การวิเคราะห์ไอเอสเอสอาร์ของเปล้าน้อย *Croton sublyratus* Kurz. ในประเทศไทย

นางสาวรัฐพร กลิ่นบรรทม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ISSR ANALYSIS OF *Croton sublyratus* Kurz. IN THAILAND

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ความแตกต่างทางพันธุกรรมของเปล้าน้อยจำนวน 37 ตัวอย่างที่ได้จากแหล่งต่าง ๆ ในประเทศไทยซึ่งได้แก่ จังหวัดประจวบคีรีขันธ์ นครพนม และปราจีนบุรี นำมาวิเคราะห์ด้วยเทคนิคอาร์เอพีดี พบว่ามี 3 ไพรเมอร์ คือ GGCACTGAGG, ACGACCGACG และ GATGACCGCC ที่สามารถใช้สร้างเครื่องหมายโมเลกุลแบบอาร์เอพีดีแต่ไม่ให้รูปแบบคงเดิมเมื่อทำซ้ำ จึงเปลี่ยนมาวิเคราะห์ด้วยเทคนิค ไอเอสเอสอาร์พบว่ามี 3 ไพรเมอร์คือ $(CA)_8G$, $BSC(GA)_8$ และ $CRN_2(CTT)_5$ ที่สามารถใช้สร้างเครื่องหมายโมเลกุลแบบไอเอสเอสอาร์ที่ให้รูปแบบคงเดิมเมื่อทำซ้ำ แถบไอเอส-เอสอาร์ที่รวบรวมได้มีทั้งสิ้น 146 แถบ ประกอบด้วยแถบดีเอ็นเอที่ไม่มีความหลากหลายจำนวน 50 แถบ (34%) และแถบดีเอ็นเอที่มีความหลากหลายจำนวน 96 แถบ (66%) เมื่อประมาณค่าความแตกต่างทางพันธุกรรม และสร้างแผนภูมิความสัมพันธ์ทางพันธุกรรมโดยใช้วิธี Unweighted Pair Group Method with Arithmatic Mean (UPGMA) พบว่าต้นเปล้าน้อยประกอบด้วยกลุ่มย่อย 2 กลุ่ม ซึ่งสอดคล้องกับแหล่งกำเนิด

การศึกษาลักษณะทางกายภาพของใบของตัวอย่างต้นเปล้าน้อยพบว่า มีลักษณะใบที่แตกต่างกันตามแหล่งกำเนิด ซึ่งสอดคล้องกับการจัดกลุ่มความแตกต่างทางพันธุกรรมของต้นเปล้าน้อย การวิเคราะห์หาปริมาณเปลาโนทอลในใบเปล้าน้อย ได้ใช้ตัวอย่างใบจำนวน 37 ตัวอย่าง ผลการทดลองแสดงให้เห็นว่าปริมาณเปลาโนทอลในใบเปล้าน้อยพบอยู่ในช่วง 0.11-1.01% (w/w) โดยน้ำหนักแห้ง และพบว่า ไพรเมอร์ $(CA)_8G$ ให้แถบดีเอ็นเอที่สามารถบอกความแตกต่างเบื้องต้นระหว่างต้นเปล้าน้อยที่มีปริมาณเปลาโนทอลสูงกับต่ำได้ ผลการทดลองยังแสดงให้เห็นว่าต้นเปล้าน้อยจากแหล่งกำเนิดต่างกันจะมีปริมาณเปลาโนทอลในใบต่างกัน ซึ่งสอดคล้องกับความแตกต่างทางพันธุกรรม

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....

ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ทีปรึกษาร่วม.....

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KEY WORD: *Croton sublyratus* Kurz / dendrogram / genotype / ISSR / plaunotol / UPGMA

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Inter-simple sequence repeat (ISSR) analysis was used to determine the level of genetic diversity among 37 samples of *Croton sublyratus* Kurz from various sources, including Prachuapkhirikhan, Nakhonphanom and Prachinburi. Three RAPD primers (GGCACTGAGG, ACGACCGACG and GATGACCGCC) were used to study genetic diversity but they gave non-reproducible results. Three of ISSR primer ($(CA)_8G$, $BSC(GA)_8$ and $CRN_2(CTT)_5$) gave reproducible ISSR patterns. One hundred and forty six reproducible ISSR bands were generated across the investigated species of these, 50 band (34%) were monomorphic band and 96 band (66%) were polymorphic band. Genetic variation of 37 samples of *C. sublyratus* were analyzed by Unweighted Pair Group Method with Arithmatic Mean (UPGMA) and the dendrogram divied *C. sublyratus* into 2 groups was indicated with geographical location.

This study indicated that the genetic variability is higher in the species as genotypes did show distinctive leaf morphological characters under uniform conditions of growth for most of the attributes. Thus, the alteration of responses seems to be genetic variation rather than environmentally induced for *C. sublyratus*. In the evaluation of plaunotol content in the leaves of *C. sublyratus*. The analysis revealed that *C. sublyratus* leaves contained plaunotol in the rang 0.11-1.01% (w/w) dry weight. A specific ISSR fragment can be used for primary screening to select for high and low plaunotol content in *C. sublyratus* with $(CA)_8G$. The result would suggest that the variation between *C. sublyratus* in the plaunotol content from various geographical location is governed by genetic variation.

Field of study.....Biotechnology..... Student's signature.....

Academic year 2004..... Advisor's signature.....

Co-advisor's signature.....

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A, T, C, G	nucleotide containing the base adenine, thymine, cytosine and guanine, respectively
ATP	adenosine triphosphate
bp	base pair
°C	degree Celcius
CTAB	hexadecetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
EDTA	ethylenediamine tetraacetic acid
GC	gas chromatography
HCl	hydrochloric acid
ISSR	inter simple sequence repeat
Kb	kilobase
KCl	potassium chloride
$MgCl_2$	magnesium chloride
ml	millilitre
mM	millimolar
mtDNA	mitochondrial DNA
mRNA	messenger ribonucleic acid
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RnaseA	ribonuclease A
rpm	revolution per minute

UV	ultraviolet
v	volume
V	volt
W	watt
W/V	weight/volume
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I

INTRODUCTION

Croton sublyratus Kurz or Plau-noi (in Thai) is the plant belonging to the family Euphorbiaceae. It is distributed extensively in tropical areas, especially those near by the Andaman sea such as the Indonesia, Malasia, Thailand, Myanmar and the southern part of China. In Thailand , *C. sublyratus* is found to be native to the Thai provinces of Prachinburi, Prachuap khiri khan and the border near Myanmar of Kanchanaburi. (ณรงค์ เพ็งปรีชา, 2530). *C. sublyratus* is a deciduous shrub or tree, 2-3.5 m hight, shoots rusty-scurfy. The leaves are simple, alternate, 4-6 cm wide, 10-15 cm long. The flowers are small, perfect and receme. Flowering is up the scar of leaf with near shoot. The fruits are small capsules 3 lobed crustaceous sparsely pubescent and 3-5 mm long. The seeds are 2-3 mm long, white-brown and smooth (ลีนา ผู้พัฒนพงศ์ และธวัชชัย วงศ์ประเสริฐ, 2530).

C. sublyratus as a medicinal plant has been used in folk medicine for a long time. Its leaves have been used as a Thai folk medicine for anthelmitic and dermatologic agent for skin disease. For its chemical constituents, *C. sublyratus* has been reported to contain many groups of compounds, such as diterpene lactone, furanoid diterpenes, diterpene alcohols and plaunotol, the active antipeptic ulcer substance (Ogiso *et al*., 1978). Plaunotol is an acyclic diterpene alcohol present in the leaves of *C. sublyratus* are used as material for extracting an antipeptic ulcer substance with broad spectrum inhibition against the gastic ulcer. Plaunotol enhances the mucosal protective factors by increase in gastic mucosal blood flow, promotion of mucous and prostaglandin production in the gastric mucosa and increase the gastic mucosal resistance (ณรงค์ เพ็งปรีชา, 2530; วีณา วิรัจฉัยกูล และคณะ, 2533). Its chemical name is (*E*, *Z*, *E*)-7-hydroxymethyl-3, 11, 15-trimethyl-2, 6, 10, 14-hexadecateraen-1-ol or 18-hydroxygeranylgeraniol. It has a formular of $C_{20}H_{34}O_2$ and molecular weight of 306.256 (Ogiso *et al*., 1978)

There is a few information of the genetic relatedness of *C. sublyratus* in Thailand, particularly the level of genetic diversity among land races and contemporary cultivars. Morphological variation may not reliably reflect the real genetic variation because of genotype-environment interaction and largely unknown genetic control of polygenically inherited

morphological and agronomic traits (Smith, 1992). The genetic structure of plant populations reflects the interactions of different processes including long-term evolutionary history of the species (shifts in distribution, habitat fragmentation, and population isolation), mutation, genetic drift, mating system, gene flow, and selection (Slatkin, 1987 ; Schaal *et al*., 1998). All these factors can lead to complex genetic structuring within populations, which is often difficult to resolve. Nevertheless, Inter-simple sequence repeat (ISSR) analysis has provided powerful tools for the investigation of genetic variation within a species and can facilitate understanding of such complexities.

C. sublyratus from various locations in Thailand have been reported to contain different amount of plaunotol. For examples, *C. sublyratus* from Prachuapkhirikhan has more anti-ulcer substances than those form Prachinburi and the leaves contain higher content than the stems, branches and roots (ณรงค์ เพ็งปรีชา, 2530; พนิดา แสงทอง, 2528; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; วีณา วิรัจฉริยากูล และคณะ, 2533). It is not well understood that why there is so much difference of plant morphology and plaunotol content in *C. sublyratus*. Since genetic analysis of this plant has never been reported before, therefore, inter-simple sequence repeat (ISSR) and randomly amplified polymorphic DNA analyses may provided powerful tools for the investigation of genetic variation of *C. sublyratus*.

Thus the objectives of this research are the following:

1. To examine the genetic variation of *C. sublyratus* Kurz in Thailand by RAPD and ISSR analyses.
2. To study the relationship between genetic variation of *C. sublyratus* Kurz in Thailand and plaunotol content.

CHAPTER II

LITERATURE REVIEW

2.1 General characterization of the plants in the Genus Croton

The genus Croton comprises 700 species of trees or shrubs. Leaves are usually alternate with 2-glandular stipule at the base. Their flowers are solitary or clustered in the rhachis of a terminal raceme and bracts are small. Male flowers contain 5-calyx and 5-petals. There are many stamens inserted on a hairy receptacle. In female flowers, sepals are usually more ovate than the male, petals are smaller than the sepals of missing and disk annular of 4-6 glands are opposite the sepals. There are three ovaries with solitary ovule in each cell. Seeds are smooth, albumen copious and broad cotyledons.

2.2 General characterization of *Croton sublyratus* Kurz

Croton sublyratus Kurz or plau-noi (in Thai) is in the family Euphorbiaceae. The plant is a deciduous shrub or tree, 2-3.5 m high, shoots rusty-scurfy. The leaves are simple, alternate, 4-6 cm wide, 10-15 cm long; cordate at the narrowed base, very shortly petioled obovate to almost lyrate oblong obtuse or acuminate repand-serrulate beneath glabrous or with scabrous nerves and racemes stellate-tomentose. Young leaves are dark brown and inflorescense. Petiole is stout, 6-12 mm long. The flowers are small, perfect and raceme. Flowering is up the scar of leaf with near shoot. Staminate flower has five lanceolate with acuminate sepal, five petal with stellate rim, long stellate base and stamens 15-20 glabrous. Pistillate flower is similar to staminate flower, no petal and ovary is densely stellate-tomentose, brown-yellow with short styles. The fruits are capsules with small 3 loabed crustaceous sparsely pubescent and 3-5 mm long. The seeds are 2-3 mm long, white-brown and smooth (ลีนา ผู้พัฒนพงศ์ และธวัชชัย วงศ์ประเสริฐ, 2530; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; Hooker, 1973).

C. sublyratus is found extensively in tropical areas, especially those near by the Andaman sea such as Indonesia, Malasia, Thailand, Burma and the south of China. The survey on various plants related to *C. sublyratus* was relatively extensive in Thailand. There are several species belonging to the genus *Croton* in Thailand, including Plau-noi (*C. sublyratus* or *C . joufra*

or *C. kerrii*), Plau-luat (*C. hatchisonianus*), Plau-yai (*C. oblongifolius* or *C. poilanei*), Plau-namngern or Plau-kradat (*C. cascarilloides*). These plants grow naturally in every part of Thailand. However, only *C. sublyratus* has been found to contain anti-ulcer substances (Ogiso *et al*., 1981). Thai *C. sublyratus* or Plau-noi is found to be native to the Thai provinces of Prachin Buri, Prachuap khiri khan and the border near Myanmar of Kanchanaburi (ลีนา ผู้พัฒนพงศ์ และ ธวัชชัย วงศ์ประเสริฐ, 2530; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535)

The propagation of *C. sublyratus* includes budding (to form plantlet from root), seedling, and cutting. It has been reported that *C. sublyratus* plant from Prachuap khiri khan have more anti-ulcer substances than the plant form Prachin Buri and that leaves contain higher content than the stems, branches and roots (ณรงค์ เพ็งปรีชา, 2530; พนิดา แสงทอง, 2528; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; วีณา วิรัจฉริยากูล และคณะ, 2533).

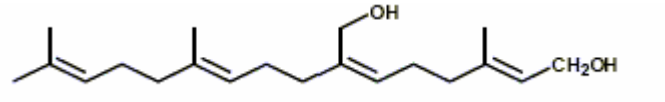
2.3 Medicinal property

C. sublyratus (Plau-noi) is a Thai Flok medicine for anthelmintic and dermatologic agent for skin disease. The plant parts of stem, bark and leaf have been used as antidiarrheal and normalize menstruation wheres its flower is used as anthelmintic. Firewood or Plau-noi is used for postpartum. In addition, it has been reported that Plau-noi and Plau-yai (*C. oblongifolius* Roxb.) are used joinly in many Thai drugs, such as stomachic, anthelmintic, emmenagogue, digestant, transquilizer, carminative, treatment of lymph, pruritic, leprosy, tumor and yaws (ประเสริฐ พรหมมณี และคณะ, 2531; นันทวัน บุณยะประภัศร, บรรณาธิการ, 2532).

The leaves of *C. sublyratus* are used as material for extracting an antipeptic ulcer substance, namely Plaunotol. Plaunotol enhances the mucosal protective factors by increase in gastic mucosal blood flow, promotion of mucous and prostaglandin production in the gastric mucosa and increase in gastic mucosal resistance.

2.4 Plaunotol

Plaunotol is an acyclic diterpene alcohol present in the leaves of *C. sublyratus.* Its chemical name is (*E*, *Z*, *E*)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecateraen-1-ol or 18-hydroxygeranylgeraniol. It has a formular of $C_{20}H_{34}O_2$ and molecular weight of 306.256 (Ogiso *et al*., 1978). The structure of plaunotol is shown in Scheme 1.



Scheme 2.1 The chemical structure of plaunotol.

Plaunotol occurs as pale yellow to light brown viscous liquid, having a slightly characteristic odor and a bitter taste. It is soluble in methanol, ethanol, acetone, ethyl acetate, dioxane, ether, chloroform, toluene or vegetable oil, but is practically insoluble in water (Department of Medicinal Information, Sankyo Co., Ltd., 1993).

2.5 Molecular genetic markers

Molecular markers are useful for various genetic studies. These markers generally include protein and DNA markers. The former refers to markers generally form allozyme and isozyme. The latter is composed of those obtained from several approaches including restriction analysis (restriction fragment length polymorphism, RFLP; DNA fingerprinting and polymorphism of single copy nuclear DNA), PCR-base techniques (e.g. randomly amplified polymorphic DNA, RAPD and microsatellites) and DNA sequencing.

2.5.1. Protein markers

Protein polymorphism is regarded as one of the genetic markers detected by electrophoresis of proteins (usually enzyme). Proteins move with different rate through a gel matrix when exposed to an electric field (Avise, 1994). The protein bands can be visualized by a specific histochemical stain of the particular enzyme.

Analysis of protein polymorphism is a technique of choice to starting with when any molecular data of a species under investigation has not been reported. The majority of protein markers are represented by allozymes, which are different molecular forms of an enzymes coded by different alleles at one gene locus. The allozyme markers are transmitted in a co-dominant manner; therefore, heterozygosity of individuals within populations of species can be determined. The advantages of allozyme approach are it cost-effectiveness, less tedious and time-consuming technique as compared to other molecular genetic techniques. Therefore, large numbers of samples can be processed within the limiting period of time. However, allozyme analysis has some limitations. For instance, synonymous mutation can not be detected. Likewise, nucleotide

substitution changing one non-polar amino acid to another does not alter the electrophoretic mobility of proteins. Scoring of gels can be complicated and need experienced scientist to interpret the precise results from electrophoresed gel (Kocher and Stephen, 1997).

2.5.2 DNA markers

DNA can be compared in several different ways. Genetic variations at the DNA level can be genelized roughly into two categories: base substitution and insertion/deletions. The polymorphism is represented by differences of extrachromosomal DNA and chromosomal DNA

2.5.3 Extrachromosomal DNA

There are two types of plant extrachromosomal, chloroplast DNA (cpDNA) has been the molecular choice for molecular phylogenetic studies (Palmer and Herbon, 1988). Plant cpDNA has the advantage over mtDNA for evolutionary and phylogenetic study because cpDNA is relatively abundant, thus facilitating extraction and analysis. The second advantage is due to extensive background of molecular information of the chloroplast genome. For example, complete DNA sequence of three cpDNA genomes are known (liverwort, *Marchatia polymorpha*; tobacco, *Nicotina tabacum*; and rice, *Oryza dativa*) (Soltis *et al*., 1992).

Chloroplast DNA of photosynthetic land plants are circular DNA molecules ranging in size from 120 to 217 kb, but plant mtDNAs are abnormally large and variable in size. Plant mtDNAs may have ranging in size from 330 kb (*Citrullus lanatus*) to 2,500 kb (*Cucumis melo*) because it has two regions of homologous repetitive DNA that cause intra-and intermolecular recombination in mtDNA molecule. This phenomenon mark mtDNA in plant cell to have more than one size (heterogenouse mtDNA) per individual, this is a major disadvantage for using mtDNA to study plant systematic (Soltis *et al*., 1992).

However, plant mtDNA can be use for study in some case such as cytoplasmic male sterile (CMS). These plants can not produce fertile pollen because of unusual mtDNA. Examples are the study about relationship between normal mtDNA organization and mtDNA organization in cytoplasmic male sterile type T in maize (Fauron *et al*., 1989; Fauron *et al*., 1990) and study of unusual mtDNA organization in cytoplasmic male sterile common bean (Janska and Mackenzie, 1993)

2.5.4 Chromosomal DNA

Chromosomal DNA contains both unique single copy and repititive regions. The single copy region generally codes for a particular gene product. The structure of protein coding genes in the nuclear genome consists of coding regions (exons) and non-coding regions (introns or intervening sequences). Typically, exon is highly conserved. In contrast, much higher polymorphism can be conserved in introns (Krawczak and Schmidtke, 1994).

Repetitive DNA consists of core sequences that are repeated in varying degrees. They may be made up of coding segments such as the ribosomal RNA (rRNA) genes, or non-coding tandem repeated units (Parker *et al*., 1998).

Variable number of tandem repeats (VNTRs) are composed of satellite, minisatellite and microsatellite DNA. Large repetitive units of satellite DNA are often associated with heterochromatin located near the centromere of chromosomes. Smaller regions (made up of repeated units < 65 base pair) are known as minisatellite DNA and can occur throughout the genome (Jeffrey *et al*., 1985) whereas microsatellite DNA has consecutive repeated units of only 1-6 base pair (Tautz, 1989).

Plant genomes are differed from animal and fungi due to polyploidy which cause it to vary in DNA content. Polyploidy can also occurred during plant development. It has been estimated that 50% of angiosperm were polyploidy resulting from the doubling of chromosomes within a species of the hybridization of two species without chromosome reduction (Foster and Twell, 1996). The second major source of variation in plant chromosomal DNA content comprises a huge number of families of repeated sequences. The proportion of repetitive DNA exceed 50% in genome larger than 1.5 pg and approach 95% in vary large genomes (Flavell, 1980). High proportions of repeats have major implications for exploring genomes via molecular genetics.

2.6 DNA techniques commonly used in genetic variation studies

2.6.1 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) analysis is one of the initial techniques widely used to indirectly detect genetic variation at the DNA level. It examines size variation of specific DNA fragment after digestion with restriction enzymes.

Conventional RFLP analysis is carried out by digestion of genomic DNA with a specific endonuclease. Products are then size fractionated using gel electrophoresis and transferred onto a membrane. The investigated fragment(s) is identified by hybridization with the specific radiolabelled probe. In more comprehensive studies, restriction sites, rather than sharing of fragment length are scored (Kocher and Stephen, 1997). WatanabeAraki (1998) studied in vitro propagation, restriction fragment length polymorphism,of Angelica plants. The resulte is genetic similarities of several *Angelica* plants were investigated by restriction fragment length polymorphism (RFLP).

2.6.2 PCR-RFLP

The polymerase chain reaction (PCR) in an effective approach for population genetic and systematic studies. The method is base on *in vitro* enzymatic amplification of DNA. Million copies of a particular target DNA fragment are produced from a vary low amount of starting template DNA (mostly in the nanogram range). PCR is a simple but powerful technique. The PCR reaction contains the target double stranded DNA, two primers that hybridize to flanking sequence, deoxyribonucleotide triphosphates and a *Taq* DNA polymerase. The amplification reaction consists of three steps: denaturation, primer annealing and elongation step. The cycle is repeated for 25-50 times. In each cycle newly synthesized strands act as the templates for subsequent replication resulting in exponential increasing of the specific product within only a few hours. The products obtained are usually electrophoretically analyzed using agrose gels.

The PCR-amplified fragment can be further analyzed for restriction site and/or fragment length polymorphism. After specific DNA is amplified through the PCR, the product is then digested with restriction endonuclease before being electrophoretically analyzed. The most important advantage of this technique is that hybridization of labeled DNA probes to the target restricted DNA is not necessary. This technique is much simple than the conventional RFLP approach.

2.6.3 DNA sequencing analysis

Polymorphism at the DNA level can be studied by several methods but the most direct strategy is determination of nucleotide sequences of a defined region. The sequences

obtained can be aligned by comparing with an orthologous region of related organisms (or population).

PCR-amplified fragments can be directly sequenced using the typical chain termination reaction (Sanger *et al*., 1977) or alternatively by cycle-sequencing. Nevertheless, more accurate sequences of DNA fragment are obtained through the cloning approach.

However, DNA sequencing is tedious, time consuming and expensive. The number of base, which can be determined by sequencing usually cover a few hundred bases. Increasing the length of investigated DNA with large number of samples are prohibited by several factors a fore mentioned. With an introduction of automatric DNA sequencers, the experiment can be carried out much faster than that based on manual sequencing thus allowing its wider applications for genetic studies on long DNA sequence, i.e. genome project.

2.6.4 Randomly amplified polymorphic DNA (RAPD) analysis

Randomly amplified polymorphic DNA (RAPD) analysis concurrently developed by Williams *et al*. (1990). Technically, it is a simple method for determination of genetic variations using arbitrarily primer PCR-base technique. The amplification conditions of RAPD differ from that of the standard PCR in that only random primer (e.g. 10 mer with GC content usually at least 50 %) is employed. RAPD amplified target DNA on the basis that the nuclear genome contains several priming sites closed to one another that is located in an invert orientation. Accordingly, the primer is utilized to scan genome for the small inverted sequences resulting in amplification of DNA segment of variable length. The amplification products are separated on agarose gels and detected by staining with ethidium bromide.

The advantages of using RAPD markers are as follows: firstly, RAPD analysis is a simple, rapid and expensive method for detecting DNA polymorphism. Secondly, RAPD does not require knowledge of the genome under investigation. Thirdly, RAPD is a PCR-based method. It requires the use of radiolabelled probes for hybridization. Finally, unlimited numbers of RAPD primers can be screened for suitable molecular markers of various applications within a short period of time.

There are some disadvantages of the RAPD approach for population genetics, genetic mapping and taxonomic studies too. Fragment (especially those arising from mispairing

of a primer with the genomic DNA) may not be reproducible among different laboratories because amplifications are sensitive to slight changes in amplification conditions.

The allele distribution of RAPD amplified fragment is treated in a dominant fashion. Accordingly, the presence of amplified fragment may reflect either a homozygouse (AA) or heterozygouse (Aa) situation. Only the absence of the fragment reveals the aa genotype. This disadvantage of RAPD results in an inability to estimate heterozygosity and the actual status of interested alleles because homozygotic can not be dissociated from heterozygotic states. Zeng Yu. *et al* (2002) studied RAPD marker in diversity detection and variety indentification of Tibetan Hulless barley. The resulte is RAPD is good technique to use for hulless variety identification and can be used to select for high and low β -glucan content varieties.

2.6.5 PCR amplification of specific sequence and microsatellite primers

Microsatellite are short core (1-6 bp) tandem repeats sequences (200up to 500 bp long) distributed along the genome. Polymorphism due to length variation and base changes in these repeats occur at higher rate than in the coding region. Microsatellite regions are screened by the insertion of up to 500 bp genomic fragments into plasmids, subsequently amplified using competent cells. Following the detection of suitable colonies, inserts are sequenced and the conserved flanking regions, identified in this method, are used to design/locus specific microsatellite primers. The primers are used in the PCR reaction, involves the incorporation of a radioisotope into the resultant PCR fragment. The samples are run on a polyacrylamide sequencing gel and visualized by autoradiography (Queller *et al*., 1993). The utility of a PCR approach allow the possible processing of a large number of samples generally required for population surveys and large breeding programs (Brooker *et al*., 1994).

Micosatellite or simple sequence repeats (SSRs) consist of tandemly repeated core sequence that often vary in the repeat numbers and are flanked by conserved DNA sequences. Microsatellites contain short motifs (usually 1-6 nucleotides per repeat) and repeat up to about 100 times. Microsatellites are relatively highly variable but tend to have fewer alleles than do minisatellites making them useful for genetic studies in many circumstances for example, highly polymorphic loci may be used for parental analysis whereas the loci having lower number of alleles can be used to investigate intraspecific subdivisions or in a few cases, at interspecific levels, (Krawczak and Schmidtlee, 1994; Mavghan *et al*., 1994; O'Reilly and Wright, 1995).

Microsatellites are abundant, widespread distributed throughout the chromosome and are highly polymorphic in eukaryotic genomes therefore, they are probably the most effective marker for mapping of the genome (Tautz, 1989).

Polymorphism at microsatellite loci was firstly demonstrated by Tautz (1989) and Weber & May (1989). Microsatellite arrays are generally short, 20 to 300 bp. These ranges are well within the capabilities to be amplified using conventional PCR. Generally, each of microsatellite DNA is flanked in a unique sequence. As a result, locus specific primers can be developed complementary to such flanking regions. The microsatellite DNA of individual can be amplified through the PCR. After electrophoretically fractionated, the sizes of microsatellite alleles at a particular locus can be estimated by compared to the DNA standard.

2.7 Genetic studies of *C. sublyratus*

Department of Botany, University of Wisconsin-Madison were extracted DNA from 87 specimens representing seven outgroup species and 78 *Croton* species. They cover about 22 of the 40 sections treated by Webster (1993) and most of the large sections. Taxa were selected based on several criteria a broad sampling of sections and geographical regions of the globe, using herbarium material at Missouri Botanical Garden, and material that we were able to collect fresh or in silica gel ourselves or that we obtained from collaborators. They successfully sequenced 64 taxa for the internal transcribe spacer (ITS region) and 65 taxa for trnL-F. Both regions are sufficiently variable to provide high statistical support for many clades found in the tree. ITS, with 39% of sites potentially informative, appears better suited to resolve relationships among very closely related species, whereas the more slowly evolving trnL-F region, with 12% of sites potentially informative, may be better at resolving early branching clades. The ITS and trnL-F data sets had 58 taxa in common, and these are congruent with each other according to the incongruence length difference test (p=0.143; Farris et al. 1994). The combined data set resolves many clades in the tree with bootstrap supports well above 50% . The main differences between the ITS and cpDNA data sets are the positions of *Croton setigerus* (sect. Eremocarpus) and *C. michauxii* (sect. Crotonopsis), both of which fall in different positions in the cpDNA from the ITS tree and the combined tree, where they are near the base. It is possible that they are being forced to the base of the tree due to long branch attraction (Felsenstein 1978), but we should be able to resolve the positions of these morphologically distinct, small sections by adding a more slowly

evolving cpDNA region such as ndhF. It was difficult to align outgroup taxa with ingroups using trnL-F and ITS, both of which are composed largely of noncoding sequences, and there were also some early branching nodes in the single and combined trees with low bootstrap support. This suggests that more slowly evolving coding regions will be useful for examining relationships among major clades within *Croton* and its outgroups. They have begun to sequence the chloroplast ndhF region, and using a subset of five divergent species from the combined data set, we found 14% of nucleotide positions to be parsimony-informative, with the same relationships as those obtained using trnL-F data.

Many studies of genetic variation in family Euphorbiaceae were done by molecular analysis. Frank R. Blattner *et al* (2001) studied the molecular analysis of phylogenetic relationships among Myrmecophytic *Macaranga* Species (Euphorbiaceae) by RAPD and microsatellite primed PCR (MP-PCR) techniques. The result is best resolutions for individual clades phenetic analysis were obtained by RAPD and MP-PCR. Kenneth M. Olsen and Barbara A. Schaal (2000) studied genetic variation at five microsatellite loci was used to investigate the evolutionary and geographical origins of cassava (*Manihot esculenta* subsp. *esculenta*) and the population structure of cassava's wild relatives. The resulte is two hundred and twelve individuals were sampled, representing 20 crop accessions, 27 populations of cassava's closest wild relative (*M. esculenta* subsp. *flabellifolia*), and six populations of a potentially hybridizing species (*M. pruinosa*). Neeraj Jain (2001) studied molecular diversity in *Phyllanthuys amarus* assessed through PCR-RFLP analysis. The resulte is *Phyllanthuys amarus* was made from various part of India to determine the extent of genetic variability using analysis at DNA level.

CHAPTER III

EXPERIMENTS

3.1 Instruments and equipments

3.1.1 Instruments

1. Autoclave Model HV-50: Hirayama Manufacturing Co., Japan
2. Automatic micropipette P20 and P200:Gilson Medical Electronics S.A., France
3. Electronic balance Model Alsep EY22A: A&D Co. Ltd., Japan Electrophoresis apparatus: Horizontal gel electrophoresis apparatus (9×12 cm)
4. -20°C Freezer: National Co., Thailand
5. -70°C Ultra low temperature freezer Model MDF 790AT: Sanyo Electric Co. Ltd., Japan
6. High speed micro refrigerated centrifuge Model MTX-150: Tomy-Seiko, Japan
7. Incubator Model BM-600: Memmert GambH, Germany
8. Microwave oven Model NE 7670: National Co. Ltd., Japan
9. Power supply: Amersham Bioscience, U.K.
10. Thermal cycle: Applied Biosystems Model 2700, U.S.A.
11. UV transiluminator Model 2011 Macrovue: San Gabriel California, U.S.A

3.1.2 Inventory supplies

1. Microcentrifuge tubes, 0.5 and 1.5 ml: Axygen Hayward, USA.
2. Pipette tips, 20 and 200 µl: Axygen Hayward, USA.
3. Thin-wall microcentrifuge tubes (domed cap), 0.2 ml: Axygen Hayward, USA.

3.2 Chemicals

1. Absolute ethanol: Merck, Germany
2. Agarose: Sigma Chemical Co., USA.
3. 100 base pair DNA ladder: Promega Co., USA.
4. Boric acid: Merck, Germany
5. Bromophenol blue: Merck, Germany
6. Cetyltrimethylammonium bromide: CTAB: Merck, Germany
7. Chloroform: Merck, Germany
8. Ethidium bromide: Sigma Chemical Co., USA.
9. Ethylene diamine tetraacetic acid, disodium salt dihydrate: Fluka, Switzerland
10. 10 mM each dATP, dCTP, dGTP and dTTP: Promega Co., USA.
11. Isoamyl alcohol: Merck, Germany
12. 2-mercapto ethanol: Fluka, Switzerland
13. Polyvinyl-pyrrolidone: PVP-40: Sigma Chemical Co., USA.
14. Sodium chloride: Merck, Germany
15. Tris-base: Sigma Chemical Co., USA.

3.3 Enzymes

1. RNase A: Sigma Chemical Co., USA.
2. *Taq* DNA polymerase: Promega Co., USA.

3.4 Sample collection

Leaves from thirty-seven samples of *Croton sublyratus* Kurz. were collected from the same plot at Chulalongkorn University planting area. All samples were stored at -70°C until used for DNA extraction. The details of sample origin are shown in Table 3.1.

Table 3.1 Locality of 37 samples of *Croton sublyratus* Kurz. used in this study.

Locality	Abbreviation of *C. sublyratus*
Amphoe ThaUtane, Nakhonphanom Province	TA-1
Amphoe ThaUtane, Nakhonphanome Province	TA-2
Amphoe ThaUtane, Nakhonphanome Province	TA-3
Amphoe ThaUtane, Nakhonphanome Province	TA-4
Amphoe ThaUtane, Nakhonphanome Province	TA-6
Amphoe ThaUtane, Nakonphanome Province	TA-9
Amphoe ThaUtane, Nakhonphanome Province	TA-10
Amphoe Muang, Prachuapkhirikhan Province	IBGE-0
Prachinburi Province	IBGE-1
Prachinburi Province	IBGE-2
Amphoe Muang, Prachuapkhirikhan Province	IBGE-4
Amphoe Muang, Prachuapkhirikhan Province	IBGE-5
Amphoe Muang, Prachuapkhirikhan Province	IBGE-6
Amphoe Muang, Prachuapkhirikhan Province	IBGE-7
Amphoe Muang, Prachuapkhirikhan Province	IBGE-8
Amphoe Muang, Prachuapkhirikhan Province	IBGE-9
Amphoe Muang, Prachuapkhirikhan Province	IBGE-10
Amphoe Muang, Prachuapkhirikhan Province	IBGE-13
Amphoe Muang, Prachuapkhirikhan Province	IBGE-14
Amphoe Muang, Prachuapkhirikhan Province	IBGE-16
Amphoe Muang, Prachuapkhirikhan Province	IBGE-17
Amphoe Muang, Prachuapkhirikhan Province	IBGE-18
Amphoe Muang, Prachuapkhirikhan Province	IBGE-19
Amphoe Muang, Prachuapkhirikhan Province	IBGE-20
Amphoe Muang, Prachuapkhirikhan Province	IBGE-21
Amphoe Muang, Prachuapkhirikhan Province	IBGE-22
Amphoe Muang, Prachuapkhirikhan Province	IBGE-23
Amphoe Muang, Prachuapkhirikhan Province	IBGE-2

Tabel 3.1 (continue)

Locality	Abbreviation of *C. sublyratus*
Amphoe Muang, Prachuapkhirikhan Province	IBGE-25
Hard Nawakorn National Park, Prachuapkhirikhan Province	NP-6
Hard Nawakorn National Park, Prachuapkhirikhan Province	NP-7
Hard Nawakorn National Park, Prachuapkhirikhan Province	NP-8
Hard Nawakorn National Park, Prachuapkhirikhan Province	NP-14
Hard Nawakorn National Park, Prachuapkhirikhan Province	NP-20
Hard Nawakorn National Park, Prachuapkhirikhan Province	NP-23
Amphoe Muang, Prachuapkhirikhan Province	Tiwa
Amphoe Muang, Prachuapkhirikhan Province	Tone

3.5 DNA extraction

Genomic DNA was extracted from 1 g of leaf tissue of each *Croton sublyratus* Kurz. following the method of Murray and Thompson (1980).

One gram of leaf tissue was ground to powder in the presence of liquid nitrogen. Immediately after that, the powder was transferred into a 2 ml-tube containing CTAB buffer (2% CTAB, 2% polyvinylpyrrolidone (PVP-40), 1.4 M NaCl, 2% 2-mercaptoethanol, 0.2 M EDTA, pH 8.0 and 0.1 M Tris-HCl, pH 8.0) and the solution was then incubated at 60°C for 1 hour. Next, 2 ml of chloroform/isoamyl alcohol (24/1) was added to the solution mixture. The solution mixture was mixed gently and centrifuged at 13,000 rpm for 10 minutes. The upper aqueous phase was transferred to new tube and chloroform/isoamyl alcohol (24/1) extraction was repeated again. Next, one volume of chloroform were added to the solution, mixed gently and centrifuged at 13,000 rpm for 10 minutes. The upper aqueous phase was transferred to new tube and added 1/10 volume of 3M sodium acetate, pH5.2, mixed gently. Next, 0.6 volume of isopropanol were added to the solution, mixed gently and incubated in -20°C for 1 hour or overnight. The solution was centrifuged at 13,000 rpm for 10 minutes and the aqueous solution was discarded and the pellet was washed with 75% ethanol and dried at room temperature. Then the pallet was dissolved

in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and followed by adding 2 µl 5mg/ml RNase A. The solution was incubated at 37°C for 30 minutes. After incubation, the solution was transferred to a new microcentrifuge tube. Next, 2 ml of chloroform/isoamyl alcohol (24/1) was added to the solution mixture. The solution was mixed gently and centrifuged at 10,000 rpm for 15 minutes. The upper aqueous phase was removed into a new microcentrifuge tube and chloroform/isoamyl alcohol (24/1) extraction was repeated again. Next, 1/10 volume of 3 M sodium acetate, pH5.2, was added and DNA was precipitated by the addition of 2 volume of cold absolute ethanol. The solution was then incubated at -20°C for1 hour or overnight. The precipitated DNA was recovered by centrifugation at 10,000 rpm for 15 minutes. The DNA pallet was washed with 75 % ethanol and air-dried. Finally, the pallet was completely redissolved by 200 µl of TE buffer and incubated at room temperature for an hour. The solution was kept at 4°C until further used.

3.6 Measurement of DNA concentrations

3.6.1 Spectrophotometry

The amount of DNA was estimated by determination of the optical density at 260 nm. The OD value at 260 allowes calculation of total nucleic acids whereas the value reading at 280 nm determine the amount of protein in the sample.

An OD at 260 nm corresponds to approximately 50 µg/ml for double stranded DNA (Maniatis *et al*., 1982). The ratio between OD 260/280 provides a roughly estimate for the purity of extracted DNA. A pure preparation of DNA has a 260/280 ratio of 1.8 – 2.0 (Kirby, 1992). To determine DNA concentration, 10 µl of extracted DNA was transferred to Eppendorf tube contaning 990 µl of TE buffer. The tube was shaken vigorously. The diluted DNA solution was transferred to a UV cuvette containing 1 ml of TE to be served as the reagent blank. DNA concentration is estimated in µg/ml using the following equation;

$$[DNA] = OD_{260} \times \text{Dilution factor} \times 50$$

3.7 DNA analysis

3.7.1 Amplification condition

Twenty-two arbitrary primer and 8 anchored microsatellite primer (Table 3.2)

were synthesized by Bio Basic Inc., Canada. The RAPD reaction was performed in 10 μl volume of reaction mixture containing 0.1 unit of *Taq* DNA polymerase, 25 ng of genomic DNA, 0.1 mM of each dATP, dTTP, dCTP and dGTP, 20 μM of a primer and 3.3 mM $MgCl_2$. RAPD reaction was performed in the DNA Thermal Cycle (Applied Biosystem Model 2400) programmed for 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 52°C for 1 minutes and extension at 70°C for 2 minutes. The final extension was carried out at 70°C for 8 minutes. ISSR reaction were amplified with touch-down protocol with the following program for 95°C for 3 minutes, 10 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 57°C (minus 0.5°C/cycle) for 1 minutes and extension at 70°C for 2 minutes, and 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 52°C for 1 minutes and extension at 70°C for 2 minutes. The final extension was carried out at 70°C for 8 minutes.

Table 3.2 Sequence of the 10 RAPD primer and 8 anchored microsatellite primer.

Primer name	Primer sequence
E 05	TCAGGGAGGT
E 20	AACGGTGACC
F 01	ACGGATCCTG
F 08	GGATATCGGC
G 02	GGCACTGAGG
G 08	TCACGTCCAC
G 13	CTCTCCGCCA
G 17	ACGACCGACG
OPC 02	GATGACCGCC
OPD 05	TGAGCGGACA

Table 3.2 (continue)

Primer name	Primer sequence
M 1	$(CA)_8G$
M 2	$(TC)_8C$
M 3	$AC(CA)_6CYG$
M 4	$A(GA)_7GT$
M 5	$(GT)_8C$
M 6	$BSC(GA)_8$
M 7	$CRN_2(CTT)_5$
M 8	$GR(TC)_5$

3.8 Agarose gel electrophoresis

Amplified products were analyzed by electrophoresis through 1.5 % agarose gels in 0.5x TBE buffer. The solution was boiled in a microwave oven to complete solubilization, and cooled down to 60°C before being poured into the chamber set with an inserted comb. When the gel solidified, the comb was carefully removed. The agarose gel was submerged in a chamber containing ample amount of 0.5x TBE buffer.

Ten microliters of each PCR product was mixed with 2 μl of loading dye (0.25% bromophenol blue and 40% sucrose) and loaded into the well. The 100 bp DNA ladders was used as standard DNA markers. Electrophoresis was operated at 60 volts for 3.30 hour. The gel was stained with a solution containing 10 ng/μl ethidium bromide for 10 minutes and destained in distilled water for 15 minutes to remove unbound ethidium bromide from agrose gels. The DNA fragments were visualized as fluorescent band under an UV transilluminator and photographed with the gel document system.

3.9 Genetic analysis

The amplified patterns are photographed and evaluated. Each amplified fragment was treated as an independent character. Accordingly, the presence (1) and absence (0) of an amplified fragment was treated in a dominant fasion.

Nei's distance is formulated for an infinite isoalleles model of mutation, in which there is a rate of neutral mutation and each mutant is to a completely new alleles. It is assumed that all loci have the same rate of neutral mutation, and that the genetic variability initially in the population is at equilibrium between mutation and genetic drift, with the effective population size of each population remaining constant.

Nei's distance is

$$D = -\ln\left(\frac{\sum_m \sum_i p_{1mi}\, p_{2mi}}{\sqrt{\left[\sum_m \sum_i p_{1mi}^2\right]\left[\sum_m \sum_i p_{2mi}^2\right]}}\right)$$

where m is summed over loci, i over alleles at the m-th locus, and where p1mi is the frequency of the i-th allele at the m-th locus in population 1. Subject to the above assumptions, Nei's genetic distance is expected, for a sample of sufficiently many equivalent loci, to rise linearly with time.

A dendrogram based on thedistance approach was constructed using the unweight-pair group method with arithmetric average (UPGMA) implemented in Phylip Version 3.57c (Felsenstein, 1996).

3.10 Extraction of plaunotol from *C. sublyratus* leaves.

Extraction of plaunotol from *C. sublyratus* leaves (Nilubol *et.al*., 1994). Sample of *C. sublyratus* leaves were dried overnight in a hot air oven at 50°C. The dried leaves were ground to fine powder in the grinder. Three grams of the ground leaves were extracted with 50 ml 95% ethanol at 70°C for 90 min. Leaf extract was filtrated by whatman No. 1 and added with 95% ethanol to final volumn of 50 ml.

3.11 Sample preparation for determination of plaunotol.

An aliquot of ethanolic extract (2 ml) was evaporated to dryness using rotary evaporator and dried in a hot air oven at 50°C for 90 min. The residue was added with 2 ml 50% ethanol and 0.4 ml of 10% sodium hydroxide and incubated at 70°C in a water bath for 30 min. After cooled, it was extracted with 3 ml of n-hexane three times. The hexane layers were pooled together and evaporated to dryness. The residue was dissolved in 250 μl of 2000 μg/ml of n-octacosane in chloroform before injected (1.0 μl) into the Gas chromatography (GC) sytem.

3.12 Determination of plaunotol.

Gas chromatography used for determination of plaunotol (Nilubol *et.al*., 1994) was performed by using glass column packed with 2% silicone OV-17 on uniport 60/80 mesh carrier gas N_2 and detector FID injection temperature is 230°C column temperature is 210°C and detector temperature is 230°C. This condition retention time of standard plaunotol for 7 min and retention times of internal standard for 11.4 min. The determination of plaunotol in *C. sublyratus* leaves with the calibration curve of standard plaunotol shown in Figure 6, which was obtained by plotting the peak areas against plaunotol concentrations is shown in Figure 7.

3.13 Leaf morphology measurements

Thirty-six of *C. sublyratus* characters are used to describe the size and shape of the leaves. Each character has to be measured on each plant in the sample. Leaf shape is assessed as leaf apex and leaf margin. Leves five were analyzed from the middle third of each plant. The lengths and widths of each leaf are measured and recorded and the variety is described by the mean values over the ten leaves. Leaf length was measured from base of petiole to leaf tip, while leaf width was recorded as maximum width of the blade. Petiole length taken from the point of attachment to the base of the first leaflet insertion. Leaf base angles were measured on base of each leaf. The number of lateral vein on each side of midrib was counted. The remaining characters are assessed by visual comparison with a key for each character the sample is assessed as a whole and any marked variability across the sample is recorded in a supplementary note.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 DNA extraction

Genomic DNA was extracted from leaf tissue of each *C. sublyratus* by CTAB DNA extraction method. The quality of extracted genomic DNA was electrophoretically determined in 1.2% agarose (w/v) gel while the concentration of the DNA was estimated spectrophotometrically. DNA concentrations were determined by measuring the optical density at 260 nm (1 OD_{260} unit was equivalent to 50 μg DNA/ml). The ratio of OD_{260}/OD_{280} was 1.8-2.0 indicating a possible contamination of RNA in the DNA samples Nevertheless, this contaminant did not interfere subsequent PCR amplification. Approximately 25 μg DNA were usually obtained from each specimen.

4.2 Primer screening

DNA from leaves of 37 *C. sublyratus* were tested for the amplification against 10 decanucleotide primers using and 8 anchored microsatellite primers. After screening, 3 anchored microsatellite primers of 18 primers (M-1, M-6 and M-7; Table 2.2) which grave reproducible ISSR patterns were included for further analysis of genetic diversity of 37 *Croton sublyratus* Kurz. Examples of the amplified patterns were those shown in Figures 4.1 to 4.3 showed the ISSR patterns of those selected primers.

Figure 4.1-4.3 ISSR patterns obtained from 37 *C. sublyratus* with primer M-1, M-6 and M-7

Lane M = 100 bp DNA ladder

Lane 1-11 = *C. sublyratus* TA-1, TA-2, TA-3, TA-4, TA-6, TA-9, TA-10, IBGE-0, IBGE-1, IBGE-2 and IBGE-4

Lane 12-22 = *C. sublyratus* IBGE-5, IBGE-6, IBGE-7, IBGE-8, IBGE-9, IBGE-10, IBGE-13, IBGE14, IBGE-16, IBGE-17 and IBGE-18

Lane 23-33 = *C. sublyratus* IBGE-19, IBGE-20, IBGE-21, IBGE-22, IBGE-23, IBGE-24, IBGE-25, NP-6, NP-7, NP-8 and NP-14

Lane 34-37 = *C. sublyratus* NP-20, NP-23, Tiwa and Tone

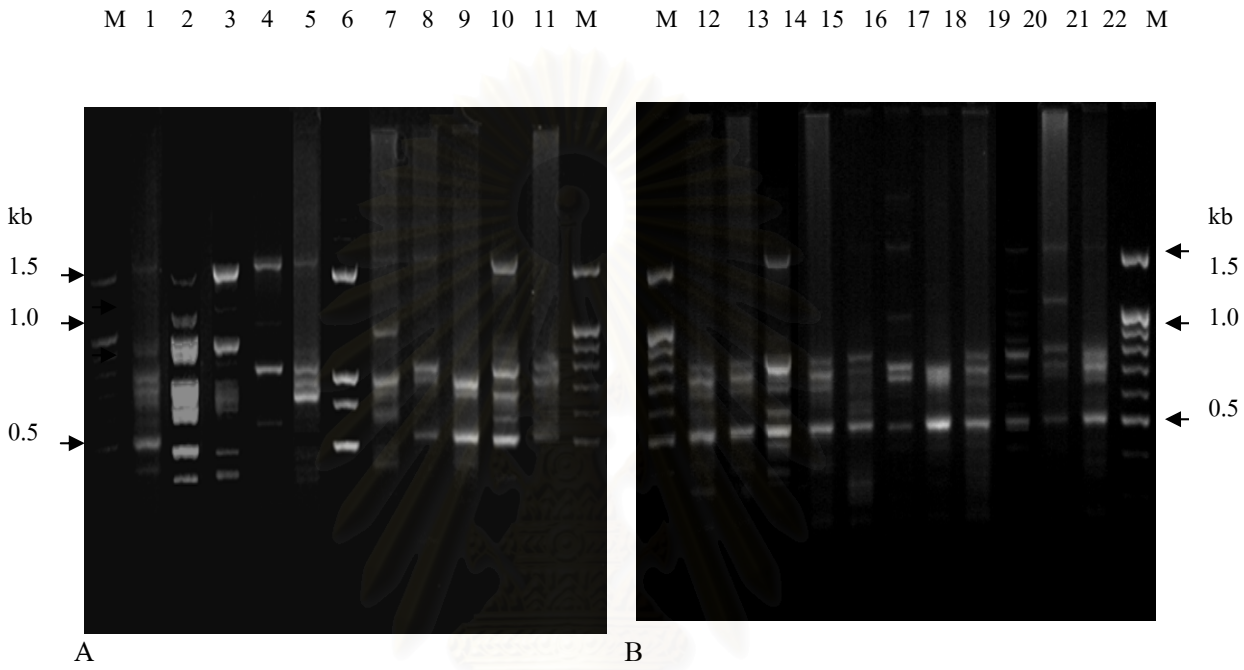


Figure 4.1.1 ISSR patterns of 22 *C. sublyratus* with primer M-1 Lane M: DNA ladder

(A) Lane 1-11 = *C. sublyratus* TA-1, TA-2, TA-3, TA-4, TA-6, TA-9, TA-10, IBGE-0, IBGE-1, IBGE-2 and IBGE-4

(B) Lane 12-22 = *C. sublyratus* IBGE-5, IBGE-6, IBGE-7, IBGE-8, IBGE-9, IBGE-10, IBGE-13, IBGE14, IBGE-16, IBGE-17 and IBGE-18

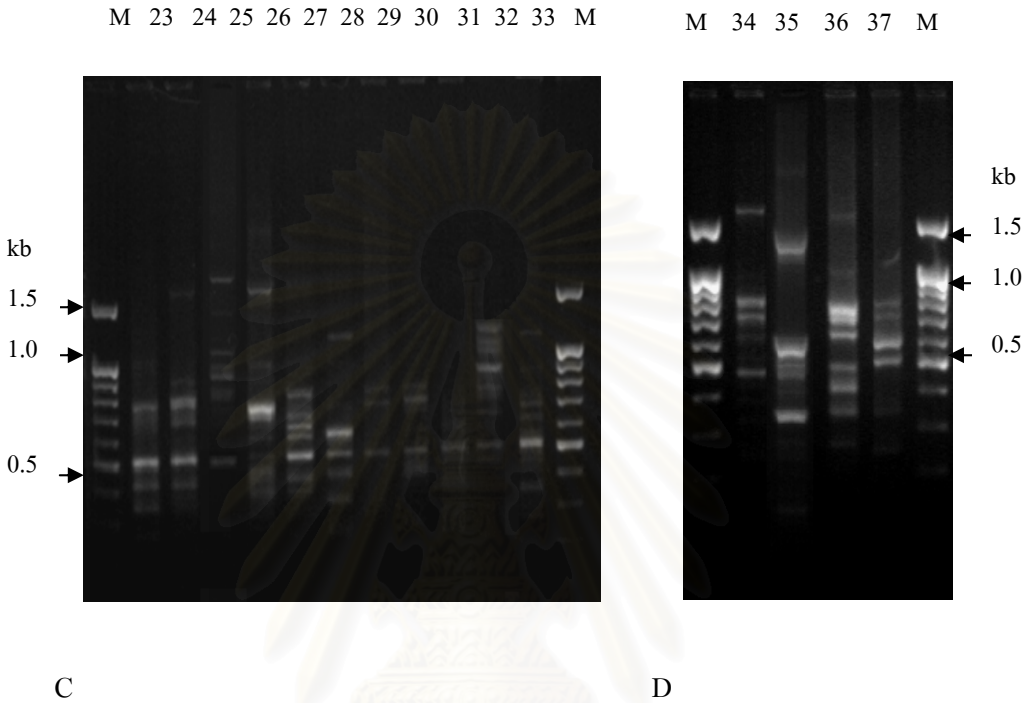


Figure 4.1.2 ISSR patterns of 15 *C. sublyratus* with primer M-1 Lane M: DNA ladder

(C) Lane 1-11 = *C. sublyratus* IBGE-19, IBGE-20, IBGE-21, IBGE-22, IBGE-23, IBGE-24, IBGE-25, NP-6, NP-7, NP-8 and NP-14

(D) Lane 12-22 = *C. sublyratus* NP-20, NP-23, Tiwa and Tone

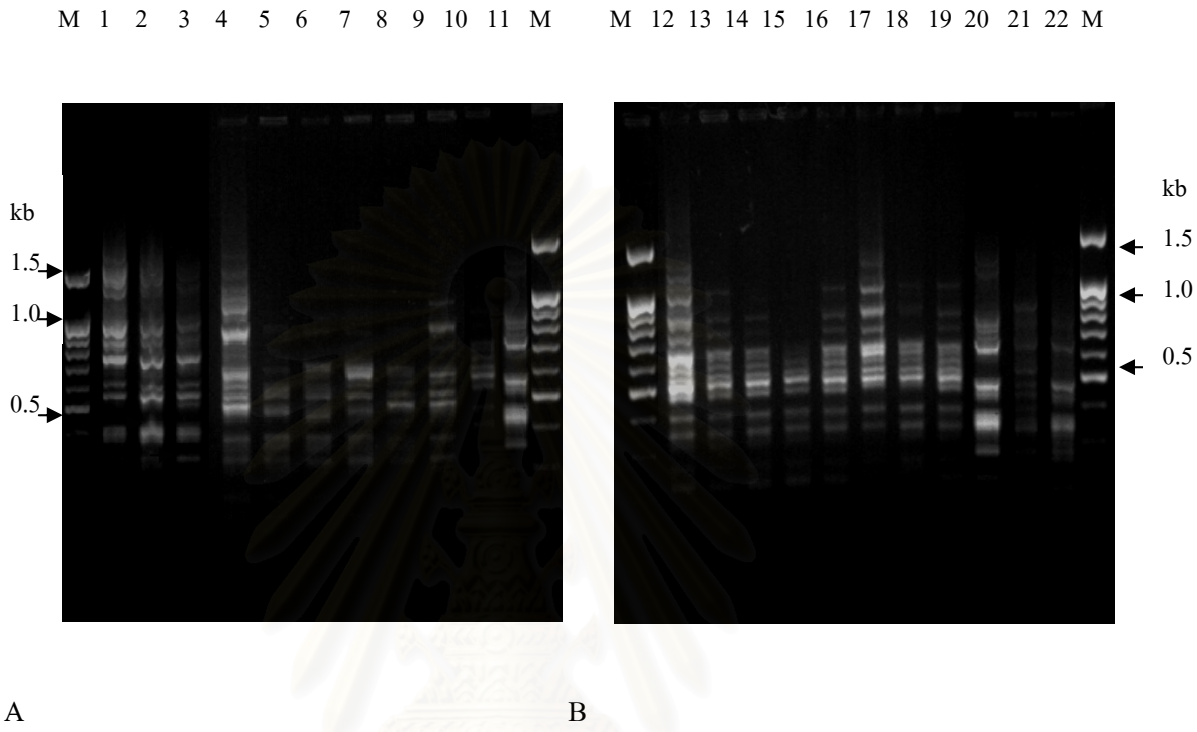


Figure 4.2.1 ISSR patterns of 22 *C. sublyratus* with primer M-6 Lane M: DNA ladder

(A) Lane 1-11 = *C. sublyratus* TA-1, TA-2, TA-3, TA-4, TA-6, TA-9, TA-10, IBGE-0, IBGE-1, IBGE-2 and IBGE-4

(B) Lane 12-22 = *C. sublyratus* IBGE-5, IBGE-6, IBGE-7, IBGE-8, IBGE-9, IBGE-10, IBGE-13, IBGE14, IBGE-16, IBGE-17 and IBGE-18

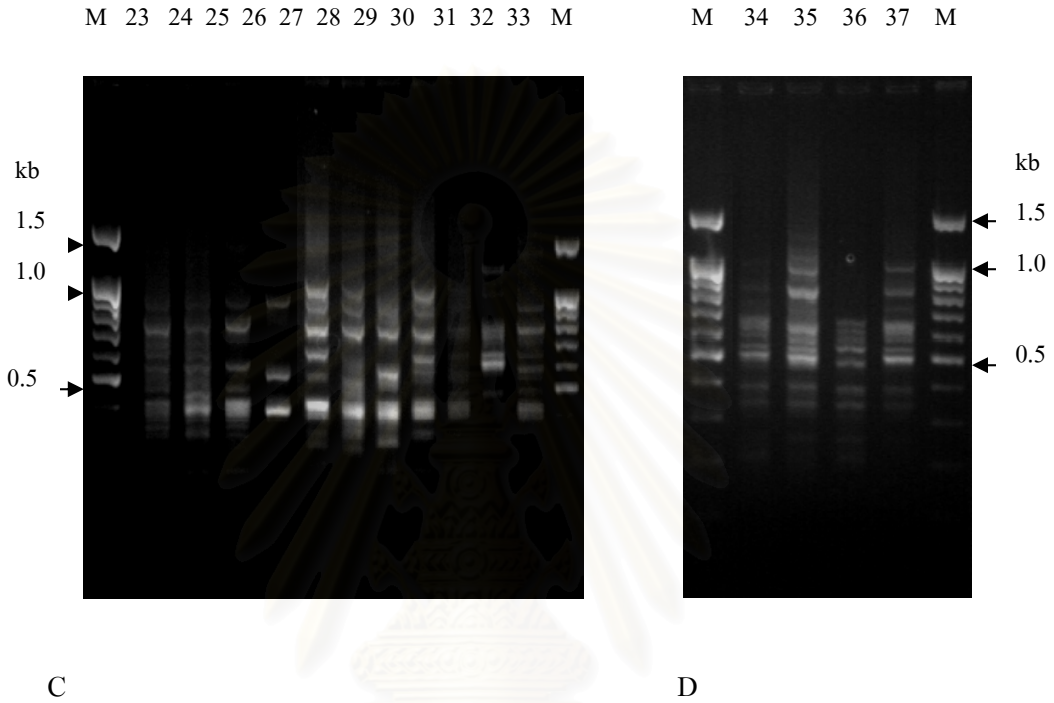


Figure 4.2.2 ISSR patterns of 15 *C. sublyratus* with primer M-6 Lane M: DNA ladder
(C) Lane 1-11 = *C. sublyratus* IBGE-19, IBGE-20, IBGE-21, IBGE-22, IBGE-23, IBGE-24, IBGE-25, NP-6, NP-7, NP-8 and NP-14
(D) Lane 12-22 = *C. sublyratus* NP-20, NP-23, Tiwa and Tone

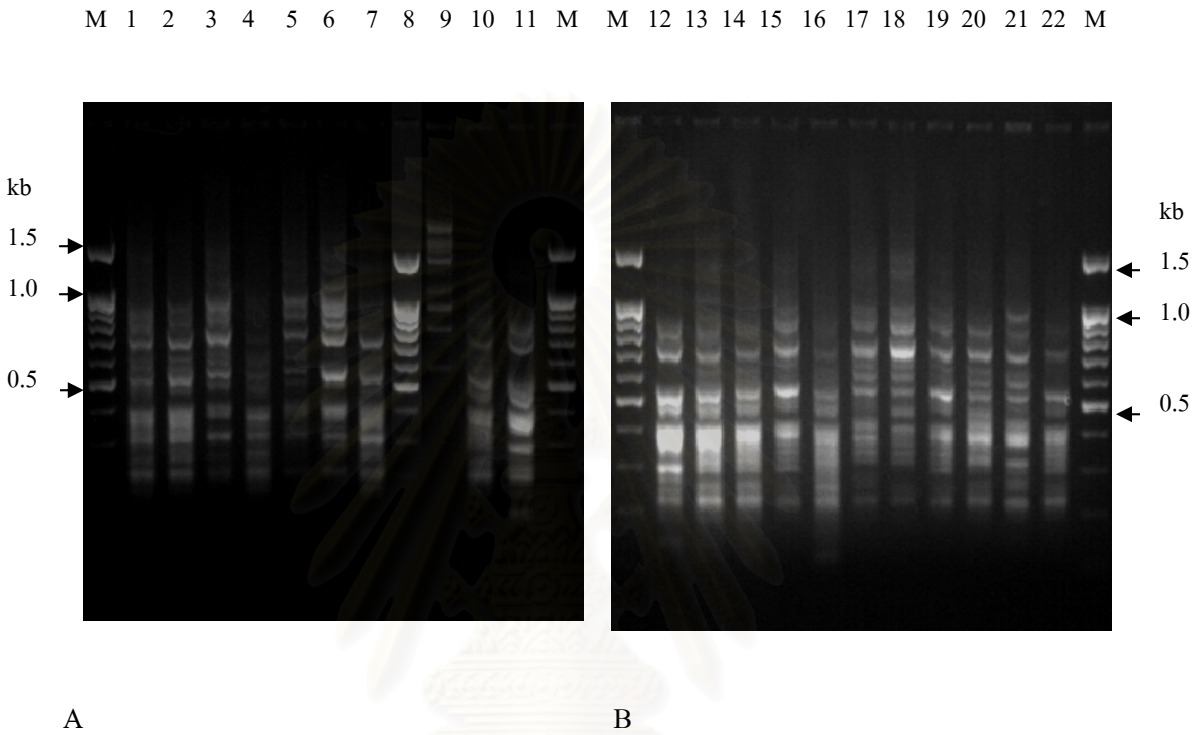


Figure 4.3.1 ISSR patterns of 22 *C. sublyratus* with primer M-7 Lane M: DNA ladder
(A) Lane 1-11 = *C. sublyratus* TA-1, TA-2, TA-3, TA-4, TA-6, TA-9, TA-10, IBGE-0, IBGE-1, IBGE-2 and IBGE-4
(B) Lane 12-22 = *C. sublyratus* IBGE-5, IBGE-6, IBGE-7, IBGE-8, IBGE-9, IBGE-10, IBGE-13, IBGE14, IBGE-16, IBGE-17 and IBGE-18

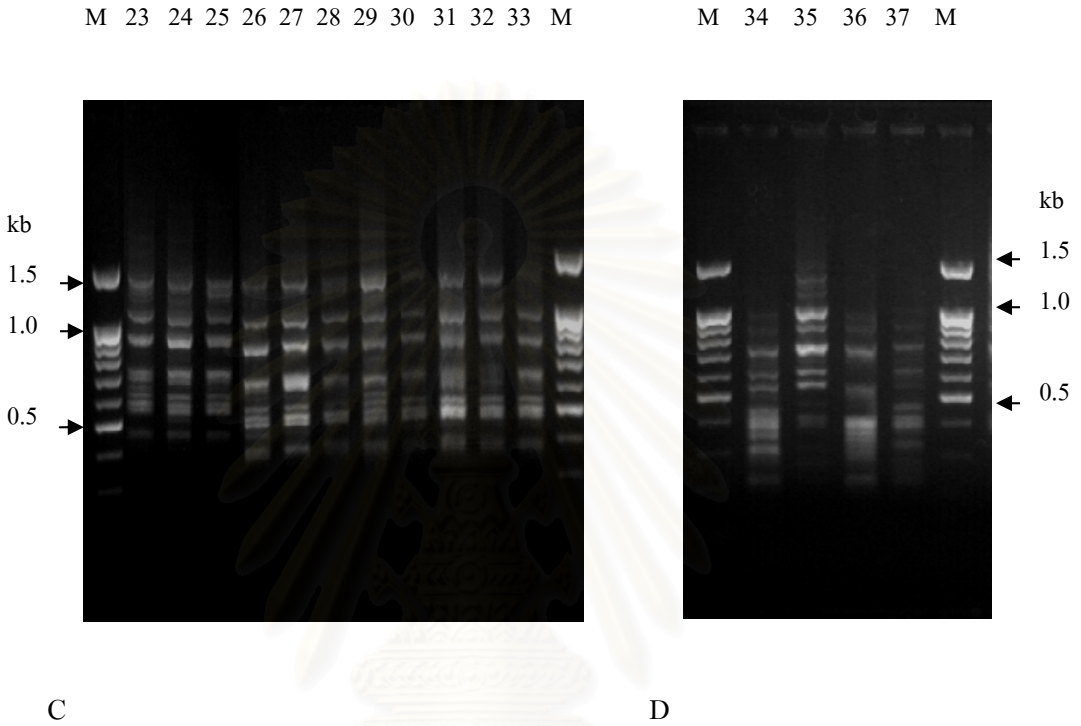


Figure 4.3.2 ISSR patterns of 15 *C. sublyratus* with primer M-7 Lane M: DNA ladder

(C) Lane 1-11 = *C. sublyratus* IBGE-19, IBGE-20, IBGE-21, IBGE-22, IBGE-23, IBGE-24, IBGE-25, NP-6, NP-7, NP-8 and NP-14

(D) Lane 12-22 = *C. sublyratus* NP-20, NP-23, Tiwa and Tone

4.3 Determination of genetic diversity of *Croton sublyratus* Kurz using ISSR analysis

Only reproducible bands were scored for presence (1) or absence (0) in each individual. A total of 146 ISSR fragment from the three primers (M-1, M-6 and M-7) were consistently generated. Primer M-1 gave ISSR bands ranging from 1.5 kb to 251 bp. It gave total 40 consistent and reproducible ISSR bands compose of 15 monomorphic and 25 polymorphic bands. Primer M-6 gave ISSR bands ranging from 1.46 kb to 220 bp. It gave total 53 consistent and reproducible ISSR bands compose of 17 monomorphic and 36 polymorphic bands. Primer M-7 gave ISSR bands ranging from 1.5 kb to 237 bp. It gave total 53 consistent and reproducible ISSR bands compose of 18 monomorphic and 35 polymorphic bands. The numbers of consistent and reproducible bands are summarized in Table 4.1

Table 4.1 Total numbers of bands, monomorphic and polymorphic band within 37 samples of *C. sublyratus* by ISSR analysis using primer M-1, M-6 and M-7

Primer name	No. of total bands	No. of monomorphic bands	No. of polymorphic bands
M-1	40	15 (37%)	25 (63%)
M-6	53	17 (32.0%)	36 (68.0%)
M-7	53	18 (33%)	35 (67%)
Total	146	50 (34%)	96 (66%)

4.4 Data analysis on the genetic relationships of similarities and distances in *C. sublyratus*

All investigated taxa were divided into 37 operational taxonomic units (OTUs). The matches between samples (M) of the three primers were calculated as the average of all possible comparisons of individuals between pairs of samples. Genetics distance (D) were converted from the index of similarity between samples (D = 1-M).

The amplified fragments were scored for the presence and absence of share fragment in the ISSR profiles. The data of shared fragment lengths for *C. sublyratus* for M-1, M-6 and M-7 primers were combined. The genetic distance value were put in to PHYLIP computer program (Feisentein, 1993). The best tree constracted using the method of UPGMA is show in Figure 4.4 The genetic distances were made to calculate similarity indices using the method of Nei and Li. The average of similarity matrics was used to generate a tree by UPGMA. Dendrogram, which was constructed from average genetic distance within *C. sublyratus* indicated that the seperated between the investigated *C. sublyratus* was clearly separate. At the intraspecific level, dendrogram showed that *C. sublyratus* can be divided into two groups based on ISSR data. Group A (30 *C. sublyratus*), while the other 7 *C. sublyratus* were another distinct group, defind as group B.

Figure 4.4 Dendrogram of 37 *C. sublyratus* based on ISSR data using three primers (M-1, M-6 and M-7) with UPGMA cluster analysis.

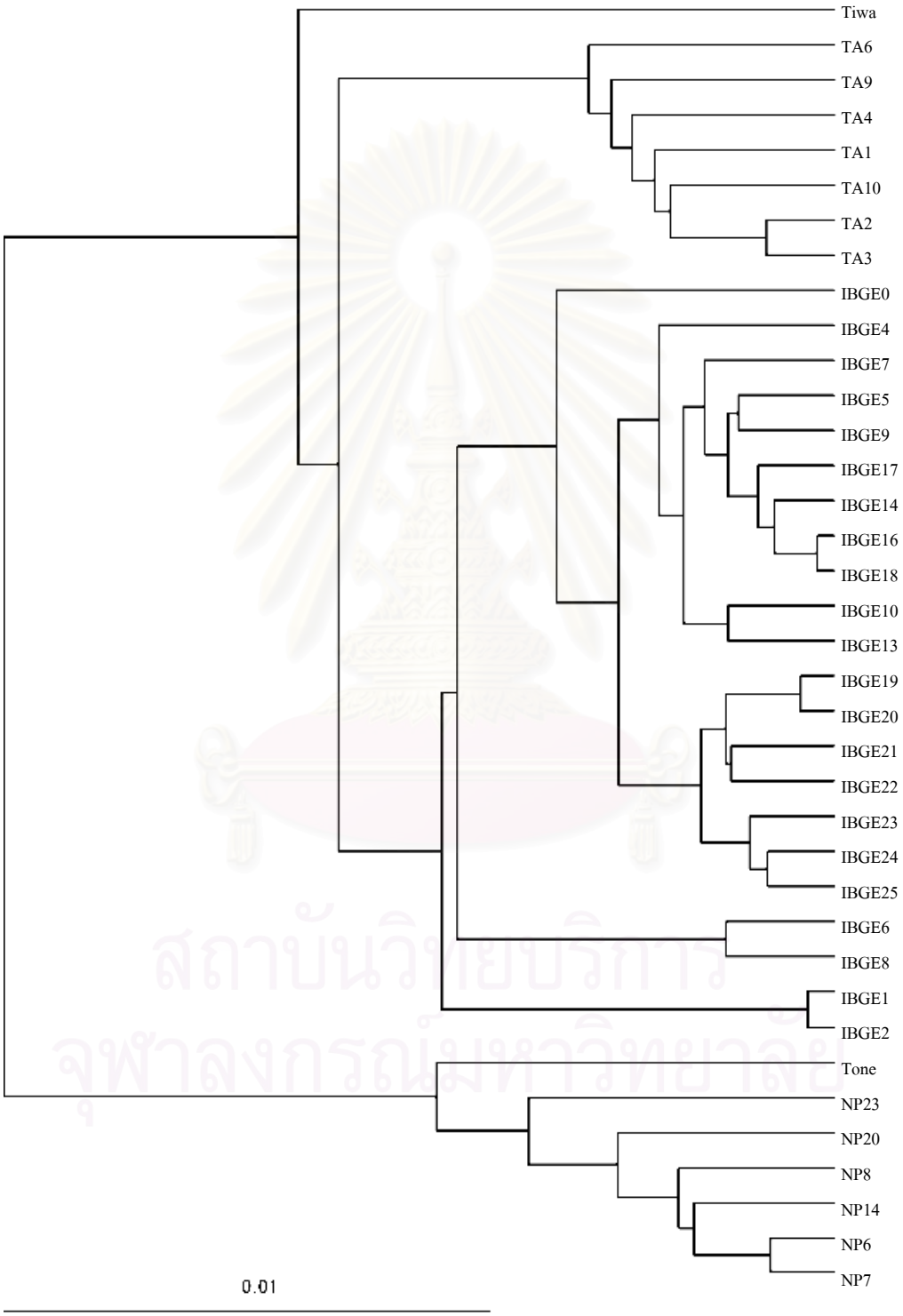


Table 4.2 Distance matrix values based on ISSR data between 37 samples of *C.sublyratus*.

	TA-1	TA-2	TA-3	TA-4	TA-6	TA-9	TA-10	IBGE-0	IBGE-1	IBGE-2	IBGE-4	IBGE-5	IBGE-6
TA-1	0.000000	0.006771	0.006757	0.008680	0.008680	0.008990	0.007062	0.013096	0.019695	0.017065	0.021204	0.021039	0.019227
TA-2	0.006771	0.000000	0.002805	0.008680	0.010873	0.008990	0.008104	0.014517	0.019695	0.017065	0.018105	0.021039	0.020984
TA-3	0.006757	0.002805	0.000000	0.006740	0.007722	0.008047	0.007062	0.016743	0.025920	0.019695	0.020391	0.022170	0.022017
TA-4	0.008680	0.008680	0.006740	0.000000	0.009899	0.010228	0.009353	0.016743	0.029180	0.024258	0.020391	0.018135	0.018322
TA-6	0.008680	0.010873	0.007722	0.009899	0.000000	0.011440	0.012045	0.016743	0.020748	0.024258	0.024104	0.030661	0.024170
TA-9	0.008990	0.008990	0.008047	0.010228	0.011440	0.000000	0.009716	0.018989	0.021116	0.020032	0.022427	0.024954	0.022324
TA-10	0.007062	0.008104	0.007062	0.009353	0.012045	0.009716	0.000000	0.014868	0.023943	0.022390	0.020680	0.020391	0.022522
IBGE-0	0.013096	0.014517	0.016743	0.016743	0.016743	0.018989	0.014868	0.000000	0.018259	0.014172	0.017016	0.013096	0.026296
IBGE-1	0.019695	0.019695	0.025920	0.029180	0.020748	0.021116	0.023943	0.018259	0.000000	0.004442	0.017016	0.011770	0.023840
IBGE-2	0.017065	0.017065	0.019695	0.024258	0.024258	0.020032	0.022390	0.014172	0.004442	0.000000	0.014048	0.009302	0.018931
IBGE-4	0.021204	0.018105	0.020391	0.020391	0.024104	0.022427	0.020680	0.017016	0.017016	0.014048	0.000000	0.005028	0.012656
IBGE-5	0.021039	0.021039	0.022170	0.018135	0.030661	0.024954	0.020391	0.013096	0.011770	0.009302	0.005028	0.000000	0.012415
IIBGE-6	0.019227	0.020984	0.022017	0.018322	0.024170	0.022324	0.022522	0.026296	0.023840	0.018931	0.012656	0.012415	0.000000
IBGE-7	0.022324	0.018633	0.019446	0.017706	0.021372	0.015000	0.019695	0.011808	0.021039	0.013949	0.007295	0.007906	0.015930
IBGE-8	0.019227	0.017637	0.018322	0.018322	0.022017	0.022324	0.018461	0.023840	0.023840	0.017336	0.014670	0.014824	0.001932
IBGE-9	0.021116	0.021116	0.022390	0.017893	0.025151	0.025547	0.020391	0.013949	0.012415	0.009560	0.006299	0.004131	0.011808
IBGE-10	0.026296	0.021687	0.020709	0.022861	0.028081	0.021039	0.023522	0.012415	0.016743	0.013352	0.010759	0.008238	0.018208
IBGE-13	0.024954	0.022522	0.023884	0.021467	0.023884	0.024258	0.024756	0.011111	0.018989	0.014854	0.009767	0.010052	0.020391
IBGE-14	0.019755	0.019755	0.020709	0.017065	0.025298	0.025627	0.021116	0.010052	0.015170	0.010972	0.007539	0.004229	0.011876
IBGE-16	0.018633	0.014242	0.014670	0.014670	0.019446	0.016436	0.017791	0.010666	0.019118	0.015284	0.007295	0.007906	0.015930

Table 4.2 (continue)

	TA-1	TA-2	TA-3	TA-4	TA-6	TA-9	TA-10	IBGE-0	IBGE-1	IBGE-2	IBGE-4	IBGE-5	IBGE-6
IBGE-17	0.022625	0.020680	0.021687	0.018018	0.023840	0.020072	0.022170	0.013352	0.016122	0.013008	0.006837	0.005766	0.016207
IBGE-18	0.018792	0.018792	0.019695	0.016073	0.024258	0.020032	0.017893	0.012748	0.014172	0.010052	0.006112	0.003387	0.012133
IBGE-19	0.023196	0.019118	0.020032	0.020032	0.022170	0.022522	0.022778	0.009420	0.019695	0.012726	0.009406	0.008599	0.019227
IBGE-20	0.023522	0.021372	0.022522	0.020391	0.022522	0.018792	0.023156	0.009741	0.020032	0.010666	0.010521	0.008899	0.019523
IBGE-21	0.020072	0.016743	0.017388	0.014370	0.019118	0.017706	0.021467	0.007361	0.025298	0.016436	0.011016	0.010477	0.020090
IBGE-22	0.018792	0.015492	0.014517	0.016073	0.019695	0.016407	0.022390	0.010228	0.021467	0.013740	0.010021	0.010364	0.022625
IBGE-23	0.020032	0.014854	0.015386	0.015386	0.018989	0.019345	0.024360	0.012063	0.023156	0.014517	0.011387	0.010873	0.024170
IBGE-24	0.020748	0.018633	0.019622	0.019622	0.019622	0.022390	0.029692	0.010649	0.019227	0.014670	0.012415	0.009560	0.020709
IBGE-25	0.020391	0.018259	0.019227	0.019227	0.019227	0.024756	0.025404	0.010276	0.016743	0.012775	0.013290	0.009218	0.022522
NP-6	0.051475	0.051475	0.050043	0.040854	0.050043	0.050537	0.047468	0.034039	0.040371	0.041779	0.036028	0.042235	0.023522
NP-7	0.050043	0.050043	0.048530	0.039380	0.048530	0.049045	0.061892	0.032571	0.048010	0.049548	0.046351	0.050043	0.025151
NP-8	0.033461	0.026296	0.028378	0.025151	0.037148	0.028777	0.041318	0.027979	0.052383	0.037954	0.030714	0.038350	0.021039
NP-14	0.037548	0.032681	0.031445	0.031445	0.042686	0.036713	0.034039	0.027158	0.031020	0.028378	0.030086	0.032681	0.024954
NP-20	0.038350	0.029553	0.032274	0.032274	0.043542	0.032681	0.041318	0.031875	0.043125	0.037954	0.038033	0.038350	0.02562
NP-23	0.027034	0.021830	0.023156	0.025920	0.025920	0.029553	0.025627	0.023840	0.043125	0.039880	0.038350	0.034962	0.025627
tiwa	0.022017	0.027979	0.023522	0.026728	0.023522	0.023943	0.028293	0.020391	0.026296	0.024360	0.022017	0.022017	0.030296
tone	0.043975	0.037548	0.036292	0.036292	0.042686	0.043125	0.049548	0.031020	0.051475	0.032274	0.037368	0.032681	0.020391

Table 4.2 (continue)

	IBGE-7	IBGE-8	IBGE-9	IBGE-10	IBGE-13	IBGE-14	IBGE-16	IBGE-17	IBGE-18	IBGE-19	IBGE-20	IBGE-21	IBGE-22	IBGE-23
TA-1	0.022324	0.019227	0.021116	0.026296	0.024954	0.019755	0.018633	0.022625	0.018792	0.023196	0.023522	0.020072	0.018792	0.020032
TA-2	0.018633	0.017637	0.021116	0.021687	0.022522	0.019755	0.014242	0.020680	0.018792	0.019118	0.021372	0.016743	0.015492	0.014854
TA-3	0.019446	0.018322	0.022390	0.020709	0.023884	0.020709	0.014670	0.021687	0.019695	0.020032	0.022522	0.017388	0.014517	0.015386
TA-4	0.017706	0.018322	0.017893	0.022861	0.021467	0.017065	0.014670	0.018018	0.016073	0.020032	0.020391	0.014370	0.016073	0.015386
TA-6	0.021372	0.022017	0.025151	0.028081	0.023884	0.025298	0.019446	0.023840	0.024258	0.022170	0.022522	0.019118	0.019695	0.018989
TA-9	0.015000	0.022324	0.025547	0.021039	0.024258	0.025627	0.016436	0.020072	0.020032	0.022522	0.018792	0.017706	0.016407	0.019345
TA-10	0.019695	0.018461	0.020391	0.023522	0.024756	0.021116	0.01779	0.022170	0.017893	0.022778	0.023156	0.021467	0.022390	0.024360
IBGE-0	0.011808	0.023840	0.013949	0.012415	0.011111	0.010052	0.010666	0.013352	0.012748	0.009420	0.009741	0.007361	0.010228	0.012063
IBGE-1	0.021039	0.023840	0.012415	0.016743	0.018989	0.015170	0.019118	0.016122	0.014172	0.019695	0.020032	0.025298	0.021467	0.023156
IBGE-2	0.013949	0.017336	0.009560	0.013352	0.014854	0.010972	0.015284	0.013008	0.010052	0.012726	0.010666	0.016436	0.013740	0.014517
IBGE-4	0.007295	0.014670	0.006299	0.010759	0.009767	0.007539	0.007295	0.006837	0.006112	0.009406	0.010521	0.011016	0.010021	0.011387
IBGE-5	0.007906	0.014824	0.004131	0.008238	0.010052	0.004229	0.007906	0.005766	0.003387	0.008599	0.008899	0.010477	0.010364	0.010873
IIBGE-6	0.015930	0.001932	0.011808	0.018208	0.020391	0.011876	0.015930	0.016207	0.012133	0.019227	0.019523	0.020090	0.022625	0.024170
IBGE-7	0.000000	0.014670	0.005350	0.008455	0.006508	0.005299	0.003813	0.006806	0.004504	0.008806	0.006526	0.007872	0.008525	0.010972
IBGE-8	0.014670	0.000000	0.014370	0.019810	0.022324	0.014132	0.017296	0.017571	0.014533	0.019227	0.019523	0.020090	0.022625	0.022017
IBGE-9	0.005350	0.014370	0.000000	0.007383	0.006086	0.002516	0.007062	0.006478	0.002307	0.007722	0.008047	0.009741	0.012063	0.012775
IBGE-10	0.008455	0.019810	0.007383	0.000000	0.004391	0.004081	0.007603	0.004864	0.006220	0.006508	0.008525	0.010038	0.010972	0.014048
IBGE-13	0.006508	0.022324	0.006086	0.004391	0.000000	0.003677	0.006508	0.005224	0.005880	0.005350	0.006478	0.007950	0.009741	0.011440
IBGE-14	0.005299	0.014132	0.002516	0.004081	0.003677	0.000000	0.005299	0.004200	0.002613	0.004951	0.005986	0.006526	0.008899	0.010364
IBGE-16	0.003813	0.017296	0.007062	0.007603	0.006508	0.005299	0.000000	0.005376	0.005224	0.008806	0.009082	0.006299	0.006790	0.008899

Table 4.2 (continue)

	IBGE-7	IBGE-8	IBGE-9	IBGE-10	IBGE-13	IBGE-14	IBGE-16	IBGE-17	IBGE-18	IBGE-19	IBGE-20	IBGE-21	IBGE-22	IBGE-23
IBGE-17	0.006806	0.017571	0.006478	0.004864	0.005224	0.004200	0.005376	0.000000	0.003420	0.005039	0.005299	0.008132	0.007906	0.010188
IBGE-18	0.004504	0.014533	0.002307	0.006220	0.005880	0.002613	0.005224	0.003420	0.000000	0.006478	0.005934	0.008238	0.008990	0.010552
IBGE-19	0.880612	0.019227	0.007722	0.006508	0.005350	0.004951	0.008806	0.005039	0.006478	0.000000	0.001388	0.003813	0.004102	0.006757
IBGE-20	0.065261	0.019523	0.008047	0.008525	0.006478	0.005986	0.009082	0.005299	0.005934	0.001388	0.000000	0.005500	0.004391	0.007062
IBGE-21	0.007872	0.020090	0.009741	0.010038	0.007950	0.006526	0.006299	0.008132	0.008238	0.003813	0.005500	0.000000	0.004229	0.005142
IBGE-22	0.008525	0.022625	0.012063	0.010972	0.009741	0.008899	0.006790	0.007906	0.008990	0.004102	0.004391	0.004229	0.000000	0.004747
IBGE-23	0.010972	0.022017	0.012775	0.014048	0.011440	0.010364	0.008899	0.010188	0.010552	0.006757	0.007062	0.005142	0.004747	0.000000
IBGE-24	0.010873	0.020709	0.011287	0.012748	0.010071	0.009100	0.009741	0.010052	0.010419	0.004631	0.005817	0.005570	0.004308	0.003644
IBGE-25	0.014517	0.022522	0.010891	0.011111	0.009716	0.008771	0.011770	0.007689	0.008877	0.004308	0.005500	0.006138	0.005750	0.003311
NP6	0.053254	0.026296	0.039380	0.036713	0.041318	0.036713	0.053254	0.033073	0.041779	0.027158	0.027561	0.037148	0.030589	0.029692
NP7	0.051943	0.028378	0.046919	0.041779	0.039880	0.041779	0.051943	0.043125	0.049548	0.029692	0.030149	0.035852	0.029246	0.033073
NP8	0.024954	0.021039	0.035852	0.045586	0.043975	0.034226	0.024954	0.040254	0.033073	0.033461	0.026664	0.024602	0.020748	0.019999
NP14	0.039141	0.024954	0.030149	0.029553	0.036713	0.029553	0.034226	0.034600	0.032274	0.022778	0.025920	0.026664	0.025151	0.027561
NP20	0.039880	0.028440	0.042235	0.045586	0.043975	0.039141	0.034962	0.035325	0.037954	0.029553	0.029941	0.034600	0.029180	0.032274
NP23	0.032073	0.033847	0.027034	0.022170	0.025298	0.021467	0.023156	0.024360	0.027158	0.013264	0.011975	0.008525	0.016743	0.012415
Tiwa	0.017091	0.034226	0.017173	0.018259	0.018829	0.018259	0.021116	0.015732	0.017130	0.019622	0.017893	0.023156	0.027561	0.026728
tone	0.039141	0.020391	0.034962	0.038350	0.036713	0.033461	0.034226	0.030661	0.032274	0.028777	0.029180	0.029941	0.028378	0.031445

Table 4.2 (continue)

	IBGE-24	IBGE-25	NP-6	NP-7	NP-8	NP-14	NP-20	NP-23	Tiwa	Tone
TA-1	0.020748	0.020391	0.051475	0.050043	0.033461	0.037548	0.038350	0.027034	0.022017	0.043975
TA-2	0.018633	0.018259	0.051475	0.050043	0.026296	0.032681	0.029553	0.021830	0.027979	0.037548
TA-3	0.019622	0.019227	0.050043	0.048530	0.028378	0.031445	0.032274	0.023156	0.023522	0.036292
TA-4	0.019622	0.019227	0.040854	0.039380	0.025151	0.031445	0.032274	0.025920	0.026728	0.036292
TA-6	0.019622	0.019227	0.050043	0.048530	0.037148	0.042686	0.043542	0.025920	0.023522	0.042686
TA-9	0.022390	0.024756	0.050537	0.049045	0.028777	0.036713	0.032681	0.029553	0.023943	0.043125
TA-10	0.029692	0.025404	0.047468	0.061892	0.041318	0.034039	0.041318	0.035852	0.028293	0.049548
IBGE-0	0.010649	0.010276	0.034039	0.032571	0.027979	0.027158	0.031875	0.020391	0.020391	0.031020
IBGE-1	0.019227	0.016743	0.040371	0.048010	0.052383	0.031020	0.043125	0.043975	0.026296	0.051475
IBGE-2	0.014670	0.012775	0.041779	0.049548	0.037954	0.028378	0.037954	0.029941	0.024360	0.032274
IBGE-4	0.012415	0.013290	0.036028	0.046351	0.030714	0.030086	0.038033	0.034666	0.022017	0.037368
IBGE-5	0.009560	0.009218	0.042235	0.050043	0.038350	0.032681	0.038350	0.039141	0.022017	0.032681
IBGE-6	0.020709	0.022522	0.023522	0.025151	0.021039	0.024954	0.025627	0.023840	0.030296	0.020391
IBGE-7	0.010873	0.014517	0.053254	0.051943	0.024954	0.039141	0.039880	0.025627	0.017091	0.039141
IBGE-8	0.020709	0.022522	0.026296	0.028378	0.021039	0.024954	0.028440	0.023840	0.034226	0.020391
IBGE-9	0.011287	0.010891	0.039380	0.046919	0.035852	0.030149	0.042235	0.043125	0.017173	0.034962
IBGE-10	0.012748	0.011111	0.036713	0.041779	0.045586	0.029553	0.045586	0.039880	0.018259	0.038350
IBGE-13	0.010071	0.009716	0.041318	0.039880	0.043975	0.036713	0.043975	0.038350	0.018829	0.036713
IBGE-14	0.009100	0.008771	0.036713	0.041779	0.034226	0.029553	0.039141	0.034962	0.018259	0.033461

Table 4.2 (continue)

	IBGE-24	IBGE-25	NP-6	NP-7	NP-8	NP-14	NP-20	NP-23	Ttiwa	Tone
IBGE-16	0.009741	0.011770	0.053254	0.051943	0.024954	0.034226	0.034962	0.025627	0.021116	0.034226
IBGE-17	0.010052	0.007689	0.033073	0.043125	0.040254	0.034600	0.035325	0.032073	0.015732	0.030661
IBGE-18	0.010419	0.008877	0.041779	0.049548	0.033073	0.032274	0.037954	0.033847	0.017130	0.032274
IBGE-19	0.004631	0.004308	0.027158	0.029692	0.033461	0.022778	0.029553	0.027034	0.019622	0.028777
IBGE-20	0.005817	0.005500	0.027561	0.030149	0.026664	0.025920	0.029941	0.022170	0.017893	0.029180
IBGE-21	0.005570	0.006138	0.037148	0.035852	0.024602	0.026664	0.034600	0.025298	0.023156	0.029941
IBGE-22	0.004308	0.005750	0.030589	0.029246	0.020748	0.025151	0.029180	0.021467	0.027561	0.028378
IBGE-23	0.003644	0.003311	0.029692	0.033073	0.019999	0.027561	0.032274	0.023156	0.026728	0.031445
IBGE-24	0.000000	0.002736	0.032571	0.031020	0.026728	0.029692	0.030589	0.024360	0.021775	0.034512
IBGE-25	0.002736	0.000000	0.023522	0.030479	0.034962	0.025404	0.030149	0.027158	0.021328	0.034039
NP-6	0.032571	0.023522	0.000000	0.002613	0.006658	0.005817	0.009269	0.013264	0.037269	0.017706
NP-7	0.031020	0.030479	0.002613	0.000000	0.005383	0.005728	0.007972	0.011975	0.044590	0.013949
NP-8	0.026728	0.034962	0.006658	0.005383	0.000000	0.007062	0.008990	0.008525	0.040371	0.018018
NP-14	0.029692	0.025404	0.005817	0.005728	0.007062	0.000000	0.009512	0.016743	0.039380	0.019523
NP-20	0.030589	0.030149	0.009269	0.007972	0.008990	0.009512	0.000000	0.012415	0.034039	0.015930
NP-23	0.041779	0.045586	0.029553	0.045586	0.039880	0.018259	0.038350	0.000000	0.041318	0.013193
Tiwa	0.021775	0.021328	0.037269	0.044590	0.040371	0.039380	0.034039	0.041318	0.000000	0.064768
Tone	0.034512	0.034039	0.017706	0.013949	0.018018	0.019523	0.015930	0.013193	0.064768	0.000000

4.5 Plaunotol content in *C. sublyratus* leaves.

Thirty-seven samples of *C. sublyratus* leaves were dried and ground to powder and determined for its plaunotol content by gas chromatography. The results of plaunotol content in *C. sublyratus* leaves are summerized in Table 4.3. It can be seen that 37 samples of *C. sublyratus* leaves contained plaunotol content ranging from 0.11% to 1.01% (w/w) dry weight. This results showed that 37 samples of *C. sublyratus* leaves had significant difference in the content of plaunotol. There were highly significant ($F=49$; $p<0.05$) differences in plaunotol content, although the *C. sublyratus* leaves from Prachinburi and Prachuapkhirikhan contained higher plaunotol content than *C. sublyratus* leaves from Nakhonphanom.

Table 4.3 Plaunotol content in 37 samples of *C. sublyratus* leaves.

Sample of *C. sublyratus* leaves	Plaunotol content (%w/w dry weight)
TA-1	$0.23^{a1}\pm0.03$
TA-2	$0.16^{a2}\pm0.02$
TA-3	$0.15^{a2}\pm0.02$
TA-4	$0.11^{a2}\pm0.03$
TA-6	$0.20^{a1}\pm0.02$
TA-9	$0.24^{a1}\pm0.08$
TA-10	$0.16^{a2}\pm0.02$
IBGE-0	$0.44^{b2}\pm0.05$
IBGE-1	$0.37^{b1}\pm0.04$
IBGE-2	$0.78^{b5}\pm0.04$
IBGE-4	$0.33^{b1}\pm0.03$
IBGE-5	$0.43^{b2}\pm0.07$
IBGE-6	$0.60^{b4}\pm0.04$
IBGE-7	$0.40^{b2}\pm0.06$

Table 4.3 (continued)

Sample of *C. sublyratus* leaves	Plaunotol content (%w/w dry weight)
IBGE-8	$0.38^{b1}\pm0.01$
IBGE-9	$0.49^{b3}\pm0.02$
IBGE-10	$0.40^{b2}\pm0.06$
IBGE-13	$0.41^{b2}\pm0.05$
IBGE14	$0.36^{b1}\pm0.02$
IBGE-16	$0.38^{b1}\pm0.04$
IBGE-17	$0.46^{b2}\pm0.04$
IBGE-18	$0.48^{b3}\pm0.01$
IBGE-19	$0.37^{b1}\pm0.06$
IBGE-20	$0.44^{b2}\pm0.06$
IBGE-21	$0.51^{b3}\pm0.04$
IBGE-22	$0.36^{b1}\pm0.01$
IBGE-23	$0.33^{b1}\pm0.08$
IBGE-24	$0.33^{b1}\pm0.03$
IBGE-25	$0.47^{b3}\pm0.08$
NP-6	$0.47^{b3}\pm0.02$
NP-7	$0.52^{b2}\pm0.07$
NP-8	$0.42^{b2}\pm0.04$
NP-19	$0.79^{b5}\pm0.04$
NP-20	$1.01^{b6}\pm0.11$
NP-23	$0.57^{b4}\pm0.03$
Tone	$0.37^{b1}\pm0.05$
Tiwa	$0.32^{b1}\pm0.02$

Statistical significance was evaluated using ANOVA followed by Least Significant Difference (LSD) test. 37 samples of *C. sublyratus* leaves were significantly ($p<0.05$) different for plaunotol content.

4.6 Leaf morphology in samples of *C. sublyratus*.

Differences in leaf morphology between the thirty six individuals of *C. sublyratus* were examined for five quantitative characters, with leaf length, leaf width, petiole length, number of lateral and leaf base angle are summerized in Tabel 4.4.

Table 4.4 Leaf characters measurements in 36 samples of *C. sublyratus*.

Sample of *C.sublyratus*	Leaf length	Leaf width	Petiole length	No.of lateral	Leaf base angle
TA-1	14.34±0.27	6.2±0.35	3.38±0.26	15.6±0.27	47.8±0.36
TA-2	14.02±0.13	5.68±0.25	2.72±0.37	15.8±0.35	44.8±0.16
TA-3	14.26±0.21	5.86±0.34	2.62±0.31	15.8±0.15	49.2±0.34
TA-4	14.02±0.28	6.0±0.18	3.02±0.26	15.8±0.38	48.8±0.28
TA-6	14.24±0.33	5.7±0.36	3.12±0.27	16.2±0.19	47.0±0.28
TA-9	13.96±0.16	6.24±0.31	3.34±0.23	16.2±0.21	48.0±0.22
TA-10	13.82±0.24	6.1±0.22	3.2±0.33	16.2±0.26	48.0±0.17
IBGE-0	12.76±0.25	7.4±0.36	2.6±0.35	13.6±0.36	61.2±0.43
IBGE-1	13.16±0.31	6.2±0.15	1.96±0.24	13.2±0.27	55.8±0.28
IBGE-2	13.06±0.36	6.1±0.18	1.78±0.17	13.8±0.12	55.4±0.36
IBGE-4	18.24±0.25	5.4±0.36	2.68±0.36	14.4±0.22	37.8±0.37
IBGE-5	18.64±0.18	5.98±0.35	2.98±0.29	14.4±0.35	37.4±0.34
IBGE-6	18.2±0.26	5.6±0.32	2.64±0.30	15.2±0.19	38.0±0.26
IBGE-7	17.42±0.37	5.4±0.41	3.0±0.27	16.6±0.24	37.6±0.28
IBGE-8	17.58±0.24	5.48±0.26	2.56±0.41	15.0±0.36	38.8±0.16
IBGE-9	17.72±0.41	5.56±0.36	2.82±0.26	15.8±0.19	36.8±0.27
IBGE-10	17.14±0.34	5.64±0.18	2.82±0.14	16.2±0.18	38.4±0.22
IBGE-13	17.62±0.29	5.32±0.24	2.98±0.34	15.6±0.31	39.0±0.36
IBGE14	17.64±0.37	5.28±0.45	2.46±0.36	16.2±0.35	38.2±0.31
IBGE-16	17.42±0.26	5.56±0.38	2.46±0.27	16.0±0.37	39.2±0.35

Table 4.4 (continued)

Sample of *C.sublyratus*	Leaf length	Leaf width	Petiole length	No.of lateral	Leaf bottom angle
IBGE-17	13.3±0.34	7.36±0.29	2.2±0.41	14.6±0.28	60.4±0.24
IBGE-18	17.48±0.21	5.56±0.19	2.62±0.31	15.6±0.18	38.4±0.34
IBGE-19	17.48±0.31	5.48±0.27	2.56±0.21	16.0±0.26	37.7±0.32
IBGE-20	17.42±0.23	5.6±0.23	2.7±0.21	14.6±0.32	37.2±0.26
IBGE-21	17.58±0.27	5.44±0.24	2.56±0.26	14.4±0.27	37.6±0.37
IBGE-22	17.2±0.26	5.68±0.17	2.68±0.28	14.4±0.26	38.0±0.16
IBGE-23	17.36±0.25	5.32±0.26	2.62±0.35	14.6±0.48	38.1±0.25
IBGE-24	17.320.45	5.36±0.36	2.76±0.36	14.6±0.15	38.0±0.26
IBGE-25	17.94±0.36	5.36±0.37	2.58±0.33	14.8±0.26	37.4±0.31
NP-6	15.3±0.35	6.44±0.33	2.52±0.32	15.8±0.35	49.6±0.26
NP-7	15.3±0.26	6.26±0.38	2.58±0.27	16.2±0.27	47.2±0.36
NP-8	15.84±0.27	6.5±0.24	2.54±0.28	16.8±0.36	46.4±0.34
NP-19	15.38±0.31	6.26±0.26	2.46±0.46	16.2±0.31	49.2±0.33
NP-20	15.62±0.36	6.56±0.41	2.58±0.24	16.8±0.32	45.6±0.41
NP-23	15.64±0.22	6.76±0.26	2.62±0.26	16.4±0.35	46.6±0.25
Tone	16.16±0.15	7.32±0.27	2.04±0.15	15.6±0.27	60.6±0.24

Each value represented the mean±SD is across five leaves.

T_r plaunotol is 7.71 min.

T_r octacosane (internal standard) is 11.91 min.

Both locality and genotype had a difference impact on leaf morphology in 36 samples of *C. sublyratus* can be divieded into six groups (Appendix C). Thus while leaf morphology depended on genotype, there was still an overriding and highly significant effect of locality. The variation within genotypes depended on the character, with variation in leaf shape, leaf apex, leaf margin and are summerized in Table 4.5. The variation within genotype depended on the character with variation in leaf indument are summerized in Table 4.6.

Table 4.5 Leaf morphology in 36 samples of *C. sublyratus*.

Sample of *C.sublyratus*	Leaf shape	Leaf apex	Leaf margin	Locality
IBGE-1, IBGE-2	obovate	acute	serrate	Prachinburi
IBGE-0, IBGE-17	elliptical	acute	serrate	Prachuapkhirikhan
other IBGE	oblanceolate	acuminate	dentate	Prachuapkhirikhan
TA	obovate	acute	dentate	Nakhonphanom
NP	lanceolate	acute	entire	Prachuapkhirikhan
Tone	elliptical	acute	serrulate	Prachuapkhirikhan

Table 4.6 Leaf indument in 36 samples of *C. sublyratus*.

Sample of *C. sublyratus*	Leaf indument		Locality
	upper surface	under surface	
IBGE-1, IBGE-2	glabrous	glabrous	Prachinburi
IBGE-0, IBGE-17	pubescent	covered	Prachuapkhirikhan
other IBGE	pubescent	covered	Prachuapkhirikhan
TA	pubescent	covered	Nakhonphanom
NP	glabrous	covered	Prachuapkhirikhan
Tone	pubescent	covered	Prachuapkhirikhan

4.7 Genetic different between 37 *C. sublyratus* and plaunotol content.

Based on their plaunotol content, the 37 *C. sublyratus* may be arbitrarily classifield into 2 groups. They are (a) low plaunotol contents of *C. sublyratus* (0.11-0.24%) (b) high plaunotol contents of *C. sublyratus*. The low plaunotol contents group includes *C. sublyratus* from Nakhon-phanom Province. The high plaunotol contents group includes *C. sublyratus* from Prachinburi and Prachuapkhirikhan Province. In dendrogram the lowest plaunotol contents include of *C. sublyratus* from Nakhon-phanom Province (TA-4) and highest (NP-20) includes of *C. sublyratus* from Nakhonphanom Province plaunotol contents belong to different group. The low plaunotol contents of *C. sublyratus* cluster into one phylogenetic group with very small genetic distances between them.

ISSR analysis generated phylogenetic tree shows a close genetic relation among the 37 samples of *C. sublyratus*. The estimated genetic distances reflect the degree of genetic differentiation between *C. sublyratus* from different parts of Thailand. The results show that ISSR analysis may be used to study the genetic differentiation of *C. sublyratus* from various parts of Thailand. It also shows that phylogenetic trees may be used for the study of genetic relatedness between 37 samples of *C. sublyratus* with different plaunotol contents.

The 37 samples of *C. sublyratus* were from different parts of Thailand. Low plaunotol content of *C. sublyratus* were collected from Nakhonphanom Province. High plaunotol content of *C. sublyratus* were collected from Prachinburi and Prachuapkhirikhan Province.

In the dendrogram constructed using phylogenetic distances, the 37 samples of *C. sublyratus* clustered into 2 groups. The low plaunotol contents of *C. sublyratus* cluster into a group with close genetic distances between them. The highest plaunotol contents of *C. sublyratus* shows substantial genetic differentiation from lowest plaunotol contents *C. sublyratus* and they are from different genetic group.

The results would suggest that the variation in plaunotol content of 37 samples *C. sublyratus* is governed by a strong genetic factor has occurred during evolution. The result show that the ISSR analysis may be used to generate reliable molecular markers to distinguish between 37 *C. sublyratus* different in plaunotol content. A larger selection of primer sequences, a greater number of *C. sublyratus* samples of various parts of Thailand and of different plaunotol content would improve significantly the resolution of experimental results.

CHAPTER V

CONCLUSION

Three RAPD primer (GGCACTGAGG, ACGACCGACG and GATGACCGCC) were used to study genetic diversity among 10 samples of *C. sublyratus*, but they gave non-reproducible results. ISSR analysis was used to determine genetic diversity among 37 samples of *C. sublyratus*. Three of ISSR primer ($(CA)_8G$, $BSC(GA)_8$ and $CRN_2(CTT)_5$) gave reproducible ISSR pattern. Genetic variation of 37 samples of *C. sublyratus* were analyzed by Unweighted Pair Group Method with Arithmatic Mean (UPGMA) and the dendrogram could be divided into 2 groups from different original location.

Determination of plaunotol in leaf samples collected in January contained lower plaunotol content than usual. The leaves of *C. sublyratus* obtained from various sources of Thailand contained different level of plaunotol, ranging from 0.11 to 1.01% (w/w) dry weight. The majority of samples contain plaunotol in the range of 0.3 to 0.4% (w/w) dry weight. Morphological study of leaf samples of various *C. sublyratus* revealed that leaf samples collected from different location had different leaf characteristics. Meanwhile, leaf samples from *C. sublyratus* within the same area did not exhibit any major difference in leaf morphology. Therefore, physical characteristics of *C. sublyratus* leaves are likely to be due to genetic background, not growing environments.

A specific ISSR fragment suggested that a 500 pb DNA fragment could be used for primary screening to select for high and low plaunotol content in *C. sublyratus* using ISSR analysis with primer $(CA)_8G$.

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APPENDICES

APPENDIX A

ISSR genotype generated from three primers in all 37 samples of *Croton sublyratus* Kurz.

A. primer M-1

SIZE (bp)	251	270	280	291	340	354	370	398	415	432	443	449	472	489	500	520	595	610	637	645	650	665	675	685	726	730	760	778	795	806	813	852	872	880	905	921	938	1000	1160	1250
TA-1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0
TA-2	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1
TA-3	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1
TA-4	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
TA-6	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
TA-9	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0
TA-10	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
IBGE-0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
IBGE-1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-2	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
IBGE-4	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
IBGE-5	0	0	1	0	1	0	1	0	1	1	0	0	0	1	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
IBGE-6	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-7	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
IBGE-8	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0
IBGE-9	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
IBGE-10	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
IBGE-13	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A. primer M-1 (continue)

SIZE (bp)	251	270	280	291	340	354	370	398	415	432	443	449	472	489	500	520	595	610	637	645	650	665	675	685	726	730	760	778	795	806	813	852	872	880	905	921	938	1000	1160	1250
IBGE-16	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-17	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
IBGE-18	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
IBGE-19	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0
IBGE-20	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
IBGE-21	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-22	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-23	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-24	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-25	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
NP-6	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
NP-7	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
NP-8	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
NP-14	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
NP-20	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
NP-23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Tiwa	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Tone	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0

B. primer M-6

Size(bp)	220	243	271	320	350	400	452	464	475	480	487	500	518	531	540	557	577	580	592	600	613	633	638	656	665	669	680
TA-1	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0
TA-2	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0
TA-3	1	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
TA-4	1	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
TA-6	1	0	1	0	1	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0
TA-9	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
TA-10	1	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
IBGE-0	1	0	1	0	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
IBGE-1	1	0	1	0	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
IBGE-2	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-4	1	0	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0
IBGE-5	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-6	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-7	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-8	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-9	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-10	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-13	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-14	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0

B. primer M-6 (continue)

SIZE (bp)	706	723	734	739	752	762	790	796	861	842	948	961	973	1025	1051	1065	1111	1134	1164	1224	1240	1256	1304	1338	1343	1462
TA-1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
TA-2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
TA-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TA-4	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0
TA-6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
TA-9	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TA-10	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-2	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-4	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-5	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0
IBGE-6	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-7	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-9	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
IBGE-13	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
IBGE-14	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

B. primer M-6 (continue)

Size(bp)	220	243	271	320	350	400	452	464	475	480	487	500	518	531	540	557	577	580	592	600	613	633	638	656	665	669	680
IBGE-16	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0
IBGE-17	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0
IBGE-18	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
IBGE-19	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0
IBGE-20	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0
IBGE-21	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0
IBGE-22	1	0	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0
IBGE-23	1	0	1	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0
IBGE-24	1	0	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0
IBGE-25	1	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0
NP-6	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
NP-7	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
NP-8	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1
NP-14	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
NP-20	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1
NP-23	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1
Tiwa	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Tone	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0

B. primer M-6 (continue)

SIZE (bp)	706	723	734	739	752	762	790	796	861	842	948	961	973	1025	1051	1065	1111	1134	1164	1224	1240	1256	1304	1338	1343	1462
IBGE-16	0	0	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-17	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
IBGE-18	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-19	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-20	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-21	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-22	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-23	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-24	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-25	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NP-6	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP-7	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP-8	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP-14	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP-20	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
NP-23	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Tiwa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tone	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

C. primer M-7

Size(bp)	237	250	260	280	300	332	336	380	394	400	420	437	461	480	500	530	540	557	564	580	600	607	634	645	677	684
TA-1	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0
TA-2	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0
TA-3	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0
TA-4	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0
TA-6	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0
TA-9	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TA-10	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
IBGE-0	1	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	1	0	0
IBGE-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
IBGE-2	1	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0
IBGE-4	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0
IBGE-5	1	1	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
IBGE-6	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0
IBGE-7	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0
IBGE-8	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0
IBGE-9	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
IBGE-10	1	0	1	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0
IBGE-13	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0
IBGE-14	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0

C. primer M-7 (continue)

SIZE (bp)	700	730	736	749	765	778	783	831	850	880	902	919	941	959	971	988	1000	1028	1060	1098	1135	1200	1256	1300	1400	1462	1500
TA-1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
TA- 2	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
TA-3	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
TA-4	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
TA-6	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0
TA-9	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0
TA-10	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
IBGE-1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0
IBGE-2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-4	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-5	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-6	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-7	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-8	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
IBGE-9	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-10	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
IBGE-13	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
IBGE-14	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1

C. primer M-7 (continue)

Size(bp)	237	250	260	280	300	332	336	380	394	400	420	437	461	480	500	530	540	557	564	580	600	607	634	645	677	684
IBGE-16	1	0	1	1	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0
IBGE-17	1	0	1	1	1	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0
IBGE-18	1	0	1	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
IBGE-19	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	1	0	0
IBGE-20	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	1	0	0
IBGE-21	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	1	0	0
IBGE-22	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	1	0	0
IBGE-23	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0
IBGE-24	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0
IBGE-25	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0
NP-6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1
NP-7	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1
NP-8	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1
NP-14	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1
NP-20	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0
NP-23	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0
Tiwa	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Tone	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0

C. primer M-7 (continue)

SIZE (bp)	700	730	736	749	765	778	783	831	850	880	902	919	941	959	971	988	1000	1028	1060	1098	1135	1200	1256	1300	1400	1462	1500
IBGE-16	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-17	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
IBGE-18	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IBGE-19	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0
IBGE-20	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0
IBGE-21	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0
IBGE-22	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
IBGE-23	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
IBGE-24	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
IBGE-25	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
NP-6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
NP-7	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
NP-8	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
NP-14	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
NP-20	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP-23	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
Tiwa	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Tone	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0

APPENDIX B

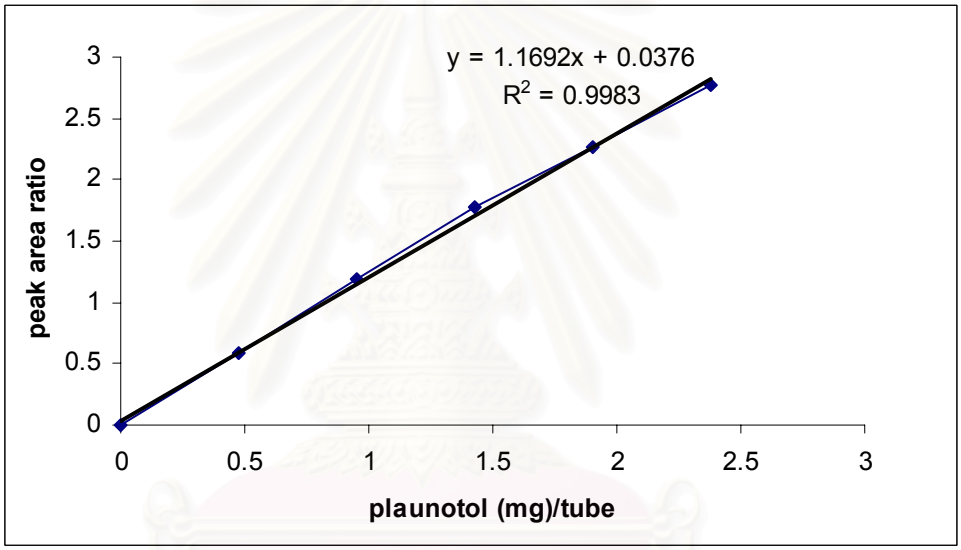

Figure 5. The standard calibration curve of plaunotol

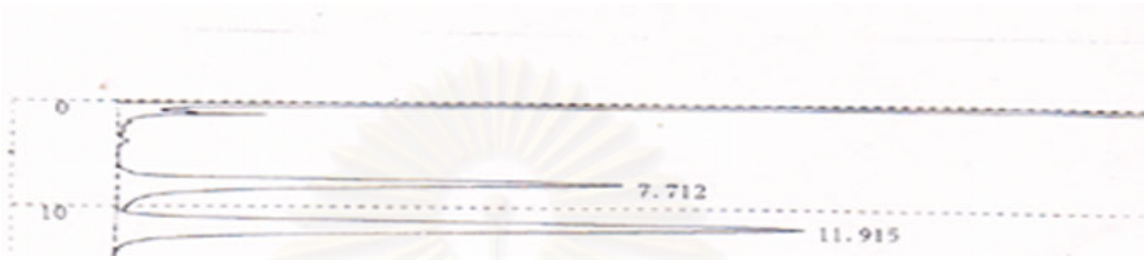

Figure 6. . GC chromatogram of standard plaunotol and n-octacosane or internal standard.

Figure 7. GC chromatogram of plaunotol in leaves extract from *C. sublyratus*.

APPENDIX C

Leaf morphology variation in 36 samples of *C.sublyratus* can be divieded into six groups.

Figure 8. Leaf morphology of *C. sublyratus* (IBGE-0).

Figure 9. Leaf morphology of *C. sublyratus* (IBGE-17).

Figure 10. Leaf morphology of *C. sublyratus* (IBGE-1 and IBGE-2).

Figure 11. Leaf morphology of *C. sublyratus* (other IBGE).

Figure 12. Leaf morphology of *C. sublyratus* (TA).

Figure 13. Leaf morphology of *C. sublyratus* (NP).

Figure 14. Leaf morphology of *C. sublyratus* (Tone).

VITAE

Miss. Rattaporn Klinbantom was born on November 12, 1979 in Lopburi Province, Thailand. She graduated with Bachelor Degree of Science in May 2002 from the Faculty of Science, Chulalongkorn University. Sinc 2003, she has studied for a Master Degree in the field of Biotechnology, Faculty of Science, Chulalongkorn University and completed the program in May 2005.