การพัฒนาเครื่องหมายโมเลกุลแบบเอเอฟแอลพีเพื่อพิสูจน์เอกลักษณ์พันธุ์ใบยาสูบที่ผ่านการบ่มแล้ว

นางสาวเธียรรัตน์ พิธีการณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DEVELOPMENT OF AFLP MOLECULAR MARKER FOR CULTIVAR IDENTIFICATION OF CURED TOBACCO LEAVES

Miss Tianrat Piteekan

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Thesis Advisor	Assistant Professor Jessada Denduangboripant, Ph.D.

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เธียรรัตน์ พิธีการณ์ : การพัฒนาเครื่องหมายโมเลกุลแบบเอเอฟแอลพีเพื่อพิสูจน์เอกลักษณ์พันธุ์ใบยาสูบที่ผ่านการบ่มแล้ว. (DEVELOPMENT OF AFLP MOLECULAR MARKER FOR CULTIVAR IDENTIFICATION OF CURED TOBACCO LEAVES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. เจษฎา เด่นดวงบริพันธ์, 136 หน้า.

ยาสูบที่มีการเพาะปลูกในประเทศไทยนั้น แบ่งแยกออกเป็นสองกลุ่มใหญ่ คือ พันธุ์ยาสูบพื้นเมืองและพันธุ์ยาสูบนำเข้าจากต่างประเทศ อย่างไรก็ตาม วิธีการในปัจจุบันที่ใช้แยกแยะความแตกต่างระหว่างสายพันธุ์ยาสูบยังไม่มีความแม่นยำเพียงพอโดยเฉพาะอย่างยิ่งในใบยาสูบบ่มแห้งแล้ว ในการศึกษาครั้งนี้จึงได้นำเอาเทคนิคเอเอฟแอลพีมาใช้ในการศึกษาความแตกต่างทางพันธุกรรมระหว่างตัวอย่างใบยาสูบบ่ม 23 สายพันธุ์ ผลการทำเอเอฟแอลพีพบว่าคู่ไพรเมอร์ E_{AAG}/M_{CAA}, E_{AAG}/M_{CGC} และ E_{ACT}/M_{CAG} เกิดแถบดีเอ็นเอทั้งหมด 139 แถบ ซึ่งเป็นแถบดีเอ็นเอที่แตกต่างกันในตัวอย่างยาสูบจำนวน 103 แถบ (74.1%) จากคู่ไพรเมอร์ดังกล่าวพบว่าคู่ไพรเมอร์ E_{ACT}/M_{CAG} ให้ทั้งจำนวนแถบดีเอ็นเอ (59 แถบ) และเกิดแถบดีเอ็นเอที่แตกต่างกัน (83.1%) มากที่สุด ยิ่งกว่านั้นคู่ไพรเมอร์ดังกล่าวให้แถบดีเอ็นเอที่มีความจำเพาะต่อยาสูบสายพันธุ์เวอร์ยิเนีย ซึ่งแถบดีเอ็นเอดังกล่าวน่าจะมีประโยชน์ในการจำแนกและยืนยันจีโนไทป์ของยาสูบสายพันธุ์เวอร์ยิเนีย ในการซื้อขายใบยาได้ แผนภูมิต้นไม้ความสัมพันธ์ทางพันธุกรรมโดยเทคนิคเอ็นเจและยูพีจีเอ็มเอได้แสดงการจัดกลุ่มของใบยาสูบที่คล้ายคลึงกัน ส่วนใหญ่ยาสูบพื้นเมืองมีความสัมพันธ์ใกล้ชิดกันเองและใกล้ชิดกับยาสูบนำเข้าสายพันธุ์เบอร์เล่ย์และเตอร์กิช แนวโน้มการจัดกลุ่มย่อยของยาสูบพื้นเมืองนั้นขึ้นอยู่กับพื้นที่การเพาะปลูก ในส่วนของยาสูบสายพันธุ์นำเข้า นอกจากมีแนวโน้มการจัดกลุ่มตามพื้นที่การเพาะปลูกแล้วยังมีการจัดกลุ่มตามกลุ่มย่อย (เวอร์ยิเนีย เบอร์เล่ย์ และ เตอร์กิช) อีกด้วย ส่วนยาสูบสายพันธุ์เวอร์ยิเนีย นั้นแยกจากยาสูบพื้นเมืองและยาสูบนำเข้าสายพันธุ์อื่นๆ ได้อย่างชัดเจน โดยสรุปแล้วเครื่องหมายโมเลกุลแบบเอเอฟแอลพีนี้มีความสามารถในการแยกแยะยาสูบสายพันธุ์เวอร์ยิเนียออกจากยาสูบสายพันธุ์อื่นๆ ที่มีการเพาะปลูกในประเทศไทยได้ และผลการศึกษาที่ได้นี้สามารถเป็นข้อมูลพื้นฐานสำหรับการศึกษาพันธุกรรมของยาสูบในอนาคต

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: AFLP/ CULTIVAR IDENTIFICATION/ MOLECULAR MARKER/ TOBACCO

TIANRAT PITEEKAN: DEVELOPMENT OF AFLP MOLECULAR MARKER FOR CULTIVAR IDENTIFICATION OF CURED TOBACCO LEAVES. ADVISOR: ASST. PROF. JESSADA DENDUANGBORIPANT, Ph.D., 136 pp.

Tobacco cultivars grown in Thailand are separated to 2 major groups: local and imported cultivars. However, current approaches to distinguish tobacco cultivars are not accurate enough, particularly for dry-cured tobacco. In this study AFLP molecular marker was introduced to investigate genetic differences between cured leaf-materials of 23 tobacco cultivars. The AFLP result showed that three primer-pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) gave a total of 139 AFLP fragments, of which 103 (74.1%) were polymorphic bands. Among these primers, E_{ACT}/M_{CAG} primer gave both highest number of AFLP-PCR fragments (59 bands) and polymorphic bands (83.1%). Furthermore, this primer-combination produced one cultivar-specific band of Virginia cultivars. This cultivar-specific marker would be useful in identification and confirmation of Virginia genotype in tobacco trade. Genetic relationship trees based on NJ and UPGMA techniques revealed clusterings similar to each other. Almost all local cultivars were closely related with each other and also with Burley and Turkish imported cultivars. The supgrouping of local cultivars tended to form following their cultivating regions. Among the imported cultivars, their clusterings were not only based on cultivating area but also on the imported cultivar groups (Virginia, Burley and Turkish). Virginia cultivars were clustered separately from other local and imported cultivars. In conclusion, this AFLP marker technique could separate Virginia cultivars from other cultivars grown in Thailand. And these results could be a fundamental information for other tobacco genetic researches in the future.

Field of Study : Biotechnology Student's Signature Tianrat Piteekan

Academic Year : 2009 Advisor's Signature Jessada Denduangboripant

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
^{o}C	Degree Celsius
µg	Micromolar
µl	Microlitre
µM	Micromolar
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleotide
EDTA	Ethylene diamine tetraacetic acid (disodium salt)
EtBr	Ethidium bromide
ISSR	Inter Simple Sequence Repeat
$MgCl_2$	Magnesium chloride
mg	Miligram
ml	Millilitre
mM	Milimolar
ng	nanogram
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
TBE	Tri-boric-ethylene diamine tetraacetic acid
Tris	Tris (hydroxyl methy) aminomethane
Volt	Voltage

CHAPTER I
INTRODUCTION

Tobacco has been cultivated worldwide for thousands of years and has served as a raw material for cigarette and cigar industries of many countries. Almost all of the commercial tobaccos produced in the world are *Nicotiana tabacum*. It is also one of the most important crops of Thailand. Tobacco was first introduced into Thailand in the 16th century. By Thai law, all tobacco cultivars cultivated in Thailand are defined to two cultivar groups: local cultivars and imported (Virginia, Burley and Turkish) cultivars. This cultivar group separation leads to some differences in tariff-collecting and crop-growing regulations between the local and imported tobacco cultivars. Moreover, if tobacco leaves from either of the two cultivar-groups are dry-cured, they could not be distinguished by any accurate approach whether the leaves are of the local or imported cultivars.

From such problem, some molecular markers had been introduced to determine genetic differences between tobacco cultivars (for example, Del Piano *et al*., 2000; Rossi *et al*., 2001; Zhang *et al*., 2006). Molecular markers, or genetic markers, have become useful tools to provide a relatively unbiased estimation of genetic diversity in plants (Clegg, 1997). Even though many different tobacco cultivars have been grown in Thailand, there is not such genetic information available. Developing molecular marker for genetic relationship studies of Thai tobacco cultivars is therefore very important. Recently, Denduangboripant *et al*. (2008) have successfully developed ISSR (Inter-Simple Sequence Repeat) molecular markers to specifically distinguish some local tobacco cultivars which may have been cultivated in Thailand for a long time. However, these ISSR markers were suitable only for fresh leaf materials, not for dry-cured tobacco leaves.

AFLP (Amplified Fragment Length Polymorphism) is another molecular marker which has been successfully used in polymorphism analysis, crop cultivar identification, and phylogenetic evaluation. The AFLP technique would be a better method for a narrow-genetic basis of cultivated crops like tobacco than other molecular marker

techniques. Generally, AFLP produced more polymorphic loci per primer in any diversity study than RFLP, SSR or RAPD techniques (Bogani *et al*., 1997). Previously, Rossi *et al.* (2001) studied AFLP markers of tobaccos and found that AFLP appeared to be an appropriate technique for genetic fingerprinting of both fresh and processed tobacco leaves. In addition, AFLP genetic-diversity study among flue-cured tobacco (Virginia cultivar-group) revealed that the flue-cured tobacco germplams commonly grown in China have narrow genetic diversity among the cultivars (Zhang *et al*., 2006). In 2008, Siva *et al.* studied genetic polymorphism of Indian tobaccos using AFLP and found that the flue-cured cultivars were clustered separately from the air-cured type (Burley cultivar-group). The AFLP markers of Siva *et al.* were also found being useful for a genotypic identification in trades and commerces.

From these reviewed literatures and the fact that genetic relationship analysis of Thai tobacco cultivars using AFLP technique has never been done before, in this thesis I then introduced AFLP and developed molecular markers to examine genetic differences between local and imported tobacco cultivars grown in Thailand.

Research objective

To develop and improve suitable AFLP molecular markers for determining genetic differences between imported and local tobacco cultivars from cured-leaf samples.

CHAPTER II
BACKGROUND

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

Most cultivated tobaccos belong to the species *Nicotiana tabacum* L. It is a natural amphidiploid (2n= 48) derived from hybridisation of wild progenitor species: *N. sylvestris* and *N. tomentosiforimis* (Gerstel, 1960; Gerstel, 1963). Tobacco originated in the tropical America and it can now be found growing from about 60°N to 45°S (Akehurst, 1981). The *N. tabacum* plant is a perennial herbaceous plant and it is found only in cultivation. Its leaves are commercially processed as an ingredient of cigarettes and cigars.

Tobacco is in the genus *Nicotiana* which is a genus of herbs and shrubs of the family Solanaceae. Solanaceae is also a family of pepper, petunia, potato and tomato and has been divided into three subgenera (*Rustica, Tabacum* and *Pentuniodes*). The genus *Nicotiana* contains about 64 different species (Goodspeed, 1954; Narayan 1987). At the present time, almost all of the commercial tobaccos produced in the world are *N. tabacum* and it has been one of major economic forces in at least 97 industrial and developing countries around the world (Ren and Timko, 2001).

2.1.1 Morphology of tobacco

Tobacco is an unbranched annual crop (Figure 2.1 A). The plant-height ranges from 100 to 600 cm. Its stems are strong, erect, 3 to 6 cm thick, and terminate in a conspicuous inflorescence. Tobacco leaves are big, rough and ovate-elliptic in shape, and sessile on the stem. The size of leaves may vary from 50 to 70 cm long and 12 to 20 cm broad (Figure 2.1 B) (Brücher, 1989). The leaves and stems are covered with glandular hairs. The glandular hairs produce resinous fluid and are sticky to the touch. Inflorescences consist of 100-150 flowers in one raceme. Corollas are pink or white, 3 to 5 cm long (Figure 2.1 C). The flower has five stamens which are attached to the base of a corolla tube. Calyxs are green and cover its fruits. The fruit is a capsule containing

numerous very small seeds (estimation 1 million seeds per plant). The colour of seed varies from dark brown to light brown.

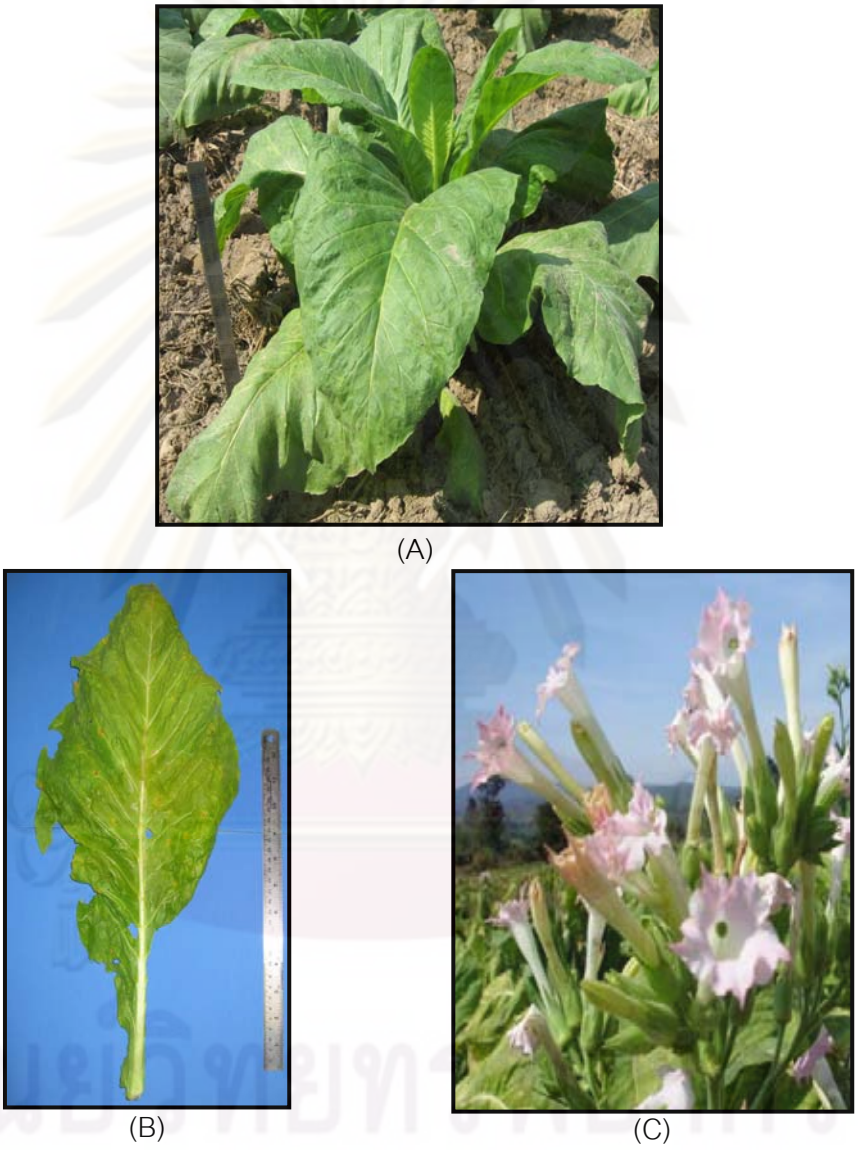


Figure 2.1 Morphological characteristics of tobacco. (A) A tobacco plant. (B) A tobacco leaf. (C) Tobacco flowers.

2.1.2 History of growing

Tobacco is the most widely-grown, commercially non-food crop in the world (Capehart and Grice, 1994). Tobacco has grown natively in North and South America continents since 6000 BC. The use of tobacco originated with the American Indians around 3000 BC (David and Nelson, 1999). Tobacco is used in many different ways, such as inhaling and medicinal practices. The plant had spread over North American before the coming of the white man. In 1492, Christoper Columbus was offered dried tobacco leaves from the American Indians that he encountered.

In 1559, Jean Nicot, for whom the plant was named *Nicotiana*, brought tobacco back to France. Soon after that, the tobacco was being grown all over Europe, Africa, Asia and Australia (Albert, 1996). During the 20th century tobacco has become one of the most economically important agricultural crops in the international marketplace. At this current stage, tobacco has been cultivated in subtropical and temperate regions of the world by approximately 33 million farmers. An example of tobacco plants growing in a cultivation field is shown in Figure 2.2.

Figure 2.2 Tobacco plants growing in a cultivation field in Sukhuthai province of Thailand.

2.2 Tobacco cultivars grown in Thailand

In Thailand, tobacco is believed to be introduced into the country since the 16th century. From Annals of Monsieur De La Loubare, both Thai men and women were seen inhaling tobaccos which were from Manila island, China and inland of Thailand. In Sukhothai era, Thai tobaccos were widely cultivated in the northern and the northeastern regions of the country (อุทิศ, 2534). In 1935, British American Tobacco (B.A.T) company introduced Virginia tobacco cultivars into Thailand. Thereafter, the Royal Thai government bought all tobacco-operations and found Thailand Tobacco Monopoly in 1939 under Ministry of Finance (ประกิต และ กรองจิต, 2457). In addition, during 1958-1959, Burley and Turkish tobacco cultivars were firstly cultivated in Sukhothai and Roi Et provinces, respectively.

At present, Thai farmers cultivate tobacco in various regions of Thailand such as in the North, the South, the Northeast and the Central. There are two major types of commercial tobacco cultivars grown in Thailand: imported tobacco cultivars and local cultivars.

2.2.1 Imported tobacco cultivars

Since 1939 Thailand Tobacco Monopoly has imported tobacco seeds from foreign countries for experiment and development of tobacco cultivation in Thailand. Thailand Tobacco Monopoly assigns tobacco seeds to its regional tobacco stations around the country and also provides knowledge and financial support to farmers (วรวิทย์ และคณะ, 2549). Maejo Tobacco Experiment Station was also set in Sansai district of Chiang Mai province in 1933 to serve as a research and development post for high-quality tobacco (สงวน, 2524). All imported tobacco cultivars can be separated to three groups: Virginia, Burley and Turkish. These three cultivar groups are classified mainly by different methods of curing, i.e. a flue-curing method for Virginia, light air-curing for Burley and sun curing for Turkish.

2.2.1.1 Virginia cultivar group (or flue-cured tobacco)

Cured leaves of Virginia tobacco cultivars are the major component of most blended cigarettes (Collins and Hawks, 1993). Virginia cultivar (Figure 2.3 A) is mostly cultivated in the upper northern region (for example, Chiang Rai, Lamphun, Phrae and Phayao provinces) and the northeastern region (Nong Khai and Nakhon Phanom) of Thailand. A flue-curing method is used for tobacco leaves of Virginia cultivars, therefore sometimes called flue-cured or bright tobacco. The main feature of flue-curing method is a leaf-drying process under controlled conditions. Leaves are taken into a closed curing-house (Figures 2.3 B-C) and heated by hot flue. The heat source supplies controlled heat to the leaves until their starch is converted into sugar and the green fresh leaves become bright-yellow colour, with aromatic smell and fine leaf texture. This curing method also produces smooth smoking properties with medium to high levels of nicotine. Some of Virginia cultivars popularly grown in Thailand are Coker-187 hick, Coker-347 and K-326 (วรวิทย์ และคณะ, 2549).

(A)

(B)

(C)

Figure 2.3 Virginia tobacco cultivar. (A) Virginia tobacco cultivar growing in Lumphun province. (B) A flue-curing house. (C) Virginia leaves hung in a curing house.

2.2.1.2 Burley cultivar group (or light air-cured tobacco)

These cultivars are used as a blended component of cigarettes, inferior to Virginia cultivars. Burley cultivars are grown in the lower northern region of Thailand (for example, in Sukhothai and Phetchabun provinces). Burley cultivars usually cultivated in Thailand are B1 special, KY14, TN86 and TN90 (วรวิทย์ และ คณะ, 2549). Burley tobaccos (Figure 2.4 A) are lightly air cured. In the light air-curing method, the

source of energy is an atmospheric heat carried through air. This method requires an open barn in which tobacco leaves are hung and protected from direct wind and sun light (Figure 2.4 B). After cured, tobacco leaf colour are changed from green to yellow and then to medium brown (Figure 2.4 C) while leaves and stems are dried solely. Light air-cured tobacco is low in sugar with sweet flavour and high nicotine.

(A)

(B)

(C)

Figure 2.4 Burley tobacco cultivar. (A) Burley tobacco cultivar growing in the crop field in Sukhothai province. (B) An air-curing barn (C) Dried Burley tobacco after air-curing process.

2.2.1.3 Turkish cultivar group (or sun-cured tobacco)

Turkish cultivars are a very short plant with small-sized leaves (Figure 2.5 A). Their leaves are 2 or 3 inches wide. Turkish cultivars are used in blended cigarettes and have unique aroma smell, with low sugar and nicotine. Its unique aromatic and other properties are a result of an adaptation of the plants to poor soil and stressful climatic condition of cultivated areas. These Turkish cultivars are also called oriental tobaccos and grown in the northeast region of Thailand (for example, Nakhon Phanom, and Nong Khai provinces). The best known cultivars of Turkish tobaccos are Basma, Samsun and Xantiyaka (Murray, 2009). A sun-curing method is commonly used for Turkish cultivars and the source of energy for sun-curing method is direct solar-heat. When being cured, the tobacco leaves are stung onto cotton strings. Once each string is completely filled, this bundle of leaves is then attached to a bamboo pole at either end of the pole and dried uncovered under the sun (Figure 2.5 B). The heat of the sun directly changes the leaf colour from yellow to orange (Figure 2.5 C).

(A)

(B)

(C)

Figure 2.5 Turkish tobacco cultivar. (A) Turkish tobacco cultivar growing in the crop field in Nakhon Phanom province. (B) Leaves drying in the sun. (C) Dried leaves of Turkish cultivar after sun-curing process.

2.2.2 Local cultivar group (or air- and sun-cured tobacco)

Local tobacco cultivars (Figure 2.6 A) of Thailand are similar to Virginia and Burley imported cultivars in high stalks and large leaves (วรวิทย์ และ คณะ, 2549). Local cultivars are cultivated in crop fields all over the country such as the northern region (for example, Phrae, Phayao and Sukhothai provinces), the southern region (Nakhon Si Thammarat), the northeastern region (Nakhon Phanom and Nong Khai) and the central region (Kanchanaburi, Suphan Buri and Lop Buri) of Thailand. The terminal buds of local tobacco plants are picked during cultivation, or called a topping method, to keep the leaf strength. Local tobaccos are usually used to make a “roll-your-own” (RYO) cigarettes (Figure 2.6 B) which is a locally dried tobacco product. The air- and sun-curing method includes three steps. In the first step, the tobacco leaves are air-cured about four-to-five days. Secondly, the air-cured leaves are cut into 1 to 2 inches strips by hand or machine. Then the strips are spread on wooded racks and sun-dried for about two days (Figure 2.6 C). Examples of local tobacco cultivars are E-dum (in Phetchabun province), Petkhangsink (Sukhothai) and Kan (Suphan Buri).

(A)

(B)

(C)

Figure 2.6 Local tobacco cultivar. (A) Hangkai local cultivar growing in the crop field in Phrae province. (B) Roll-your-own (RYO) tobacco. (C) Local tobacco was sun-dried on wooden racks.

2.4 Plant DNA markers

Molecular markers have played a major role in genetic characterisation and improvement of many plant crops. DNA-based markers are powerful tools for unbiased estimation of genetic evaluation and breeding, identification of cultivars, phylogenetic evaluation and study of genetic diversity (Souframanien and Gopalakrishna, 2004). The greatest advantage of molecular marker techniques is their capability to detect genetic diversity at a higher level of resolution than other conventional methods. The DNA-based assays are usually accurate and speedy. Furthermore, molecular information can be obtained from little amounts of plant materials, and is not effected by environmental conditions (Sergio and Gianni, 2005).

One of early DNA-based marker techniques is Restriction Fragment Length Polymorphism (RFLP). RFLP is based on genomic DNA which is digested with restriction enzymes and separated by electrophoresis on a gel. The gel is then blotted onto a membrane and the specific fragments are made visible by hybridisation with a labeled probe (Weising *et al*, 1995). RFLP had been a basis method for most works in plant genetic studies for many years, even though this RFLP marker is rather difficult to prepare.

The invention of polymerase chain reaction (PCR) in 1985 to amplify target DNA gave rise to the generation of faster and less expensive PCR-based markers. The PCR-based markers involve an *in vitro* amplification of a particular DNA sequence with specific or arbitrary primers. PCR basically amplifies DNA template sequence by primers of complementary DNA strands to thousand copies. The amplified products are then separated by electrophoresis. One of 20 to 30 cycles of the PCR reaction consists of three basic steps (Figure 2.7):

1) Denaturing step: a double-stranded DNA template is denatured at high temperature to make the template accessible for primers and DNA polymerase.

2) Annealing step: primers bind to the complementary sequence on the template at lower temperature.

3) Extension step: DNA polymerase syntheses new DNA strands which are complementary to the DNA template strands by adding dNTPs.

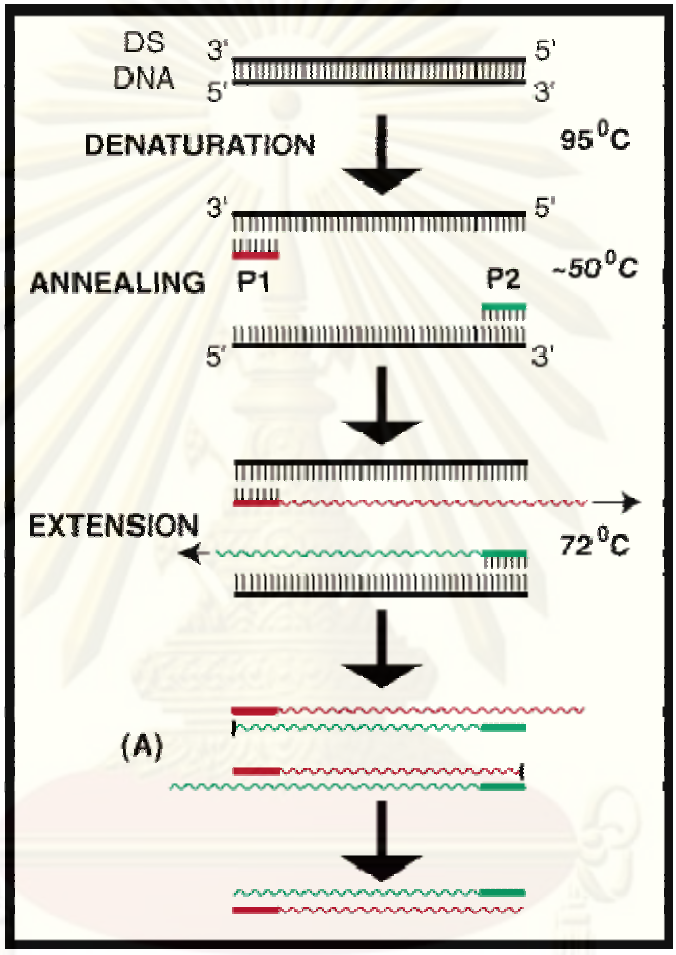


Figure 2.7 Three steps of a Polymerase Chain Reaction
(Source: http://flmnh.ufl.edu/cowries/PCR.gif&imgrefurl)

PCR-based DNA techniques have been very useful in assessment of biodiversity, study of plant populations and identification of plant cultivars. The most common techniques of these PCR-based systems are Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR) and Amplified Fragment Length Polymorphism (AFLP). The comparison of these three PCR-based techniques that relate to their characteristics are summarised in Table 2.1.

Table 2.1 Comparison of RAPD, ISSR and AFLP PCR-based marker systems in plants.

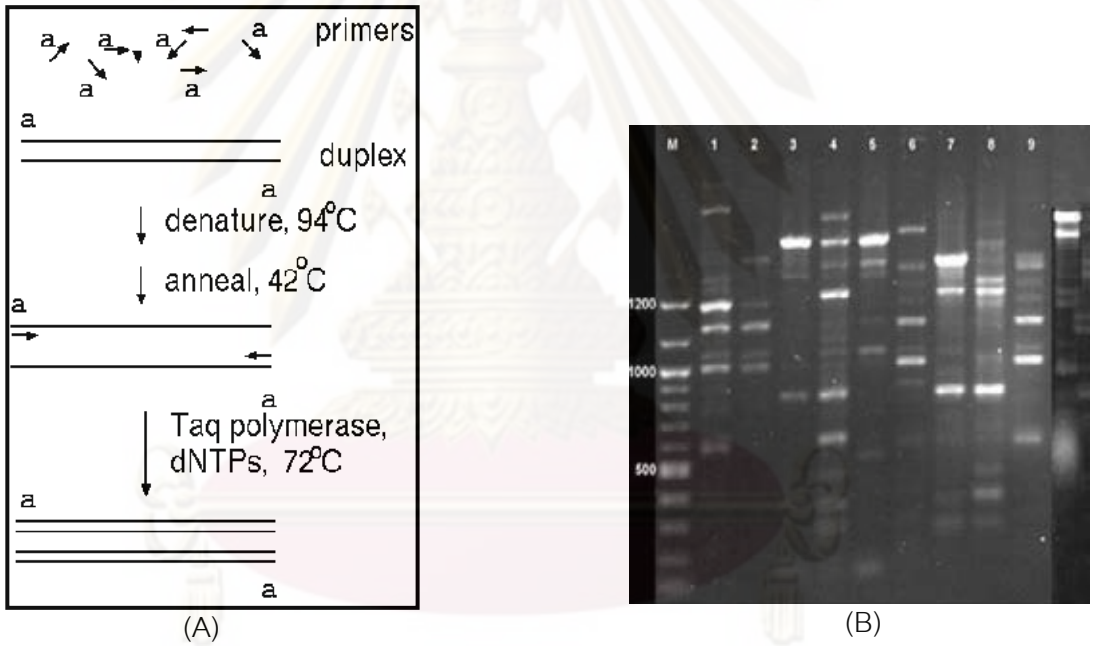
Marker / Characteristics	RAPD	ISSR	AFLP
Principle	DNA amplify with random primers	Regions between two SSRs amplify with 3'-anchored primers	Endonuclease restriction, adapter ligation, PCR
Cause of polymorphism	Point mutations, Insertions, Deletions	Point mutations, Insertions, Deletions	Point mutations, Insertions, Deletions
Level of template	Low	Low	Low
Quality of DNA	High	Medium	Medium
Level of amplified product	Medium	Medium	High
Level of polymorphism	Medium	Medium	High
Reliability	Low	Medium	High
Information of DNA sequence	No	No	No
Type of marker	Dominant	Dominant	Dominant
Radioactivity	No	No	Yes/No
Development cost	Low	Medium	High
Cost per analysis	Low	Low	Medium

2.4.1 RAPD (Random Amplified Polymorphic DNA)

RAPD marker is a dominant marker system which is simple, rapid and capable to scan and detect genetic polymorphism. In 1990, this method to generate PCR amplification products randomly from genomic DNA was introduced by Williams *et al.* (1990). DNA segments are amplified with short random primers (8-12 nucleotides) of arbitrary nucleotide sequences. The short primers are annealed in the first PCR cycles at low stringency. One of the advantages of RAPD is that no prior sequence information of the target gene is required because the RAPD-primers would bind anywhere in the sequence (Figure 2.8 A). RAPD polymorphism results from changes in primer-binding sites in the DNA sequence. The polymorphic PCR products

can be separated by gel electrophoresis (Figure 2.8 B). In theory, the total number of PCR products per primer depends on size of template, PCR conditions, primer sequences and base-mismatching between primers and the template (Bussell *et al*., 2005).

The advantages of the RAPD marker technique make this method very popular in plant genetic analysis, although band profiles of RAPD can not be interpreted in terms of loci and alleles. RAPD has been used for many purposes, ranging from studies at the individual level to closely-related species. It also has been applied to study genetic differences between species, population genetics and phylogenetics.



(A) (B)

Figure 2.8 Random Amplified Polymorphic DNA (RAPD) technique. (A) A principle of RAPD. (B) An example of a RAPD band pattern.
(Source: http://manitoba.ca/afs/plant_science/courses/bioinformatics/lec08/rapd.gif 20/05/2009)

2.4.2 ISSR (Inter-Simple Sequence Repeat)

ISSR is one of PCR-based fingerprinting techniques. ISSR markers were first studied by Zietkiewicz *et al*. in 1994. This method has been reported to produce more complex marker patterns than the RAPD approach (Parsons *et al*., 1997; Chowdhury *et al*., 2002). The ISSR technique exploits abundant distribution of

simple sequence repeats (SSRs) or microsatellietes in plant genomes (Figure 2.12 A). The SSR regions are commonly distributed throughout the genomes and have been utilised in numerous ways to screen genomic variability (Weising *et al*., 1995). ISSR markers use simple sequence repeat primers (e.g. $[AC]_n$) to amplify regions between their target SSR sequence (Kahl, 2001). ISSR primers are designed to be longer than RAPD primers which allow a higher annealing temperature to be used. This marker technique normally amplifies 25 to 50 fragments of the PCR products which could be visualised in either agarose or polyacrylamide gels (Figure 2.9 B). Nowadays, ISSR analysis has been widely used for cultivar identification in numerous plant species.

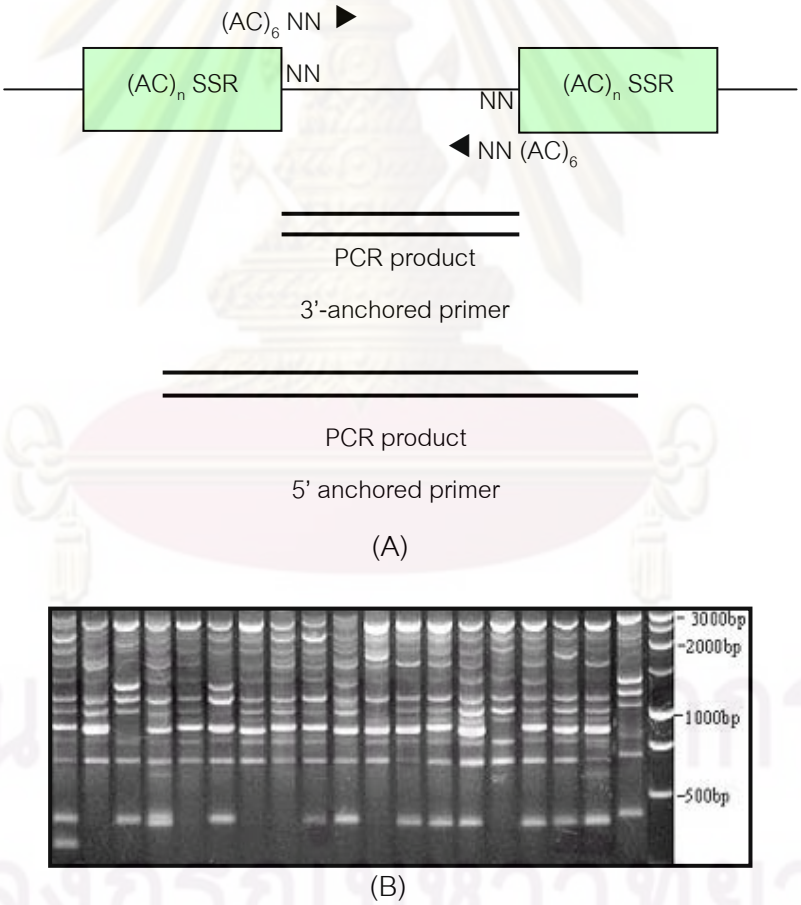


Figure 2.9 Inter Simple Sequence Repeat (ISSR) technique. (A) A principle of ISSR. (B) An example of an ISSR band pattern.

(Source: http://210.212.212.7:9999/PHP/SILKSAT/index.php?f=protocol_issr)

2.4.3 AFLP (Amplified Fragment Length Polymophism)

AFLP, another DNA fingerprinting technique, was developed by Vos *et al*. in 1995. AFLP is essentially a combination of RFLP and PCR techniques. This method is based on a PCR amplification of restriction fragments digested from total genomic DNA. The AFLP technique involves three steps. Genomic DNA is first digested with two restriction enzymes: an infrequent cutter (i.e. *Eco*RI with a six-cut-bases recognition sequence) and a frequent cutter (i.e. *Mse*I; four-bases recognition sequence). A ligation of oligonucleotide adapters specific to the ends of the restriction fragments provides a stringent priming template for amplification of the restriction-fragments (Figure 2.10). Secondly, subsets of restriction fragments are selectively amplified using a combination of selective primers. This selective amplification is achieved by using selective nucleotides internal to the restriction sites of the fragments. And thirdly, the AFLP amplified products are analysed on a manual polyacrylamide gel electrophoresis with an autoradiography (Figure 2.11).

AFLP analysis is alike to RAPD in that no prior sequence information of the genome is needed. Therefore, it can be applied to any DNA sample. In general, AFLP detects a greater number of loci per primer than RFLP, RAPD and ISSR (Bogani, 1997). AFLP method produces very high number of polymorphic bands and it is also a highly sensitive method for DNA fingerprinting. AFLP has been used as a powerful molecular marker technique for a construction of linkage map, marker-assisted breeding, genetic distance analysis, molecular typing and genetic fingerprinting. AFLP is being used in genetic diversity analysis of crop plants because of its maximum coverage of the genome in a short time.

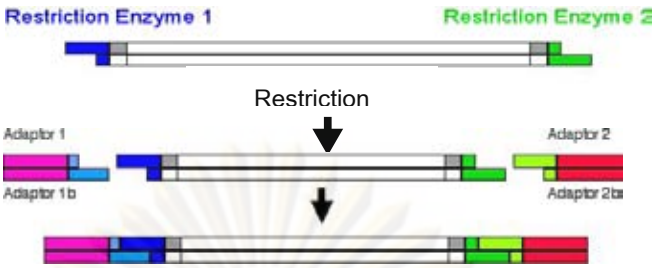


Figure 2.10 DNA is digested with two restriction enzymes and AFLP adapters are ligated to the sticky ends of the restriction fragment. (Source: http://insilico.ehu.es/AFLP/aflp3.jpg)

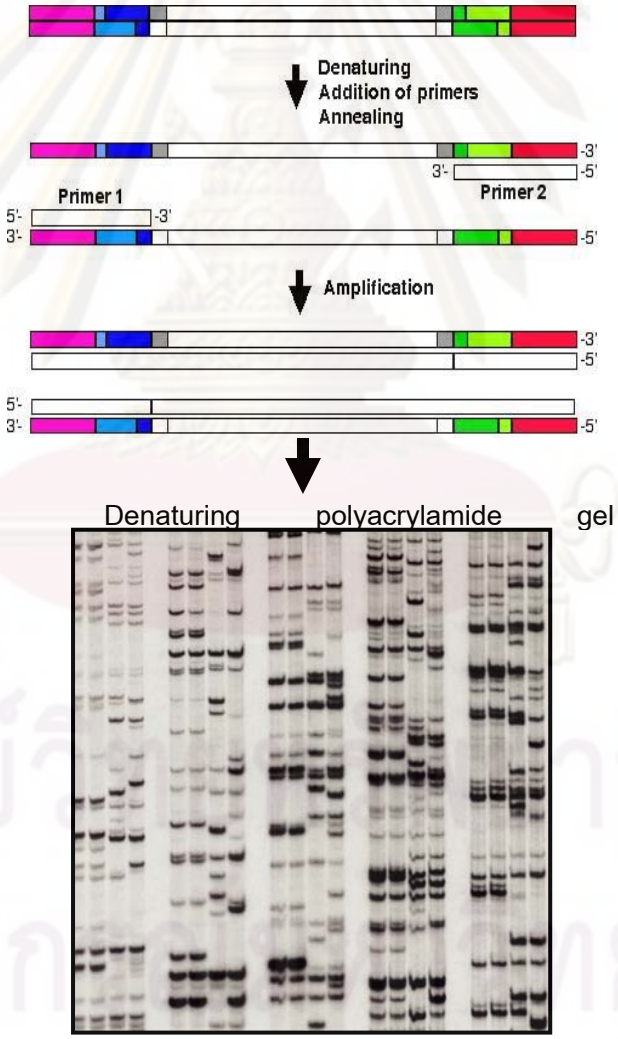


Figure 2.11 AFLP-PCR with primer-matching adapters and an example of AFLP band pattern. (Source: http://insilico.ehu.es/AFLP/aflp3.jpg)

2.5 Molecular marker studies of tobaccos

During the last two decades, molecular markers such as RAPD, ISSR and AFLP have acted as versatile tools for genetic improvement in different fields of agricultural research. Recently, these molecular markers have also been employed to study evolutionary genetics and breeding of tobacco. RAPD markers have been used in early studies on molecular genetic relationships among tobaccos. Filippis, Hoffman and Hampp (1996) used RAPD technique to identify some somatic hybrids of tobaccos. Two parental tobacco species and six somatic hybrids were successfully analysed for their genetic relationship. Their study suggests that RAPD technology is a versatile, precise, sensitive and cost effective method for genetic analysis of tobaccos. In 2000, Del Piano *et al*. carried out a preliminary analysis of genetic diversity in 12 cultivars of *N. tabacum* using three random RAPD primers. Relationships of 31 flue-cured tobacco cultivars in China were also detected by RAPD (He *et al*., 2001) and their genetic diversity was found being rather narrow.

In 2001, Rossi *et al*. applied RAPD markers for tobacco variety identification and found that tobacco cultivars appear to have low level of genetic diversity. Similarly, genetic and geographic polymorphisms of Mus and flue-cured cultivars (FCV) in Turkey using RAPD technique were found having smallest genetic distances among cultivated tobaccos studied (Arslan and Okumus, 2005). Furthermore, Sarala and Rao (2008) have recently examined genetic diversity in Indian flue-cured (or FCV) and Burley tobacco cultivars based on RAPD. Their result showed a clear pattern of division among the flue-cured tobaccos based on geographic origin and clearly distinguished between Burley and FCV cultivars. Another RAPD analysis of genetic diversity among flue-cured tobacco cultivars has been performed by Zhang *et al*. (2008). However, their results did not indicate any clear pattern of division among tobacco accessions.

In addition, ISSR molecular markers had also been used in genetic fingerprinting of *Nicotiana* species (Del Piano *et al*., 2004). Their result showed that the technique is a useful tool to develop molecular markers to characterise all *Nicotiana* species. Yang *et al*. (2005) studied genetic diversity of 24 flue-cured tobaccos and found low genetic

diversity among the flue-cured cultivars and also indicated that ISSR analysis was suitable for cultivar identification. In 2008, Denduangboripant *et al.* have recently developed ISSR markers to determine local cultivars of tobacco grown in Thailand. Their result showed that two of 13 Thai local cultivars were distantly separated from some other imported cultivars.

AFLP is another molecular marker having been used to analyse tobacco cultivars. In 2001, Ren and Timko resolved genetic polymorphism and evolutionary relationships among cultivated and wild *Nictiana* species using AFLP technique. The amount of genetic polymorphism among cultivated tobacco lines presenting in their study was limited. Genetic polymorphism existing among wild species of *Nicotiana* species was found greater than among cultivar forms and the cultivated tobaccos were grouped based upon geographic origin and manufacturing quality traits. In addition, Rossi *et al*. (2001) evaluated the performance between RAPD and AFLP techniques for tobacco cultivar identification and showed that AFLP appears to be an appropriate technology for a genetic fingerprinting of both green and cured tobacco leaves. The AFLP research of Zhang *et al*. (2006) on genetic diversity among flue-cured tobacco in China revealed that the present-day commonly grown flue-cured tobacco has narrow genetic diversity among all other cultivars and their clustering were also based on geographic origin.

In India, Siva *et al*. (2008) used AFLP to study genetic polymorphism of 54 Indian tobacco types belonging to two cultivated species of *Nicotiana* (*N. tabacum* and *N. rustica*). Genetic polymorphism presenting among Indian cultivars was low and their clusters were formed on the basis of species and manufacturing quality traits of the cultivars. They also found that the cultivated flue-cured cultivars were clustered separately from other air-cured types. Furthermore, Zhang *et al*. (2008) assessed AFLP and RAPD markers for genetic diversity among 28 flue-cured tobacco cultivars. They reported that AFLP generated larger number of bands than RAPD and the flue-cured tobacco cultivars were grouped together suggesting their shared common ancestry.

From these reviewed literatures and the fact that genetic analysis of Thai tobacco cultivars using AFLP molecular marker has never been done before, the AFLP technique was then introduced in this thesis to examine genetic differences between local and imported tobacco cultivars grown in Thailand.

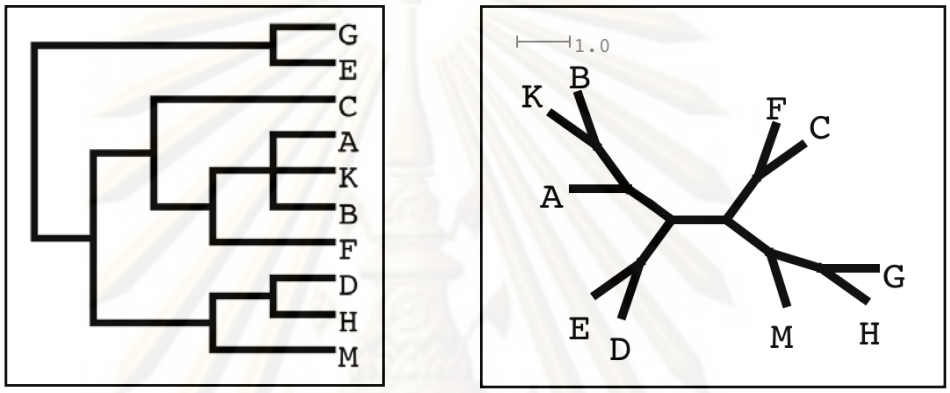
2.6 Phylogenetic tree reconstruction methods

2.6.1 Phylogenetic tree reconstruction

Phylogenetics is one branch of systemetics and it is a field of biology concerning with identifying and understanding the evolutionary relationships among many different kinds of organisms. In phylogenetic studies, the most convenient way of visually presenting evolutionary relationships among a group of organisms is through an illustration called “a phylogenetic tree” on “a phylogeny”. A phylogenetic tree describes a pattern of relationships among taxa which helps to understand the history of all life, genealogical of organisms and representation of hypothesised ancestor/descendant relationships. Phylogenies are reconstructed using all kinds of data such as morphological character data from either living or fossillised organisms, molecular data, metabolic data, geographical and geological data (Swofford *et al*., 1996). Reconstruction of a phylogenetic tree is driven by computer operations. Many phylogenetic computer programs such as PHYLIP (Felsenstein, 1981), NTSYS (Rohlf, 1994) and PAUP* (Swofford, 1998) are now easily to conduct a phylogenetic tree.

A phylogenetic tree is a tree diagram showing evolutionary relationships among various biological species that are believed to have a common ancestor (Figure 2.12). Each phylogenetic tree is composed of nodes and branches. Each node represents a taxonomic unit (species, population and individual). Internal nodes are generally called “hypothetical taxonomic units” (HTUs), as they cannot be directly observed, and terminal nodes are often called “operation taxonomic units” (OTUs). A branch of the tree defines relationships between the taxonomic units in term of descent and ancestry. A pattern of all branches on the tree is called a topology. A branch length represents a number of changes that have occurred in the tree branch. The branches can be unscaled if the branch length is not proportional to the number of

changes that has occurred. Phylogenetic trees may also be either rooted or unrooted. In a rooted tree (Figures 2.12 A), there is a unique node called a root. The tree root is a common ancestor of all taxa from which a unique path leads to any other node. An unrooted tree (Figure 2.12 B) only specifies relationships among species without identifying a common ancestor.



(A) (B)

Figure 2.12 Phylogenetic trees. (A) A rooted tree. (B) An unrooted tree. (Source: www.embl-heidelberg.de/~seqanal/courses/wtacM)

2.6.2 Evaluation of DNA fragment patterns

Some commonly employed molecular marker methods, especially RAPD, ISSR and AFLP, generate a fingerprinting pattern obtained from a particular DNA material. Fingerprinting patterns originated from different samples have to be compared from one pattern to another pattern. Evaluation and comparison of marker patterns required individual DNA bands from each sample and the different bands between samples are scored for a presence of co-migrating bands. Polymorphisms between the fingerprinting patterns of individuals are scored as presence (1) or absence (0) of particular sized fragments. Correctness and accuracy of band-scoring strongly depend on several parameters, such as DNA quality, completely digestion of genomic DNA, electrophoretic conditions and means of signal detection (Weising *et al*., 1995). Molecular weight markers are typically used to estimate size of bands and also serve as standards.

Only unambiguously scorable DNA bands should be considered for a phylogenetic analysis. Moreover, DNA bands that cannot be scored accurately throughout all lanes should be excluded from the analysis. The fingerprinting patterns can be evaluated by eye-and-hand or by an automated method. Fragment sizing and matching can be scored directly on a photo of electrophoresis gel with help of a transparent ruler.

2.6.3 Similarity index

As the first step of similarity analysis, multilocus band-patterns are applied to various procedures to quantify a pairwise similarity of genotypes represented in the different fingerprinting patterns. Commonly, a similarity index is calculated from band-sharing data of each pair of the fingerprints (Weising *et al*., 2005). These similarity indices can be used to quantify the amount of genetic variation between pairs of samples directly. They can be grouped agreeing to the origin of the compared samples and depicted in a frequency table. More often, a matrix of pairwise similarity (or genetic distance) is used as an input file for subsequent multivariate analyses.

There are many similarity coefficients used in molecular marker analysis. The coefficient formula can acquire any value between 0 (= no bands in common) and 1 (= pattern is identical). Examples of the similarity coefficients are as follow:

Jaccard's coefficient: J= a/ (a+b+c) (1)

Here, a = the number of 1-1 matches, b = the number of 1-0 matches and c = the number of 0-1 matches (1 = band present, 0 = band absent).

Nei and Li's coefficient: N= 2a/ (a+b) (a+c) (2)

Here, a = the number of 1-1 matches, b = the number of 1-0 matches and c = the number of 0-1 matches (1 = band present, 0 = band absent).

In the formulas of Jaccard's and Nei and Li's coefficients are derived from comparing the number of bands shared between individuals or populations (Jaccard, 1908; Nei and Li, 1979). However, coefficient of Nei and Li puts more weight on positive matches. An advantage of these two methods is that the band absence can be

excluded from the analysis and there are no assumptions of Hardy-Weinberg equilibrium.

Dice's coefficient as in Formula 3 (Dice, 1945) is similar to Nei and Li's coefficient but gives twice the 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). Jaccard's, Nei and Li's and Dice's coefficients do not consider the number of 0-0 matches.

Dice's coefficient: D= a/ (2a+b+c) **(3)**

Here, a = the number of 1-1 matches, b = the number of 1-0 matches and c = the number of 0-1 matches (1 = band present, 0 = band absent).

In the case of the Simple Matching coefficient (Formula 4) (Sneath and Sokal, 1973) is similar to Jaccard's coefficient, but this method incorporates band absence (d) in the formula.

Simple Matching coefficient: SM= (a+d)/ (a+b+c+d) **(4)**

Here, a = the number of 1-1 matches, b = the number of 1-0 matches, c = the number of 0-1 matches and d = the number of 0-0 matches (1 = band present, 0 = band absent).

For UPGMA and NJ reconstruction method is calculated based on presente/absente data for pairwise comparisons between lanes. One of most commonly used similarity indices is Nei and Li's coefficient, which avoid including shared absences of fragments in the calculation of similarity, and often yield closely correlated results. Therefore, this coefficient was chosen to use in this thesis.

2.6.4 Tree construction using distance matrix method

Distance method try to fit a tree to a matrix of pairwise genetic distance (Felsenstein, 1985). Distance is often defined as the fraction of mismatches at aligned positions, with gaps either ignored or counted as mismatches. The distance matrix uses evaluated distances in a matrix from between all pairs of species (or genes) in a data set to reconstruct a phylogenetic tree. This method is computationally fast; however,

before a distance matrix can be analysed, any data set of nucleic acid or fingerprinting data must first be converted into estimated distances in a matrix form. The main distance matrix method is a cluster analysis and minimum evolution (Peer, 2000). Moreover, this method may produce a rooted and an unrooted tree, depending on the algorithm used to calculate them. The main advantage of distance-matrix methods is simple method to implement and they are much less computer-intensive, which is important for comparison of many taxa. However, disadvantage of this method is their inability to efficiently use an information about local high-variation regions that appear across multiple subtrees. Presently, Unweighted Pair Group Method of Arithmatic Mean (UPGMA) and Neighbour-Joining (N-J) method are mostly used distance methods for phylogenetic tree reconstruction.

2.6.4.1 UPGMA (Unweighted Pair Group Method of Arithmatic Mean) method

UPGMA was originally developed for constructing taxonomic phenograms which are trees that reflect the phenotypic similarities between operational taxonomic units (OTUs) (Sneath and Sokal, 1973). This method involves clustering of closely species. At each stage of clustering, tree branches are being built and the branch lengths are calculated. UPGMA assumes a constant evolutionary rate, and so the two species in a cluster are given the same branch length from the node. UPGMA employs a sequential clustering algorithm in which local topological relationships are identified in order of similarity, and the phylogenetic tree is build in a stepwise manner. It is a simple and fast method of tree construction. However, because of an assumption of relatively constant rate of evolution, it often produces incorrect topologies when an assumption is not met. An example of UPGMA tree is shown in Figure 2.13.

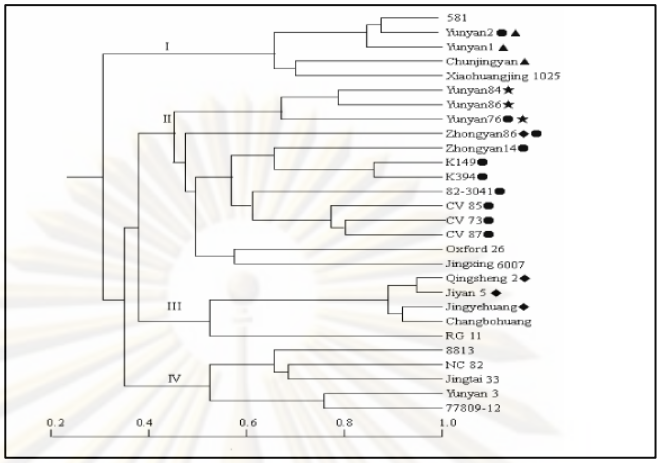


Figure 2.13 An example of a UPGMA dendrogram of 24 Flue-cured tobacco cultivars based on data from RAPD and AFLP (Zhang *et al*., 2008).

2.6.4.2 NJ (Neighbour-Joining) method

The neighbour-joining method by Saitou and Nei (1987) is a wildely used method for constructing phylogenetic trees. It is a distance-based method and does not require the data to be ultrametric. The raw data is provided as a distance matrix and the tree is constructed by linking the least distant pair of nodes in this modified matrix. The NJ method consists in clustering of neighbour species that are joined together by one node. Neighbor-joining method applies general data-clustering techniques to sequence analysis using genetic distance as a clustering metric. The simple neighbor-joining method produces unrooted trees, but it does not assume a constant rate of evolution (i.e., a molecular clock) across lineages. Rooted trees (for example, Figure 2.14) can be created by using an outgroup and the root can then effectively be placed on the rooting point in the tree where the edge from the outgroup connects. Furthermore, neighbor-joining is statistically consistent under many models of evolution. Then, given data of sufficient lengths, neighbor-joining method would reconstruct the true tree with high probability.

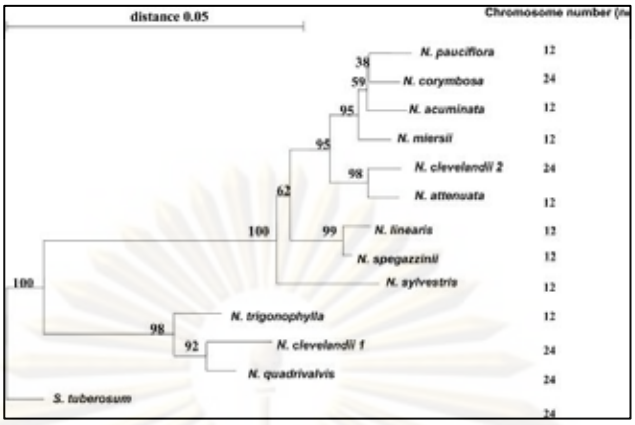


Figure 2.14 An example of a neighbour-joining tree of 12 North American *Nicotiana* species (Qu *et al*., 2004)

2.6.5 Tree evaluation using bootstrap analysis

A bootstrap analysis is a simple and effective computer-based technique for assessing the accuracy of almost any statistical estimate. It is one of tree evaluation method with provide measure of support for each branch in phylogenetic tree. Felsenstein (1985) first introduced the use of the bootstrap analysis in the estimation of confidence intervals for phylogenetic tree inferred from sequence data. This technique has been widely used and can take quite some time. Moreover, bootstrap analysis can be basically applied to all tree-reconstruction method.

A bootstrap data matrix is created by randomly selecting a column from the original matrix with replacement. Pseudoreplicate datasets are generated by randomly sampling the original character matrix to create new matrices of the same size as the original. The whole process is repeated independently a large number of times (approximately 100-1000 replications). A bootstrap value is a count (or percentage) of how often each branch presents in the resampled trees. For each new tree, numbers of branch points which correspond to the original tree are counted. If all trees show the same branch points, the bootstrap value is 100%. The bootstrap values can be shown on the phylogenetic trees and proportions of the bootstrap trees that agree with the original tree are calculated. These proportions or bootstrap confidence values can be considered as a reasonable assessment of errors for the estimated tree.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Fresh leaves of 34 tobacco cultivars and cured leaves of 24 cultivars were used in this study. These cultivars comprised of different groups of tobacco defined by methods of curing (i.e. Virginia group: flue-curing method; Burley: light-air curing; and Turkish: sun curing). Fresh leaf specimens of nine imported and 15 local tobacco cultivars were sampled in crop fields from 10 different provinces around Thailand (Table 3.1) with support of Thailand Tobacco Monopoly, Ministry of Finance. The fresh leaf samples were cut to small pieces (approximately 3x3 cm^2) and put into silica gel bags (Figure 3.1 A). Additionally, five imported and five local tobacco cultivars were obtained from a greenhouse of Maejo Tobacco Experiment Station, Chiang Mai province (Table 3.1).The leaf specimens of these eight cultivars were sampled from young plants in seed beds (Figure 3.1 B).

For cured leaf samples, seven imported and 14 local tobacco cultivars were collected from 10 different provinces. Cured leaves of three more imported tobacco cultivars were obtained from Maejo Tobacco Experiment Station (Table 3.2). Collecting localities of the tobacco cultivars are given in Figure 3.2. Fresh and cured tobacco leaves were kept separately in silica gel bags and stored at room temperature until used for genomic DNA preparation.

Table 3.1 Fresh leaf samples of tobacco cultivars used in this study.

Cultivar name	Cultivar group	Area of collection (province)
K187	Imported (Virginia)	Nakhon Phanom
K326	Imported (Virginia)	Lamphun
PVH03	Imported (Virginia)	Phayao
PV09	Imported (Virginia)	Chiang Rai
Coker326	Imported (Virginia)	Phrae
B1 special	Imported (Burley)	Sukhothai
KY14	Imported (Burley)	Sukhothai
Samsun	Imported (Turkish)	Nakhon Phanom
Xantiyaka	Imported(Turkish)	Nakhon Phanom
K190	Imported (Virginia)	Maejo Tobacco Experiment Station, Chiang Mai
HBO04P	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
HB01	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
TN90	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
TN97	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
Yamueang	Local	Phayao
K326-phuenmueang	Local	Nong Khai
Kariang	Local	Kanchanaburi
Kan	Local	Suphan Buri
Kan-kiw	Local	Suphan Buri
Kan-kiw Dok-chom-phu	Local	Suphan Buri
Kan-kiw Dok-khao	Local	Suphan Buri
Laodong	Local	Kanchanaburi
Meao	Local	Kanchanaburi
Hangkai	Local	Phayao
E-dam	Local	Petchabun
Phu	Local	Nong Khai
Yahan	Local	Nakorn Phanom
Petkhangsing	Local	Sukhothai
Petmakhuea	Local	Sukhothai
Padang	Local	Maejo Tobacco Experiment Station, Chiang Mai
Pasak	Local	Maejo Tobacco Experiment Station, Chiang Mai
Linchang	Local	Maejo Tobacco Experiment Station, Chiang Mai
Chorlare1	Local	Maejo Tobacco Experiment Station, Chiang Mai
Nisan	Local	Maejo Tobacco Experiment Station, Chiang Mai

Table 3.2 Cured leaf tobacco cultivars used in AFLP analysis.

Cultivar name	Cultivar group	Area of collection (province)
K187	Imported (Virginia)	Nakhon Phanom
K326	Imported (Virginia)	Lamphun
PV09	Imported (Virginia)	Chiang Rai
PVH03	Imported (Virginia)	Phayao
KY14	Imported (Burley)	Sukhothai
Samsun	Imported (Turkish)	Nakhon Phanom
Xantiyaka	Imported (Turkish)	Nakhon Phanom
TN86	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
TN90	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
TN97	Imported (Burley)	Maejo Tobacco Experiment Station, Chang Mai
Whitegold	Local	Nong Khai
K326-phuenmueang	Local	Nong Khai
E-dum	Local	Phetchabun
E-lueang	Local	Phetchabun
Kariang	Local	Kanchanaburi
Yamueang	Local	Phayao
Kan	Local	Suphan Buri
Kan-kiw	Local	Suphan Buri
Laodong	Local	Suphan Buri
Hangkai	Local	Phayao
Phu	Local	Nong Khai
Ya-glai	Local	Nakorn Si Thammarat
Bai-lia	Local	Lop Buri
Bia-tang	Local	Lop Buri

(A)

(B)

Figure 3.1 Collecting of tobacco leaf samples. (A) Young tobacco leaves were sampled from a tobacco crop and put into a plastic bag. (B) Young tobacco plants in seed beds at Maejo Tobacco Experiment Station.

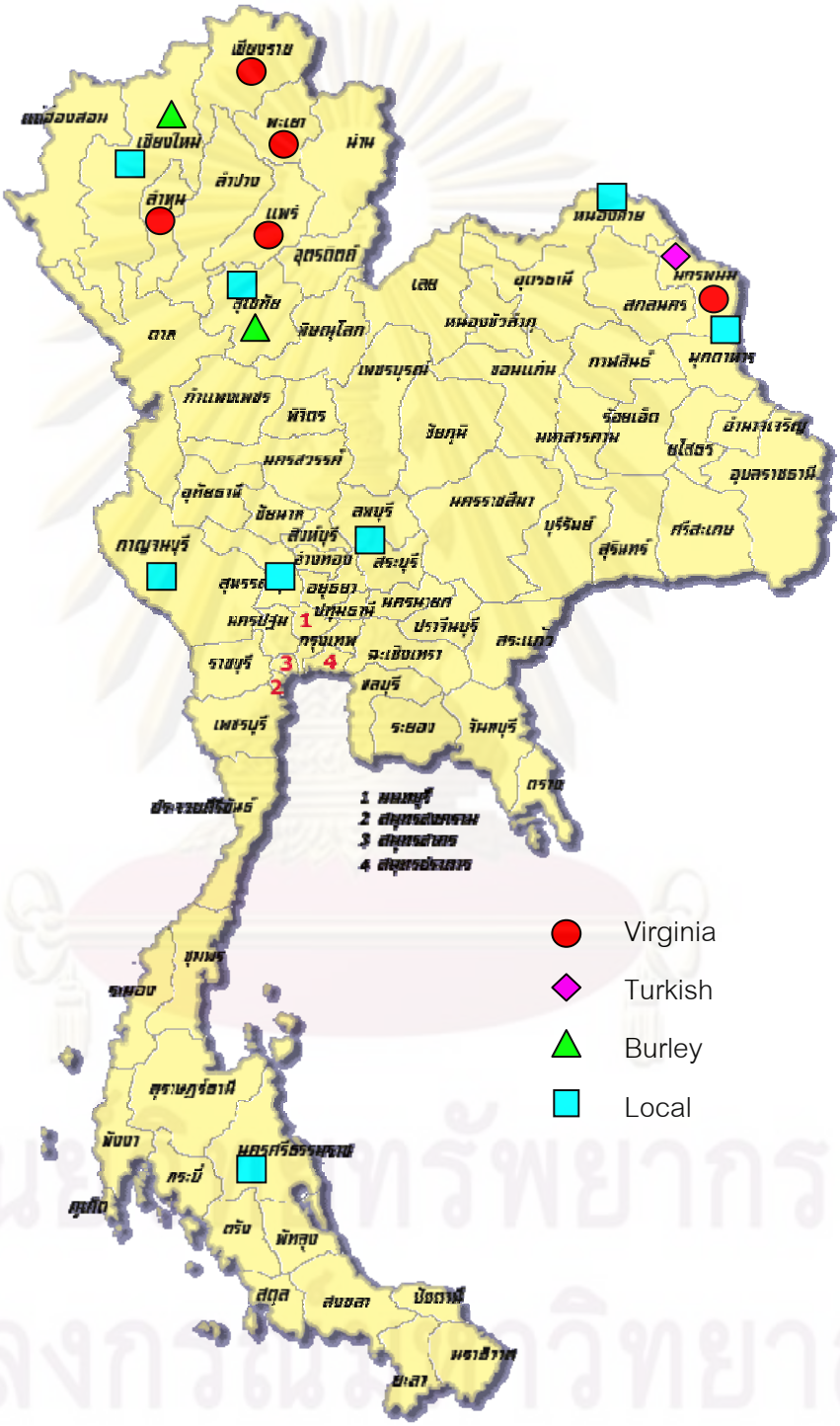


Figure 3.2 Collecting localities of tobacco cultivars in 12 different provinces around Thailand.

3.1.2 Equipments

- Autoclave: model Conbraco (Conbraco Ind. Inc., USA)
- Automatic micropipettes (P10, P20, P200 and P1000) (Gilson, France)
- Centrifuge/vortex mixer: model centrifuge FVL-2400 (Biosan, Latvia)
- Electronic UV transilluminator (Ultra Lum Inc., USA)
- Vertical sequencing apparatus: model DYCZ – 20C (Beijing Liuyi Instrument Factory, China)
- Electrophoresis chamber set: model Mupid (Advance Co., Ltd., Japan)
- Kimwipe tissue paper (Kimberly-clark, USA)
- Microcentrifuge tubes (0.5 and 1.5 ml) (Axygen Scientific, Inc., USA.)
- Microcentrifuge: model centrifuge Sorvall® pico D-37520 Osterode (Kendro Laboratory Products, Germany)
- 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)
- AC/DC power supply: model EC570-90 LVD CE (E-C Apparatus corporation, USA)
- Syringe (10 ml)
- Vortex mixer: model MS I Minishaker (IKA-Works, Inc., USA)

3.1.3 Chemicals

- 10x TBE buffer (Tris-base 108 g, boric acid 55 g, 0.5 M EDTA (pH 8.0) 80 ml and distilled water added upto 1 litre)
- 37% formaldehyde solution, analysis grade (Merck, Germany)
- 6x loading dye (glycerol 4 ml, bromophenal blue 25 mg and 1x TBE buffer upto 100 ml)

- 98% formamide loading dye (98% formamide, 0.5 M EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol)
- 99.5% (v/v) glycerol ($C_3H_80_3$), M.W. = 92.10 (Research Organics,USA)
- Absolute Ethanol (CH_3CH_2OH), M.W. = 46.07 (Merck, Germany)
- Acetic acid (glacial) anhydrous (CH_3COOH) (Merck, Germany)
- 4x Acrylamide (C_3H_5NO) (Bio Basic, Inc., USA.)
- Agarose gel (Research Organics,USA)
- Ammonium persulfate (APS) ($(NH_4)_2S_2O_8$) (Bio Basic, Inc., USA.)
- Bind-silane (Amersham Biosciences, Sweden)
- Bis-acrylamide ($C_7H_{10}O_2N_2$) (Bio Basic, Inc., USA.)
- Boric acid (Research Organics, USA)
- Bromophenol blue ($C_{19}H_{10}Br_4O_5S$), M.W. = 670 (Research Organics,USA)
- 50 bp DNA ladder marker (New Englang BioLab Inc.)
- 100 bp DNA ladder marker (SibEnzyme, Russia)
- Dynazyme^{TM} II *Taq* DNA polymerase, Mg^{+2}-free (Finnzyme, Finland)
- *Eco*RI (12 U/µl) system Lot# 256224 (Promaga, USA)
- 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.)
- Formamide (CH_3NO) (Bio Basic, Inc., USA.)
- Genomic DNA Mini Kit (Plant) (Geneaid Biotech Ltd., Taiwan)
- Isopropyl alcohol (Bio Basic, Inc., USA.)
- Multi-core^{TM} 10x buffer (250 mM Tris acetate (pH 7.8 at 25^0C), 1 M potassium acetate, 100 mM magnesium acetate and 10 mM DTT) system Lot# 253563 (Promaga, USA)
- Repel-silane (Amersham Biosciences, Sweden)
- Silver nitrate, analysis grade (Merck, Germany)

- Sodium carbonate (anhydrous), analsis grade (Na_2CO_3) (Merck, Germany)
- Sodium hydroxide (pellets), analsis grade (NaOH) (Merck, Germany)
- Sodium thiosulfate pentahydrate (ACS) ($Na_2S_2O_3.5H_2O$) (Polskie Odczynniki Chemiezne S.A., Gliwice)
- T4 DNA ligase (3 U/µl) system Lot# 256078 (Promaga, USA)
- TEMED ($C_6H_{16}N_2$) (Bio Basic, Inc., USA.)
- Tris-base (Research Organics, USA)
- *Tru*9I (10 U/µl) system Lot# 245503 (Promaga, USA)
- Urea, (Research organics, Inc., USA)
- Xylene cyanol (Research organics, Inc., USA)

3.1.4 Oligonucleotide primers

AFLP primers, *Eco*RI adapter and *Mse*I adapter were synthesised by Bio Basic, Inc., USA.

3.2 Methods

AFLP marker development started with genomic DNA extraction from fresh and cured leaf materials. Then, a quality of the extracted genomic DNA was tested and AFLP-PCR amplification was performed based on the protocol of Vos *et al.* (1995) with some modification. The PCR condition was further optimised for higher PCR specificity. AFLP profiles were generated from various primer combinations. Genetic relationship of tobacco cultivars was analysed from the scored AFLP profiles.

3.2.1 Genomic DNA extraction

Tobacco leaf samples were ground to fine powder with liquid nitrogen and total genomic DNA was extracted using Plant Genomic DNA Mini kit (Geneaid, Taiwan) following an instruction of the manufacturer. The leaf powder was transferred to a 1.5 ml microcentrifuge tube. 400 µl of GP1 buffer and 5 µl of RNase A solution were added into the sample tube and mixed by vortex. The mixture was incubated at 65°C for

10 minutes. During incubation, the mixture tube was inverted every 5 minutes. 100 µl of GP2 buffer were added to the mixture tube, mixed and incubated on ice for 3 minutes. After that, the mixture was transferred to a filter column and centrifuged for 1 minute at 13,000 rpm. The flow-through was carefully transferred to a new 1.5 ml microcentrifuge tube. 1.5 volumes of GP3 buffer were added to the lysate and the tube was vortexed immediately for 5 seconds. 700 µl of the mixture were transferred to a GD column and the column was centrifuged for 2 minutes. The flow-through was discarded from a collection tube. The remaining mixture was added to the GD column and centrifuged. The flow-through was discarded and the GD column was placed back in the collection tube. 400 µl of W1 buffer were added to the column and centrifuged for 1 minute. The column was washed with 600 µl of wash buffer, centrifuged for 1 minute and centrifuged again for 3 minutes to dry the column matrix. 400 µl of absolute ethanol were added to the column and centrifuged for 1 minute. The flow-through was discarded and centrifuged again to dry the column matrix. The dried column was transferred to a clean 1.5 ml microcentrifuge tube. 100 µl of 65^{o}C preheated elution buffer were added to the center of the column matrix. The column was left for 3-5 minutes at room temperature and then centrifuged for 1 minute to elute the purified DNA. The extracted genomic DNA was maintained in a -20^{o}C freezer until used.

3.2.2 Agarose gel electrophoresis

The extracted genomic DNA was electrophoresed through 1% agarose gel. Agarose gel powder was weighted and mixed with 1x TBE buffer. An agarose-gel solution was boiled in a microwave oven until completely solubilised and left at room temperature until warm (around 60^{o}C) before poured into a gel mould. The gel was left at room temperature for 30 minutes to be completely solidified. When used, a comb of the gel mould was gently removed. The gel was placed in an electrophoresis chamber containing adequate amount of 1x TBE buffer, covering the gel for approximately 0.5 cm.

5 µl of the DNA sample were mixed with 2 µl of a loading dye solution and then loaded into each well of the gel. 100 bp DNA ladder was used as standard DNA marker. An electrophoresis was carried out at 100 voltages for 45 minutes. After electrophoresed, the gel was stained with 0.5 mg/ml ethidium bromide (EtBr) for 5 minutes. The gel was then submerged in distilled water for 15 minutes to remove EtBr. DNA bands were visualised under UV light using a UV transilluminator.

3.2.3 Amplified fragment length polymorphisim (AFLP) analysis

3.2.3.1 Restriction enzyme digestion and adapter ligation

Each individual genomic DNA (approximately 250 ng) was completely digested with 6 units of *Eco*RI in a 25 µl reaction mixture containing 1x Multi-core™ buffer and incubated at 37°C for 3 hours. The mixture was agitated every hour during the incubation. At the end of the incubation period, 3 units of *Tru*9I (an isochizomeric enzyme of *Mse*I) were added to the reaction mixture. The mixture was incubated at 65°C for 3 hours and agitated every hour. Oligonucleotide adapters of *Eco*RI (5'-CTC GTA TGC GTA CC-3') and *Mse*I (5'-AAT TGG TAC GCA GTC TAC-3') were subsequently ligated to the digested DNA fragments in a total volume of 20 µl reaction. 10 µl of an adapter-ligating solution composed of 1x ligation buffer, 1.5 µl each of *Eco*RI and *Mse*I adapters and 1 unit of T4 DNA ligase. The reaction was incubated at 4°C for approximately 16 hours.

3.2.3.2 Pre-selective amplification

The pre-selective amplification step was carried out using adapter-specific primers with a single selective nucleotide on each primer. The reaction volume was 25 µl containing 1x PCR buffer, 2 µM of dNTP, 1.5 mM $MgCl_2$, 50 ng of E_{+A} and 50 ng of M_{+C} primers (Table 3.3), 1.5 units of DyNazyme™ II DNA Polymerase (Finnzymes) and 2 µl of the adapter-ligated DNA. A PCR reaction was performed in a GeneAmp® PCR system 9700 thermocycler. The pre-selective amplification condition used following cyclic parameters: 20 cycles of denaturing at 94°C for 30 seconds, annealing at 56°C for 60 seconds and extension at 72°C for 60 seconds.

3.2.3.3 Selective amplification

For a preliminary AFLP study on fresh leaf samples of 30 tobacco cultivars (12 imported and 18 local cultivars), 32 selective AFLP primer-pairs (Table 3.3) were screened against all tobacco samples. The pre-selectively amplified DNA was diluted in a ratio of 1:10 and then was used as a template for a selective amplification step. This amplification was carried out using +3 primers of *Eco*RI and *Mse*I adapters in a 25 µl reaction containing 1x PCR buffer, 2 µM of dNTP, 1.5 mM $MgCl_2$, 30 ng of E_{+3} and 30 ng of M_{+3} primers, 1.5 units of DyNazyme™II DNA Polymerase (Finnzymes) and 5 µl of the pre-selectively amplified product. The PCR was performed in a GeneAmp® PCR system 9700 thermocycler and the cycling parameters of this step were: one cycle of denaturing at 94°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 60 seconds; followed by 12 cycles of a touchdown phase with decreasing of the annealing temperature for 0.7°C in every cycle; and 23 cycles of extension at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 60 seconds. The selective AFLP-PCR products were electrophoresed through 2.0% agarose gel as described previously.

After screening for suitable AFLP primers, four (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) primers were selected to use. Genomic DNA extracted from cured leaf samples of 23 tobacco cultivars (nine imported and 14 local cultivars) were pre-selectively amplified with a pre-selective primers. The pre-selectively amplified product was diluted in 1:20 and then selectively amplified using the four selected primers and following these cyclic parameters: one cycle of 30 seconds at 94°C, 30 seconds at 65°C and 60 seconds at 72°C; lowering the annealing temperature by 0.7°C per cycle for another 12 cycles; and followed by 23 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 60 seconds. Moreover, the AFLP-PCR was optimised for higher PCR specificity and clearer fragment profile by increasing an annealing temperature. The annealing temperature of AFLP-PCR condition was therefore increased from 65°C to 67°C.

Table 3.3 Names and nucleotide sequences of AFLP primers used in this study.

Primer name	Sequence
Preselective amplification primer	
E_A	5'-GAC TGA GTA CCA ATT CA-3'
M_C	5'-GAT GAG TCC TGA GTA AC-3'
Selective amplification primer	
E_{AAC}	5'-GAC TGA GTA CCA ATT CAAC-3'
E_{AAG}	5'-GAC TGA GTA CCA ATT CAAG-3'
E_{ACA}	5'-GAC TGA GTA CCA ATT CACA-3'
E_{ACT}	5'-GAC TGA GTA CCA ATT CACT-3'
M_{CAA}	5'-GAT GAG TCC TGA GTA ACAA-3'
M_{CAC}	5'-GAT GAG TCC TGA GTA ACAC-3'
M_{CAG}	5'-GAT GAG TCC TGA GTA ACAG-3'
M_{CAT}	5'-GAT GAG TCC TGA GTA ACAT-3'
M_{CCA}	5'-GAT GAG TCC TGA GTA ACCA-3'
M_{CCC}	5'-GAT GAG TCC TGA GTA ACCC-3'
M_{CCG}	5'-GAT GAG TCC TGA GTA ACCG-3'
M_{CCT}	5'-GAT GAG TCC TGA GTA ACCT-3'
M_{CGA}	5'-GAT GAG TCC TGA GTA ACGA-3'
M_{CGC}	5'-GAT GAG TCC TGA GTA ACGC-3'
M_{CGG}	5'-GAT GAG TCC TGA GTA ACGG-3'
M_{CGT}	5'-GAT GAG TCC TGA GTA ACCT-3'
M_{CTA}	5'-GAT GAG TCC TGA GTA ACTA-3'
M_{CTC}	5'-GAT GAG TCC TGA GTA ACTC-3'
M_{CTG}	5'-GAT GAG TCC TGA GTA ACTG-3'
M_{CTT}	5'-GAT GAG TCC TGA GTA ACTT-3'

3.2.3.4 Denaturing polyacrylamide gel electrophoresis

Two glass plates of a vertical sequencing apparatus were cleaned many times with tap water and detergent, and rinsed with distilled water. The inner side of both glass plates was thoroughly cleaned with 1 ml of 95% ethanol on Kim-wipe tissue paper for 3 times. Afterwards, the inner side of the long glass plate was

coated with freshly-prepared bind-silane solution (5 µl of bind-silane, 1000 µl 95% ethanol and 5 µl of glacial acetic acid, mixed by inverting). For the notched glass plate, its inner side was cleaned and coated with repel-silane in the same manner. Both glass plates were let standing for 10 minutes to dry with air. Excessive silane was removed with 95% ethanol on kim-wipe tissue paper for 3 times. The two cleaned glass plates were assembled together with a pair of 0.4 mm spacers. A space within the plates was called “gel room”. The bottom of the gel room was sealed with plastic tape and spring clips.

6% denaturing polyacrylamide gel were prepared by combining 100 ml of acrylamide solution (19:1 acrylamide:bis-acrylamide with 7 M Urea in 1X TBE buffer) with 100 µl of TEMED and 500 µl of freshly prepared 10% ammonium persulphate. The acrylamide solution was gently mixed by a stirring rod and poured into the gel room using a bottle with moderate pressure. The filled gel room was left in a horizontal position. An electrophoresis comb was inserted into the gel room with its teeth facing up (approximately 5 mm deep). The gel was allowed to be polymerised at room temperature for at least 4 hours (or overnight).

The spring clips and sealing tape were carefully removed before running electrophoresis. The assembled gel unit was placed into a lower buffer tank against the main tank plate of a vertical sequencing apparatus model DYCZ – 20C (Beijing Liuyi, China) following an instruction of the manufacturer. A “T”-shape spacer block was put into the lower tank and all of the screws were then screwed for safety. The assembled gel unit was fixed with “U”-shape spacer blocks at the left and right sides and the screws were screwed. After finished assembling the sequencing apparatus, 1x TBE buffer solution was poured into the lower tank until the surface of buffer solution was approximately 1 cm above the bottom of the gel room. The top buffer chamber was also filled with 1x TBE buffer until about 1 inch high above the notched glass plate. The comb was removed from the gel room and the gel surface was flushed with 1x TBE buffer using a needled syringe. The sharktooth side of the comb was reinserted into the gel until the teeth were just sided down approximately 1 mm into the gel. 6 µl of a 98%

formamide loading dye was loaded into each well. The gel was pre-run at 800 voltages for 30 minutes. After pre-run, 6 µl of the amplified products were mixed with 3 µl of the loading dye. The sample was carefully loaded into each well. 50 bp and 100 bp DNA ladder markers were also loaded to estimate sizes of the AFLP fragments. The electrophoresis was carried out at 800 voltages for 3 hours and 30 minutes.

After finished running the electrophoresis, the glass plates were carefully separated using a spatula. The acrylamide gel was still on the long glass plate. The gel was fixed with 2 litres of a fix/stop solution (10% acetic acid) in a plastic tray and agitated well for 30 minutes. The gel was washed with distilled water by shaking 3 times for 5 minutes. 1.5 litres of a staining solution (0.1% silver nitrate) was poured into another plastic tray. The gel plate was transferred into a staining solution and shaken for 30 minutes. The gel was submerged in 1.5 litres of distilled water and shaken by hand around 10 seconds (forward-and-backward agitation for 10 times). This step is important and too much rinsing time will result in weak staining. The gel was then immediately placed in a plastic tray containing 1.5 litres of a cold developing solution. The gel was shaken until the first band was appeared on the gel (usually 1-2 minutes). After that, the gel was transferred to another 1.5 litres of a cold developing solution. The gel was agitated until bands from every lane were observed (usually 1- 2 minutes). 2 litres of a fix/stop solution was directly added to the developing solution and continued shaking for approximately 3 minutes. The stained gel was rinsed 2 times with distilled water for about 5 minutes. The gel was left drying at room temperature.

3.2.4 Genetic relationship analysis

The AFLP bands were treated as dominant markers. Only bright, clearly-resolved AFLP fragments were scored for presence (1) or absence (0) of the bands. The scored data was transformed into a 0/1 binary-character matrix for each primer combination. Nei and Li's coefficient analysis (1979) was used to calculate pairwise band-similarity values of the samples using a program PAUP* 4.0b10 (swofford, 2002). A cluster analysis was performed to construct a dendrogram using Unweighted Pair

Group Method of Arithmatric Mean (UPGMA) (Sneath and Sokal, 1973) and Neighbor-Joining (NJ) (Saitou and Nei, 1987) methods. Reliability of the clusters was estimated by bootstrap analysis with 1,000 replications to show the degree of confidence of each branch on the tree. The bootstrap analysis was summerised with a 50% majority-rule concensus tree. Only bootstrap values over 50% were considered significant and mentioned on the dendrogram. Clustering patterns on the tree diagram were compared with other information of the tobacco cultivars.

CHAPTER IV
RESULTS

4.1 DNA extraction

4.1.1 Fresh tobacco leaf samples

Genomic DNA of fresh leaves of 34 tobacco cultivars was extracted using Plant Genomic DNA Mini kit. Most of the extracted genomic DNA was in high yield, but containing RNA contamination observed as fainted smear (Figure 4.1). Some extracted DNA samples were found degraded (cultivars K190, HBO01, HBO04P and Pasak as lanes 3, 8, 9 and 18 in Figure 4.1, respectively). The estimated concentration DNA varied from 20 to 200 ng/µl and the quality of the extracted DNA was acceptable to be further used.

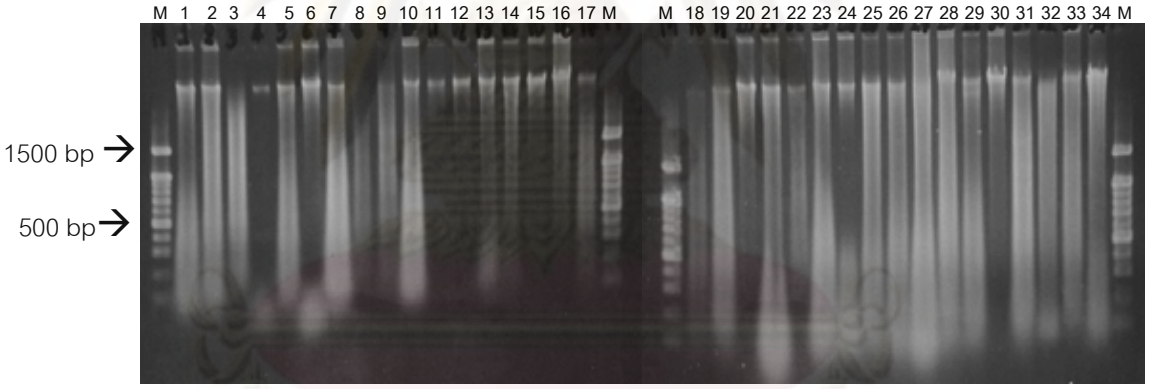


Figure 4.1 Genomic DNA bands from fresh leaf samples of 34 imported and local tobacco cultivars compared with 1.5 kb + 100 bp DNA marker (M= DNA marker, no. 1-34= Coker326, K187, K190, K326, PV09, PVH03, B1 special, HBO01, HBO04P, KY14, TN90, TN97, Samsun, Xanthiyaka, Chorlare1, Nisan, Padang, Pasak, Petmakhuea, Petkhangsing, Yamueang, Linchang, Phu, Hangkai, Yahan, E-dum, K326 phuen-mueang, Kan, Kan-kiw, Kan-kiw dok-khao, Kan-kiw dok-chom-phu, Kariang, Laodong and Meao, respectively).

4.1.2 Cured tobacco leaf samples

An electrophoretic analysis of the genomic DNA extraction from cured tobacco leaves (Figures 4.2 and 4.3) revealed low quantity of the total extracted genomic DNA and some fainted smear. Concentration of the extracted genomic DNA from cured samples was approximately 30 to 120 ng/µl. Almost all of the extracted genomic DNA was sheared and some tobacco cultivars gave fainted DNA (cultivars KY14, TN86 and TN90 as lanes 5, 6 and 7 in Figure 4.2; Phu, Hangkai and Bai-lai as lanes 5, 6, and 12 in Figure 4.3, respectively). One sample of the imported tobacco cultivars (K187 as lane 1 in Figure 4.2) and two samples of the local cultivars (E-lueang and Yamueang as lanes 4 and 7 in figure 4.3, respectively) failed in DNA extraction. However, the low DNA quality and quantity of these tobacco cultivars, though rather low was acceptable for further experiments.

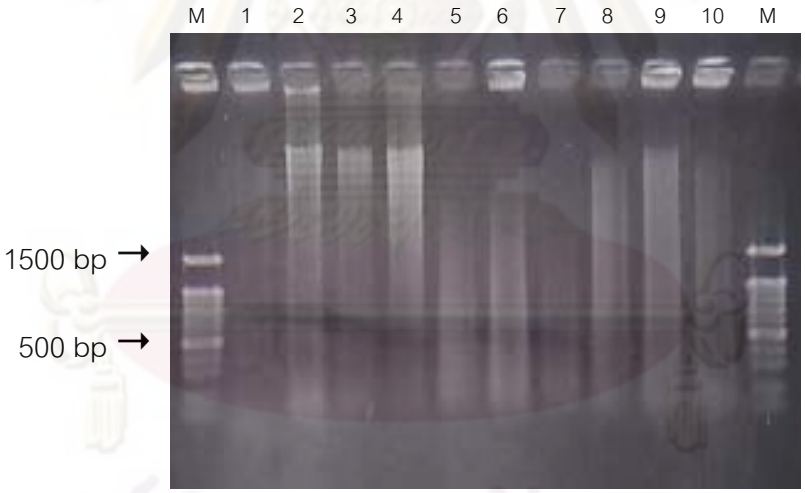


Figure 4.2 Genomic DNA bands from cured leaf samples of 10 imported tobacco cultivars compared with 1.5 kb + 100 bp DNA marker (M= DNA marker, no. 1-10= K187, K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun and Xanthiyaka, respectively).

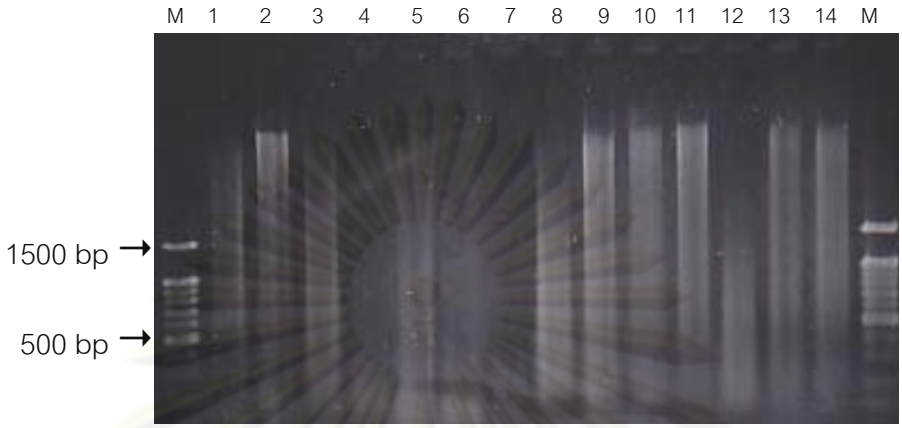


Figure 4.3 Genomic DNA bands of some cured leaf samples of 14 local tobacco cultivars compared with 1.5 kb + 100 bp DNA marker (M= DNA marker, no.1-14= White gold, K382 phuen-mueang, E-dum, E-lueang, Phu, Hangkai, Yamueang, Ya-glai, Kariang, Kan, Bai-tang, Bai-lai, Loadong and Kan-kiw, respectively).

4.2 AFLP-PCR amplification

A preliminary AFLP study on fresh leaves of 30 tobacco cultivars (12 imported cultivars and 18 local cultivars) were performed using E_A/M_C primers in the pre-selective amplification step. The amplified products ranged in size from 100 to 1000 bp and the major size was between 300 to 500 bp (Figure 4.4). The pre-selective amplification results showed that the genomic tobacco DNA was successfully digested and ligated. The pre-selective amplification products were diluted to 1:10 ratio and were further applied to the selective amplification step with +3 AFLP primers. The amplification results were visualised using an agarose gel electrophoresis. Totally 32 selective AFLP primer-pairs were screened against all tobacco samples. Four primer-combinations (E_{ACT}/M_{CTA}, E_{ACA}/M_{CAG}, E_{ACA}/M_{CAT} and E_{ACA}/M_{CTA}) did not give any amplified products while six primer-combinations generated smeared DNA patterns of less than 300 bp amplified products. Another 16 primer pairs produced very smeared patterns while two other primer-combinations gave very fainted products (summarised in Table 4.1). Only the four primer-pairs left (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) could be suitably used as +3 primers for the selective AFLP-PCR step.

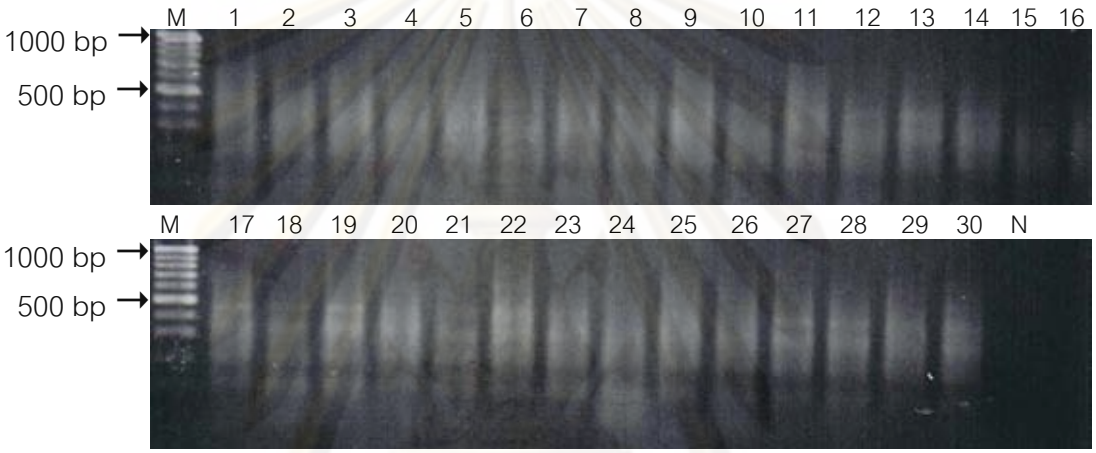


Figure 4.4 Pre-selective amplification products of 30 tobacco cultivar samples using E_A/M_C pre-selective primers (lanes no. 1-30 = tobacco cultivars K187, K326, PVH03, PV09, B1 special, KY14, HBO04P, TN90, TN97, Samsun, Xanthiyaka, Coker326, Chorlare1, Nisan, Pasak, Padang, Petkhangsink, Petmakhua, Yamuang, Linchang, Hangkai, Phu, Yahun, K326 phun-mueang, Kariang, Kan, Kan-kiw dok-chom-phu, Kan-kiw dok-khao, Laodong, and Maeo, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

Table 4.1 Amplification results of the preliminary screening for suitable AFLP selective primers.

Primer	Result
E_{AAC}/M_{CAC}	Very smeared
E_{AAC}/M_{CAG}	Very fainted
E_{AAC}/M_{CAT}	Very smeared
E_{AAC}/M_{CTA}	Very smeared
E_{AAC}/M_{CTG}	Smear pattern <300 bp
E_{AAC}/M_{CTT}	Smear pattern <300 bp
E_{AAC}/M_{CGT}	Very smeared
E_{AAC}/M_{CGC}	Very smeared
E_{AAC}/M_{CCA}	Very smeared
E_{AAC}/M_{CCC}	Smear pattern <300 bp
E_{AAC}/M_{CGG}	Smear pattern <300 bp
E_{AAG}/M_{CAA}	+++
E_{AAG}/M_{CAT}	+++
E_{AAG}/M_{CTA}	Very smeared
E_{AAG}/M_{CTC}	Smear pattern <300 bp
E_{AAG}/M_{CTG}	Smear pattern <300 bp
E_{AAG}/M_{CTT}	Very fainted
E_{AAG}/M_{CGA}	+++
E_{AAG}/M_{CGC}	Very smeared
E_{AAG}/M_{CCA}	Very smeared
E_{AAG}/M_{CCT}	Very smeared
E_{AAG}/M_{CCG}	Very smeared
E_{AAG}/M_{CCC}	Very smeared
E_{ACA}/M_{CAA}	Very smeared
E_{ACA}/M_{CAC}	Very smeared
E_{ACA}/M_{CAG}	–
E_{ACA}/M_{CAT}	–
E_{ACA}/M_{CTA}	–
E_{ACT}/M_{CAA}	Very smeared
E_{ACT}/M_{CAC}	Very smeared
E_{ACT}/M_{CAG}	+++
E_{ACT}/M_{CTA}	–

– = negative amplification, +++ = positive amplification suitable for further use.

The four chosen selective primer-pairs produced only 13, 34, 50 and 27 clear AFLP bands, respectively, on smeary backgrounds (Table 4.2). These DNA fingerprinting results gave a total number of 124 scorable AFLP fragments. Sizes of all amplified bands from the four chosen primers ranged from 150 to 700 bp with an average number of 31 fragments per primer-pair. The primer-pair E_{AAG}/M_{CGC} could yield the highest number of the amplified fragments (50 bands), and also give less smeared background than the others (Figure 4.5). The lowest number of the amplified fragments was by the E_{AAG}/M_{CAA} pair, which were only 13 bands. Among the total 124 amplified bands, 69 amplified bands (55.7%) were polymorphic bands and 55 bands (44.3%) were monomorphic. The average number of polymorphic loci per primer-combination was 17 bands per primer-pair and the average polymorphic percentage was 55%. The primer-combination E_{AAG}/M_{CAA} gave the highest polymorphism across all cultivars (84.6%) whereas the E_{AAG}/M_{CGC} pair gave the lowest polymorphic percentage (40%).

Table 4.2 The numbers of amplified bands and degrees of polymorphism revealed from AFLP analyses on the fresh leaf samples.

Primer combination	Total band	Polymorphic band	Monomorphic band	Polymorphic percentage (%)
E_{AAG}/M_{CAA}	13	11	2	84.6
E_{AAG}/M_{CAT}	34	21	13	67.8
E_{AAG}/M_{CGC}	50	19	31	40.0
E_{ACT}/M_{CAG}	27	18	9	66.7
Total	124	69	55	55.7
Average	31	17	14	55.0

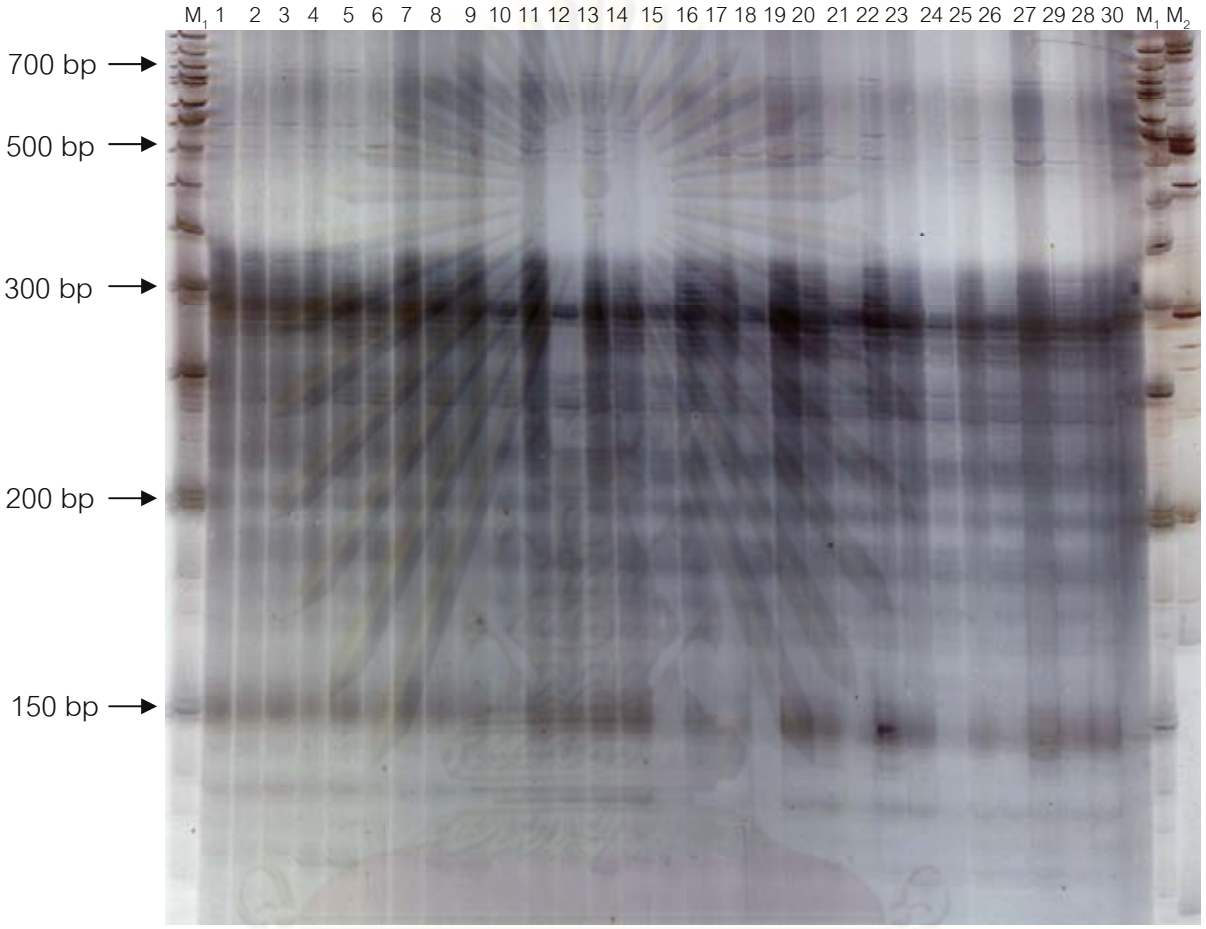


Figure 4.5 An AFLP profile of 30 tobacco cultivar samples using E_{AAG}/M_{CGC} selective primers (lanes no. 1-30 = tobacco cultivars K187, K326, PVH03, PV09, B1special, KY14, HBO04P, TN90, TN97, Samsun, Xanthiyaka, Coker326, Chorlare, Nisan, Pasak, Padang, Petkhangsink, Petmakhua, Yamuang, Linchang, Hangkai, Phu, Yahun, K326 Phun-mueang, Kariang, Kan, Kan-kiw dok-chom-phu, Kan-kiw dok-khao, Laodong and Maeo, respectively). 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2) were used.

From the AFLP study on cured leaf samples, totally 23 tobacco cultivars (nine imported and 14 local cultivars) were pre-selectively amplified with 1:20 diluted DNA templates and E_A/M_C pre-selective primers. An electrophoretic analysis revealed smeared PCR products with sizes ranged approximately from 200 to 1000 bp (Figure 4.6). Results of some tobacco cultivars were found as fainted smear. The four selective primer-pairs from the preliminary screening experiment generated AFLP products with sizes ranged from 200 to 1000 bp (Figures 4.7 - 4.10). The AFLP-PCR products were estimated through 6% denaturing polyacrylamide gel electrophoresis as shown in the Figures 4.11 - 4.14.

The results from these AFLP experiments on the 1:20 diluted templates showed clearer patterns and higher numbers of scorable bands than those of the previous results 1:10 dilution experiment. The E_{AAG}/M_{CAA} combination gave 51 AFLP fragments with 39 polymorphic bands. Three tobacco cultivars, TN97, K326 phun-mueang and E-lueang (lanes 6, 10 and 12 of Figure 4.11, respectively) could produce only some faint bands from this primer. Thirty-seven AFLP bands (with 22 polymorphic bands) and 92 AFLP fragments (with 67 polymorphic bands) were produced from the primer-pairs E_{AAC}/M_{CAT} and E_{AAG}/M_{CGC}, respectively. The last primer-combination E_{ACT}/M_{CAG} gave 34 AFLP fragments with 23 in Table 4.3 polymorphic bands. The cured leaf samples of Yamueang, E-lueang, and TN90 tobacco cultivars were found giving only faint bands on the AFLP polyacrylamide gel of primers E_{AAG}/M_{CAT} (lanes 5, 11 and 18 of Figure 4.12, respectively) and also on those of the primers E_{AAG}/M_{CGC} (lanes 5, 11 and 18 of Figure 4.13, respectively) and E_{ACT}/M_{CAG} (lanes 6, 13 and 19 of Figure 4.14, respectively).

The fingerprinting results gave a total number of 214 scorable AFLP-fragments from the four primer-combinations with an average of 54 fragments per primer-pair. Fragment sizes of the AFLP-PCR products of these four primer-pairs ranged from approximately 100 to 900 bp. The highest number of the amplified fragments per primer-pair was 92 bands by the E_{AAG}/M_{CGC} with less smeared background than the others (Figure 4.13). The lowest number of the amplified fragments (34 bands) was by

the E_{ACT}/M_{CAG} primer pair. There were totally 151 amplified polymorphic bands (70.6% of total 214 bands) and the average polymorphism degree was 38 polymorphic loci per-primer. The average polymorphic percentage was 70.4% (Table 4.3). The primer-combination E_{AAG}/M_{CAA} gave the highest polymorphism (76.5%) across all cultivars whereas the E_{AAG}/M_{CAT} pair gave the lowest score (59.5%).

Table 4.3 The numbers of amplified bands and degrees of polymorphism revealed from AFLP analyses on the cured leaf samples.

Primer combination	Total band	Polymorphic band	Monomorphic band	Polymorphic percentage (%)
E_{AAG}/M_{CAA}	51	39	12	76.5
E_{AAG}/M_{CAT}	37	22	15	59.5
E_{AAG}/M_{CGC}	92	67	25	72.8
E_{ACT}/M_{CAG}	34	23	11	67.6
Total	214	151	63	70.6
Average	54	38	16	70.4

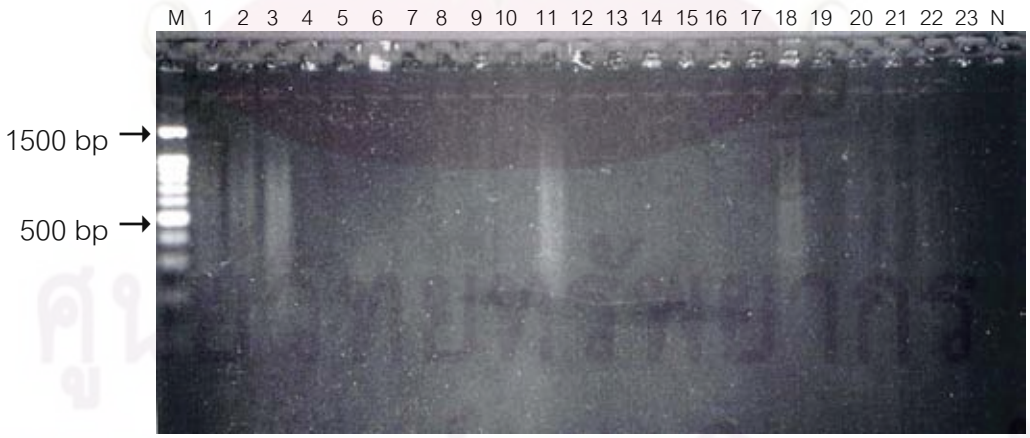


Figure 4.6 Pre-selective amplification products of 23 tobacco cultivar samples using E_A/M_C pre-selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

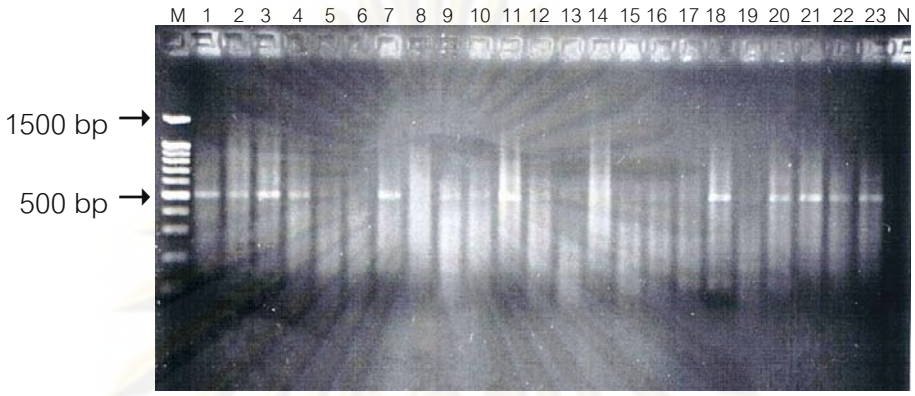


Figure 4.7 Selective amplification products of 23 tobacco cultivar samples using E_{AAG}/M_{CAA} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

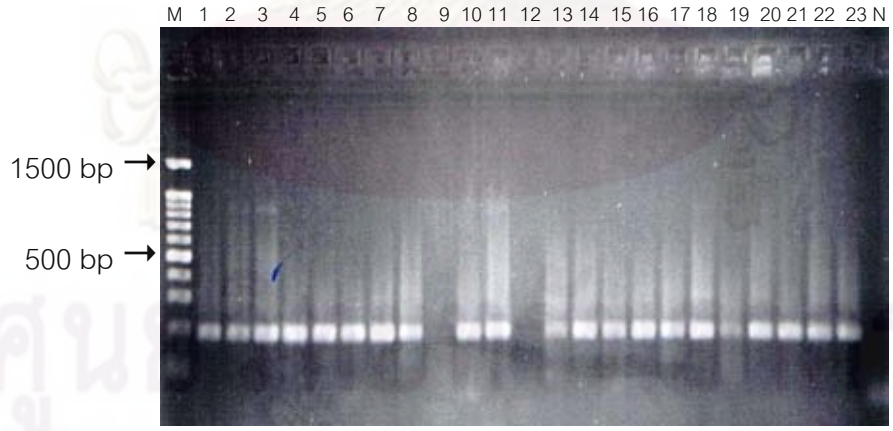


Figure 4.8 Selective amplification products of 23 tobacco cultivar samples using E_{AAG}/M_{CAT} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

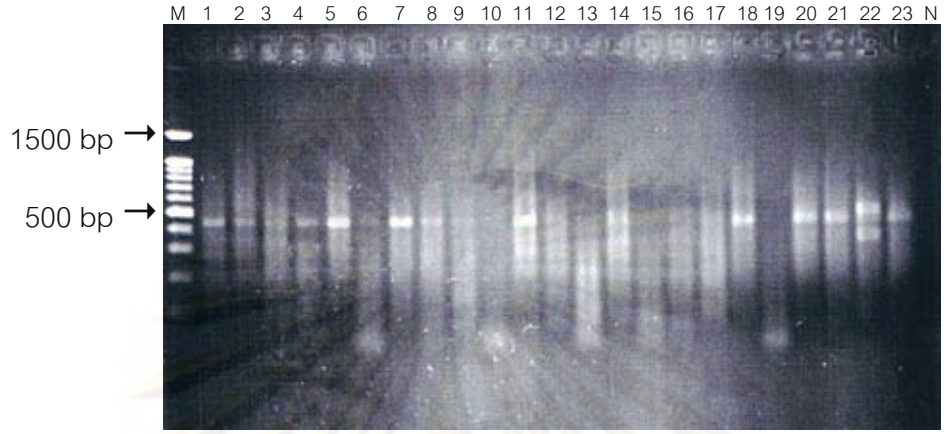


Figure 4.9 Selective amplification products of 23 tobacco cultivar samples using E_{AAG}/M_{CGC} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

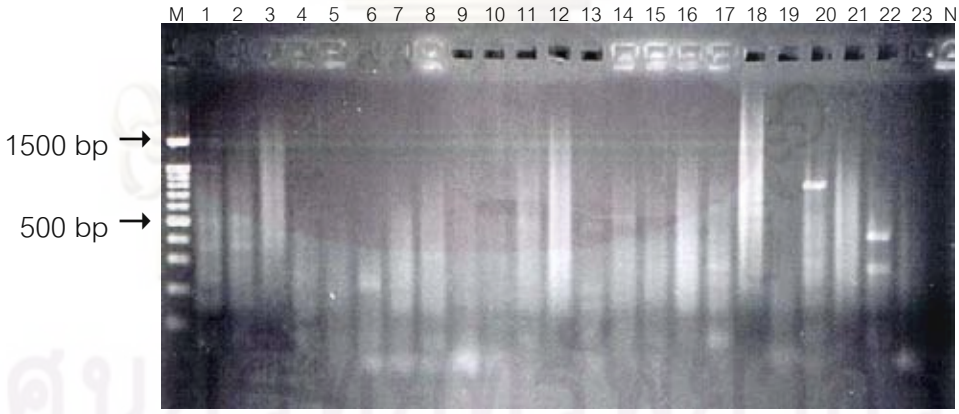


Figure 4.10 Selective amplification products of 23 tobacco cultivar samples using E_{ACT}/M_{CAG} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

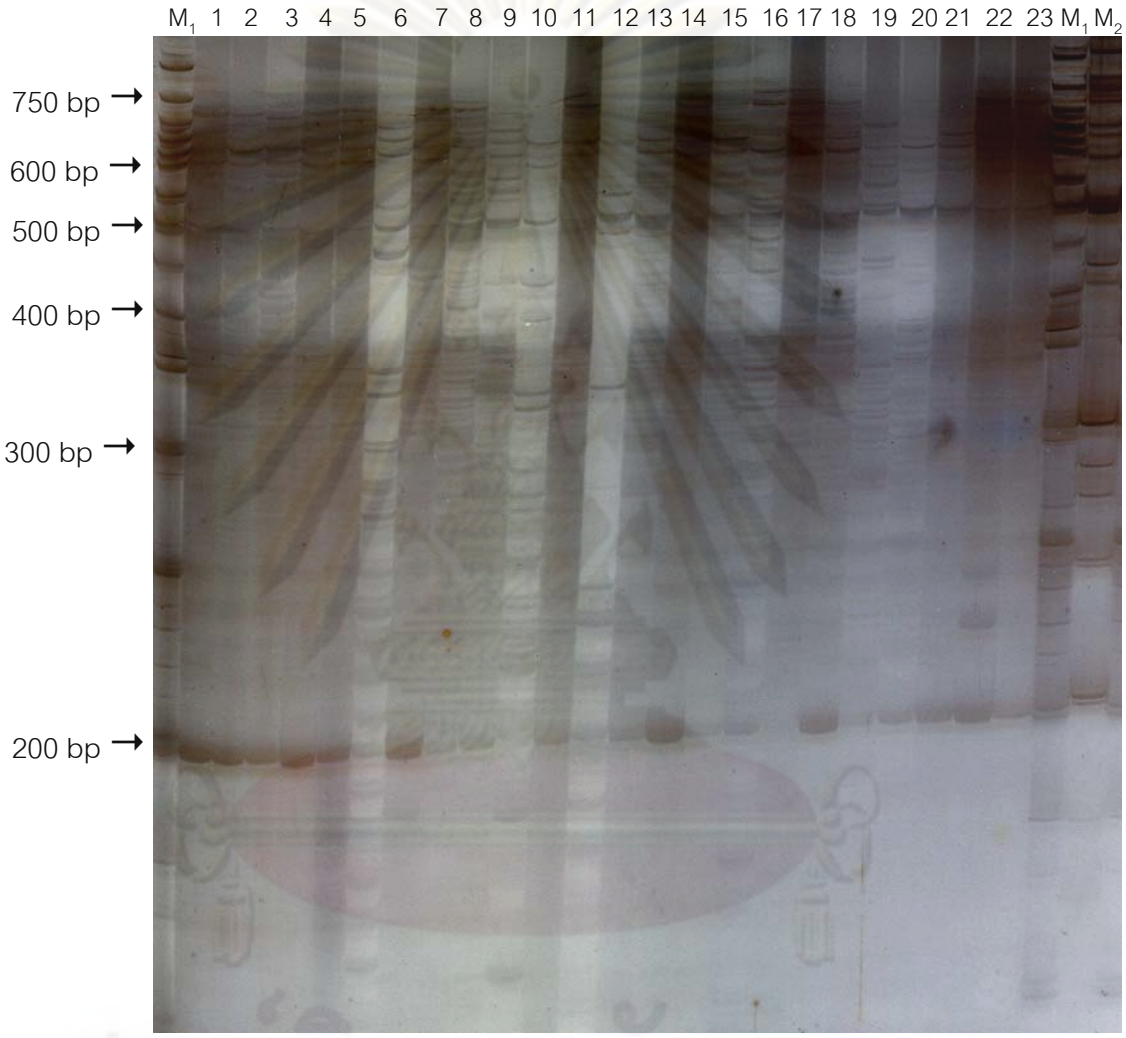


Figure 4.11 An AFLP profile of cured leaf samples of 23 tobacco cultivars using E_{AAG}/M_{CAA} selective primers (lanes no. 1-23 = tobacco cultivars PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong, Kan-kiw and K326, respectively). 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2, respectively) were used.

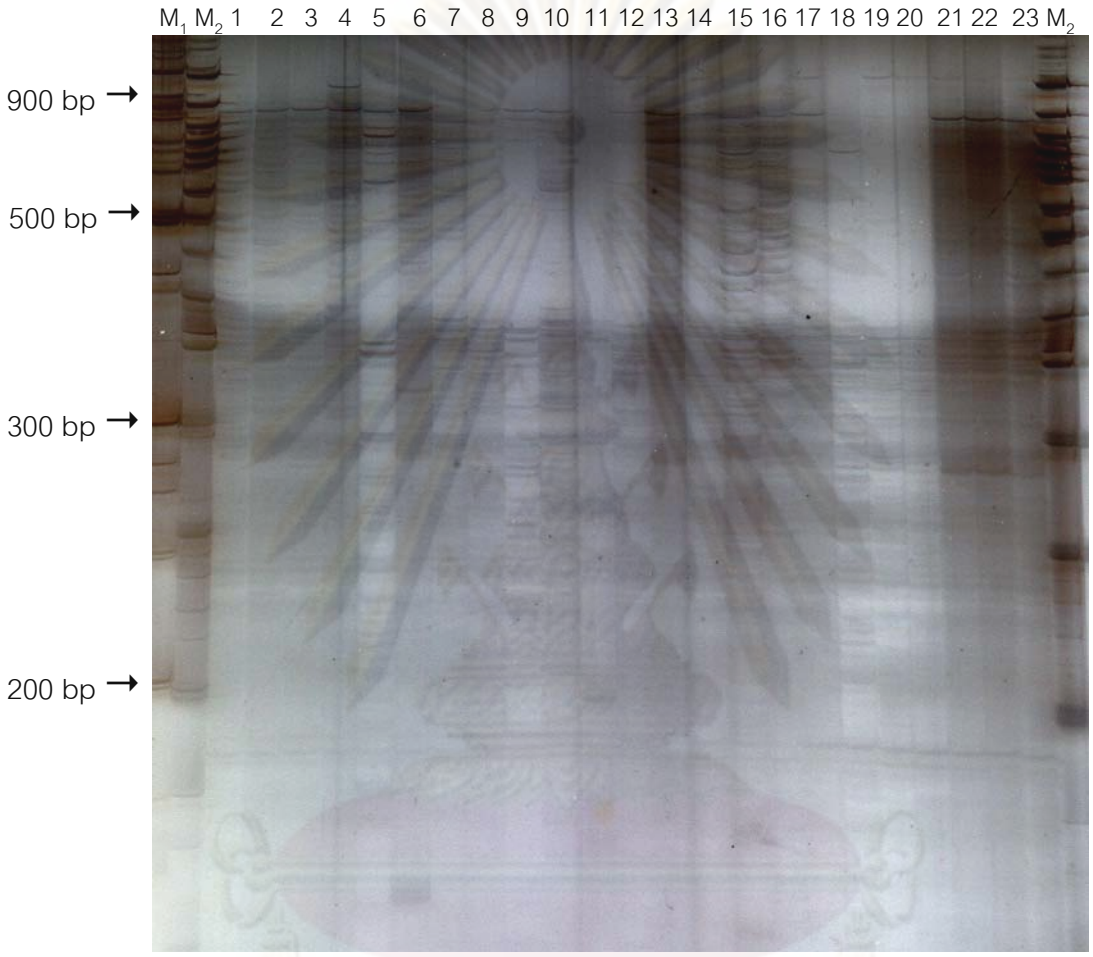


Figure 4.12 An AFLP profile of cured leaf samples of 23 tobacco cultivars using E_{AAG}/M_{CAT} selective primers (lanes no. 1-23 = tobacco cultivars Kan-kiw, Laodong, Bai-tang, Kan, Yamueang, Kariang, Bia-lai, Ya-glai, Hangkai, Phu, E-lueang, E-dum, K326 phun-mueang, White gold, Xanthiyaka, Samsun, TN97, TN90, TN86, KY14, PV09, PVH03 and K326, respectively). 100 bp and 50 bp DNA ladder markers (lane M_1 and M_2, respectively) were used.

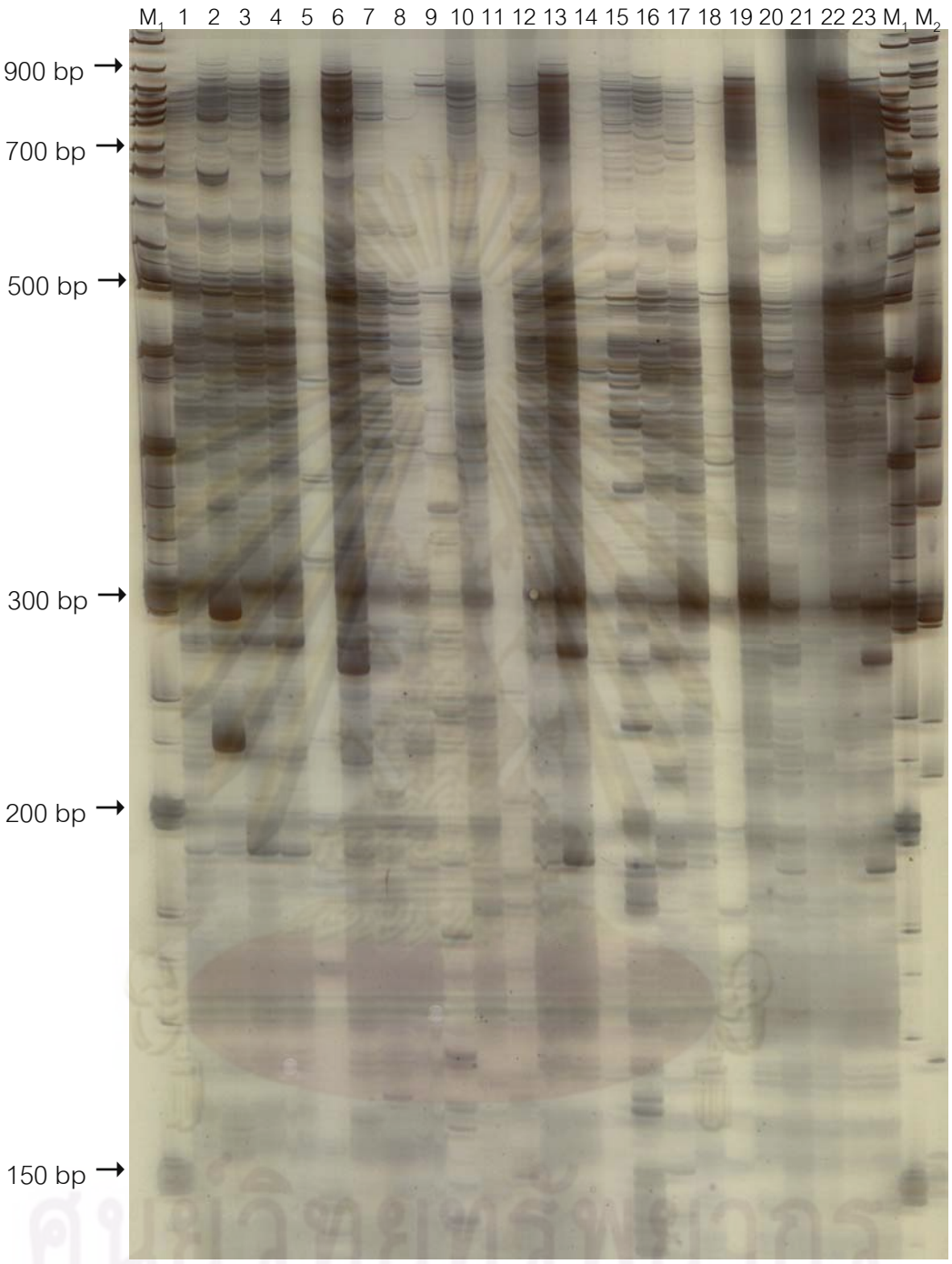


Figure 4.13 An AFLP profile of cured leaf samples of 23 tobacco cultivars using E_{AAG}/M_{CGC} selective primers (lanes no. 1-23 = tobacco cultivars Kan-kiw, Laodong, Bai-tang, Kan, Yamueang, Kariang, Bia-lai, Ya-glai, Hangkai, Phu, E-lueang, E-dum, K326 phun-mueang, White gold, Xanthiyaka, Samsun, TN97, TN90, TN86, KY14, PV09, PVH03 and K326, respectively). 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2, respectively) were used.

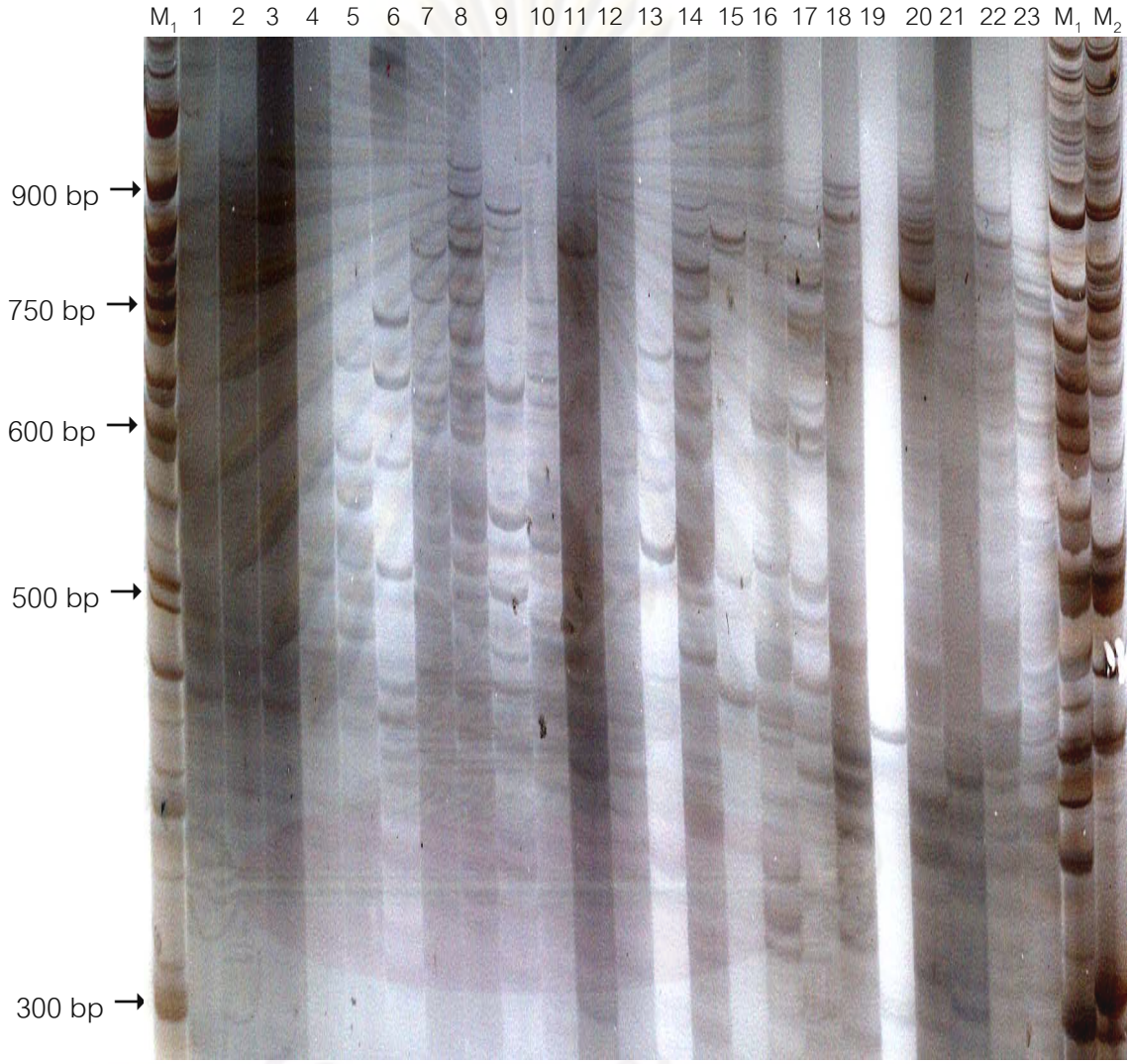


Figure 4.14 An AFLP profile of cured leaf samples of 23 tobacco cultivars using E_{ACT}/M_{CAG} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively). 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2, respectively) were used.

To optimise the selective amplification step, an annealing temperature of the selective amplification step was raised from 65°C to 67°C. The optimisation experiment was performed on nine imported and 14 local tobacco cultivars using the same four chosen AFLP primers (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}). The pre-selective amplification result shown in the Figure 4.15 gave smear PCR products ranged from 100 to 1000 bp. Almost all selective PCR reactions, except that of E_{AAG}/M_{CAT} primer-pair, were successfully done and gave smear products of 200-1000 bp on agarose gel. The fingerprint patterns on 6% denaturing polyacrylamide gel using E_{AAG}/M_{CAA}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG} primers were shown in Figures 4.19-4.21.

The acrylamide gel results of these three primer-pairs showed that the PCR optimisation using 67°C annealing temperature gave clearer AFLP profiles than those using 65°C. From the result of E_{AAG}/M_{CAA} primer-combination, E-lueang, Hangkai and Yamueang local cultivars (lanes 13, 15 and 19 of Figure 4.19, respectively) could give only some faint products. Likewise, the result of E_{AAG}/M_{CGC} primer-pair showed that, Yamueang also gave faint product but KY14 and E-luaeng cultivars did not show any amplified bands. This was similar to the result of the E_{ACT}/M_{CAG} primer-pair which E-lueang and Yamueang cultivars (lanes 13 and 19 in Figure 4.21, respectively) produced faint PCR products and the reaction of Kan-kiw cultivar (lane 23 in Figure 4.21) was failed. Interestingly, the selective amplification with the E_{ACT}/M_{CAG} primers could give one scorable band (approximately 150 bp) which may be a specific band for Virginia tobacco cultivars (for example K326, PV09 and PVH03 as lanes 1, 2 and 3 in Figure 4.21).

A total of 139 scorable bands were obtained from 19 tobacco cultivars with three primer-pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}). The amplified bands of each primer-pair were 35, 45 and 59 fragments, respectively, with an average of 46 fragments per pair (Table 4.4). The highest number of the amplified fragments (59 fragments) was from the primer-combination E_{ACT}/M_{CAG} whereas the lowest number of fragments was from E_{AAG}/M_{CAA} (35 fragments). Sizes of the AFLP fragments amplified with the three primer-pairs ranged from 100 to 750 bp. Among 139 scorable AFLP bands, the numbers

of polymorphic bands found from these cured-leaf tobacco samples with the primer-pairs E_{AAG}/M_{CAA}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG} were 19, 35 and 49 bands, respectively (Table 4.4). The highest degree of polymorphism (83.1%) was generated by the E_{ACT}/M_{CAG} primer-pair while the E_{AAG}/M_{CAA} primer gave the lowest degree of polymorphism (54.3%).

Table 4.4 The numbers of amplifiied bands and degrees of polymorphism revealed from AFLP analyses on cured leaf samples.

Primer combination	Total band	Polymorphic band	Monomorphic bands	Polymorphic percentage (%)
E_{AAG}/M_{CAA}	35	19	16	54.3
E_{AAG}/M_{CGC}	45	35	10	77.8
E_{ACT}/M_{CAG}	59	49	10	83.1
Total	139	103	36	74.1
Average	46	34	12	73.9

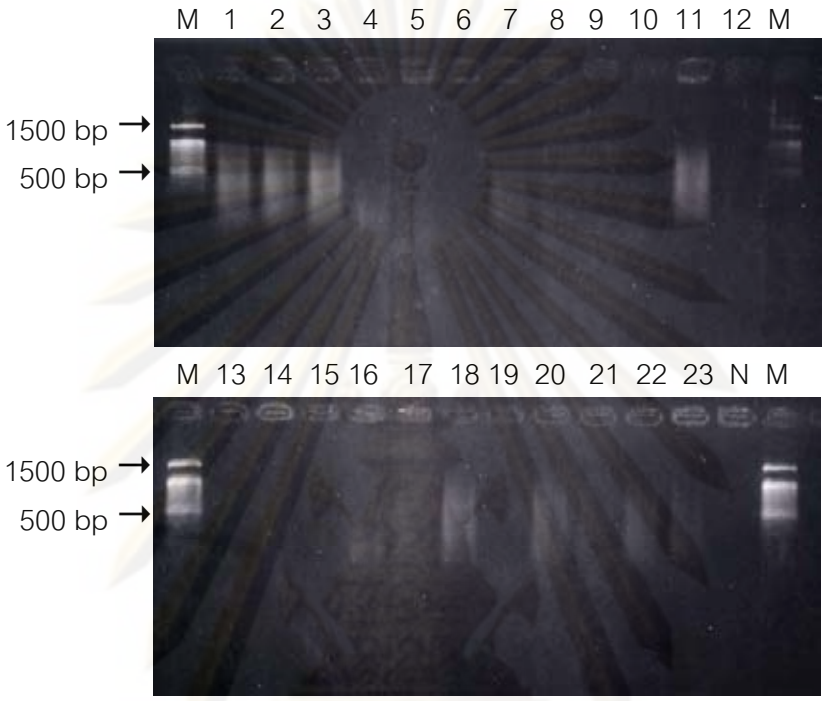


Figure 4.15 Pre-selective amplification products of 23 tobacco cultivar samples using E_A/M_C pre-selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

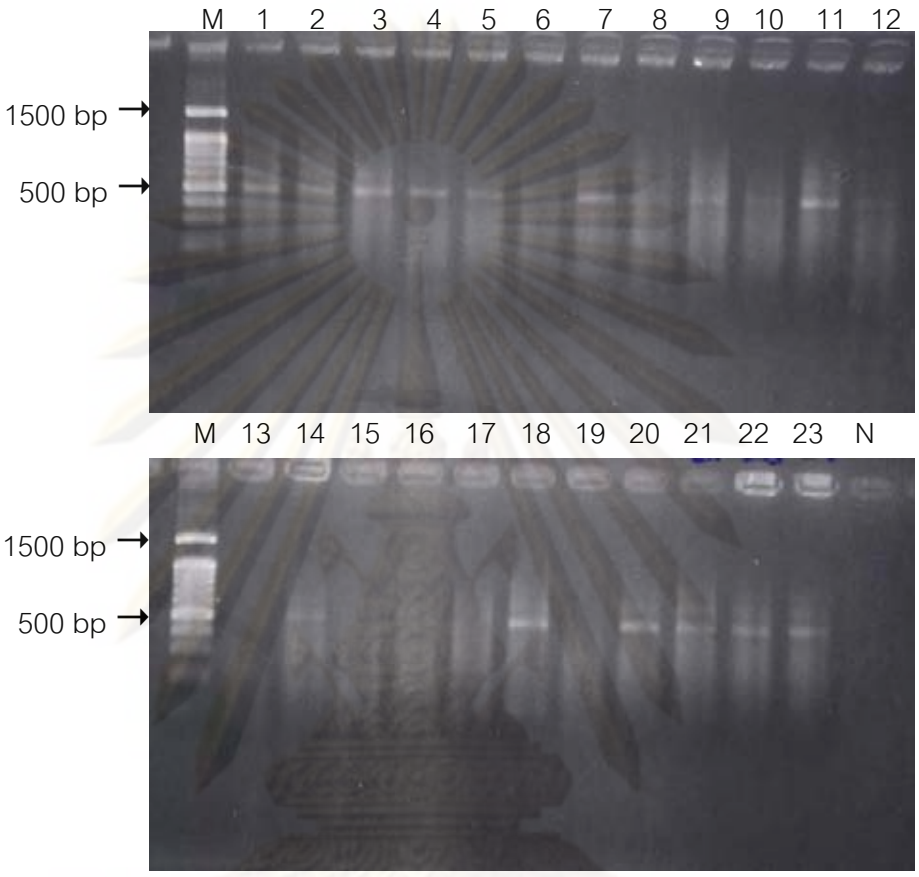


Figure 4.16 Selective amplification products of 23 tobacco cultivar samples using E_{AAG}/M_{CAA} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

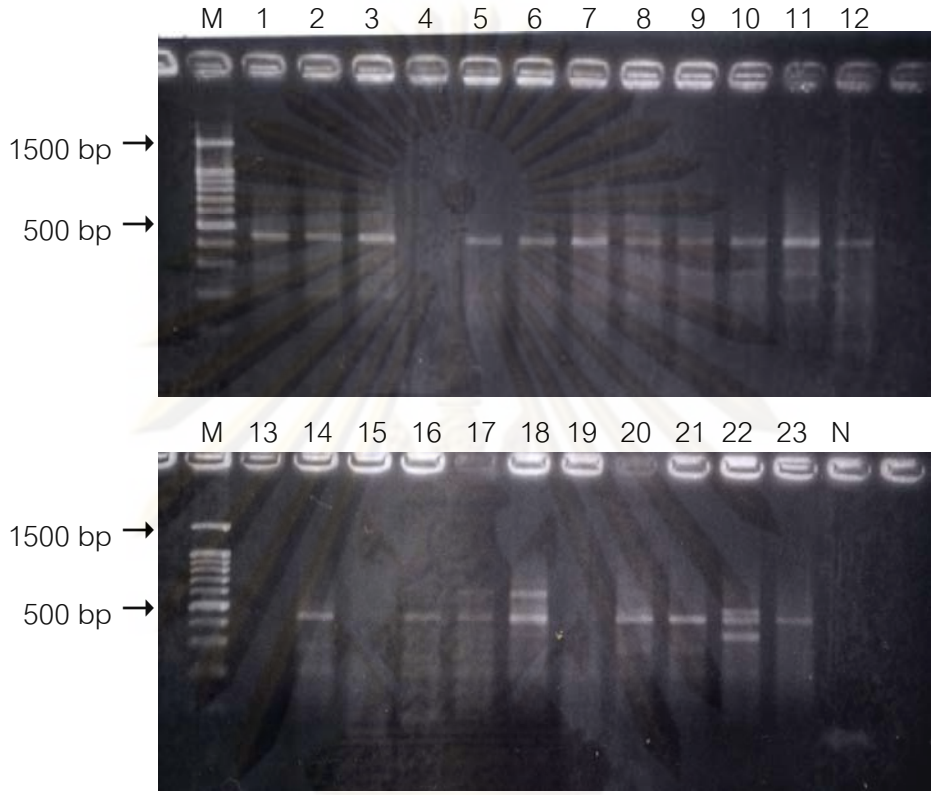


Figure 4.17 Selective amplification products of 23 tobacco cultivar samples using E_{AAG}/M_{CGC} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

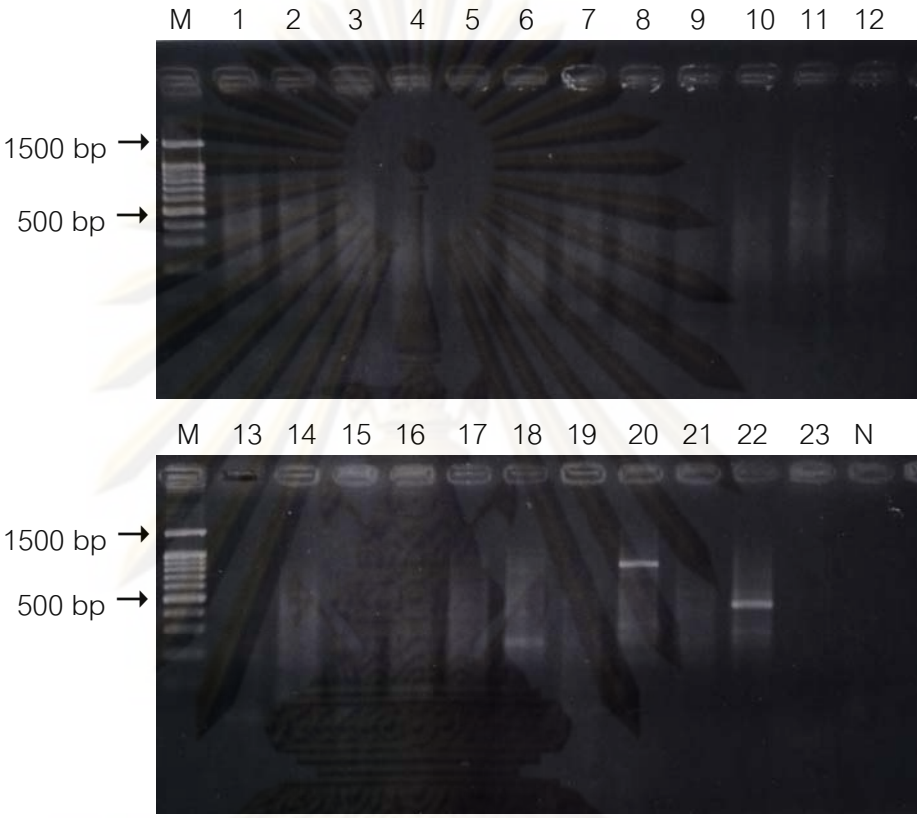


Figure 4.18 Selective amplification products of 23 tobacco cultivar samples using E_{ACT}/M_{CAG} selective primers (lanes no. 1-23 = tobacco cultivars K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively, and N = Negative control). 100 bp DNA ladder marker (lane M) was used.

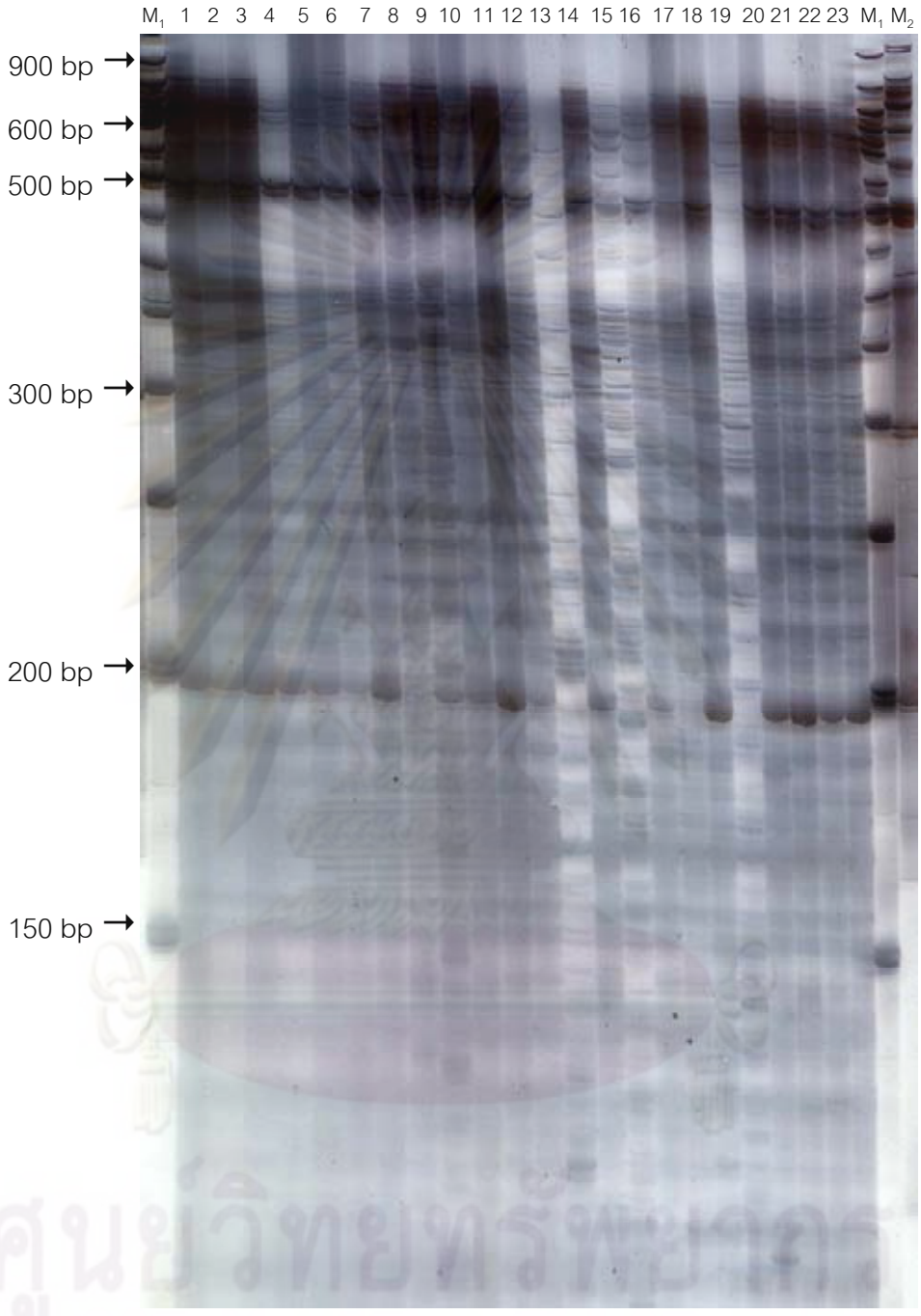


Figure 4.19 An AFLP profile of cured leaf samples of 23 tobacco cultivars using E_{AAG}/M_{CAA} selective primers (lanes no. 1-23 = tobacco cultivars : K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively). 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2, respectively) were used.

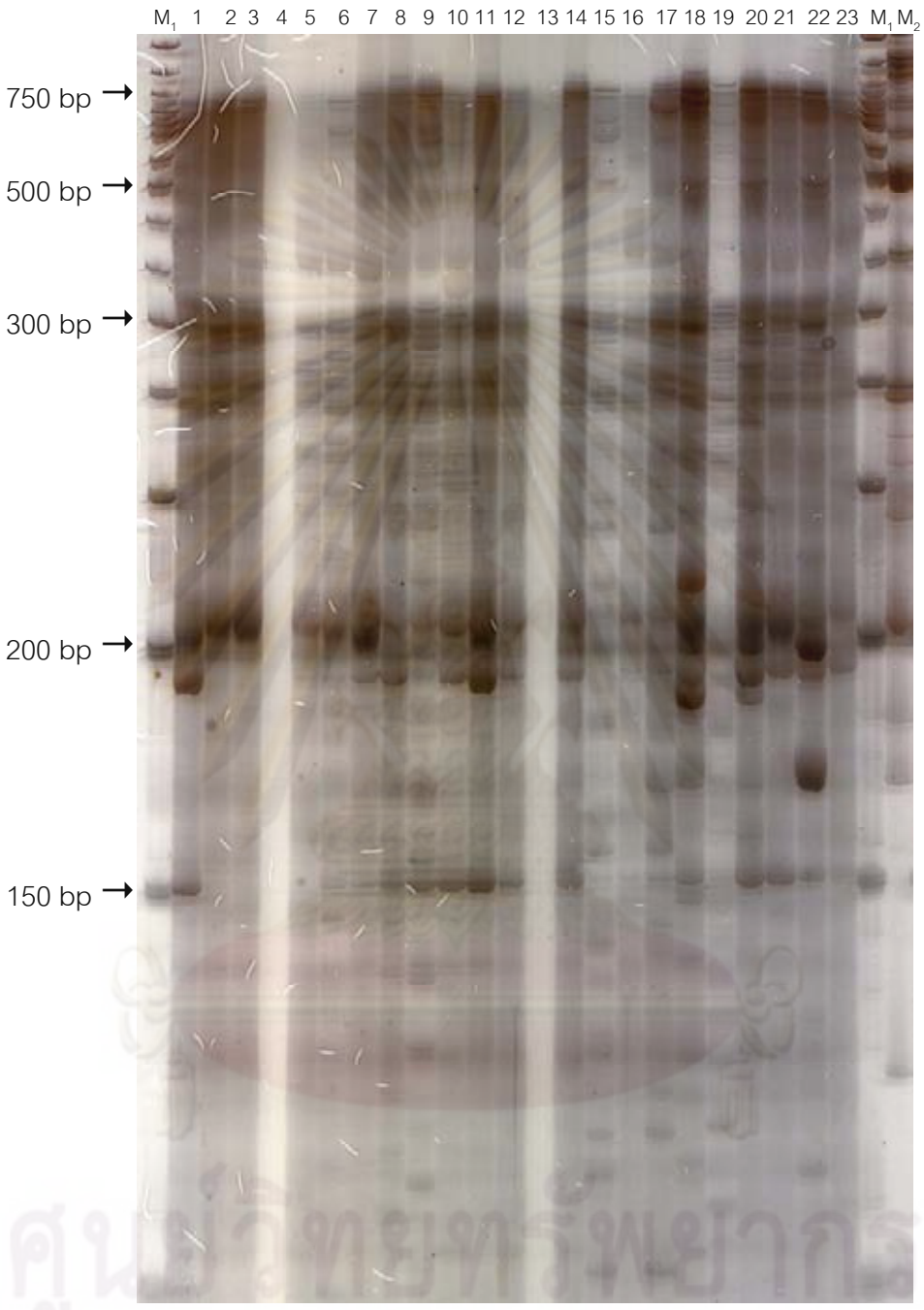
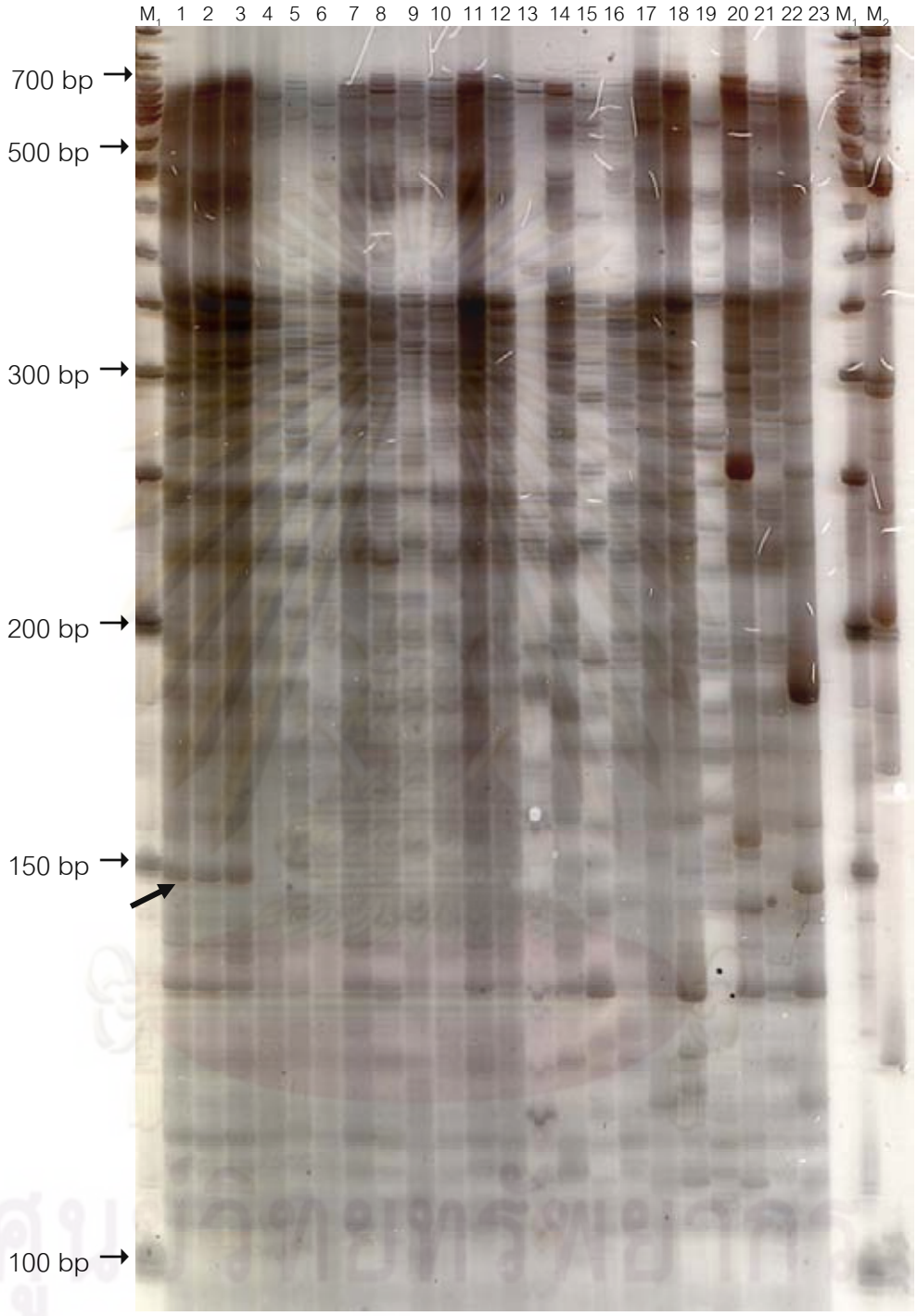


Figure 4.20 An AFLP profile of cured leaf samples of 23 tobacco cultivars using E_{AAG}/M_{CGC} selective primers (lanes no. 1-23 = tobacco cultivars : K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively). 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2, respectively) were used.



Figuer 4.21 An AFLP profile of cured leaf samples of 23 tobacco cultivars using E_{ACT}/M_{CAG} selective primers (lanes no. 1-23 = tobacco cultivars : K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun, Xanthiyaka, White gold, K326 phun-mueang, E-dum, E-lueang, Phu, Hangkai, Ya-glai, Bai-lai, Kariang, Yamuang, Kan, Bai-tang, Laodong and Kan-kiw, respectively). 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2, respectively) were used. The arrow mark the specific bands AFLP fragment in Virginia cultivars.

4.3 Phylogenetic relationship analyses of tobacco cultivars in Thailand

From the AFLP-PCR experiments of 23 cured-leaf samples, three tobacco cultivars (Yamueang, E-lueang and TN90) were found giving only fainted bands on all polyacrylamide gels and were then excluded from phylogenetic relationship analyses. Both neighbor-joining (NJ) and UPGMA analyses gave similar groupings of the 20 cultivars left on the tree diagrams.

The NJ tree analysis (Figure 4.22) revealed that nine of 12 local tobacco cultivars (Bai-lai, Bai-tang, Hangkai, Ya-glai, Kan, Kan-kiw, Kariang, Laodong and Phu) were grouped together, separated from the imported cultivars. However, the group of the other three local cultivars (White gold, E-dam and K326 phuen-mueang) was placed closely to the imported cultivars. Within the group of these nine local cultivars, some subgroupings were formed following their cultivating regions. For example, most of the local cultivars from the central region of Thailand (Laotong, Kan, Bai-tang and Kan-kiw) were clustered together and within this subgroup Bai-tang and Kan-kiw were paired together with 60% bootstrap supporting value. Apparently, Hangkai cultivar was rather different from the other local cultivars.

Among the eight imported cultivars, their clusterings on the NJ tree were not only based mostly on cultivating areas but also on the imported cultivar groups (Virginia, Burley, and Turkish cultivar groups). All three Virginia cultivars (K326, PVH03 and PV09) were distinguished from the other five imported cultivars with 100% bootstrap supporting value. Two Burley cultivars (KY14 and TN86) were also paired together with high bootstrap percentage (99%). The other two Turkish cultivars (Samsun and Xanthiyaka) were closely grouped together with 74% bootstrap support but also had TN97 cultivar of Burley group joined within their cluster.

The UPGMA dendrogram (Figure 4.23) showed some similar groupings of tobacco cultivars as on the NJ tree. Almost all local cultivars from the Northeast and Central of Thailand were grouped together on the UPGMA tree. Some of the local cultivars from the central region were also clustered together. For example, Kan, Bai-tang, Kan-kiw and Laodong cultivars were grouped together with 62% bootstrap support

and Bai-tang and Kan-kiw cultivars were paired together with 67% bootstrap, similar to their positions on the NJ tree (Figure 4.22). Moreover, K326 phuen-mueang and E-dum local cultivars were also clustered together. Like the result in the NJ tree, Hangkai cultivar was found rather different from the other local cultivars with 87% bootstrap support.

In the case of the imported tobacco cultivars, some clusterings of the imported cultivars were found on the UPGMA tree, which were mostly based on the imported cultivar groups and the cultivating regions as well. K326, PVH03 and PV09 of Virginia cultivar group were strongly clustered together with 100% bootstrap supporting value and could be distinguished from the other imported cultivar groups (Burley and Turkish groups). KY14 and TN86 cultivars of Burley group were not only paired together with very high bootstrap percentage (99%) but also had Ya-glai local cultivar placed beside them with 60% bootstrap support. The other two Turkish cultivars (Samsun and Xanthiyaka) were placed together too with 88% high bootstrap percentage, even though this pair was positioned closely to the major cluster of the local cultivars.

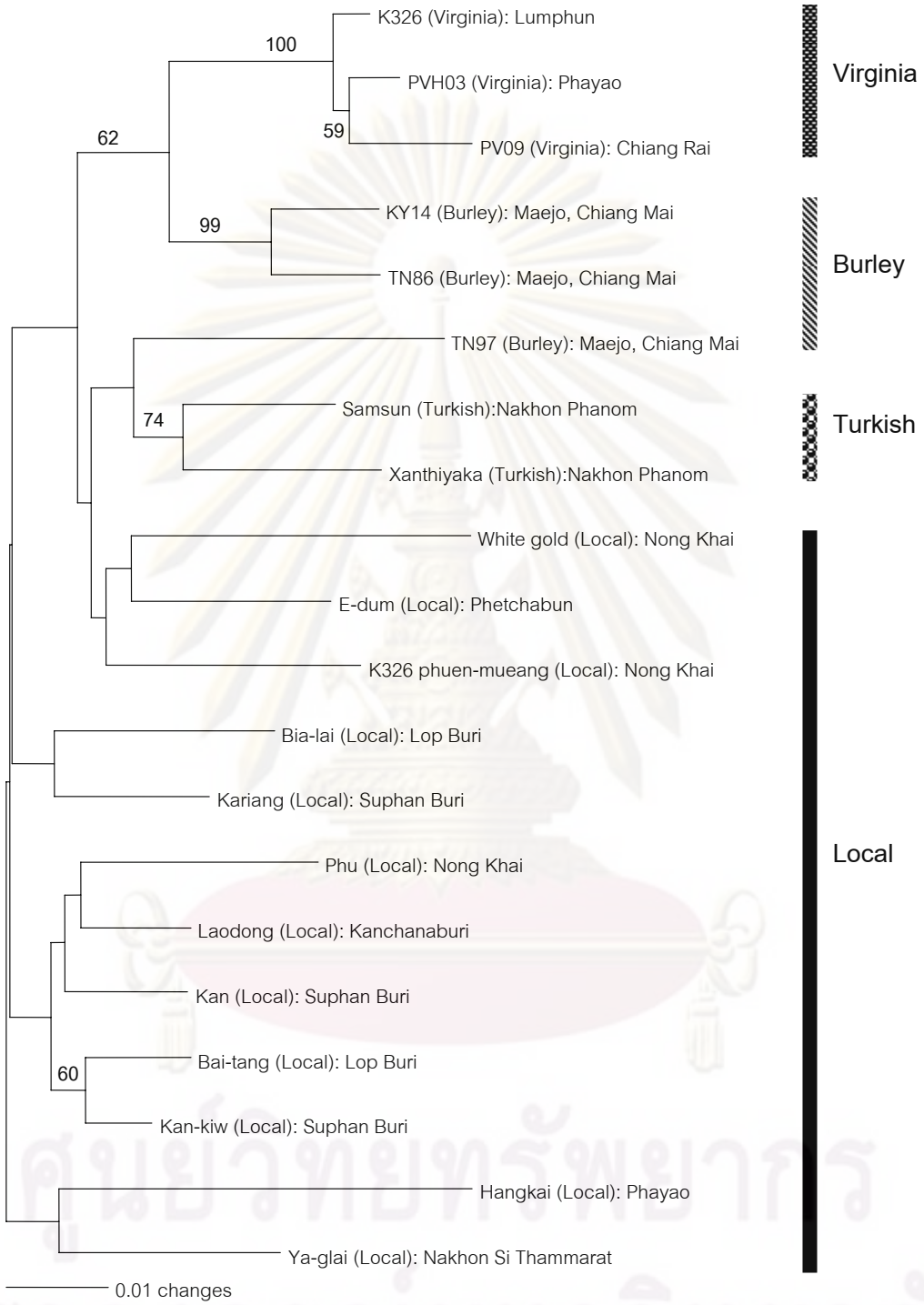


Figure 4.22 Genetic relationship tree from total 214 AFLP bands of 20 tobacco cultivar using Neighbour-joining (NJ) technique based on Nei and Li's similarity coefficient. Numbers along branches are bootstrap-supporting values generated after 1,000 replications. The bootstrap values less than 50% are not shown on the tree.

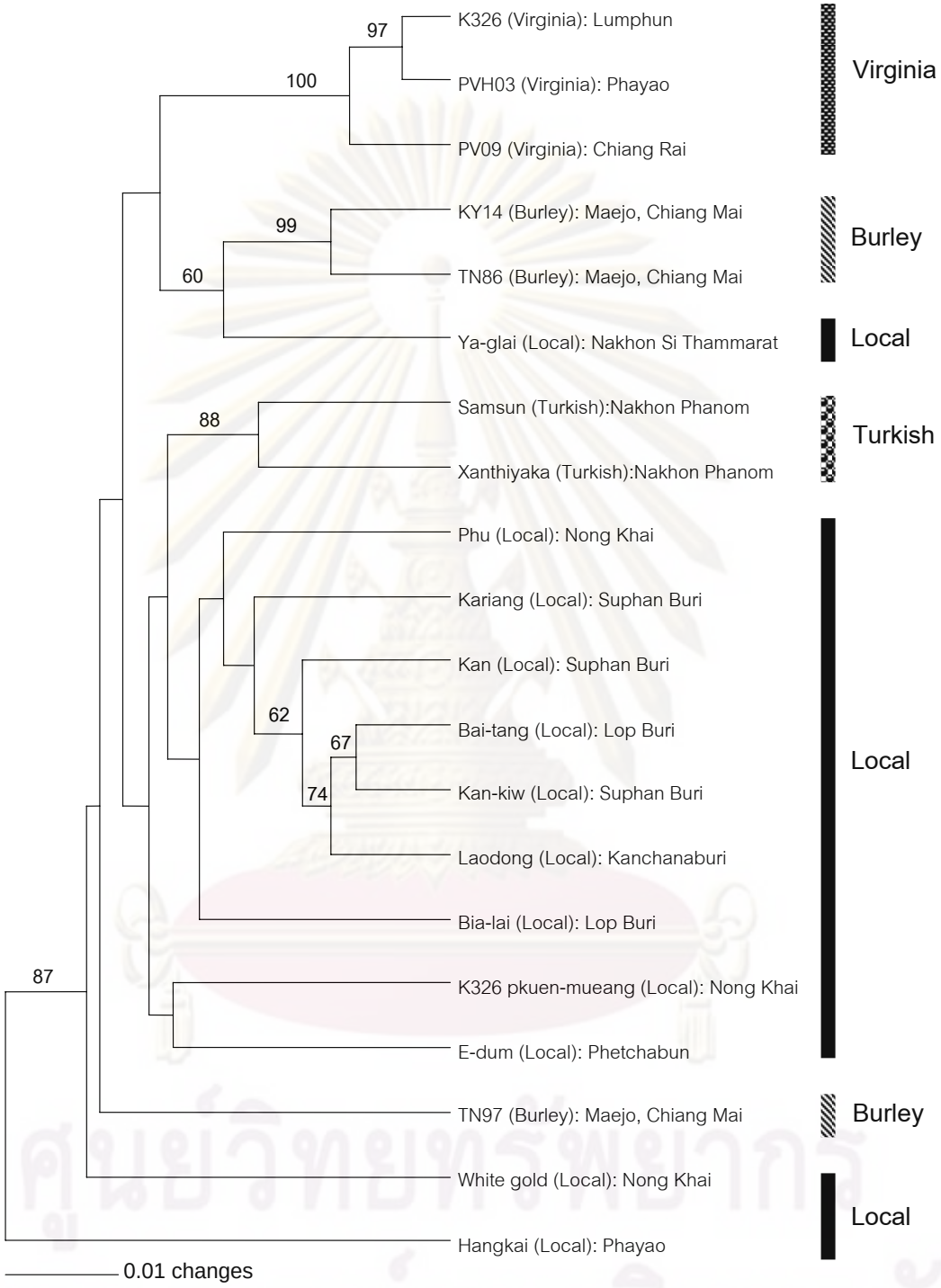


Figure 4.23 Genetic relationship tree from total 214 AFLP bands of 20 tobacco cultivar using UPGMA technique based on Nei and Li's similarity coefficient. Numbers along branches are bootstrap-supporting values generated after 1,000 replications. The bootstrap values less than 50% are not shown on the tree.

After performing on optimisation of the AFLP-PCR reactions of 23 cured leaf samples (nine imported and 14 local cultivars), the results found that eight imported and 11 local cultivars could give higher numbers of AFLP-fragments and suitable for further experiments. However, four tobacco cultivars (KY14, Kan-kiw, E-lueang and Yamueang) still could not generate AFLP profiles since their amplified products were too faint from all three primer-pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}). Therefore these four cultivars were not used for further phylogenetic relationship analyses. The NJ and UPGMA tree diagrams were constructed with combined data scored from the three primer-combinations. The NJ tree (Figure 4.24) showed groupings of the tobacco cultivars similarly to those found on the UPGMA tree (Figure 4.25). Both NJ and UPGMA trees were slightly different from those before the PCR optimisation, although some minor differences appeared especially within the clusters of the imported cultivars.

Based on the NJ analysis, almost all local tobacco cultivars were grouped together, except K326 phuen-mueang cultivar which was placed near Virginia imported cultivars. Five local cultivars from the Central of Thailand, (Bai-lai, Bai-tang, Kan, Kariang and Laodong) were clustered together with 54% bootstrap supporting value. This cluster of the cultivars from the central region was similar to that found in the previous experiment before optimisation. Within this group, Bai-lai, Kan and Kariang were clustered together with 66% bootstrap value and a pair of Bai-tang and Laodong cultivars strongly supported with 98% bootstrap value was also found. K326 phuen-mueang was placed near the group of Virginia cultivars as well. White gold and E-dum local cultivars were paired together and Ya-glai cultivar from Nakhon Si Thammarat was placed near them. Phu and Hangkai local cultivars from Phayao and Nong Khai provinces, respectively, were paired together with 84% bootstrap support.

Among the imported tobacco cultivars analysed in this optimised experiment, all of Virginia cultivars (K326, PV09 and PVH03) were strongly clustered together with 100% bootstrap value. This Virginia group was separated from the other imported tobacco cultivars. However, the other five imported cultivars did not form clusters based on their subgrouping of imported cultivars. Two Burley cultivars (TN86 and TN90) were paired

together with 61% bootstrap value, but also had Xantiyaka (Turkish) cultivar and then a mixed pair of TN97 (Burley) and Samsun (Turkish) cultivars with 61% bootstrap values, placed close to them.

On the UPGMA tree diagram (Figure 4.25), almost all local cultivars were clustered together on the tree except Hangkai. Five local tobacco cultivars sampled from the central region of Thailand were grouped together as well as their grouping found on the NJ tree. The result showed that Bai-lai, Kan and Kariang cultivars were grouped with 54% bootstrap supporting value while a pair of Bai-tang and Laodong was supported with high bootstrap value (93%). Hangkai cultivar was rather different from the other local cultivars.

In the case of the imported tobacco cultivars, two Burley cultivars (TN86 and TN90) were paired together and placed near the local cultivars. TN97 (Burley) and Samsun (Turkish) cultivars were paired together with very high bootstrap value (95%) while Xanthiyaka (Turkish) cultivar was also placed near them. Last, all three Virginia cultivars (K326, PV09 and PVH03) were grouped together on the tree with 100% otstrap support.

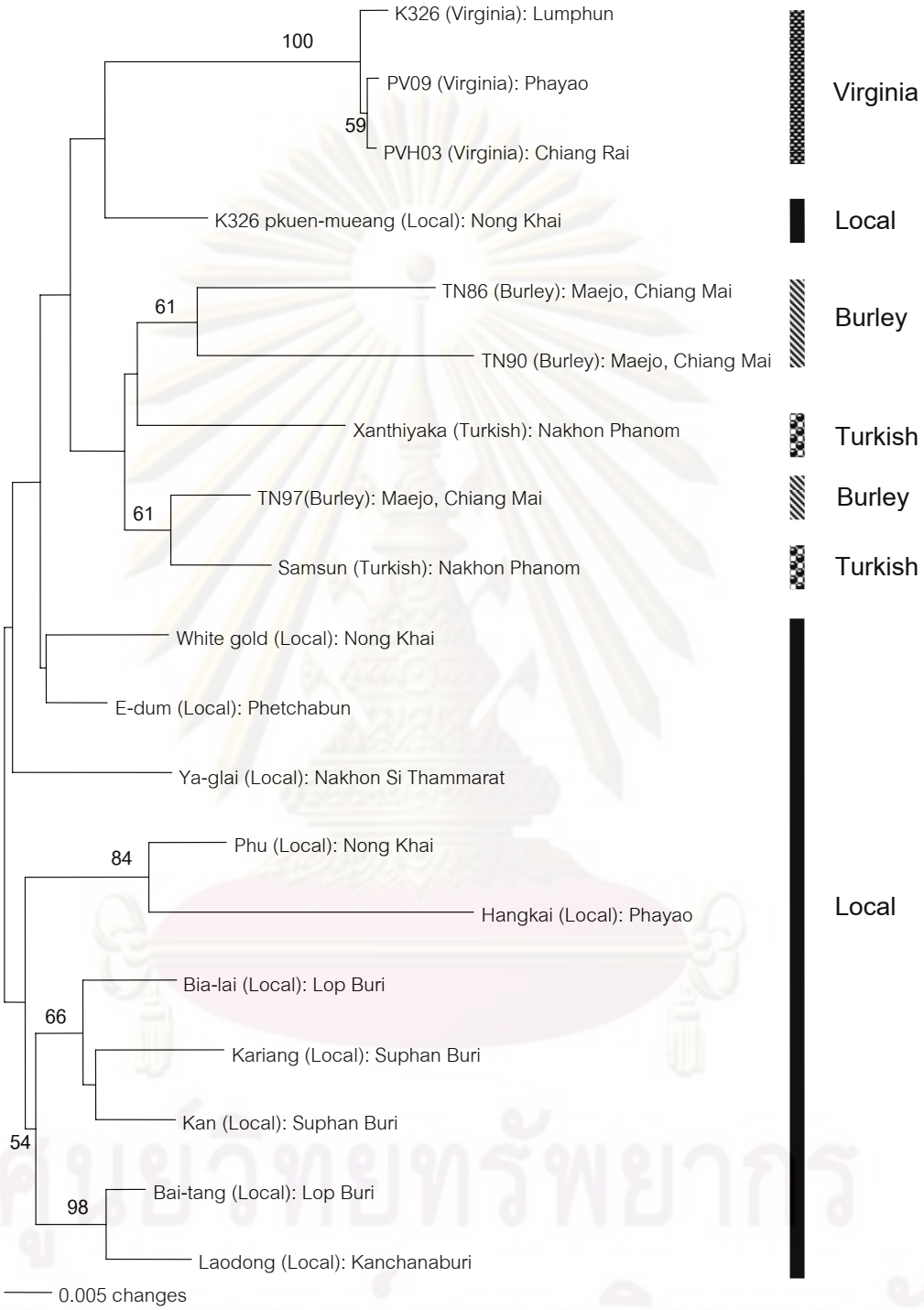


Figure 4.24 Genetic relationship tree from total 139 optimised AFLP bands of 19 tobacco cultivars using Neighbour-joining (NJ) technique based on Nei and Li's similarity coefficient. Numbers along branches are bootstrap-supporting values generated after 1,000 replications. The bootstrap values less than 50% are not shown on the tree.

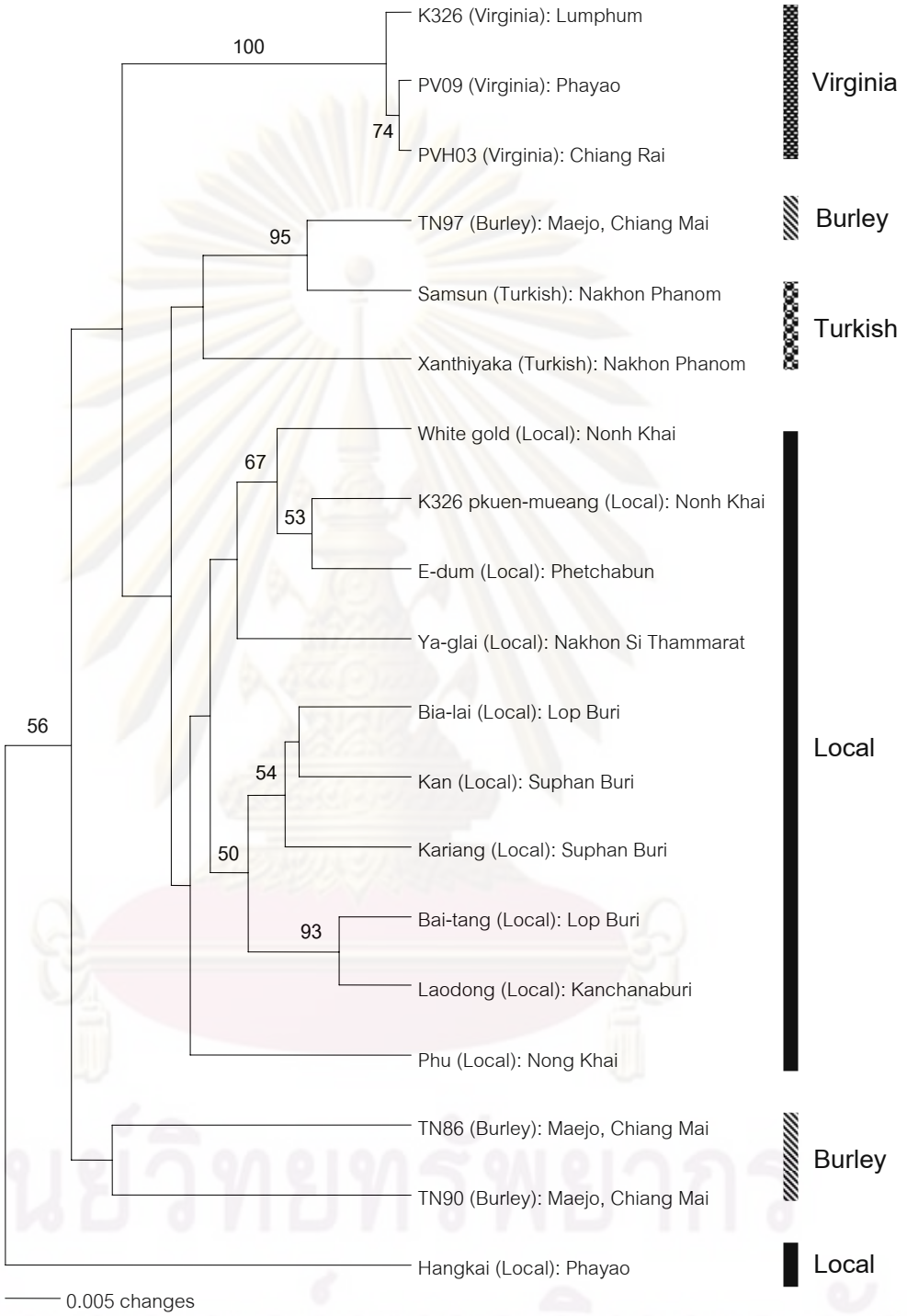


Figure 4.25 Genetic relationship tree from total 139 optimised AFLP bands of 19 tobacco cultivars using UPGMA technique based on Nei and Li's similarity coefficient. Numbers along branches are bootstrap-supporting values generated after 1,000 replications. The bootstrap values less than 50% are not shown on the tree.

CHAPTER V
DISCUSSIONS

5.1 Sample collection

In this study, most of the tobacco leaf samples collected from crop fields in the upper-northern region (Chiang Rai, Lumphun and Phrae provinces) of Thailand were in Virginia cultivar-group. Information about growing history of the tobaccos were given by local farmers and regional officers of Thailand Tobacco Monopoly. They explained the majority of Virginia cultivars over there that Thailand Tobacco Monopoly has promoted some Virginia cultivars to be grown in the region for a long time. Moreover, the officers of the tobacco regional stations ensured me about the name of most of the collected leaf samples to be of K326 cultivar. This was confirmed by the fact that the regional officers annually germinate Virginia tobacco seeds by their own and distribute 30-day old seedlings to the local farmers supported by the company. However, I was not so sure about the name of Coker326, another tobacco cultivar collected in the upper-northern-region. There was a disagreement between the regional officers whether this Coker326 was of Burley or Virginia cultivar group. I decided to describe Coker326 as Virginia cultivars because the plants looked more similar to most Virginia plants than to Burley. Moreover, the name “Coker” was usually used for Virginia cultivars in other publications (วรวิทย์ และคณะ, 2547; Yang et al., 2005; Zhang et al., 2006).

K326 and Coker326 cultivars were not the only Virginia cultivars grown in the Upper North, there were also some other Virginia cultivars, such as PV09 and PVH03, grown over there. These two Virginia cultivars were supported by private leaf-processing companies instead of Thailand Tobacco Monopoly while these private leaf-processing plants have hired some local farmers to grow tobacco especially for them. The private companies bought the mature tobacco leaves back and then flue-cured the leaves. To control the tobacco quality, the private companies usually imported pure seed of such cultivars directly and also planted the seed in seed-beds before sending the seedlings to their contract farmers. By the information given from the regional officers, I was also

sure about the names of PV09 and PVH03 cultivars and their status as another representatives of Virginia cultivar group.

Not only the samplings of four tobacco cultivars by myself in crop fields, but eleven tobacco cultivars were also collected from Meajo Experiment Station, Chiang Mai province. At the time of samplings, the tobacco seedling cultured in seed beds were one-to-two weeks old. For imported tobacco cultivars, I collected one Virginia (K190) and five Burley cultivars (HB01, HBO04P, TN86, TN90 and TN97), and the cultivars names of these tobacco seedlings were affirmed by the officers of Meajo station. Additionally, five local cultivars (Chorelare1, Padang, Pasak, Linchang and Nisan in Table 3.1) were also sampled from this station, but the officers were not so sure or knew about the origin and history of cultivation of these local cultivars, except Chorelare1. They suggested that this cultivar has been cultivated in Chiang Mai province since a long time ago and therefore Chorelare1 cultivar may be the best representative local cultivars grown in Chiang Mai. Another representative of local cultivars, Hangkai, was collected in the collecting trip to Phayao province. It was cultivated in the field near local farmers' houses. They did not know about the origin of this cultivar but believed that Hangkai cultivar also had a long-history of growing in Phayao province. I therefore expected that Chorelare1 and Hangkai local cultivars may have different genetic characteristics from other cultivars and could be hypothesised whether they should be called "true" local cultivars.

About the sample collection in Sukhothai and Petchabun provinces, the lower northern region, this province was supported by Thailand Tobacco Monopoly to be the first cultivating area of Burley tobacco cultivars around 50 years ago (ประกิต และ กรองจิต, 2547). Most of Burley cultivars promoted by the company were KY14 and B1 special. In the case of local cultivars in this area, I collected about two-week old samples of two local cultivars named Pechmakhuea and Petkhangsing. The officers of Sukhothai tobacco station commented that Pechmakhuea cultivar was originally grown in Phetchabun, a nearby province of Sukhothai, and Petkhangsing cultivar was simply known to be descended from Pechmakhuea. Petkhangsing cultivar got its named from

its stem which is similar to the shin of a lion by having many stipules around the main stem. These two local cultivars may have different genetic characteristics from other tobacco cultivars, which made them having such unique morphology.

Unlike the distribution procedure used with Virginia cultivars in the upper-northern region, the regional officers in Sukhothai did not grow and distribute Burley seedlings to local farmers, but let the farmers keep and germinate Burley seed by themselves for the next cultivating season. This less-strict procedure may cause problem to a certainty of the cultivar names in this region. I believe that some of Burley seed may have been passed to other local farmers in Sukhothai and surrounding areas. If they had cultivated in local areas for a period of time, they were probably named in Thai and incorrectly recognised as local cultivars. This could being a confusing to the casa of row-your-own (RYO) tobacco products which must make from cured leaves of local cultivars only.

For Turkish tobacco cultivars, they were collected only from the northeastern region of Thailand, and Samsum and Xanthiyaka cultivars were cultivated the most in the region. Turkish cultivars have been grown specifically in the Northeast because Thailand Tobacco Monopoly needed strong oriental-smell of these cultivars and such oriental smell would be achieved only by growing Turkish plants on poor soil and stressful climate of this area. Although Turkish cultivars are small-sized plants and easily to recognise, I still wondered whether the distinctive morphological characteristics of Turkish cultivars were in fact influenced mainly from stress conditions of the cultivating area. Some regional officers in Nong Khai and Nakhon Phanom provinces also questioned that if growing Turkish cultivars in other regions, would it be similar to other tobacco cultivars, particularly Burley cultivars.

Some of local tobacco cultivars were also found in the Northeastern region, such as Whitegold, K326-phuenmueang and Phu. Some of the regional officers hypothesised that these northeastern local cultivars may not originally be local cultivars of the area because their morphological characteristics were very similar to those of Burley and Virginia, especially the leaf shape. Moreover, the names of Whitegold and K326-

phuenmueang also suggested their similarity to Virginia plants. If their hypothesis is true, I assumed that these local cultivars would actually have descended from some imported cultivars in Thailand.

While most of the imported tobacco cultivars were usually grown in the North and Northeast of Thailand, most of the local tobacco cultivars were cultivated in the central region. Six local cultivars - Kariang, Kan, Bia-tang, Bai-lai, Kan-kiw and Laodong - were collected in this area. From the local farmers' comments, Laodong and Kariang were the most preferable cultivars and this may be because the two cultivars had stronger aromatic-smell than the others. Moreover, Mrs. Ang Siwaprapa, a local farmer in Mueang district of Kanchanaburi province, mentioned that Kariang cultivar was firstly cultivated in the province probably around 100 years ago. This was similar to the believe of some regional officers of Chiang Mai tobacco station that all local tobaccos in Thailand were first cultivated in Kanchanaburi and Lop Buri provinces though without any evidence of this suggestion. Following their idea, I then proposed that most of the local tobacco cultivars grown in the Central of Thailand may have unique genetic characteristics from long history of cultivation, and if such, could also be pronounced as "true" local cultivars.

5.2 DNA extraction

In this experiment, genomic DNA was extracted from different fresh leaf samples of 34 tobacco cultivars (14 imported and 20 local cultivars). Following the experiment on the fresh leaf samples, genomic DNA of 24 cured leaf materials was extracted (10 imported and 14 local cultivars). Most of the genomic DNA extracted from the fresh leaf samples were in high quantity (Figure 4.1). However, large amount of smear background also appeared on the gel electrophoresis results. This smear background may be the result of some DNA degradation and RNA contamination. I proposed that the degradation of the tobacco DNA may have come from too much humidity still left in the leaf tissue before DNA extraction. Such humidity may have activated DNase (DNA endonuclease) within the plant cells to digest the genomic DNA. For example, four

tobacco cultivars of fresh leaf meterial, K326, TN90, TN97 and Chorlare1 (lanes 4, 11, 12 and 15 in Figure 4.1, respectively), gave higher quantity of total DNA than the other 30 cultivars and this may be because the leaf samples of these four cultivars seemed to be perfectly dried compared to the others. Normally, quality and yields of plant DNA preparations are mostly influenced by the condition of starting materials. The quality of the isolated DNA will generally decrease with the duration of the desiccation process. Moreover, water stress and humidity in connection with wounding cells induce the accumulation of phenolic compounds, which may interfere severely with the isolation of high quality (Savolainen *et al*. 1995). The rapid drying of plant tissues can be done with the help of desiccation agents (usually silica gel) which was first suggested by Liston *et al*. (1990) and Chase and Hills (1991).

Although fresh tobacco leaf samples were left drying in silica gel bags, I had to wait for 2-3 days before brought them back from the crop fields to the laboratory. The samples were also stored in a cupboard at room temperature without any special desiccating facility except a few amount of silica gel in each sample bag. This may have led to the low quality of some of my extracted DNA. In fact, Weising *et al*. (2005) suggested that DNA isolation should be performed from fresh or young plant tissue harvested immediately before the isolation. The samples need to be completely dried within 24 hours to ensure the high DNA quality. Therefore, their recommendation to cut a tobacco leaf into small pieces and keep them in a dry silica-gel bag with the weight ratio of silica gel to plant tissue exceed 10:1 is advisable for my further work. Moreover, the samples should be processed as soon as possible upon return to the laboratory and the silica gel should be replaced with the new one when the colour of indicating silica gel changes from blue to pinkish-purple or colourless.

In the case of RNA contamination, it may have resulted from my first decision not to add RNase (RNA endoneuclease) into the sample tubes in prior to the lysis step of DNA extraction. I previously thought that RNA contamination would not have any effect on the AFLP amplification. However, after finished the DNA extraction of the fresh leaf samples, I found that a contamination of RNA in the DNA extract had been reported

to possibly alter the enzyme digestion levels of DNA template with two restriction enzymes (Incirli *et al*., 2001). The level of restriction enzyme digestion may reduce when having too much RNA in the reaction and thus RNase should be added to prevent the RNA contamination. Therefore, I added RNase into the reaction tubes of the DNA extraction from the cured-leaf specimens. And, for the further work adding RNase in the lysis step of the genomic DNA extraction would rather be recommended. Moreover, the DNA extraction should perform on young plant leaves only to give high DNA yield since they contain less phenolic compounds than either old or cured leaves (Dabo *et a*l., 1993; Michiels *et al*., 1994).

In the case of the DNA extraction from cured tobacco leaf materials, the quantity of the extracted genomic DNA was mostly less than that from the fresh leaf samples. That would be even worse if the samples passed a curing-process. Both heat and humidity from a curing-process could damage or reduce the quality and quantity of the extracted genomic DNA. I assumed that the DNA extraction problem of the cured leaf samples probably have not caused only by the improper desiccation of the materials (discussed before), but also by various parameters of the curing-methods such as heat, humidity and period of the process. Apparently, the quality and quantity of the extracted genomic DNA of Burley, Turkish and local cultivars were lower than that of Virginia cultivars whereas Burley cultivars gave even lower DNA quality than Turkish and local cultivars. This may be because an air-curing method of Burley cultivars takes much longer time than the other three curing methods. The humidity of the air-curing method is also higher than that of a sun-curing method of Turkish cultivars. Therefore, DNase could digest the genomic DNA of the air-cured Burley samples more rapidly than that of the others. On the other hand, Virginia tobacco cultivars gave the highest quantity of the total extracted genomic DNA. Although a flue-curing method of Virginia cultivars uses very high temperature to dry and cure tobacco leaves, it processes under low humidity and the period of curing process is rather short (only 5 days) compared to the other methods (30-40 days for air curing, 13-15 days for sun curing and 8-10 days for air-and-

sun curing). This would have reduced the chance of the flue-cured Virginia genomic DNA being damaged by DNase.

Nevertheless, though with low amount of DNA in some samples, the quality of almost all of the extracted DNA was still suitable for the further experiment. The AFLP reaction could give some certain amount of AFLP-PCR products even in the case of some cured leaf samples which gave quite low quantity of the extracted DNA. Their genomic DNA was pure enough to be successfuly used in the AFLP experiment, and the purity of my extracted DNA from both fresh and cured leaf materials may have resulted from high efficiency of the commercial DNA extraction kit used in the study. From the DNA extraction kit's handbook, it insures that an extracted DNA from using the kit should be pure enough for using in common PCR, real-time PCR, Southern bloting and fingerprinting analysis. My decision to use the commercial kit was also based on its other advantages, i.e. its fast procedure and no requirement for special laboratory equipments or toxic reagents such as phenol and chloroform.

5.3 AFLP-PCR amplification

The preliminary AFLP study of 30 tobacco cultivars using fresh leaf samples revealed only a small number of scorable fragments per primer (only 31 bands per primer) while having a lot of DNA smear in each lane (see an example in Figure 4.5). Ren and Timko (2001) previously reported that their AFLP study of 46 cultivated tobacco accessions successfully detected as high as 92 bands per primer, which is almost three-time more than my result. In addition, Zhang *et al*. (2006) produced 82 AFLP-PCR fragments per primer from the experiment on 51 flue-cured (Virginia) tobacco cultivars. Moreover, another study of nine AFLP primers using 54 tobacco cultivars in India also amplified AFLP-PCR products in the ratio of 84 fragments per primer (Siva *et al*., 2008). I suspected that the PCR condition which I used in the selective-primer screening might not have been best suitable and the condition was needed to be optimised. This led to the optimisation experiment which an annealing temperature was raised up and a concentration of the DNA template was adjusted possibly to give a clearer fragment profile.

After diluting the DNA template concentration to 1:10 and 1:20 ratios to optimise the AFLP profile, the amplifications of all four selective primers gave clearer fragment profiles with a higher number of scorable AFLP fragments than the previous result. The smear background was also decreased (see Figures 4.11-4.14). Actually, the dilution of DNA template could be increased to the ratio of 1:50, similar to the concentration used in the two previous AFLP studies of tobacco (Ren and Timko, 2001; Siva *et al*., 2008) However, the two studies performed on young fresh-leaf materials unlike my AFLP study which was on the cured-leaf samples, I have rather decided to keep using the dilution ratio of 1:20 for all later AFLP experiments in this thesis.

Not only optimising the concentration of the DNA template, but the starting annealing-temperature of the selective amplification step was also increased to achieve a better AFLP-PCR result. Shen *et al*. (2007) suggested that higher annealing temperatures significantly improve the efficiency of a touchdown PCR amplification. They recommended to do touchdown PCR at a higher starting temperature which was able to overcome their PCR bias problem. Likewise, the increment of the annealing temperature from 65^{o}C to 67^{o}C in my experiment gave much clearer AFLP profiles with sharper amplified bands and reduced smear background in almost all AFLP selective amplifications (Figures 4.19-4.21), except that of E_{AAG}/M_{CAT} primer which still produced many fainted PCR bands (data not shown). Therefore, I concluded that in this case the starting annealing-temperature of 67^{o}C was more suitable for primer binding than 65^{o}C.

After optimising the AFLP-PCR reactions by diluting the template concentration and increasing the annealing temperature, the average number of scorable AFLP fragments was increased to 54 fragments per primer. Although these optimised AFLP experiments gave a higher number of AFLP bands, it was still lower than the previous AFLP results of Ren and Timko (2001), Zhang *et al*. (2006) and Siva *et al*. (2008). I suspected that this low number of the scorable AFLP fragments in my study may have resulted from low yield of the genomic DNA extracted from the cured leaf materials, whereas those three studies used young fresh-leaf materials.

From the AFLP profiles after increasing the annealing temperature, the amplified band patterns (Figures 4.19-4.21) were somewhat similar to those of the previously unoptimised experiment (Figures 4.11-4.14), but clearer and sharper. Furthermore, the AFLP patterns of most local tobacco cultivars were similar to those of Burley and Turkish cultivars. Interestingly, the AFLP profile from E_{ACT}/M_{CAG} primer-pair could give one cultivar-specific band, approximately 150 bp, which was a specific band to the three Virginia cultivars (Figure 4.24) and not found in the other imported and local cultivars. Although Siva *et al*. (2008) revealed as many as 34 species-specific markers of the species *N. tabacum*, but they could not find any cultivar-specific band. Likewise, Ren and Timko (2001) also admitted that AFLP analysis would not be effective in analysing polymorphism at the subspecies level in *Nicotiana*. I therefore proposed that the single 150 bp AFLP band newly found in my study would be very useful for implementing as a novel specific marker for Virginia cultvars.

5.4 Phylogenetic relationship analyses of tobacco cultivars in Thailand

After performing the optimisation of AFLP-PCR reactions, NJ and UPGMA tree analyses of genetic relationship among 19 tobacco cultivars from cured-leaf samples (Figures 4.24 and 4.25) revealed that most of the local cultivars in Thailand were closely related with each other and also with Burley and Turkish imported cultivars. Both NJ and UPGMA trees were not too much different from the trees constructed from the AFLP results before the PCR optimisation (Figures 4.22 and 4.23). Subgroupings of the local cultivars tend to follow their cultivated areas. Likewise, the imported cultivars formed several clusters based mostly on their cultivar groups (Virginia, Burley and Turkish groups). There are some previous AFLP studies on tobacco cultivars reporting similar results to mine. For example, Ren and Timko (2001) reported that 46 tobacco cultivars representing 18 different countries around the world could be divided primarily based on geographic origins and manufacturing quality traits. In their study, all tobacco cultivars grown in the United States were contained in the same group and all cigar-types from Central America were grouped together, supporting to my results that

genetic relationships of tobacco cultivars tend to be based on their geographic origin and cultivating areas.

In the AFLP study of Zhang *et al*. (2006), 51 flue-cured (Virginia) cultivars with desirable agronomic characteristics (such as high leaf yield, low nicotine content and resistance to various diseases) also formed groupings based on their geographic origin. Moreover, Siva *et al*. (2008) reported that 16 cultivars of flue-cured cultivars, which were used in cigarette manufactures, were grouped together in their study and almost all of the bidi types (a tobacco product that originated in India) were also grouped together. Although the results of most AFLP studies support the idea that tobacco cultivars tend to be grouped based on their geographic origin, this hypothesis disagrees with the finding of Zhang *et al.* (2008) whose dendrogram generated with combined RAPD and AFLP data did not indicate any clear pattern of clusters among the flue-cured tobacco based on geographic origin. This disagreement may be because small numbers of 125 RAPD amplified bands were used in the RAPD analyses compared with a much higher number of 561 AFLP-PCR bands normally used. Therefore, AFLP results should be more preferable than RAPD data in a construction of phylogenetic tree.

Following the hypothesis of geographic-based genetic relationships among tobacco cultivars, Bai-lai, Kariang, Kan, Bai-tang and Laodong local cultivars from the Central of Thailand were also clearly grouped together on my NJ and UPGMA trees. I proposed that the close genetic relationship of these local cultivars was possibly due to their common origin of cultivation. From the interview with Mrs. Ang Siwaprapa, a local tobacco farmer in Kanchanaburi province, Kariang local cultivar was first grown in that province around 100 years ago. Likewise, officers of the regional tobacco station of Chiang Mai province suggested that the local tobaccos cultivated in Kanchanaburi and Lop Buri provinces may have been grown there for a long time. From these reasons, I proposed that some local cultivars from the Central of Thailand might be descended from the same parentage and they have been cultivated in the area for a long time.

Unlike those five local cultivars which were closely grouped together, White gold, K326 phuen-mueang, E-dum, Phu and Ya-glai cultivars were placed near the

groups of Turkish and Burley imported cultivars. This result was like the previous finding of Setaphan (2007) using ISSR analysis to study genetic relationship of 40 tobacco cultivars grown in Thailand. In her study, most of all 13 local cultivars were grouped with some Burley and Turkish imported cultivars. I suspected that these four local cultivars may have been originated from some imported cultivars, either Burley or Turkish. This could have happened because Burley and Turkish cultivars have been imported by Thailand Tobacco Monopoly since 50 years ago and given to their contact farmers for growing. The officers of Chiang Mai and Sukhothai regional stations also mentioned that if any promoted tobacco produced highly satisfying yield, the farmers might have kept the tobacco's seed on their own for the next cultivating season. The genetic relationship result was also supported with another suggestion of officers that some local cultivars were morphologically similar to imported cultivars. They then assumed that some local cultivars were probably not truely local cultivars. If this hypothesis is true, some local cultivars should be pronounced as "imported" cultivar instead, although they have Thai names and have been cultivated in local areas for quite a long time.

Interestingly, Hangkai, the other local cultivar left in this study, was found on both NJ and UPGMA trees to position distantly from the other ten local cultivars. It means that this cultivar may be genetically much different from the other local cultivars. The tobacco farmers in Phayao province told me that they were not so sure about the origin of Hangkai cultivar but believed that it has been grown there for a very long time. I therefore suggested that Hangkai local cultivar may have long history of growing in Thailand and they could be pronounced as a "true" local cultivar of Thailand.

While Hangkai local cultivar was separated from the other ten local cultivars, K326, PV09 and PVH03 Virginia cultivars were even distantly separated from all eleven local cultivars and the other imported cultivar groups (Burley and Turkish). Furthermore, all of the three Virginia cultivars were found having a single 150 bp cultivar-specific band on the AFLP profile produced by E_{ACT}/M_{CAG} primer-pair (Figure 4.24). I assumed that Virginia cultivars should have very different genetic characteristics from other cultivar groups grown in Thailand. This hypothesis agrees well with the results of Siva *et*

al. (2008) in which their cultivated flue-cured (Virginia) cultivars were clustered separately from air-cured (Burley) cultivars. My result was also supported by the RAPD analysis of Sarala and Rao (2008). Their analysis clearly distinguished two Burley cultivars from eight flue-cured cultivars.

However, this is not the case when compared with the previous ISSR marker analysis of Setaphan (2007). Her result suggested that K399, Coker319, Coker371 and NC37NF Virginia cultivars were grouped with TN97 and KY10 Burley cultivars with low bootstrap support. This incongruity between the ISSR and AFLP results may be because of the fact that ISSR technique reveals less amplified fragments than AFLP method. Moreover, ISSR analysis has lower capability to produce polymorphic bands from a tobacco genome than AFLP method since the tobacco genome is large and then require the use of AFLP for wide genome coverage, which provides a more realistic measure of genetic diversity (Siva *et al*., 2008). This is supported with ISSR and AFLP analyses on *Nicotiana attenuate* of Bahulikar *et al*. (2004) showing that the AFLP analysis gave higher percentages of polymorphic loci than the ISSR analysis. For these reasons, Virginia cultivars should be assumed to have a very distinct genetic characteristics among all tobacco cultivars and AFLP marker technique should be the most powerful tool to clearly separate Virginia cultivars from other cultivars grown in Thailand.

Furthermore, the 150 bp Virginia-specific marker from E_{ACT}/M_{CAG} AFLP profile may be further developed to be a Sequence Charecterised Amplified Region (SCAR) marker. Normally, SCAR marker is developed from any DNA fragment of interest, which is amplified by PCR reaction and the specific primers are designed for such specific DNA fragment. A previous study of Julio *et al.* (2006) using AFLP markers successfully developed seven SCAR markers linked to three disease resistant genes (blue-mold, Potato Virus Y and black root rot diseases) within *N. tabacum*. Following that procedure, the 150 bp Virginia-specific AFLP-PCR fragment could be converted to a SCAR marker for Virginia cultivars. If successfully developed, this marker should be easily used to identify any unknown tobacco plant or germplasm whether it is a Virginia cultivar.

Additionally, such Virginia-specific marker could be applied to investigate an illegal mixing of Virginia cultivars into roll-your-own (RYO) products, which legally must be made only from cured leaves of local cultivars. If any RYO sample is sent to investigate in a laboratory and then produces an amplified product similar in-size to that 150 bp Virginia-specific fragment, we could infer that the tested RYO sample may have been illegally mixed with Virginia cultivars.

Although the AFLP technique could generate a Virginia-specific marker, there was still some weak points found in this study. For example, not all of local cultivars grown in Thailand had been analysed with this AFLP technique, and more number of both imported and local tobacco samples are needed to confirm an efficacy of the Virginia-specific marker. Moreover, additional screening of other selective-primer combinations should be performed to find a better combination which can yield a sufficient number of highly polymorphic AFLP-PCR bands. Using more specifically selective primers such as +4 nucleotide primers should be considered. Furthermore, the acrylamide-gel electrophoresis conditions should be optimised since they can affect the result scoring for absence or presence of AFLP bands. To have clearer visualised AFLP bands on the acrylamide gel, other factors such as the gel staining and developing methods should also be optimised. For example, a silver staining method could cause a visual problem with its strong artefact background of silver precipitation. Thus, the gel developing step of this silver staining method should not take longer time than 5 minutes because it could result in too strong background. Furthermore, ultrapure water must be used in all steps of the silver staining method to avoid any unexpected reaction of silver with ion trace or any impurity in the water, resulting in very high background after staining. Though very expensive, an automated florescence dye method may be used instead of the silver staining to give clear background staining and the tedious procedure of silver staining can be avoided.

Last but not least, my genetic relationship analysis of tobacco cultivars grown in Thailand using AFLP results could be a fundamental information for other tobacco genetic researches in the future. The results from this research would be an important

source for improvement of tobacco germplasm, population genetics and genotyping of tobacco cultivars and parental choice for breeding purposes. In addition, the Virginia cultivar-specific marker identified in this study should be useful for identification and confirmation of Virginia genotypes in trade and commerce, such as being a simple, but robust quality control of the production line of Thailand Tobacco Monopoly from seed germination to cured-leaf buying.

CHAPTER VI
CONCLUSION

1. In this thesis, the preliminary experiment to select suitable AFLP primers was performed with the extracted DNA from fresh leaf materials of 12 imported cultivars and 18 local cultivars. Four primer-pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) were selected to be the suitable +3 primers for the selective AFLP-PCR step. These four primers were used further to study genetic relationships based on the genomic DNA extracted from nine imported and 14 local cultivars cured leaf samples. The selective AFLP-PCR reactions were optimised by raising the annealing temperature and adjusting the concentration of the templates. Almost all of the selective AFLP-PCR reactions, except that of E_{AAG}/M_{CAT} primer-pair, were successfully done and gave clearer AFLP profiles than the results of the previous unoptimised reactions, with a higher number of the scorable AFLP bands.

2. After the NJ and UPGMA trees were reconstructed from the AFLP-PCR results of eleven local and eight tobacco cultivars, subgroupings of the local cultivars tended to form following their cultivating regions. The clusterings of the imported cultivars were not only based mostly on cultivating areas, but also on their cultivar groups (Virginia, Burley, and Turkish groups). Interestingly, most of the local cultivars in Thailand were closely related with each other and also with Burley and Turkish imported cultivars. On the other hand, all three Virginia cultivars (K326, PVH03 and PV09) were distinguished from the other five imported cultivars, suggesting that this AFLP technique could be used to separate Virginia cultivars from any other imported and local cultivars.

3. The AFLP-PCR result also revealed that the selective amplification with E_{ACT}/M_{CAG} primer gave one clear band (approximately 150 bp) which could be the specific marker for Virginia cultivars. This Virginia-specific amplified fragment would be

further developed as a Sequence Charecterised Amplified Region (SCAR) marker to identify any unknown tobacco plant or germplasm whether it is a Virginia cultivar. Additionally, this marker could be applied to investigate an illegal mixing of Virginia cultivars into roll-your-own (RYO) products, and to identify and confirm Virginia genotypes in a quality control of the tobacco production line from seed germination to cured-leaf buying.

4. Finally, all of these research results should be a fundamental information for other tobacco genetic researches in the future. It would be an important source for improvement of tobacco germplasm, population genetics and genotyping of tobacco cultivars and parental choice for breeding purposes.

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APEENDICES

APPENDIX A

A1. Scored fragments from AFLP-PCR amplification with four primer-pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) in all 30 local and imported of fresh tobacco cultivars.

1) E_{AAG}/M_{CAA} primer-pair

Cultivar	Scored-AFLP band
1. K187	1 0 0 0 0 1 0 1 1 1 1 1 1 1
2. K326	1 0 0 0 0 1 0 1 1 1 1 1 1 1
3. PVH03	1 0 0 0 0 1 0 1 1 1 1 1 1 1
4. PV09	1 0 0 0 0 1 0 0 0 0 0 0 1 1
5. B1 special	1 0 0 0 0 1 0 0 0 0 0 0 1 1
6. KY14	1 1 0 0 0 1 0 1 1 1 1 1 1 1
7. HBO04P	1 1 0 0 0 1 0 0 1 1 1 0 0
8. TN90	1 0 0 0 0 1 0 0 0 0 0 0 0
9. TN97	0 0 0 0 0 1 0 0 0 0 0 1 0

10. Samsun	0 0 1 0 0 1 1 0 1 1 1 1 1
11. Xanthiyaka	0 0 0 1 0 1 1 1 1 1 1 1 1
12. Coker326	0 0 0 1 0 1 0 1 1 1 1 1 1
13. Chorelare1	0 0 0 1 0 1 0 1 1 1 1 1 1
14. Nisan	0 0 0 1 0 1 0 0 1 1 1 1 1
15. Pasak	0 0 0 1 0 1 0 0 0 0 0 1 1
16. Padang	0 0 0 1 0 1 1 1 1 1 1 1 0
17. Petkhangsink	0 0 0 1 0 1 1 1 1 1 1 1 0
18. Petmakhuea	0 0 0 1 1 1 1 0 0 0 0 1 0
19. Yamueang	0 0 0 1 0 1 1 1 0 0 0 1 1
20. Linchang	0 0 0 1 0 1 1 1 0 0 0 1 1
21. Hangka	0 0 0 1 0 1 1 1 0 0 0 1 1
22. Yahan	0 0 0 1 0 1 1 1 1 1 1 1 1
23. Phu	0 0 0 0 0 1 0 0 0 0 0 1 1
24. K326 phuen-mueang	0 0 0 0 0 1 0 0 0 0 0 1 0
25. Kariang	0 0 0 0 0 1 0 0 0 0 0 1 0
26. Kan	0 0 0 0 0 1 0 0 0 0 0 1 0

27. Kan-kiw Dok-khao	0 0 0 0 0 1 0 0 0 0 0 1 0
28. Meao	0 0 0 0 0 1 0 0 0 0 0 1 0
29. Laodong	0 0 0 0 0 1 0 0 0 0 0 1 0
30 Kan-kiw Dok-chom-phu	0 0 0 0 0 1 0 0 0 0 0 0 0

2) E_{AAG}/M_{CAT} primer-pair

Cultivar	Scored-AFLP band
1. K187	0 1 1 0 0 0 0 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1
2. K326	0 1 1 0 0 0 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
3. PVH03	0 1 1 1 1 0 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
4. PV09	0 1 1 1 1 0 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
5. B1 special	0 1 1 1 1 1 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
6. KY14	0 1 1 1 1 0 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
7. HBO04P	0 1 1 1 1 0 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
8. TN90	0 0 0 0 0 0 0 0 1 1 1 0 1 1 0 0 0 0 1 1 0 1 1 1 1 1 1 1 1 1 0 0 1 1

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9. TN97	0 0 0 1 1 0 1 0 1 1 1 0 1 1 0 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1
10. Samsun	0 0 0 0 0 0 0 0 1 1 1 0 1 1 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 1
11. Xanthiyaka	0 0 0 1 1 1 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
12. Coker326	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1
13. Chorelare1	0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1
14. Nisan	1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
15. Pasak	1 1 1 0 0 0 1 0 1 1 1 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
16. Padang	0 0 0 0 0 0 0 0 1 1 1 1 1 1 0 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 0 0 0 1
17. Petkhangsink	0 0 0 0 0 0 0 0 1 1 1 1 1 1 0 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 0 0 0 1
18. Petmakhuea	0 0 0 1 0 0 0 0 0 0 1 0 0 1 0 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1
19. Yamueang	0 0 0 1 0 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
20. Linchang	0 0 0 1 0 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
21. Hangka	0 0 0 1 0 0 0 0 1 1 1 0 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
22. Yahan	0 0 0 1 1 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
23. Phu	1 1 1 0 0 0 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
24. K326 phuen-mueang	1 1 1 0 0 0 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
25. Kariang	0 0 0 0 0 0 1 0 1 1 1 0 0 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 0 0 1 1

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26. Kan	0 0 0 0 0 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
27. Kan-kiw Dok-khao	0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
28. Meao	0 0 0 0 0 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
29. Laodong	0 0 0 0 0 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
30 Kan-kiw Dok-chom-phu	0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1

3) E_{AAG}/M_{CGC} primer-pair

Cultivar	Scored-AFLP band
1. K187	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 0 1 0 0 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 1 0 1 1 1 1 0
2. K326	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 0 1 0 0 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 1 0 1 1 0 1 0
3. PVH03	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 0 1 0 0 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 1 0 1 1 1 1 0
4. PV09	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 0 1 1 0 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 1 0 0 1 1 1 1
5. B1 special	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 1 0 1 1 1 1 1
6. KY14	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 0 0 0 1 1 1 0
7. HBO04P	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 1 0 1 1 0 1 0
8. TN90	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 0 0 0 1 0 1 1

9. TN97	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 1 1 1 1 1 0 1 0 0 1 0 1 0 1 1 0 0 1 1 0 1 1
10. Samsun	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 1 1 0 0 0 0 0 0 0 1 0 0 0
11. Xanthiyaka	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 1 1 1 0 1 1 1 0 1 1 1 1 0
12. Coker326	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 1 0 1 1 0 1 1 0 1 1 0 1 1 1 0 1 0 0 0 1 1 0 0 0
13. Chorleare1	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 0 1 1 1 0 1 1 1 1 1
14. Nisan	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 0 1 1 1 0 1 1 1 1 0
15. Pasak	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 0 0 0 0 0 0 0 0 0 0 1
16. Padang	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 0 1 0 0 0 1 1 0 0 0
17. Petkhangsink	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 0 1 1 1 0 1 0 0 0 1 1 0 0 0
18. Petmakhuea	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 1 0 0 0 0 0 0 0 0 0 0
19. Yamueang	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 0 1 1 1 0 1 1 1 1 0
20. Linchang	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 0
21. Hangka	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 0 0 0 0 0 0 0 1 0 0 0
22. Yahan	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0
23. Phu	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 0 1 1 0 0 0 1 1 1 1
24. K326 phuen-mueang	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 1 0 1 1 1 0 1 0 0 0 0 1 1 1 0
25. Kariang	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 0 0 1 1 0 1 1 1 1 0

26. Kan	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 0 0 1 1 0 1 0 0 0 0 1 1 1 0
27. Kan-kiw Dok-khao	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 0 1 1 1 0
28. Meao	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 0 0 1 1 0 1 1 1 1 1 1 0 1 0
29. Laodong	1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 0 1 1 1 0 1 1 1 1 1 1 1 0
30 Kan-kiw Dok-chom-phu	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 0 1 1 1 0 0 1 0 0 0 0 0 0 1 0

4) E_{ACT}/M_{CAG} Primer-pair

Cultivar	Scored-AFLP band
1. K187	0 0 0 0 0 0 0 1 0 1 0 1 0 1 1 1 1 1 1 1 1 0 1 1 0 1 1
2. K326	0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 1 1 0 1 1 1 0 1 1 0 1 1
3. PVH03	1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1
4. PV09	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1
5. B1 special	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 0
6. KY14	1 0 0 0 0 1 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 0 1 1 0 1 0
7. HBO04P	1 0 0 0 0 1 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
8. TN90	1 0 0 0 0 1 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 0 1 0

9. TN97	1 0 0 0 0 1 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 0 0 1 0 1 0
10. Samsun	1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0
11. Xanthiyaka	0 0 0 0 1 1 1 0 0 0 0 1 1 0 1 1 1 0 1 1 0 0 0 1 0 1 0
12. Coker326	0 0 0 0 1 1 1 0 0 0 0 1 1 0 1 1 1 0 1 1 0 0 0 1 0 1 0
13. Chorelare1	1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0
14. Nisan	1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0
15. Pasak	1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0
16. Padang	0 0 0 0 1 1 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
17. Petkhangsink	0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
18. Petmakhuea	0 0 0 0 1 1 1 1 0 0 1 1 0 1 1 1 1 0 1 1 1 0 0 1 0 1 0
19. Yamueang	0 0 0 0 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
20. Linchang	0 0 0 0 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
21. Hangka	1 0 0 0 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
22. Yahan	1 0 0 0 1 1 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
23. Phu	1 0 0 0 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
24. K326 phuen-mueang	1 0 0 0 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 1 0
25. Kariang	1 0 0 0 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0

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26. Kan	1 0 0 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0
27. Kan-kiw Dok-khao	0 0 0 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0
28. Meao	0 0 0 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0
29. Laodong	0 0 0 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 0 1 0
30 Kan-kiw Dok-chom-phu	0 0 0 0 1 1 1 1 0 0 1 1 0 1 1 1 1 0 1 1 0 0 0 1 0 1 0

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A2. Scored fragments from AFLP-PCR amplification with four primer-pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) in all 23 local and imported of cured tobacco cultivars.

1) E_{AAG}/M_{CAA}primer-pair

Cultivar	Scored-AFLP band
1. K326	1 1 1 0 1 0 0 1 0 1 0 0 0 0 1 0 0 0 0 0 0 0 1 1 0 0 0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
2. PV09	0 0 1 0 0 0 0 1 1 1 0 0 0 0 1 1 0 0 0 0 0 0 1 1 0 0 0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
3. PVH03	0 1 1 0 0 0 0 1 1 1 0 0 0 0 1 1 0 0 0 0 0 0 1 1 0 0 0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
4. KY14	0 0 1 1 1 0 0 1 1 1 0 0 0 0 1 0 0 0 1 1 1 0 1 1 0 0 0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
5. TN86	0 1 1 0 1 1 0 1 1 0 0 0 0 0 1 1 0 0 1 1 1 0 1 1 0 0 0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
6. TN90	0 1 1 0 1 1 0 1 1 0 0 0 0 0 1 0 0 0 1 1 1 0 1 1 1 0 0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 1 1 1 0 0 0
7. TN97	0 1 0 0 1 1 0 1 1 0 0 1 0 0 1 0 1 1 1 1 1 0 0 1 0 0 0 0 1 1 0 0 1 1 1 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1
8. Samsun	0 0 1 1 1 0 0 1 0 1 1 0 0 0 1 1 0 1 1 1 1 0 1 1 0 1 1 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0
9. Xanthiyaka	1 1 1 1 1 0 0 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0
10. Whitegold	0 1 0 0 0 0 1 1 0 0 1 1 0 0 1 0 0 0 0 0 1 0 1 1 0 0 0 1 1 1 1 1 1 0 1 0 0 0 0 0 0 0 1 1 1 0 0 0 0 0 0
11. K326 phuen-mueang	0 0 0 0 0 0 1 1 0 1 1 0 0 0 1 0 0 0 0 0 1 0 0 0 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 0 1 0 0 1 0 0 1 1 1

12. E-dum	0 0 1 0 1 0 1 1 0 0 0 0 0 1 0 0 0 0 0 0 1 1 0 0 0 0 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
13. E-luaeung	0 1 0 0 0 0 1 0 0 0 1 0 0 0 1 1 1 0 0 0 0 0 0 0 1 0 0 0 0 1 1 1 1 0 0 1 0 0 0 1 1 1 1 0 0 1 0 0 0 0 0
14. Phu	0 0 0 0 0 0 1 1 0 1 0 0 0 0 1 1 0 1 0 0 0 0 1 1 0 0 0 0 0 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
15. Hangkai	0 0 1 0 1 0 1 1 0 1 0 0 0 0 1 0 0 0 1 1 1 0 1 1 0 0 0 0 0 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
16. Ya-glai	1 1 1 0 1 0 1 1 0 1 0 1 0 0 1 1 0 0 1 1 1 0 1 1 0 0 0 0 0 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
17. Bai-lai	1 1 1 0 1 0 0 1 0 1 0 0 1 1 1 1 1 0 1 1 1 0 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 0 0 0 0 0 1 0 0 1 1 1 1 0
18. Kariang	1 1 1 0 1 0 1 1 0 1 0 0 0 0 1 0 0 0 1 1 1 0 1 1 0 1 0 0 0 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
19. Yamueang	1 1 1 0 1 0 1 1 0 1 0 0 0 0 1 1 0 0 1 1 1 0 1 1 0 0 0 0 0 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
20. Kan	0 1 0 0 1 0 0 1 0 1 0 1 0 1 1 0 0 1 1 1 1 0 1 1 0 1 0 0 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
21. Bai-tang	0 0 0 0 0 0 0 1 0 1 0 0 0 0 1 0 0 0 1 1 1 0 0 1 0 0 0 0 0 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
22. Kan-kiw	0 0 0 0 0 0 0 1 0 1 0 0 0 0 1 0 0 0 1 1 1 0 1 1 0 0 0 0 0 1 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
23. Laodong	1 1 1 0 1 0 0 1 0 1 0 0 0 0 1 0 0 0 1 1 1 0 1 1 0 0 0 0 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0

2) E_{AAG}/M_{CAT} primer-pair

Cultivar	Scored-AFLP band
1. K326	1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 0 0 1 1 1 1 0 0 0 1 1 0
2. PV09	1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 0 0 1 1 1 1 0 0 0 1 1 0
3. PVH03	1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 0 0 1 1 1 1 0 0 0 1 1 0
4. KY14	1 0 1 0 0 0 0 0 0 0 0 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0
5. TN86	1 0 1 0 0 0 0 0 0 0 0 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 1 1 1 1 1 1 1 0 0 1 1 0
6. TN90	0 0 1 0 1 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 1 0 1 1 1 1 1 0
7. TN97	1 0 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 0 1 1 0 0 0 0 1 1 1 1 0 0 1 0 0 0 0 1 1 0
8. Samsun	0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 1 0 0 0 0 1 1 0
9. Xanthiyaka	0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 1 1 1 1 1 0 0 0 0 1 1 0
10. Whitegold	1 0 1 0 0 0 1 1 1 0 0 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 1 1 1 1 1 0 0 0 0 1 1 0
11. K326 phuen-mueang	1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 1 1 1 1 0 1 0 0 0 0 1 1 0
12. E-dum	1 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 0 0 0 0 1 1 0 0 1 1 1 0 0 0 0 1 1 0
13. E-luaeung	0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 0 0 1 0 0 0 0 0 1 1 0 0 0 0 1 1 0 0 0 1 1 0
14. Phu	0 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 0 0 1 1 1 0 0 0 0 1 1 0

15. Hangkai	0 0 1 1 1 1 0 0 0 0 0 0 1 1 0 0 0 0 1 1 1 0 0 1 1 1 1 1 1 0 1 0 1 1 1 1 1 0
16. Ya-glai	0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 0 0 1 0 0 0 0 0 1 1 0 1 1 0 1 0 0 0 0 1 1 0
17. Bai-lai	0 0 1 1 1 1 1 1 0 0 0 1 1 0 1 1 1 0 0 1 1 0 0 0 0 1 1 0 1 1 0 1 0 0 0 0 1 1 0
18. Kariang	0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 1 1 1 0 0 0 0 1 1 0 1 1 0 1 0 0 0 0 1 1 1
19. Yamueang	0 0 1 1 1 1 1 1 0 0 0 0 0 1 1 0 1 0 0 1 0 1 1 1 1 1 1 0 1 1 0 1 0 1 1 1 1 1 0
20. Kan	0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 1 0 1 1 0 1 1 0 0 0 1 1 0
21. Bai-tang	0 0 1 1 1 1 0 0 0 0 0 1 1 1 1 0 1 0 0 1 1 0 0 0 0 1 1 0 1 1 1 1 1 0 0 0 1 1 0
22. Kan-kiw	0 0 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 0 0 1 1 0 0 0 0 1 1 0 1 1 1 1 1 0 0 0 1 1 0
23. Laodong	0 0 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 0 0 1 1 0 0 0 0 1 1 0 1 1 1 1 1 0 0 0 1 1 0

3) E_{AAG}/M_{CGC} primer-pair

Cultivar	Scored-AFLP band
1. K326	0101111010101100000000001110000011111010011111111111110110100010000000101011111100001010000000
2. PV09	0101111010101100000000001110000011111011111111111111110110100010000000101011011100001000000000
3. PVH03	0000010000001000000000001110000011010000001110101111101101000100000001010110111000010000000000

4. KY14	00000000000000000000000111111111111101001111011111111010110111110010101011111110001010000000
5. TN86	00010110001001000000000111000101111101001111111110011010100011110010101011111110001000000000
6. TN90	01000100001000000000001000001011000000001100000101011100100111100001010000100000010000000010
7. TN97	00000110101001000011001111111111111101111111111111111111011111111100101011010110101110100000
8. Samsun	00011110001010100110001110011011111101000111111111111111011101111111010101101011110001010011
9. Xanthiyaka	00111111111111101110111000111111110010001111111111111010111010011111100011110000001110101111
10. Whitegold	00010110010100010000000110000111111000110111011011111110001100010000010001110000000010000000
11. K326 phuen-mueang	01011111110001000000000110000111111001010111011111111111010001111000010101101111000001000000000101111111000100000000011000011111100101011101111111111010001111000010101101111000001000000000
12. E-dum	00000110000001010000000000000000100000000000000000000000000000000000010000000000000001000001001
13. E-luaeung	01011110111111011110001110011001010000000111111110100110100011100000101111011110000000000000
14. Phu	00011110000110000110000010011001000000011111000001001000100111111000010001101100000000011100000
15. Hangkai	00000110010100000000000110011101111001001111011110011111010001111001110101101110000000000000000
16. Ya-glai	00111111111111000110000110011101111101001111010111111111100011111010100010110110011000010010
17. Bai-lai	10111111100111000110000110111101111000001111011111111111011001111111010111111011000001000000000
18. Kariang	00000110000000000000000000000000100000000000011000000000110000001100000000000000000000000000000
19. Yamueang	11111111111111000110000110011001111000001111011111111111010011111000110111101111000000000000000

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20. Kan	00011111111101000000000110011001111001001111011111111001000111100001000110111100010000000000
21. Bai-tang	01111111111111010110100110111001111111000111011111111010100111110110111111011100000010000000
22. Kan-kiw	00011111111101010110100110011001111000011111011111111000100011110100100011011110001000000000
23. Laodong	

4) E_{ACT}/M_{CAG} primer-pair

Cultivar	Scored-AFLP band
1. K326	0 0 0 1 1 1 0 0 0 0 0 0 0 0 1 1 1 1 0 1 0 1 0 1 1 1 1 0 0 0 0 0 0 0 1 1 1
2. PV09	1 0 0 1 1 1 0 0 0 0 0 0 0 0 1 1 1 1 0 1 0 1 0 1 1 1 1 0 0 0 0 0 0 0 1 1 1
3. PVH03	0 0 0 1 1 1 0 0 0 0 0 0 0 0 1 1 1 1 0 1 0 1 1 1 1 1 1 0 0 0 0 0 0 0 1 1 1
4. KY14	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 0 1 1 1 0 1 1 1 1 0 0 0 0 0 0 0 1 1 1
5. TN86	0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 1 0 1 1 1 0 1 1 1 1 0 0 0 0 0 0 0 1 1 1
6. TN90	1 1 1 0 0 0 0 0 0 1 0 0 0 1 0 0 1 1 1 1 1 0 0 1 1 1 1 0 0 0 0 0 0 0 1 0 0
7. TN97	0 0 0 0 0 0 0 1 1 0 1 1 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 0 0 0 0 0 1 1 1
8. Samsun	0 0 0 0 0 1 1 1 1 1 1 1 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 0 0 0 0 1 1 1
9. Xanthiyaka	0 0 0 0 0 0 1 1 0 0 1 0 0 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 1 1 0 0 0 0 1 0

10. Whitegold	0 0 0 0 0 0 0 0 1 0 1 1 0 1 0 0 1 1 0 1 1 1 1 1 1 1 1 0 0 0 0 0 0 0 1 1 1
11. K326 phuen-mueang	0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 1 0 1 1 1 0 1 1 1 1 0 0 0 0 0 0 0 0 0 0
12. E-dum	0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 1 0 1 1 0 1 1 1 1 1 0 0 0 0 0 0 0 0 0 1
13. E-luaeung	0 0 0 0 0 0 0 0 0 1 0 1 1 0 0 0 1 1 0 0 0 1 1 0 1 1 1 0 1 0 0 1 0 0 0 1 0
14. Phu	1 0 0 0 0 0 1 1 1 1 1 1 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 1 0 0 1 1 1 1 1 1
15. Hangkai	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 1 0 1 1 0 1 1 1 1 1 0 1 0 0 0 0 0 0 1 0
16. Ya-glai	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 1 0 0 0 0 0 1 0 1
17. Bai-lai	0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 0 1 1 1 0 1 1 1 1 0 1 0 0 0 0 0 0 1 0
18. Kariang	0 0 0 1 1 1 0 0 0 0 1 0 0 0 0 0 0 0 0 1 1 1 0 1 1 1 1 0 1 0 0 0 0 0 0 0 0
19. Yamueang	0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 0 1 1 0 0 0 0 0 0 1 1 1 0 1 0 0 0 0 0 0 0 0
20. Kan	0 0 0 0 1 1 1 1 0 1 1 1 1 0 0 0 1 1 0 1 1 1 0 1 1 1 1 1 1 0 0 0 0 0 0 0 0
21. Bai-tang	0 0 0 0 1 1 0 0 0 1 1 1 1 0 0 0 1 1 0 1 1 1 0 1 1 1 1 0 1 0 0 0 0 0 1 1 1
22. Kan-kiw	0 0 0 0 0 1 0 0 0 1 1 1 1 0 0 0 1 1 0 1 1 1 0 1 1 1 1 0 1 0 0 0 0 0 1 1 1
23. Laodong	0 0 0 0 0 1 0 1 0 1 1 1 1 0 0 0 1 1 0 1 1 1 0 1 1 1 1 0 1 0 0 0 0 0 1 1 1

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A3. Scored fragments from AFLP-PCR amplification with three primer-pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) in all 19 local and imported of cured tobacco cultivars.

1) E_{AAG}/M_{CAA} primer-pair

Cultivar	Scored-AFLP band
1. K326	0 1 0 1 0 1 1 0 0 0 0 1 0 0 0 0 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 0 0 0 1
2. PV09	0 1 0 1 0 1 1 0 0 0 0 1 0 0 0 0 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 0 0 0 1
3. PVH03	0 1 0 1 0 1 1 0 0 0 0 1 0 0 0 0 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 0 0 0 1
4. TN86	0 1 0 1 1 1 0 1 1 0 0 1 1 0 0 0 0 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 1 1 0 1
5. TN90	1 0 0 1 0 1 0 0 0 1 1 1 1 1 1 0 1 1 0 0 1 1 1 1 0 0 1 0 1 1 1 1 1 0 1
6. TN97	0 1 1 1 0 1 0 1 0 0 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 1 0 0 1
7. Samsun	1 1 1 1 1 1 0 1 1 0 0 1 1 0 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 0 0 1
8. Xanthiyaka	1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 1 0 0 0
9. Whitegold	0 1 1 1 0 1 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 1 1 1 1 0 1
10. K326 phuen-mueang	0 1 1 1 0 1 0 1 0 0 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 0 0 1 1 1 1 1 1

11. E-dum	0 1 1 1 0 1 0 1 1 0 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 0 0 1 1 1 1 0 1
12. Phu	1 1 1 1 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 1 1 1 0 0 1
13. Hangkai	1 1 1 1 0 1 0 1 1 1 1 1 1 1 0 0 1 1 1 1 1 0 1 1 1 1 0 1 0 1 1 1 1 1 0
14. Ya-glai	0 1 1 1 0 1 0 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 1 1
15. Bai-lai	0 1 1 1 0 1 0 1 1 0 0 1 0 1 0 0 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 1
16. Kariang	0 1 1 1 0 1 0 1 0 0 0 1 0 1 0 0 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 1
17. Kan	0 1 1 1 1 1 0 0 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 0 1
18. Bai-tang	0 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 0 1
19. Kan-kiw	0 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 0 1

2) E_{AAG}/M_{CGC} primer-pair

Cultivar	Scored-AFLP band
1. K326	1 1 1 0 0 0 0 0 0 0 1 0 0 0 1 0 0 1 1 1 1 0 1 0 1 1 0 1 0 0 1 0 1 1 1 1 0 0 0 0 0 0 1 0 1
2. PV09	1 1 1 0 0 0 0 0 0 0 1 0 0 0 1 0 0 1 1 1 1 0 1 0 1 0 0 1 0 0 1 0 1 1 1 1 0 0 0 0 0 0 1 1 0
3. PVH03	1 1 1 0 0 0 0 0 0 0 1 0 0 0 1 0 0 1 1 1 1 0 1 0 1 0 0 1 0 0 1 0 1 1 1 1 0 0 0 0 0 0 1 1 0

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4. TN86	1 1 1 0 0 1 0 0 0 1 0 1 0 0 0 1 0 1 1 1 0 1 1 1 0 1 0 0 1 0 1 1 0 1 1 1 1 0 0 0 0 0 1 1 1
5. TN90	1 1 1 1 1 0 0 0 1 0 1 0 0 0 1 0 0 1 1 1 0 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 0 1 1 0 0 1 1 1
6. TN97	1 1 0 0 0 1 0 1 1 1 1 0 0 0 1 0 0 1 1 1 1 1 1 0 1 0 0 1 0 1 1 1 1 1 1 1 1 1 0 1 0 0 0 1 1 1
7. Samsun	1 1 0 0 0 1 0 1 1 0 1 0 0 0 1 0 0 1 1 1 1 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 1
8. Xanthiyaka	1 1 1 1 1 1 0 1 1 0 1 0 0 0 1 1 0 1 1 1 1 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1
9. Whitegold	1 0 1 1 1 0 0 0 0 0 1 0 0 0 0 0 1 1 1 1 1 1 1 1 0 1 0 0 1 0 0 1 1 1 1 1 1 0 1 0 1 1 0 1 1 1 1
10. K326 phuen-mueang	1 1 1 0 1 0 0 0 0 0 1 0 0 0 1 0 0 1 1 0 1 1 1 0 1 0 0 1 0 0 1 1 0 1 1 0 1 0 0 1 0 0 1 1 1
11. E-dum	1 1 1 1 1 0 0 0 0 0 1 0 0 0 1 0 0 1 1 1 1 1 1 1 0 1 0 0 1 0 0 1 1 0 1 1 0 1 0 0 1 0 0 1 1 1
12. Phu	1 1 0 0 0 1 1 0 0 0 1 0 0 0 1 0 0 1 1 1 1 0 1 1 1 0 0 1 0 0 1 0 1 1 0 0 1 1 1 0 0 0 1 1 1
13. Hangkai	1 1 1 1 1 1 1 0 0 0 1 1 0 0 1 0 0 1 1 1 1 0 1 1 1 0 0 0 1 1 1 0 0 0 1 1 0 1 1 1 1 1 0 1 0
14. Ya-glai	1 1 1 0 1 1 0 0 0 0 1 1 0 0 0 1 0 1 1 1 1 1 1 1 1 0 0 1 0 0 1 0 0 0 1 0 0 0 0 1 1 1 0 1 1
15. Bai-lai	1 1 1 1 1 0 1 1 1 0 1 1 0 0 1 0 0 0 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 1 0 1 0 0 1 0 1 0 0 0 1 1
16. Kariang	0 1 1 1 0 0 1 1 1 0 1 0 1 1 1 0 0 0 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 1 0 1 0 1 0 0 1 1 1 1 1 0
17. Kan	1 1 1 1 1 1 1 1 1 1 0 1 0 0 0 1 0 0 1 1 1 0 1 1 0 1 0 0 0 1 0 1 0 1 0 1 0 0 0 0 1 0 0 1 1 0
18. Bai-tang	1 1 1 1 1 1 1 0 1 1 0 1 0 0 0 1 0 0 1 1 1 1 1 1 1 0 1 0 0 0 1 0 1 0 1 0 1 0 0 0 0 1 0 0 1 0 1
19. Kan-kiw	1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 0 0 1 1 1 1 1 1 1 0 1 0 0 0 1 0 1 0 0 0 1 0 0 0 0 1 0 0 1 1 1

3) E_{ACT}/M_{CAG} primer-pair

Cultivar	Scored-AFLP band
1. K326	0 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 0 1 0 0 0 0 1 1 1 0 1 1 1 1 1 1 0 0 0 0 0 0 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1
2. PV09	0 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 0 0 0 0 1 1 1 0 1 1 1 1 1 1 0 0 0 0 0 0 1 1 1 0 0 1 1 0 1 1 1 1 0 1 1 1 1 0 1
3. PVH03	0 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 0 0 0 0 1 1 1 0 1 1 1 1 1 1 0 0 0 0 0 0 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1
4. TN86	0 0 0 0 0 0 0 0 1 0 1 0 0 0 1 0 0 0 0 1 0 0 0 1 0 1 1 0 0 1 1 1 1 1 1 1 1 0 1 0 0 1 1 0 0 0 1 1 1 1 0 1 0 0 0 1 0 1 0 0
5. TN90	0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 1 0 1 0 0 1 0 1 1 1 0 0 1 1 1 1 1 0 1 0 1 1 1 0 1 0 0 0 1 1 0 1 0 0 0 0 0 0 0 0 0
6. TN97	0 0 0 0 0 0 0 0 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 0 1 0 1 0 0 0 1 1 0 0 1
7. Samsun	0 0 0 0 0 0 1 1 1 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 0 1 0 0 0 0 1 1 1 0 0 1
8. Xanthiyaka	0 0 0 0 0 1 1 0 1 0 1 1 1 1 0 1 0 0 0 1 0 0 0 0 1 1 1 0 1 0 1 1 1 1 1 0 0 1 1 1 1 0 0 0 1 0 1 0 1 0 0 0 1 0 0 0 1 0 0
9. Whitegold	0 0 0 0 0 0 0 1 0 0 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 0 1 1 1 1 1 1 1 0 1 0 1 0 0
10. K326 phuen-mueang	0 0 0 0 0 0 1 1 1 0 1 1 1 1 1 1 0 0 0 1 0 0 0 0 1 0 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 1 1 1 0 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1
11. E-dum	0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 0 1 1 1 0 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 1 0 1 0 0 1 1
12. Phu	0 0 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0 1 1 1
13. Hangkai	0 0 0 0 1 0 0 0 0 1 0 1 1 1 1 0 0 0 0 0 0 1 1 0 0 1 1 1 1 1 0 0 0 1 0 0 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 1 1 1 1 0 0 0 0 0 0 0 1 0 1 0 1

14. Ya-glai	0 0 0 0 0 0 0 1 1 1 1 1 1 1 0 1 1 1 1 1 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 0 1 0 1 0 1 0 0 0 1 0 0 1 0
15. Bai-lai	0 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 0 0 0 0 0 1 0 0 0 0
16. Kariang	0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 1 1 0 1 0 1 1 1 0 0 0 1 1 0 1 1
17. Kan	1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 0 0 0 1 1 1 1 1
18. Bai-tang	0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 0 1 1 0 0 1 1 1 1 1 0 1
19. Kan-kiw	0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 1 1 1 0 1 1 1

APPENDIX B

B1. Distance metric using Nei and Li's similarity coefficient of 20 cured-leaf samples

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. K326																				
2. PVH03	0.007																			
3. PV09	0.019	0.016																		
4. KY14	0.044	0.047	0.047																	
5. TN86	0.039	0.040	0.048	0.021																
6. TN97	0.072	0.071	0.085	0.050	0.053															
7. Samsun	0.055	0.057	0.064	0.052	0.058	0.045														
8. Xanthiyaka	0.062	0.070	0.078	0.067	0.069	0.058	0.034													
9. Whitegold	0.067	0.068	0.067	0.061	0.060	0.064	0.067	0.060												
10. K326 pkuen-mueang	0.053	0.052	0.064	0.061	0.063	0.054	0.054	0.061	0.058											
11. E-dum	0.054	0.056	0.058	0.056	0.060	0.055	0.056	0.065	0.052	0.049										
12. Phu	0.053	0.050	0.061	0.081	0.073	0.073	0.049	0.061	0.067	0.061	0.049									
13. Hangkai	0.099	0.098	0.098	0.079	0.081	0.102	0.078	0.083	0.091	0.089	0.078	0.072								
14. Ya-ghai	0.065	0.069	0.065	0.041	0.038	0.067	0.065	0.065	0.058	0.063	0.050	0.058	0.062							
15. Bia-lai	0.063	0.069	0.080	0.064	0.059	0.057	0.052	0.044	0.081	0.049	0.064	0.060	0.070	0.051						
16. Kariang	0.050	0.060	0.066	0.058	0.064	0.067	0.046	0.045	0.069	0.051	0.048	0.054	0.068	0.046	0.036					
17. Kan	0.051	0.055	0.064	0.066	0.065	0.062	0.046	0.047	0.072	0.047	0.046	0.035	0.064	0.048	0.039	0.027				
18. Bai-tang	0.060	0.059	0.073	0.057	0.056	0.067	0.059	0.070	0.068	0.059	0.061	0.050	0.065	0.046	0.056	0.049	0.036			
19. Laodong	0.048	0.049	0.063	0.051	0.057	0.063	0.044	0.050	0.066	0.053	0.048	0.033	0.065	0.050	0.047	0.034	0.025	0.034		
20. Kan-kiw	0.047	0.050	0.059	0.054	0.051	0.058	0.047	0.048	0.060	0.052	0.045	0.036	0.057	0.045	0.038	0.034	0.024	0.027	0.018	

B2. Distance metric using Nei and Li's similarity coefficient of 19 cured-leaf samples

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. K326																			
2. PV09	0.004																		
3. PVH03	0.004	0.002																	
4. TN86	0.056	0.054	0.056																
5. TN90	0.073	0.075	0.077	0.054															
6. TN97	0.045	0.043	0.045	0.040	0.052														
7. Samsun	0.056	0.057	0.059	0.051	0.051	0.018													
8. Xanthiyaka	0.061	0.065	0.064	0.056	0.054	0.042	0.033												
9. Whitegold	0.052	0.053	0.052	0.049	0.053	0.034	0.041	0.047											
10. K326 pkuen-mueang	0.040	0.041	0.037	0.049	0.067	0.035	0.038	0.044	0.029										
11. Edum	0.046	0.046	0.043	0.049	0.055	0.029	0.036	0.042	0.019	0.018									
12. Phu	0.060	0.058	0.054	0.066	0.073	0.041	0.037	0.047	0.042	0.048	0.035								
13. Hangkai	0.105	0.099	0.101	0.096	0.073	0.078	0.076	0.065	0.069	0.073	0.060	0.042							
14. Ya-glai	0.059	0.057	0.056	0.063	0.076	0.042	0.049	0.054	0.031	0.037	0.026	0.040	0.065						
15. Bia-lai	0.060	0.058	0.060	0.063	0.063	0.040	0.039	0.052	0.037	0.043	0.028	0.042	0.055	0.031					
16. Kariang	0.058	0.053	0.053	0.074	0.077	0.042	0.047	0.061	0.043	0.040	0.033	0.045	0.069	0.039	0.024				
17. Kan	0.053	0.049	0.048	0.068	0.060	0.042	0.045	0.056	0.041	0.038	0.029	0.039	0.054	0.040	0.020	0.021			
18. Bai-tang	0.042	0.045	0.045	0.059	0.065	0.037	0.031	0.048	0.032	0.037	0.027	0.032	0.067	0.035	0.028	0.033	0.019		
19. Laodong	0.051	0.052	0.048	0.068	0.066	0.043	0.041	0.051	0.039	0.040	0.030	0.033	0.067	0.040	0.035	0.038	0.022	0.013	

APPENDIX C

C. Manuscript presented in the 3rd Biotanical Conference of Thailand, 25th-27th March, Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand.

การพัฒนาเครื่องหมายโมเลกุลแบบเอเอฟแอลพีเพื่อการตรวจสอบสายพันธุ์ยาสูบ

DEVELOPMENT OF AFLP MOLECULAR MARKER FOR VARIETY IDENTIFICATION OF TOBACCO

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บทคัดย่อ

ยาสูบเป็นพืชอีกชนิดหนึ่งที่มีความสำคัญอย่างมากต่อเศรษฐกิจของประเทศไทยโดยเฉพาะอย่างยิ่งในอุตสาหกรรมการผลิตบุหรี่ ยาสูบที่มีการเพาะปลูกในประเทศไทยนั้น แบ่งแยกออกเป็นสองกลุ่มใหญ่ คือ พันธุ์ยาสูบพื้นเมืองและพันธุ์ยาสูบนำเข้าจากต่างประเทศ อย่างไรก็ตาม วิธีการในปัจจุบันที่ใช้แยกแยะความแตกต่างระหว่างสายพันธุ์ยาสูบยังไม่มีความแม่นยำเพียงพอ โดยเฉพาะอย่างยิ่งในใบยาสูบบ่มแห้งแล้ว ในการศึกษาครั้งนี้จึงได้นำเอาเทคนิคเอเอฟแอลพีมาใช้ในการศึกษาความแตกต่างทางพันธุกรรมระหว่างสายพันธุ์ยาสูบที่ปลูกในประเทศไทย ผลการทำเอเอฟแอลพีพบว่ามีไพรเมอร์สี่คู่ (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} และ E_{ACT}/M_{CAG}) ที่มีความเหมาะสมที่สุดในขั้นการเพิ่มปริมาณเอเอฟแอลพีพีซีอาร์แบบคัดเลือก โดยที่คู่ไพรเมอร์ E_{AAG}/M_{CGC} ให้จำนวนของผลิตภัณฑ์เอเอฟแอลพีพีซีอาร์มากที่สุดและเกิดรอยเปื้อนน้อยกว่าคู่ไพรเมอร์อื่น แผนภูมิแสดงความสัมพันธ์ทางพันธุกรรมทั้งแบบเอ็นเจและยูพีจีเอ็มเอให้ผลการจัดกลุ่มพันธุ์ยาสูบที่คล้ายกัน โดยที่สายพันธุ์นำเข้าเกือบทั้งหมดถูกจัดกลุ่มอยู่ด้วยกัน อย่างไรก็ตาม ยาสูบสายพันธุ์นำเข้า 3 สายพันธุ์ (Coker326, Samsun และ Xanthiyaka) ได้ถูกจัดรวมกลุ่มกับยาสูบสายพันธุ์พื้นเมือง จากการศึกษาเบื้องต้นในใบยาสูบสดนี้แสดงให้เห็นว่าเครื่องหมายโมเลกุลเอเอฟแอลพีมีศักยภาพสำหรับการพัฒนาเป็นเครื่องมือสำหรับใช้ศึกษาความแตกต่างทางพันธุกรรมระหว่างยาสูบสายพันธุ์พื้นเมืองของไทยและสายพันธุ์นำเข้าได้ต่อไป ในขณะนี้กำลังอยู่ในระหว่างการปรับปรุงปฏิกิริยาพีซีอาร์ให้มีความเหมาะสมในการศึกษาตัวอย่างใบยาสูบบ่มแห้ง

คำสำคัญ: การตรวจสอบสายพันธุ์ เครื่องหมายโมเลกุลแบบเอเอฟแอลพี ประเทศไทย ยาสูบ

Abstract

Tobacco is one of important economic crops in Thailand, especially in cigarette industry. Tobacco varieties grown in Thailand are separated to 2 major groups: local and imported varieties. However, current approaches to distinguish tobacco varieties are not accurate enough, particularly for dry-cured tobacco. In this study AFLP molecular marker was introduced to investigate genetic differences between tobacco varieties grown in Thailand. The AFLP result showed that four primer pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) could be the most suitable primers for selective AFLP-PCR amplification step. Among these primers, E_{AAG}/M_{CGC} primer gave the highest number of AFLP-PCR fragments with less smeared background than the others. Genetic relationship trees based on NJ and UPGMA techniques revealed similar clusterings. Almost all imported varieties were clustered together. However, 3 imported varieties (Coker326, Samsun and Xanthiyaka) were grouped with the local varieties. This preliminary study using fresh leaf materials suggested that AFLP molecular marker method has potential to be further developed as tool for the study of genetic differences between Thai local and imported tobacco varies. At the moment, we are improving the PCR condition which would be suitable for dry-cured leaf samples.

Keywords: Variety Identification, AFLP Molecular Marker, Thailand, Tobacco

Introduction

Tobacco has been cultivated for thousands of years and has served as a raw material for cigarette and cigar industries of many countries. Almost all of the commercial tobaccos produced in the world are *Nicotiana tabacum*. It is also one of the most important crops of Thailand. Tobacco was first introduced into Thailand in the 16th century. By Thai law, all tobacco varieties cultivated in Thailand are defined to two variety-groups: local varieties and imported varieties (Virginia, Burley & Turkish sub groups) varieties. This variety separation leads to some differences in tariff-collecting and crop-growing regulations between the local and imported tobacco varieties. Moreover, if tobacco leaves from either of the two variety groups are dry-cured, there would not be any accurate approach to distinguish whether the leaves are of the local or

imported varieties. From such problem, some molecular markers have been introduced to determine genetic differences between tobacco varieties (for example, Del Piano *et al*., 2000; Rossi *et al*., 2000; and Zhang *et al*., 2006). Molecular markers (or genetic markers) have become useful tools to provide a relatively unbiased estimation of genetic diversity in plants (Clegg, 1997). The genetic relationship studies of Thai tobacco varieties are so important because there had not been such genetic information available before; even though many different commercial varieties were developed in Thailand. Recently, our group has successfully developed ISSR (Inter-Simple Sequence Repeat) molecular markers to distinguish some local tobacco varieties which might have been cultivated in Thailand for a long time (Denduangboripant *et al*., 2008). However, these ISSR makers were unfortunately suitable only for fresh leaf samples, not dry-cured tobacco leaves.

AFLP (Amplified Fragment Length Polymorphism) is another molecular marker which has been successfully used in polymorphism analysis, crop cultivar identification, and phylogenetic evaluation. The AFLP technique is also a favourable method for cultivated crops like tobacco cultivars in which the genetic distances are too small for some molecular marker techniques. The AFLP technique is based on the PCR (Polymerase Chain Reaction) amplification of a subset of genomic restriction fragments. The AFLP system is composed of four successive enzymatic-reaction steps: digestion of DNA with restriction endonucleases, ligation of two specific adapters to the restriction fragments, pre-selective PCR amplification, and selective PCR amplification. The reason why AFLP has been extensively used in genetic diversity analyses of crop plants is its maximum coverage of the whole-genome in a short time. Generally, AFLP produced more polymorphic loci per primer in any diversity study than RFLP, SSR or RAPD techniques (Bogani *et al*., 1997).

Previously, Rossi *et al.* (2000) studied AFLP markers of tobaccos and found that AFLP appeared to be an appropriate technique for genetic fingerprinting of both fresh and processed tobacco leaves. Another AFLP analysis of genetic polymorplisms and evolutionary relationships among cultivated and wild *Nicotiana* species showed that the

genetic polymorphism presenting among cultivated tobacco lines (*N. tabacum*) was limited (Ren and Timko, 2001). In addition, AFLP genetic-diversity study among flue-cured tobacco (*N. tabacum*) revealed that the flue-cured tobacco germplams commonly grown in China have narrow genetic diversity among the cultivars (Zhang *et al*., 2006). In 2008, Siva *et al.* studied genetic polymorphism of Indian tobaccos using AFLP and found that the cultivated flue-cured varieties were clustered separately from the air-cured type. The AFLP markers of Siva *et al.* were also found being specific to some true hybrid varieties and can be used in a genotypic identification in trades and commerces. From these reviewed literatures and the fact that no genetic analysis of Thai tobacco varieties using AFLP has been done before, in this study we then introduced AFLP molecular marker technique to examine genetic differences between local and imported tobacco varieties grown in Thailand.

Materials and methods

Plant materials

Fresh leaf specimens of nine imported and 13 local varieties were sampled in the crop fields from 10 different provinces around Thailand (Table 1) with the help of Thailand Tobacco Monopoly, Ministry of Finance. Additionally, three imported and five local varieties were obtained from the cultivating greenhouse of Maejo Tobacco Experiment Station, Chiang Mai province (Table 1). For cured leaf samples, six imported and 14 local varieties were collected from 10 different provinces and three imported varieties were obtained from Maejo Tobacco Experiment Station, Chiang Mai province (Table 1). All imported tobacco varieties were separated into three sub groups (Virginia, Burley and Turkish). These varieties were classified by methods of curing. (Virginia: flue curing, Burley: light air curing, and Turkish: sun curing). The tobacco leaves were kept separately in silica gel bags and stored at room temperature until used for genomic DNA preparation.

Genomic DNA Extraction

The leaf samples were ground to fine powder with liquid nitrogen and total genomic DNA was extracted using Plant Genomic DNA Mini kit (Geneaid, Taiwan) following an instruction of the manufacturer. The concentration of the extracted DNA was estimated on 0.8% agarose gel electrophoresis using 100 bp ladder as a standard DNA marker. The extracted genomic DNA was maintained at -20°C until used.

PCR-AFLP marker

The AFLP marker amplification was performed based on the protocol of Vos *et al.* (1995) with some modification. The extracted genomic DNA (approximately 250 ng) was digested completely with *Eco*RI and *Tru*9I restriction enzymes in a total volume of 25 µl. *Tru*9I is an isoschizomer of *Mse*I. *Eco*RI and *Mse*I oligonucleotide adapters were subsequently ligated to the digested DNA fragments. *Eco*RI primers were 5'-GAC TGC GTA CCA ATT C-3' and *Mse*I primers were 5'-GAT GAG TCC TGA GTA A-3'. The pre-amplification step was first carried out using adapter-specific primers with a single selective nucleotide on each primer: *Eco*RI primer (E_A) and *Mse*I primer (M_C). In pre-selective amplification, the following cycling parameters were employed: 20 cycles of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. The pre-amplified DNA was used as a template for selective amplification using *Eco*RI and *Mse*I adapter-specific primers with three selective nucleotides on each primer (+3 primers). On this step, thirty-two selective AFLP primer-pairs were screened whether any of them could produce polymorphic band from "all" tobacco samples. The cycling parameters of this step were: the first cycle of 30 s at 94°C, 30 s at 65°C and 60 s at 72°C, and lowering the annealing temperature by 0.7°C per cycle for another 11 cycles, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. The AFLP amplified products were separated by electrophoresis on 6% denaturing polyacrylamide gel containing 7M urea. The separated AFLP-PCR products were visualised by silver staining. Sizes of the fragments were estimated using 50 bp and 100 bp DNA ladder markers.

Table 1 Tobacco varieties used in this study

Fresh leaf samples			Cured leaf samples		
Variety name	Variety group	Area of collection (province)	Variety name	Variety group	Area of collection (province)
K187	Imported(Virginia)	Nakhon Phanom	K326	Imported(Virginia)	Lamphun
K326	Imported(Virginia)	Lamphun	PVH03	Imported(Virginia)	Phayao
PVH03	Imported(Virginia)	Phayao	PV09	Imported(Virginia)	Chiang Rai
PV09	Imported(Virginia)	Chiang Rai	KY14	Imported (Burley)	Sukhothai
Coker326	Imported (Burley)	Phrae	Samsun	Imported(Turkish)	Nakhon Phanom
B1 special	Imported (Burley)	Sukhothai	Xantiyaka	Imported(Turkish)	Nakhon Phanom
KY14	Imported (Burley)	Sukhothai	TN86	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
Samsun	Imported (Turkish)	Nakhon Phanom	TN97	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
Xantiyaka	Imported(Turkish)	Nakhon Phanom			
HBO04P	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai			
TN90	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai			
TN97	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai			
Yamueang	Local	Phayao	K326 phuen-mueang	Local	Nong Khai
K382-phuenmueang	Local	Nong Khai	E-dum	Local	Phetchabun
Kariang	Local	Kanchanaburi	Kariang	Local	Kanchanaburi
Kan	Local	Suphan Buri	Kan	Local	Suphan Buri
Kan-kiw Dok-chom-phu	Local	Suphan Buri	Kan-kiw	Local	Suphan Buri
Kan-kiw Dok-khao	Local	Suphan Buri	Laodong	Local	Suphan Buri
Laodong	Local	Kanchanaburi	Hangkai	Local	Phayao
Meao	Local	Kanchanaburi	Phu	Local	Nong Khai
Hangkai	Local	Phayao	Ya-glai	Local	Nakorn Si Thammarat

Phu	Local	Nong Khai	Bai-lia	Local	Lop Buri
Yahan	Local	Nakorn Phanom	Bia-tang	Local	Lop Buri
Petkhangsing	Local	Sukhothai			
Petmakhuea	Local	Sukhothai			
Padang	Local	Maejo Tobacco Experiment Station, Chiang Mai			
Pasak	Local	Maejo Tobacco Experiment Station, Chiang Mai			
Linchang	Local	Maejo Tobacco Experiment Station, Chiang Mai			
Chorlare	Local	Maejo Tobacco Experiment Station, Chiang Mai			
Nisan	Local	Maejo Tobacco Experiment Station, Chiang Mai			

AFLP Data analysis

Only the bright, clearly-resolved AFLP fragments generated from each primer combination were scored for presence (1) or absence (0) of the bands of the 30 tobacco varieties. Nei and Li's coefficient analysis (1979) was used to calculate pairwise band similarity values of the samples using program PAUP* 4.0b10. Cluster analysis and dendrogram construction were performed using Unweighted Pair Group Method of Arithmatric Mean (UPGMA) and Neighbor-Joining (NJ) methods. Reliability of the clusters was estimated by bootstrap analysis with 1,000 replications.

Results

AFLP-PCR amplification

From our preliminary AFLP study on fresh leaf samples of 30 tobacco varieties, thirty-two selective AFLP primer-pairs were screened against all tobacco samples. Only four pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) could be suitably used as +3 primers in the selective AFLP-PCR step. The sizes of bands amplified by each primer ranged from 150 to 700 basepairs (bp). In the case of cured leaf samples of 23 tobacco varieties, the fingerprinting results provided a total number of 219 AFLP fragments from the combination of the four primers with an average of 54 fragments per primer combination. The size of PCR products ranged from 100-900 bp. The average of AFLP polymorphism degree were 44 polymorphic loci per primer. There were totally 177 (80.2%) amplified polymorphic bands and 42 (19.2%) monomorphic bands (Table 2). The average polymorphic percentage was 81.5%. The highest number of the amplified fragments was by the primer pair E_{AAG}/M_{CGC} (92 bands), which also having less smeared background than the others (Fig.1). The lowest number of the amplified fragments was by the E_{ACT}/M_{CAG} pair (37 bands). The primer combination E_{AAG}/M_{CAA} gave the highest polymorphism (86.9%) across all varieties, whereas the E_{ACT}/M_{CAG} pair gave the lowest (67.6%) score.

Table 2 Number of bands and degrees of polymorphism revealed by combined AFLP results of 23 cured-leaf samples.

Primer combinations	Total bands	Polymorphic bands	% Polymorphism
E_{AAG}/M_{CAA}	51	41	80.3
E_{AAG}/M_{CAT}	39	29	74.4
E_{AAG}/M_{CGC}	92	82	86.9
E_{ACT}/M_{CAG}	37	25	67.6
Total	219	177	80.2
Average	54	44	81.5

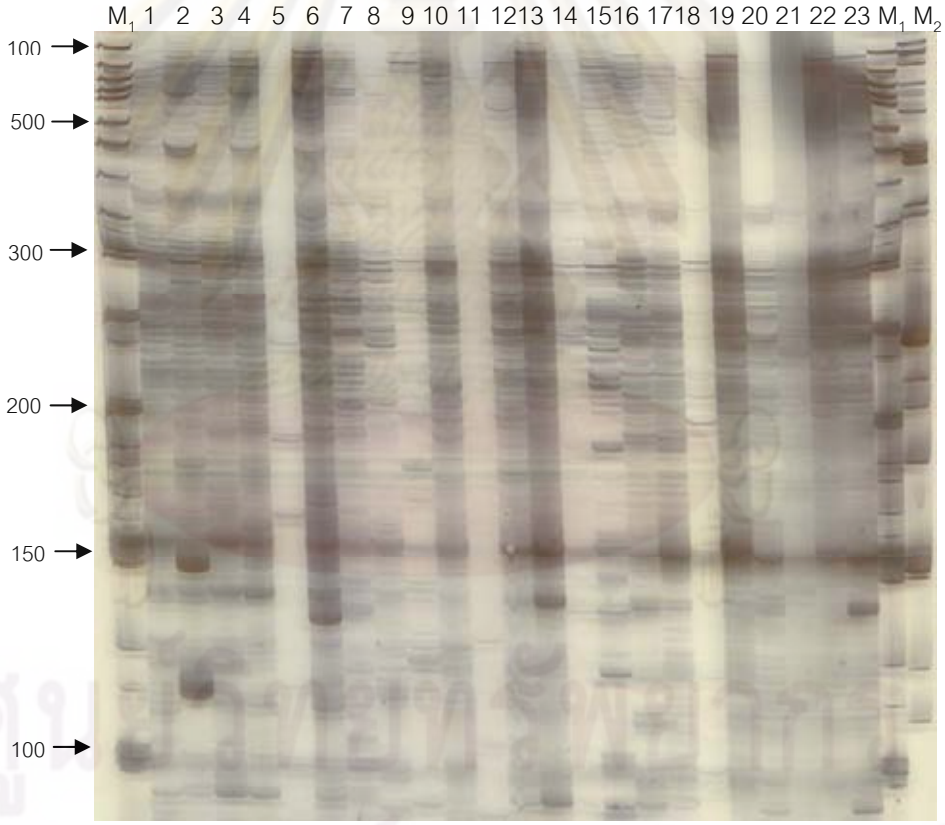

Fig. 2 An AFLP pattern of cured leaf samples of 23 tobacco varieties using E_{AAG}/M_{CGC} selective primers (lane no. 1-23 = tobacco varieties : Kan-kiw, Laodong, Bai-tang, Kan, Yamueang, Kariang, Bia-lai, Klai, Hangkai, Phu, E-lueang, E-dum, K326 phun-mueang, White gold, Xanthiyaka, Samsun, TN97, TN90, TN86, KY14, PV09, PVH03 and K326, respectively. 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2, respectively) were used.

Genetic relationship analyses

Since there were three tobacco varieties (Yamueang, E-lueang, and TN90; lane 5, 11 and 18 in Fig. 1, respectively) which gave only fainted bands on all four AFLP acrylamide gels of the cured-leaf samples, they were exclude from the genetic relationship analyses. Neighbor-joining (NJ) and UPGMA analyses gave similar groupings of tobacco varieties on both genetic tree diagrams. The NJ tree (Fig. 2) revealed that nine of 12 local varieties were grouped together, separating from the imported varieties. The other three local varieties (White gold, E-dam, and K326 phuen-mueang) were clustered together and placed closely to the imported varieties. Within the group of local varieties, some subgroupings were formed possibly following their cultivated areas. For example, most of the local varieties from the central region of Thailand (Bai-tang, Kan, Kan-kiw, and Laotong) were clustered together while some Northeastern local varieties were grouped in another cluster. Hangkai local variety was apparently distinguished from the other local and imported varieties. This finding was the same as that in the UPGMA tree (data not shown).

Among the imported varieties, their clusterings were based mostly on sub groups (Virginia, Burley, and Turkish) and cultivated areas. All three Virginia varieties (K326, PVH03 and PV09) were distinguished from the other five imported varieties with 100% bootstrap supporting values. Two Burley varieties (KY14 and TN86) were paired together with high bootstrap percentage (99%) too. Both Turkish varieties (Samsun and Xanthiyaka) were closely grouped together with 74% bootstrap support, but also had TN97 of Burley varieties placed near them.

Discussions

Although tobacco is one of important economic crops in the world, only a few studies of genetic diversity and polymorphisms of tobacco varieties have been done before. For instance, preliminary analysis of a genetic diversity in 12 varieties of *N. tabacum* was done using three random RAPD primers (Del Piano *et al*., 2000). AFLP technique has also been used to analyse genetic diversity of tobacco (Ren and Timko, 2001; Rossi *et al*., 2000; Zhang *et al*., 2006; and Siva *et al*., 2008).

Our preliminary AFLP study using fresh leaf samples showed that four selective primer pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) gave clearer amplified bands than the other tested primers. Nevertheless, this acrylamide gel results still showed a lot of smeared background. Therefore, the AFLP-PCR condition was improved to give a clearer fragment profile. For example, we found that an optimization of the template concentration could give clearer fragment profiles and higher number of scorable AFLP fragments because it decreased the smeared background.

In this study, a total of 219 AFLP amplified bands were obtained from cured leaf samples of all 23 tobacco varieties growing in Thailand. Our average number of AFLP fragments per primer was 54. This AFLP study therefore gave higher number of bands than the previous AFLP report of Ren and Timko (2001) which were 92 bands from 46 cultivated tobacco accessions. In addition, Siva *et al*. (2008) and Zhang *et al*. (2006) produced 107.4 and 249 AFLP-PCR fragments of tobacco varieties per primer, respectively. These are much higher than all of previous RAPD results and clearly indicate a greater power of AFLP analysis. For instance, RAPD study of Del Piano *et al*. (2000) could detect only 8.15 fragments per primer (106 RAPD fragments in 20 tobacco lines).

Our NJ and UPGMA trees from AFLP analyses of cured-leaf samples of 20 tobacco varieties revealed genetic relationships among them that most of the local varieties in Thailand were closely related with each other. The subgroupings of the local varieties tend to follow their cultivated areas. Likewise, the imported varieties were clustered together mostly based on sup groups of varieties (Virginia, Burley and Turkish). There are some previous studies reporting similar results. For example, Ren and Timko (2001) reported that cultured tobacco groups could be divided primarily based on geographic origins and manufacturing quality traits. In their study, polymorphism among cultivated tobacco lines was also limited as evidenced by the high degree of similarity. This agrees well with our result as one of Burley varieties (TN97) were placed beside the pair of Turkish (Samsun and Xanthiyaka) on the NJ tree. Moreover, three local varieties (E-dum, K326 phuen-mueang, and White gold) were found closely related to this Turkish and Burley cluster. We suspect that these three

local tobacco varieties somehow may have been originated from imported varieties, either Burley or Turkish.

Interestingly, the three Virginia varieties were distantly separated from other tobacco varieties especially all local varieties. Therefore, this finding has demonstrated that AFLP marker technique could be used as a powerful tool to separate Virginia varieties from other imported and local varieties grown in Thailand. Our results also agree well with the AFLP analysis of Siva *et al*. (2008). Their cultivated flue-cured (Virginia) varieties were clustured separately from air-cured (Burley) varieties. Therefore, we suggest to implement AFLP technique as an effective polymorphism analysis for closely-related tobacco cultivars such as those grown in Thailand.

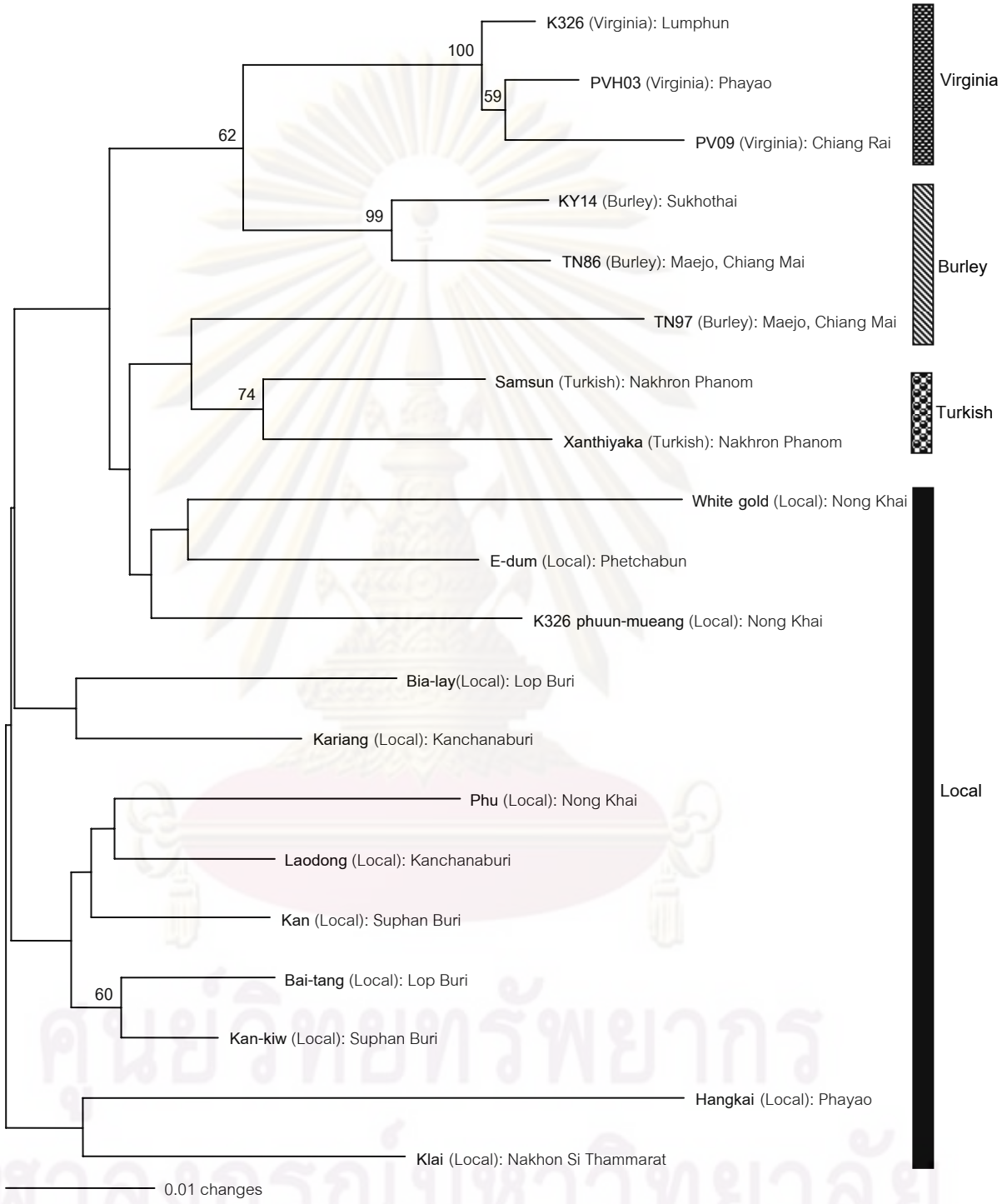

Fig. 3 A genetic relationship tree from total AFLP fragment data of cured leaf samples based on Nei and Li's similarity coefficient using Neighbour-Joining (NJ) methods. Numbers along branches are %bootstrap-supporting values (only >50%) generated after 1,000 replications.

BIOGRAPHY

Miss Tianrat Piteekan was born on June 26th, 1982 in Nakhon Sri Thammarat province. She completed her high school level from Triam Udom Suksa School of the South, Nakhon Sri Thammarat, in 2000. She received bachelor degree of science in applied biology (biology) from Department of Biology, Faculty of Science, Chandrakasem Rajabhat University, Bangkok, in 2004. She has taken Master degree of Science in the programme of Biotechnology, Chulalongkorn University, since 2007.

Research presentation

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