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APPENDIX

APPENDIX

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

การคัดเลือกลำดับดีเอ็นเอในคลอโรพลาสต์ที่แปรผันสูงเพื่อศึกษาความสัมพันธ์ทางพันธุกรรมของสายพันธุ์ยาสูบในประเทศไทย

SELECTION OF HIGHLY VARIABLE CHLOROPLAST DNA SEQUENCES FOR GENETIC RELATIONSHIP STUDY OF TOBACCO VARIETIES IN THAILAND

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บทคัดย่อ

ในประเทศไทย ต้นยาสูบสายพันธุ์พื้นเมืองและสายพันธุ์นำเข้าจากต่างประเทศมีกฎเกณฑ์ในการจัดเก็บภาษีที่แตกต่างกัน อย่างไรก็ตาม การตรวจวิเคราะห์เพื่อแยกแยะสายพันธุ์ทั้งสองกลุ่มออกจากกันยังคงเป็นปัญหาใหญ่อยู่ การวิจัยนี้จึงได้นำข้อมูลลำดับดีเอ็นเอมาใช้ศึกษาความสัมพันธ์ทางพันธุกรรมระหว่างยาสูบสายพันธุ์พื้นเมืองและ สายพันธุ์นำเข้า (กลุ่มสายพันธุ์เวอร์ยิเนีย เตอร์กิช และเบอร์เลย์) ที่เพาะปลูกในประเทศไทย โดยสกัดจีโนมิกดีเอ็นเอของยาสูบจากตัวอย่างใบสด นำมาเพิ่มปริมาณดีเอ็นเอบริเวณที่ไม่ได้ถอดรหัสและมีความแปรผันสูงจากดีเอ็นเอใน คลอโรพลาสต์ จำนวน 9 บริเวณ (*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$^{(GGU)}$, *psb*J-*pet*A, 3′*rps*16–5′*trn*K$^{(UUU)}$, *atp*I-*atp*H, และ *pet*L-*psb*E) ผลการอ่านลำดับดีเอ็นเอพบว่า ในจำนวน 9 บริเวณนั้นมี 6 บริเวณ (*rpl*32-*trn*L$^{(UAG)}$, *ndh*F-*rpl*32, *trn*T-*psb*D, 3′*trn*V$^{(UAC)}$-*ndh*C, *psb*J-*pet*A, และ *atp*I-*atp*H) ที่แสดงความแตกต่างของลำดับดีเอ็นเอทั้งความยาวของลำดับเบส (แตกต่างกัน 1 ถึง 66 คู่เบส) และการแทนที่ของเบส (0.37% ถึง 0.82%) ยาสูบสายพันธุ์นำเข้ากลุ่มเวอร์ยิเนียทั้งหมดมีลำดับดีเอ็นเอที่แตกต่างจากสายพันธุ์อื่นๆ และเป็นที่น่าสนใจว่าบริเวณ *rpl*32-*trn*L ยังสามารถแยกแยะยาสูบสายพันธุ์พื้นเมืองบางสายพันธุ์ได้อีกด้วย โดยมีดีเอ็นเอขนาดใหญ่ 66 คู่เบส แทรกในผลิตภัณฑ์พีซีอาร์

คำสำคัญ : ดีเอ็นเอในคลอโรพลาสต์ ความสัมพันธ์ทางพันธุกรรม ลำดับที่แปรผันสูง ประเทศไทย ยาสูบ

Abstract

In Thailand, local and imported tobacco varieties are subject to different regulations in tariff collection. However, there is a major technical problem as to how to distinguish between two groups of varieties. In this study, DNA sequences were applied for the study of genetic relationships between local and imported tobacco varieties (Virginia, Turkish, and Burley variety groups) cultivated in Thailand. Genomic DNA of tobacco was extracted from fresh leaf samples. Nine highly variable noncoding regions of chloroplast DNA (*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$^{(GGU)}$, *psb*J-*pet*A, 3′*rps*16–5′*trn*K$^{(UUU)}$, *atp*I-*atp*H, and *pet*L-*psb*E) were amplified by PCR. The sequence results revealed that six out of nine regions (*rpl*32-*trn*L$^{(UAG)}$, *ndh*F-*rpl*32, *trn*T-*psb*D, 3′*trn*V$^{(UAC)}$-*ndh*C, *psb*J-*pet*A, and *atp*I-*atp*H) showed polymorphic sequence characteristics, both in sequence lengths (from 1 to 66 basepair differences) and the amounts of base substitutions (from 0.37% to 0.82%). All tobacco samples of the imported Virginia variety-group were different in nucleotide sequences from the others. Interestingly, the *rpl*32-*trn*L region could distinguish some local tobacco varieties with a large 66 bp insertion within their PCR products.

Keywords: chloroplast DNA, genetic relationship, highly variable sequence, Thailand, tobacco

Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the most important economic crops of the world as raw material for cigarette industry. This plant originated in the tropical America (Hawks, 1970). From there, it was rapidly spread over Europe, Africa, Asia, and Australia (Albert, 1996). Tobacco first arrived in Thailand in the late 16th century and has been developed to be several local varieties. Nowadays, tobacco plants are mostly grown in the northern and northeastern parts of the country. Tobacco varieties cultivated in Thailand can be separated to two major groups: local varieties and imported varieties

(further separated to three minor groups: Virginia, Turkish, and Burley). Both variety groups have different regulations in tariff collection, i.e. the tariff for local varieties is much less than that of the imported ones. However, how to legally and technically separate the two groups apart is still a major problem. For example, only the tobacco varieties which have been cultivated in the country "for a long time" could be legally called "local" varieties, even though there is neither chemical nor physical standard method to determine their cultivation history.

Recently, several molecular markers, such as Simple Sequence Repeat (SSR) (Morgante and Olivieri, 1993), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz et al., 1994), Random Amplified Polymorphic DNA (RAPD) (William et al., 1990), and Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995), have been developed and increasingly used as modern techniques to distinguish genotypes of organisms. These DNA fragment markers have been successfully used in polymorphism analysis, crop cultivar identification, and phylogenetic evaluation in many plants. Each marker technique has its own advantages and disadvantages. Common benefits from most markers include rapid analyses, highly informative results, and being independent on environmental factors. However, DNA fragment amplification markers also have some limitations in the data analysing step. For instance, DNA band results may not be clear enough for the analysis and some PCR amplified fragments may not be repeatable due to a low quality of the genomic DNA. These problems particularly lead to an uncertainty when analysing the genetic distance between organisms, especially in the case of cultivated crops. To avoid such problems, DNA sequencing technique would rather be used as an alternative molecular marker than DNA fragment markers.

The DNA sequencing technique has been widely used in many aspects in the recent years. For plant genomes, nucleotide sequences of chloroplasts have been proved to be a primary source of data for molecular genetic relationship studies. Many early publications usually focused on several coding-regions of chloroplast DNA (cpDNA) sequences such as *rbc*L, *mat*K, *atp*B and *ndh*F genes to elucidate genetic relationships among higher-level taxa (e.g. Chase et al., 1993; Olmstead and Sweere, 1994; Steele and Vilgalys, 1994). In tobaccos (*Nicotiana*), Clarkson et al. (2004) have

recently shown the genetic relationships in the genus inferred from multiple plastid DNA regions and Yukawa et al. (2005) have reported the complete nucleotide sequence of the *Nicotiana tabacum* chloroplast DNA and its gene organization. Not only the coding sequences of chloroplasts, but noncoding cpDNA sequences (introns and intergenic spacers) have also been used in plant molecular systematic research for more than fifteen years (since the *trn*L-*trn*F intron work of Taberlet et al. in 1991). Noncoding regions of the cpDNA have been proved to be more suitable for lower-level taxonomic studies than the coding regions (Gielly and Taberlet, 1994). This is under the assumption that noncoding regions should be under less functional constraint than coding regions and then provide greater levels of variation for analyses. In 1998, Small et al. have tested the relative utility of seven noncoding cpDNA regions and a pair of homologous nuclear genes for resolving recent divergences, using tetraploid cottons (*Gossypium*) as a model system. Recently, Shaw et al. (2005) have evaluated the amplification and phylogenetic utility of twenty-one different noncoding cpDNA regions in a wide range of seed plant lineages. They have proposed that there are many more variable noncoding regions which have rarely been employed. They also showed that the most widely-used regions (such as *trn*L-*trn*F intron) are among the least variable. Moreover, in 2007 the same group has later used the best regions of their previous work (Shaw et al., 2005) as a baseline and successfully identified another thirteen unexplored noncoding regions and evaluated them more thoroughly (Shaw et al., 2007). They showed that at least nine newly explored regions offered the levels of variation even higher than those of the most variable regions identified in their 2005 work. That study led us to propose that these highly variable regions of chloroplast genomes could be evaluated for their potential applicability in genetic relationship study of tobacco varieties.

Thus, the aims of this study were to examine some of Shaw et al. (2007)'s primer pairs for PCR amplification of highly variable regions of tobacco chloroplast DNA and then to select the suitable primers for genetic relationship analyses of tobacco varieties cultivated in Thailand.

Materials and methods

Plant materials and DNA extractions

Fresh tobacco leaves of eight imported and 35 local varieties (27 fresh leaves, seven cured leaves, and one processed tobacco) were sampled from many cultivated crops around Thailand: North (Chiang Mai, Chiang Rai, Phayao, Lamphun and Nan provinces), Northeast (Nong Khai, Nakhon Phanom and Ubon Ratchathani provinces), West (Kanchanaburi province), Central (Phetchabun, Sukhothai, Suphan Buri and Lop Buri provinces) and South (Nakhon Si Thammarat province). The names of all tobacco varieties used in this study are in Table 1. Leaf material of each variety was cut to small pieces and kept separately in silica-gel bags until DNA extraction. Genomic DNA was extracted from the dried leaf tissue using Dneasy Plant Mini Kit (QIAGEN, Germany) to give rapid extraction and high quality extracted DNA. The extracted DNA was UV-visualised by 1% agarose gel electrophoresis after stained with ethidium bromide.

Table 1 Imported and local tobacco varieties sampled from many cultivated crops around Thailand for using in this study.

Variety name	Variety group	Collecting locality (districts/provinces)
Samsun	Imported (Turkish)	That Phanom, Nakhon Phanom
Xanthiyaka	Imported (Turkish)	That Phanom, Nakhon Phanom
PVH03	Imported (Virginia)	Pong, Phayao
K326	Imported (Virginia)	Ban Thi, Lamphun
PV09	Imported (Virginia)	Thoeng, Chiang Rai
B1 special	Imported (Burley)	Si Samrong, Sukhothai
Ky14	Imported (Burley)	Si Samrong, Sukhothai
TN90	Imported (Burley)	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-Nan	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
K326 local*	Local	Mueang, Nong Khai
Ya-glai*	Local	Tha Sala, Nakhon Si Thammarat
E-dum*	Local	Lhom Sak, Phetchabun
Kan-kiw Dok-khao*	Local	Nhong Yasai, Suphan Buri
Kan*	Local	Dan Chang, Suphan Buri
Baitung*	Local	Khok Samrong, Lop Buri
Bailai*	Local	Khok Samrong, Lop Buri
Maesomsong**	Local	Mueang, Sukhothai

* cured leaf

** processed tobacco

Highly variable chloroplast DNA region amplifications

Nine highly variable regions were selected from thirteen 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$^{(GGU)}$, *psb*J-*pet*A, 3′*rps*16–5′*trn*K$^{(UUU)}$, *atp*I-*atp*H, and *pet*L-*psb*E. *Nicotiana* cpDNA map is shown in Figure 1. Sequences of the forward and reverse primer for each region are presented in Table 2. The extracted genomic DNA of 43 sampled tobacco specimens were PCR amplified with the nine primer pairs. PCR reaction mixtures of 50 µl volumes were prepared with the following reaction component: 1 µl of template DNA (approximately 500-1900 ng), 2 units of Dynazyme thermostable DNA polymerase (Finnzyme, Finland) and its optimised enzyme buffer (with 1.5 mM $MgCl_2$), 1 µl of 2.5 mM mixed dNTP and 5 µl of each 10 µM primer. The PCR cycling condition used in this study is called "slow and cold" condition which was modified from that of Shaw et al. (2007). DNA amplifications were 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 51°C for 1 minute (optimised from 50°C originally suggested for higher PCR specificity) 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. Only the primers which could produce a single, clear DNA band were selected for DNA sequence analysis.

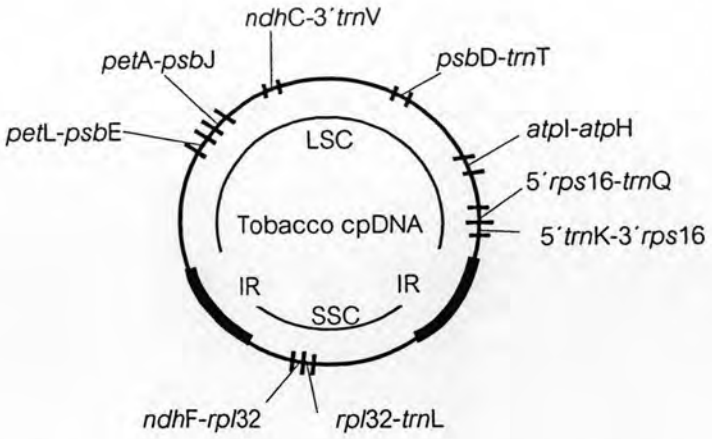

Figure 1 An approximate map of nine non-coding chloroplast DNA regions examined in this study (modified from Yukawa et al., 2005 and Shaw et al., 2007). The circle represents the chloroplast genome, with shaded regions representing two inverted repeats (IR). LSC and SSC are large and small single copy regions, respectively.

Table 2 Details of nine primer pairs used for PCR amplification and sequencing.

Region	Primer name	Primer sequence (5'-3')	Tm (°C)	%GC
*rpl*32F-*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*32R	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	atpI	TAT TTA CAA GYG GTA TTC AAG CT	53.04	30.43
	atpH	CCA AYC CAG CAG CAA TAA C	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-3′*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

psbJ-petA	psbJ	ATA GGT ACT GTA RCY GGT ATT	52.16	33.33
	petA	AAC ART TYG ARA AGG TTC AAT T	48.89	22.73
petL-psbE	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

PCR purification and DNA sequencing

The PCR amplified products were purified prior to a sequencing step with either the QIAquick PCR Purification Kit (QIAGEN, Germany) or the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan) to give higher quality purified DNA. The purified PCR products were sent to Macrogen Inc. (Korea) for DNA sequencing service. All DNA sequencing reactions were incorporated with BigDye™ fluorescence dyes (Applied Biosystems, USA). The sequencing products were electrophoresed and detected on an ABI3730XL automated sequencer (Applied Biosystems, USA).

Sequence analysis

Chromas Lite program (Technelysium Pty Ltd, Tewantin, Australia) was used to check and compare nucleotide sequence data between the forward and reverse sequencing reactions. The raw sequence data was changed to FASTA format to prepare a DNA data matrix. ClustalX (Thompson et al., 2001) was used to align all nucleotide sequences in the data matrix with each other (so called multiple alignments). Neighbour-Joining (NJ) analysis was performed with "Total character difference" standard distance option implemented in PAUP* version 4.0b10 (Swofford, 2002). For phylogenetic analysis, gaps in the alignment were coded as binary characters with numerically as "0" or "1". Bootstrap supporting values were estimated using 1,000 replicates to show the degree of confidence of each branch on the tree. Only the bootstrap values over 50% were considered as significant and mentioned on the diagram.

The proportion of observed mutational events for each noncoding cpDNA region was estimated using the formula used in Shaw et al. (2007). The proportion of mutational events (or % variability) is equal to [(NS+ID+IV)/L] x 100, when NS = the

number of nucleotide substitutions, ID = the number of indels (insertions/deletions), IV = the number of inversions, and L = the aligned sequence length.

Results

PCR amplification

In this study, the genomic DNA of eight imported and 35 local tobacco varieties was successfully extracted with good yield and quality enough for PCR amplification. The PCR results revealed that from nine examined regions of the tobacco cpDNA, eight regions, except *pet*L-*psb*E, were amplified from the genomic DNA of every sample. We also found that four regions (*rpl*32-*trn*L$^{(UAG)}$, 5´*rps*16-*trn*Q$^{(UUG)}$, *psb*D-*trn*T$^{(GGU)}$, and 5´*trn*K$^{(UUU)}$-3´*rps*16) gave several non-specific PCR bands. An optimisation of the annealing temperature by raising the temperature from 50°C to 51°C effectively improved the PCR specificity, decreasing amount of the non-specific DNA bands of only *rpl*32-*trn*L$^{(UAG)}$ and *psb*D-*trn*T$^{(GGU)}$ regions. Lengths of the total PCR products of these selected six regions were approximately 850-1,300 basepairs (bp).

DNA sequencing and genetic relationship analysis

DNA sequences of the six selected regions (*rpl*32-*trn*L$^{(UAG)}$, *ndh*F-*rpl*32R, *trn*T-*psb*D, *ndh*C-3´*trn*V$^{(UAC)}$, *pet*A-*psb*J, and *atp*H-*atp*I) showed polymorphism in nucleotide sequence characteristics among different tobacco varieties shown in Table 3, both in sequence lengths (from 1 to 66 bp differences) and amounts of base substitutions (from 0.37% to 0.82%). The nucleotide sequences of all three imported Virginia tobacco (PVH03, K326 and PV09 varieties) were different from the other variety groups (Burley, Turkish, and local varieties). Interestingly, only the *rpl*32-*trn*L primer could distinguish eight local varieties (Hangkai, Kan (fresh and cured leaves), Petmakhuea, Ubon Ratchathani, Petkhangsink, Baitung (cured leaf), Bailai (cured leaf) and Maesomsong (processed tobacco)) from the other local and imported varieties with a large 66 bp insertion within their PCR fragments (Figure 2). The phylogram based on Neighbour-Joining (NJ) analysis of the *rpl*32-*trn*L sequence data clearly illustrated a cluster of Virginia varieties and a special grouping of these eight local varieties (Figure 3).

Table 3 Degree of polymorphism in nucleotide sequence characteristics of the six selected chloroplast non-coding regions.

Region	Average sequence length	Aligned sequence length	Base substitution	Indel	% variability
*rpl*32-*trn*L	817 bp (627-1,062)	716 bp	9 bp (0.72%)	66 bp	1.4
*pet*A-*psb*J	947 bp (750-1,058)	753 bp	3 bp (0.40%)	20 bp, 3 bp	0.7
*ndh*F-*rpl*32	756 bp (751-768)	769 bp	4 bp (0.52%)	13 bp, 6 bp	0.8
*atp*H-*atp*I	1,033 bp (802-1,162)	811 bp	3 bp (0.37%)	9 bp	0.5
*ndh*C-3'*trn*V	924 bp (666-1,055)	685 bp	3 bp (0.44%)	8 bp, 7 bp	0.7
*trn*T-*psb*D	1,113 bp (730-1,238)	731 bp	6 bp (0.82%)	1 bp	1.0

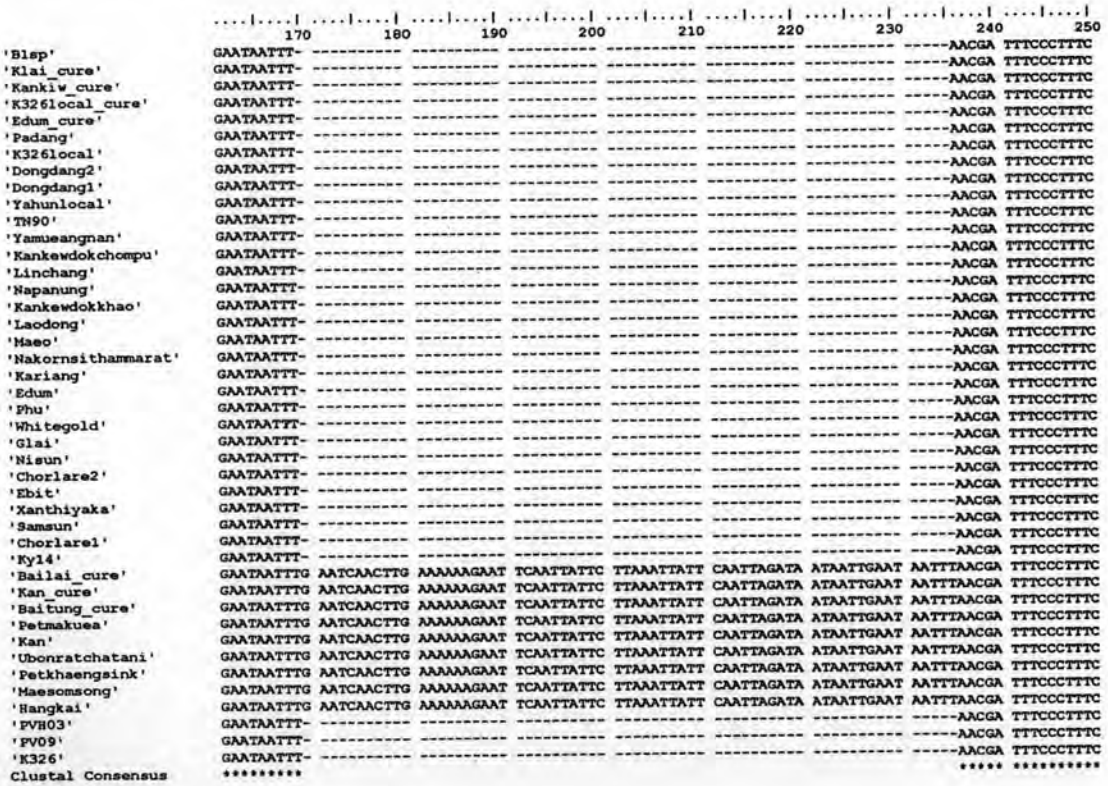

Figure 2 One part of the alignment of *rpl*32-*trn*L sequences of the tobacco samples examined in this study, showing a large 66 bp insertion of some local varieties.

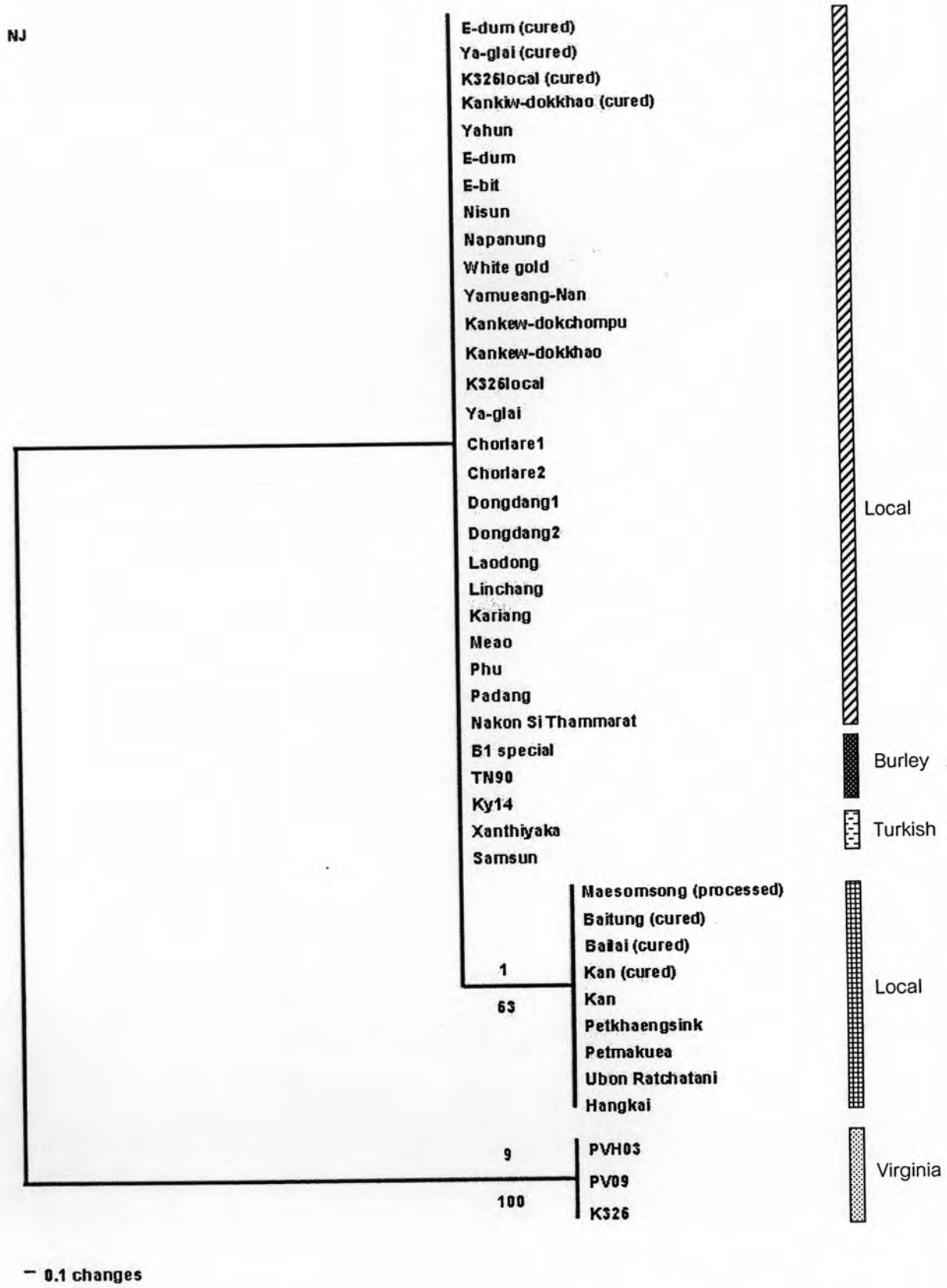

Figure 3 NJ tree from the *rpl*32-*trn*L sequence data of forty-three tobacco varieties. Branch lengths (with 1-gap included) are shown above each branch while bootstrap-supporting percentages are shown below.

Discussions and Conclusions

In our study of non-coding chloroplast sequences of tobacco varieties grown in Thailand, six highly variable regions (*rpl*32-*trn*L$^{(UAG)}$, 3′*trn*V$^{(UAC)}$-*ndh*C, *ndh*F-*rpl*32, *psb*D-*trn*T$^{(GGU)}$, *psb*J-*pet*A, and *atp*I-*atp*H) were successfully amplified from all tobacco samples. These non-coding cpDNA regions could give high Potentially Informative Characters (PICs) as suggested by Shaw et al. (2007). They are also fairly new markers that have not yet been widely utilised in plant genetic relationship study, although having the most variation among other universal angiosperm primers (Shaw et al., 2007). Therefore, we expected to see these regions being a potential molecular marker for the problem of how to distinguish between local and imported tobacco varieties cultivated in Thailand.

*rpl*32-*trn*L$^{(UAG)}$ is the best choice among the six primer pairs examined in this sequence polymorphic study. This region is an intergenic spacer in a small single-copy (SSC) region of the chloroplast genome (Figure 1). In our work, *rpl*32-*trn*L$^{(UAG)}$ could be amplified in all tobacco samples and its average sequence length is 817 bp, and it ranges from 627-1,062 bp. This is the highest polymorphic region of all six selected regions in our study within its 1.4 percentages of variability (Table 3). We also observed a single 66 bp large indel in the sequence result of eight local varieties (Hangkai, Kan (fresh and cured leaves), Petmakhuea, Ubon Ratchathani, Petkhangsink, Baitung (cured leaf), Bailai (cured leaf) and Maesomsong (processed tobacco)) (Figure 2). Therefore, we proposed that these eight local varieties presumably had a long-history of growing in Thailand and they could be legally pronounced as "true" local varieties (Figure 2 and 3). On the other hand, all other 26 local varieties were placed closely to the imported tobacco varieties (Burley and Turkish) on the tree (Figure 3). These genetic relationship results agreed well with the fact that they were morphologically similar to some imported varieties. We proposed that these local varieties were descended from some imported varieties industrially promoted and given by Thailand Tobacco Monopoly to farmers 20-40 years ago. If this hypothesis is true, these tobaccos should not be classified as local varieties, although they have their own Thai names. This region was noted as the best region surveyed for low-taxonomic-level molecular studies by Shaw et al. (2007) and they also showed a large indel (approximately 52-70 bp in size) from

their low-taxonomic-level molecular survey. Timme et al. (2007) noted too that this region is highly variable, even though it has hardly been used in any sequence-based investigations.

Practically, our genetic relationship study of 43 tobacco varieties grown in Thailand based on six highly variable non-coding regions of chloroplast DNA could be used to distinguish Virginia imported tobacco varieties from any local varieties. This finding is a great benefit as a solution for the "local" vs "imported" variety problem. Hopefully, it would help distinguishing tobacco variety contamination when analysing uncertain tobacco specimens. Moreover, the *rpl*32-*trn*L region could also easily separate eight local varieties from the other locals. This therefore suggested that we may be able to use such highly variable cpDNA region to determine "true" local tobacco varieties which had been cultivated in Thailand for along time, as defined by law. At the moment, we are trying to improve the DNA extraction technique and the PCR condition to be able to determine processed tobacco specimens. Multiplex PCR technique will be introduced to reduce the time and cost of the tobacco variety analyses efficiently.

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