เครื่องหมายโมเลกุลเพื่อตรวจสอบสายพันธุ์ยาสูบ *Nicotiana tabacum* L.

นางสาวสรสุดา เสตพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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MOLECULAR MARKERS FOR DETERMINING TOBACCO *Nicotiana tabacum* L. VARIETIES

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for the Degree of Master of Science Program in Biotechnology
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สรสุดา เสตพันธ์: เครื่องหมายโมเลกุลเพื่อตรวจสอบสายพันธุ์ยาสูบ *Nicotiana tabacum* L. (MOLECULAR MARKERS FOR DETERMINING TOBACCO *Nicotiana tabacum* L. VARIETIES) อ.ที่ปรึกษา: ผศ.ดร. เจษฎา เด่นดวงบริพันธ์, 116 หน้า.

ประเทศไทยมีอัตราการจัดเก็บภาษีระหว่างยาสูบสายพันธุ์พื้นเมือง และสายพันธุ์นำเข้าจากต่างประเทศที่แตกต่างกัน อย่างไรก็ตาม ยังพบว่ามีปัญหาใหญ่ทั้งทางด้านกฎหมายและด้านวิธีการตรวจวิเคราะห์เพื่อแยกแยะสายพันธุ์ยาสูบทั้งสองประเภทนี้ออกจากกัน ในการศึกษาครั้งนี้เทคนิคไอเอสเอสอาร์ (Inter-Simple Sequence Repeat, ISSR) ได้ถูกนำมาใช้เพื่อศึกษาความสัมพันธ์ทางพันธุกรรมระหว่างยาสูบพันธุ์นำเข้าและพันธุ์พื้นเมืองรวม 66 สายพันธุ์ที่ปลูกในประเทศไทย โดยจากที่ได้ทดลองคัดเลือก 20 ไพรเมอร์พบว่า ไพรเมอร์ UBC-810 UBC-813 UBC-817 และ UBC-818 ถูกพบว่าให้รูปแบบพีซีอาร์ที่แตกต่างกันระหว่างสายพันธุ์ และถูกเลือกมาใช้หาความสัมพันธ์ทางพันธุกรรมระหว่างยาสูบพันธุ์นำเข้า 24 สายพันธุ์ ซึ่งถึงแม้ว่าผลที่ได้จะไม่สามารถแยกแยะความสัมพันธ์ได้อย่างชัดเจน แต่พบว่าสายพันธุ์เตอร์กิช (Turkish) ส่วนใหญ่มีความสัมพันธ์ใกล้ชิดกันเองมากกว่าสายพันธุ์เบอร์เลย์ (Burley) และสายพันธุ์เวอร์จิเนีย (Virginia) นอกจากนี้ พบว่ามีอยู่ 2 ไพรเมอร์ ได้แก่ UBC-807 และ UBC-836 ที่ให้แถบพีซีอาร์จำเพาะเจาะจง ซึ่งสามารถแยกยาสูบพันธุ์พื้นเมืองสองพันธุ์คือ ช่อแล 1 และช่อแล 2 ออกจากยาสูบพันธุ์นำเข้า 53 พันธุ์ได้ ส่วนการหาสภาวะที่เหมาะสมต่อการทำปฏิกิริยาพีซีอาร์พบว่าสามารถเพิ่มความจำเพาะเจาะจงของปฏิกิริยาได้ แผนภูมิต้นไม้ความสัมพันธ์ทางพันธุกรรมโดยใช้หลักการของ NJ และ UPGMA ได้แสดงการจับกลุ่มของสายพันธุ์ยาสูบได้เพียง 4 คู่ ที่มีค่าการสนับสนุนของบูทสแตร็ปมากกว่าร้อยละ 50 ได้แก่ ช่อแล 1 และช่อแล 2 นิสันและเพชรแช่งสิงห์ ยาและพื้นเมืองนครศรีธรรมราช และ NC37NF (Virginia)และ Ky-10 (Burley) จากยาสูบสายพันธุ์พื้นเมืองที่ถูกรวบรวมและศึกษาทั้งหมด 13 พันธุ์ มีเพียงคู่ของช่อแล 1 และช่อแล 2 ที่ถูกแยกห่างออกจากสายพันธุ์นำเข้าอื่นๆ แสดงให้เห็นว่าสายพันธุ์ยาสูบดังกล่าวอาจมีการปลูกในประเทศไทยมาเป็นระยะเวลานานแล้ว ส่วนยาสูบพันธุ์พื้นเมืองที่เหลืออีก 11 พันธุ์พบว่ามีลักษณะทางสัณฐานวิทยาและทางพันธุกรรมคล้ายคลึงกับสายพันธุ์ต่างประเทศบางพันธุ์ ซึ่งยาสูบพื้นเมืองเหล่านี้อาจมีต้นกำเนิดมาจากสายพันธุ์นำเข้าดังกล่าว โดยสรุปแล้วแม้ว่าเทคนิคไอเอสเอสอาร์จะไม่สามารถอธิบายความสัมพันธ์ระหว่างยาสูบสายพันธุ์ที่ศึกษาได้อย่างชัดเจน แต่เทคนิคนี้น่าจะถูกพัฒนาให้เป็นเครื่องหมายโมเลกุลที่ง่ายและมีประสิทธิภาพในการแยกแยะสายพันธุ์พื้นเมืองบางพันธุ์ที่ถูกเพาะปลูกมานานแล้ว ออกจากสายพันธุ์นำเข้าได้

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....สรสุดา เสตพันธ์.....

ปีการศึกษา.....2550.....ลายมือชื่ออาจารย์ที่ปรึกษา.....Jessada Denduangboripant.....

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KEYWORDS: MOLECULAR MARKER, ISSR, AND TOBACCO

SORNSUDA SETAPHAN: MOLECULAR MARKERS FOR DETERMINING TOBACCO *Nicotiana tabucum* L. VARIETIES. THESIS ADVISOR: ASST. PROF. JESSADA DENDUANGBORIPANT, Ph.D., 116 pp.

In Thailand, imported and local tobacco varieties have different regulations in tariff collection. However, there are major legal and technical problems on how to distinguish between the two groups of tobacco varieties. In this study, Inter-Simple Sequence Repeat (ISSR) method was introduced to study genetic relationships among totally 66 imported and local tobacco varieties grown in Thailand. Of 20 screened primers, UBC-810, UBC-813, UBC-817, and UBC-818 primers were found giving polymorphic PCR patterns and were selected to determine genetic relationships among 24 imported varieties. Although the relationships among them were not so clear from the ISSR results, most of Turkish varieties were found more closely related to each other than to Burley or Virginia varieties. Moreover, the two best ISSR primers (UBC-807 and UBC-836) generated specific PCR bands which could separate two local tobacco varieties (Chorlare 1 and Chorlare 2) from 53 imported varieties. PCR optimisations were also performed to increase PCR specificity. Genetic relationship trees based on NJ and UPGMA methods revealed only four pairs of tobacco varieties which had Bootstrap supporting-values higher than 50%, i.e. Chorlare 1 and Chorlare 2, Nison and Petkhangsink, Ya and Local Nakhon Si Thammarat, and NC37NF (Virginia) and Ky-10 (Burley). Among all 13 local tobacco varieties collected and examined, only the pair of Chorlare 1 and 2 was distantly separated from other imported varieties, suggesting their long-history of being grown in Thailand. The other 11 local varieties were found morphologically and genetically similar to some imported varieties and could have been originated from such varieties. In conclusion, although ISSR technique could not give clear relationships between tobacco varieties in this study, it would be developed to be a simple, effective molecular marker to distinguish some long-time cultivated local varieties from the imported ones.

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

Academic year.....2007.....Advisor's signature.....Jessada Denduangboripant.....

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
?C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid (disodium salt)
EtBr	Ethidium bromide
ISSR	Inter Simple Sequence Repeat
Kb	Kilobase pair
$MgCl_2$	Magnesium chloride
mg	Milligram
ml	Milligram
mM	Millimolar
mmol	Millimole
ng	nanogram
OD	Optimal density
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
SSR	Simple Sequence Repeat
TBE	Tris-boric-ethylene diamine tetraacetic acid
Tris	Tris (hydroxyl methyl) aminomethane
Volt	Voltage

CHAPTER I
INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) belongs to the family Solanaceae. It is one of important economic crops of Thailand, as raw material for cigarette industry. Tobacco is also used for other industries such as paints and vanishes, insecticides, and herbal medicines. This plant originated in the tropical America and had been first introduced into Thailand since the 16th century. Tobacco has then been developed to many local varieties in the country. Nowadays, most of tobacco plants are cultivated in the northern and northeastern parts of Thailand.

Tobacco contains nicotine, a powerful neurotoxin which can stimulate the central nervous system. Nicotine can also cause several bad effects to the cigarette smokers such as nausea, dizziness, hallucination, lung cancer, and heart disease. Thus, the government has set up a strict taxation to control production and consumption of tobacco. However, while the taxation of imported tobacco varieties is high, that of the local varieties has been reduced to support Thai local farmers. These different rates of taxation could cause a great problem to the Excise Department on how to distinguish between local and imported varieties. This problem comes in terms of a difficulty in the identification procedure and also an unclear definition of the term “local varieties”. The legal definition of the local tobacco varieties is not sufficiently accurate enough: “the tobacco which has been grown in Thailand for a long time and the color of their leaves are changed to brown after passed a dry-curing process”. Therefore, it is a need to find more suitable techniques use to solve the identification of tobacco varieties.

Recently, molecular or genetic markers have been increasingly used as a modern technique to distinguish genotypes of organisms. The techniques provide powerful tools for DNA polymorphism analysis, genetic and phylogenetic evaluation, and genetic diversity assessment. A number of molecular marker techniques, such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR) and Inter-Simple Sequence Repeat (ISSR), have been developed for crop plants genetic studies. Each marker technique has its own advantage and

disadvantages. Common advantages of most markers include rapid analyses, highly informative results, and being independent on environmental factors.

The genetic marker chose for this study to solve problem on tobacco variety identification is the ISSR technique. The ISSR marker is based on an amplification of 100 -3,000 bp. regions between inversely oriented SSR (or microsatellites). The ISSR marker is simple, reproducibility, require small amounts of DNA, and does not require information on DNA sequence. The primer used for ISSR markers is designed from SSR motif. This technique can be undertaken for any plant species that contains a sufficient number and distribution of SSR motif, which are very polymorphic and wide spread in plant genomes. The potential supply of ISSR markers depend on the variety and frequency of SSR motif within the species. Therefore, the ISSR technique has been widely used in many aspects such as to study genetic diversity in rice *Oryza*, to evaluate genetic relationships among coffee species, to analyze polymorphism in cotton, to investigate molecular variation and fingerprinting of *Leucadendron* cultivars, and to identify olive (*Olea europaea* L.) cultivars. Since genetic analysis of tobacco (*Nicotiana tabacum* L.) varieties in Thailand has never been done before, therefore, ISSR analysis should provide new powerful tools for the determination and investigation of genetic relationships between tobacco varieties.

Research objective

This thesis aims to select Inter-Simple Sequence Repeat (ISSR) primers as the suitable genetic markers to distinguish tobacco varieties and determine genetic relationships between tobacco varieties in Thailand.

CHAPTER II

BACKGROUND

2.1 Characteristics of Tobacco (*Nicotiana tabacum* L.)

Tobaccos (*Nicotiana* spp.) are related to many other plants which include vegetables, flowers, weeds and poisonous herbs like potatoes, tomatoes and petunias. The genus *Nicotiana* is member of the family Solanaceae and has been divided into three subgenera (*Rustica*, *Tabacum*, and *Petunioides*) containing over 64 recognised species (Arslan and Okumus, 2006).

Some 100 species of tobaccos are known, but one of these are of commercial importance. *Nicotiana tabacum* is the source of most of the tobaccos in use at the present time. It is originated in the tropical America and grown commercially in at least 97 countries around the world (Ren and Timko, 2001). *Nicotiana tabacum* is a natural amphidiploid (2n=48) derived from an hybrid ancestor of *N. sylvestris* and *N. tomentosiforimis* (Yukawa, Tsudzuki, and Sugiura, 2005).

Morphology

Tobacco stems are strong, about 100-600 cm. high and 3-6 cm. thick. Most leaves are usually borne directly (sessile) on the main stem (50-70 cm. long and 12-20 cm. broad) (Br?cher, 1989). The stems and leaves are covered with glandular hairs which secrets a resinous fluid and are very sticky to the touch. Flowers are pink or white (Figure 2.1). Inflorescences are rich-bearing with 100-150 flowers in one raceme. Colloras show 3-5 cm. long, with a long slender style and five stamens which are attached to the base of the corolla tube. Fruits are capsules with numerous very small seeds (Albert, 1996).

History

The use of tobacco has long history. The Maya who were native Americans inhaled the tobacco smoke for medicinal purpose more than 2,000 years ago before Europeans arrived in North and South America (วรวิทย์และคณะ, 2549). In 1492,

Christopher Columbus encountered a native man on San Salvador with dry tobacco leaves and was first to note the use of tobacco to cure many illness. In 1559, Jean Nicot , for whom the plant was name *Nicotiana*, was chiefly responsible for its exploitation in France. From there, it has rapidly been spread over the rest of Europe, Africa, Asia, and Australia (Albert, 1996). In Asia, tobacco was first introduced to the Phillipines, and then to India, China, and Indonesia, respectively.

(A) (B)

(C)

Figure 2.1 Tobacco (*Nicotiana tabacum* L.) in the field.
(A) Morphological characteristics of tobacco in Nakhon Phanom province.
(B) Flowers of tobacco (*Nicotiana tabacum* L.).
(C) Local tobacco varieties cultivated along riverside area near Khong river at Nong Khai province.

2.2 Tobacco (*Nicotiana tabacum*) in Thailand

In Thailand, people in various regions recognise *Nicotiana tabacum* as in different local names such as “Yasub” (ยาสูบ), “Ja-lour” (จาลัว) in Surin province, and etc. There are two major types of commercial tobacco varieties cultivated in Thailand.

2.2.1 Imported tobacco varieties

Since 1939, Thailand Tobacco Monopoly, Ministry of Finance, has imported tobacco seeds from many world’s finest tobacco sources for experiment and development of tobacco cultivation in Thailand. For example, the company has set up Maejo Tobacco Experiment Station in Sansai district of Chiang Mai province to serve as a research and development post for tobacco leaf stains. It also provides support to tobacco farmers through each step from the growing process to harvest process. The identification of imported varieties types was classified by curing methods and tobacco barn design.

2.2.1.1 Virginia varieties (or flue-cured tobacco)

Virginia tobaccos are mostly cultivated in the upper northern region (for example, Chiang Rai, Chiang Mai, Lumpang, Lamphun, Phrae, and Nan) and the northeastern region (Nong Khai, Nakhon Phanom) of Thailand. Virginia varieties develop a characteristic bright yellow colour with high sugar contents in leaves. The leaves are dried in tobacco-curing barns under fire heat of charcoals or hardwoods (Figure 2.2). The tobacco-curing barns have flues which run from externally-fed fire boxes, heat-curing the tobacco without exposing it to smoke, and slowly raising the temperature over the course of curing. The process will generally take about a week. Many popular varieties of virginia tobaccos are grown in Thailand such as Coker-187 hick, Coker-347, K-326, and etc. (Davis and Nielsen, 1999).

(A) (B)

(C) (D)

Figure 2.2 Virginia tobacco varieties.
(A) Virginia tobacco varieties in Nong Khai province.
(B) Dried virginia leaves after passed fire-curing process.
(C) The curing barn of Virginia varieties in Nong Khai province.
(D) Virginia leaves were held in tobacco curing barn.

2.2.1.2 Burley varieties (or light air-cured tobacco)

Burley tobaccos are air-cued by being hung in well-ventilated barns and allowed to dry over a period of four to eight weeks (Figure 2.3). This curing process is slowly carried on under virtually natural conditions in which the temperature and humidity can be carefully controlled. These varieties are grown in the lower-northern region (for example, Sukhothai) of the country. Burley varieties represent 5% of tobacco cultivation in Thailand. Examples of Burley varieties usually cultivated in Thailand are Burley-21, Burley-37, and etc. (วรวิทย์และคณะ, 2549).

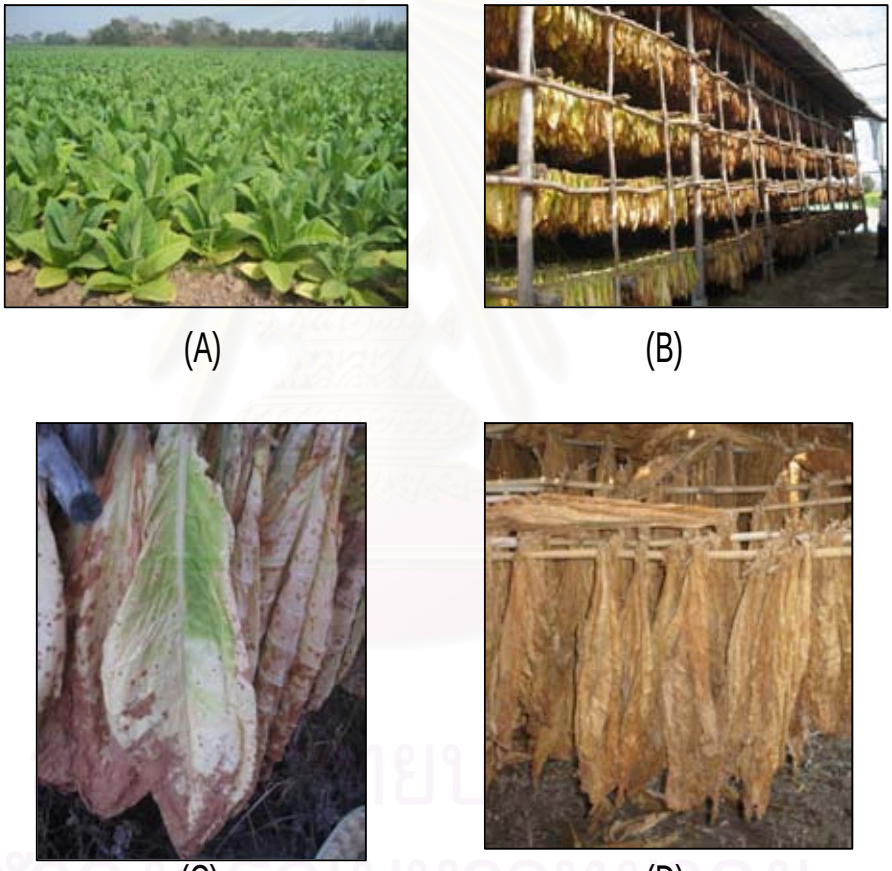


Figure 2.3 Burley tobacco varieties.
(A) Burley tobacco varieties in the field.
(B) The air curing-barn of burley varieties in Nong Khai province.
(C) The leaves were changed from green to yellow.
(D) Dried burley varieties after air curing processed.

2.2.1.3 Turkish varieties (or oriental tobacco)

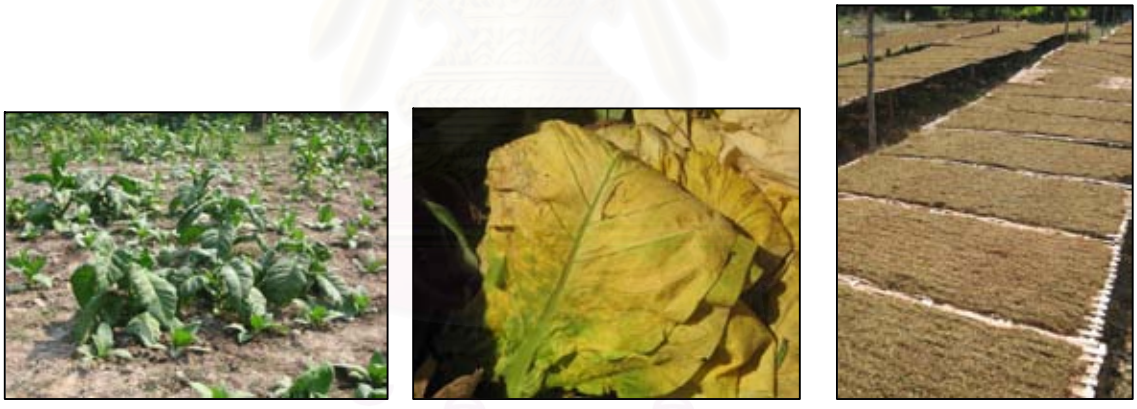
Turkish varieties or oriental tobaccos are generally very short. Leaves are two or three inches wide. Oriental tobaccos are grown in Northeast of Thailand (for example, Roi Et, Maha Sarakham, Nakhon Phanom, Khon Kaen and Kalasin). Their leaves contain high aroma and low in both sugar and nicotine. Leaves are dried by being strung out on racks and exposed to the sun (Figure 2.4). The whole process takes from 12 to 30 days. The sun's direct heat fixes the leaves at a yellow to orange colour resulting from the high sugar content in the leaves. The best known varieties of Turkish tobaccos are Samsun, Basma, and Izmir (Murray, 2007).

(A) (B) (C)

Figure 2.4 Turkish tobacco varieties.
(A) Morphological characteristics of Turkish varieties in the field in Nakhon Phanom province.
(B) Leaf characteristics of Xanthiyaka varieties.
(C) Turkish leaves drying in the sun at Na Njam (นางาม) village of Nakhon Phanom province.

2.2.2 Local tobacco varieties (or air-and sun-cured tobacco)

Local tobaccos usually have a stalk with large leaves drooping off the main stem similar to Virginia and Burley varieties. These local varieties are grown in the northern region (for example, Phrae, Uttaradit, and Sukhothai), the southern region (Nakhon Si Thammarat) and the central (Kanchanaburi) of Thailand. The tobacco farmers usually cultivate the plants in lightly sandy loams near riverside areas (Figure 2.5). During cultivation of the tobaccos, the terminal buds are removed, an operation known as topping, in order to keep the leaf strength. The curing process includes three steps. In the first step, the leaves were air-cured about four-to-five days, cut into one-to-two inches strips, and sun-dried about two days on racks called “gra-tae” (กระแตะ) or “pang” (แผง). Local tobaccos are usually used to make “ya-sen” (ยาเส้น), a dried local tobacco product. Examples of popular varieties of the local tobaccos are “E-dum” (Phetchabun), “Petkhangsink” (Sukhothai), “Kanchanaburi” (Kanchanaburi), and etc.



(A) (B) (C)

Figure 2.5 The local tobacco varieties.
(A) Morphological characteristics of local varieties in the field at Nakhon Phanom province.
(B) The leaves were changed to yellow after air-curing processed.
(C) Ya-sen (ยาเส้น) was sun-dried on “gra-tae” (กระแตะ) in Nong Khai province.

2.3 Molecular markers

Analyses of genomic DNA are commonly performed in plants studies. Precise genotypic identification and assessment of genetic diversity germplasms are especially important for plant breeding purpose. Traditional identification methods have been based on morphological traits of leaf, flower, and fruit. This approach is problematic since closely related cultivars often cannot be distinguished by observation of their morphology. Expression of morphological traits is also affected by environmental factors (Kuras, Korbin, and Zurawicz, 2004).

For these reasons, distinguishing and identification of plant cultivars need modern methods which are more effective, rapid, informative, and independent on environmental factors than the traditional one. Among various techniques developed over the past two decades, molecular or genetic markers can be more powerful than morphological or characters for plant cultivar identification (Terzopoulos et al., 2005).

2.3.1 Biochemical markers

Biochemical markers, such as isozyme electrophoresis, have been successfully utilised to study molecular phylogenetics in Zingiberaceae (Vanijajiva, 2001). However, the isozyme techniques is less useful than DNA markers (2.3.2) in classifying plant specimens because of the small number of available loci providing limited coverage of the genome (Brantestem et al., 2004).

2.3.2 DNA markers

DNA markers provide a powerful tool for genetic evaluation and breeding of crops, especially for identification of cultivars and species, phylogenetic evaluation, and analysis of genetic diversity and DNA variation (Souframanien and Gopalakrishna, 2004).

The first DNA marker ever applied was Restriction Fragment Length Polymorphism (RFLP) and served as reliable markers for genetic analyses in many plants (Awasthi et al., 2004). RFLP 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 (Figure 2.6). Conventional RFLP

analysis is carried out by digestion of genomic DNA with a specific endonuclease. Sizes of the products are fractionated using gel electrophoresis. This gel is Southern blotted onto a membrane, and specific fragments are made visible by hybridisation with a labeled probe (Kinbantom, 2004).

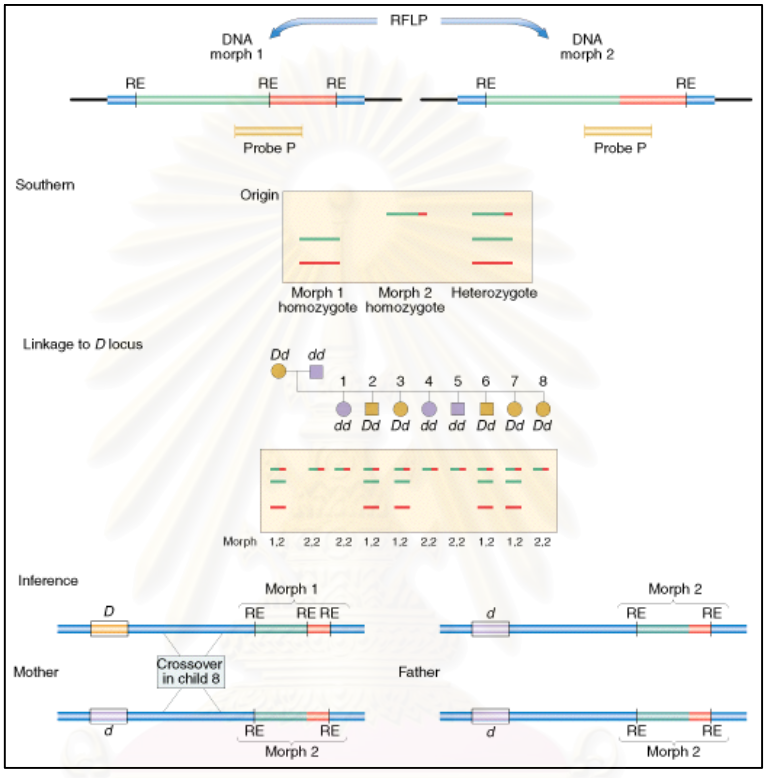


Figure 2.6 Principle of RFLP (Restriction Fragment Length Polymorphism). (Source: http://www.usask.ca)

In 1985, a new technique was introduced which revolutionised molecular biology methods: the Polymerase Chain Reaction (PCR). This technique allows us to amplify any DNA sequence of interest to high copy numbers (Weising et al., 1995). The amplification reaction consists of three steps (Figure 2.7):

1) denaturation: double-stranded DNA is denatured at high temperature to form single strand (template).

2) primer annealing: short oligonucleotide primers bind at a lower temperature to the single-stranded complementary templates.

3) extension: the temperature is raised for synthesis of targeting sequence by primer extension.

The cycle is repeated for 25-50 times. PCR is effective approach for population genetic and systematic studies because sometimes the source of DNA from herbarium specimens is ranged quality from normal to several degraded.

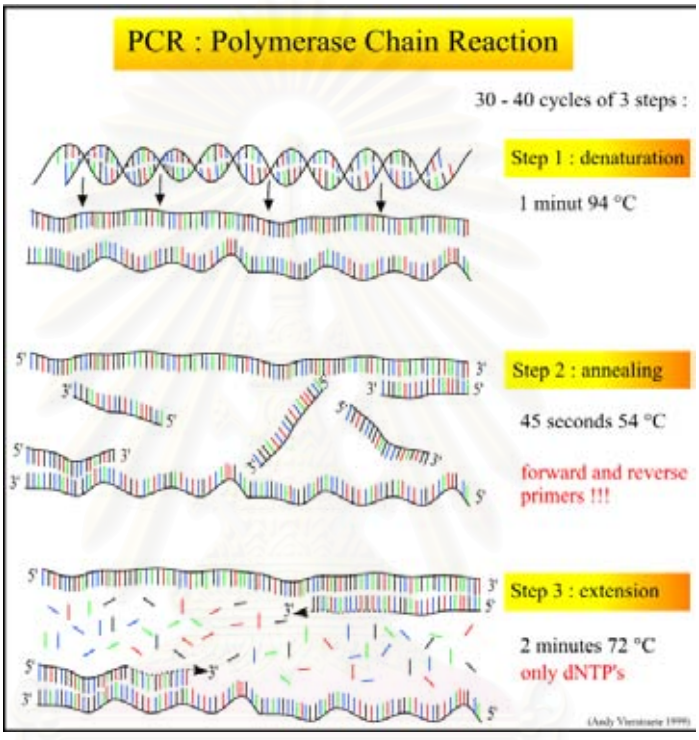


Figure 2.7 Three steps of PCR (Polymerase Chain Reaction).
(Source: http://users.ugent.be/avierstr/principles/pcr.htm)

With the development of PCR techniques, molecular marker systems based on PCR have emerged as major molecular tools for various genetic analyses. Common PCR-based marker systems include Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter Simple Sequence Repeat (ISSR) and Amplified Fragment Length Polymorphism (AFLP) analysis (Kumar et al., 2001). Each marker technique has its own advantages and disadvantages.

2.3.2.1 RAPD (Random Amplified Polymorphic DNA)

In 1990, William et al. reported a novel identification technique based on random amplification of genomic DNA sequences by PCR using single, short arbitary primers (usually ten base-pairs). This technique is commonly called RAPD-PCR (Figure 2.8). RAPD priming sites are thought to be randomly distributed throughout a genome and the DNA polymorphisms in these priming regions result in differing amplified products (Filippis, Hoffmann, and Hampp, 1996). Some other advantages of this technique include no knowledge of the plant's DNA needed, its ability to scan and detect extensive polymorphism, its simplicity and rapidity, no radioactivity used, and very small amounts of genomic DNA required. However, the RAPD technique is often criticised for its lack of reproducibility over time as well as between laboratories (Arcade et al., 2000). RAPD technique has been widely applied in phylogenetics, population genetics, and species different studies (Sreekumar and Renuko, 2006).

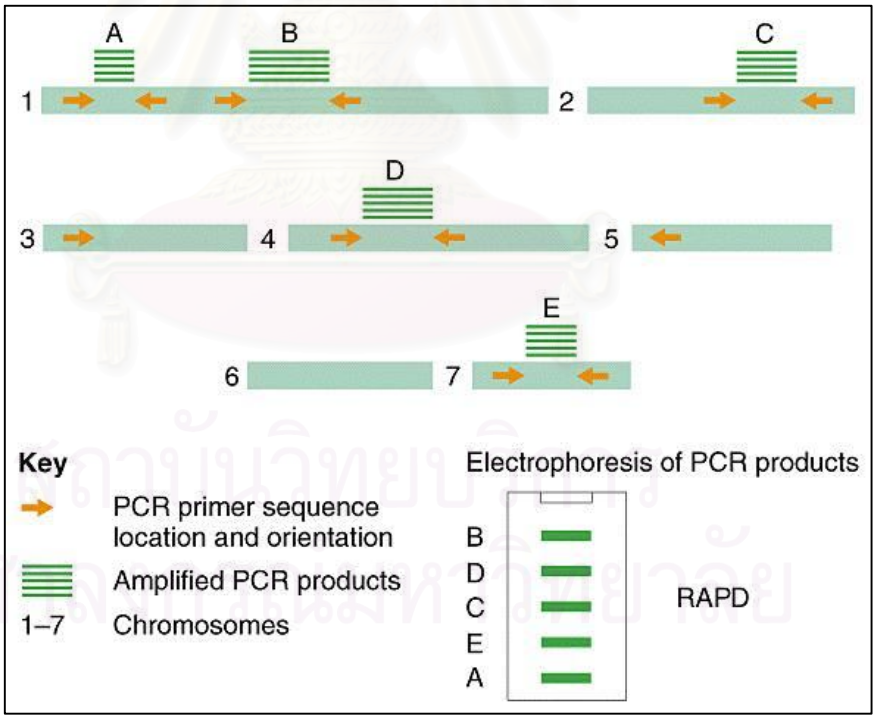


Figure 2.8 Principle of RAPD (Random Amplified Polymorphic DNA).
(Source: http://www.usask.ca/agriculture/plantsci/classes/plsc416/projects_2002/pawlin/resources/rapds.html)

2.3.2.2 SSR (Simple Sequence Repeat)

Simple sequence repeats (SSRs) or microsatellites are short (1-5 bp.), tandemly repeated DNA sequences that are abundant and randomly interspersed in eukaryotic genomes. Changes in number of the repeats give the length polymorphism which could be revealed by PCR primers designed for the sequences flanking the microsatellite repeat motif (Figure 2.9).

The SSR-PCR method requires very small quantity of DNA (~5 ng/ reaction). In addition, this method provides co-dominant, highly polymorphism, rapidly typed via PCR (Maughan, Maroof, and Buss, 1995). However, an analysis of the SSR-PCR requires prior characterisation of the flanking sequences to allow the primer design for PCR amplification. High development cost is a major impediment to a routine application of SSR-PCR in the genetic study to identify SSR markers located in the interested chromosomal regions of non-commercial species (Hayden and Sharp, 2001).

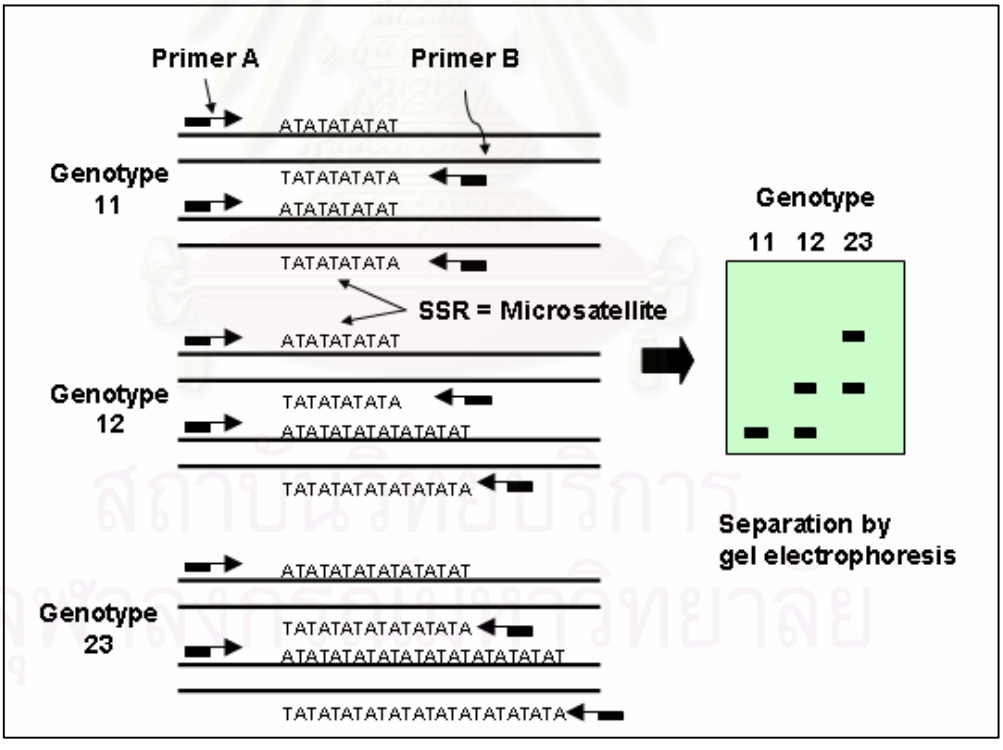


Figure 2.9 Principle of SSR (Simple Sequence Repeat).
(Source: http://www.nal.usda.gov/gpdic/Probe/v2n1/simple.html)

2.3.2.3 AFLP (Amplified Fragment Length Polymorphism)

AFLP, a promising new technique, was developed by Vos et al. in 1995. It is based on the principle of selective amplification of a subset of restriction fragments from a complex mixture of DNA fragments (Figure 2.10). The restriction fragments obtain after a digestion of the genomic DNA with restriction enzymes and then a ligation with adapters using a pair of primers with two-to-three selective nucleotides. AFLP fragments can be resolved with either a manual polyacrylamide gel electrophoresis or with the help of an automated genotyper. AFLP is considered to be more stringent than RAPD (2.3.2.1) due to the use of longer primers, precise anchoring sequences and higher annealing temperatures (Bussell et al., 2005).

This AFLP technique has been widely used to genetic-mapping construction, fingerprinting, analysis of genetic relationship and genetic diversity (Baydar and Debener, 2004; Loh et al., 2000). The main disadvantage of AFLP-PCR is a difficulty in identifying homologous markers (alleles), rendering this method to be less useful for studies that require precise assignment of allelic states, such as heterozygosity analyses. This high-resolution AFLP method also requires training and some laboratory set-up costs (Mueller and Wolfenbarger, 1999).

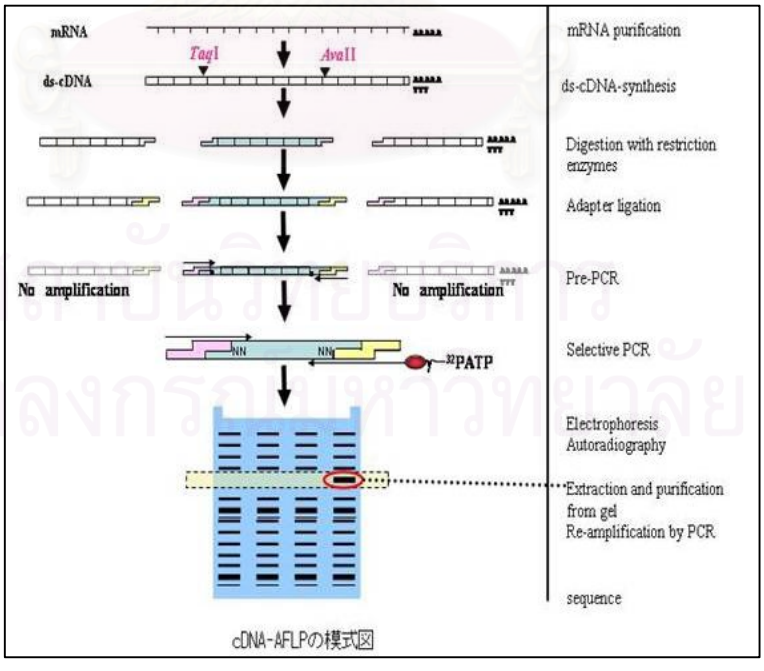


Figure 2.10 Principle of AFLP (Amplified Fragment Length Polymorphism). (Source: http://www.agbi.tsukuba.ac.jp/~tatsujin/akihiro/htmlfiles/main.htm)

2.3.2.4 ISSR (Inter-Simple Sequence Repeat)

As mentioned before (2.3.2.2), Simple Sequence Repeat (SSRs) or microsatellites are usually proportionally dispersed in the genome. However, the regions with a greater abundance of these SSR sequences have been found and are named “SSR hot spots”. Such regions can serve as a source of ISSR marker.

The first studies employing ISSR markers were published by Zietkiewicz et al. in 1994. The ISSR technology is based on the amplification of 100-3000 bp. region between inversely oriented, closely spaced microsatellites (Figure 2.11). The ISSR technique is similar to that for RAPD, except that the ISSR primer consists of di- or trinucleotide (15-30 bp.) simple sequence repeat motif with a 5’ or 3’ anchoring sequence by two-to-four arbitrary selective nucleotides (Bussell, Waycott, and Chappill, 2005). The ISSR technique usually amplifies 25 to 50 PCR products in one reaction. The ISSR-PCR products generated thus reveal multilocus profiles which could be resolved with either agarose or polyacrylamide gels. The number of product bands was produced by the ISSR primer with the given microsatellite repeat should reflect the relative frequency of that motif in a given genome (Bahulikar et al., 2004).

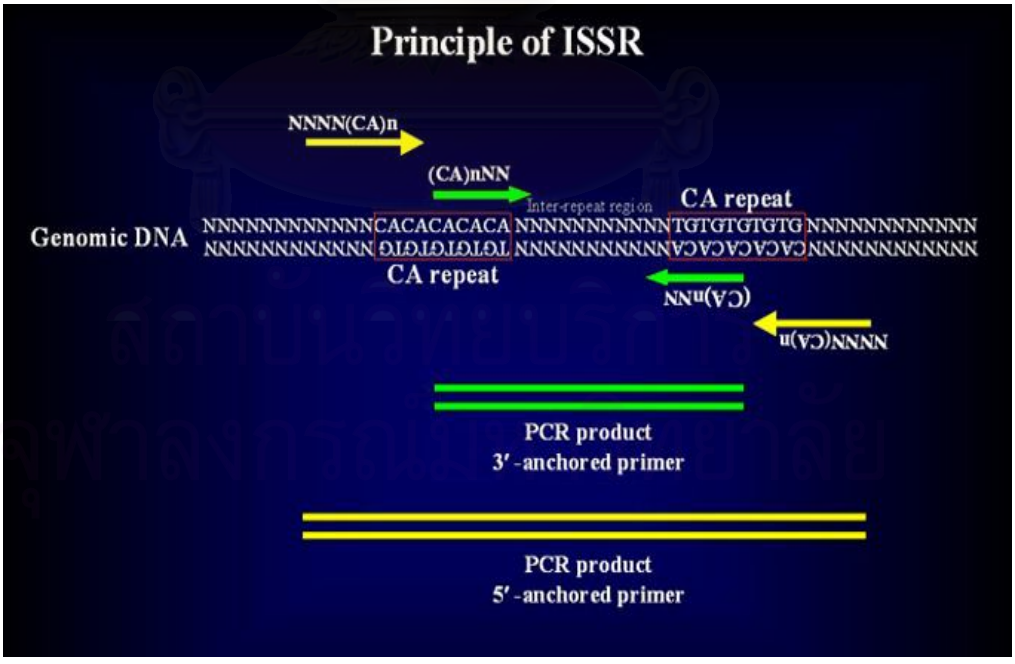


Figure 2.11 Principle of ISSR (Inter-Simple Sequence Repeat).
(Source: http://210.212.212.7:9999/PHP/SILKSAT/index.php?f=protocol_issr)

The major advantage of this ISSR-PCR method is the fact that it does not require a time-consuming and expensive step of genomic library construction. In spite of the fact that the ISSR regions are mostly inherited as dominant, or rarely as codominant, genetic markers, they are thought to be highly useful for many different purposes (Trojanowska and Bolibok, 2004). They are random-typed DNA markers as the length of the intervening space between the microsatellites has randomly changed. Another benefit of the ISSR markers is a possibility to study SSR abundance and distribution in the genome.

Compared with other marker techniques, the ISSR primer sequences are usually larger, allowing for a higher primer-annealing temperature. This results in a greater band-reproducibility than RAPD markers, highly polymorphic and informative degree, therefore generating a reliable information for DNA analysis with necessary sensibility to distinguish among genetically related individuals. Moreover, this ISSR method requires no prior knowledge of the genome, cloning or specific primer-design and the cost of the DNA profile analyses is lower than that of AFLP (Lu et al., 2005; P?rez de la Torre and Escand?n, 2006).

For all of these reasons, ISSRs have been broadly and successfully used in many studies on genetic diversity, phylogenetics, genetic mapping and evolutionary evaluation in a wide range of plant species.

2.4 Molecular marker studied of Tobacco (*Nicotiana tabacum* L.)

Molecular techniques have been used for comparing the genotypes in many plants such as rice, barley, soybean, and also *Nicotiana* species. Numerous methods have been performed: RAPD, AFLP, and ISSR for this purpose.

Early works on molecular genetic relationships of tobaccos come from those of Filippis, Hoffman, and Hampp (1996). They used RAPD-PCR to analyse the relationship between two parental species of tobaccos, and six somatic hybrids produced as a result of fusion of vacuolated and evacuolated protoplast, and subsequent culture. The result showed that RAPD technology is a versatile, precise, sensitive, and cost effective method for genetic analysis of tobacco hybrids. In 2001, He et al. investigated genetic relationships of germplasms of 31 flue-cured tobacco cultivars also using RAPD.

Likewise, genetic and geographic polymorphisms of cultivated tobaccos (*N. tabacum*) in Turkey using RAPD method were studied by Arslan and Okumus (2006).

Furthermore, other molecular markers have been used to examine in greater detail of the evolutionary origin of tobaccos. In 2001, Ren and Timko developed AFLP markers to determine the degree of intra-and inter-specific genetic variation in the genus *Nicotiana*. Other AFLP markers were generated to identify bacterial-wilt-resistant tobacco breeds (Nishi et al. , 2003) and to conduct on set of 92 *N. tabacum* accessions from diverse types (flue-cured, dark air-cured, burley, oriental, and cigar wrapper) and breeding origin to identify markers associated with three disease resistance (blue mold, potato virus Y, and black root rot) (Julio et al., 2006). In 2000 and 2001, Rossi and Timko assessed RAPD and AFLP markers for investigation of the genetic diversity and identification of cultivated tobacco varieties. Their results indicate that tobacco varieties have low level of genetic diversity. The capability of AFLP to generate large number of polymorphic markers in a short period of time was suggested to be used in the future to construct a tobacco genetic map.

ISSR, is also one of molecular marker techniques studied in tobaccos. The evaluation of genetic diversity of flue-cured tobacco varieties based on the ISSR marker was studied by Yang (2005). These results of cluster analysis, UPGMA dendrogram of 24 varieties were divided into 5 major groups. In 2006, Qi et al. used ISSR to detect genetic diversity and genetic relatives analysis of tobacco germplasms. Sixteen primers were selected from 70 ISSR primers, and DNA fragments were amplified from 30 samples including 30 wild species and 27 cultivated species. The results indicated that the ISSR technique has better stability than RAPD, and is useful in revealing the genetic diversity and genetic relationship among tobacco species.

Since genetic analysis of tobacco (*Nicotiana tabacum* L.) varieties in Thailand have never been done before, therefore ISSR analysis should provide new powerful tools for the determination and investigation of genetic relationships between tobacco varieties.

2.5 Phylogenetic tree reconstruction method

Phylogenetic is the branch of systematics concerning with a reconstructing of a phylogenetic tree or phylogeny. A phylogeny is a genealogical history of a group of organisms, representating of hypothesised ancestor/descendant relationships. The phylogeny of organism is usually visualised as a branching pattern which can be determined by an analysis of characters from either living or fossillised organisms. Phylogenetic is a dynamic field in it own right, utilising phylogenetic principle and methodology with empirical advances driven by the computer revolution. A phylogeny is now easily to construct with several commercial programs such as PHYLIP, NTSYS, and PAUP* (Radford, 1986; Doyle and Gaut, 2000).

2.5.1 Phylogenetic tree

Phylogenetic tree is a particular kind of graph containing nodes connected by lines, usually called braches (for example, Figure 2.12). A particular node may be selected as a rooting point of the phylogenetic tree; but, this is not necessary since the tree may be rooted or unrooted. An unrooted tree shows a topology of relationships between organisms but not the pattern of ancestor/descend pathway. A Rooted phylogenetic tree is a move implicitly, directed graph, implying the ancestor-descendant relationship along the direction of each branch. The total length of all braches through the phylogenetic tree is the sum of the tree lengths. In phylogenetic trees, branch lengths signify either some measure of the similarity (dissimilarity) between two species, or the length of time since their separation (Lesk, 2002).

2.5.2 Evaluation of DNA fragment patterns

A fingerprint pattern originating from different DNA samples has to be compared to each other. Individual bands within a lane are assigned to particular positions and different lanes are screened for comigrating bands. The preciseness and accuracy of band-scoring are critically dependent on methodological parameters, including DNA quality, completeness of PCR reaction, and electrophoresis conditions (Weising, 1995).

DNA fragment patterns may either be evaluated by the eyes and hands of the investigator or by automated methods. Fragment sizing and matching by eyes are most often done by scoring a photo of the UV-illuminated gel directly, usually with the help of a transparent ruler. The DNA banding patterns are scored in term of presence (1) or absence (0) of polymorphic bands in each population (Ren and Timko, 2001).

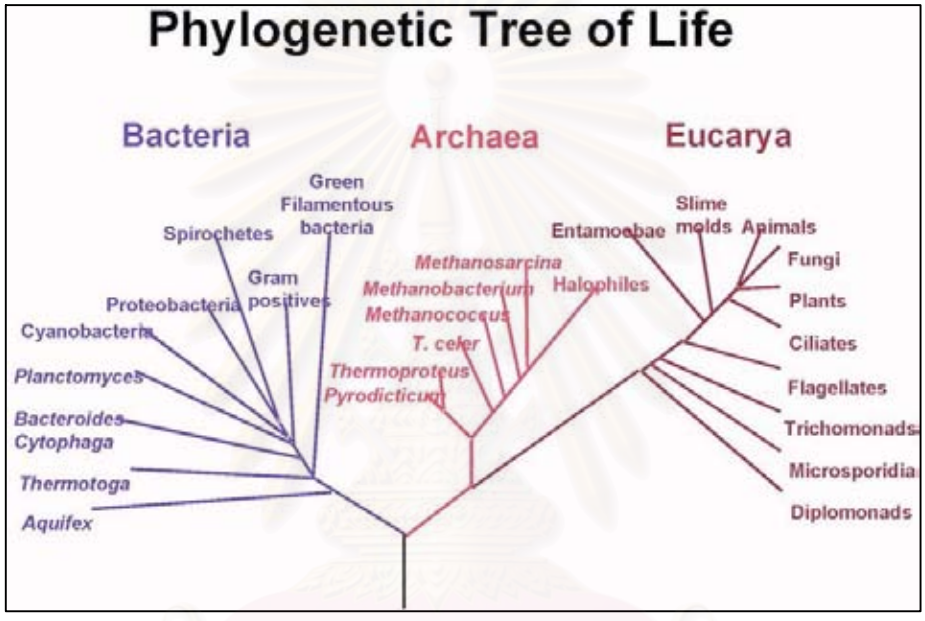


Figure 2.12 A phylogenetic "tree of life" constructed by computer analysis of cytochrome-C molecules in the organism.
(Source: http://darwin.nmsu.edu/~mol470/fall2005/projects/pan)

2.5.3 Similarity index

Once the fingerprint patterns have been generated and scorable bands are assigned to specific positions in all lanes to be compared, different strategies may be followed to quantify the pairwise similarity of the genotypes represented in different lanes. Most commonly, a "similarity index" is calculated from band-sharing data of each pair of fingerprints (Weising, 1995). The similarity matrix is used to assess relationships among species and populations with a dendrogram (tree diagram) by cluster analysis.

There are several similarity coefficients use in molecular marker analysis. The coefficient formula can acquire any value between 0 and 1, where 0 means “no bands in common” and 1 means “pattern is identical”. Examples of the similarity coefficients are as follow:

1) Jaccard’s coefficient (J)

J = a / (a+b+c)

where: a = the number of 1-1 matches
b = the number of 1-0 matches
c = the number of 0-1 matches
(1 = band present, 0 = band absent)

2) Nei’s and Li’s coefficient (N)

N = 2a / (a+b) (a+c)

where: a = number of bands present in both individuals.
b = number of bands present in individual A absent in individual B
c = number of bands present in individual B absent in individual B

3) Dice’s coefficient (D)

D = a / (2a+b+c)

where: a = number of bands present in both individuals.
b = number of bands present in individual A absent in individual B
c = number of bands present in individual B absent in individual B

4) Simple Matching coefficient (SM)

SM = (a+d) / (a+b+c+d)

where: a = the number of 1-1 matches
b = the number of 1-0 matches
c = the number of 0-1 matches
d = the number of 0-0 matches
(1 = band present, 0 = band absent)

The coefficient of Jaccard (J) and that of Nei and Li (N) compare the number of bands shared between individuals or populations. Jaccard's coefficient is the most simple but the coefficient of Nei and Li puts more weight on positive matches. These methods are advantageous because the band absence can be excluded from analyses and there are no assumptions of Hardy-Weinberg equilibrium (Cully, 2007).

Dice's coefficient (D) gives more weight to matches than to mismatches. It also has a direct biological meaning: it is an estimate of the expected portion of amplified fragments shared by two samples due to inheritance from a common ancestor (Soltis, 2000). Simple Matching coefficient (SM) is similar to Jaccard's coefficient, but SM method incorporates band absence (d) in the formula.

2.5.4 Tree construction

The aim of reconstructing a phylogenetic tree (or dendrogram) is to give the best estimate of relationship and past evolutionary history among individuals, cultivars or populations within species. The three commonly used optimal criteria for phylogeny reconstructions are maximum parsimony, maximum likelihood, and distance matrix.

2.5.4.1 Maximum Parsimony

Maximum parsimony method can be viewed simply as an optimality criterion for which the optimal tree is a tree with the smallest number of mutational changes. Parsimony assumes that trees with the minimum number of evolutionary changes are the most preferable trees. This method bases on the number of character-state changes to construct all possible trees and give each a score. The most parsimonious tree is that with fewest character-state changes (for example, Figure 2.13).

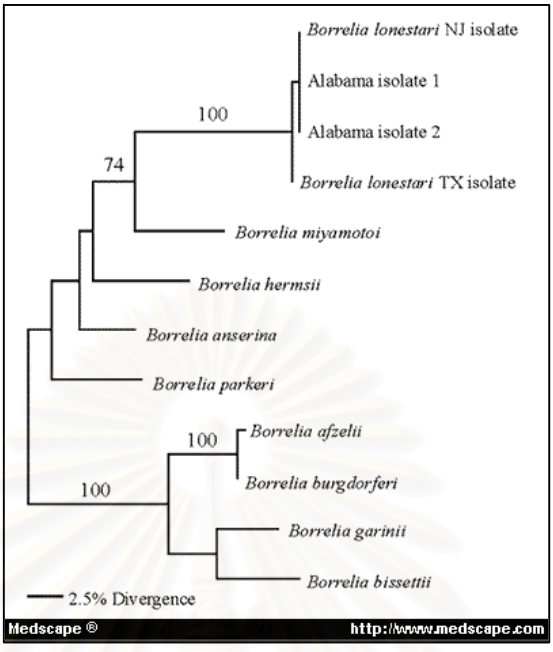


Figure 2.13 Example of maximum-parsimony phylogenetic tree generated by using PAUP*.
(Source: http://www.medscape.com/viewwarticle/414446_2)

2.5.4.2 Maximum Likelihood

Maximum likelihood is a family of statistical approaches commonly used throughout the biological sciences. Maximum likelihood uses an explicit model of evolution to reconstruct a phylogeny. It uses statistical tools to evaluate a hypothesis about evolutionary history, that is, it constructs all possible trees of evolutionary history from an observed data set and assumes that a preferred history is that with highest probability of reaching the observed states.

2.5.4.3 Distance matrix

This method is usually referred to as a cluster analysis. The distance method is an approach to reconstruct a phylogeny begining with an estimation of pairwise distances between data. Only one optimal dendrogram is given and there is no method for comparing or ranking suboptimal dendrograms. The clustering methods are computationally simple and can be used with large data sets (Weising, 1995). These

approachs are not explicitly character-based because a pairwise distance matrix, rather than changes at individual nucleotide position, is used in the tree building process (Doyle and Gaut, 2000).

2.5.4.3.1 Unweighted Pair Group Method with Arithmetic mean (UPGMA)

UPGMA is the simplest approach among the clustering methods. Its original purpose was to construct taxonomic phenograms, which are trees that reflect the phenotypic similarities between operational taxonomic units (OTUs). It uses clustering approach and unrooted data to build a tree (for example, Figure 2.14). This method is only suitable with datasets consisting lineages with relatively constant rates of evolution. The algorithm used to utilise a UPGMA distance matrix constructed from an allelic profile data only (Hoang, 2005).

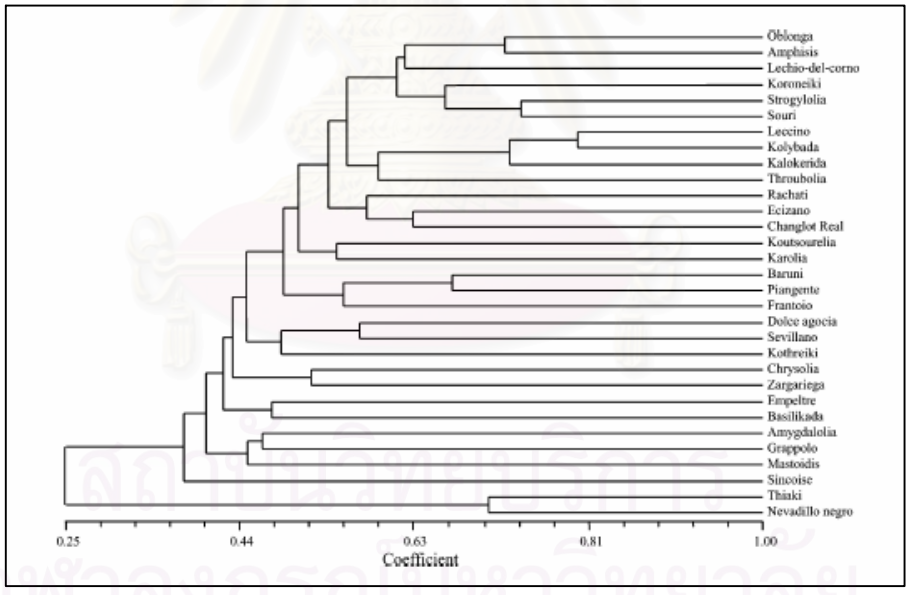


Figure 2.14 UPGMA dendrogram of 31 cultivars of olive trees based on data from ISSR primers UBC-849 and UBC-818 combined.
(Source: Terzopoulos et al., 2005)

Advantages

- Simple and fast method.
- Extensively used in literature.

Disadvantages

- Used to construct phylogenetic tree of only taxa with the relatively constant rate of evolution.
- Do not reflect evolutionary descent.

2.5.4.3.2 Neighbour-Joining (NJ)

Neighbour-joining techniques is a star decomposition method, that is the phylogenetic tree is constructed from a star-like tree by grouping OTUs with the shortest distance of a branch length together (for example, Figure 2.15). This method is very suitable with dataset consisting descendants with largely varying rates of evolution. The very fast neighbour-joining algorithm provides a good approximation of the minimum evolution tree and is available in many software packages, such as MEGA and PAUP*. NJ method is preferred for distance analyses over the older and even more widely available UPGMA algorithm because, unlike UPGMA, it does not assume that all the sequences evolve at the same rate (Doyle and Guat, 2000).

Advantages

- Relatively rapid, so it is suitable for analysing a large dataset.
- Calculate the branch length.
- Allow to correct multiple sequences.
- Construct only one possible tree.

Disadvantages

- Yield a biased tree under some condition.
- Compress sequence information.
- Depend heavily on the evolutionary model applied.

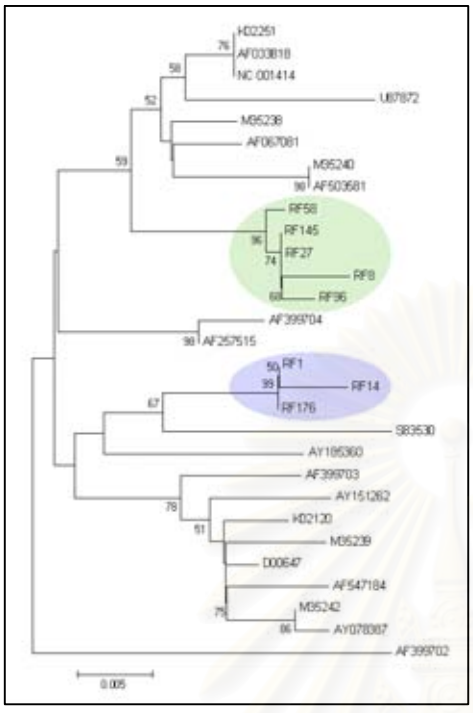


Figure 2.15 Neighbour-Joining (NJ) phylogram of *Bruella indicadas*. (Source: http://www.inia.cl/bioanimal/bovdis.html)

2.5.5 Tree evaluation

The bootstrap analysis is one of tree evaluation methods used to provide some measure of support for each branch in the tree, which introduced by Felsenstein (1985). Bootstrap values are upon statistically resampling (with replacement) analyses from the original data matrix. Other characters are randomly chosen to compose a matrix with the same size as the original one. The effect is to delete some characters randomly and to reweight others randomly with the constraint that the sum of the weights for all characters aquals to the number of characters in the original matrix. The replicates are then subject to phylogenetic analysis, yielding a series of bootstrap trees. Those results are conventionally summarised with a majority-rule component consensus of the bootstrap trees. Group frequencies greater than 50% are indicated as posterior probability (%) nodes. A bootstrap value of 90% along any branch of the tree diagram means that the branch (or clade) appeared in 90% of the resamples, and presumably that branch is better statistically supported than another branch with a boostrap value of 80%. The benefit of this method is that the bootstrap analysis is easy to calculate and widely used.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Fresh tobacco leaves of 53 imported and two local varieties were obtained from Maejo Tobacco Experiment Station, Chiang Mai province (Table 3.1). The seeds of imported tobacco varieties were planted in seed beds and the young plants were collected for this experiment when they had aged about 3-4 weeks. Additionally, 12 local cultivars were sampled from four provinces (Table 3.2) (Sukhothai, Nong Khai, Nakhon Phanom and Nakhon Si Thammarat) during January 23th - Febuary 7th, 2007 with help of Thailand Tobacco Monopoly, Ministry of Finance, Thailand (Figure 3.1). The collecting localities of the local tobacco varieties are showed in Figure 3.2. Fresh leaf materials of each variety were cut to small pieces (approximately 3x3 cm.) and kept separately in silica gel bags until DNA extraction.

Table 3.1 Imported and local tobacco varieties from Maejo Tobacco Experiment Station, Chiang Mai province.

Variety type	Number	Variety name
Turkish	9	Bafra, Basma, Basma xanthiyaka, Izmir, Samsun bafra, Samsun evkaf, Samxun maden, Xanthiyaka, and Zichan
Virginia	19	Coker-139, Coker-206, Coker-254, Coker-319, Coker-347, Coker-371 gold, K-149, K-317, K-326, K-346, K-394, K-399, Speight G-52, Speight G-70, Speight G-140, Speight NC-82, NC-2326, NC 37 NF, and NC 89
Burley	17	Burley-21, Burley-64, Ky-9, Ky-10, Ky-15, Ky-17, Ky-8959, Ky-907, Ky-908, MS Ky-14 x L-8, TN-86, TN-90, TN-97, Va-182, Va-509, Va-528, and VS Burley-21 x Ky-9
Local	2	Chor-lare 1 and Chor-lare 2
Unclassified	8	MC Nair 135, Baisee, Blanket A1, C.N.T, C.S.T, CDL 28, Dimon 1, and E-bit

Table 3.2 Local tobacco varieties sampled from four provinces in Thailand.

Variety name	Cultivated areas	Collecting locality (district, province)
1. Nison (นิสัน)	Riverside of Yom river	Si Samrong, Sukhothai
2. Petkhangsink (เพชรแข้งสิงห์)	Riverside of Yom river	Mueang, Sukhothai
3. Pu 001 (พู 001)	Riverside of Khong river	Phon Phisai, Nong Khai
4. Pu 002 (พู 002)	Riverside of Khong river	Phon Phisai, Nong Khai
5. White gold (ไวท์โกลด์)	Riverside of Khong river	Tha Bo, Nong Khai
6. Napanang (นาพนัง)	Riverside of Khong river	That Phanom, Nokhon Phanom
7. E-bit (อีบิด)	Riverside of Khong river	That Phanom, Nokhon Phanom
8. Ya-glai (ยากลาย)	Edge of reservoir	Tha Sala, Nakhon Si Thammarat
9. Ya-chun (ยาจุน)	Edge of irrigating canel	Srichon, Nakhon Si Thammarat
10. Ya (ยา) (Local x Kaset)	Edge of irrigating canel	Srichon, Nakhon Si Thammarat
11. Local Nakhon Si Thammarat	Roadside of Asia road (highway no. 41)	Chulabhorn, Nakhon Si Thammarat

(A) (B)

Figure 3.1 Tobacco leaf sample collection from one tobacco crop in Sukhothai province. (A) Sample collection with help from staffs of the provincial tobacco office (B) Collection of young tobacco leaves into a plastic bag.

Figure 3.1 Collecting localities of the local tobacco varieties sampled from five provinces in Thailand. (1= Chiang Mai, 2= Sukhothai, 3= Nong Khai, 4= Nakhon Phanom and 5= Nakhon Si Thammarat)

3.1.2 Equipments

- Autoclave: model Conbraco (Conbraco Ind. Inc., USA)

- Automatic micropipette, P10, P20, P200 and P1000 (Gilson, France)

- Centrifuge/vortex: model centrifuge FVL-2400 (Biosan, Latvia)

- Electronic UV transilluminator (Ultra Lum Inc., USA)

- Electrophoresis chamber set: model Mupid (Advance Co., Ltd., Japan)

- Microcentrifuge: model centrifuge Sorvall? pico D-37520 Osterode (Kendro Laboratory Products, Germany)

- Microcentrifuge tubes (0.5 and 1.5 ml.) (Axygen Scientific, Inc., USA.)

- Microwave oven: model Sharp Carousel R7456 (Sharp, Thailand)

- PCR machine: model GeneAmp? PCR system 9700 (Applied Biosystem, Singapore)

- pH meter: model Cybersean 500 (Eutech Cybernatics, Singapore)

- Pipette tips (10, 200, and 1,000 μl) (Axygen Scientific, Inc., USA.)

- Polaroid camera: model Direct screen instant camera DS 34 H-34 (Peca products, UK)

- Power supply: model EC570-90 LVD CE (E-C Apparatus corporation, USA)

- Vortex: model MS I Minishaker (IKA-Works, Inc., USA)

3.1.3 Chemicals

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

- Agarose gel (Research Organics,USA)

- Boric acid (Research Organics, USA)

- Bromophenol blue, $C_{19}H_{10}Br_4O_5S$, M.W. = 670 (Research Organics,USA)

- DNA Ladder Marker 100 bp (SibEnzyme, Russia)

- DNeasy? P lant Mini Kit (QIAGEN UmbH, (Germany)

- EDTA (Ethylene diamine tetra-acetic acid), $C_{10}H_{14}N_2O_8Na_2.H_20$, M.W. = 372.24 (Bio Basic, Inc., USA)

- Ethidium bromide, M.W. = 934.32 (Bio Basic, Inc., USA.)

- HotstarTaq DNA polymerase (QIAGEN UmbH, (Germany)

- Metaphor? Agarose (Lonza, USA.)

- NuSieve? 3:1 Agarose (Cambrex Bio Science Rockland, Inc., USA.)

- Dynazyme Taq DNA polymerase (Finnzyme, Finland)

- Tris-base (Research Organics, USA)

- 6x loading dye (Glycerol 4 ml, Bromophenal blue 25 mg, and 1X TBE buffer upto 100 ml)

- 10x TBE buffer (Tris-base 108g, Boric acid 55g, 0.5 M EDTA (pH 8.0) 80 ml, and distilled water upto 1 Litre)

- 99.5% (v/v) Glycerol ($C_3H_80_3$) M.W. = 92.10 (Research Organics,USA)

3.1.4 Oligonucleotide primers

- Primer set No. 9, Biotechnology Laboratory, University of British columbia, Canada

- Bio Basic custom-synthesised primer, name UBC-807 and UBC-823 (Proligo Co., Ltd., Germany)

- BSU custom-synthesised primer, name UBC-836 (Bio Service Unit, Thailand)

3.2 Molecular marker experiments

For molecular marker developments, we began with genomic DNA extraction from the leaf materials, quality tests of the extracted genomic DNA, and ISSR-PCR amplification. After the PCR amplification experiment, the PCR reaction was optimised for higher specificity, and the banding data from PCR product patterns was then analysed genetic relationship in varieties.

3.2.1 DNA extraction

Genomic DNA extraction of all studied tobaccos was performed using Dneasy Plant Mini Kit to give rapid extraction and high quality extracted DNA. The extraction kit uses a DNeasy mini spin column filled with silica-gel membrane to isolate very pure DNA, free from inhibitory contaminants. Leaf powder grounded under liquid nitrogen was transformed to a 1.5 ml Eppendorf microcentrifuge tube and added with 400 μl of AP1 buffer and 4 μl of 100 mg/ml RNase A stock solution. The mixture was vortexed and incubated at 65?C for 10 minutes, also mixed several times by inverting the tube during incubation. One-hundred thirty microlitres of buffer AP2 were added to the tube, mixed, and incubated for 5 minutes on ice. The lysate was applied to a QIAshredder mini spin column set and centrifuged (14,000 rpm) for 2 minutes. A flow-through fraction was transferred to a new tube and 1.5 volumes of AP3 solution were added. Six-hundred fifty microlitres of the mixture were applied to the DNeasy mini spin column set, centrifuged (8,000 rpm) for 1 minute, and the flow-through was discarded.

The remaining sample was added to the spin column and centrifuged for another minute. The column was placed in a new tube and 500 μl of AW buffer were added. It was then centrifuged for one more minute, added with another 500 μl of AW buffer, and centrifuged (14,000 rpm) for 2 minutes. The spin column was transferred to a new tube and 50 μl of 65?C-preheated AE buffer were pipetted directly to the membrane of the spin column. The column was incubated for 2 minutes at room temperature and then centrifuged for one minute to elute the extracted DNA. Another 50 μl of the preheated buffer were added to elute the DNA for the second time. The extracted genomic DNA was then stored in -20?C freezer until used.

3.2.2 Agarose gel electrophoresis

The extracted genomic DNA was analysed by electrophoresis through 1% agarose gel in 1xTBE buffer. The agarose gel solution was boiled in a microwave oven to complete solubilisation, and cooled down to at least 60?C before being poured into an electrophoresis tray set with a plastic comb. When the gel was solidified, the comb was carefully removed. The agarose gel was submerged into an electrophoresis chamber containing ample amount of 1xTBE buffer.

Five microlitres of each genomic DNA were mixed with 2 μl of a loading dye and loaded into a well of the gel. One-hundred basepair ladder DNA was used as a standard DNA marker. An electrophoresis was normally operated at 100 Volt. for 45 minutes. The gel was stained with 0.5 mg/ml ethidium bromide solution and destained in ultra pure water. DNA bands were then be visualised and photographed under UV light with a UV transilluminator.

3.2.3 ISSR marker technique

3.2.3.1 Preliminary selection of suitable ISSR primers

After analysed the quantity with gel electrophoresis, the extracted genomic DNA was used for preliminary selection of suitable PCR primers. Twenty ISSR-PCR primers (as shown in Table 3.3) were screened to amplify ISSR regions of the genomic DNA of the tobacco varieties studied in two different aspects:

1) ISSR primers to determine relationship between imported varieties

First, sixteen primers (UBC-808, UBC-809, UBC-810, UBC-811, UBC-812, UBC-813, UBC-814, UBC-815, UBC-816, UBC-817, UBC-818, UBC-819, UBC-820, UBC-821, UBC-857, and UBC-873) were used to analyse genetic relationships between 24 pre-selectively imported tobacco varieties. These 24 imported tobacco varieties included four varieties of Turkish variety (Basma xanthiyaka, Xanthiyaka, Samsun maden, and Samsun evkaf), 11 varieties of Burley variety (Burley-21, Burley-64, Ky-15, Ky-8959, Ky-907, Ky-908, MC Nair 135, MS Ky-14 x L-8, Va-182, Va-528, and VS Burley-21 x Ky-9), and nine varieties of Virginia variety (Coker-139, Coker206, Coker-254, Coker-319, K-317, K-346, K-394, Speight G-52, and Speight NC-82).

2) ISSR primers to investigate band differences between imported and local tobacco varieties

Secondly, seventeen primers were used to distinguish between local and imported tobacco varieties. These were 11 of tobacco varieties tested in this experiment including two representative varieties of local tobaccos (Chorlare-1 and Chorlare-2), three Turkish varieties (Bafra, Basma xanthiyaka, and Samsun bafra), three Burley varieties (Ky-17, TN-86, and Va-116), and three Virginia varieties (Coker-48, K-326, and Speight G-140). The ISSR primers used in this experiment were UBC-807, UBC-808, UBC-809, UBC-810, UBC-813, UBC-814, UBC-815, UBC-816, UBC-817, UBC-818, UBC-819, UBC-820, UBC-823, UBC-835, UBC-836, UBC-857, and UBC-873.

The condition for all ISSR-PCR reactions in both primer selection studies was as follows: PCR reaction mixtures of 20 µl contained approximately 20 ng of template DNA, 2.5 unit of Dynazyme thermostable DNA polymerase and its optimised enzyme buffer (with 1.5 mM $MgCl_2$), 0.5 µl of 2.5 mM mixed dNTP, and 1 µl of each 15 µM ISSR-primer. When a number of reactions were performed in the same time, a master mix comprising all the reagents except the target DNA template was prepared and aliquoted to the reaction tubes. The DNA template was thereafter added as the last component before thermal cycling.

PCR amplification of the ISSR region was carried out in 0.2 ml microcentrifuge tubes in a PCR machine. Each PCR reaction was proceeded as follows: initial denaturation at 94?C for 5 minutes; 45 cycles of denaturation at 94?C for 1 minute, annealing at 42?C for 45 seconds, and extension at 72?C for 2 minutes; and final extension at 72?C for 5 minutes. The PCR products were detected by gel electrophoresis with 1.8% agarose gel with 1x TBE buffer at 80 Volt. for about 1 hour 10 minutes, stained with 0.5 mg/ml of ethidium bromide and viewed under ultra-violet light. Each PCR amplification reaction was repeated twice to ensure reproducibility. Only those primers that produce clearly unique DNA bands two or more times were selected for further study.

Table 3.3 Primers used in ISSR analysis and their sequences.

No.	Primer name	Sequence (5' to 3')	T_m (?C)
1	UBC-807	AGA GAG AGA GAG AGA GT	45.25
2	UBC-808	AGA GAG AGA GAG AGA GC	47.18
3	UBC-809	AGA GAG AGA GAG AGA GG	46.27
4	UBC-810	GAG AGA GAG AGA GAG AT	43.87
5	UBC-811	GAG AGA GAG AGA GAG AC	45.31
6	UBC-812	GAG AGA GAG AGA GAG AA	44.20
7	UBC-813	CTC TCT CTC TCT CTC TT	44.15
8	UBC-814	CTC TCT CTC TCT CTC TA	43.10
9	UBC-815	CTC TCT CTC TCT CTC TG	45.28
10	UBC-816	CAC ACA CAC ACA CAC AT	49.33
11	UBC-817	CAC ACA CAC ACA CAC AA	49.62
12	UBC-818	CAC ACA CAC ACA CAC AG	50.24
13	UBC-819	GTG TGT GTG TGT GTG TA	48.61
14	UBC-820	GTG TGT GTG TGT GTG TC	50.27

Table 3.3 Primers used in ISSR analysis and their sequences (continued)

No.	Primer name	Sequence (5' to 3')	T_m (?C)
14	UBC-820	GTG TGT GTG TGT GTG TC	50.27
15	UBC-821	GTG TGT GTG TGT GTG TT	49.61
16	UBC-823	TCT CTC TCT CTC TCT CC	46.31
17	UBC-835	AGA GAG AGA GAG AGA GYC	47.12-50.22
18	UBC-836	AGA GAG AGA GAG AGA GYA	45.41-49.32
19	UBC-857	ACA CAC ACA CAC ACA CYG	52.17-54.91
20	UBC-873	GAC AGA CAG ACA GAC A	46.00

*Y = (C,T)

3.2.3.2 PCR optimisation

After screening of suitable, highly polymorphic ISSR primers that can distinguish between local and imported tobacco varieties, PCR reaction was optimised using hotstart-PCR method with HotstarTaq DNA polymerase and Q-solution (QIAgen) for higher PCR specificity. The PCR condition was also improved by increasing annealing temperature. Five selected primers (UBC-807, UBC-809, UBC-813, UBC-823, and UBC-836) were used in this experiment to amplify the genomic DNA of two local varieties (Chorlare 1 and Chorlare 2) and nine imported tobacco varieties. The imported tobaccos were including three Turkish varieties (Bafra, Basma xanthiyaka, and Samsun bafra), three Burley varieties (Ky-17, TN-86, and Va-116), and three Virginia varieties (Coker-48, K-326, and Speight G-140).

Three steps of PCR optimisations were performed. First, HotstarTaq DNA polymerase was used for its benefit of having hotstart PCR ability by being in an inactive state with no polymerase activity at ambient temperature until incubated at 95?C for 15 minutes. This prevents the formation of misprimed non-specific PCR products and primer-dimers at low temperature. Secondly, the annealing temperature of the PCR condition was increased from 42?C to 45? to reduce non-specific amplification products. Last, the Q-solution of HotstarTaq DNA polymerase was added to the PCR reaction to reduce smear and to increase amplified yields of the PCR products. The Q-solution is a

PCR additive that facilitaties amplification of templates by modifying the particular primer-template pair.

Furthermore, two kinds of special, high resolution agarose gels were tested to improve the gel electrophoresis step. These two types of agorose gels chosen for this experiment were Nusieve 3:1 agarose (Cambrex Bio Science Rockland, Inc.) and Metaphor agarose (Lonza). They were used to increase clearness of the PCR band patterns after gel electrophoreses.

3.2.4 Genetic relationship analysis

Only those bands that were clear and reproducible were scored for genetic relationship analysis. ISSR bands were coded numerically as “1” when present and “0” when absent. Data sets derived from the respective PCR banding patterns were used to generate a data matrix for each reaction. Nei and Li’s coefficient (1979) was employed to calculate pairwise band similarities for samples using the PAUP* 4.0b10 software. Cluster analysis was used to construct a dendrogram using Unweighted Pair Group Method of Arithmatic Mean (UPGMA) and Neighbour-Joining (NJ) methods. Bootstrap analysis was also calculated based on 1,000 replicates to show the degree of confidence of each branch on the dendrogram. Bootstrap analysis was usually summarised with a 50% majority-rule component consensus tree. Only bootstrap values over 50% were considered significant and mentioned on the dendrogram. The constructed dendrograms revealed genetic relationships between tobacco varieties and the clustering patterns on the tree diagram were compared with other tobacco variety information.

CHAPTER IV
RESULTS

4.1 Collection of the local tobacco samples

Eleven local tobacco varieties were collected from four provinces of Thailand (Sukhothai, Nong Khai, Nakhon Phanom, and Nakhon Si Thammarat) during 23th January to 7th Febuary 2007. The morphological characteristics of each local tobacco variety are shown in Table 4.1.

Two local tobacco varieties found in Sukhothai were named “Nison” (นิสัน) and “Petkangsink” (เพชรแข้งสิงห์). Tobacco farmers and the officers of the provincial tobacco office gave some information of the local tobacco varieties. Nison’s leaf characteristics were similar to those of most Burley varieties. On the other hand, the characteristics of Petkangsink’s leave were like those of Turkish varieties as shown in Figure 4.1. Both Nison and Petkangsink varieties were mostly cultivated near riverside areas of Yom river.

In Nong Khai province, two local other tobacco varieties collected from Phon Phisai (โพนพิสัย) and Tha Bo (ท่าบ่อ) districts were “Pu” (พู) and “White gold” (ไวท์โกลด์) varieties, respectively. The characteristics of these two local varieties were similar to those of Virginia varieties (especially K-326 variety) as shown in Figure 4.2. Both tobacco varieties were usually cultivated near riverside areas of Khong river.

At That Phanom (ธาตุพนม) district of Nakhon Phanom province, “Napanang” (นาพนัง) and “E-bit” (อีบิด) were sampled as the representative local varieties of the area. They were also planted in tobacco crops nearby the Khong river. Napanang and E-bit varieties had leaves of Virginia (especially coker 187 hick) and Burley (especially Burley-14) varieties, respectively. Characteristics of these two local varieties were shown in Figure 4.3.

Last, four local varieties were taken from tobacco plantations in Nakhon Si Thammarat province, the southern part of Thailand, and were named “Ya-glai” (ยาก

ลาย), “Ya-chun” (ยาจุน), “Ya” (ยา) (Local x Kaset), and Local Nakhon Si Thammarat. Chactacteristics of these varieties were presented in Figure 4.4 Tobacco farmers usually grew these tobaccos at the edge of irrigating canels or reservoirs of the villages.

Table 4.1 Characteristics of local tobacco varieties collected from Sukhothai, Nong Khai, Nakhon phanom, and Nakhon Si Thammarat.

Variety	Characteristics	Similar to
1. Nison (นิสัน)	The plants aged about 3-4 weeks. Leaves were lanceolate and entire shaped with approximately 17-18 inches long.	Burley
2. Petkhansink (เพชรแข้งสิงห์)	The plants aged about 3-4 weeks. The shape of leaf was cordate and entire (approximately 6-7 inches long).	Turkish
3. Pu 001 (พู 001)	The collected leaf samples sprouted after the terminal buds were removed. These young leaves were lanceolate with 4-5 inches long.	Virginia: coker-411
4. Pu 002 (พู 002)		
5. White gold (ไวท์โกลด์)	The collected young leaf grew after the tobacco products were harvested. The plants aged about 4-5 months. Leaves were linear/lanceolate-shaped about 8-9 inches long.	Virginia: K-326
6. Napanang (นาพนัง)	The plants aged about 3-4 months and the terminal buds were removed. The young leaf samples grew on the top of the plants. The leaf shape was undulate and landceolate with approximately 5-6 inches long.	Virginia: Coker 187 hick

Table 4.1 (continued)

Variety	Characteristics	Similar to
7. E-bit (อีบิด)	The terminal buds of the plants were picked. The young leaves spouted between large leaves of the main stem were collected.These collected leaves were lanceolate in shape and oblique at the leaf base (approximately 6-7 inches long).	Burley: Ky-14
8. Ya-glai (ยากลาย)	The collected samples were the plantlets which were planted in seed beds near the reservoir of Glai (กลาย) village, Tha Sala district. They aged about one week and produced 1-3 leaves per plant (approximately 1-4 inches long).The leaves were ovate in shape and yellow-green in colour.	N/A
9. Ya-chun (ยาฉุน)	These plants were cultivated on para-rubber fields at the edge of irrigating canel of the village in Sichon district. The plants aged about 1-2 months. Leaves were lanceolate-shaped with 4-5 inches long.	Virginia
10. Ya (LocalxKaset) (ยา: พื้นเมืองxเกษตร)	The terminal buds of the plants were removed and they were ready to be harvested. The young leaves were grown on top of the plants. The shape of the collected leaves was non-symmetry lanceolate with approximately 4-5 inches long.	
11. Local Nakhon Si Thammarat (พื้นเมืองนครศรีธรรมราช)	The plant was found growing along the roadside of Asia road. Leaf characteristics were the same as Viginia's leaf. The leaf was lanceolate-shaped like Pu, Napanang, and Ya-chun varieties (approximately 3-4 inches in length).	Virginia & Burley

(A)

(B)

Figure 4.1 Morphological characteristics of the two local tobacco varieties collected in Sukhothai province: (A) Nison and (B) Petkhangsink.

(A)

(B)

(C)

Figure 4.2 Morphological characteristics of the two local tobacco varieties collected in Nong Khai province: (A) Pu 001, (B) Pu002, and (C) White gold.

(A)

.

(B)

Figure 4.3 Morphological characteristics of the two local tobacco varieties collected in Nakhon Phanom province: (A) Napanang and (B) E-bit.

(A) (B)

(C) (D)

Figure 4.4 Morphological characteristics of the four local tobacco varieties collected in Nakhon Si Thammarat province: (A) Ya-glai, (B) Ya-chun, (C) Ya (LocalxKaset), and (D) Local Nakhon Si Thammarat.

4.2 DNA extraction

Genomic DNA of young tobacco leaves was extracted using DNeasy? Plant Mini kit, which is easy, quick and convenience method. The extracted genomic DNA bands were sharp with high yields. Some extracted DNA samples were found (example Chorlare 1, Chorlare 2, and Va-509) containing RNA contamination visualised as smear band at the bottom of the agarose gel. Approximately 10-20 ng DNA were usually obtained from each specimen as shown in Figures 4.6 and 4.7

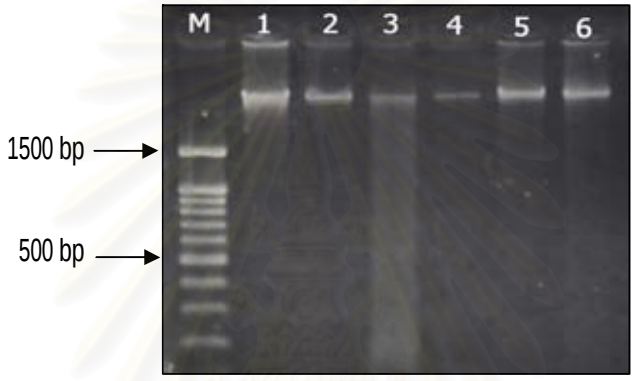


Figure 4.6 Genomic DNA bands of some imported and local tobacco varieties compared with 1.5 kb + 100 bp DNA marker (M = DNA marker, no. 1-6 = Chorlare 1, Chorlare 2, Va-509, Va-116, NC-89, and NC-2326, respectively).

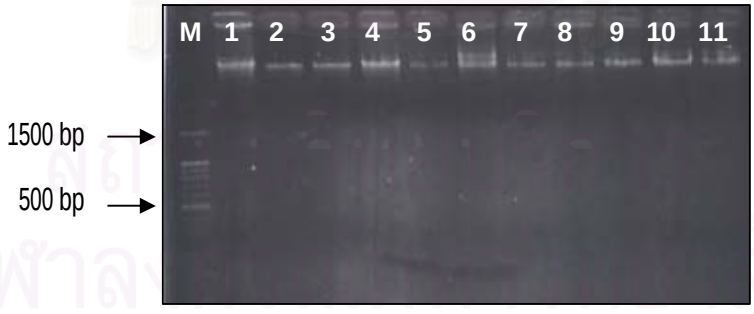


Figure 4.7 Genomic DNA bands of some local tobacco varieties compared with 1.5 kb + 100 bp DNA marker (M = DNA marker , No. 1-11 = Nison, Petkhangsing, Pu 001, Pu 002, Ya-glai, Ya (LocalxKaset), White gold, Napanang, E-bit, Ya-chun, and Local Nakhon Si Thammarat, respectively).

4.3 Selection of molecular markers using ISSR-PCR

4.3.1 Preliminary selection of suitable ISSR primers

4.3.1.1 ISSR primers to determine relationships between imported varieties

The genomic extracted DNA from leaves of 24 imported tobacco varieties were tested for the ISSR-region amplification with 3'-anchored microsatellite primers. Of 16 primers used, three primers did not produce any distinct band on the smeary background (UBC-809, UBC-811, and UBC-819) and another three primers resulted as very faint bands (UBC-814, UBC-815, and UBC-816). The number of polymorphic bands produced by each primer varied from one to 12 bands. The PCR fragment sizes ranged from 200 to 1600 bp. The highest number of polymorphic bands (8-12 bands) was produced by primers UBC-809 and UBC-818, whereas the one band was produced by the CAC ACA CAC ACA CAC AT repeat (primer UBC-816). Of all amplified profiles, four best primers (UBC-810, UBC-813, UBC-817, and UBC-818) giving highly polymorphic patterns were selected for further analysis of the genetic relationships among these 24 imported tobacco varieties.

From the amplification with four selected primers, the UBC-810 primer gave nine ISSR fragments (bands) which were estimated as 280, 400, 450, 550, 650, 800, 900, 1000, and 1200 bp. (Figure 4.8). Among all 24 samples, Samsun maden (lane 3), Va-182 (lane 13), and Va-528 (lane 14) tobacco varieties did not produce any amplified product at all. In the case of UBC-813 primer, seven ISSR fragments were generated and scored as 350, 450, 580, 600, 800, 900, and 1300 bp. (Figure 4.9). Most PCR patterns of both Turkish (lane 1-4) and Burley (lane 5-15) varieties are very much alike. Only two tobacco varieties, Coker-139 (lane 16) and Speight G-52 (lane 23) did not produce any amplified product.

Ten ISSR bands were generated by UBC-817 primer. They were approximately 450, 520, 580, 650, 800, 900, 1000, 1200, and 1300 bp. (Figure 4.10), whereas the UBC-818 primer gave 12 PCR fragments which were 280, 350, 400, 480, 520, 550, 650, 700, 800, 900, 1000, 1200, and 1400 bp. (Figure 4.11). From 24 tested tobacco samples almost all imported varieties were able to be amplified with the primer UBC-817, except Speight G-52 (lane 23, Figure 4.10) while the primer UBC-818 could

not amplify one variety: Samsun evkaf (lane 4, Figure 4.11). The number of amplifiable tobacco samples and number of PCR bands of each primer are summarised in Table 4.2.

Table 4.2 PCR products obtained from ISSR-PCR amplification with four selected primers suitable to determine genetic relationships between 24 imported tobacco varieties.

Primer name	Number of amplifiable tobacco samples	Number of PCR bands	Band sizes (basepairs)
UBC-810	22	9	280, 400, 450, 550, 650, 800, 900, 1000, and 1200
UBC-813	22	7	350, 450, 580, 600, 800, 900, and 1300
UBC-817	23	10	450, 520, 580, 650, 800, 900, 1000, 1200, 1300, and 1500
UBC-818	23	12	280, 350, 400, 480, 520, 550, 700, 800, 900, 1000, 1200, and 1400

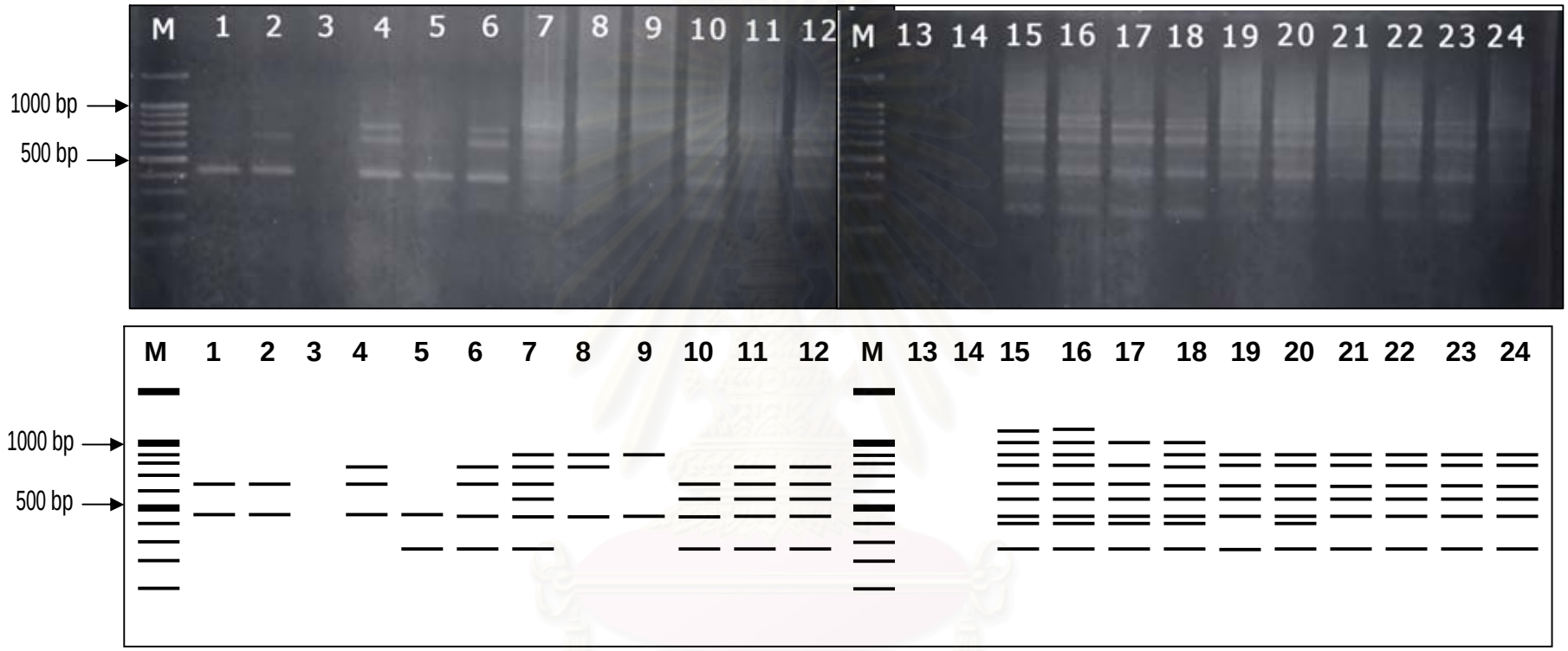


Figure 4.8 ISSR patterns obtained from 24 imported tobacco varieties amplified with primer UBC- 810 (Lane M = 1.5 kb + 100 bp DNA marker , No. 1-4 = Turkish varieties: Basma xanthiyaka, Xanthiyaka, Samsun maden, and Samsun evkaf, No. 5-15 = Burley varieties: Burley-21, Burley-64, Ky-15, Ky-8959, Ky-907, Ky-908, MC Nair 135, MS Ky-14 x L-8, Va-182, Va-528, and VS Burley-21x Ky-9, and No. 16-24 = Virginia varieties: Coker-139, Coker-206, Coker-254, Coker-319, K-317, K-346, K-394, Speight G-52, and Speight NC-82).

Figure 4.9 ISSR patterns obtained from 24 imported tobacco varieties amplified with primer UBC- 813 (Lane M = 1.5 kb + 100 bp DNA marker , No. 1-4 = Turkish varieties: Basma xanthiyaka, Xanthiyaka, Samsun maden, and Samsun evkaf, No. 5-15 = Burley varieties: Burley-21, Burley-64, Ky-15, Ky-8959, Ky-907, Ky-908, MC Nair 135, MS Ky-14 x L-8, Va-182, Va-528, and VS Burley-21x Ky-9, and No. 16-24 = Virginia varieties: Coker-139, Coker-206, Coker-254, Coker-319, K-317, K-346, K-394, Speight G-52, and Speight NC-82).

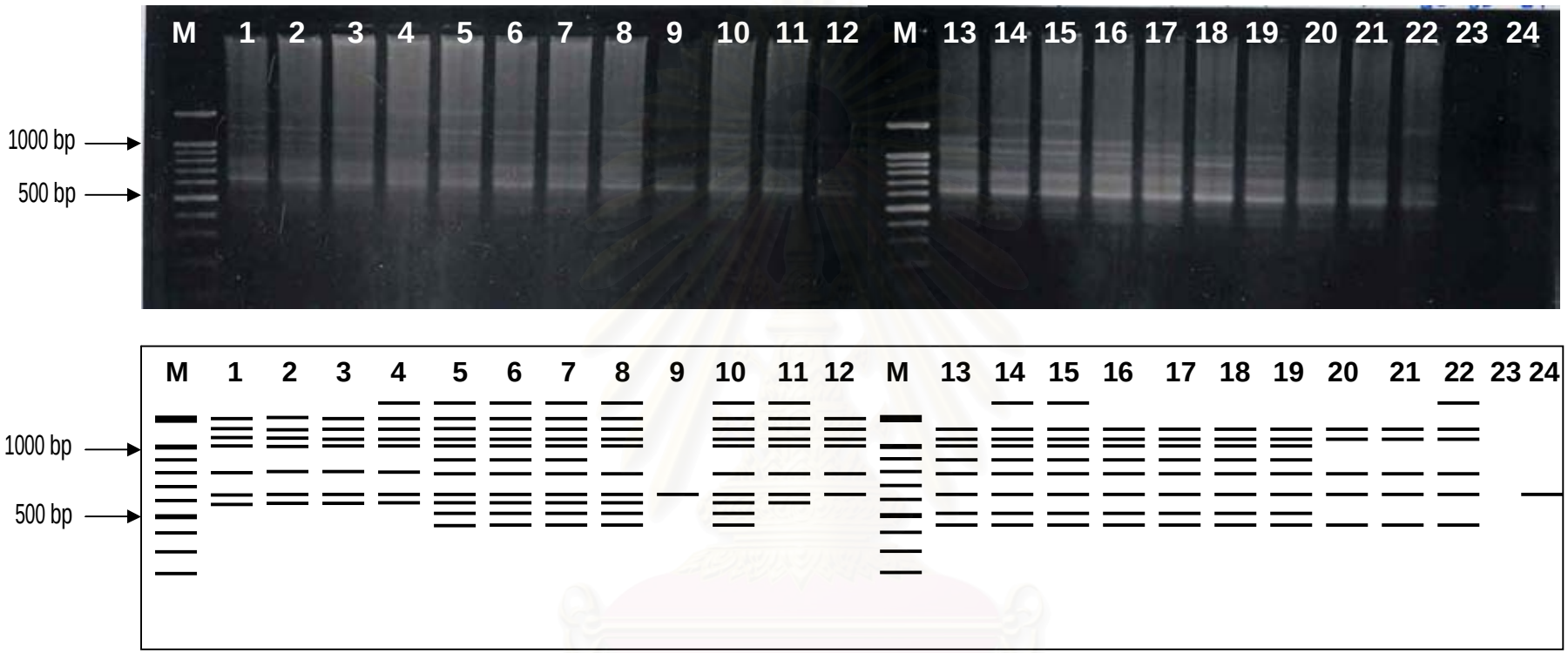


Figure 4.10 ISSR patterns obtained from 24 imported tobacco varieties amplified with primer UBC- 817 (Lane M = 1.5 kb + 100 bp DNA marker , No. 1-4 = Turkish varieties: Basma xanthiyaka, Xanthiyaka, Samsun maden, and Samsun evkaf, No. 5-15 = Burley varieties: Burley-21, Burley-64, Ky-15, Ky-8959, Ky-907, Ky-908, MC Nair 135, MS Ky-14 x L-8, Va-182, Va-528, and VS Burley-21x Ky-9, and No. 16-24 = Virginia varieties: Coker-139, Coker-206, Coker-254, Coker-319, K-317, K-346, K-394, Speight G-52, and Speight NC-82).

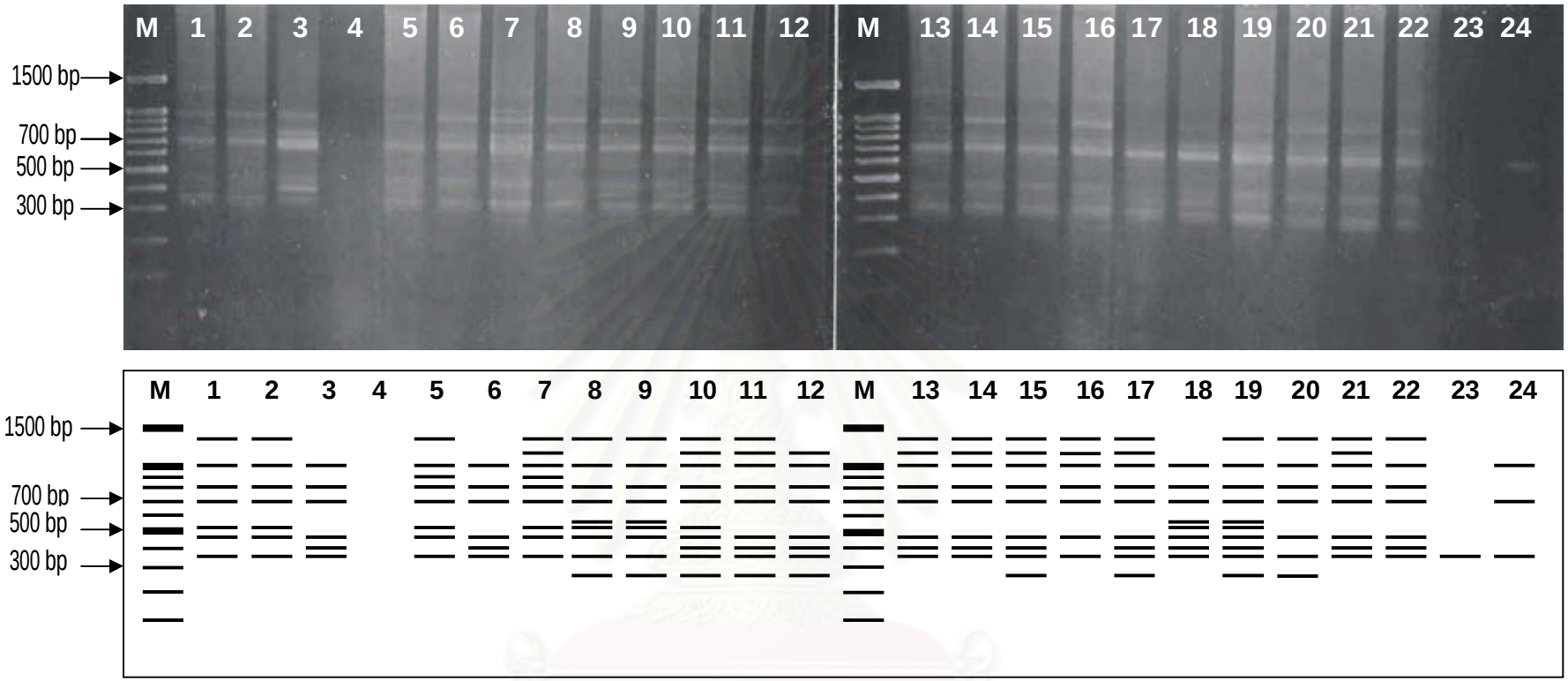


Figure 4.11 ISSR patterns obtained from 24 imported tobacco varieties amplified with primer UBC- 818 (Lane M = 1.5 kb + 100 bp DNA marker , No. 1-4 = Turkish varieties: Basma xanthiyaka, Xanthiyaka, Samsun maden, and Samsun evkaf, No. 5-15 = Burley varieties: Burley-21, Burley-64, Ky-15, Ky-8959, Ky-907, Ky-908, MC Nair 135, MS Ky-14 x L-8, Va-182, Va-528, and VS Burley-21x Ky-9, and No. 16-24 = Virginia varieties: Coker-139, Coker-206, Coker-254, Coker-319, K-317, K-346, K-394, Speight G-52, and Speight NC-82).

4.3.1.2 ISSR primers to investigate band differences between imported and local tobacco varieties

The 3'-anchored microsatellite primers were used for the DNA amplification of nine imported and two local tobacco samples. The 17 ISSR primers generated total 128 bands. The sizes of PCR bands ranged from 280 to 1,600 bp. The number of polymorphic bands generated varied between one and 11 bands, with on average of five bands per primer. Six primers (UBC-810, UBC-813, UBC-814, UBC-818, UBC-835, and UBC-836) were able to produce clear PCR bands. Another six primers (UBC-808, UBC-809, UBC-810, UBC-815, UBC-817, and UBC-823) produced faint bands while the primer UBC-819 did not produce any band. The five selected primers (UBC-807, UBC-809, UBC-813, UBC-823, and UBC-836) generated highly reproducible bands and were possible to be used to investigate the relationship between local and imported tobacco varieties.

Eleven ISSR fragments (bands) were generated by the primer UBC-807. They were scored as 520, 580, 680, 720, 800, 950, 1000, 1100, 1200, 1300, and 1400 bp (Figure 4.12). All of local and imported tobacco samples could be amplified. One scorable band (about 680 bp.) is a specific band only for Chorlare 1 and Chorlare 2 varieties. In the case of the amplification with UBC-809 primer, the PCR products were found on a smeary background. Sizes of the bands were estimated as 280, 480, 500, 600, 750, and 1500 bp. (Figure 4.13). The specific marker band generated from Chorlare 1 and Chorlare 2 is 750 bp. Moreover, the UBC-813 primer gave eight PCR fragments, which were as 350, 450, 580, 650, 850, 900, 950, and 1200 bp. One fragment approximately 900 bp. was revealed as a specific band for Chorlare 1 and Chorlare 2 (Figure 4.14). Interestingly, Chorlare 1 and Chorlare 2 did not give two fragments (850 and 950 bp.) which were found in all imported tobacco varieties.

From the UBC-823 primer amplification, 11 fragments were produced as approximately 400, 500, 550, 620, 700, 800, 900, 950, 1100, 1200, and 1500 bp. The specific bands of Chorlare 1 and Chorlare 2 were found as 700 and 800 bp. (shown in Figure 4.15). In the case of UBC-836 primer, 11 PCR products were as 260, 280, 320,

380, 420, 520, 700, 800, 1200, and 1500 bp. (Figure 4.16). The 380 bp. PCR fragment was found in all tobacco varieties, except Chorlare 1 and Chorlare 2. The PCR products given from PCR-amplification with the five selected primers were summarised in Table 4.3.

Table 4.3 PCR products obtained from ISSR-PCR amplification with five selected primers suitable to investigate different bands between nine imported and two local tobacco varieties.

Primer name	Number of amplifiable tobacco samples	Number of PCR bands	Different bands (bp.)	Band sizes (bp.)
UBC-807	11	11	680	520, 580, 680, 720, 800, 950, 1000, 1100, 1200, 1300, and 1400
UBC-809	11	6	750	280, 480, 500, 600, 750, and 1500
UBC-813	11	8	900	350, 450, 580, 650, 850, 900,950, and 1200
UBC-823	11	11	700 and 800	400, 500, 550, 620, 700, 800, 900, 950, 1100, 1200, and 1500
UBC-836	11	11	380	260, 280, 320, 380, 420, 520, 700, 720,800, 1200, and 1500

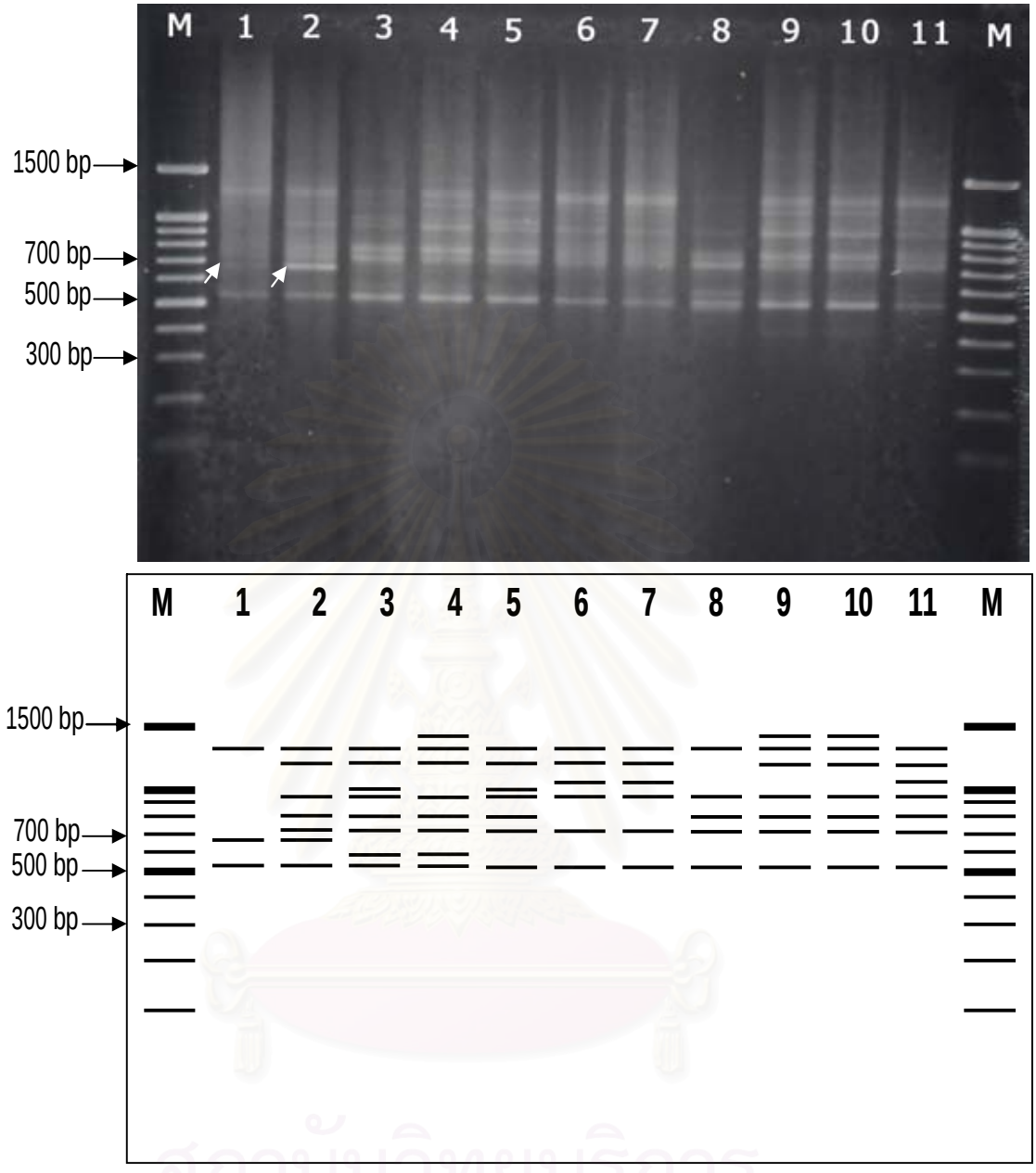


Figure 4.12 ISSR patterns obtained from two local and 11 imported tobacco varieties amplified with primer UBC-807 (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

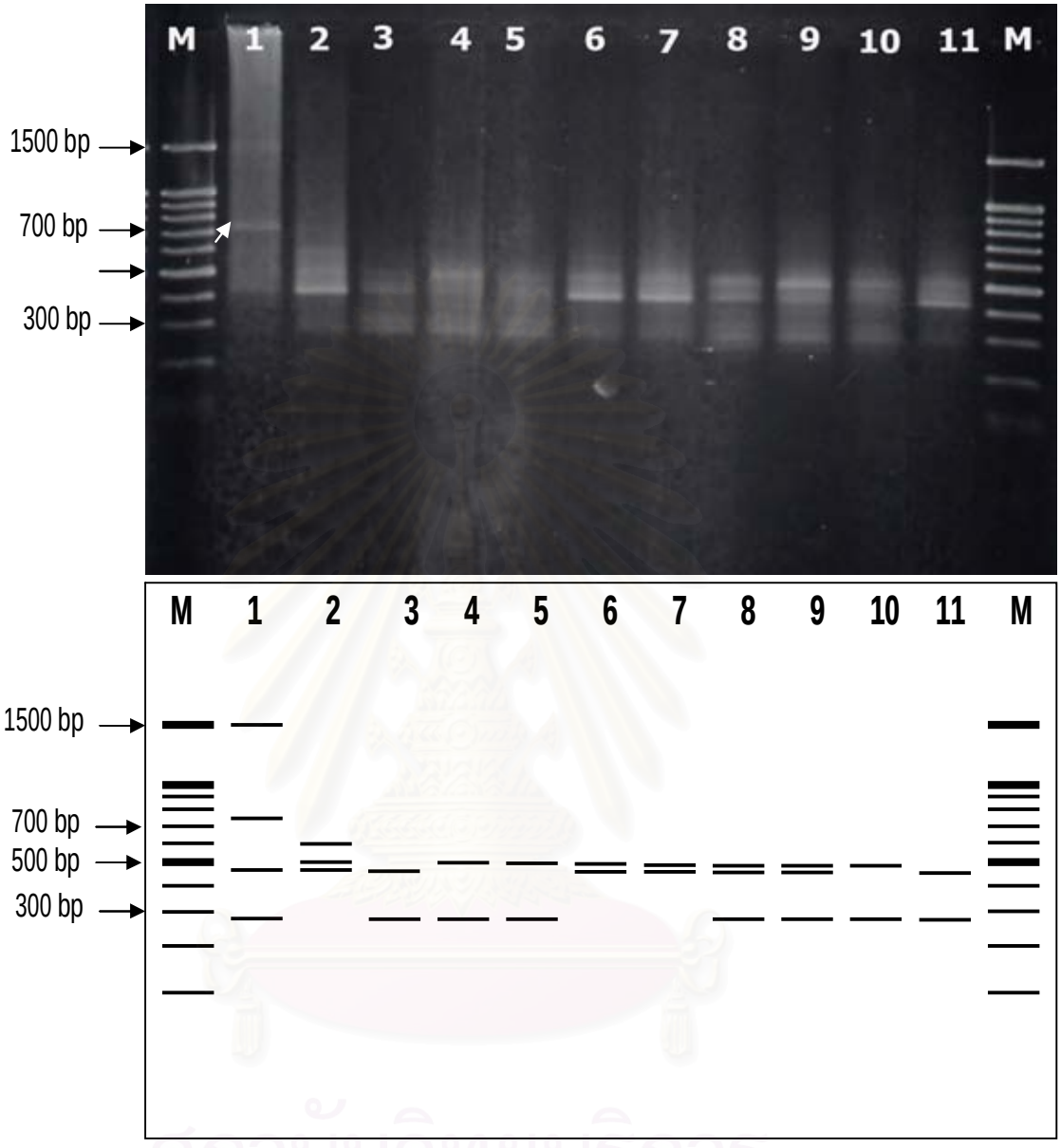


Figure 4.13 ISSR patterns obtained from two local and 11 imported tobacco varieties amplified with primer UBC-809 (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

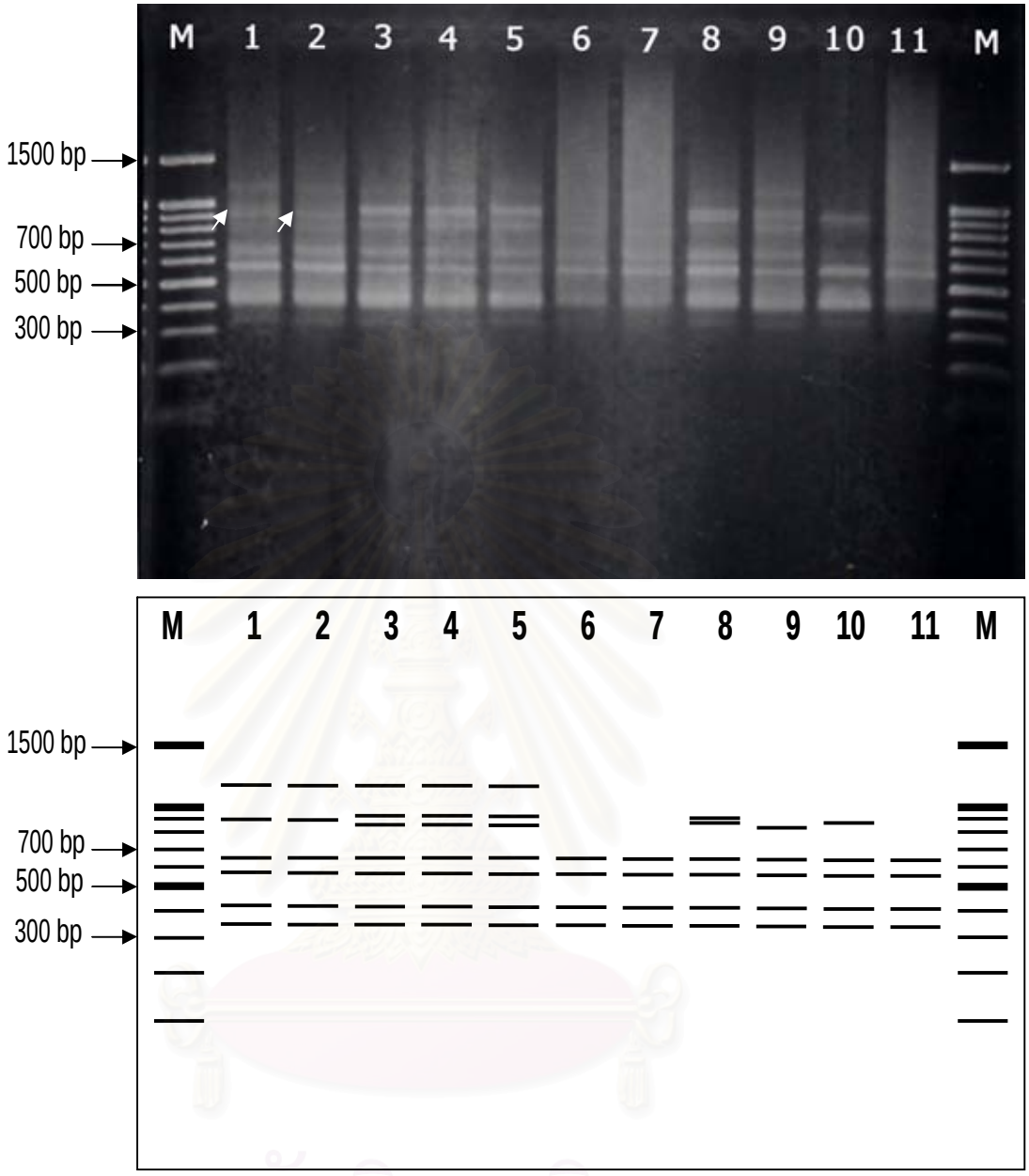


Figure 4.14 ISSR patterns obtained from two local and 11 imported tobacco varieties amplified with primer UBC-813 (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

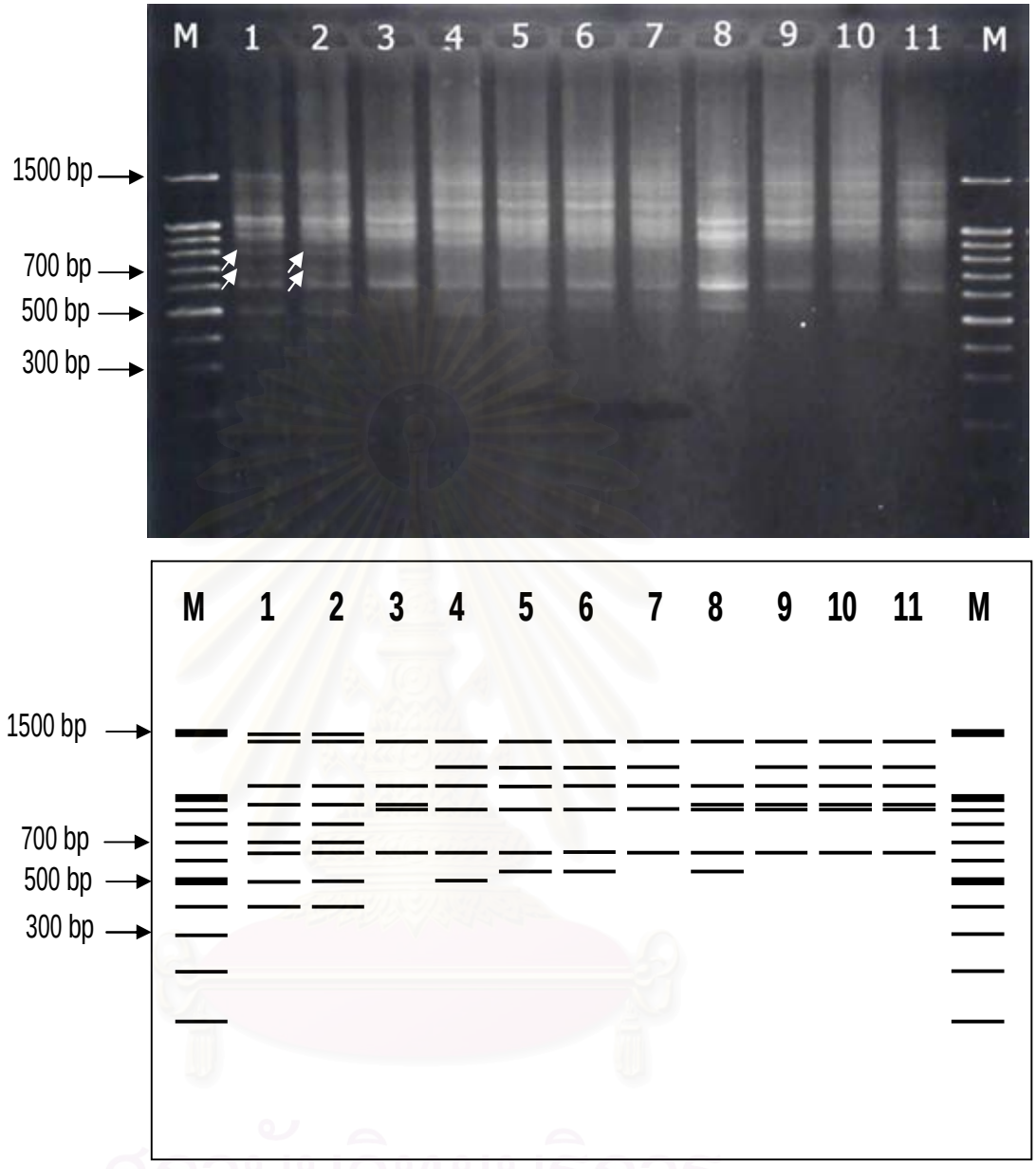


Figure 4.15 ISSR patterns obtained from two local and 11 imported tobacco varieties amplified with primer UBC-823 (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

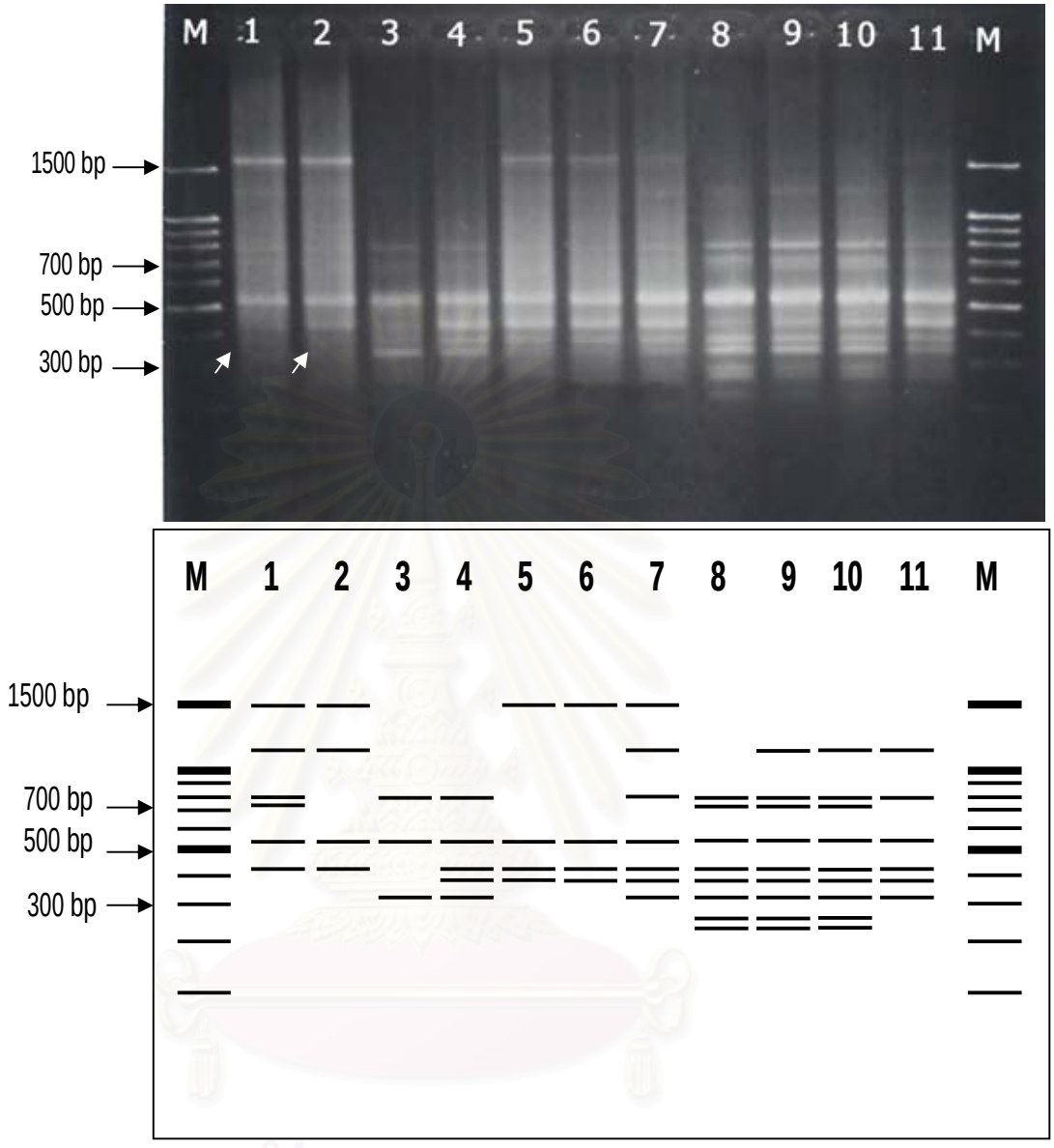


Figure 4.16 ISSR patterns obtained from two local and 11 imported tobacco varieties amplified with primer UBC-836 (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

4.3.2 Optimisation of PCR reaction

From the preliminary screening results, we selected two suitable ISSR primers (UBC-807 and UBC-836) to use in the further study. The aims of the next step were to do a reproducibility test, to optimise the PCR reaction, and also to use the selected primers to investigate genetic differences between the local and imported tobacco varieties.

4.3.2.1 Reproducibility of PCR reaction

Genomic DNA of two local and nine imported tobacco varieties were amplified with the two selected primers (UBC-807 and UBC-836) again to examine the repeatability of ISSR-PCR reaction. The tests proved that the amplified patterns were still repeatable. In the case of the reproducibility test using primer UBC-807, we found that all eleven samples were amplified and gave ten fragments as 380,420, 500, 520, 580, 680, 720, 800,950, 1200, 1300, and 1400 bp. (shown in Figure 4.17A). The PCR patterns from almost all samples were alike. Since the PCR products were revealed on smeary background, the specific band of Chorlare varieties and some other bands ranging from 600-800 bp. were not cleary seen.

From of UBC-836 reproductcibility test, ten fragments were generated agian as 320, 420, 520, 700, 720, 800, 850, 1000, 1200, and 1600 bp (shown in Figure 4.17B). The PCR patterns were also presented on smeary background, especially the fragments ranging from 600-1000 bp. All three distingtively major bands (approximately 420, 520, and 1600 bp.) were still found. From this result, the DNA patterns of most samples after PCR amplified were similar to each other.

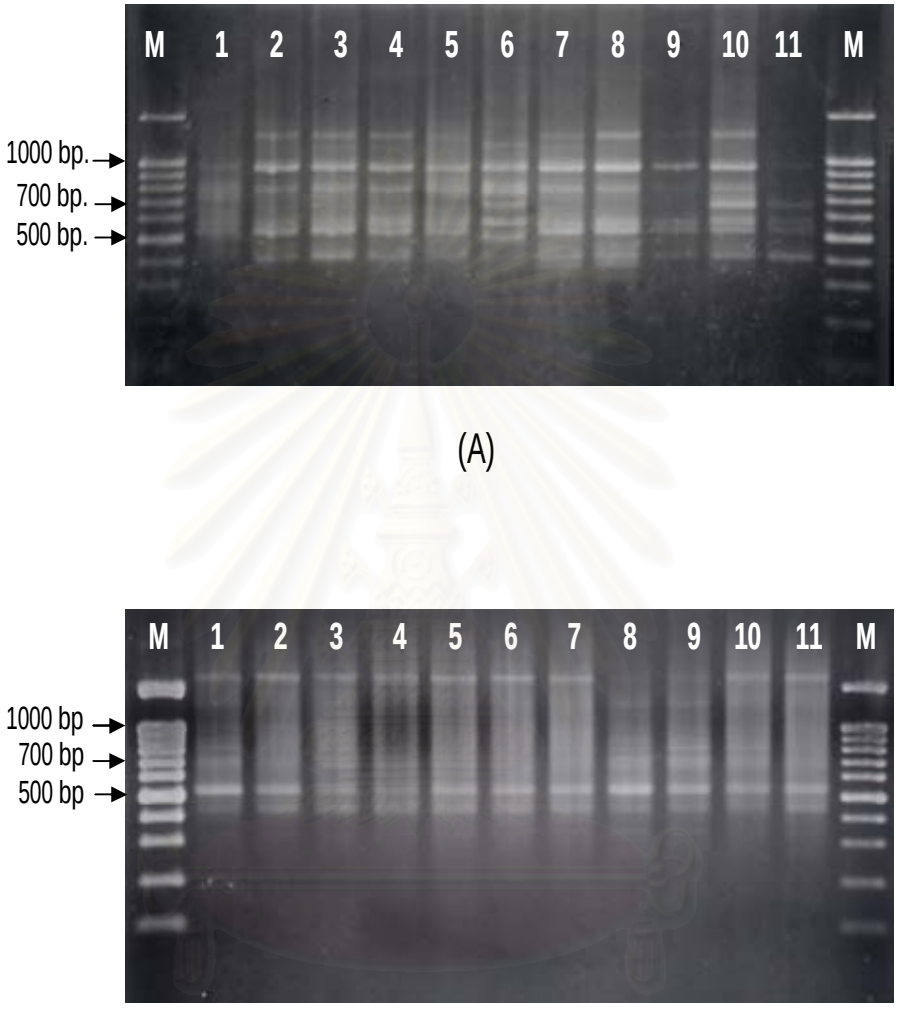


Figure 4.17 DNA patterns of ISSR-PCR amplification with (A) UBC-807 and (B) UBC-836 primers, respectively, from the reproducibility test (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

4.3.2.2 Temperature optimisation of PCR reaction

Two selected ISSR primers (UBC-807 and UBC-836) were used for PCR annealing-temperature optimisation tested. These experiments were performed with two local and nine local tobacco varieties. After the annealing temperature of the ISSR-PCR reaction were increased from 42?C to 45?C, most nonspecifically amplified products were reduced. The PCR patterns from both primers were more distinctive and clearer than those with 42?C. When the primer UBC-807 was used for ISSR-PCR, ten fragments were still generated generated 420, 520, 580, 680, 720, 800, 950, 1000, 1200, and 1500. The specific band produced specific band approximately 680 bp. of Chorlare 1 and Chorlare 2 was still found (lane 1 and lane 2) (shown in Figure 4.18).

Figure 4.18 Specific bands (indicated with arrows) of Chorlare 1 and Chorlare 2 tobacco varieties after amplified with the UBC-807 primer and increased the annealing temperature to 45?C (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

From the ISSR-PCR amplification with primer UBC-836, all eleven tobacco samples were amplified and gave PCR bands ranging from 1-8 fragments. Figure 4.19 showed ten fragments which were estimated as 320, 380, 420, 520, 720,800, 850, 950, 1200, and 1600 bp. Furthermore, the major bands approximately 500 and 1600 bp. were consistently generated. The specific bands of Chorlare varieties were shown as faint bands

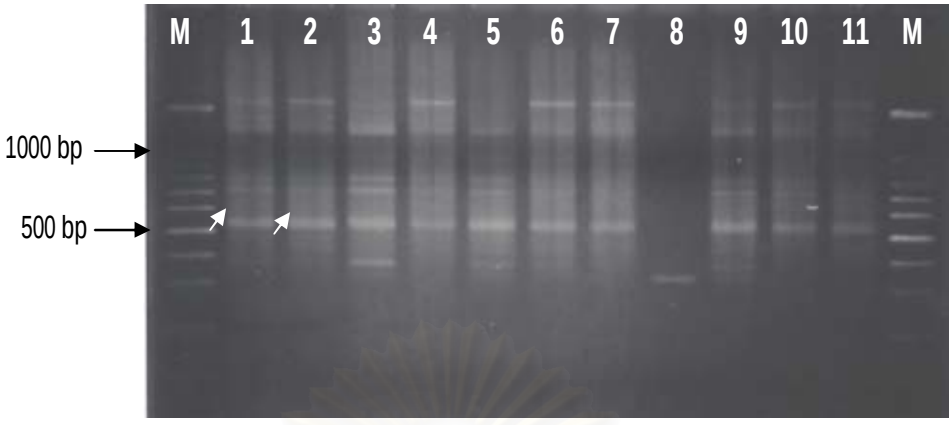
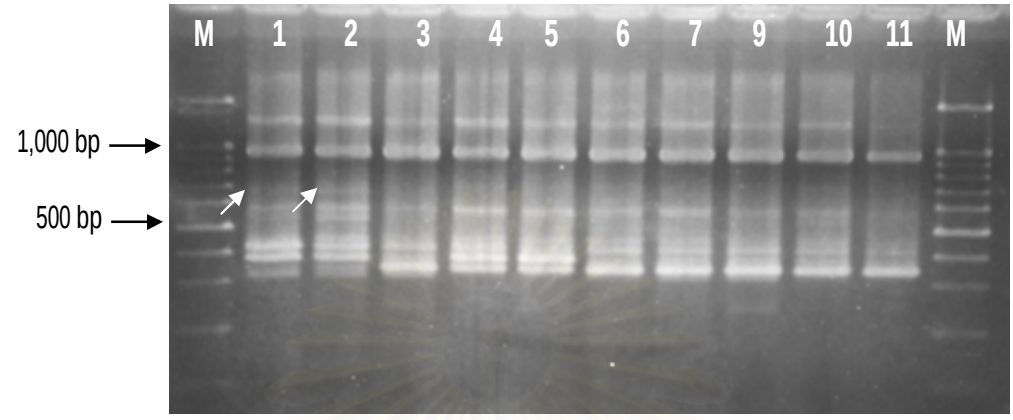


Figure 4.19 Specific bands (indicated with arrows) of Chorlare 1 and Chorlare 2 tobacco varieties after amplified with the UBC-836 primer and increased the annealing temperature to 45?C (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

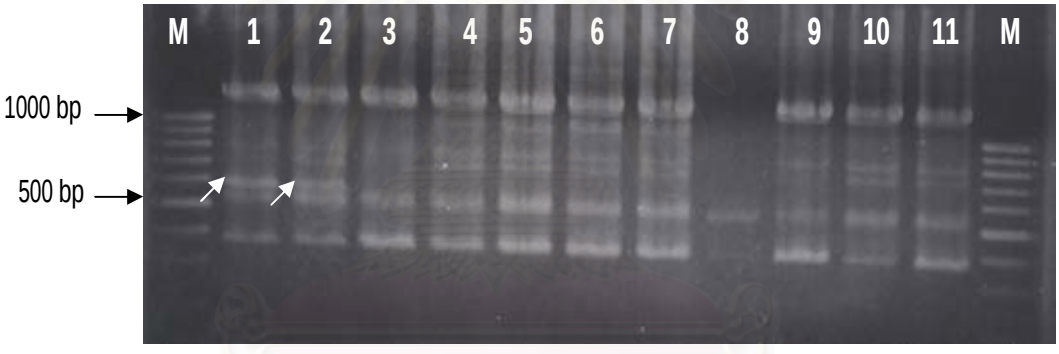
4.3.2.3 Hot start-PCR reaction

A hotstart-PCR technique was introduced to increase amplification specificity. The result after using HotstarTaq DNA polymerase (QIAgen) in the PCR condition with UBC-807 primer showed an increasing amplification yield while reducing nonspecific bands. The PCR patterns still had the 680 bp-specific band of Chorlare 1 and Chorlare 2 showing on a smear background while all ten PCR-product fragments were also generated as 320, 350, 380, 420, 450, 520, 580, 680, 950, and 1300 bp (Figure 4.20A).

From the results of primer UBC-836, all expected PCR products were found with reduced smeary background. Eight fragments were amplified from the tobacco samples providing 380, 520, 580, 720, 800, 1000, 1200, and 1400 bp fragments. Figure 4.20B also showed the 580 bp-specific band of Chorlare 1 and Chorlare 2 (lane 1 and lane 2). The amplified patterns of each tobacco sample were as same as those of the previous experiment (see Figure 4.12).



(A)



(B)

Figure 4.20 Specific bands of Chorlare 1 and Chorlare 2 were produced after amplified with the (A) UBC-807 and (B) UBC-836 primers and also used hotstart-PCR technique (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

4.3.2.4 Addition of Q-solution to PCR condition

After adding Q-solution (QIAgen) to the PCR reactions with UBC-807 primer, nine from ten genomic DNA samples were amplified and provided ISSR fragments ranging from 2-9 bands. The smeary background and nonspecific bands of the PCR products were reduced. Chorlare 1 and Chorlare 2 varieties still generated a 680 bp variety-specific band with fainted as shown in Figure 4.21.

In the PCR result with UBC-836 primer, the amplified products of nine from 11 samples were generated. The number of fragment presented in each sample ranging from 4-8 bands. Total ten amplified fragments were found and scored 380, 420, 520, 580, 720, 800, 1200, 1300, 1400, and 1500 bp. Both Chorlare varieties produced a clear and intensive 580 bp-specific band (Figure 4.22).

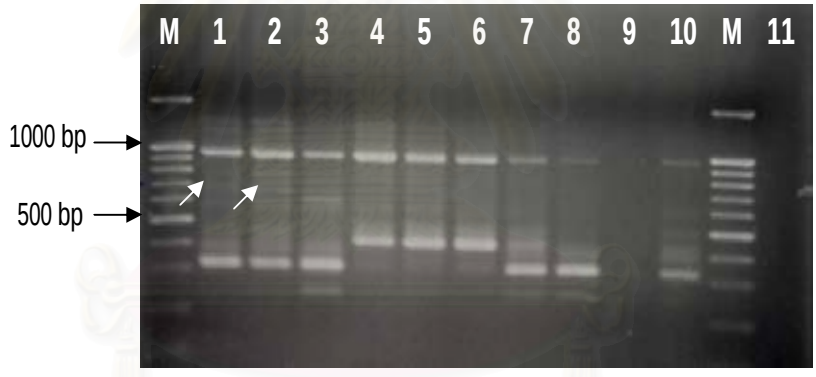


Figure 4.21 Specific bands (indicated with arrows) of Chorlare 1 and Chorlare 2 were amplified with the UBC-807 primer after added Q-solution to the PCR reactions (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

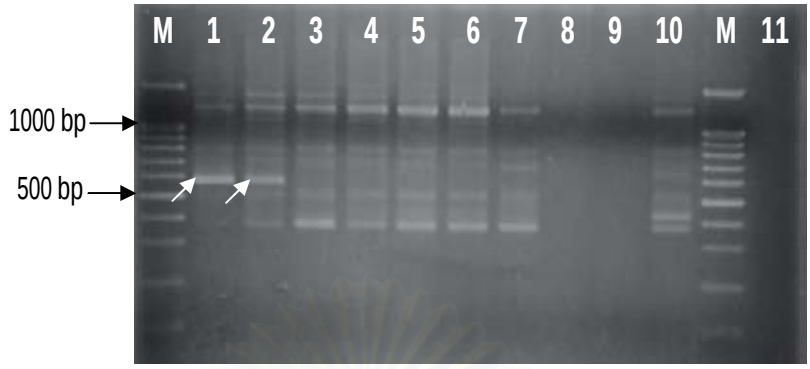


Figure 4.22 Specific bands (indicated with arrows) of Chorlare 1 and Chorlare 2 were amplified with the UBC-836 primer after added Q-solution to the PCR reactions (M = 1.5 kb + 100 bp DNA marker, No. 1-2 = local varieties: Chorlare 1 and Chorlare 2, No. 3-5= Turkish varieties: Bafra, Basma xanthiyaka, and Samsun bafra, No. 6-8 = Burley varieties: Ky-17, TN-86, and Va-116, and No. 9-11 = Virginia: Coker-48, K-326, and Speight G-140).

4.3.2.5 Band seperation tests with high-resolution agarose gel

Eleven tobacco DNA samples were used to test separation capabilities of two high-resolution agarose gels: Nusieve 3:1 agarose and MetaPhor agarose. A 1.8% concentration of Nusieve 3:1 agarose was difficult to prepare since its very fine powder was hardly dissolved. The time spent for gel solidification was longer than that of the normal agarose gel while giving a softer gel. Mobility distances of the PCR products passed through the Nusieve 3:1 agarose gel were shorter after running with the same period of time. The PCR patterns produced with UBC-807 and UBC-836 primers were mostly the same as previously found from normal agarose gel electrophoresis, but presenting as sharp and clear bands. Interestingly, Nusieve 3:1 agarose could reveal some more minor bands, especially in the UBC-807 PCR products which showed 5-6 minor bands ranging between 400-1000 bp (showed in lane 2 of Figure 4.23A). However, it did not show the specific bands of Chorlare varieties. Unlike

UBC-836, the 580 bp variety-specific band of Chorlare varieties were found as showed in Figure 4.23B.

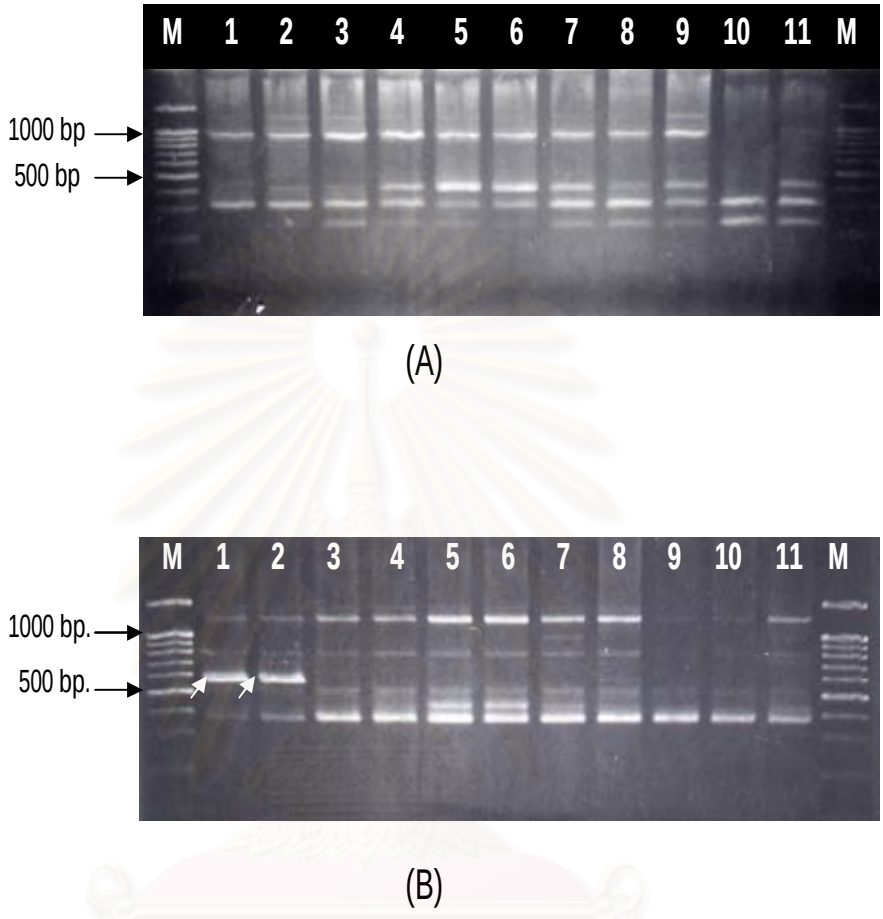
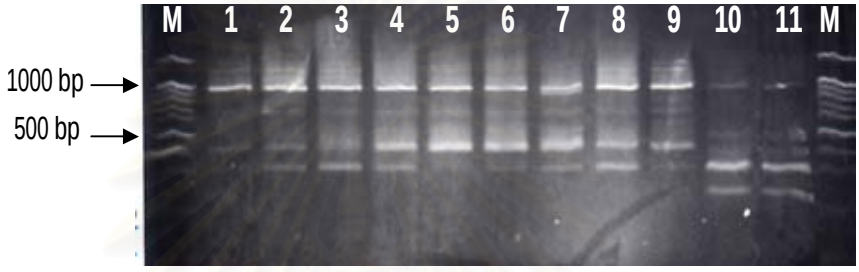


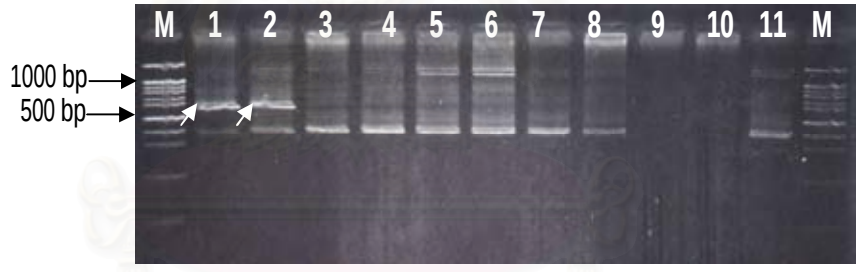
Figure 4.23 ISSR patterns of PCR amplification with the (A) UBC-807 and (B) UBC-836 primers running in Nusieve 3:1 agarose gel. (M = 1.5 kb + 100 bp DNA marker, lane 1-11 Chorlare 1 and Chorlare 2, Nison, Petkhangsink, Pu 001, Napanang, Ya-glai, Local Nakhon Si Thammarat, Xanthiyaka (Turkish), Ky-10 (Burley), and Coker-319 (Virginia)).

Compared to Nusieve 3:1 agarose, preparation of 1.8% concentration of MetaPhor agarose was even harder because its solidified gel was softer than other agarose gels. The MetaPhor gel was almost too soft to handle as it tore a part easily. The PCR fragments mobilised through MetaPhor agarose gel much slower than through Nusieve 3:1 agarose and normal agarose gels. The PCR pattern from ISSR-PCR amplification with UBC-807 primer produced 4-5 clearly minor bands ranging from

1000-1500 bp as shown in lane 2 and lane 3 of Figure 4.24A. On the other hand, the PCR patterns of UBC-836 primer in MetaPhor agarose gel were like the patterns in Nusieve 3:1 and agarose gel, but showed very sharp and clear bands. Moreover, it also gave strongly intensive specific-bands of Chorlare varieties (shown in lane 1 and lane 2 of Figure 4.24B)



(A)



(B)

Figure 4.24 ISSR patterns of PCR amplification with the (A) UBC-807 and (B) BC-836 primers running in MetaPhor agarose gel. Arrows indicate the specific bands of Chorlare varieties. (M = 1.5 kb + 100 bp DNA marker, lane 1-11 Chorlare 1 and Chorlare 2, Nison, Petkhangsink, Pu 001, Napanang, Ya-glai, Local Nakhon Si Thammarat, Xanthiyaka (Turkish), Ky-10 (Burley), and Coker-319 (Virginia)).

4.3.3 PCR amplification to distinguish between local and imported tobacco varieties

After the PCR optimisation had been performed, total 53 tobacco varieties including all three imported variety groups (Turkish, Virginia, and Burley) were tested aganist two local tobacco varieties (Chorlare 1 and Chorlare 2) using hotstart-PCR technique. From the PCR amplification with primer UBC-807, we found that almost all genomic DNA of imported and local varieties were amplified. The PCR patterns generated were sharp and clear, with brighter bands than before optimised. Figure 4.25 showed variety-specific band (approximately 680 bp) of Chorlare 1 and Chorlare 2 (lane C1 and lane C2). Most of the PCR patterns of Virginia and Burley varieties were very much alike (for example, NC 89 (lane 28) and Burley-21 (lane29)), whereas the PCR patterns of Turkish varieties (lane 1-9) were different from the other two since they did not produce two fragments, approximately 720 and 800 bp.

In the case of UBC-836 primer, 52 genomic DNA from 55 tobacco samples were amplified. Va-182 (lane 40), VS Burley-21xKy-19 (lane 44), and C.S.T (lane 50) failed to give any amplified product (shown in Figure 4.26). The PCR products were seen as clear, strong-intensity bands on only a little smeary background. The PCR patterns of most imported tobacco varieties were similar to each other, especially all of them did not have the 580-bp specific band of Chorlare 1 and Chorlare 2 (lane C1 and lane C2).

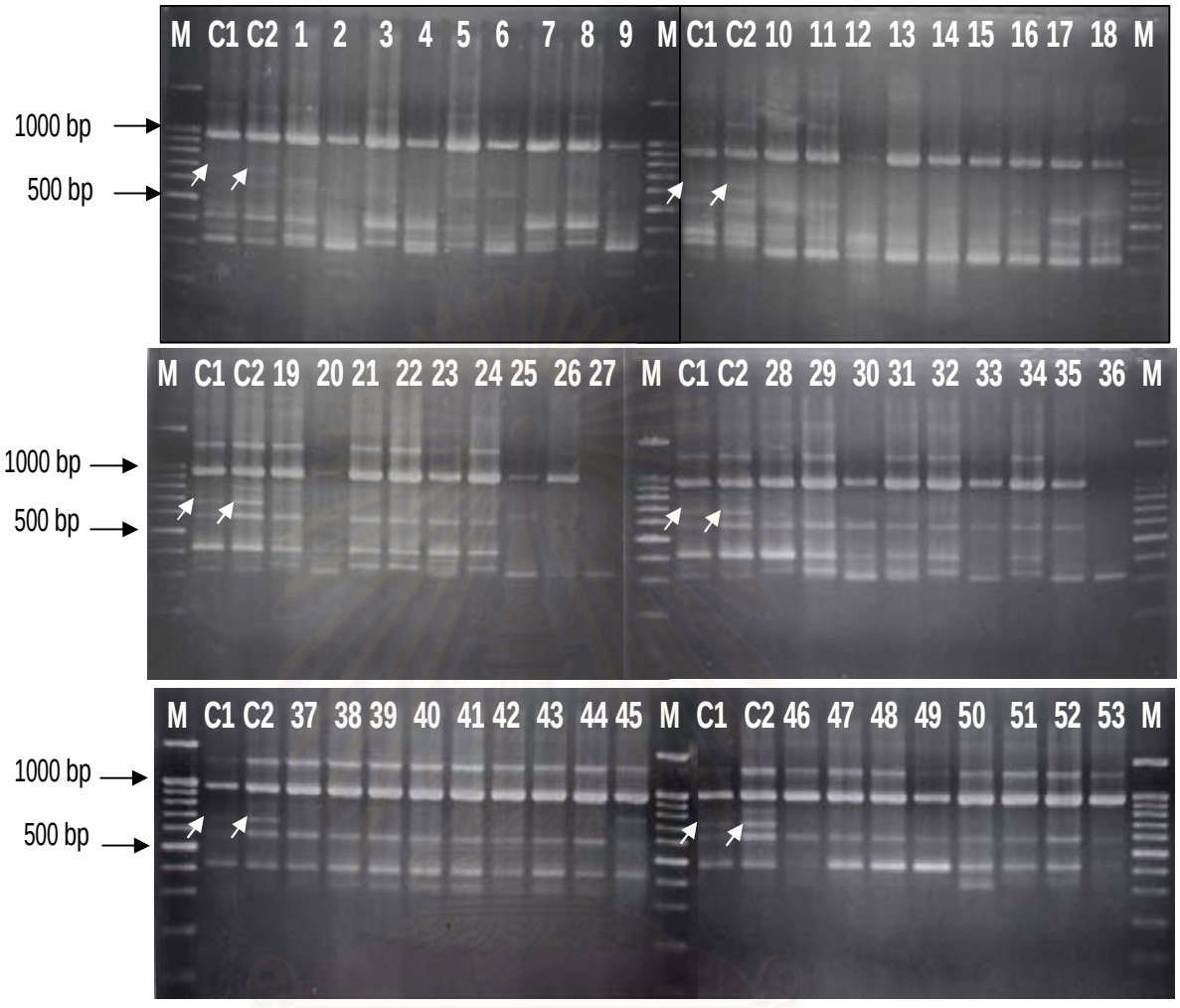


Figure 4.25 Specific bands of Chorlare 1 (C1) and Chorlare 2 (C2) amplified with UBC-807 primer (indicated with arrow).

(M = 1.5 kb + 100 bp DNA marker, lane C1 and C2 = local varieties: Chorlare 1 and Chorlare 2,
No. 1-9 = Turkish varieties: Bafra, Basma, Basma xanthiyaka, Izmir, Samsun bafra, Samsun evkaf, Samxun maden, Xanthiyaka, and Zichan,
No. 10-28 = Burley varieties: Coker-139, Coker-206, Coker-254, Coker-319, Coker-347, Coker-371 gold, K-149, K-317, K-326, K-346, K-394, K-399, Speight G-52, Speight G-70, Speight G-140, Speight NC-82, NC-2326, NC 37 NF, and NC 89,
No. 29-45 = Virginia varieties: Burley-21, Burley-64, Ky-9, Ky-10, Ky-15, Ky-17, Ky-8959, Ky-907, Ky-908, MS Ky-14 x L-8, TN-86, TN-90, TN-97, Va-182, Va-509, Va-528, and VS Burley-21 x Ky-9,
No. 46-53: Unclassified varieties: MC Nair 135, Baisee, Blanket A1, C.N.T, C.S.T, CDL 28, Dimon 1, and E-bit).

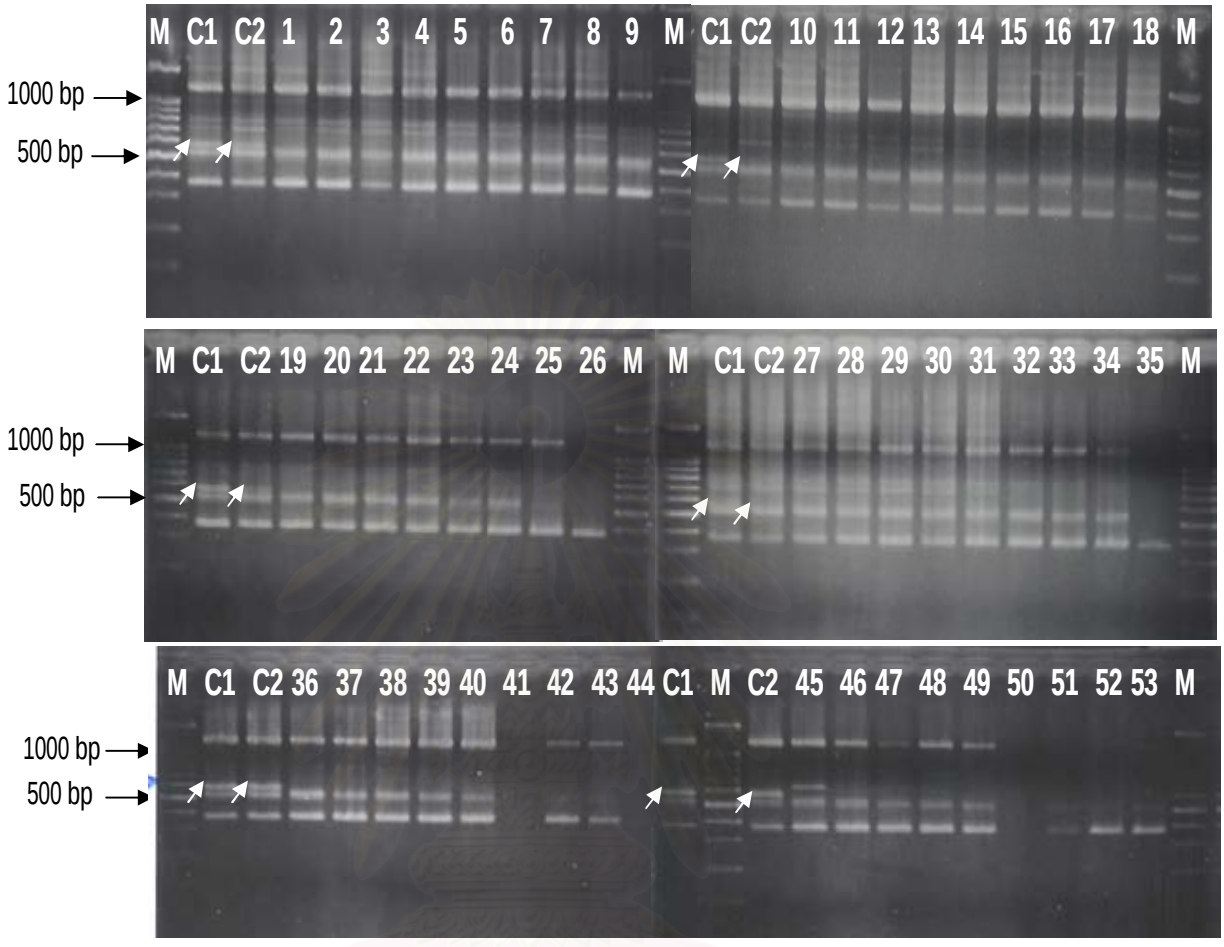


Figure 4.26 Specific bands of Chorlare 1 (C1) and Chorlare 2 (C2) amplified with UBC-836 primer (indicated with arrow).

(M = 1.5 kb + 100 bp DNA marker, lane C1 and C2 = local varieties: Chorlare 1 and Chorlare 2,
No. 1-9 = Turkish varieties: Bafra, Basma, Basma xanthiyaka, Izmir, Samsun bafra, Samsun evkaf, Samxun maden, Xanthiyaka, and Zichan,
No. 10-28 = Burley varieties: Coker-139, Coker-206, Coker-254, Coker-319, Coker-347, Coker-371 gold, K-149, K-317, K-326, K-346, K-394, K-399, Speight G-52, Speight G-70, Speight G-140, Speight NC-82, NC-2326, NC 37 NF, and NC 89,
No. 29-45 = Virginia varieties: Burley-21, Burley-64, Ky-9, Ky-10, Ky-15, Ky-17, Ky-8959, Ky-907, Ky-908, MS Ky-14 x L-8, TN-86, TN-90, TN-97, Va-182, Va-509, Va-528, and VS Burley-21 x Ky-9
No. 46-53: Unclassified varieties: MC Nair 135, Baisee, Blanket A1, C.N.T, C.S.T, CDL 28, Dimon 1, and E-bit).

4.4 Genetic relationships of tobacco varieties in Thailand

4.4.1 Genetic relationship analysis using ISSR-PCR

Genomic DNA of 40 varieties was amplified with five selected primers (UBC-807, UBC-809, UBC-813, UBC-823, and UBC-836) and their PCR patterns were transformed to a band-scoring matrix to construct genetic relationship trees. All 40 local and imported tobacco samples were successfully amplified. PCR patterns obtained from the ISSR-PCR amplification with UBC-807 primer showed 2-8 bands per sample. All eight bands produced were 280, 340, 420, 550, 680, 800, 950, and 1300 bp The bands were distinct, clear and consistency. Faint bands of 680 bp were found from Chorlare 1 and Chorlare 2 (shown in lane 1 and lane 2 of Figure 4.27). Most of the PCR patterns of tobacco samples were similar to each other.

The UBC-809 primer gave 12 ISSR fragments which were estimated as 200, 220, 250, 280 320, 400, 450, 480, 520, 600, 750, and 1400 bp (Figure 4.28). Moreover, the 750 bp fragment was clearly found in Chorlare 1 and Chorlare 2. In the case of UBC-813 primer, PCR products were found as approximately 280, 300, 480, 550, 600, 700, and 950 bp as shown in Figure 4.29.

Six ISSR bands were generated by UBC-823 primer. They were approximately 550, 620, 750, 850, 1100, and 1400 bp. The PCR patterns from the ISSR-PCR amplification of all local and imported tobacco samples were very much alike by this UBC-823 primer (Figure 4.30). Two fragments of PCR products (approximately 620 and 750 bp) were produced in some samples as very faint bands. In the case of UBC-836 primer, the sizes of PCR bands have ranges from 380 to 1400 bp. Nine fragments were totally produced as 380, 450, 520, 580, 720, 800, 1000, 1200, and 1400 bp as shown in Figure 4.31. Most of the patterns of all amplified samples were similar to each other, except those of Chorlare varieties. One scorable band about 580 bp was revealed as a specific band for Chorlare 1 and Chorlare 2 (lane 1 and lane 2). DNA patterns of the three unclassified tobacco varieties (Blanket A1, Dimon 1, and E-bit) were observed as faint bands.

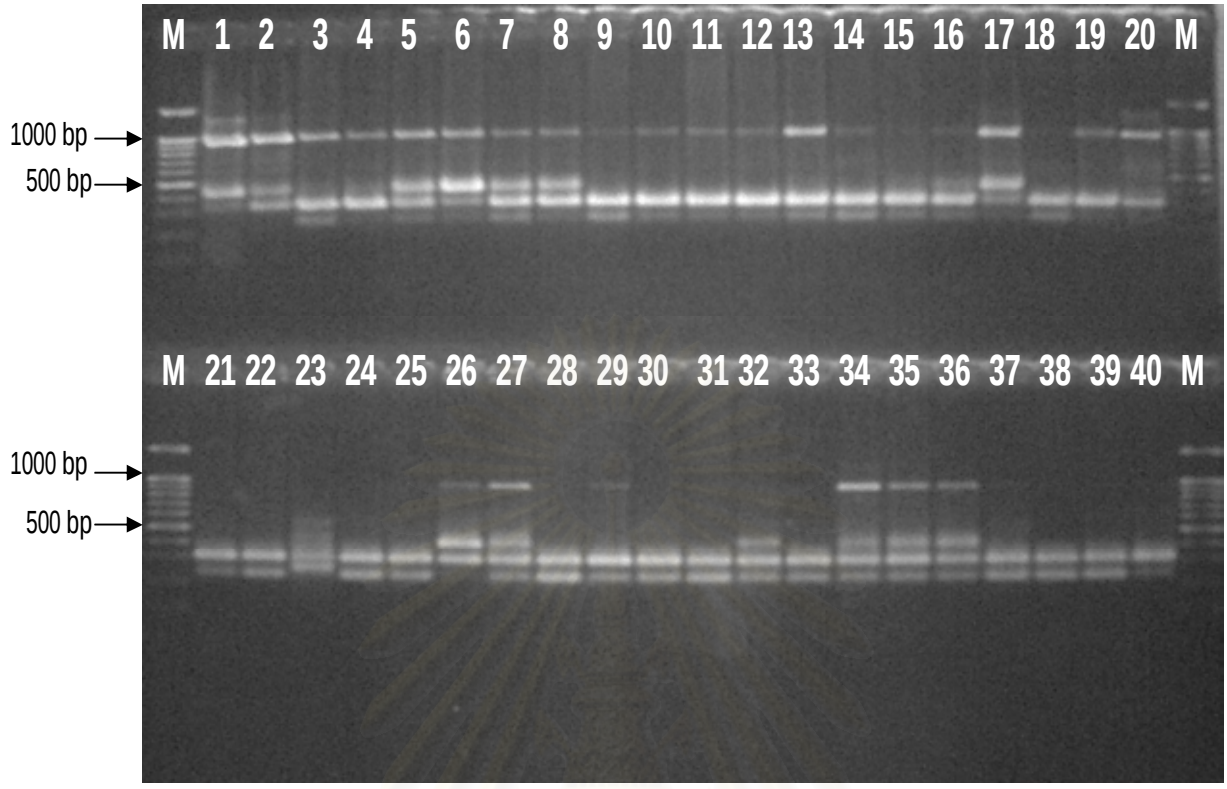


Figure 4.27 PCR patterns of 40 local and imported tobacco samples after ISSR-PCR amplification with UBC-807 primer.

(No.1-13 = Local varieties: Chorlare 1, Chorlare 2, Nison, Petkhangsink, Pu 001, Pu 002, White gold, Napanang, E-bit, Ya-glai, Ya (LocalxKaset), Local Nakhon Si Thammarat, and Ya-chun.
No. 14-21 = Turkish varieties: Bafra, Basma, Izmir, Samsun bafra, Samsun maden, Samsun maden, Xanthiyaka, and Zichan.
No. 22-29 = Virginia varieties: Coker-139, Coker-347, Coker-371 gold, K-394, K-399, Speight G-70, NC 37 NF, and NC 89.
No. 30-37 = Burley varieties: Ky-9, Ky-10, Ky-15, TN-86, TN-90, TN-97, Va-509, and Va-528.
No. 38-40 = Unclassified varieties: Blanket A1, Dimon 1, and E-bit).

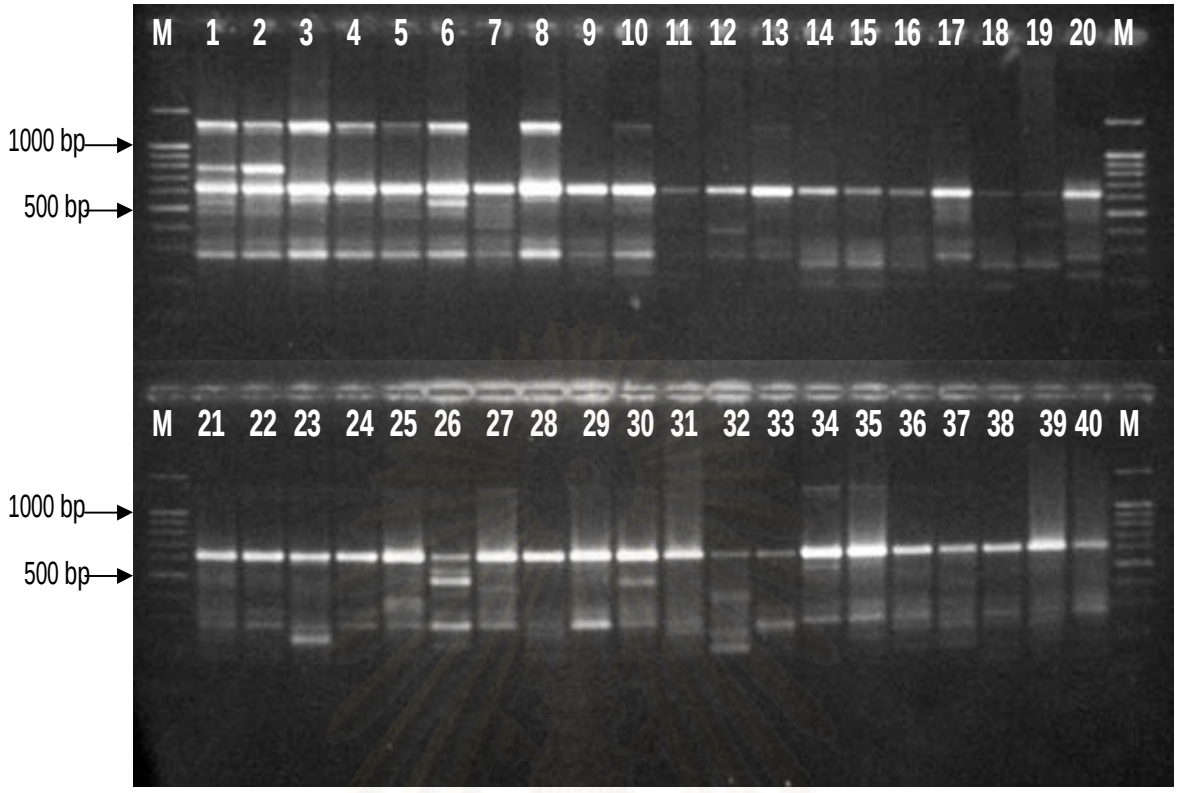


Figure 4.28 PCR patterns of 40 local and imported tobacco samples after ISSR-PCR amplification with UBC-809 primer.

(No.1-13 = Local varieties: Chorlare 1, Chorlare 2, Nison, Petkhangsink, Pu 001, Pu 002, White gold, Napanang, E-bit, Ya-glai, Ya (LocalxKaset), Local Nakhon Si Thammarat, and Ya-chun.
No. 14-21 = Turkish varieties: Bafra, Basma, Izmir, Samsun bafra, Samsun maden, Samsun maden, Xanthiyaka, and Zichan.
No. 22-29 = Virginia varieties: Coker-139, Coker-347, Coker-371 gold, K-394, K-399, Speight G-70, NC 37 NF, and NC 89.
No. 30-37 = Burley varieties: Ky-9, Ky-10, Ky-15, TN-86, TN-90, TN-97, Va-509, and Va-528.
No. 38-40 = Unclassified varieties: Blanket A1, Dimon 1, and E-bit).

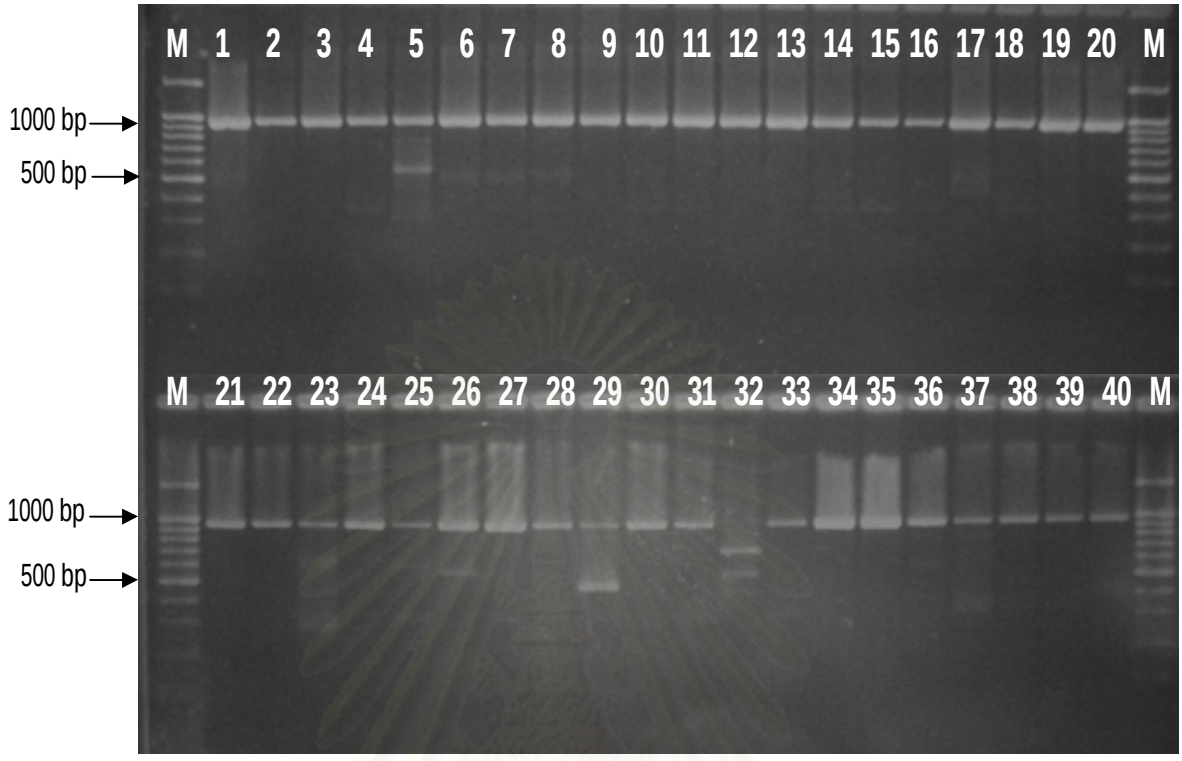


Figure 4.29 PCR patterns of 40 local and imported tobacco samples after ISSR-PCR amplification with UBC-813 primer.

(No.1-13 = Local varieties: Chorlare 1, Chorlare 2, Nison, Petkhangsink, Pu 001, Pu 002, White gold, Napanang, E-bit, Ya-glai, Ya (LocalxKaset), Local Nakhon Si Thammarat, and Ya-chun.
No. 14-21 = Turkish varieties: Bafra, Basma, Izmir, Samsun bafra, Samsun maden, Samsun maden, Xanthiyaka, and Zichan.
No. 22-29 = Virginia varieties: Coker-139, Coker-347, Coker-371 gold, K-394, K-399, Speight G-70, NC 37 NF, and NC 89.
No. 30-37 = Burley varieties: Ky-9, Ky-10, Ky-15, TN-86, TN-90, TN-97, Va-509, and Va-528
No. 38-40 = Unclassified varieties: Blanket A1, Dimon 1, and E-bit).

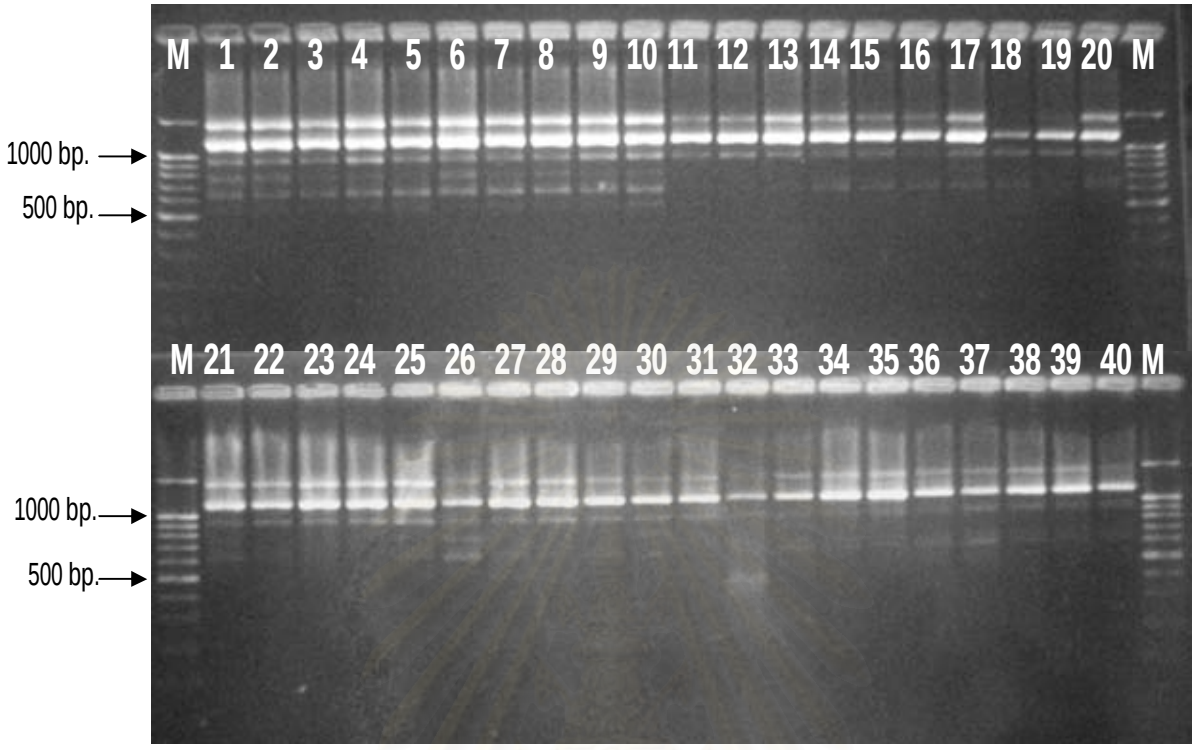


Figure 4.30 PCR patterns of 40 local and imported tobacco samples after ISSR-PCR amplification with UBC-823 primer.

(No.1-13 = Local varieties: Chorlare 1, Chorlare 2, Nison, Petkhangsink, Pu 001, Pu 002, White gold, Napanang, E-bit, Ya-glai, Ya (LocalxKaset), Local Nakhon Si Thammarat, and Ya-chun.
No. 14-21 = Turkish varieties: Bafra, Basma, Izmir, Samsun bafra, Samsun maden, Samsun maden, Xanthiyaka, and Zichan.
No. 22-29 = Virginia varieties: Coker-139, Coker-347, Coker-371 gold, K-394, K-399, Speight G-70, NC 37 NF, and NC 89.
No. 30-37 = Burley varieties: Ky-9, Ky-10, Ky-15, TN-86, TN-90, TN-97, Va-509, and Va-528
No. 38-40 = Unclassified varieties: Blanket A1, Dimon 1, and E-bit).

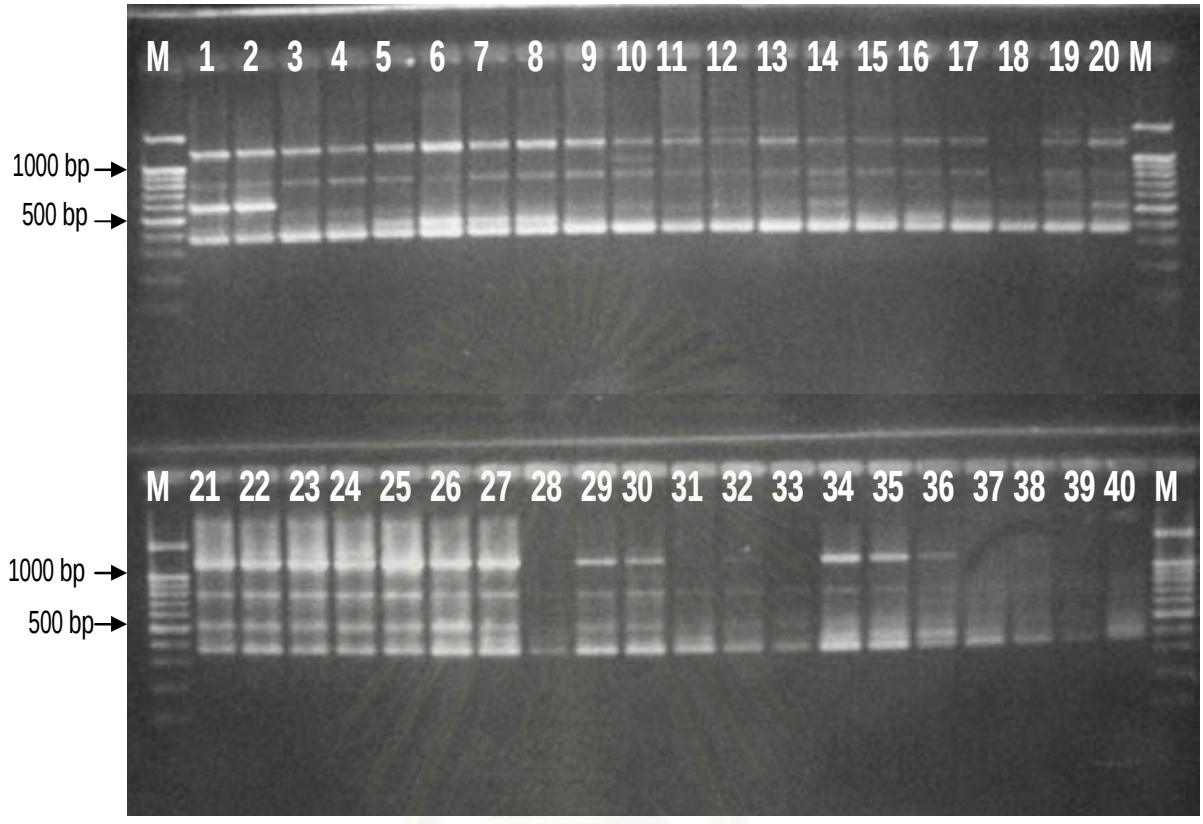


Figure 4.31 PCR patterns of 40 local and imported tobacco samples after ISSR-PCR amplification with UBC-836 primer.

(No.1-13 = Local varieties: Chorlare 1, Chorlare 2, Nison, Petkhangsink, Pu 001, Pu 002, White gold, Napanang, E-bit, Ya-glai, Ya (LocalxKaset), Local Nakhon Si Thammarat, and Ya-chun.
No. 14-21 = Turkish varieties: Bafra, Basma, Izmir, Samsun bafra, Samsun maden, Samsun maden, Xanthiyaka, and Zichan.
No. 22-29 = Virginia varieties: Coker-139, Coker-347, Coker-371 gold, K-394, K-399, Speight G-70, NC 37 NF, and NC 89.
No. 30-37 = Burley varieties: Ky-9, Ky-10, Ky-15, TN-86, TN-90, TN-97, Va-509, and Va-528.
No. 38-40 = Unclassified varieties: Blanket A1, Dimon 1, and E-bit).

4.4.2 Genetic relationship tree analysis

This experiment was performed to study genetic relationships between local and imported tobacco varieties using amplified fragments obtained from five ISSR primers (UBC-807, UBC-809, UBC-813, UBC-823, and UBC-836). Genetic relationship trees were reconstructed using NJ and UPGMA methods with Nei and Li's similarity coefficient. A phylogram based on NJ analysis revealed only four groups of tobacco varieties which had bootstrap values higher than 50% as shown in Figure 4.32. Group I consisted of both Chorlare varieties, considered as very closely related with 100% bootstrap support. Group II included Nison and Petkhangsink which were grouped with 58% bootstrap. Ya (LocalxKaset) and Local Nakhon Si Thammarat varieties were paired as Group III with 65% bootstrap. NC 37 NF (Virginia) and Ky-10 (burley) were also grouped together with 68% bootstrap. Other pairwised branches with < 50% bootstrap supporting values were also labeled, although they did not indicate any significant relativeness between the varieties.

A UPGMA dendrogram revealed the same four groupings of some tobacco varieties found in the NJ tree as shown in Figure 4.33. Group I also consisted of Chorlare 1 and Chorlare 2 distantly separated from the others with very high 99% bootstrap value. Group II of Nison and Petkhangsink had 54% bootstrap while the other two local tobacco varieties, Ya (LocalxKaset) and Local Nakhon Si Thammarat, were clustered together as Group III with 53% bootstrap support. Last, group IV consisted of NC 37 NF (Virginia) and Ky-10 (Burley) were separated from the other tobaccos with 61% bootstrap. Noted that four varieties of the Turkish variety (Bafra, Izmir, Samsun maden, and Samsun evkaf) were grouped together in both NJ and UPGMA trees, but with very low bootstrap supporting values.

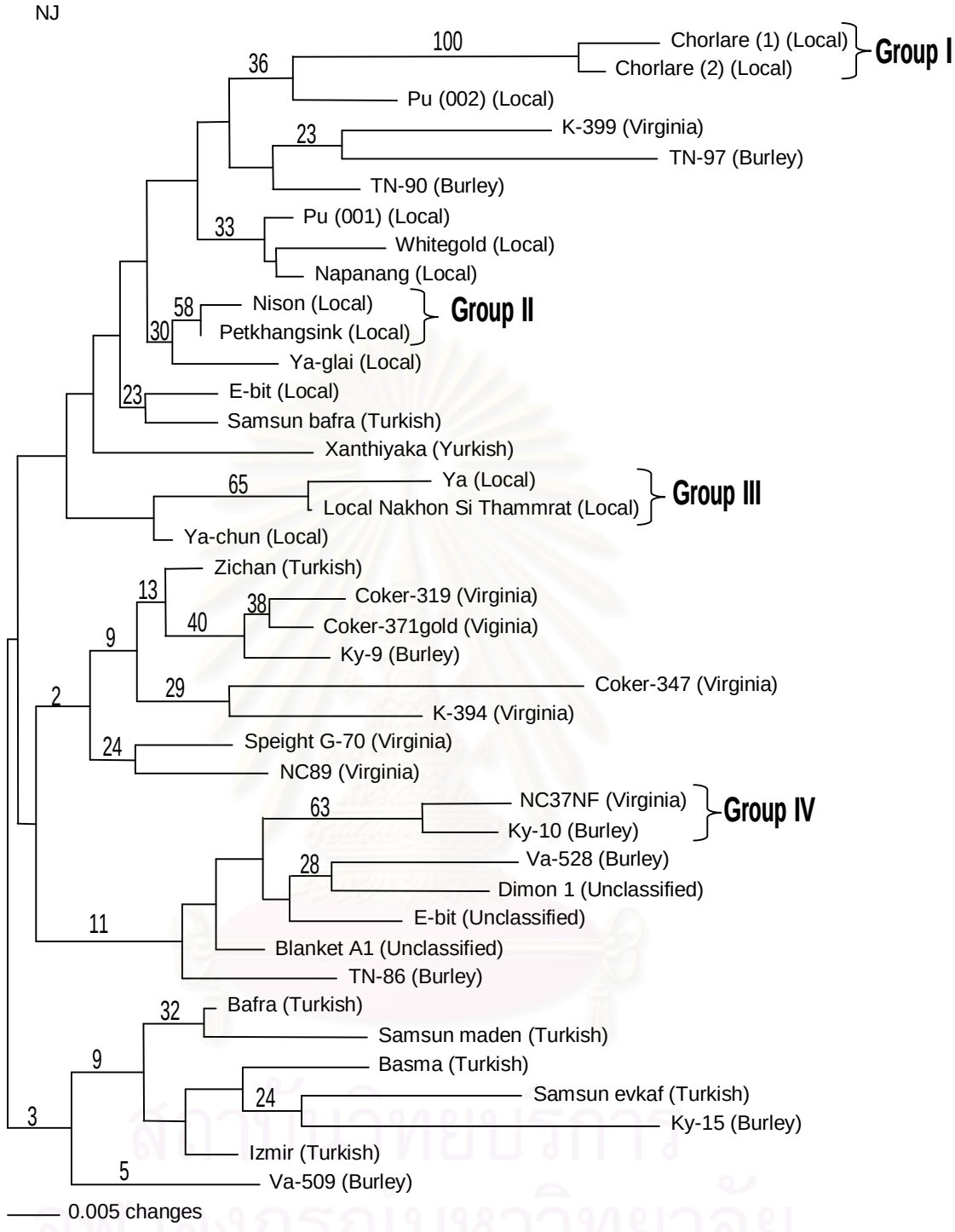


Figure 4.32 A neighbour-joining tree from whole ISSR data among 40 tobacco samples based on Nei and Li's similarity coefficient. Numbers along branches are bootstrap-supporting values (%) generated after 1000 replications.

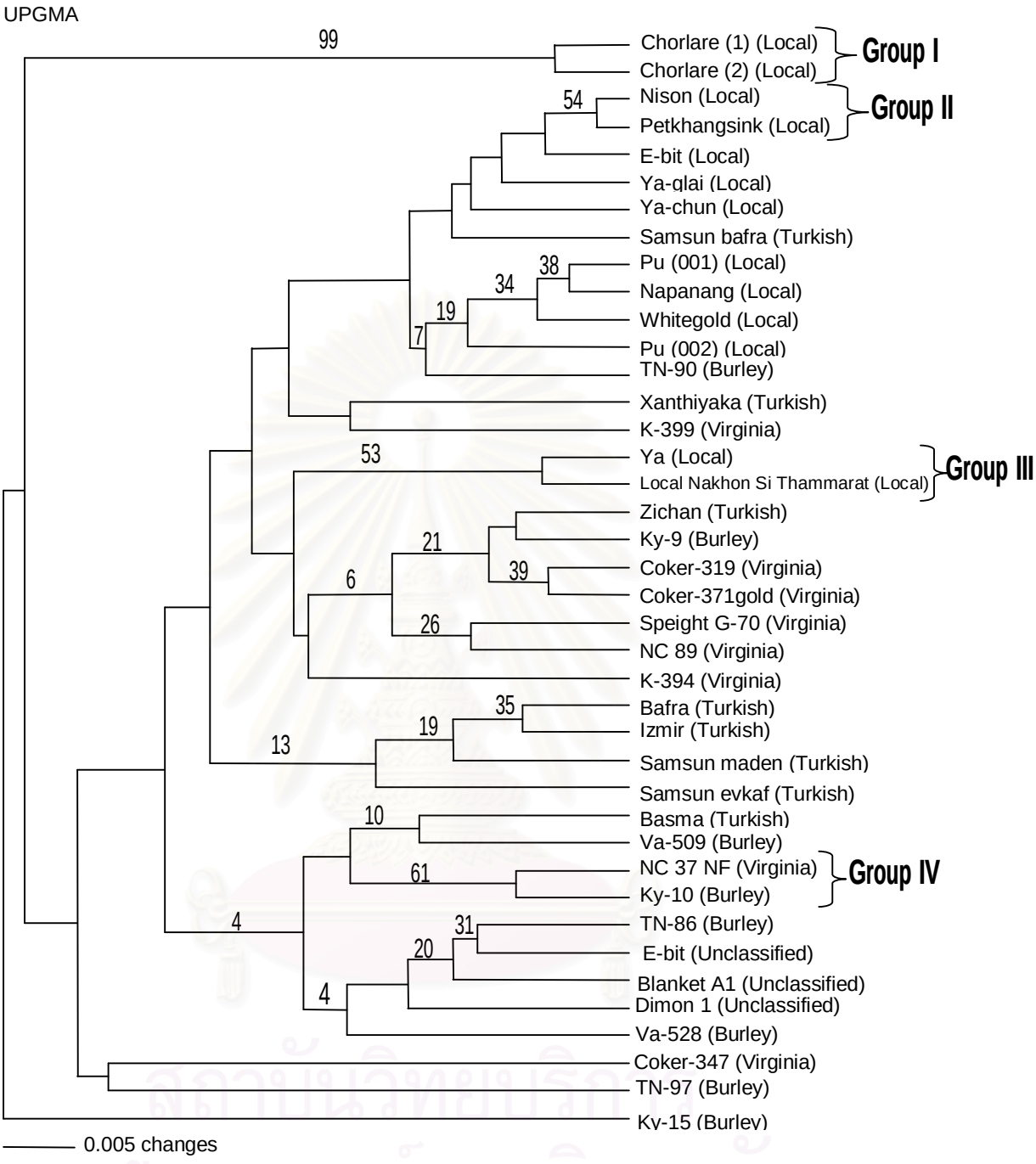


Figure 4.33 A UPGMA tree from whole ISSR data among 40 tobacco samples based on Nei and Li's similarity coefficient. Numbers along branches are bootstrap-supporting values (%) generated after 1000 replications.

CHAPTER V
DISCUSSION

5.1 Collection of the local tobacco samples

I went collecting local tobacco samples at Sukhothai, Nong Khai, and Nakhon Phanom provinces with officers of the tobacco product analysis subdivision, Department of Research, Thailand Tobacco Monopoly. Along the trips, we got so much help from the officers of the tobacco regional stations and many tobacco farmers in each province, who gave us information about tobacco cultivation and background history.

In Sukhothai province, we chose Si Samrong and Mueang districts to collect samples of local tobacco varieties since they were growing tobaccos the most in Sukhothai. As expected, the tobacco farmers usually cultivated the plants near riverside areas of Yom river. We found two local tobacco varieties named Nison and Petkhangsink, which were about one to two months old. The officers of Sukhothai tobacco office suggested that the large, long leaf characteristics of Nison were like those of Burley varieties. Although, tobacco farmers in the areas did not know about the history of Nison variety, but they were quite sure that this variety had been taken to the districts for decades. Unlike Nison, Petkhangsink variety was known to be improved from Petmakhur (เพชรมะเขือ) variety of Phetchabun province. The name Khangsink means the shin of a lion to which its stem is similar: having many small branches around the main stem. We agreed that this variety may be related to Turkish varieties since the leaves were cordate-, entire-, and heart-shaped like those of Sumsun variety (Turkish), but larger.

Likewise, the officers of the tobacco regional offices in the other two provinces, Nong Khai and Nakhon Phanom assumed that local tobacco varieties found over there were probably not originally local varieties. Morphological characteristics of the varieties collected in these provinces were as same as those of Burley and Virginia tobacco varieties. For example, Pu, Whitegold, and Napanang local varieties were similar to Virginia varieties while the leaf shape of E-bit local variety was lanceolate like Burley's leaves. In fact, the name E-bit came from its semi-twisted leaf base

characteristics. The officers proposed that these local varieties descended from some imported tobacco varieties industrially promoted and given from Thailand Tobacco Monopoly to farmers10-20 years ago. Thus, if there were any healthy, high yield, and disease-resistant plants left, the farmers might have secretly kept tobacco seeds for their own growing in the next cultivating season. Should this hypothesis is true and has been practiced for generations, it could explain why many local tobacco varieties in Thailand are similar to imported varieties, although they have had their own Thai name and been recognized as local areas.

When we had a collecting trip to Nakhon Si Thammarat in the South, we got a little bit of problems as there was not any regional tobacco office in the province. Therefore, in this trip we had to coordinate with the province chief-officer of Ministry of Agriculture and staff of the administrative authority of each district. Although we did not have a tobacco expert with us to suggest how close the local tobacco varieties in this province were to any imported variety, the agricultural officers of Nakhon Si Thammarat were very helpful on organising our visiting to tobacco fields and pointed the areas which usually grew tobaccos the most in this province were the Srichon and Tha Sala districts. Interestingly, the farmers in these areas planted tobacco seeds at the different periods of time, unlike in the Northeastern provinces where must tobacco farmers usually grew the plants at almost the same time.

In Tha Sala district, we found only Ya-glai variety, which was named following its originally cultivated areas, Glai tambon (sub-district). At that time, the farmers had just planted tobacco seeds and the seedlings were only one-week old. These seedlings had only 3-4 pale greenish, ovate-shaped leaves on each one. Normally, the plantlets would be grown in seed beds until about one-month old and then transplanted to field crops near a reservoir of the village.

In Srichon district, we found three local tobacco varieties: Ya-chun, Ya, and Local Nakhon Si Thammarat. Ya-chun (or previously called Kaset-16) variety were sampled when they aged about one to two months. Unlike other varieties, Ya-chun was found being grown between rows of para-rubber tree fields, near the bank of an irrigating canel passing through the village. From comments of the interviewed farmers, they believed that this tobacco variety had been cultivated there for a long time and got

the name Ya-chun from its strong smell of smoked rolled-up cigarettes. Moreover, the tobacco farmers also mentioned that Ya-chun variety might have been taken from Glai tumbon too. Since their leaf shape was lanceolate which large size, we hypothesised that they might be related to same Virginia varieties.

For Ya variety of Sichon district, crop owners mentioned the history of this variety that it was a crossed hybrid between some unknown local and Kaset varieties. We found that most of the plants in these fields aged three to four months old and, interestingly, their terminal buds had just been removed - the technique known as topping. This terminal-bud removal technique was performed to prevent the plants from flowering and seeding; therefore, they could only grew the leaves and increased nicotine content with them. This led to the stronger smell than usual of Ya-chun variety.

The last variety sampled from Nakhon Si Thammarat was named as Local Nakhon Si Thammarat by ourselves. That was because this variety was not found in any proper tobacco field, but growing along the roadside of Asia road (highway No. 41) in Chulabhorn district. There seemed to be no owner of this tobacco plantation and then we hypothesised that the plants might be grown for local cigarette consumption, not for any larger-scale production. We also recognized that the cultivation pattern of this local variety was too dispersed to be intentionally planted for agricultural purpose. Surprisingly, while the leaf-arrangement pattern around the stem of this local variety was similar to those of Burley varieties, the shape of leaves was however as same as Virginia varieties. Therefore, we assumed that this variety would be a crossed bred between some Virginia and Burley varieties.

5.2 Preliminary selection of suitable ISSR primers

ISSR-PCR technique is an arbitrarily amplified dominant (AAD) marker, which analysed dominant inheritant of species based on PCR technique with arbitrarily primers like RAPD (Ramdon Amplified Fragment DNA) and AFLP (Amplified Fragment Length Polymorphism) (Bussell et al., 2005). Morever, ISSR uses a longer (15-30 bp) single-simple sequence repeat (SSR) primer which allows for a higher annealing temperature and results in greater reproducibility of amplified bands. In most genetic diversity

analyses, selection of suitable primers is a very important step for the PCR reaction. Needed properties of suitable ISSR primers are to be able to give highly polymorphic PCR products, high reproducibility, and more informative. One successful example of using ISSR-PCR to study genetic variation is the work of Pharmawati, Yan, and Finnegen (2005). They used 64 ISSR primers to screen against genomic DNA of three *Leucadendron* cultivars. Two ISSR primers (UBC-856 and UBC-857) were selected to construct a DNA fingerprinting. They concluded that ISSR profiling was a powerful method for doing identification and molecular classification of *Leucadendron* cultivars.

Although tobaccos are one most importantly agricultural plants, there are only a few studies of genetic diversity and relationship of tobacco using molecular markers, especially ISSR techniques. Yang et al. (2005) screened 100 ISSR primers to amplify tobacco genomic DNA. Ten primers were found producing reproducibly amplified products. Compared to the results in this thesis, four ISSR primers (UBC-810, UBC-813, UBC-817, and UBC-818) from 16 screened primers could determine genetic relationship between 24 imported tobacco varieties studied (Figure 4.8-4.11). These four primers gave higher numbers of polymorphic bands than the other 12 primers which did not produce any band or could produce only some faint bands. For example, the ISSR-PCR amplification with UBC-811 primer gave only two faint bands with highly smeary background (the data not shown). However, Sarla, Neeraja, and Siddiq (2005) reported a different result as they revealed that the UBC-811 primer could give high numbers of amplified bands from rice varieties and used the primer to assess genetic diversity of rice. Therefore, the UBC-811 primer and probably other problematic primers might not be a suitable primer for the genomic DNA of tobaccos. Furthermore, the PCR condition used in the screening experiment might not be best suitable and needed to be optimised. This led to the optimisation experiment which annealing temperature was raised up and some chemicals were changed or added to achieve high specificity of the reaction.

To preliminarily investigate a possibility of using ISSR marker to distinguish local tobacco varieties from imported ones, the officers of Meajo Tobacco Experiment Station (Chiang Mai province) sent leaf samples of two Chorlare varieties to be representatives

for local varieties. They proposed that Chorlare were one best Chiang Mai representative varieties. Although they did not know about the history of this variety, they were sure that the varieties have been cultivated in the province since long time ago. From 17 ISSR primers screened on nine imported and two Chorlare varieties, five primers were found being possible to differentiate between imported and local tobacco varieties. The two best selected primers, UBC-807 and UBC-836 primers could give clearer and sharper bands of Chorlare varieties than the other these primers (680 bp and 580 bp, respectively). Interestingly, this finding was not expected by Dr. Ramsey Lewis (Crop Science Department, North Carolina State University, USA.), a foreign scientific advisor for Thailand Tobacco Monopoly. He had commented the proposal of this thesis that identification of genetic polymorphisms between tobacco varieties with simple molecular markers would be very difficult. In his opinion, tobacco has little genetic variation between varieties and it would be revealed at only very low rate, except using advanced markers such as AFLP (Amplified Fragment Length Polymorphism). Zhang et al. (2006) had also presented that tobacco germplasms have narrow genetic diversity among cultivars.

Even though there were a few precautions about a failure in using ISSR to differentiate between local and imported tobacco varieties, at least UBC-807and UBC-836 ISSR primers could be repeatedly used to separate Chorlare 1 and Chorlare 2 varieties from more than 53 imported varieties. Moreover, we proposed that some tobacco varieties cultivated in Thailand such as Chorlare, could be officially pronounced as “true” local varieties if they have unique genetic characteristics which are different from other varieties and suggest their long-history of growing in the country. Nevertheless, one caution is needed to be noted: this primer screening experiment could be successful in only fresh leaf samples. Fresh leaves of any plant sample usually give much better high quality of genomic DNA than dried leaves. That would be even worse if the leaves are passed a curing process, both heat-cured and air-cured, which could reduce or damage the quality of genomic DNA. In fact, tobacco samples sent to the laboratory of Research Department, Thailand Tobacco Monopoly for variety identification were usually passed a drying and curing processes. Thus, the low quality

and quantity of genomic DNA from cured leaves may cause a great problem in ISSR-PCR reactions. In the end, it is necessary to try finding other techniques, for example, AFLP analysis which give greater ability of genetic polymorphism.

5.3 Optimisation of PCR reaction

ISSR technique is similar to RAPD that it does not require either prior knowledge of genome, cloning, or specific primer design; yet it has higher reproducibility than RAPD. Filippis et al. (1996) commented the importance of doing a reproducibility test. He advised that genetic markers usually have limitations mainly because reproducibility from sample to sample is difficult. Therefore, the ISSR-PCR reactions performed in my thesis were also repeated twice to ensure reproducibility. The results showed that all distinctively major ISSR bands were still reproduced (shown in Figure 4.17), confirming the assumption that the two ISSR primers selected from the screening experiment could specifically combine to SSR-regions within the tobacco genome. This finding agreed well with the works of Bahulikar et al. (2004). They tested reproducibility of the ISSR-PCR amplification in *Nicotiana attenuate* and assumed that ISSR method could produce reproducible bands.

An annealing temperature is also another important parameter that is usually optimised to achieve a better PCR result. Increasing or reducing an annealing temperature would have an impact on the pattern quality and reproducibility of ISSR fingerprints. Some researchers suggested to do an annealing temperature optimisation for a minimum range of 5 temperatures covering a melting temperature (Tm) of the primers (Bornet and Branchared, 2001). However, in this thesis the annealing temperature was examiningly with only one increased temperature, from 42?C to 45?C, to reduce nonspecific amplified products. That was because the annealing temperature for preliminary selection of suitable ISSR primers was at 42?C which is a little lower than Tm of most primers screened. The preliminary results demonstrated that 42?C could produce the PCR patterns, although there were many faint bands on smeary backgrounds. Fortunately, raising the annealing temperature from 42?C to 45?C resulted

in giving sharper bands, specially the specific bands of Chorlare varieties (shown in lane 1 and lane 2 of Figure 4.18 and Figure 4.19). I assumed that this improved result dued to the annealing temperature of 45?C which was closer to the Tm of the two primers (UBC-807: 45.25?C and UBC-836: 45.41-49.32?C) than of 42?C. Although PCR patterns from ISSR amplification with both UBC-807 and UBC-836 primers were improved, some faint bands on smeary background were still observed. From the project of Pharmawati et al. (2005), they proposed that such failure of some primers to give clear banding patterns may be because those primers require special amplification condition, such as alternative chemical stabilisers.

Taq DNA polymerase is probably the most important chemical in PCR reaction. Moreover, there are some cautions of using *Taq* DNA polymerase, such as it could possess unexpected residual activity not only at high temperature but also at low temperature. Nonspecific PCR products thus can be amplified during thermal cycling and this would lead to lowered yield of the desired products with high background. From previous experiments, we used Dynazyme *Taq* DNA polymerase (Finnzyme, Finland) for PCR amplification and some faint bands and highly smeary background were found. To solve that problem, hot-start PCR method using Hotstar*Taq* DNA polymerase (QIAgen) was introduced for higher PCR specificity. Hot-start PCR has been the subject of numerous publications over the last decade. For example, Dahiya et al. (1995) used TaqStart antibody (Taq DNA polymerase antibody) heated in the thermal cycler with hot-start technique to inhibit nonspecific products and primer-dimers formation in PCR reaction. Likewise, hotstart *Taq* polymerase of QIAgen, which was used to optimise PCR reaction in my thesis, can prevent the formation of misprimed products and primer-dimers at low temperature. That was because it would be activated only by incubating at 95?C for 15 minutes. When the ISSR-PCR reactions were optimised using Hotstart *Taq* polymerase, the results with UBC-807 and UBC-836 primers (Figure 4.20) showed an increasing in the amplified yield while reducing the smeary background compared with previous results (Figure 4.12). For example, the optimised ISSR pattern of UBC-836 primer showed four fragments (approximately 380, 520, 580, and 1600 bp) which were sharper and brighter than before optimised. But the other four faint fragments (approximately 320, 420, 850, and 950 bp) seen in the previous results

were disappeared. Therefore, I assumed that using hot-start *Taq* DNA polymerase could also help increasing specificity of the PCR reactions.

Not only changing *Taq* DNA polymerase and PCR method to Hotstar*Taq* polymerase, but one additive chemical, Q-solution (QIAgen), was also experimentally added to the reactions. The company (QIAgen) recommended to use Hotstar*Taq* DNA polymerase with Q-solution to facilitate amplification of difficult templates, by modifying the melting behaviour of DNA. Normally, some DNA may be entitled as “difficult template”, even though it had high quality and free of any potential inhibitory contaminants. The reasons of these difficult templates could be 1) having GC-rich (over 65% GC), 2) containing di- or more nucleotide repeats, 3) having strong hairpins, poly-A/poly-T, or other homopolymer regions. Adding DMSO is one most common technique used to help amplifying through the GC-rich regions and a particular primer-template pair (Soltis et al., 2000). Like DMSO, the Q-solution reagent was claimed to be able to improve a suboptimal PCR which would have caused by some difficult templates having a high degree of secondary structure or being GC rich. However, unlike DMSO and other commonly used PCR additives, Q-solution is nontoxic and PCR purity is guarunteed.

After optimised the results by adding Q-solution to the PCR reactions with Hotstar*Taq* polymerase, it could reduce smear and increase amplified yields of PCR products. After adding Q-solution to the reactions with UBC-807 and UBC-836 primers, the amplified-band patterns of each tobacco sample were similar to those of the previous experiment, but clearer and brighter. Moreover, smeary backgrounds and non-specific bands of the PCR products were also reduced. Although the Q-solution used in this study did not produce sharper bands than using only Hotstar*Taq*, it could give brighter and were intensive bands. Therefore, I concluded that adding Q-solution can help increasing specificity of ISSR-PCR reaction and most PCR patterns were not different from the previous patterns.

The last experiment performed to improve ISSR-PCR results was the separation test with some different agarose gels. Normally, I used 1% concentration of common agarose gel to test quality of genomic DNA and 1.8% concentration to separate the PCR

products. Agarose gel is polysaccharide extracted from seaweed and may be the most widely-preferred medium matrix for routine gel electrophoresis to resolve DNA fragments. It is typically used at various concentrations from 0.5 to 2% and has a large range of separation (100 bp-23 kb), but with relatively low resolution (He et al., 2007). This may help explaining the low resolution of the patterns of UBC-807 primer even after testing with hot-start PCR technique (Topic 4.3.2.5, Figure 4.20A) of which the DNA bands 600-1000 bp were unclear. Thus, changing the electrophoresis medium from common agarose gel to high resolution gel would have been helpful to increase sharpness and clearness of the ISSR products. Two kinds of the gel matrix usually used in electrophoresis nowadays are agarose gel and polyacrylamide gel. Although these two matrices are different in chemical ingredients and preparations, they work in the same way as moblility of any fragment or molecule under a high-voltage electric current depends on the concentration of the gel, and size and conformation of the molecule. First, polyacrylamide gel was considered to be a better choice to improve the resolution of gel, but it is neurotoxin when absorbed though the skin and would give a symptom of muscular weakness. Moreover, polyacrylamide gel is suitable a separation of small fragments less than about 500 bp but the PCR fragments found in this study ranged from 200-1500 bp. Thus, I chose two kinds of special high-resolution agarose gels, Nusieve 3:1 agarose and Metaphor agarose, to be tested and hopefully it may have given better result than normal agarose gel. The company (Cambrex Bio Science Rockland, Inc.) suggested that Nusieve 3:1 agarose could ensure fine resolution of DNA fragments from 10 bp upto 1,500 bp. Nagaraju et al. (2002) repeated their successful study of using Nusieve agarose mixed with Sigma agarose to resolve PCR products of silkworm samples. Likewise, the results from band separation with Nusieve 3:1 agarose in this study could reveal some minor bands ranging between 400-1000 bp (Figure 4.23). However, Nusieve 3:1 agarose gel was more difficult to prepare than common agarose gel. The time spent for gel solidification was longer than normal agarose. Furthermore, mobility distances of the PCR products were shorter after running electrophoresis in the same time. The cost of Nusieve 3:1 agarose was also more expensive than common agarose gel.

In the case of Metaphore gel, the company (Lonza) claimed that this high-resolution agarose gel could resolve DNA fragments in the range of 200 to 800 bp as good as polyacrylamide gel. Metaphor agarose was used in the work of He et al. (2007) whom studied the resolution of Metaphore compared with polyacrylamide gel and vinyl-polymer of polyacrylamide. They found that the resolution of Metaphore agarose gel and polyacrylamide gel was not as high as that of vinyl-polymer of polyacrylamide gel which was much sharper and tighter. The results in this band separation test after using Metaphore revealed that the Metaphor gel preparation was even more difficult than using Nusieve 3:1 and normal agarose gels. The solidified gel was too soft and uneasy to handle, although the PCR patterns showed very clear ISSR-PCR amplified bands. Noted that the result from UBC-836 primer produced sharper and clearer bands than the patterns on Nusieve 3:1 agarose (Figure 4.24A).

From the band separation tests with two kinds of high-resolution agarose gels (Nusieve 3:1 agarose and Metaphor agarose), I assumed that both agarose gels could help resolving any unclear PCR band, although they had disadvantages of the difficulty in preparation and handling. Moreover, these gels were very expensive and were not stocked in the company much enough for my genetic relationship experiments of tobacco varieties. Thus, I decided to continue using normal agarose gel with high enough concentration to study the genetic relationships of tobacco varieties in Thailand.

5.4 Genetic relationships of tobacco varieties in Thailand

After the ISSR-PCR amplifications were performed with five selected primers to analyse genetic relationships among 27 imported and 13 local tobacco varieties, I found that most of primers gave a number of amplified DNA fragments that were enough for reconstructing genetic relationship trees. The exceptional primer was UBC-813 which provided low numbers of DNA fragments (Figure 4.29). This problem may have come from an unsuitable PCR condition for UBC-813 primer and should be optimised later. Although there was the problem with UBC-813 reaction found, the data from all amplified PCR fragments was scored and used to make genetic relationship trees. The

PCR patterns of all three groups of the imported tobacco varieties (Turkish, Burley, and Virginia) were similar to each other. The PCR pattern suggested that they were genetically closely related to each other. These results were as same as the study of Yang et al. (2005), they suggested that 24 flue-cured tobacco varieties were closely related and had low genetic diversity.

Furthermore, the PCR patterns of most local tobacco varieties were found being similar to those of some imported varieties, except Chorlare 1 and Chorlare 2 varieties which had their own specific bands. I proposed that some local tobaccos such as Chorlare varieties might have genetic characteristics different from any other varieties. Likewise, genetic relationship trees based on NJ and UPGMA methods also supported the distinctiveness of both Chorlare varieties since they had 100% bootstrap supporting-values higher along the branch (Figure 4.32). Then I suggested that Chorlare varieties may had long-history of growing in Thailand and they could be officially pronounced as “true” local varieties of the country. Unlike Chorlare, the other 11 tobacco varieties were grouped with the imported varieties (Figure 4.32 and 4.33). The genetic relationship results from PCR analyses agreed well with the previous suggestions that most of the 11 local varieties were morphologically similar to some imported varieties. Such hypothesis came from the officers of the tobacco regional offices in Nongkhai and Nakhon Phanom provinces. They assumed that some local tobacco varieties were probably not truely local varieties. Addtionally, three unclassified tobacco varieties: Blanket A1, Dimon 1, and E-bit were another targets of study whether they were either Turkish, Burley, and Virginia. Even through the three varieties were paired with some imported varieties, their bootstrap values were too low (less than 50%) to ensure to which variety group each of them belonged.

Although the ISSR-PCR markers selected in this thesis could not clearly indicate the genetic relationships between imported and local tobacco varieties growing in Thailand, some of them would be developed to be an effective molecular marker to distinguish some long-cultivated local varieties from the other varieties. The results from this research should be an important source for the study and development of tobacco germplasms in the future. Moreover, these putative variety-specific markers (for

example, the 580 bp band of Chorlare from UBC-836 primer) may be further converted to SCAR (Sequence Characterised Amplified Region) markers, which are DNA fragments amplified by the PCR reactions using specific 15-30 bp primers and are designed for specific nucleotide sequences. Last but not least, some more advanced molecular marker techniques, such as AFLP (Amplified Fragment Length Polymorphism) which is a highly sensitive method for detecting polymorphisms in DNA, could be introduced to give a higher resolution of the genetic relationships between imported and local tobacco varieties.

CHAPTER VI
CONCLUSIONS

1. From the excursions to collect local tobacco samples in Thailand, 11 local varieties were sampled from four provinces across the country (Sukhothai, Nong Khai, Nakhon Phanom, and Nakhon Si Thammarat). Morphological characteristics of these varieties were studied and some of them found being particularly similar to some imported varieties.

2. Preliminary selections of suitably polymorphic ISSR-PCR primers were firstly performed and four primers (UBC-810, UBC-813, UBC-817, and UBC-818) were selected as they could possibly determine genetic relationships between 24 imported tobacco varieties, although the true relationships among them were not so clear.

3. Then two best polymorphic ISSR primers (UBC-807 and UBC-836) were found being able to generate unique, variety-specific bands for two local varieties (Chorlare 1 and Chorlare 2) which were suggested from Thailand Tobacco Monopoly to be main representatives for local tobacco varieties. These specific ISSR-PCR markers were successfully tested against 53 imported tobacco varieties.

4. Moreover, the PCR reactions were optimised in many ways, for instance, using hot-start PCR technique, raising an annealing temperature, adding Q-solution chemical, and testing with high-resolution agarose gels. These optimisation approaches improved the PCR results as they could increase specificity of the reactions.

5. After NJ and UPGMA genetic relationship trees were reconstructed from the data matrices of the ISSR-PCR scored results of 13 local and 27 imported tobacco varieties with five selected primers, the trees showed that only the two Chorlare varieties were distantly separated from all others. Another two pairs of Nison and Petkhangsink and Ya and Local Nakhon Si Thammarat were also found but positioned close to some imported

tobacco varieties. The other seven local varieties were also found genetically similar to imported tobacco varieties.

6. Combined with morphological characteristics, these 11 local varieties might have not been grown in Thailand for so long times but could have recently been developed from some imported varieties.

7. Unlike the uncertaining 11 local varieties, Chorlare 1 and Chorlare 2 varieties from Chiang Mai province could be officially pronounced as “true” local varieties if they really have been grown in Thailand for long times till they had unique genetic characteristics as found from the genetic tree results.

8. The “true local” status of Nison-and-Petkhangsink and Ya-and-Local-Nakhon-Si-Thammarat pairs should be tested and confirmed but propably with some more advanced marker techniques, such as AFLP (Amplified Fragment Length Polymorphism).

9. Finally, this thesis then concluded that, although the ISSR marker technique could not clearly indicate the genetic relationships between imported and local tobacco varieties grown in Thailand, it would be developed to be a powerful tool for identification of some long-cultivated local varieties from the imported ones.

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APPENDICES

APPENDIX A

A1. ISSR fragments generated from PCR amplification with five primers (UBC-807, UBC-809, UBC-813, UBC-823, and UBC-836) in all 40 local and imported tobacco varieties.

1) Primer UBC-807

size	280	340	420	550	680	800	950	1300
1. Chorlare 1	0	1	1	1	1	1	1	1
2. Chorlare 2	0	1	1	1	1	1	1	1
3. Nison	1	1	0	0	0	0	1	0
4. Petkhangsink	1	1	0	0	0	0	1	0
5. Pu 001	1	1	1	0	0	0	1	0
6. Pu 002	1	1	1	0	0	0	1	0
7. Whitegold	1	1	1	0	0	0	1	0
8. Napanang	1	1	1	0	0	0	1	0
9. E-bit (local)	1	1	0	0	0	0	1	0
10. Ya-glai	1	1	0	0	0	0	1	0
11. Ya (localxkaset)	1	1	0	0	0	0	1	0
12. local Nakhon Si Thammarat	1	1	0	0	0	0	1	0
13. Ya-chun	1	1	0	0	0	0	1	0
14. Bafra	1	1	0	0	0	0	1	0
15. Basma	1	1	0	0	0	0	0	0
16. Izmir	1	1	1	0	0	0	1	0
17. Samsun bafra	1	1	1	0	0	0	1	0
18. Samsun evkaf	1	1	0	0	0	0	0	0
19. Samsun maden	1	1	0	0	0	0	0	0
20. Xanthiyaka	1	1	0	1	0	0	1	1
21. Zichan	1	1	0	0	0	0	0	0
22. Coker-319	1	1	0	0	0	0	0	0
23. Coker-347	0	1	1	1	0	0	0	0
24. Coker-371 gold	1	1	0	0	0	0	0	0

25. K-394	1	1	0	0	0	0	0	0
26. K-399	0	1	1	0	0	0	1	0
27. Speight G-70	1	1	1	0	0	0	1	0
28. NC 37 NF	1	1	0	0	0	0	0	0
29. NC 89	1	1	0	0	0	0	1	0
30. Ky-9	1	1	0	0	0	0	0	0
31. Ky-10	1	1	0	0	0	0	0	0
32. Ky-15	1	1	1	0	0	0	0	0
33. TN-86	1	1	0	1	0	0	0	0
34. TN-90	1	1	1	0	0	0	1	0
35. TN-97	1	1	1	0	0	0	1	0
36. Va-509	1	1	1	0	0	0	1	0
37. Va-528	1	1	0	0	0	0	0	0
38. Blanket A1	1	1	0	0	0	0	0	0
39. Dimon 1	1	1	0	0	0	0	0	0
40. E-bit (unknown)	1	1	0	0	0	0	0	0

2) Primer UBC-809

size	200	220	250	280	320	400	450	480	520	600	750	1400
1. Chorlare 1	0	0	0	1	1	1	0	1	1	1	1	1
2. Chorlare 2	0	0	0	1	1	0	0	1	0	1	1	1
3. Nison	0	0	0	1	1	0	0	0	1	1	0	1
4. Petkhangsink	0	0	0	1	1	0	0	0	0	1	0	1
5. Pu 001	0	0	0	1	1	0	1	0	0	1	0	1
6. Pu 002	0	0	0	1	1	0	1	1	1	1	0	1
7. Whitegold	0	0	0	1	1	1	1	0	0	1	0	0
8. Napanang	0	0	0	1	1	0	0	0	0	1	0	1
9. E-bit (local)	1	0	0	1	1	0	0	0	0	1	0	0
10. Ya-glai	0	1	0	1	1	0	0	1	0	1	0	1

11. Ya (localxkaset)	0	0	0	0	0	1	0	0	0	1	0	0
12. local Nakhon Si Thammarat	0	0	0	1	0	0	0	0	0	1	0	0
13. Ya-chun	0	0	0	1	1	0	0	0	0	1	0	1
14. Bafra	0	0	1	0	0	0	0	0	0	1	0	0
15. Basma	1	0	1	0	0	0	0	0	0	1	0	0
16. Izmir	1	0	1	0	0	0	0	0	0	1	0	0
17. Samsun bafra	1	0	0	1	1	0	0	1	0	1	0	0
18. Samsun evkaf	1	0	1	0	0	0	0	0	0	1	0	0
19. Samsun maden	0	0	1	0	0	0	0	0	0	1	0	0
20. Xanthiyaka	0	1	0	1	1	0	0	1	0	1	0	0
21. Zichan	0	0	0	1	1	0	0	1	0	1	0	0
22. Coker-319	1	0	0	1	1	0	0	1	0	1	0	0
23. Coker-347	0	0	1	1	0	0	0	1	0	1	0	0
24. Coker-371 gold	0	0	0	1	1	0	0	0	0	1	0	0
25. K-394	0	0	0	1	0	1	0	0	0	1	0	0
26. K-399	0	1	0	1	1	0	0	1	1	1	0	0
27. Speight G-70	0	0	0	1	1	0	1	0	0	1	0	0
28. NC 37 NF	1	0	1	1	0	0	0	0	0	1	0	0
29. NC 89	0	0	0	1	1	0	0	0	0	1	0	0
30. Ky-9	0	0	0	1	1	0	0	1	0	1	0	0
31. Ky-10	0	0	1	1	0	0	0	0	0	1	0	0
32. Ky-15	1	0	1	0	0	1	0	0	0	1	0	0
33. TN-86	0	0	0	1	1	0	0	0	0	1	0	0
34. TN-90	0	0	0	1	1	0	0	0	1	1	0	1
35. TN-97	0	1	0	1	1	0	0	0	1	1	0	1
36. Va-509	1	0	1	1	0	0	0	0	0	1	0	0
37. Va-528	1	0	0	0	1	0	0	0	0	1	0	0
38. Blanket A1 (Unclassified)	0	0	1	1	1	0	0	0	0	1	0	0
39. Dimon 1 (Unclassified)	0	0	0	0	0	0	0	0	0	1	0	0
40. E-bit (Unclassified)	0	0	0	1	1	0	0	0	0	1	0	0

3) Primer UBC-813

size	280	300	480	550	600	700	950
1. Chorlare 1	0	0	0	0	0	0	1
2. Chorlare 2	0	0	0	0	0	0	1
3. Nison	0	0	0	0	0	0	1
4. Petkhangsink	0	0	0	0	0	0	1
5. Pu 001	0	0	0	0	0	0	1
6. Pu 002	0	0	0	0	1	0	1
7. Whitegold	0	0	0	0	0	0	1
8. Napanang	0	0	0	0	0	0	1
9. E-bit (local)	0	0	0	0	0	0	1
10. Ya-glai	0	0	0	0	0	0	1
11. Ya (localxkaset)	0	0	0	0	0	0	1
12. local Nakhon Si Thammarat	0	0	0	0	0	0	1
13. Ya-chun	0	0	0	0	0	0	1
14. Bafra	0	0	0	0	0	0	1
15. Basma	0	0	0	0	0	0	1
16. Izmir	0	0	0	0	0	0	1
17. Samsun bafra	0	0	0	0	0	0	1
18. Samsun evkaf	0	0	0	0	0	0	1
19. Samsun maden	0	0	0	0	0	0	1
20. Xanthiyaka	0	0	0	0	0	0	1
21. Zichan	0	0	0	0	0	0	1
22. Coker-319	0	0	0	0	0	0	1
23. Coker-347	1	0	0	0	1	0	1
24. Coker-371 gold	0	0	0	0	0	0	1
25. K-394	0	1	0	0	1	0	1
26. K-399	0	0	0	1	0	0	1
27. Speight G-70	0	0	0	0	0	0	1
28. NC 37 NF	0	0	0	0	0	0	1
29. NC 89	0	0	1	0	0	0	1
30. Ky-9	0	0	0	0	0	0	1

31. Ky-10	0	0	0	0	0	0	1
32. Ky-15	0	0	1	0	0	1	1
33. TN-86	0	0	0	0	0	0	1
34. TN-90	0	0	0	0	0	0	1
35. TN-97	0	0	0	0	0	0	1
36. Va-509	0	0	0	0	0	0	1
37. Va-528	0	1	0	0	0	0	1
38. Blanket A1 (Unclassified)	0	0	0	0	0	0	1
39. Dimon 1 (Unclassified)	0	0	0	0	0	0	1
40. E-bit (Unclassified)	0	0	1	0	0	0	1

4) Primer UBC-823

size	550	620	750	800	1100	1400
1. Chorlare 1	0	1	1	1	1	1
2. Chorlare 2	0	1	1	1	1	1
3. Nison	1	1	1	1	1	1
4. Petkhangsink	1	1	1	1	1	1
5. Pu 001	1	1	1	1	1	1
6. Pu 002	0	1	1	1	1	1
7. Whitegold	1	1	1	1	1	1
8. Napanang	1	1	1	1	1	1
9. E-bit (local)	1	1	1	1	1	1
10. Ya-glai	1	1	1	1	1	1
11. Ya (localxkaset)	0	0	0	1	1	1
12. local Nakhon Si Thammarat	0	0	0	1	1	1
13. Ya-chun	0	1	0	1	1	1
14. Bafra	0	1	1	1	1	1

15. Basma	0	1	1	1	1	1
16. Izmir	0	1	1	1	1	1
17. Samsun bafra	0	1	1	1	1	1
18. Samsun evkaf	0	1	1	1	1	0
19. Samsun maden	0	1	0	1	1	0
20. Xanthiyaka	0	1	1	1	1	1
21. Zichan	0	1	1	1	1	1
22. Coker-319	0	0	0	1	1	1
23. Coker-347	0	0	0	1	1	1
24. Coker-371 gold	0	0	0	1	1	1
25. K-394	0	1	0	1	1	1
26. K-399	0	1	1	1	1	1
27. Speight G-70	0	0	0	1	1	1
28. NC 37 NF	0	0	0	1	1	1
29. NC 89	0	0	0	1	1	1
30. Ky-9	0	0	1	1	1	1
31. Ky-10	0	0	0	1	1	1
32. Ky-15	0	1	0	1	1	0
33. TN-86	0	1	1	1	1	1
34. TN-90	0	1	0	1	1	1
35. TN-97	0	0	0	1	1	1
36. Va-509	0	1	0	1	1	1
37. Va-528	0	1	0	1	1	1
38. Blanket A1 (Unclassified)	0	1	0	1	1	1
39. Dimon 1 (Unclassiffied)	0	1	0	1	1	1
40. E-bit (Unclassified)	0	1	0	1	1	1

5) Primer UBC-836

size	380	450	510	580	720	800	1000	1200	1400
1. Chorlare 1	1	1	0	1	0	1	0	1	0
2. Chorlare 2	1	0	0	1	0	1	0	1	0
3. Nison	1	0	1	0	0	1	0	1	0
4. Petkhangsink	1	0	1	0	0	1	0	1	0
5. Pu 001	1	1	1	0	0	1	0	1	0
6. Pu 002	1	1	0	0	0	1	0	1	0
7. Whitegold	1	1	0	0	0	1	0	1	0
8. Napanang	1	1	0	0	0	1	0	1	0
9. E-bit (local)	1	0	1	0	0	1	0	1	0
10. Ya-glai	1	0	1	0	0	1	1	1	0
11. Ya (localxkaset)	1	0	1	0	0	1	0	1	1
12. local Nakhon Si Thammarat	1	0	1	0	0	1	0	1	1
13. Ya-chun	1	0	1	0	0	1	0	1	1
14. Bafra	1	0	1	0	1	1	0	1	0
15. Basma	1	1	0	0	0	1	0	1	0
16. Izmir	1	1	1	0	1	1	0	1	0
17. Samsun bafra	1	0	1	0	0	1	0	1	0
18. Samsun evkaf	1	0	1	0	1	0	0	0	0
19. Samsun maden	1	0	1	0	1	1	0	1	1
20. Xanthiyaka	1	0	1	0	1	1	0	1	1
21. Zichan	1	0	1	0	1	0	0	1	0
22. Coker-319	1	0	1	0	1	0	0	1	0
23. Coker-347	1	0	1	0	1	0	0	1	0
24. Coker-371 gold	1	0	1	0	1	0	0	1	1
25. K-394	1	0	1	0	1	0	0	1	0
26. K-399	1	0	1	0	1	0	1	1	0
27. Speight G-70	1	1	1	0	1	0	0	1	0
28. NC 37 NF	1	0	0	0	0	0	0	0	0
29. NC 89	1	0	1	0	1	0	0	1	0
30. Ky-9	1	0	1	0	1	0	1	1	1
31. Ky-10	1	1	0	0	0	0	0	0	0
32. Ky-15	1	0	0	0	0	0	0	1	0

33. TN-86	1	0	0	0	0	0	0	0	0
34. TN-90	1	1	1	0	0	0	0	1	0
35. TN-97	1	1	1	0	0	0	0	1	0
36. Va-509	1	1	0	0	0	0	0	1	0
37. Va-528	1	1	0	0	0	0	0	0	0
38. Blanket A1 (Unclassified)	1	0	0	0	0	0	0	1	0
39. Dimon 1 (Unclassified)	1	0	0	0	0	0	0	0	0
40. E-bit (Unclassified)	1	0	0	0	0	0	0	0	0

APPENDIX B

Distance metrix using Nei and Li's similarity coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. Chorlare (1) (Local)																				
2. Chorlare (2) (Local)	0.0105																			
3. Nison (Local)	0.0571	0.05634																		
4. Petkhangsink (Local)	0.0642	0.05229	0.00457																	
5. Pu 001 (Local)	0.0542	0.05331	0.01759	0.01336																
6. Pu 002 (Local)	0.0391	0.0469	0.03428	0.03731	0.02064															
7. Whitegold (Local)	0.0506	0.06031	0.03306	0.02871	0.01268	0.02575														
8. Napanang (Local)	0.0469	0.0455	0.01857	0.01411	0.00855	0.02171	0.01336													
9. E-bit (Local)	0.0758	0.0639	0.01411	0.00953	0.02291	0.04814	0.02871	0.02425												
10. Ya-plai (Local)	0.0644	0.05331	0.01759	0.01336	0.02575	0.03927	0.04145	0.02715	0.02291											
11. Ya (Local)	0.1005	0.10343	0.05331	0.04814	0.06322	0.08265	0.05834	0.06797	0.04814	0.06322										
12. Local Nakhon Si Thammarat	0.1005	0.08729	0.03987	0.03471	0.04975	0.06797	0.05834	0.05331	0.3471	0.04975	0.01236									
13. Ya-chun (Local)	0.0719	0.05975	0.01966	0.01495	0.02871	0.04389	0.04663	0.03046	0.02575	0.02871	0.02939	0.01699								
14. Bafra (Turkish)	0.094	0.08148	0.03731	0.03245	0.04663	0.06359	0.05449	0.04975	0.03245	0.04663	0.03731	0.03731	0.03987							
15. Basma (Turkish)	0.0899	0.0918	0.05834	0.05331	0.05449	0.0591	0.04975	0.04488	0.03987	0.06797	0.06225	0.06225	0.06278	0.02939						
16. Izmir (Turkish)	0.0796	0.08047	0.05112	0.04663	0.03731	0.05229	0.04389	0.03953	0.03506	0.05975	0.05331	0.05331	0.05449	0.01495	0.0209					
17. Samsun bafra (Turkish)	0.0571	0.0455	0.02871	0.02425	0.02715	0.03128	0.03306	0.02871	0.01411	0.02715	0.05331	0.03987	0.03046	0.03731	0.04488	0.02871				
18.Samsun evkaf (Turkish)	0.1504	0.13674	0.07886	0.0735	0.08911	0.11189	0.1019	0.09668	0.05743	0.08911	0.08786	0.08786	0.08576	0.03162	0.04033	0.03471	0.6278			
19. Samsun maden (Turkish)	0.1209	0.10796	0.05834	0.05331	0.06797	0.08729	0.77089	0.073	0.05331	0.06797	0.03162	0.03162	0.03471	0.01699	0.05192	0.03245	0.05834	0.04033		
20. Xanthiyaka (Turkish)	0.0578	0.0469	0.04145	0.03731	0.04945	0.05331	0.05634	0.05229	0.03731	0.02969	0.05446	0.04203	0.03306	0.03953	0.07259	0.05229	0.03128	0.07789	0.04663	
21.Zichan (Turkish)	0.0805	0.06797	0.03731	0.03245	0.04663	0.05112	0.05449	0.04975	0.03245	0.03506	0.06797	0.05192	0.03987	0.03471	0.05743	0.04975	0.02575	0.04622	0.05743	0.02871
22. Coker-319 (Virginia)	0.1088	0.0962	0.06322	0.05834	0.07259	0.07709	0.08265	0.07789	0.04488	0.0591	0.05192	0.03731	0.03987	0.06278	0.0735	0.06322	0.03731	0.06225	0.05743	0.03953
23. Coker-347 (Virginia)	0.098	0.08576	0.09879	0.09401	0.0918	0.08148	0.10343	0.09879	0.09401	0.0918	0.0913	0.0735	0.08911	0.06797	0.09668	0.06797	0.06797	0.08576	0.07886	0.06797
24. Coker-371 gold (Virginia)	0.1167	0.10343	0.05331	0.04814	0.06322	0.08265	0.073	0.06797	0.04814	0.06322	0.04033	0.02575	0.02939	0.05192	0.08003	0.06797	0.05331	0.06797	0.04622	0.04203
25. K-394 (Virginia)	0.1088	0.11237	0.06322	0.05834	0.07259	0.07709	0.06797	0.07789	0.05834	0.07259	0.05192	0.05192	0.05331	0.04814	0.0735	0.06322	0.06322	0.06225	0.05743	0.06359
26. K-399(Virginia)	0.0644	0.06418	0.04814	0.05548	0.05634	0.04945	0.0639	0.05975	0.05548	0.03532	0.09401	0.07789	0.06359	0.0591	0.09879	0.05975	0.03731	0.08911	0.08265	0.03927
27. Speight G-70 (Virginia)	0.0845	0.08576	0.05449	0.04975	0.02871	0.04389	0.03506	0.04203	0.04975	0.06359	0.05743	0.04281	0.04488	0.05331	0.07886	0.04203	0.04203	0.08576	0.06278	0.05548
28. NC 37 NF (Virginia)	0.1414	0.12699	0.08576	0.08003	0.09668	0.1019	0.0913	0.08576	0.06225	0.09668	0.07485	0.055	0.0741	0.06797	0.04389	0.06797	0.06797	0.04814	0.08148	0.1019
29. NC 89 (Virginia)	0.1047	0.0918	0.04488	0.03987	0.05449	0.07259	0.06322	0.05834	0.03987	0.05449	0.04622	0.03162	0.03471	0.04281	0.08576	0.05834	0.04488	0.0741	0.05192	0.04663
30. Ky-9 (Burley)	0.98	0.08576	0.05449	0.04975	0.06359	0.06797	0.07259	0.06797	0.04975	0.03953	0.05743	0.04281	0.04488	0.05331	0.07886	0.06797	0.04203	0.06797	0.06278	0.03306
31. Ky-10 (Burley)	0.1213	0.12699	0.08576	0.08003	0.07886	0.08406	0.0735	0.06797	0.08003	0.09668	0.07485	0.055	0.0741	0.06797	0.04389	0.06797	0.08576	0.06797	0.08148	0.1019
32. Ky-15 (Burley)	0.1209	0.12587	0.10696	0.1019	0.09879	0.10343	0.07789	0.08911	0.08406	0.11667	0.08003	0.09996	0.09668	0.0735	0.05192	0.05834	0.073	0.05634	0.06797	0.12133
33. TN-86 (Burley)	0.0815	0.06797	0.4814	0.04281	0.05834	0.06322	0.5331	0.04814	0.04281	0.05834	0.08786	0.06797	0.05192	0.06225	0.05634	0.07886	0.04214	0.06161	0.09401	0.04975
34. TN-90 (Burley)	0.0533	0.0639	0.02425	0.03046	0.02291	0.02715	0.03953	0.02425	0.04203	0.04389	0.06278	0.04814	0.02575	0.05834	0.06797	0.04633	0.03506	0.0913	0.06797	0.05975
35. TN-97 (Burley)	0.0719	0.08576	0.06797	0.07789	0.06359	0.05548	0.07259	0.06797	0.07789	0.06359	0.0735	0.05743	0.073	0.08406	0.09668	0.06797	0.05449	0.12842	0.09668	0.06797
36. Va-509 (Burley)	0.0805	0.08148	0.6322	0.05834	0.04663	0.05112	0.04203	0.03731	0.04488	0.07259	0.06797	0.05192	0.05331	0.04814	0.02939	0.02575	0.03731	0.06225	0.05743	0.07709
37. Va-528 (Burley)	0.1124	0.11667	0.7886	0.0735	0.073	0.07789	0.06797	0.06278	0.05743	0.08911	0.08786	0.08786	0.06797	0.08003	0.04033	0.06278	0.06278	0.06161	0.09401	0.09401
38. Blanket A1 (Unclassified)	0.0962	0.08265	0.04814	0.04281	0.05834	0.06322	0.05331	0.04814	0.04281	0.05834	0.06797	0.05023	0.03731	0.04622	0.04033	0.06278	0.04814	0.06161	0.05634	0.06322
39. Dimon 1 (Unclassified)	0.1319	0.11667	0.0741	0.06797	0.08576	0.0913	0.08003	0.0741	0.06797	0.08576	0.06079	0.06079	0.06161	0.055	0.04814	0.0741	0.0741	0.05331	0.06797	0.0913
40. E-bit (Unclassified)	0.108	0.09401	0.05743	0.05192	0.06797	0.073	0.06278	0.05743	0.05192	0.06797	0.08148	0.06161	0.04622	0.0741	0.06797	0.0913	0.05743	0.07485	0.08786	0.073

Distance metrix using Nei and Li's similarity coefficient (continued)

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1. Chorlare (1) (Local)																				
2. Chorlare (2) (Local)																				
3. Nison (Local)																				
4. Petkhangsink (Local)																				
5. Pu 001 (Local)																				
6. Pu 002 (Local)																				
7. Whitegold (Local)																				
8. Napanang (Local)																				
9. E-bit (Local)																				
10. Ya-plai (Local)																				
11. Ya (Local)																				
12. Local Nakhon Si Thammarat																				
13. Ya-chun (Local)																				
14. Bafra (Turkish)																				
15. Basma (Turkish)																				
16. Izmir (Turkish)																				
17. Samsun bafra (Turkish)																				
18.Samsun evkaf (Turkish)																				
19. Samsun maden (Turkish)																				
20. Xanthiyaka (Turkish)																				
21.Zichan (Turkish)																				
22. Coker-319 (Virginia)	0.0223																			
23. Coker-347 (Virginia)	0.05331	0.05331																		
24. Coker-371 gold (Virginia)	0.0239	0.01151	0.05743																	
25. K-394 (Virginia)	0.03471	0.04814	0.05331	0.03731																
26. K-399(Virginia)	0.03506	0.0591	0.06359	0.06322	0.07259															
27. Speight G-70 (Virginia)	0.03987	0.03987	0.05834	0.02939	0.05331	0.05112														
28. NC 37 NF (Virginia)	0.06797	0.05023	0.0741	0.055	0.06797	0.11667	0.0741													
29. NC 89 (Virginia)	0.02939	0.02939	0.06278	0.01823	0.04281	0.05449	0.0223	0.06161												
30. Ky-9 (Burley)	0.01591	0.01591	0.05834	0.01699	0.05331	0.03953	0.04488	0.0741	0.03471											
31. Ky-10 (Burley)	0.06797	0.06797	0.0741	0.055	0.06797	0.11667	0.05634	0.01591	0.06161	0.0741										
32. Ky-15 (Burley)	0.0913	0.0913	0.09668	0.09996	0.0735	0.11667	0.09668	0.06161	0.08576	0.11667	0.08148									
33. TN-86 (Burley)	0.03162	0.06225	0.08576	0.05023	0.06225	0.073	0.06797	0.04814	0.05634	0.05192	0.04814	0.09401								
34. TN-90 (Burley)	0.04488	0.05834	0.07789	0.04814	0.05834	0.04389	0.02575	0.08003	0.03987	0.06322	0.06225	0.08041	0.05743							
35. TN-97 (Burley)	0.06797	0.06797	0.073	0.0735	0.08406	0.05112	0.04488	0.09401	0.06278	0.073	0.0741	0.11667	0.10571	0.03731						
36. Va-509 (Burley)	0.06278	0.06278	0.06797	0.06797	0.06278	0.07259	0.03987	0.03423	0.05743	0.08406	0.03423	0.04281	0.06225	0.03245	0.05331					
37. Va-528 (Burley)	0.06225	0.06225	0.12842	0.06797	0.06225	0.10696	0.06797	0.04814	0.0741	0.08576	0.04814	0.0741	0.04389	0.05743	0.10571	0.04622				
38. Blanket A1 (Unclassified)	0.03162	0.04622	0.06797	0.03423	0.04622	0.073	0.05192	0.03046	0.04033	0.05192	0.03046	0.05634	0.02791	0.04281	0.08576	0.03162	0.04389			
39. Dimon 1 (Unclassified)	0.055	0.07485	0.10409	0.06079	0.055	0.10571	0.08148	0.03731	0.06797	0.08148	0.03731	0.06797	0.03354	0.06797	0.10409	0.055	0.03354	0.03354		
40. E-bit (Unclassified)	0.04033	0.05634	0.9996	0.04389	0.05634	0.08406	0.06225	0.04099	0.03423	0.06225	0.04099	0.06797	0.02134	0.05192	0.09996	0.05634	0.03731	0.02134	0.02575	

APPENDIX C

C1. Morphological characteristics of Turkish tobacco varieties

Bafra

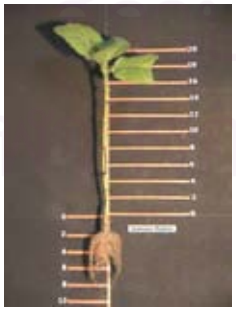
Basma

Basma Xanthiyaka

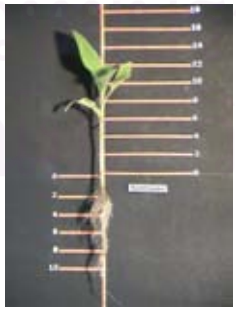
Izmir

Samsun bafra

Samsun evkaf



Samsun maden



Xanthiyaka

Zichan

C2. Morphological characteristics of some Virginia tobacco varieties

Coker-254

Coker-319

Coker-347

Coker-371 gold

K-149

K-317

K-326

K-346

K-394

K-399

Speight G-52

Speight G-140

Speight NC-82

NC-2326

NC 37 NF

NC-89

C3. Morphological characteristics of some Burley tobacco varieties

Burley-21

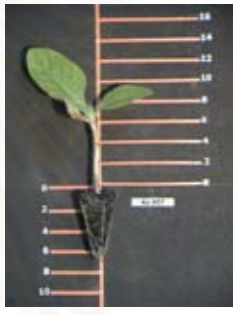
Burley-64

Ky-9

Ky-10

Ky-15

Ky-17

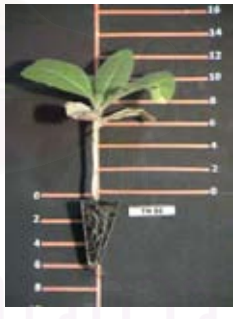


Ky-907

Ky-809

Ky-8959

MS Ky-14xL-8



TN-89

TN-90

TN-97

Va-182

Va-528

VS Burley-21xKy-9

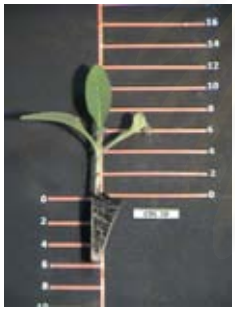
C4. Morphological characteristics of Unclassified tobacco varieties

MC Nair 135

Baisee

Blanket A1

C.N.T



CDL 28

Dimon 1

E-bit

C5. Morphological characteristics of two Chorlare varieties

Chorlare 1

Chorlare 2

APPENDIX C

Reagent preparation protocal

C1. Agarose

1.1) 1% agarose

An enough amount of ingredients for a 100 ml gel composed of:

- Agarose 1 g
- 1xTBE 100.0 ml

1.2) 1.5%and 1.8% agarose

Preparing 1.5%and 1.8% agarose is like 1% agarose but 1.5 and 1.8 g of agrose is used to dissolve in 100 ml 1xTBE buffer.

How to apply the description previously used follow:

1. For 1% Agarose, agarose powder about 1 g is mixed into 1xTBE buffer 100 ml. For 1.5% Agarose, agarose powder about 1.5 g is mixed into 1xTBE buffer 100 ml.

2. The agarose solution is solubilized by heating in a micro oven.

3. The solubilised agarose allow for cool for 50-60?C before pour into a gel mould.

4. Prepare gel mould for set the gel. When time is finished, the dissolved gel is transferred about 25.50 ml.

5. The soluble gel is poured into the gel mould which the comb is already inserted to the gel mould.

6. When the gel as completely setting. The comb was removed.

7. The gel is transferred into a gel chamber containing an enough of 1xTBE buffer that covered the gel to about 1-2 mm depth.

C2. 10xTBE Boric EDTA buffer (10xTBE)

An enough amount of ingredients for a 1000 ml composed of:

- Tris aminomethane 108.0 g
- Boric acid 50.4 g
- EDTA 7.44 g

The solution is prepared as follow:

1. Tris, Boric and EDTA are mixed into volumetric flask 1000 ml.

2. Double distilled water is added up 1000 ml.

3. Solutions stirred until completely dissolve.

4. Store in room temperature and use 1xTBE for running electrophoresis.

C3. 6xloading dye

An enough amount of ingredients for a 100 ml composed of:

-Bromophenol blue 0.125 g
-Glyceral 30 ml.
-10xTBE 60 ml.

All of ingredients mixed and add distilled water up to 100 ml and stored at -20?C.

BIOGRAPHY

Miss Sornsuda Setaphan was born on July 12th, 1983 in Bangkok. She finished her high school level from Benjamamaharat School, Ubonratchathanee Province in 2001. She received Bachelor degree of Science in Applied Biology (Biology) from Department of Biology, Faculty of Science, Chandrakesem Rajabhat University, Bangkok in 2005. She has studied for the degree of Master of Science at the program of Biotechnology, Chulalongkorn University since 2005.

Research presentation

Setaphan, S., Denduangboripant, J., 2006. Molecular marker selection to study genetic relationships of tobacco varieties in Thailand. Abstract *Research plans and development of Thailand Tobacco Monopoly,* 26th-28th September, Department of Research, Thailand Tobacco Monopoly, Bangkok, Thailand.

Setaphan, S., Denduangboripant, J., Suwanprasart, V., and Sutrarashun, D. 2006. Molecular marker selection to study genetic relationships of tobacco varieties in Thailand. Abstract. *The 11th Biological Sciences Graduate Congress,* 15th-17th December, Chulalongkorn Universtiy, Bangkok, Thailand.

Setaphan, S., Denduangboripant, J., Suwanprasart, V., and Sutrarashun, D. 2007. Molecular Marker for determining tobacco *Nicotiana tabacum* L. varieties. Abstract. *The 6th Asian Crop Science Association Conference,* 5th-9th November, Queen Sirikit National Center, Bangkok, Thailand.