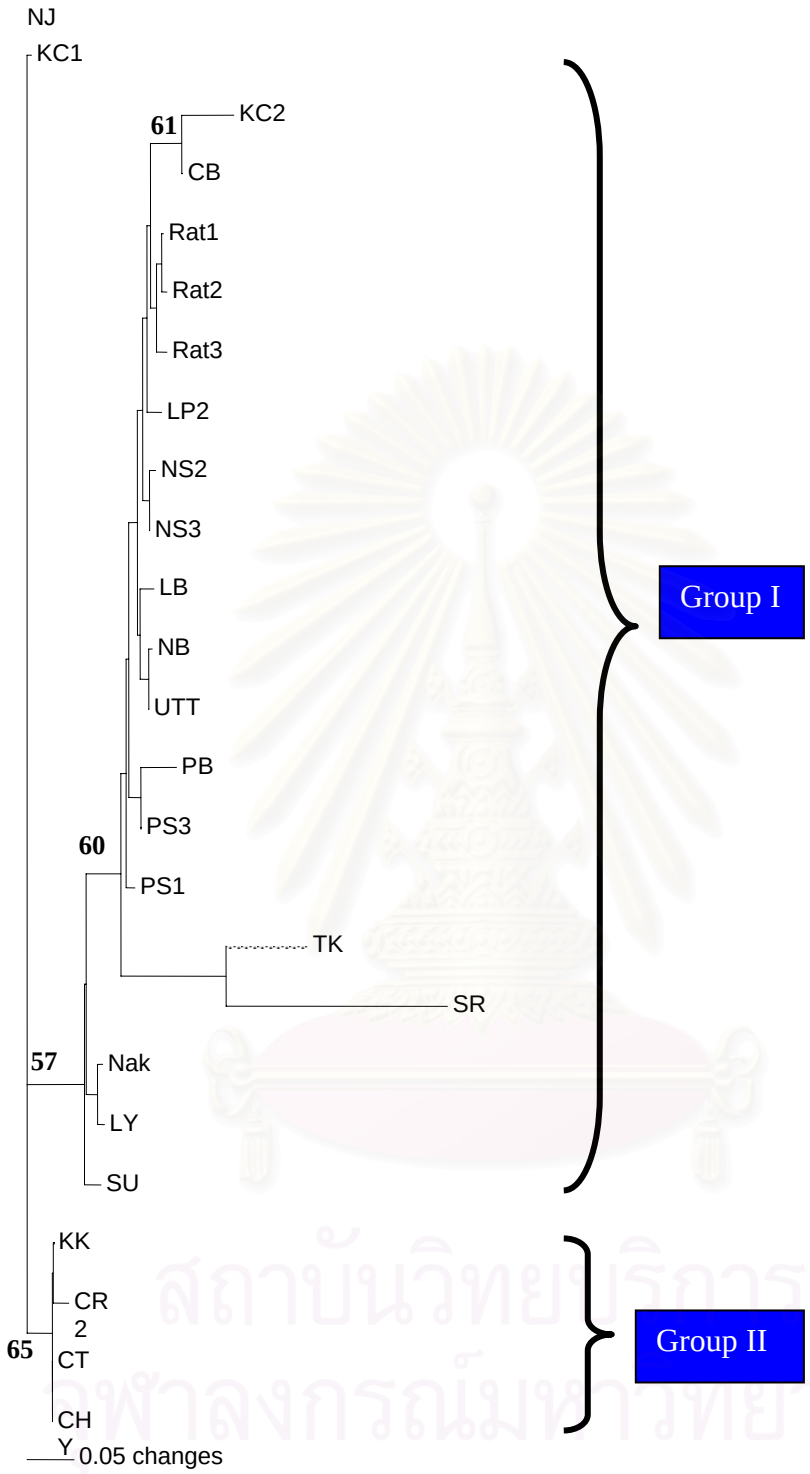


Figure 32. Neighbor-joining tree of Nei-Li genetic distance among 34 populations of *B. superba* and *B. monosperma* (Bm).

4.2.6 **RAPD Phylogram**

Phylogenetic tree of RAPD was shown in Figure 32. There are 2 separated major groups. Group I contained 19 cultivars of *B. superba* and was separated from Group II with 57% bootstrap value.

In addition, Group II contained 4 cultivars of *B. superba* was separated from others with 65% bootstrap value.

Table 16. RAPD sequence divergence based on pairwise comparisons among *B. superba* samples in Thailand (see Table 4 for abbreviated names).

Nei-Li distance matrix

	1	2	3	4	5	6	7	8	9
1 KC1	-								
2 KC2	*0.88954	-							
3 KK	0.06797	*0.88954	-						
4 CT	0.06797	*0.88954	0.00000	-					
5 CB	*0.88954	0.12191	*0.88954	*0.88954	-				
6 CHY	0.06797	*0.88954	0.00000	0.00000	*0.88954	-			
7 CR2	0.15474	*0.88954	0.03731	0.03731	*0.88954	0.03731	-		
8 TK	0.15474	0.34017	0.15474	0.15474	0.21265	0.15474	0.21265	-	
9 Nak	0.18605	*0.88954	0.18605	0.18605	0.26575	0.18605	0.15474	0.15474	-
10 NS2	0.19536	0.14468	0.26575	0.26575	0.13942	0.26575	0.37864	0.09401	0.19536
11 NS3	0.22070	0.13674	0.29143	0.29143	0.09401	0.29143	0.40626	0.11128	0.22070
12 PB	*0.88954	0.29143	*0.88954	*0.88954	0.16310	*0.88954	*0.88954	0.36844	0.23580
13 PS1	0.17621	0.28046	0.24635	0.24635	0.15474	0.24635	0.35765	0.06161	0.17621
14 PS3	0.17109	0.24290	0.29143	0.29143	0.14776	0.29143	0.27472	0.11667	0.10190
15 Rat1	0.24290	0.17330	0.31392	0.31392	0.09180	0.31392	0.43030	0.14978	0.31392
16 Rat2	0.25631	0.15897	0.32752	0.32752	0.11667	0.32752	0.44477	0.15957	0.32752
17 Rat3	0.27472	0.17621	0.27472	0.27472	0.12191	0.27472	0.38831	0.18021	0.27472
18 LB	0.23580	0.18605	0.35765	0.35765	0.12264	0.35765	0.34618	0.15474	0.16580
19 LP2	0.31392	0.17330	0.31392	0.31392	0.12587	0.31392	0.30673	0.10696	0.24290
20 LY	0.21265	*0.88954	0.21265	0.21265	0.27472	0.21265	0.18605	0.16580	0.02575
21 SR	*0.88954	*0.88954	*0.88954	*0.88954	*0.88954	*0.88954	*0.88954	0.32085	*0.88954
22 SU	0.18605	0.39750	0.18605	0.18605	0.26575	0.18605	0.15474	0.22460	0.06797
23 NB	0.21265	0.26266	0.33395	0.33395	0.11049	0.33395	0.32085	0.14049	0.14295
24 UTT	0.22460	0.26879	0.34618	0.34618	0.11667	0.34618	0.33395	0.11667	0.15474

Nei-Li distance matrix (continued)

	10	11	12	13	14	15	16	17	18
10 NS2	-								
11 NS3	0.01495	-							
12 PB	0.16310	0.18605	-						
13 PS1	0.03731	0.05331	0.10796	-					
14 PS3	0.06797	0.08786	0.08148	0.05331	-				
15 Rat1	0.07709	0.05229	0.16794	0.08265	0.12842	-			
16 Rat2	0.08576	0.06031	0.18021	0.09180	0.13942	0.01518	-		
17 Rat3	0.12191	0.08265	0.13290	0.09130	0.12362	0.04389	0.03128	-	
18 LB	0.09996	0.06278	0.18605	0.11049	0.06797	0.11189	0.12133	0.10571	-
19 LP2	0.09180	0.06390	0.20636	0.09879	0.12842	0.07581	0.08332	0.06797	0.07789
20 LY	0.20422	0.22841	0.25631	0.18605	0.11667	0.24973	0.26266	0.28327	0.17621
21 SR	*0.88954	*0.88954	*0.88954	0.34618	*0.88954	*0.88954	*0.88954	*0.88954	*0.88954
22 SU	0.26575	0.22070	0.23580	0.24635	0.17109	0.24290	0.25631	0.20422	0.16580
23 NB	0.11049	0.06797	0.16580	0.09744	0.05112	0.10190	0.11189	0.09401	0.04814
24 UTT	0.09401	0.05743	0.17621	0.08148	0.05975	0.08911	0.09879	0.08003	0.05500

Nei-Li distance matrix (continued)

	19	20	21	22	23	24
19 LP2	-					
20 LY	0.24973	-				
21 SR	*0.88954	*0.88954	-			
22 SU	0.24290	0.09401	*0.88954	-		
23 NB	0.10190	0.15474	*0.88954	0.14295	-	
24 UTT	0.08911	0.16580	*0.88954	0.15474	0.00741	-

* Undefined distance

CHAPTER V

DISCUSSION

5.1 Sampling collections

Considering sampling collections, *B. superba* is distributed in all parts of Thailand (the northern, the central, the northeastern, the eastern, and the western parts) except the southern part (Figure 11). It might be that *B. superba* can not grow in a tropical rain forest in the southern part of Thailand. Due to our survey since 1998, *B. superba* habitats are always bamboo forests, dry evergreen forests, and mixed deciduous forests. According to Table 5, some cultivars in some localities could not be collected for morphometric analysis. That involved in the natural problems such as raining season. It made ground surface very slippery. Flash flood in the northern and the central parts occurred in Thailand in 2006. That caused us to lose a lot of mature leaves, but not fresh young leaves for genetic analysis. Therefore, field trip should be performed more often. It should be better if a survey can be performed in all seasons.

5.2 Morphometry analysis

Twenty five mature leaves of each cultivar were collected. This is based on Hidalgo (2003). It would provide an experimental error below 10%. Nine morphometric parameters were measured by a ruler and a vernier caliper. The chosen parameters in this research were recommended by Perrier (1998), González-Andrés (2001), and Andrés-Agustín (2006). Only 7 morphometric parameters (PD, NPV, SPL, RL, TLB, AB^0, and PL) from total of 9 morphometric parameters (Figure 8 - 10) were analyzed according to Factor analysis using SPSS for window (Ruttner, 1988; Tilde *et al.*, 2000; Hepburn *et al.*, 2001; Chaiyavong, 2001). In addition, 7

morphometric parameters could be divided into 3 factors (Figure 12-14). For the multivariate analysis, a data matrix was prepared by mean values of all cultivars (Appendix II). Two different multivariate analyses [Principal Component Analysis (PCA) and Cluster Analysis (CA)] were carried out. A dendrogram was constructed by Hierarchical Cluster analysis of squared Euclidean distance between means of factor score (Figure 15). The results showed that *B. superba* cultivars could not be separated into groups. In this research, stronger discriminations were provided by the PCA analysis. It indicated that 9 parameters of leaf length were not enough to separate *B. cultivars* into groups.

The result of correlation of geographic trends in morphometric characteristics of *B. superba* from Thailand was shown in Table 6. Analysis of factor scores against longitude ($P<0.05$) shows clinal patterns in the characters of *B. superba* in Thailand. Considering PD, NPV, RL, and TLB parameters in factor 1, *B. superba* increases in size from the North to the South. In addition, SPL and PLL parameters in factor 2 decrease in size from the North to the South (Figure 16-21).

In our research, only leaf morphometry was used. According to Andrés-Agustín (2006), morphometry of cherimoya cultivars (*Annona cherimola* Mill) was determined by leaf, seed, flowers, and fruit parameters. Although more parameters should be used, our result is the first or beginning survey to cluster *B. superba* cultivars.

5.3 DNA sequences

This research includes the data of DNA sequences of several cultivars of *B. superba* for the first time. First of all, we had to extract DNA from *B. superba* leaf. High MW and sharp band of genomic DNA should be observed in order to indicate a good quality (Figure 22). We used 3 pairs of primers to amplify DNA (Table 2). Product of *rbc*L at 300 bp, product of *trn*LF-cd at 550 bp, and product of *trn*LF-cf at 1,000 bp were obtained, respectively (Figure 23 - 25). According to Table 7, we failed to obtain a single band from *trn*LF-cf of Phitsanulok 2 and Loei cultivars. It might be that the sequences of both cultivars are much different from others, especially the difference at the primer binding sites. Failing to obtain a sequence of PCR product as recorded in Table 8 may come from contamination of the product. Then, we aligned all obtained sequences and constructed phylograms by using NJ and MP analysis.

5.4 RAPD analysis

For further experiments, we used RAPD technique to support the result of DNA sequencing. Selected RAPD primers were based on Mienie *et al.* (1995) which performed RAPD to identify South African soybean cultivars. Those primers were used for amplification of DNA of *B. superba* because both belong to the group of Leguminosae. The obtained sequences of products amplified by the selected RAPD primers were analyzed by neighbor-joining cluster. It demonstrates the relationships among cultivars by using Nei-Li genetic distance (PAPU*4.0, Swofford, 1998). Only a reproducible band was score for presence 1 or for absence 0. Total of 48 RAPD fragments from 5 primers (OPA19, OPA12, OPA7, OPD2, and OPC15) were obtained. Polymorphic bands by each primer were 10, 10, 11, 7, and 10 bands, respectively (Table 15). The result indicates that polymorphism could be determined

among cultivars. There are high levels of genetic variation within cultivars. Primers were specific with some cultivars and were not able to amplify DNA of some cultivars. In the future, more RAPD primers should be tried.

5.5 Phylogenetic relationship

Four phylogenetic trees of *rbc*L, *trn*LF-cd, and *trn*LF-cf from DNA sequences and 1 phylogenetic tree from 5 RAPD primers were obtained. According to phylogenetic analysis by NJ and MP, *rbc*L sequences could be not separated into groups (Figure 29). It is probably that *rbc*L gene of *B. superba* came from the same ancestor. Moreover, *rbc*L gene is a coding regions and this region is expected to have low genetic variation.

In addition, phylogeny of *trn*LF-cd can separate *B. superba* cultivars into 2 major groups (Figure 30). The IA minor group in group I shows low genetic variation among *B. superba* cultivars. The IB and IC minor groups have high genetic variation. Furthermore, Group II has only Sakhonnakorn cultivar. It indicates that Sakhonnakorn cultivars are genetically different from the others

Moreover, phylogeny of *trn*LF-cf can separate *B. superba* cultivars into 2 major groups (Figure 31). The IA minor group in group I shows low genetic variation like Figure 30 although shorter sequences were obtained to construct a *trnL*F-cf tree.

Finally, phylogeny generated by RAPD primers could separate *B. superba* cultivars into 2 groups (Figure 32). Group I had high genetic variation. The tree generated by RAPD primers was different from the trees of *rbc*L, *trn*LF-cd, and *trn*LF-cf in term that the tree generated by RAPD primers could show the node of closely sequences of cultivars from the same province. For example, the node of Rat 1 and Rat 2 is close to the node of NS 2 and NS 3.

5.6 Genetic variation among *B. superba* cultivars

According to Figure 29 - 31, some cultivars can be separated from the others, especially Sakhonnakorn cultivar. Sequences amplified by *rbc*L primer are almost similar among *B. superba* cultivars. It indicated that *rbc*L primer was designed from conserved regions. That leads to low genetic variation in *B. superba* cultivars. Sequences amplified by *trn*LF-cd and *trn*LF-cf present higher genetic variation among cultivars than sequences amplified by *rbc*L. The obtained sequences can separate an outgroup from *B. superba* cultivars. SU and KC 1 cultivars are closely similar in sequences after being amplified by *trnL*F-cd. Moreover, PB and BR cultivars are closely similar in sequences of *trnL*F-cf primers. Also, they provide the bootstrap values of 86 and 99, respectively.

It can be concluded that geographic and genetic data are not related to each other. The cultivars from the same part of Thailand still have different genetic distance except some cultivars from the same province (Rat 1 - 3 cultivars) are closely related.

In the future, chemical content analysis will be obtained. The relationship among morphometric variation, genetic variation, and chemical content variation will be discovered. This information will lead to the selection of the best cultivar for commercial purpose.

CHAPTER VI

CONCLUSIONS

1. According to factor analysis, 3 factors of parameters can be distinguished. The 1st factor accounted for 34.24% of total variation was mainly associated with petiole diameter (PD), number of pairs of primary veins (NPV), rachis length (RL), and terminal leaflet breadth (TLB). The 2nd factor was accounted for 17.17% and was mainly associated with stipule length (SPL) and petiolet length (PLL). The 3rd factor was mainly associated with angle of the first leaf border (AB^0). This factor was accounted for 11.48% of total variation.

2. Considering on cluster analysis, it demonstrates that *B. superba* cultivars could not separated into groups by 9 parameter of morphometric method.

3. By correlation analysis, clinal patterns in the characters of *B. superba* in Thailand were determined. *B. superba* leaves increase in size from the North to the South of Thailand in factor 1 and decrease in size from the North to the South in factor 2.

4. Sequences of amplified *rbc*L coding gene of *B. superba* indicate low level of genetic diversity among cultivars originating from different geographic localities in Thailand.

5. Sequence of amplified *trn*LF-cd and *trn*LF-cf of *B. superba* indicated high level of genetic diversity among cultivars. Both primers can separate *P. mirifica* and *P. lobata* form *B. superba.*

6. According to MP and NJ analyses, 3 phylogenetic trees were constructed. Cultivars were separated into groups which are based on *trn*LF-cd and *trn*LF-cf regions. For example, Sakhonnakorn cultivar was clearly separated from the rest.

7. High polymorphic patterns were visible by RAPD. Phylogeny shows 2 majors group of *B. superba* cultivars. High genetic variation was in group I.

8. Due to our data, morphometry can not determine the variation of *B. superba* collected in Thailand. In contrast, RAPD is effective enough in analyzing the difference of *B. superba* cultivars in Thailand

REFERENCES

Albach, D.C., Soltis, P.S., Soltis, D.E., Olmstead, R.G. 2001. Phylogenetic analysis of asterias based on sequences of four genes. *Analysis Molecular Botany Garden*. 88: 163–212.

Andrés-Agustín, J., González-Andrés, F., Nieto-Ángle, R. and Barrientos-Priego, A. F. 2006. Morphometry of the organs of cherimoya (*Annona cherimola* Mill.) and analysis of fruit parameters for the characterization of cultivars, and Mexican germplasm selection. *Scientia horticulturae.* 107: 337-346.

Bandara, M. K., Kumar, N.S. and Samaranayake. 1989. An antifungal constituent from the stem bark of *Butea monosperma*. *Journal of Ethnopharmacology*. 25: 73-75.

Bhargava, S. K. 1986. Estrogenic and postcoial anticonceptive activity in rats of butin isolated from Butea monosperma seed. *Journal of Ethnoplarmacology*. 18: 95-101.

Boongapim, R. 2002. *Effect of the extract from Butea superba on isolated human umbilical vein*. Master's Thesis. Medical Science. Chulalongkorn University.

Caetanoanolles, G., Bassam, B.J., Gresshoff, P.M. 1993. Enhanced detection of polymorphic DNA by multiple arbitrary amplicon profiling of endonuclease-digested DNA-identification of markers tightly linked to the supermodulation locus in soybean. *Molecular Genetic.* 241: 57–64.

Chase, M.W. *et al.* 1993. Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene *rbcL. Annual Missouri Botany Garden.* 80, 528-580.

Chase, M.W., Albert, V.A., 1998. A perspective on the contribution of plastid rbcL DNA sequences to angiosperm phylogenetics. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), *Molecular Systematics of Plants II*: DNA Sequencing. Kluwer, Boston.

Cherdshewasart, W., Cheewasopit, W. and Picha, P. 2004a. The differential antiproliferation effect of white (Pueraria mirifica), red (Butea superba), and black (Mucuna collettii) Kwao Krua plants on the growth of MCF-7 cells. *Journal of Ethnopharmacology*. 93: 255-260.

Cherdshewasart, W., Cheewasopit, W. and Picha, P. 2004b. Anti-proliferation effects of the white (Pueraria mirifica), red (Butea superba), and black (Mucuna collettii) Kwao Krua plants on the growth of HeLa cells. *Journal of Scientific Research of Chulalongkorn University*. 29(1): 27-32.

Cherdshewasart, W., Kitsamai, Y. and Malaivijitnond, S. 2006. Estrogenic activity of the wild *Pueraria mirifica* evaluated by vaginal cornification assay, in preparation.

Cherdshewasart, W. and Nimsakul, N. 2003. The clinical trial of *Butea superba* as an herbal alternative treatment for erectile dysfunction. *Asian Journal of Andrology*. 5: 243-246.

Cherdshewasart, W., Subtang, S. and Dahlan, W. 2006. Diversity of major isoflavonoids of the Thai phytoestrogen rich herb, *Pueraria mirifica* in comparison with Pueraria lobata, in preparation.

Chung, J. D., Lin, T. P., Chen, Y. L., Cheng, Y. P., and Hwang, S. Y. 2007. Phylo-geographic study reveals the origin and evolutionary history of a Phodo-endron species complex in Taiwan. *Molecular Phylogenetics and Evolution*. 42: 14-24.

Clegg, M.T., Gaut, B.S., Learn, G.H.J., Morton, B.R. 1994. Rates and patterns of chloroplast DNA evolution. *Proc. Natl. Acad. Sci*. USA 91: 6795–6801.

Cuenoud, P., Martinez, M.D.P., Loizeau, P.-A., Spichiger, R., Andrews, S., Manen, J.-F. 2000. Molecular phylogeny and biogeography of the genus Ilex L. (Aquifoliaceae). *Annual of Botany*. 85: 111–122.

Downie, S.R., Watson, M.F., Spalik, K., Katz-Downie, D.S. 2000. Molecular systematics of Old world Apioideae (Apiaceae): relationships among some members of tribe *Peucedaneae* sensu lato, the placement of several island-endemic species, and resolution within the apioid superclade. *Canada Journal of Botany*. 78: 506–528.

Doyle, J.J., Doyle, J.L.1995. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemistry*. Bull. 19, 11–15.

Gonza´lez-Andre´s, F. 2001. La Caracterizacio´n Morfolo´gica. In: Gonza´lez-Andre´s, F., Pita-Villamil, J.M. (Eds.), Conservacio´n y Caracterizacio´n de Recursos Fitogene´ticos. *Publicaciones I.N.E.A. Valladolid*, Espan˜a, pp. 199–217.

Gunakkunru, A., Padmanaban, K., Thirumal, P., Pritila, J., Parimala, G., Vengatesan, N., Gnanasekar, N., Perianayagam, J. B., Sharma, S. K. and Pillai. K. K. 2005. Anti-diarrhoeal activity of *Butea monosperma* in experimental animals. *Journal of Ethonopharmacology*. 98: 241-244.

Hidalgo, R., 2003. *Variabilidad gene´tica y caracterizacio´n de especies vegetales*. In Ana´lisis estadı´stico de datos de caracterizacio´n morfolo´gica en Recursos Fitogene´ticos.

Honton, J. 2005. *Program 'complete botanica' version 2.0.* [Online]. Available from: http://www.crescentbloom.com.

Huang, J., Giannasi, D. E., and Price, R. A. 2005. Phylogenetic relationships in Ephedra (Ephedraceae) inferred from chloroplast and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*. 35: 48-59.

Ingkanian, K., Temkitthawon, P., Chuenchom K., Yuyaem, T. and Thongnoi, W. 2003. Screening for acetylcholinesterase inhibitory activity in plants used in Thai traditional rejuvenating and neurotonic remedies. *Journal of Ethnopharmacology*. 89(23): 261-264.

Lark, K.G., Evans, J., Basha, F., Bogden, R., Copeland, R., Ellison, R., Horne, D., Lee, K., McDonald, K., Pierson, J., Schuster, W., Wilhelm, P., Yu, Y. 1992. Molecular phylogeny as a genetic tool for soybean breeding. *Soybean Genetic Newslation*. 19: 174–181.

Lewis, P.A., Mishler, B.D., Vilgalys, R., 1997. Phylogenetic relationships of the liverworts (Hepaticae), a basal embryophyte lineage, inferred from nucleotide sequence data of the chloroplast gene rbcL. *Molecular Phylogenetic and Evolution*. 7: 377–393.

Loontaisong, A. 2005. Chemical constituents and biological activity of *Butea superba* Roxb. Master's Thesis, Faculty of Science, Chulalongkorn University.

Kallersjo, M., Farris, J.S., Chase, M.W., Bremer, B., Fay, M., Humphries, C.J., Petersen, G., Seberg, O., Bremer, K. 1998. Simultaneous parsimony jackknife analysis of 2538 rbcL DNA sequences reveal support for major clades of green plants, land plants, seed plants and flowering plants. *Plant Systematic. Evolution*. 213: 259–87.

Kathriarachchi, H., Hoffmann, P., Samuel, R., Wurdack, K. J., and Chase M. W. 2005. Molecular phylogenetics of Phyllanthaceae inferred from five genes

(plastid *atp*B, *mat*K, 3'*ndh*F, *rbc*L, and nuclear *PHYC*). *Molecular Phylogenetics and Evolotion*. 36: 112-134.

Käss, E., and Wink, M. 1996. Molecular Evolution of the Leguminosae: Phylogeny of the Three Subfamilies Based on *rbc*L-sequences. Biochemical Systematics and Ecology. 24(5): 365-378.

Kazan, K., Manners, J.M., Cameron, D.F. 1993. Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. *Theory Apply of Genetic*. 85: 882–888.

Kelchner, S.A. 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. *Annual Molecular Botany Garden.* 87: 482–498.

Kohne, D.E., Chiscon, J.A., Hoyer, B.H. 1972. Evolution of primate DNA sequence. *Journal of Human Evolution*. 1: 627-644.

Koller, B., Lehmann, A., McDermott, J.M., Gessler, C. 1993. Identification of apple cultivars using RAPD markers. Theor. *Application of Genetic*. 85: 901–904.

Kurz, Z. 1877. *Forest Flora of British Burma Vol. 1*. Calcutta. Office of the superintendent of government prining.

Manosroi, A., Saowadon, S. and Manosroi, J. 2001. Preliminary chronic toxicity study of red kwao krua (*Butea superba*, Roxb) and White kwao krua (*Pueraria mirifica*, Airy Shaw and Suvatabandhy) in rats. *SWU Journa ofl Pharmaceutical Scencei*. 9(1): 1-12

Martinez, I., Malmheden Yman, I. 1998. Species identification in meat products by RAPD analysis. *Food Research International*. 31: 459–466.

Matin, S.R., Vences, F.J., Saenz de miera, L.E., Barroso, M.R. and Carnide, V. 2006.

RAPD analysis of genetic diversity among and within Portuguese landraces of common white bean (*Phaseolus vulgaris* L.). *Scientia Horticulture*. 108: 133-142.

Mehta, B. K., Dubey, A., Bokadia, M. M. and Mehta, S. C. 1983. Isolation in *vitro* antimicrobial efficiency of *Butea monosperma* seed oil on human phathogenic bacteria and Phytopathogenic fungi. *Advanced Cell Technology Microbiology of Hungary*. 30:75-77.

Mienie, C., Smit, M., and Pretorius, P. 1995. Use of random amplified of South African soybean cultivars. *Field Crops Research*. 43: 43-49.

Moreno-Sánchez, M. 2004. Graphic approach for morphometric analysis of *Archaeopteria* leaves. *Annales de Palẽontologie*. 90: 161-173.

Müller, K., Borsch, T., and Hilu, K. W. 2006. Phylogenetic utility of rapidly evolving DNA at high taxonomical level: Contrasting *mat*K, *trn*T-F, and *rbc*L in basal angiosperms. *Molecular Phylogentics and Evolution.* 41: 99-117.

Negrisolo, E., Maistro, S., Incarbone, M., Moro, I., Valle, L. D., Broady, P. A., and Andreoli, C. 2004. Morphological convergence characterizes the evolution of Xanthophyceae (Heterokontophyta): evidence from nuclear SSU rDNA and plastidial *rbc*L genes. *Molecular Pylogenetics and Evolution.* 33: 156-170.

Ngamrojanavanich, N., Loontaisong, A., Pengprecha, S., Cherdshewasart, W., Pornpakakul, S., Pudhom, K., Roengsumran, S and Petsom, A. 2007. Cytotoxic constituents from *Butea superba* Roxb. *Journal of Ethno-pharmacology*. 109: 354-358.

Olmstead, R.G., Reeves, G., Yen, A.C. 1998. Patterns of sequence evolution and implications for parsimony analysis of chloroplast DNA. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), *Molecular Systematics of Plants* II: DNA

Sequencing. Kluywer, Boston.

Paiva, E.,Vilarinhos, A.D., Debarros, E.G., Sediyama, C.S., Moreira, M.A. 1994. Use of the random amplified polymorphic DNA technique to characterize soybean (*Glycine max* (L. Merrill)) genotypes. *Revista Brasileirn Genetic*. 17: 287-290.

Palmer, J.D., Jansen, R.K., Michaels, H.J., Chase, M.W., Manhart, J.R.,1998. Analysis of chloroplast DNA variation. *Annual Molecula Botany Garden* 75,1180-1206.

Panriansaen, R. 2000. *Characterization of Pueraria mirifica populations from various parts of Thailand*. Master's Thesis, Chulalongkorn University.

Perriers, X. 1998. *Analyse de la diversité génétique: Mesures de dissimilarité et representations arbores*. Thése doctorat. Université de Montpellier II. Montpellier.

Pongpanparadon, A., Aritajat, S. and Saenphet, K. 2002. The toxicology of *Butea superba*, Roxb. *Southeast Asian Journal Tropical Medicine Public Healthy*. 33: 155-158.

Prabhu, R.R., Gresshoff, P.M. 1994. Inheritance of polymorphic markers generated byDNAamplification fingerprinting and their use as genetic markers in soybean. *Plant Molecular Biology*. 26: 105–116.

Prashanth, D., Asha, M. K., Amit, A. and Padmaja, R. 2001. Anthelmintic activity of *Butea monosperma*. *Fitoterapia*. 72: 421-422.

Pulcharoen, K., Cherdshewasart, W., Rengpipat, S. 2005. *Mutagenic and antimutagenic activities of extracts from white kwao krua Pueraria mirifica, red kwao krua Butea superba and black kwao krua Mucuna collettii by ames test*. Master's Thesis, Program in Biotechnology, Faculty of Science, Chulalongkorn University. 153 pp.

Raksilp, T. 1995. *Chemical constituents of the tuberous roots of Butea superba Roxb*. Master's Thesis. Chulalongkorn University.

Raachandran, S., Sridhar, Y., Sam, S. K., Saravanan, M., Leonard, J. T., Anbalgan, N. and Sridhar, S. K. 2004. Aphrodisiac activity of *Butea frondosa* Koen. Ex Roxb. Extract in male rat. *Phytomedicine*. 11: 165-168.

Razdan, M. K., Kapila, K. and Bhide, N. K. 1969. Antifertility effect and some pharmacological actions of *Butea frondosa* seed extracts. *Indian Journal of Phytophamacology*. 13: 239-249.

Ridder-Numan, J. W. A. and van der Ham, R. W. J. M. 1997. Pollen morphology of *Butea*, *Kunstleria*, *Meizotropis* and *Spatholobus* (Leguminosae, Papilionoideae), with notes on their position in the tribes Millettieae and Phaseoleae. *Review of Palaeobotany and Palynology*. 96: 255-280.

Roengsumran, S., Petsom, A. Ngamrojnavanich, N., Rugsilp, T., Sittiwicheanwong, P., Khorphueng, P., Cherdshewasart, W. and Chaichantipyuth, C. 2000. Flavonoid and flavonoid glycoside from *Butea superba* and their cAMP Phosphodiesterase inhibitory Activity. *Journal of the Science Faculty of Chiang Mai University* 1: 170-176.

Savolainen, V., Chase, M.W., Hoot, S.B., Morton, C.M., Soltis, D.E., Bayer, C., Fay, M.F., De Bruijn, A.Y., Sullivan, S., Qiu, Y.-L. 2000. Phylogenetics of flowering plants based upon a combined analysis of plastid *atp*B and *rbc*L gene sequences. *Systematic Biology*. 49: 306–362.

Savolainen, V., Fay, M.F., Albach, D.C., Backlund, M., Van der Bank, M., Cameron, K.M., Johnson, S.A., Lledo, L., Pintaud, J.C., Powell, M., Sheanan, M.C., Soltis, D.E., Soltis, P.S., Weston, P., Whitten, W.M., Wurdack, K.J., Chase,

M.W. 1995. A nearly complete familial analysis of the *rbc*L gene sequences. *Phylogeny of the eudicots*. Kew Bull. 55: 257–309.

Soltis, D.E., Soltis, P.S., 1998. Introduction. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), *Molecular Systematics of Plants II*: DNA Sequencing. Kluywer, Boston.

Soman, I., Mengi, S. A. and Kasture, S. B. 2004. Effect of leaves of *Butea frondosa* on stress, anxiety, and cognition in rats. *Phamacology Biochemistry Behavior*. 79: 11-16.

Stiles, J.I., Lemme, C., Sondur, S., Morshidi, M.B., Manshardt, R. 1993. Using randomly amplified polymorphic DNA for evaluating genetic relationships among papaya cultivars. *Theory Apply of Genetic*. 85: 697-701.

Subba, R.V. and Seshadri, T.R. 1949. Chemical composition of the flower of *Butea superba. Journal of Science Indian Research*. 8B: 178-179.

Suntara, A. 1931. The *remedy pamphlet of Kwao Krua tuber of Luanganusarn-suntarakromkarnphiset* Chiang Mai: Upatipongsa Press Chiang Mai. pp. 1-13 (in Thai).

Sumitra, M., Manikandan, P. and Suguna, L. 2005. Efficacy of *Butea monosperma* on dermal wound healing in rats. *International Journal of Biochemistry Cell Biology.* 37: 566-573.

Sutjit, W. 2003. *The antioxidant test, Ames'test and Micronuclei test of chemical extract from White Kwao Krua (Pueraria mirifica), Red Kwao Krua (Butea superba Roxb), Black Kwao Krua (Mucuna collettii) and Kudzu (Pueraria lobata)*. Master's Thesis, Faculty of Science, Chulalongkorn University.

Tanasugarn, L. 2001. When papent rights may not be enforceable: The case of Kwao Krua patent. *Intellectual Property and International Trade Court*. Thailand.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. 1991. University primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105-1109.

Weder, J., 2002. Species Identification of Bean, Pea and Other Legumes by RAPD-PCR after DNA Isolation using Membrane Columns. Lebensm.-Wiss. *University of Technology*. 36: 277-283.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. *Acids Research*. 18: 6531-6535.

Wu, S.H., Hwang, C.Y., Lin, T.P., Chung, J.D., Cheng, Y.P., Hwang, S.Y. 2006. Contrasting phylogeographic patterns of two closely related species, *Machilus thunbergii* and *Machilus kusanoi* (Lauraceae), in Taiwan. *Journal of Biogeography*. 33: 936-947.

Yang, X., Quiros, C. 1993. Identification and classification of celery cultivars with RAPD markers. *Theory Apply of Genetic*. 86: 205-212.

Yoke-Kqueen, C and Radu, S. 2006. Random amplified polymorphic DNA analysis of genetically modified organisms. *Journal of Biotechnology*. 127: 161-166.

Yavada, R. N. and Reedly, K. I. S. 1998. A novel flavone glycoside from the stems of *Butea superba*. *FITOERAPIA*. Vol.LXIX (3): 269-270.

Yavada, R. and Tiwari, L. 2005. A poteintial antiviral flavone glycoside from the seeds of *Butea monosperma* O. Kuntze. *Journal of Asian National Productivity Research*. 7: 185-188.

Zhang, Y., Yang, J. and Rao, G. 2005. Genetic diversity of an amphicarpic species, *Amphicarpaea edgeworthii* Benth. (Leguminosae) based on RAPD markers. *Biochemical Systematics and Ecology*. 33: 1246-1257.

APPENDICES

APPENDIX I

Collection of *Butea superba* in Thailand

No	Sampling area	code	Coordinate	
			Latitude	Longitude
1	Kanchanaburi 1	KC 1	14.6251076521	99.0873080573
2	Kanchanaburi 2	KC 2	14.5104187721	99.2365880565
3	Kanchanaburi 3	KC 3	14.4365076521	99.4876580547
4	Khon Kaen	KK	16.5157476497	102.103642066
5	Chantaburi	CT	12.8166630257	102.16825325
6	Chachoengsao	CC	13.6176836419	101.41629037
7	Chonburi	CB	13.3013575092	101.307945148
8	Chaiyaphum	CHY	15.7495335472	101.958630758
9	Chiangrai 1	CR 1	19.9234756964	99.9260901905
10	Chiangrai 2	CR 2	19.9234756964	99.9260901905
11	Tak	TK	16.5991435917	98.8377214542
12	Nakhon ratchasima	NAK	15.0106952434	102.172490531
13	Nakhon sawan 1	NS 1	15.7044058077	100.092577664
14	Nakhon sawan 2	NS 2	15.7044058077	100.092577664
15	Nakhon sawan 3	NS 3	15.7044058077	100.092577664
15	Buriram	BR	14.8287900598	103.014088222
17	Prachenburi	PB	14.081945926	101.661011708
18	Phitsanulok 1	PS 1	16.8990584232	100.482408205
19	Phitsanulok 2	PS 2	16.8990584232	100.482408205
20	Phitsanulok 3	PS 3	16.8990584232	100.482408205
21	Phetchaboon	PC	16.2553390872	101.084767117
22	Ratchaburi 1	RAT 1	13.5904004023	99.5252179977
23	Ratchaburi 2	RAT 2	13.5904004023	99.5252179977
24	Ratchaburi 3	RAT 3	13.5904004023	99.5252179977
25	Ratchaburi 4	RAT 4	13.5904004023	99.5252179977
26	Lopburi	LB	14.9414112878	100.767904682
27	Lampang 1	LP 1	18.3315799986	99.5094143632
28	Lampang 2	LP 2	18.3315799986	99.5094143632
29	Loei	LY	17.4005045127	101.491640015
30	Sakhonnakorn	SK	17.3429318627	103.827286701
31	Saraburi	SR	14.7029252082	100.878847838
32	Sukhothai	SU	17.2622420621	99.6615550691
33	Nongbualamphu	NB	17.2535133858	102.253903114
34	Uttaradit	UTT	17.6696092002	100.514325475

APPENDIX II

Means and Standard Deviation of morphometric characters of *Butea suprba* in Thailand

Sample no.		PL	RL	PLL	SPL	TLL	TLB	PD	NPV	AB°
Kanchanaburi (KC1)	Mean	6.70	31.77	1.45	0.48	27.78	21.10	0.47	5.17	37.17
	Std. Deviation	0.72	2.46	0.23	0.09	1.90	1.57	0.08	0.41	4.45
	SE	0.14	0.49	0.05	0.02	0.38	0.31	0.02	0.08	0.89
Kanchanaburi (KC2)	Mean	44.18	8.68	1.13	0.76	39.87	33.07	0.66	6.5	28.3
	Std. Deviation	3.36	0.56	0.09	0.12	4.59	2.04	0.04	0.53	2.06
	SE	0.67	0.11	0.02	0.02	0.92	0.41	0.01	0.11	0.41
Khon Kaen (KK)	Mean	32.59	10.60	0.61	0.49	33.04	33.12	0.80	6.00	34.48
	Std. Deviation	1.86	0.39	0.06	0.08	1.57	1.48	0.00	0.00	1.51
	SE	0.36	0.07	0.01	0.02	0.30	0.29	0.00	0.00	0.29
Chantaburi (CT)	Mean	9.10	32.60	1.14	0.44	29.42	30.28	0.72	6.40	30.00
	Std. Deviation	0.60	2.72	0.09	0.17	4.28	3.54	0.11	0.55	4.00
	SE	0.12	0.54	0.02	0.03	0.86	0.71	0.02	0.11	0.80
Chachoengsao (CC)	Mean	8.60	35.94	1.40	0.72	33.96	34.18	0.74	7.00	15.80
	Std. Deviation	1.20	4.11	0.12	0.40	2.69	3.43	0.05	0.71	3.63
	SE	0.24	0.82	0.02	0.08	0.54	0.69	0.01	0.14	0.73
Chonburi (CB)	Mean	9.20	39.16	1.48	0.50	33.04	28.54	0.80	6.00	34.40
	Std. Deviation	1.91	3.75	0.43	0.16	2.96	3.11	0.17	0.71	3.51
	SE	0.38	0.75	0.09	0.03	0.59	0.62	0.03	0.14	0.70
Chiangrai (CR 1)	Mean	32.54	9.43	1.05	0.95	31.62	30.74	0.70	7.00	25.84
	Std. Deviation	1.04	0.41	0.14	0.13	1.04	1.46	0.00	0.00	1.80
	SE	0.21	0.08	0.03	0.03	0.21	0.29	0.00	0.00	0.36
Tak (TK)	Mean	45.14	10.68	0.84	1.04	32.26	27.28	0.68	6	25.8
	Std. Deviation	1.27	0.11	0.18	1.13	1.92	0.00	2.47	2.10	0.03
	SE	0.25	0.02	0.04	0.23	0.38	0.00	0.49	0.42	0.01

Sample no.		PL	RL	PLL	SPL	TLL	TLB	PD	NPV	AB°
Nakorn ratchasima (Nak)	Mean	41.6	12.0	1.0	0.6	42.9	39.2	0.7	7.0	13.4
	Std. Deviation	2.2	0.7	0.0	0.1	1.3	1.1	0.0	0.0	1.4
	SE	0.4	0.1	0.0	0.0	0.3	0.2	0.0	0.0	0.3
Nakhon sawan (NS 1)	Mean	36.76	0.98	0.54	10.36	31.00	7.00	35.30	31.90	0.68
	Std. Deviation	3.03	0.04	0.23	0.47	2.24	0.00	3.04	2.31	0.08
	SE	0.61	0.01	0.05	0.09	0.45	0.00	0.61	0.46	0.02
Nakhon sawan (NS 2)	Mean	32.56	9.60	0.94	0.64	31.87	26.33	0.52	5.00	30.24
	Std. Deviation	3.90	0.87	0.14	0.11	2.36	2.53	0.05	0.00	2.92
	SE	0.78	0.17	0.03	0.02	0.47	0.51	0.01	0.00	0.58
Nakhon sawan (NS 3)	Mean	30.61	6.75	0.68	0.49	28.95	24.92	0.53	5.20	37.20
	Std. Deviation	3.31	0.71	0.21	0.11	1.59	2.44	0.05	0.40	2.62
	SE	0.66	0.14	0.04	0.02	0.32	0.49	0.01	0.08	0.52
Buriram (BR)	Mean	8.72	27.46	1.56	0.46	36.8	34.84	0.8	6.4	34.4
	Std. Deviation	1.93	6.25	0.32	0.18	2.08	2.47	0.10	0.55	3.65
	SE	0.39	1.25	0.06	0.04	0.42	0.49	0.02	0.11	0.73
Prachenburi (PB)	Mean	33.06	9.21	1.00	0.20	30.49	29.21	0.70	6.00	16.44
	Std. Deviation	2.14	0.23	0.00	0.00	1.41	1.04	0.01	0.00	3.55
	SE	0.43	0.05	0.00	0.00	0.28	0.21	0.00	0.00	0.71
Phitsanulok (PS 1)	Mean	29.16	7.08	1.18	0.44	40.52	35.66	0.60	5.00	17.64
	Std. Deviation	1.10	0.23	0.08	0.05	1.19	1.86	0.00	0.00	1.75
	SE	0.22	0.05	0.02	0.01	0.24	0.37	0.00	0.00	0.35

APPENDIX II (continued)

Means and Standard Deviation of morphometric characters of *Butea suprba* in Thailand

Sample no.		PL	RL	PLL	SPL	TLL	TLB	PD	NPV	AB^0
Phitsanulok (PS 2)	Mean	47.71	11.57	1.28	0.67	39.26	34.38	0.66	6.68	40.70
	Std. Deviation	2.73	0.94	0.12	0.08	7.89	1.84	0.07	0.80	1.74
	SE	0.55	0.19	0.02	0.02	1.58	0.37	0.01	0.16	0.35
Phitsanulok (PS 3)	Mean	31.82	7.58	1.12	0.86	38.38	27.58	0.62	5.44	33.88
	Std. Deviation	3.10	0.85	0.11	0.22	8.55	3.02	0.08	0.51	1.69
	SE	0.62	0.17	0.02	0.04	1.71	0.60	0.02	0.10	0.34
Ratchaburi (Rat 1)	Mean	34.04	7.64	1.00	0.70	31.76	25.35	0.60	6.00	41.20
	Std. Deviation	3.56	0.49	0.00	0.07	1.73	1.59	0.00	0.00	0.87
	SE	0.71	0.10	0.00	0.01	0.35	0.32	0.00	0.00	0.17
Ratchaburi (Rat 2)	Mean	39.51	8.99	1.66	0.65	41.88	32.25	0.70	6.00	32.48
	Std. Deviation	1.62	0.64	0.13	0.05	1.26	1.41	0.00	0.00	1.33
	SE	0.32	0.13	0.03	0.01	0.25	0.28	0.00	0.00	0.27
Ratchaburi (Rat 3)	Mean	26.54	8.20	1.69	0.49	30.80	29.38	0.60	5.68	27.60
	Std. Deviation	0.85	0.21	0.07	0.03	0.99	0.89	0.00	0.48	1.80
	SE	0.17	0.04	0.01	0.01	0.20	0.18	0.00	0.10	0.36
	Mean	35.26	10.86	1.63	0.38	35.00	37.36	0.78	5.00	36.52
	Std. Deviation	2.01	0.73	0.14	0.09	1.83	1.52	0.08	0.00	2.87
Ratchaburi (Rat 4)	SE	0.40	0.15	0.03	0.02	0.37	0.30	0.02	0.00	0.57
	Mean	44.43	6.93	1.04	0.65	28.03	26.29	0.54	5.36	24.60
	Std. Deviation	1.74	0.43	0.11	0.05	1.57	2.93	0.05	0.49	1.78
Lopburi (LB)	SE	0.35	0.09	0.02	0.01	0.31	0.59	0.01	0.10	0.36
Lampang 1 (LP1)	Mean	32.10	8.58	1.00	0.50	34.15	32.58	0.70	6.00	34.22
	Std. Deviation	1.19	0.49	0.00	0.08	2.01	2.01	0.00	0.00	1.65
	SE	0.23	0.09	0.00	0.01	0.39	0.39	0.00	0.00	0.32

Sample no.		PL	RL	PLL	SPL	TLL	TLB	PD	NPV	AB^0
Lampang 2(LP2)	Mean	7.76	22.18	1.80	0.18	32.32	30.86	0.57	6.00	27.20
	Std. Deviation	1.28	1.21	0.16	0.03	2.35	4.10	0.08	0.71	5.07
	SE	0.26	0.24	0.03	0.01	0.47	0.82	0.02	0.14	1.01
Loei (LY)	Mean	44.90	10.57	1.40	0.34	36.51	36.99	0.69	6.72	21.96
	Std. Deviation	2.05	0.38	0.17	0.05	1.70	1.40	0.07	0.45	1.04
	SE	0.39	0.07	0.03	0.01	0.33	0.27	0.01	0.09	0.20
Sakhonnakorn (SK)	Mean	7.10	23.82	1.54	0.40	34.86	26.20	0.70	6.60	41.60
	Std. Deviation	1.30	3.66	0.36	0.12	2.65	3.06	0.07	0.55	3.21
	SE	0.26	0.73	0.07	0.02	0.53	0.61	0.01	0.11	0.64
Saraburi (SR)	Mean	8.34	34.28	1.06	0.80	25.78	26.28	0.64	6.40	24.00
	Std. Deviation	0.67	2.06	0.09	0.07	3.48	2.01	0.05	0.55	2.83
	SE	0.13	0.41	0.02	0.01	0.70	0.40	0.01	0.11	0.57
Sukhothai (SU)	Mean	45.24	10.65	1.02	0.46	43.29	35.61	0.70	6.00	21.08
	Std. Deviation	2.25	0.85	0.04	0.09	1.44	1.67	0.00	0.00	1.41
	SE	0.43	0.16	0.01	0.02	0.28	0.32	0.00	0.00	0.27
Nongbualamphu (NB)	Mean	29.08	7.33	0.96	0.29	30.38	27.83	0.70	6.00	34.08
	Std. Deviation	0.79	0.48	0.10	0.03	1.96	1.35	0.01	0.00	1.41
	SE	0.16	0.10	0.02	0.01	0.39	0.27	0.00	0.00	0.28
B. monosperma (Bm)	Mean	15.76	7.33	0.72	0.00	23.06	17.66	0.70	6.00	34.72
	Std. Deviation	1.25	0.48	0.13	0.00	1.56	1.27	0.01	0.00	1.97
Outgroup	SE	0.25	0.10	0.03	0.00	0.31	0.25	0.00	0.00	0.39

APPENDIX III

Factor analysis

9 leaf parameters of 34 cultivars of *Butea superba* and an outgroup (*Butea monosperma*).

Component Matrix[a]

	Component		
	1	2	3
Zscore(TLB)	.836		
Zscore(RL)	.805		
Zscore(TLL)	.695		
Zscore(PD)	.647		
Zscore(NPV)			
Zscore(PL)			
Zscore(PLL)		.718	
Zscore(SPL)		-.699	
Zscore(AB)			.683

Extraction Method: Principal Component Analysis.

a. 3 components extracted.

Rotated Component Matrix[a]

	Component		
	1	2	3
Zscore(PD)	.786		
Zscore(RL)	.704		
Zscore(TLB)	.689		
Zscore(NPV)	.669		
Zscore(TLL)			
Zscore(SPL)		.749	
Zscore(PLL)		-.669	
Zscore(PL)			
Zscore(AB)			-.803

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 5 iterations.

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.081	34.235	34.235	3.081	34.235	34.235	2.630	29.225	29.225
2	1.545	17.172	51.407	1.545	17.172	51.407	1.550	17.224	46.448
3	1.033	11.480	62.886	1.033	11.480	62.886	1.479	16.438	62.886
4	.872	9.684	72.571						
5	.782	8.690	81.260						
6	.594	6.596	87.857						
7	.512	5.687	93.544						
8	.352	3.916	97.460						
9	.229	2.540	100.000						

Extraction Method: Principal Component Analysis.

APPENDIX IV

Correlation analysis

Correlations

		REGR factor score 1 for analysis 1	REGR factor score 2 for analysis 1	REGR factor score 3 for analysis 1	Latitude
REGR factor score 1 for analysis 1	Pearson Correlation	1	.000	.000	.212**
	Sig. (2-tailed)		1.000	1.000	.000
	Sum of Squares and Cross-products	724.000	.000	.000	193.703
	Covariance	1.000	.000	.000	.268
	N	725	725	725	725
REGR factor score 2 for analysis 1	Pearson Correlation	.000	1	.000	-.241**
	Sig. (2-tailed)	1.000		1.000	.000
	Sum of Squares and Cross-products	.000	724.000	.000	-220.529
	Covariance	.000	1.000	.000	-.305
	N	725	725	725	725
REGR factor score 3 for analysis 1	Pearson Correlation	.000	.000	1	-.068
	Sig. (2-tailed)	1.000	1.000		.068
	Sum of Squares and Cross-products	.000	.000	724.000	-62.022
	Covariance	.000	.000	1.000	-.086
	N	725	725	725	725
Latitude	Pearson Correlation	.212**	-.241**	-.068	1
	Sig. (2-tailed)	.000	.000	.068	
	Sum of Squares and Cross-products	193.703	-220.529	-62.022	1153.845
	Covariance	.268	-.305	-.086	1.594
	N	725	725	725	725

**. Correlation is significant at the 0.01 level (2-tailed).

Correlation analysis

Correlations

		REGR factor score 1 for analysis 1	REGR factor score 2 for analysis 1	REGR factor score 3 for analysis 1	longtitude
REGR factor score 1 for analysis 1	Pearson Correlation	1	.000	.000	.020
	Sig. (2-tailed)		1.000	1.000	.595
	Sum of Squares and Cross-products	724.000	.000	.000	25.493
	Covariance	1.000	.000	.000	.035
	N	725	725	725	725
REGR factor score 2 for analysis 1	Pearson Correlation	.000	1	.000	.058
	Sig. (2-tailed)	1.000		1.000	.116
	Sum of Squares and Cross-products	.000	724.000	.000	75.256
	Covariance	.000	1.000	.000	.104
	N	725	725	725	725
REGR factor score 3 for analysis 1	Pearson Correlation	.000	.000	1	.046
	Sig. (2-tailed)	1.000	1.000		.218
	Sum of Squares and Cross-products	.000	.000	724.000	58.999
	Covariance	.000	.000	1.000	.081
	N	725	725	725	725
longtitude	Pearson Correlation	.020	.058	.046	1
	Sig. (2-tailed)	.595	.116	.218	
	Sum of Squares and Cross-products	25.493	75.256	58.999	2294.194
	Covariance	.035	.104	.081	3.169
	N	725	725	725	725

APPENDIX V

A. Reagent preparation

Agarose gel electrophoresis

1) 1% (w/v) agarose gel

- agarose	0.3	g
- 1x TBE buffer	30	ml

2) 1x Tris Boric EDTA buffer (TBE buffer), pH 8.0

- Tris aminomethane (50 mM)	108	g
- Boric acid (50 mM)	50.4	g
- EDTA (0.65 mM)	7.44	g

Adjust pH to be 8.0 and quantitate volume to be 1,000 ml.

Polyacrylamide gel electrophoresis (PAGE)

1) 8% (v/v) polyacrylamide gel

- 30% acrylamide solution (29.2% Bio-rad® acrylamide monomer: 0.8% bis-acrylamide)	4.8	ml
- 10x TBE buffer (1x)	1.2	ml
- 10% APS [$(NH_4)_2S_2O_8$] (3%)	240	µl
- TEMED (0.2%)	15	µl
- d-H_2O	17.7	ml

2) 5x loading dye

- 1 M Tris-Hcl, pH 6.8 (0.312 M)	0.6 ml
- Glycerol (50% v/v)	5.0 ml
- 10% (w/v) SDS	2.0 ml
- 2-Mercaptoethanol	0.5 ml
- 1% Bromophenol blue	0.1 g
- d-H_2O	0.9 ml

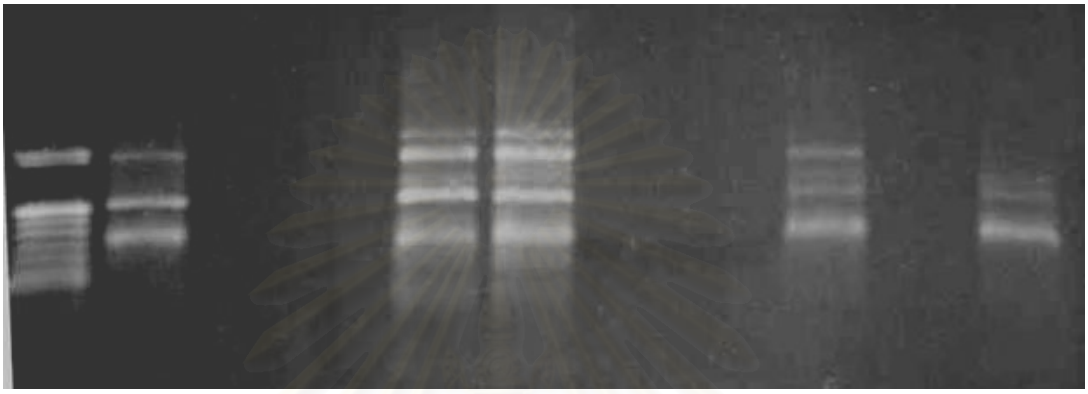
One part of sample buffer was added to four parts of sample. The mixture was heated for 5 min in boiling water before loading to the gel.

APPENDIX VI

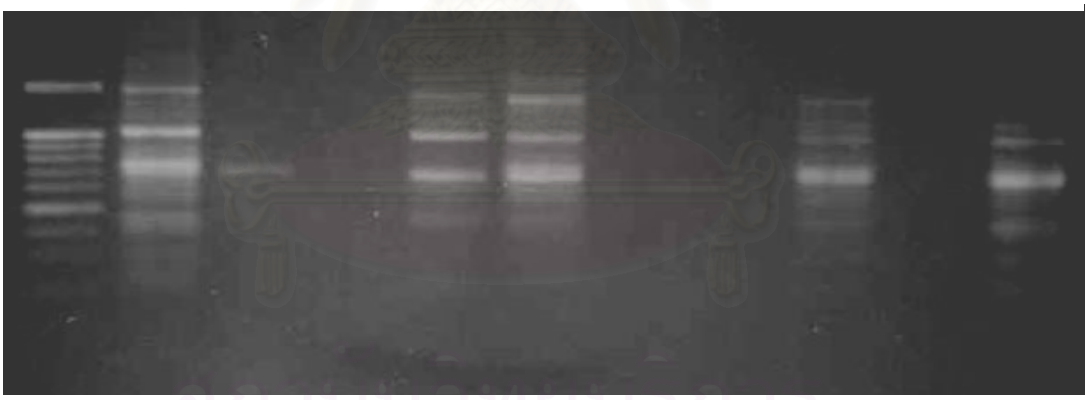
RAPD primers

OPA 7

M 1 2 3 4 5 6 7 8 9 10

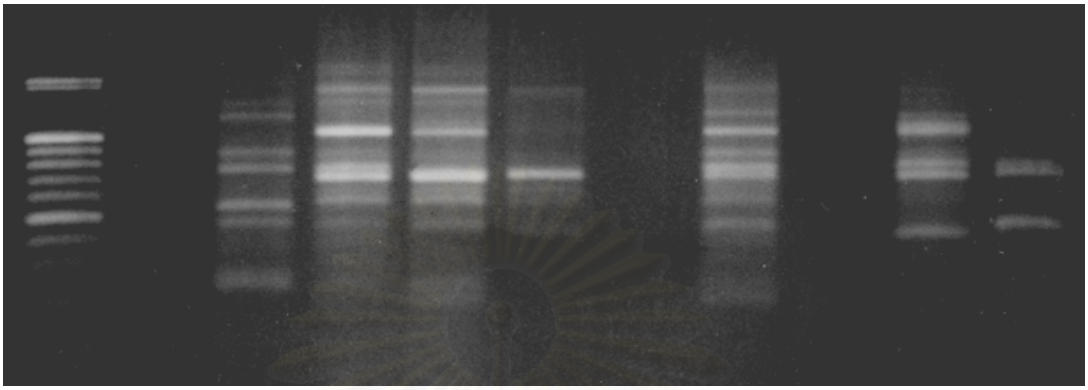


M 11 12 13 14 15 16 17 18 19 20



OPA 7

M control 21 22 23 24 25 26 27 28 29



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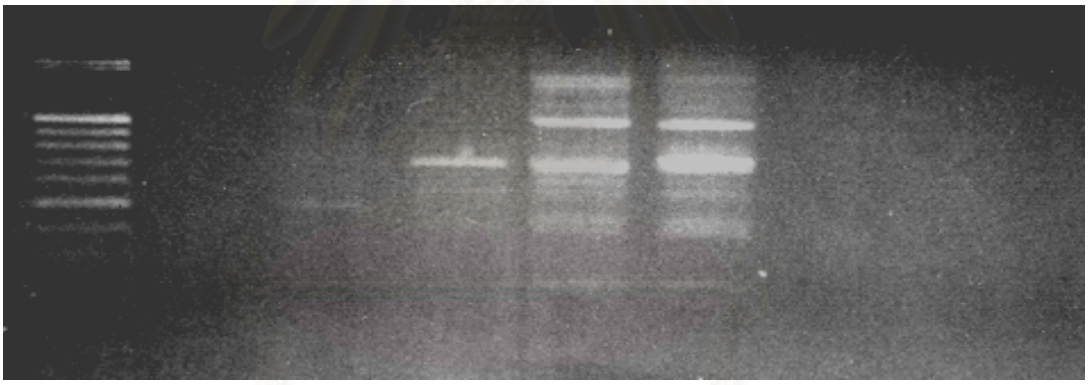

OPA 12

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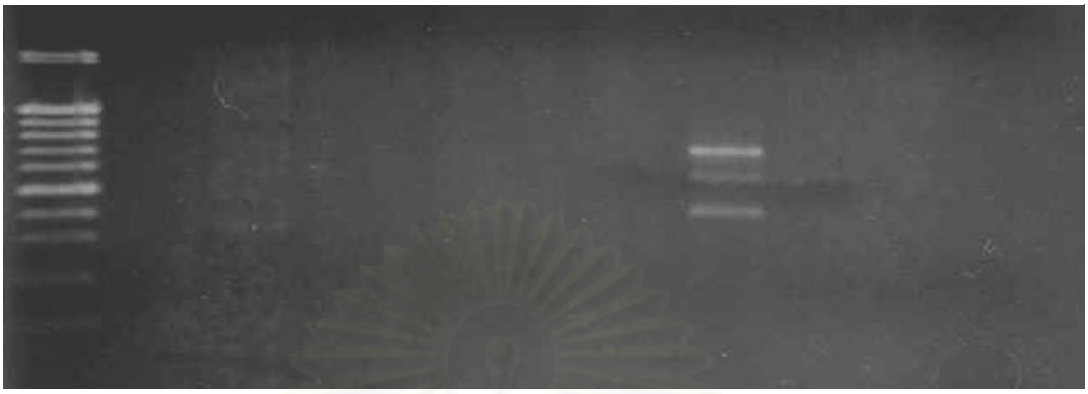

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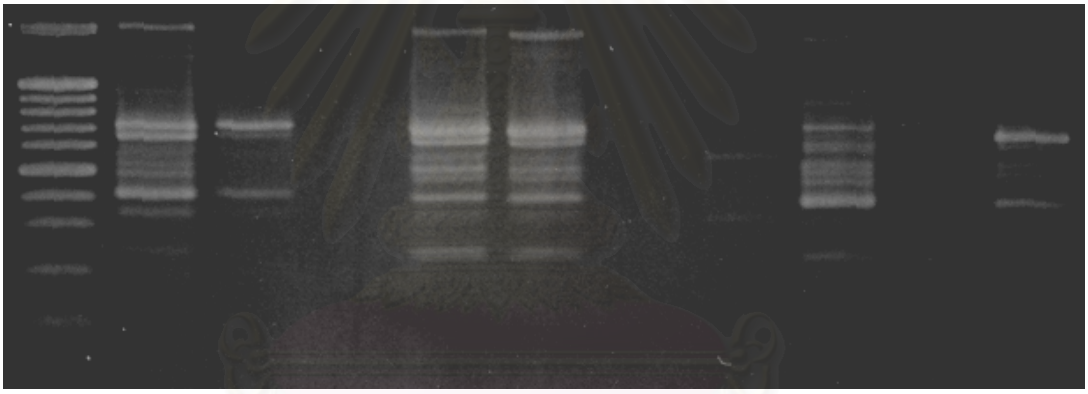

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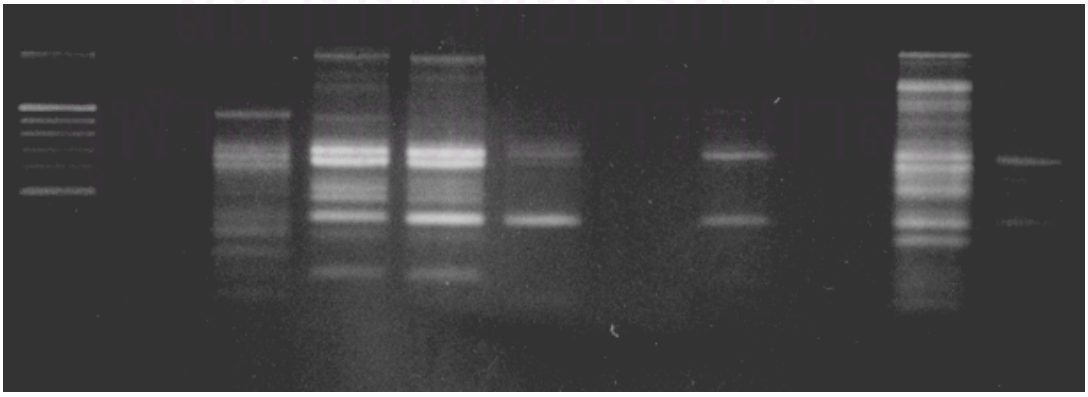

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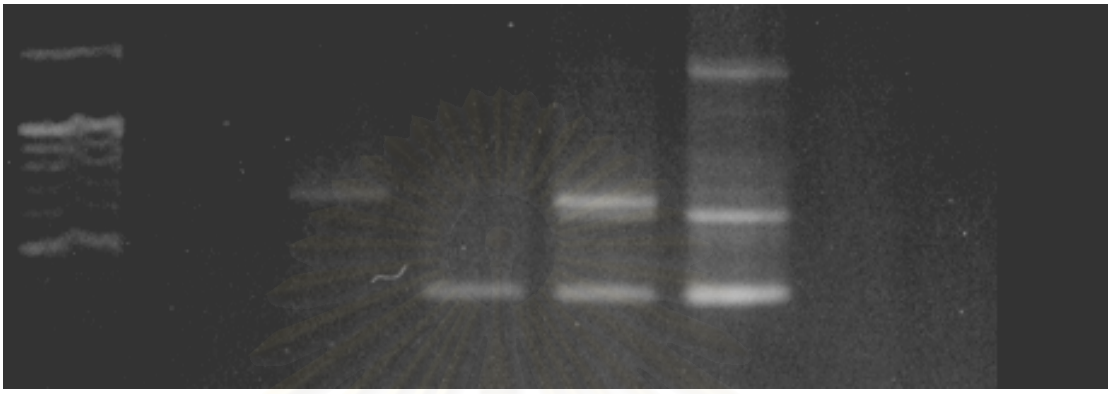

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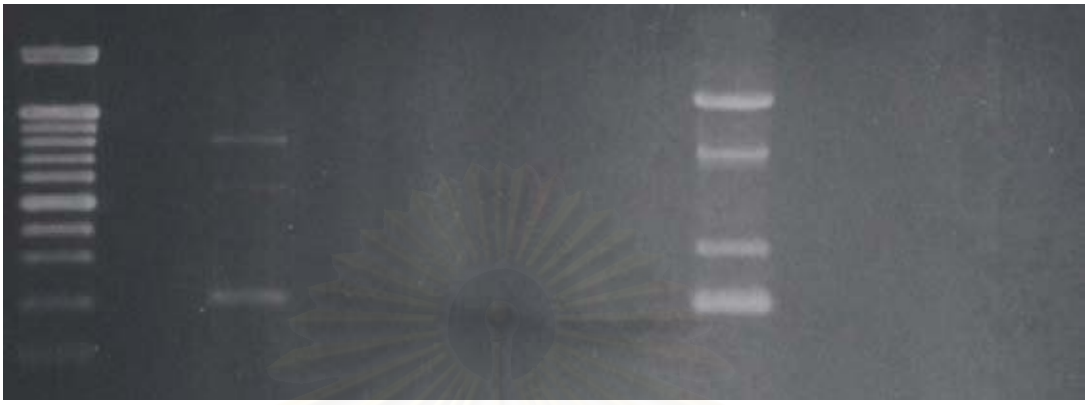

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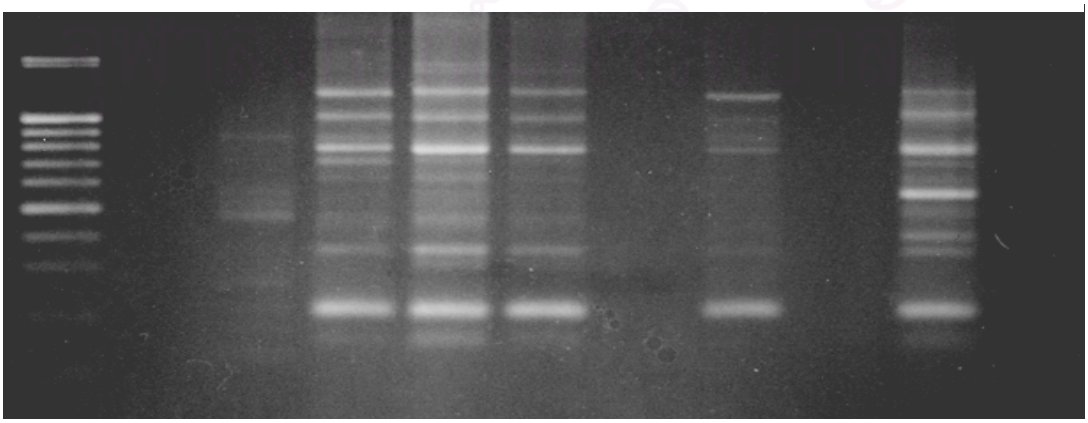
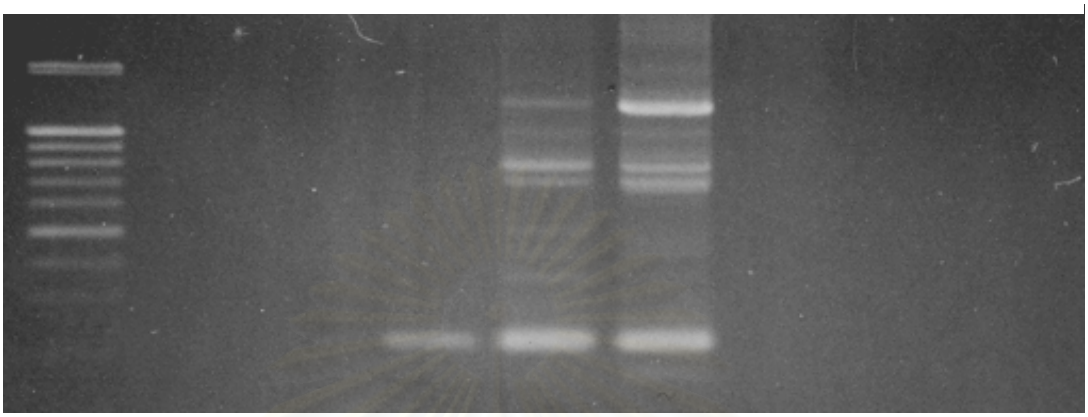


OPA 19



OPC 15

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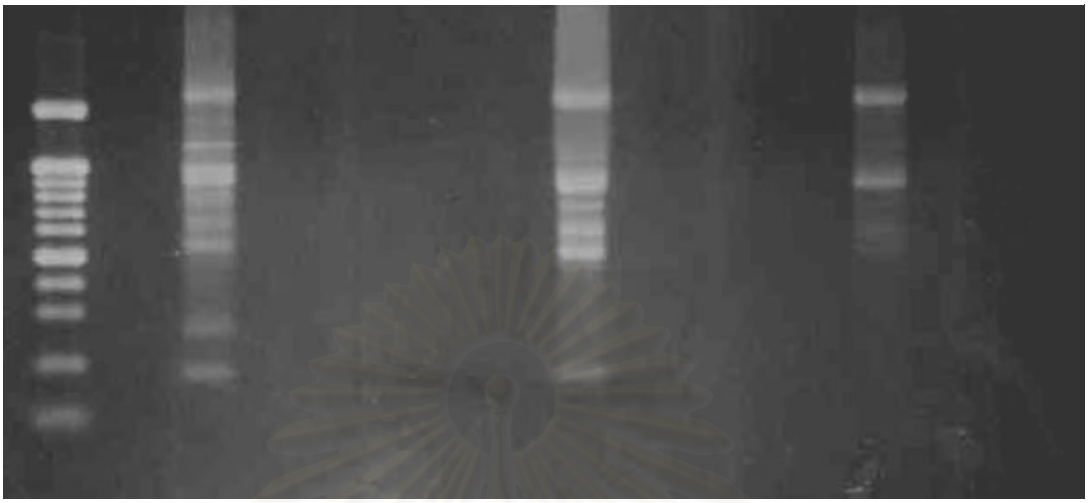

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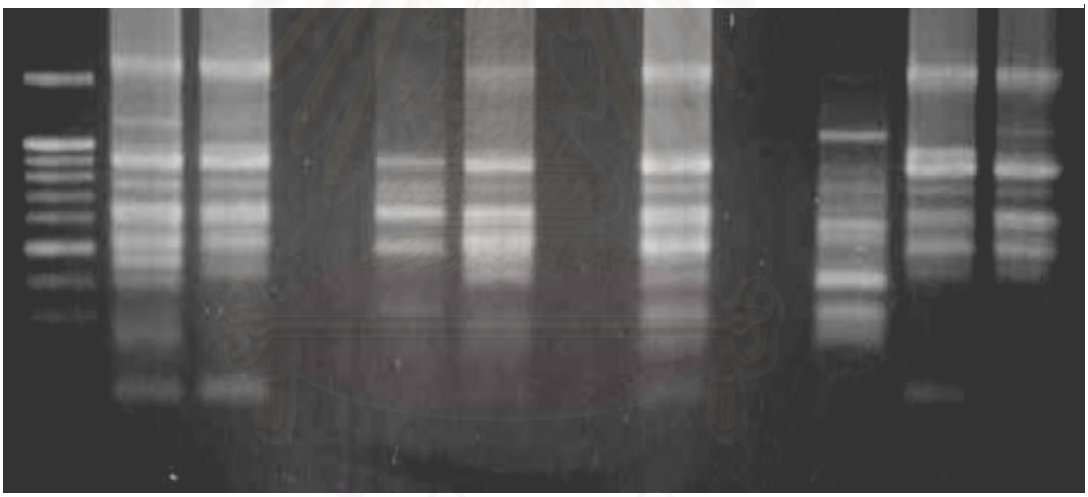
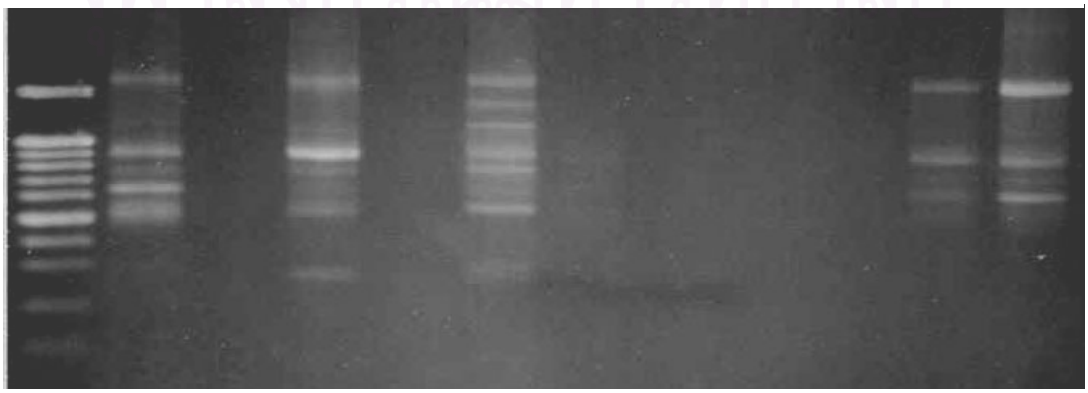
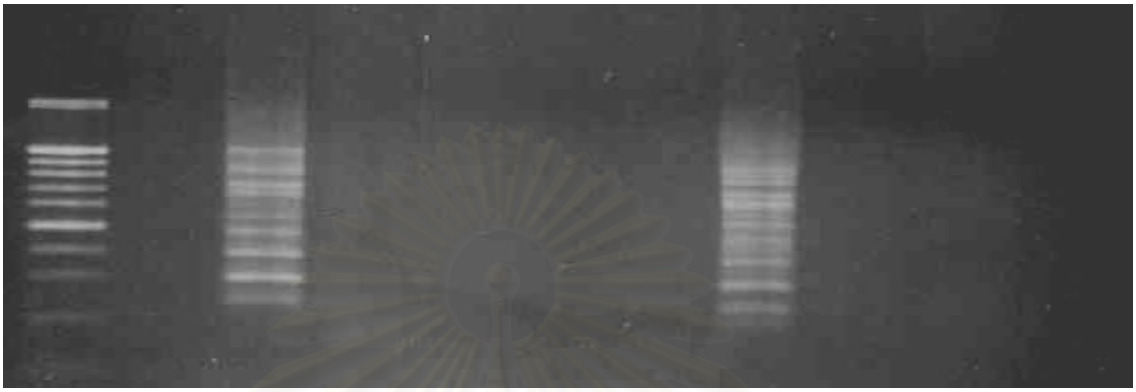


M 24 25 26 27 28 29 30 31 32 33 34



OPD 2

M 1 2 3 4 5 6 7 8 9 10



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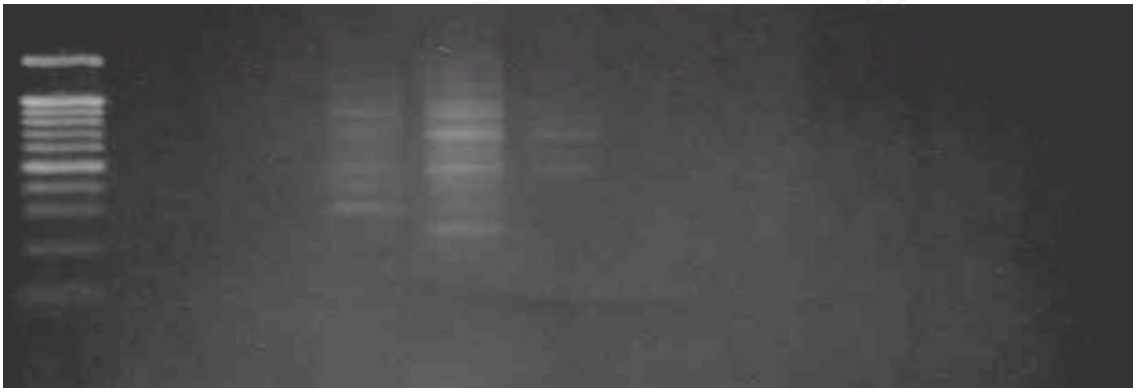

BIOGRAPHY

Miss Jirattikarn Kaewmuangmoon was born on January 6, 1982 in Lamphun province, Thailand. She finished her secondary school level from Chakkhumkanathun School in 2000, Lamphun province. After that, she got a Bachelor's Degree in Biology from Department of Biology, Faculty of Science, Chulalongkorn University in 2003. At present, she is a graduate candidate in Master's Degree in Biotechnology, Faculty of Science, Chulalongkorn University.

Research presentation:

Kaewmuangmoon, J., Chanchao, C., and Cherdshewasart, W. 2005. Leaf morphometry, Genetic variation and Phylogeny of *Butea superba* in Thailand. Abstract. *The 10th Biological Sciences Graduate Congress*, National University of Singapore, Singapore.

Kaewmuangmoon, J., Chanchao, C., and Cherdshewasart, W. 2005. Leaf morphometry, Genetic variation, and Phylogeny of *Butea superba* in Thailand. Abstract. *The KMITL International Conference on Science and Applied Science 2006,* Bangkok, Thailand.

Kaewmuangmoon, J., Chanchao, C., and Cherdshewasart, W. 2005. Leaf morphometry, Genetic variation, and Phylogeny of *Butea superba* in Thailand. Abstract. *The 14th Faculty of Science Congress, Chulalongkorn University*, Bangkok, Thailand.

Kaewmuangmoon, J., Chanchao, C., and Cherdshewasart, W. 2006. Leaf morphometry, Genetic variation, and Phylogeny of *Butea superba* in Thailand. Abstract. *The 10th BRT annual Conference*, Krabi, Thailand