CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Totally 70 tobacco samples comprised of 43 fresh leaf samples (27 local, 14 imported and two unknown tobacco cultivars), 24 cured leaf samples (14 local and 10 imported cultivars) and three roll-your-own (RYO) tobacco products were used in this research. For fresh leaf samples (Table 3.1 and 3.2), 34 samples were collected from many cultivation crops around Thailand (Figure 3.1): North (Chiang Mai, Chiang Rai, Phayao, Phrae, Lamphun and Nan provinces), Northeast (Nong Khai, Nakhon Phanom and Ubon Ratchathani), West (Kanchanaburi), Central (Phetchabun, Sukhothai, Suphan Buri and Lop Buri) and South (Nakhon Si Thammarat) during December 10th, 2007 - February 25th, 2008 with help of Thailand Tobacco Monopoly staff, Ministry of Finance. Field expeditions to collect tobacco leaf samples were done with help from staff of provincial tobacco stations. Photographs of each tobacco cultivar were taken. Their leaf samples were cut to small pieces (approximately 3x3 cm^2) and kept separately in silica gel bags for further molecular analysis (Figure 3.2).

Moreover, nine fresh leaf samples were taken from a greenhouse of Maejo Tobacco Experiment Station, Chiang Mai (Table 3.1). In this case, seeds of tobacco cultivars were planted in seed beds and fresh leaves of young plants were sampled when they were two weeks old. The collected leaves were kept in silica gel bags before used. For cured leaf samples (Table 3.3), 21 specimens were obtained from many provinces around Thailand. Another three samples - TN86, TN90 and TN97 - were taken from Maejo Tobacco Experiment Station. Three roll-your-own tobacco products were bought from rural groceries to represent processed tobacco leaves in this research (Table 3.4).

Table 3.1 Imported and local tobacco fresh-leaf samples were collected from many cultivation crops around Thailand and used in this study.

Cultivar name	Cultivar group	Collecting locality (district/province)
Samsun	Imported (Turkish)	That Phanom, Nakhon Phanom
Xanthiyaka	Imported (Turkish)	That Phanom, Nakhon Phanom
K187	Imported (Virginia)	That Phanom, Nakhon Phanom
PVH03	Imported (Virginia)	Pong, Phayao
K326	Imported (Virginia)	Ban Thi, Lamphun
PV09	Imported (Virginia)	Thoeng, Chiang Rai
Coker326	Imported (Virginia)	Rong Kwang, Phrae
B1 special	Imported (Burley)	Si Samrong, Sukhothai
KY14	Imported (Burley)	Si Samrong, Sukhothai
HB01	Imported (Burley)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
HBO04P	Imported (Burley)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
TN90	Imported (Burley)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
TN97	Imported (Burley)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
K190	Imported (Virginia)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
Chorlare1	Local	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
Chorlare2	Local	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
Padang	Local	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
Linchang	Local	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
Yamueang	Local	Pong, Phayao
Hangkai	Local	Pong, Phayao
Dongdang1	Local	Wiangsa, Nan
Dongdang2	Local	Wiangsa, Nan
Petkhangsink	Local	Mueang, Sukhothai
Petmakhuea	Local	Mueang, Sukhothai
Nisan	Local	Si Samrong, Sukhothai
E-dum	Local	Lom Sak, Phetchabun
Kan	Local	Dan Chang, Suphan Buri
Kan-kiw dok-chom-phu	Local	Nhong Yasai, Suphan Buri
Kan-kiw dok-khao	Local	Nhong Yasai, Suphan Buri
Kariang	Local	Mueang, Kanchanaburi
Laodong	Local	Mueang, Kanchanaburi

Meao	Local	Mueang, Kanchanaburi
Ya-glai	Local	Tha Sala, Nakhon Si Thammarat
Nakhon Si Thammarat	Local	Chulabhorn, Nakhon Si Thammarat
White gold	Local	Tha Bo, Nong Khai
Yahan	Local	Tha Bo, Nong Khai
Phu	Local	Mueang, Nong Khai
K326 local	Local	Mueang, Nong Khai
Napanang	Local	That Phanom, Nakhon Phanom
E-bit	Local	That Phanom, Nakhon Phanom
Ubon Ratchathani	Local	Mueang, Ubon Ratchathani

Table 3.2 Unknown tobacco fresh-leaf samples used in this study.

Cultivar name	Collecting locality (district/province)	Morphological characteristics	Similar to
Unknown1	Song, Phrae	The plant aged about 3-4 months. Young leaf samples grew on the top of the plant. Leaf shape was undulate and lanceolate with approximately 15 inches long.	Virginia
Unknown2	Song, Phrae	The plant aged about 3-4 months. Leaves were lanceolated with approximately 17-18 inches long.	Burley

Table 3.3 Imported and local tobacco cured-leaf samples used in this study.

Cultivar name	Cultivar group	Collecting locality (district/province)
Samsun	Imported (Turkish)	That Phanom, Nakhon Phanom
Xanthiyaka	Imported (Turkish)	That Phanom, Nakhon Phanom
K187	Imported (Virginia)	That Phanom, Nakhon Phanom
PVH03	Imported (Virginia)	Pong, Phayao
K326	Imported (Virginia)	Ban Thi, Lamphun
PV09	Imported (Virginia)	Thoeng, Chiang Rai
KY14	Imported (Burley)	Si Samrong, Sukhothai
TN86	Imported (Burley)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
TN90	Imported (Burley)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai

TN97	Imported (Burley)	Maejo Tobacco Experiment Station, San Sai, Chiang Mai
Hangkai	Local	Pong, Phayao
White gold	Local	Tha Bo, Nong Khai
Phu	Local	Mueang, Nong Khai
K326 local	Local	Mueang, Nong Khai
E-dum	Local	Lom Sak, Phetchabun
E-lueang	Local	Lom Sak, Phetchabun
Kariang	Local	Mueang, Kanchanaburi
Kan-kiw dok-khao	Local	Nhong Yasai, Suphan Buri
Kan	Local	Dan Chang, Suphan Buri
Bai-tung	Local	Khok Samrong, Lop Buri
Bai-lai	Local	Khok Samrong, Lop Buri
Ya-glai	Local	Tha Sala, Nakhon Si Thammarat
Yamueang	Local	Pong, Phayao
Laodong	Local	Mueang, Kanchanaburi

Table 3.4 Roll-your-own tobacco leaf samples used in this study.

Roll-your-own tobacco product	Buying locality (district/province)
Mae-somsong (white package)	Mueang, Sukhothai
Mae-somsong (red package)	Mueang, Sukhothai
Maew	Bangkok Noi, Bangkok

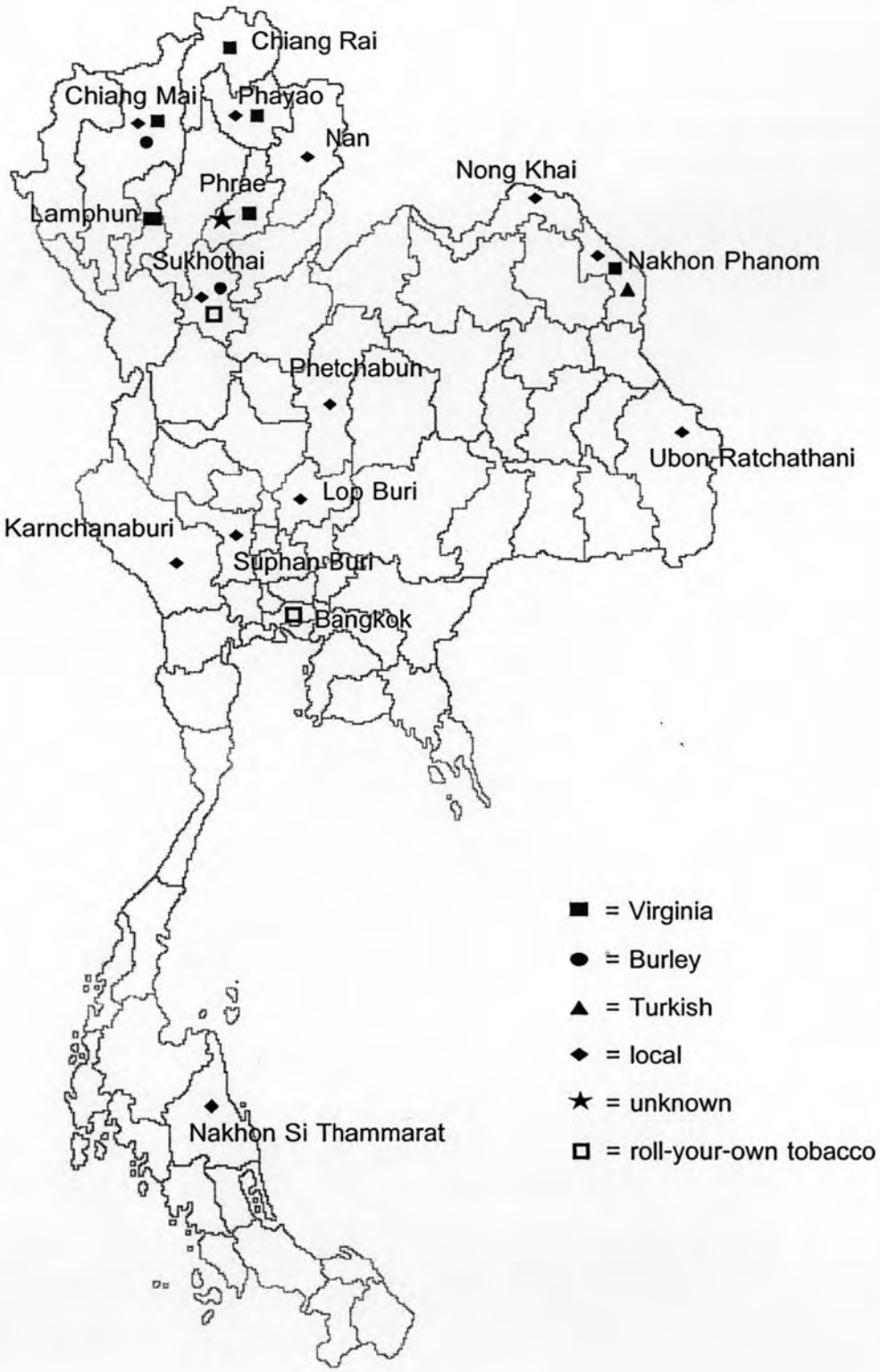

Figure 3.1 Collecting localities of tobacco samples around Thailand.

(A)

(B)

(C)

Figure 3.2 Field expeditions to collect tobacco leaf samples. (A) Tobacco crop fields. (B) Cutting tobacco leaves to small pieces. (C) Keeping the leaves separately in plastic bags with dried silica gel.

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)
- Electrophoresis chamber set: model Mupid (Advance Co., Ltd., Japan)
- Microcentrifuge: model centrifuge Sorvall® pico D-37520 Osterode (Kendro Laboratory Products, Germany)
- Microcentrifuge tubes: 0.5 and 1.5 ml (Axygen Scientific, Inc., USA.)
- Microwave oven: model Sharp Carousel R7456 (Sharp, Thailand)
- PCR machine: model GeneAmp® PCR system 9700 (Applied Biosystem, Singapore)
- pH meter: model Cybersean 500 (Eutech Cybernatics, Singapore)
- Pipette tips: 10, 200 and 1,000 µl (Axygen Scientific, Inc., USA.)
- Polaroid camera: model Direct Screen Instant Camera DS 34 H-34 (Peca Products, UK)
- AC/DC power supply: model EC570-90 LVD CE (E-C Apparatus Corporation, USA)
- Vortex: model MS I Minishaker (IKA-Works, Inc., USA)

3.1.3 Chemicals

- Absolute Ethanol (CH_3CH_2OH), M.W. = 46.07 (Merck, Germany)
- Agarose gel (Research Organics, USA)
- DNA ladder marker 100 bp (SibEnzyme, Russia)
- DNeasy® Plant Mini Kit (QIAGEN UmbH, Germany)
- Plant Genomic DNA Mini Kit (Geneaid Biotech Litd., Taiwan)
- Isopropanol (Bio Basic, Inc., USA)
- Dynazyme *Taq* DNA polymerase (Finnzyme, Finland)
- Ethidium bromide, M.W. = 934.32 (Bio Basic, Inc., USA)

- Metaphor® agarose (Lonza, USA)
- NuSieve® 3:1 agarose (Cambrex Bio Science Rockland, Inc., USA)
- Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan)
- QIAquick® PCR purification kit (QIAGEN GmbH, Germany)
- Boric acid (Research Organics, 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)
- Tris-base (Research Organics, USA)
- 10x TBE buffer (Tris-base 108 g, boric acid 55 g, EDTA 7.44 g and distilled water upto 1 litre)
- Bromophenol blue ($C_{19}H_{10}Br_4O_5S$), M.W. = 670 (Research Organics, USA)
- 99.5% (v/v) Glycerol ($C_3H_80_3$), M.W. = 92.10 (Research Organics, USA)
- 6x loading dye (glycerol 4 ml, bromophenal blue 25 mg and 1X TBE buffer upto 100 ml)
- Silica gel

3.1.4 Oligonucleotide primers

- Bio Basic custom-synthesised primer (Bio Basic Inc., USA)

3.2 Methods

Molecular marker development implemented a genomic DNA extraction from tobacco leaf materials, a quality test of the extracted genomic DNA and PCR amplification of noncoding regions of chloroplast DNA. The PCR condition was additionally optimised for higher PCR specificity. Subsequently, PCR amplified products were purified and sequenced. Computer programs were used to analyse DNA sequence data for genetic relationship of the tobacco cultivars.

3.2.1 DNA extraction and agarose gel electrophoresis

Genomic DNA of all tobacco cultivars studied was extracted from dried leaf tissues with either DNeasy® Plant Mini Kit (QIAGEN, Germany) or Plant Genomic DNA Mini Kit (Geneaid, Taiwan) to give high quality of the extracted DNA.

- Genomic DNA extraction using DNeasy® Plant Mini Kit (QIAGEN, Germany)

The extraction kit uses a DNeasy mini spin column filled with silica-gel membrane to isolate very pure DNA, free from inhibitory contaminants. Leaf powder ground under liquid nitrogen was transformed to a 1.5 ml Eppendorf microcentrifuge tube and added with 400 µl of AP1 buffer and 4 µl of 100 mg/ml RNase A stock solution. The mixture was vortexed and incubated at 65°C for 10 minutes, also mixed several times by inverting during incubation. 130 µl of buffer AP2 were added to the tube, mixed and incubated for 5 minutes on ice. The lysate was applied to a QIAshredder mini spin column set and centrifuged (14,000 rpm) for 2 minutes. A flow-through fraction was transferred to a new tube and 1.5 volumes of AP3 solution were added. 650 µl of the mixture were applied to the DNeasy mini spin column set, centrifuged (8,000 rpm) for 1 minute and the flow-through was discarded. The remaining sample was added to the spin column and centrifuged for another minute. The column was placed in a new tube and 500 µl of AW buffer were added. It was then centrifuged for 1 more minute, added with another 500 µl of AW buffer and centrifuged (14,000 rpm) for 2 minutes. The spin column was transferred to a new tube and 50 µl of 65°C preheated AE buffer were pipetted directly to the membrane of the spin column. The column was incubated for 2 minutes at room temperature and then centrifuged for 1 minute to elute the extracted DNA. Another 50 µl of the preheated buffer were added to elute the DNA for the second time. The extracted genomic DNA was then stored in a -20°C freezer until used.

- Genomic DNA extraction using Plant Genomic DNA Mini Kit (Geneaid, Taiwan)

A leaf sample was ground under liquid nitrogen to fine powder which was transferred to a 1.5 ml microcentrifuge tube. 400 µl of GP1 buffer (or GPX1 buffer) and 5 µl of RNase A were added to the tube and mixed by a vortex mixer. The mixture was incubated at 65°C for 10 minutes and the tube was inverted every 5 minutes during incubation. 100 µl of GP2 buffer were added to the mixture, mixed by a vortex mixer and incubated on ice for 3 minutes. A filter column was placed in a 2 ml collection tube. The mixture was transferred to the column and centrifuged for 1 minute at 13,000 rpm. The column was discarded and a supernatant in the tube was carefully transferred to a new 1.5 ml microcentrifuge tube. 1.5 volumes of GP3 buffer (Isopropanol added) were added to a lysate and the tube was vortexed immediately for 5 seconds. A GD column was placed in a new 2 ml collection tube. 700 µl of the mixture (including any precipitate) were transferred to the column and centrifuged at full speed for 2 minutes. The flow-through was discarded from the tube, then the remaining mixture was added to the column and the tube was centrifuged at full speed for 2 minutes. The mixture was discarded and the column was placed back in the tube. 400 µl of W1 buffer were added to the column and the tube was centrifuged at full speed for 30 seconds. The flow-through was discarded and the column was placed back in the tube. 600 µl of wash buffer (ethanol added) were added to the column and the tube was centrifuged at full speed for 30 seconds. The mixture was discarded and the column was placed back in the tube. The tube was also centrifuged again for 3 minutes at full speed to dry the column matrix. The dried column was transferred to a clean 1.5 ml microcentrifuge tube. 100 µl of 65°C preheated elution buffer or TE buffer were added to the center of the column matrix. The tube was left standing for 3-5 minutes or until the buffer was absorbed by the matrix and then centrifuged at full speed for 30 seconds to elute the purified DNA. The extracted genomic DNA was then stored in -20°C freezer.

The extracted genomic DNA was checked by electrophoresis with 0.8% (w/v) agarose gel in 1x TBE buffer as a running buffer. Normally, the electrophoresis was operated at 80 voltages for 45 minutes. 100 bp DNA ladder marker was used as a

standard DNA marker. The loading sample commonly composed of 5 µl of the extracted DNA and 2 µl of a loading dye. After that, the gel was stained with 0.5 mg/ml ethidium bromide solution and destained in distilled water. DNA bands were then visualised and photographed under UV light with a UV transilluminator.

3.2.2 Highly variable chloroplast DNA region amplification

Nine highly variable regions were selected from 13 noncoding chloroplast DNA (cpDNA) regions suggested by Shaw et al. (2007): *rpl*32-*trn*L$^{(UAG)}$, *trn*Q$^{(UUG)}$-5′*rps*16, 3′*trn*V$^{(UAC)}$-*ndh*C, *ndh*F-*rpl*32, *psb*D-*trn*T$^{(GGU)}$, *petA-psbJ*, 3′*rps*16–5′*trn*K$^{(UUU)}$, *atp*I-*atp*H, and *pet*L-*psb*E. *Nicotiana* cpDNA map is shown in Figure 3.3. Sequences of the forward and reverse primers for each region are presented in Table 3.5.

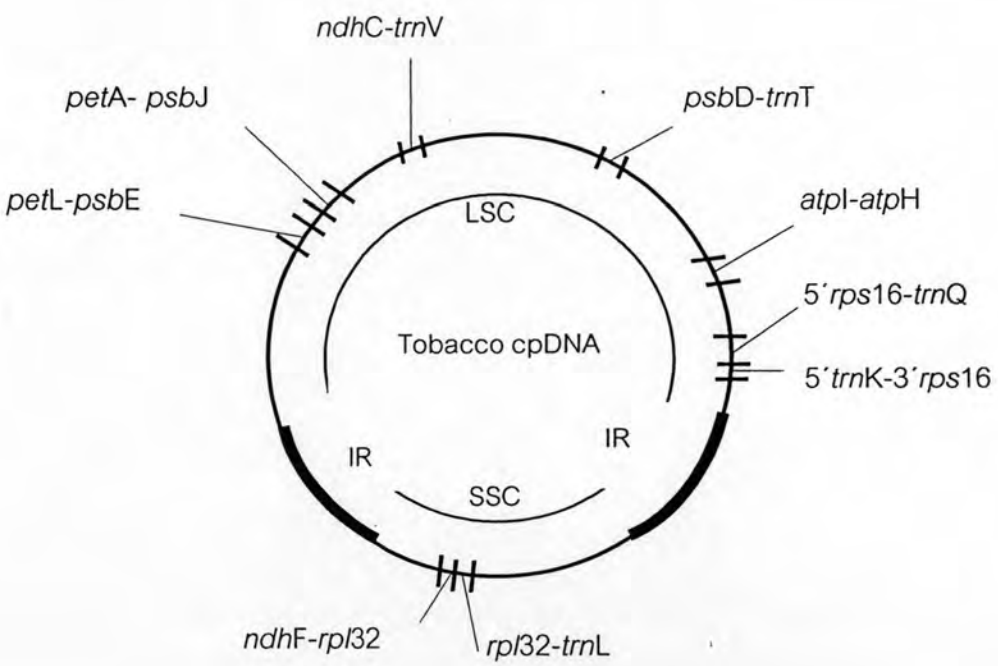

Figure 3.3 An approximate map of nine noncoding chloroplast DNA regions examined in this study (modified from Yukawa et al., 2005 and Shaw et al., 2007). The circle represents the chloroplast genome with thick lines representing two inverted repeats (IR). LSC and SSC are large and small single copy regions, respectively.

Table 3.5 Primer pairs used for PCR amplification and sequencing.

Region	Primer name	Primer sequence (5'-3')	Tm (°C)	%GC
*rpl*32-*trn*L	rpL32-F	CAG TTC CAA AAA AAC GTA CTT C	54.48	36.36
	trnL$^{(UAG)}$	CTG CTT CCT AAG AGC AGC GT	59.85	55.00
*ndh*F-*rpl*32	ndhF	GAA AGG TAT KAT CCA YGM ATA TT	51.26	26.09
	rpL32-R	CCA ATA TCC CTT YYT TTT CCA A	52.62	31.82
5'*trn*K-3'*rps*16	rpS16x2F2	AAA GTG GGT TTT TAT GAT CC	51.65	35.00
	trnK$^{(UUU)}$x1	TTA AAA GCC GAG TAC TCT ACC	56.06	42.86
5'*rps*16-*trn*Q	trnQ$^{(UUG)}$	GCG TGG CCA AGY GGT AAG GC	63.93	65.00
	rpS16x1	GTT GCT TTY TAC CAC ATC GTT T	54.48	36.36
*atp*I-*atp*H	atpH	CCA AYC CAG CAG CAA TAA C	53.04	30.43
	atpI	TAT TTA CAA GYG GTA TTC AAG CT	55.41	47.37
*psb*D-*trn*T	psbD	CTC CGT ARC CAG TCA TCC ATA	58.01	47.62
	trnT$^{(GGU)}$-R	CCC TTT TAA CTC AGT GGT AG	55.75	45.00
*ndh*C-*trn*V	trnV$^{(UAC)}$x2	GTC TAC GGT TCG ART CCG TA	57.80	50.00
	ndhC	TAT TAT TAG AAA TGY CCA RAA AAT ATC ATA TTC	54.60	18.18
*pet*A-*psb*J	petA	AAC ART TYG ARA AGG TTC AAT T	52.16	33.33
	psbJ	ATA GGT ACT GTA RCY GGT ATT	48.89	22.73
*pet*L-*psb*E	petL	AGT AGA AAA CCG AAA TAA CTA GTT A	53.78	28.00
	psbE	TAT CGA ATA CTG GTA ATA ATA TCA GC	55.68	30.77

* K = (G, T)

Y = (T, C)

M = (A, C)

R = (G, A)

3.2.2.1 Preliminary selection of suitable primers

Extracted genomic DNA of some tobacco cultivars representing three cultivar groups - Hangkai (local cultivar), K326 (Virginia imported cultivar) and B1 special (Burley) - were used for preliminary selection of suitable PCR primers. Nine PCR primer pairs (as shown in Table 3.5) were screened to amplify highly-variable noncoding regions of chloroplast DNA.

The extracted genomic DNA was amplified with a PCR mixture of 50 µl volumes. The PCR reaction component composed of 1 µl of template DNA (approximately 500-1,900 ng), 2 units of Dynazyme thermostable DNA polymerase (Finnzyme, Finland) and its optimised enzyme buffer (with 1.5 mM $MgCl_2$), 1 µl of 10 mM mixed dNTPs and 5 µl of each 10 µM primer. When a number of reactions were performed in the same time, a master mix comprising all of the reagents except the target DNA template was prepared and aliquoted to the reaction tubes. The DNA template was added as the last component before thermal cycling.

The PCR cycling condition used in this study is called "slow and cold" condition following Shaw et al. (2007). DNA amplification was carried out using a thermocycler GeneAmp® 9700 PCR system (Applied Biosystems, Singapore) as follows: initial denaturation at 80°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, primer annealing at 50°C for 1 minute and primer extension at 65°C for 4 minutes; followed by a final extension step at 65°C for 5 minutes. All reactions ended with a final 4°C hold step. PCR products were detected by 1.8% agarose gel electrophoresis with 1x TBE buffer at 80 voltages for about 1 hour. The gel was stained with 0.5 mg/ml of ethidium bromide and viewed under ultraviolet light.

After preliminary screening of suitable primers, only the primer pairs which could produce a single DNA band of all amplified DNA samples were selected. Any primer pairs giving non-specific PCR bands were optimised by raising the annealing temperature from 50°C to 51 - 52°C. This could effectively improve the PCR specificity and decrease amount of non-specific amplification products.

3.2.2.2 Selected primer pairs amplification

After selecting suitable PCR primers, the selected primer pairs were used to amplify the genomic DNA of all local and imported tobacco cultivar samples. The PCR cycling condition used in this experiment is as follows: initial denaturation at 80°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, primer annealing at 51°C (optimised from 50°C for higher PCR specificity) for 1 minute and primer extension at 65°C for 4 minutes; followed by a final extension step at 65°C for 5

minutes. PCR products were detected by 1.8% agarose gel electrophoresis with 1x TBE buffer at 80 voltages for about 1 hour. The gel was stained with 0.5 mg/ml of ethidium bromide and viewed under ultraviolet light. Only the PCR products showing clear DNA bands were selected for DNA sequence analysis.

3.2.3 PCR purification and DNA sequencing

Before sequencing the PCR products, any potential contaminants in the PCR mixture must be removed by purification. The amplified PCR products were purified with either QIAquick PCR Purification Kit (QIAGEN, Germany) or Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan) as specified by the manufacturers. These kits are designed to separate both single- and double-stranded DNA ranging from 100 bp to 10 kb from any contaminants, i.e. remaining primers, *Taq* DNA polymerase, unincorporated nucleotides and contaminated compounds introduced with the template DNA.

- PCR purification using QIAquick® PCR Purification Kit (QIAGEN, Germany)

Five volumes of PB buffer were mixed with one volume of the PCR products. The mixture was then applied to a QIAquick spin column placed in a 2 ml collection tube, and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded. 750 µl of PE buffer were added to the column, which was then centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded again and the column was centrifuged for 1 more minute at 13,000 rpm. The column was transferred to a new eppendorf tube and 50 µl of EB buffer or sterilised distilled water were added to the center of the column, which was left standing for 1 minute before centrifuged for 1 minute at 13,000 rpm.

- PCR purification using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan)

Up to 100 µl of a PCR product were transferred to a 1.5 ml microcentrifuge tube. 5 volumes of DF buffer were added to 1 volume of the sample and mixed by vortex mixer. A DF column was placed in a 2 ml collection tube and then the mixture from the previous step was transferred to the column. The tube was centrifuged at 13,000 rpm for 30 seconds, the flow-through was then discarded and the column was also placed back into the tube. 600 µl of wash buffer (ethanol added) were added into the center of the column and centrifuged at 13,000 rpm for 30 seconds. The mixture was discarded and then the column was placed back in the tube. The tube was centrifuged again for 3 minutes at full speed (13,000 rpm) to dry the column matrix. The dried column was transferred to a new 1.5 ml microcentrifuge tube and 15-50 µl of elution buffer were added into the center of the column matrix. The tube was left standing for 2 minutes or until the buffer was absorbed by the matrix and centrifuged for 2 minutes at full speed to elute the purified DNA.

After purifying the PCR products, the purified products were sent to Macrogen Inc. (Korea) for DNA sequencing service. All sequencing reactions were incorporated with BigDye™ fluorescence dye terminators (Applied Biosystems, USA). The sequencing products were electrophoresed and detected on an ABI prism 3730XL automated sequencer (Applied Biosystems, USA).

After DNA sequencing, computational analyses of the obtained DNA sequence data were performed using following computer programs. Chromas Lite (Technelysium Pty Ltd, Tewantin, Australia) was used to check and compare the nucleotide sequence data between forward and reverse sequencing reactions. The raw sequence data was changed to FASTA format file before aligned using ClustalX program (Thompson et al., 2001). A DNA data matrix was prepared by aligning all nucleotide sequences with each other (so-called a multiple alignment) until the homology of DNA data matrix became highest. ClustalX compared all sequences by left-right moving alignment with penalty scoring. Variable positions in the data matrix

were double checked against the original chromatogram files to make sure that all base calls were true at all variable positions.

3.2.4 Genetic relationship analysis of tobacco cultivars in Thailand

After completely analysis of the aligned sequences with ClustalX program, the nucleotide sequences of each region were brought to the polymorphism analysis among tobacco cultivars with the potentially informative characters (or PICs) and the proportion of mutational events or variability percentage values which followed Shaw et al. (2005). Two types of calculations were performed by:

1) The potentially informative characters (PICs)

The number of PICs were counted from the sum total of the number of nucleotide substitutions, insertion or deletion (or called indels) and inversions which found within each noncoding chloroplast DNA region of all tobacco cultivars. Any nucleotide within the indels and inversions were scored as independent, single characters.

2) The proportion of mutational events or variability percentages

Proportions of the observed mutational events for each noncoding chloroplast DNA region were estimated from the completely aligned sequences using a formula of Shaw et al. (2005).

$$\% \text{ variability} = [(NS+ID+IV) / L] \times 100$$

Where: NS = the number of nucleotide substitutions

ID = the number of indels

IV = the number of inversions

L = the aligned sequence length

After analysing the PICs and the variability percentages of the aligned sequences, then the data matrix was converted to a NEXUS file format before reconstructing a phylogenetic tree. PAUP* (Phylogenetic Analysis Using Parsimony and other methods) version 4.0b10 (Swofford, 1998) program was used to reconstruct a phylogenetic tree. Aligned nucleotide sequence matrices were analysed with unordered and initially equally weighted character states. Polymorphic characters were treated as uncertainty. Gaps or indels in the aligned matrix were coded as a separate presence/absence character with numerically as "0" or "1" (0 was coded for absence and 1 for presence) and added to the sequence data matrix. Neighbour-Joining (NJ) analysis was performed with "total character difference" standard-distance option. Bootstrap supporting values were calculated to indicate statistic-supports for internal branches. Bootstrap analysis was set to NJ option with 1,000 replicates to show the degree of confidence of each branch. Only the bootstrap values over 50% were considered as significant and mentioned on the bootstrap dendrogram.

3.2.5 Preliminary experiment of multiplex PCR technique

A multiplex PCR experiment was preliminarily performed to find a simple, fast technique to determine local and imported tobacco cultivars. If success, this method could save the cost and time for a large-scale PCR analysis in the future. PCR target regions which would be selected for the multiplex amplification should be highly polymorphic markers and be able to distinguish an individual variation of tobacco cultivars. Therefore, *rpl*32-*trn*L and *ndh*F-*rpl*32 regions were chosen to amplify the genomic DNA of the tobacco cultivars studied. The preliminary experiments were performed in two different aspects.

1) Using *rpl*32-*trn*L to determine different molecular markers between Virginia and local cultivars. The extracted genomic DNA of K326 (Virginia cultivar) and Chorlae1 (local cultivar) were amplified together with *rpl*32F-*trn*L$^{(UAG)}$ primer-pair in a single reaction tube. Another PCR reaction was performed with the extracted genomic DNA of K326 (Virginia) and Hangkai (local) amplified with *rpl*32F-*trn*L$^{(UAG)}$ primer. In this

experiment, the high-resolution agarose gels - Nusieve 3:1 agarose (Cambrex Bio Science Rockland, Inc.) and Metaphor agarose (Lonza) - were also used to improve a resolution of the gel electrophoresis step and were used to increase clearness of PCR band patterns after gel electrophoreses.

2) Using *rpl*32-*trn*L and *ndh*F-*rpl*32 to determine different molecular markers between Virginia and local cultivars. The extracted genomic DNA of K326 (Virginia) and Hangkai (local) were amplified together with two primer pairs, *rpl*32F-*trn*L$^{(UAG)}$ and *ndh*F-*rpl*32R, in a single reaction. This finding is a great benefit as a solution for the "local" and "imported" cultivar problem. Hopefully, it would help distinguishing tobacco variety contamination when analysing uncertain tobacco specimens. It will be introduced to reduce the time and cost of the tobacco cultivar analyses efficiently.

PCR reaction mixtures of both experiments were prepared with the following reaction components of 50 µl volumes: 1 µl of each template DNA, 2 units of Dynazyme thermostable DNA polymerase and its optimised enzyme buffer (with 1.5 mM $MgCl_2$), 1 µl of 10 mM mixed dNTPs and 5 µl of each 10 µM primer.

The PCR cycling condition used in this experiment is as follows: initial denaturation at 80°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, primer annealing at 51°C for 1 minute and primer extension at 65°C for 4 minutes; followed by a final extension step at 65°C for 5 minutes.