CHAPTER IV

RESULTS

4.1 DNA extraction

Genomic DNA was extracted from totally 70 tobacco samples consisting of 43 fresh-leaf samples and 24 cured-leaf samples of 47 tobacco cultivars (including two unknown cultivars) and another three roll-your-own tobaccos. Thirty nine of 43 extracted DNA from fresh-leaf samples gave high quality and quantity of yielded DNA, although some smear DNA found at the bottom of the electrophoretic agarose gels (Figures 4.1-4.3). The other four extracted DNA of K190, HB01, HBO04P (lanes 3, 8 and 9 in Figure 4.2, respectively) and Pasak (lane 1 in Figure 4.3) cultivars appeared as fainted smear on the agarose gels. However, the estimated DNA concentrations of all 43 fresh-leaf tobacco samples were suitable for subsequently PCR experiments.

Figure 4.1 Genomic DNA extracted from fresh leaf samples of two local and six imported tobacco cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-2 = local cultivars: Phu and Hangkai, no. 3-8 = imported cultivars: Samsun, Xanthiyaka, KY14, B1 special, K326 and PVH03, respectively).

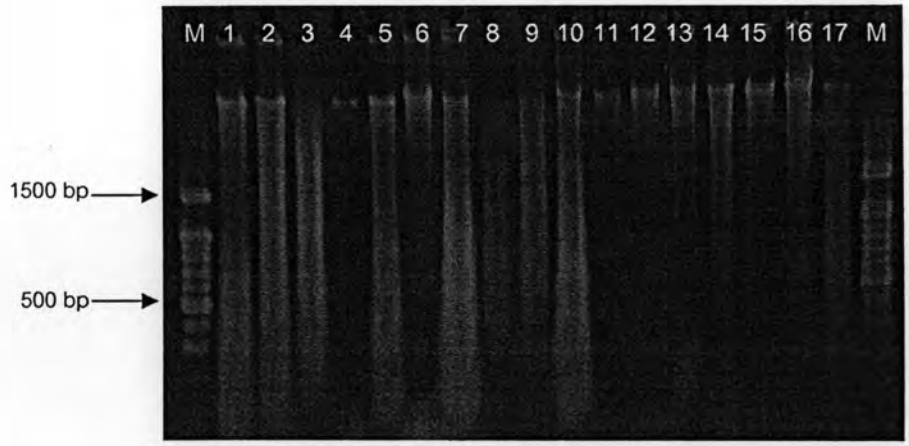

Figure 4.2 Genomic DNA extracted from fresh leaf samples of 14 imported and 3 local tobacco cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-14 = imported cultivars: Coker326, K187, K190, K326, PV09, PVH03, B1 special, HB01, HBO04P, KY14, TN90, TN97, Samsun and Xanthiyaka and no. 15-17 = local cultivars: Chorlare1, Nisan and Padang, respectively).

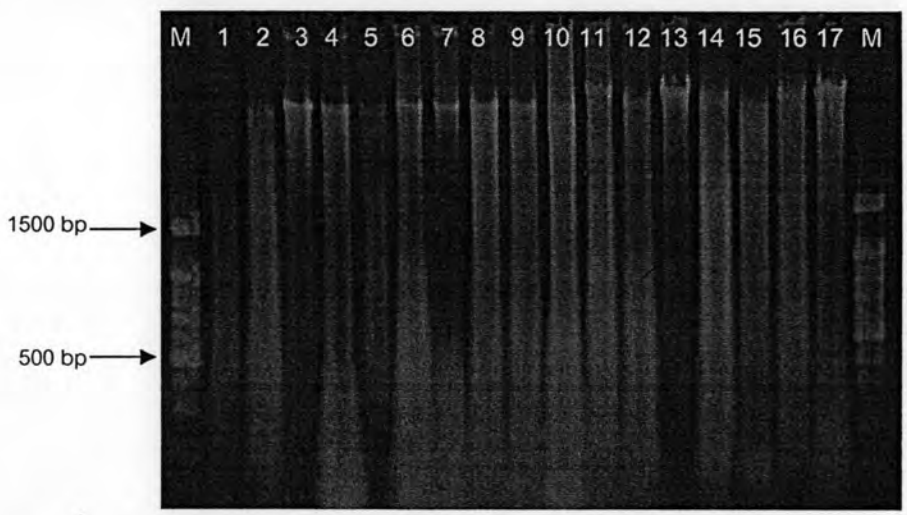

Figure 4.3 Genomic DNA extracted from fresh leaf samples of 17 local tobacco cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-17 = Pasak, Petmakhuea, Petkhangsink, Yamueang, Linchang, Phu, Hangkai, Yahan, E-dum, K326 local, Kan, Kan-kiw, Kan-kiw dok-khao, Kan-kiw dok-chom-phu, Kariang, Laodong and Meao cultivars, respectively).

From 24 cured-leaf samples, genomic DNA of 19 cured-leaf samples were successfully extracted though with some fairly fainted smear DNA on the agarose gels (Figures 4.4-4.5). However, the DNA extraction reactions of the other five cured-leaf DNA were failed (K187 and TN90 in lanes 1 and 7 of Figure 4.4; E-lueang, Hangkai and Yamueang in lanes 4, 6 and 7 of Figure 4.5, respectively) and their DNA extraction were

needed to be performed again. These five genomic DNA were successfully re-extracted and gave suitable DNA yield (the data not shown). The genomic DNA of Maew, Mae-somsong (red package) and Mae-somsong (white package) roll-your-own tobaccos (lanes 1-3 in Figure 4.6, respectively) was extracted in low yield and also appeared as fainted smear on the gel. Nevertheless, the quality and quantity of the DNA extracted from cured leaf samples and roll-your-own tobaccos were acceptable for further PCR amplification.

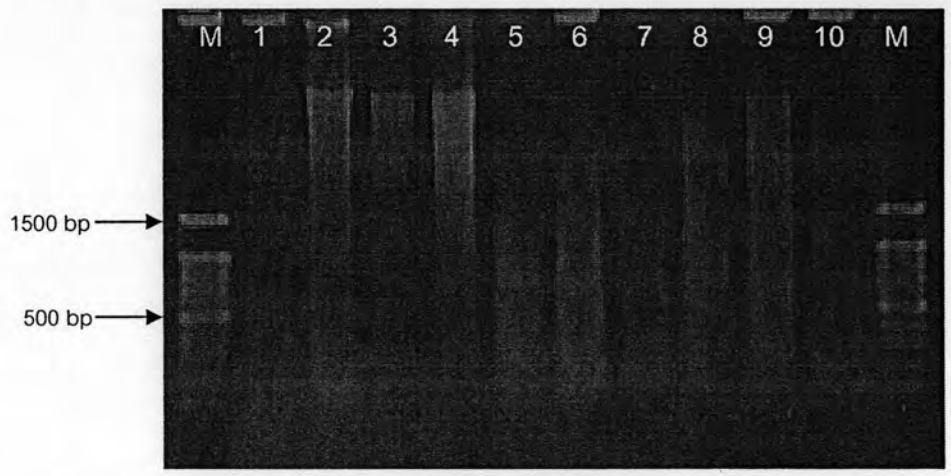

Figure 4.4 Genomic DNA extracted from cured leaf samples of 10 imported tobacco cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-10 = K187, K326, PV09, PVH03, KY14, TN86, TN90, TN97, Samsun and Xanthiyaka cultivars, respectively).

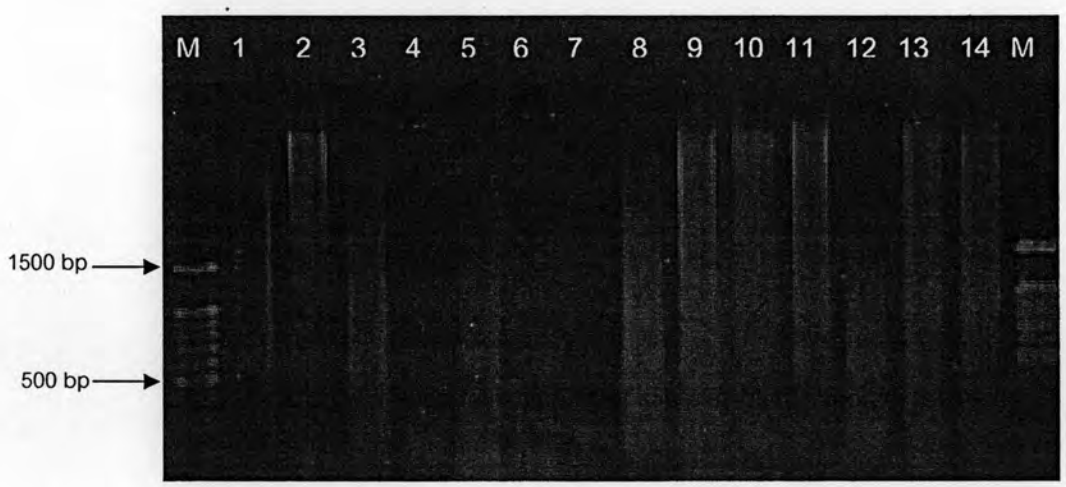

Figure 4.5 Genomic DNA extracted from cured leaf samples of 14 local tobacco cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no.1-14= White gold, K326 local, E-dum, E-lueang, Phu, Hangkai, Yamueang, Ya-glai, Kariang, Kan, Bai-tang, Bai-lai, Loadong and Kan-kiw dok-khao cultivars, respectively).

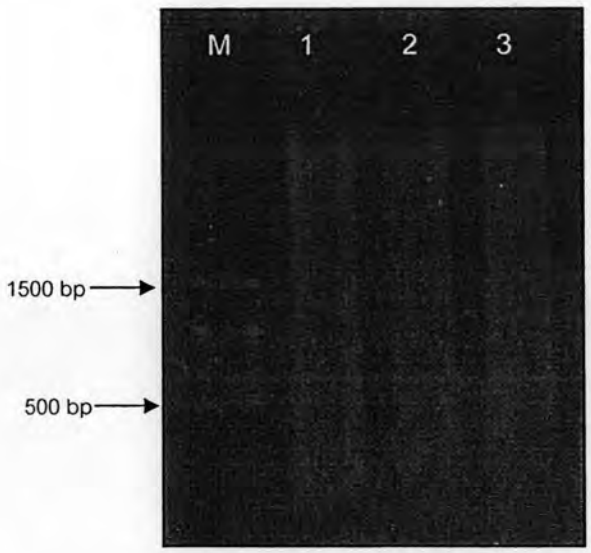

Figure 4.6 Genomic DNA extracted from three roll-your-own tobaccos (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-3 = roll-your-own tobaccos: Maew, Mae-somsong (red package) and Mae-somsong (white package), respectively).

4.2 PCR amplification and sequencing of highly-variable chloroplast DNA regions

4.2.1 Selection of suitable primers for the extracted DNA from fresh leaf samples

In the preliminary screening experiment of nine PCR primer-pairs (*rpl*32F-*trn*L$^{(UAG)}$, 5′*rps*16x1-*trn*Q$^{(UUG)}$, *ndh*C-3′*trn*V$^{(UAC)}$x2, *ndh*F-*rpl*32R, *psb*D-*trn*T$^{(GGU)}$-R, *pet*A-*psb*J, 5′*trn*K$^{(UUU)}$x1–3′*rps*16x2F2, *atp*I-*atp*H and *pet*L-*psb*E primers) with the extracted DNA of Hangkai (representing local cultivar), K326 (Virginia) and B1 special (Burley), almost all of primer pairs, except *pet*L-*psb*E (lanes 1-3 in Figure 4.7 and lanes 1-2 in Figure 4.8, respectively), successfully amplified all of the DNA samples. The sizes of the PCR products were estimated as approximately 1400 basepairs (bp) for *psb*D-*trn*T region, 1300 bp for *atp*H-*atp*I, 1400 bp for 5′*rps*16-*trn*Q, 900 bp for *ndh*F-*rpl*32, 1200 bp for *pet*A-*psb*J, 1200 bp for *ndh*C-*trn*V, 900 bp for 5′*trn*K–3′*rps*16, 1200 bp for *rpl*32-*trn*L and 1300 bp for *pet*L-*psb*E (lanes 4-15 in Figure 4.7 and lanes 3-15 in Figure 4.8, respectively). These eight effective primers gave clear and strong PCR amplified bands, even though *psb*D-*trn*T$^{(GGU)}$-R and 5′*rps*16x1-*trn*Q$^{(UUG)}$ primers also produced

non-specific PCR products (approximately 100-300 bp) as shown in the bottom of lanes 4-6 and 10-12 of Figure 4.7, respectively.

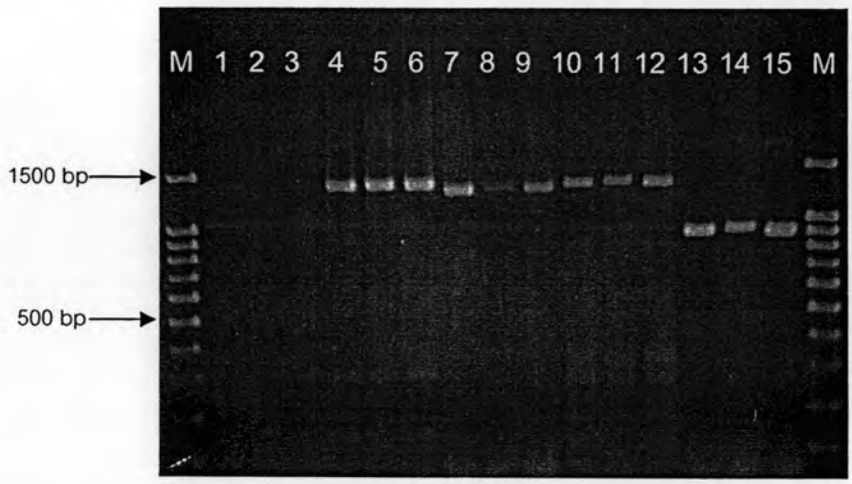

Figure 4.7 PCR products of Hangkai (local), K326 (Virginia) and B1 special (Burley) cultivars amplified with five primer-pairs and using 50°C annealing temperature (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-3 = *pet*L-*psb*E region, no. 4-6 = *psb*D-*trn*T, no. 7-9 = *atp*H-*atp*I, no. 10-12 = 5′*rps*16-*trn*Q and no. 13-15 = *ndh*F-*rpl*32; the order of cultivars in each amplified region is Hangkai, K326 and B1 special, respectively).

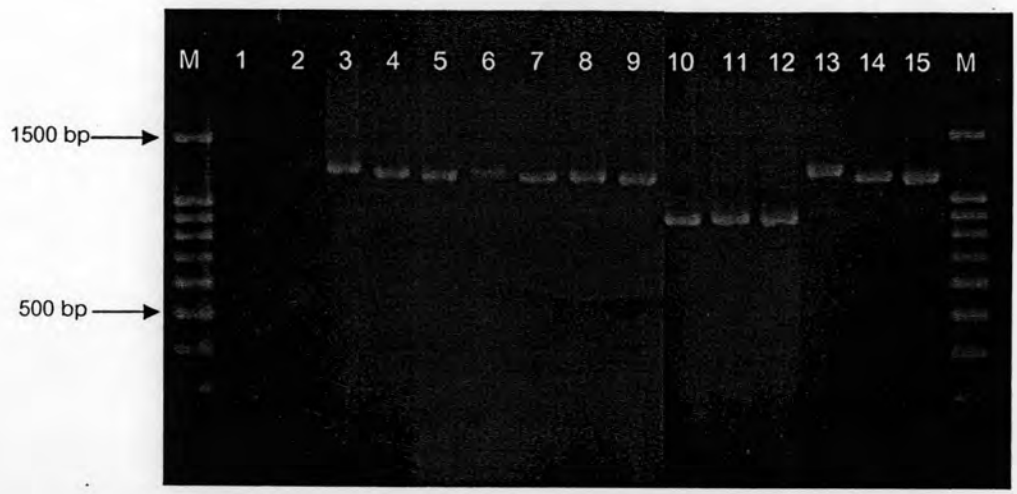

Figure 4.8 PCR products of Hangkai (local), K326 (Virginia) and B1 special (Burley) cultivars amplified with five primer-pairs and using 50°C annealing temperature (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-3 = *pet*L-*psb*E region, no. 4-6 = *pet*A-*psb*J, no. 7-9 = *ndh*C-*trn*V, no. 10-12 = 5′*trn*K-3′*rps*16 and no. 13-15 = *rpl*32-*trn*L; the order of cultivars in each amplified region is Hangkai, K326 and B1 special, respectively).

Next, an optimisation of the PCR condition for the problem of *psb*D-*trn*T and 5′*rps*16-*trn*Q regions non-specific products was done by raising the annealing temperature to 51°C and 52°C. From the first optimisation with 51°C annealing temperature, the non-specifically amplified products of both *psb*D-*trn*T$^{(GGU)}$-R and 5′*rps*16x1-*trn*Q$^{(UUG)}$ primers were decreased significantly (lanes 7-12 in Figure 4.9) compared to those of the unoptimised reactions (lanes 1-6 in Figure 4.9). Secondly, the amplified products of both primers after increasing the temperature to 52°C were also cleared without any non-specific bands observed (lanes 13-18 in Figure 4.9, respectively). However, the size of B1 special cultivar was unexpectedly decreased to about 1100 bp (lane 15 and 18 in Figure 4.9, respectively).

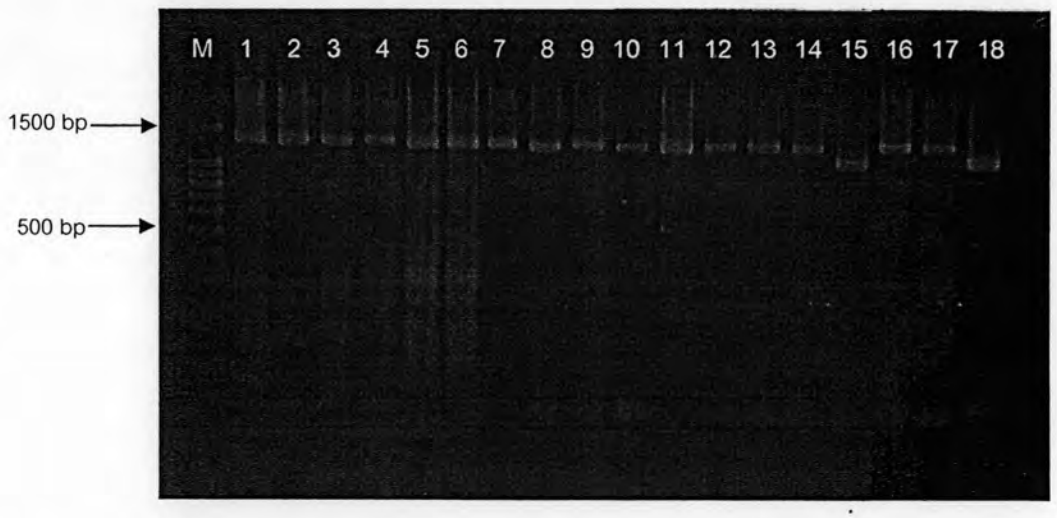

Figure 4.9 PCR products of Hangkai (local), K326 (Virginia) and B1 special (Burley) cultivars amplified with two primer-pairs and using 50°C, 51°C and 52°C annealing temperatures (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-6 = 50°C (no. 1-3 = *psb*D-*trn*T region and no. 4-6 = 5′*rps*16-*trn*Q), no. 7-12 = 51°C (no. 7-9 = *psb*D-*trn*T and no.10-12 = 5′*rps*16-*trn*Q) and no. 13-18 = 52°C (no. 13-15 = *psb*D-*trn*T and no. 16-18 = 5′*rps*16-*trn*Q); the order of cultivars in each annealing temperature is Hangkai, K326 and B1 special, respectively).

All of 24 amplified PCR products from 50°C amplification were cleaned up and sequenced. Only fifteen nucleotide sequences of *pet*A-*psb*J, *ndh*C-*trn*V, *atp*H-*atp*I, *ndh*F-*rpl*32 and *rpl*32-*trn*L regions were fairly clear with low noise from any primer-dimers or DNA contamination. The other nine sequences of *psb*D-*trn*T, 5'*rps*16-*trn*Q and 5'*trn*K-3'*rps*16 regions showed high noise signals, with the highest noise in the sequences of 5'*trn*K-3'*rps*16 region.

For the sequence results of *psb*D-*trn*T and 5'*rps*16-*trn*Q regions after 51°C and 52°C optimisation, all *psb*D-*trn*T sequences amplified with 51°C annealing temperature were clear with much lower noise than before optimised. However, the sequencing reactions were failed after amplified with 52°C. Those of 5'*rps*16-*trn*Q region were also completely failed from both of the amplification with 51°C and 52°C annealing temperatures.

Therefore, only six primer pairs (*pet*A-*psb*J, *ndh*C-3'*trn*V$^{(UAC)}$x2, *atp*H-*atp*I, *ndh*F-*rpl*32R, *rpl*32F-*trn*L$^{(UAG)}$ and *psb*D-*trn*T$^{(GGU)}$-R) were selected for further analysis with fresh-leaf samples of 23 tobacco cultivars. The 51°C annealing temperature was also chosen for the PCR condition. Almost all of *ndh*C-*trn*V and *rpl*32-*trn*L sequences were clear with very low noise signals (for example, *rpl*32-*trn*L sequence of Hangkai cultivar in Figure 4.10). Although the *ndh*C-*trn*V sequence results of PVH03, Xanthiyaka and Phu cultivars showed high noise signals (Figure 4.11, for example), these regions were sequenced again and clearer sequences were later obtained.

This phenomenon of getting a better result in the second sequencing reactions also happened in *pet*A-*psb*J region (with Samsun, Xanthiyaka, KY14, PVH03, Chorlare1, Petkhangsink, unknown1 and unknown2), *atp*I-*atp*H (with Chorlare1, KY14, Phu, unknown1 and unknown2), *ndh*F-*rpl*32 (with K326, Samsun and Chorlare1) and *psb*D-*trn*T (with PVH03, Xanthiyaka, Nisan, Ubon Ratchathani, unknown1 and unknown2). Unfortunately, repeated sequencing could not solve the problem of *pet*A-*psb*J, *atp*I-*atp*H and *psb*D-*trn*T reactions (Figure 4.12, for example) which were completely failed, showing numerous noise signals along the sequence lengths.

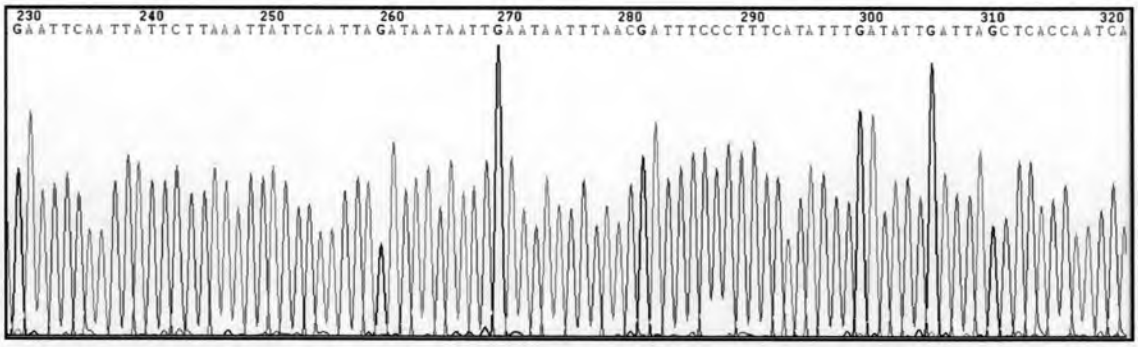

Figure 4.10 Electropherogram of *rpl*32-*trn*L sequence of Hangkai cultivar. (Four-coloured peaks represent four nucleotides: blue = cytosine (C), red = thymine (T), green = adenine (A) and cyan = guanine (G), respectively).

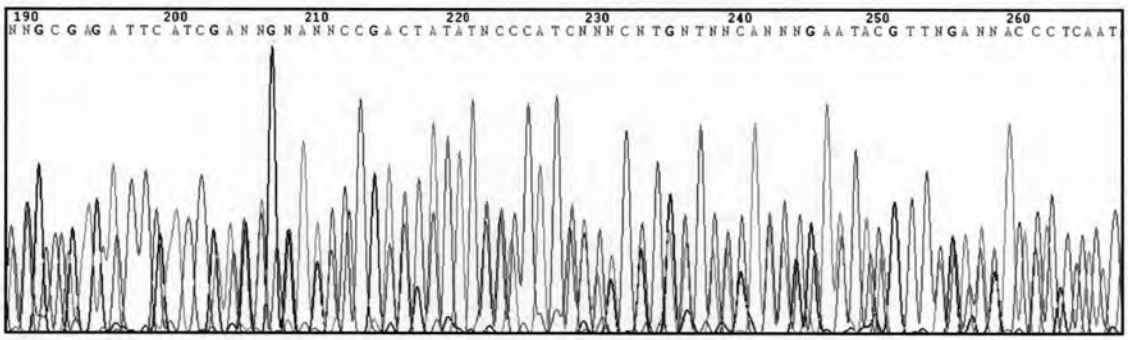

Figure 4.11 Electropherogram of *ndh*C-*trn*V sequence of Phu cultivar. (Four-coloured peaks represent four nucleotides: blue = cytosine (C), red = thymine (T), green = adenine (A) and cyan = guanine (G), respectively).

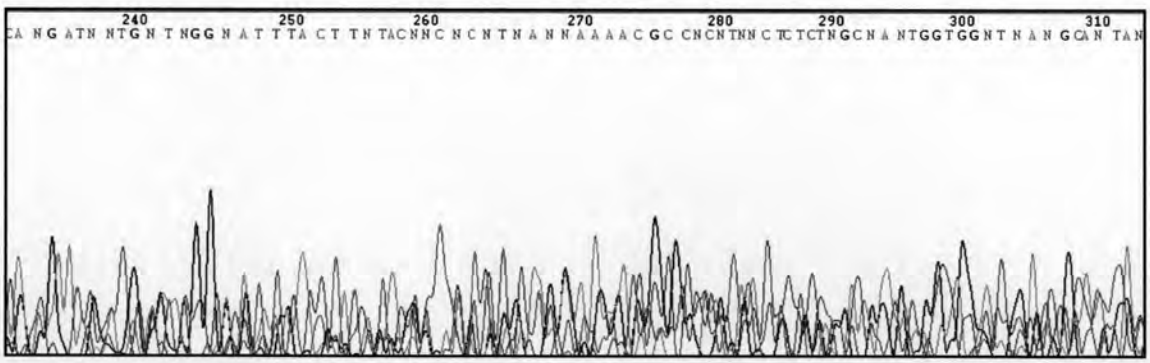

Figure 4.12 Electropherogram of *pet*A-*psb*J sequence of Chorlare2 cultivar. (Four-coloured peaks represent four nucleotides: blue = cytosine (C), red = thymine (T), green = adenine (A) and cyan = guanine (G), respectively).

In total, all of 23 genomic DNA extracted from fresh-leaf samples were successfully sequenced with *ndh*C-3′*trn*V$^{(UAC)}$x2 and *rpl*32F-*trn*L$^{(UAG)}$ primer pairs. The other three primers (*pet*A-*psb*J, *atp*I-*atp*H and *psb*D-*trn*T$^{(GGU)}$-R) could generate the sequences of only 18 DNA samples. Only five DNA samples were amplifiable with *ndh*F-*rpl*32R primer. Fortunately, these five sequences could represent all four cultivar-groups of tobacco (Hangkai and Chorlare1 of local cultivar-group; K326 of Virginia imported cultivar; Samsun of Turkish; and B1 special of Burley). The sizes and alignment lengths of these six DNA regions are shown in Table 4.1.

Table 4.1 Sizes of PCR amplified products and alignment lengths of the six DNA regions.

Region	product size (bp)	readable sequence length (bp)	aligned sequence length (bp)
*pet*A-*psb*J	1200	750-1058	753
*ndh*C-*trn*V	1200	666-1055	685
*psb*D-*trn*T	1400	730-1238	731
*atp*I-*atp*H	1300	802-1162	811
*ndh*F-*rpl*32	900	751-768	769
*rpl*32-*trn*L	1200	627-1062	716

Aligned sequence lengths of the nucleotide data matrices of *pet*A-*psb*J, *ndh*C-*trn*V, *psb*D-*trn*T, *atp*H-*atp*I, *ndh*F-*rpl*32 and *rpl*32-*trn*L regions were 753, 685, 731, 811, 769 and 716 bp, respectively (Table 4.1). Within these aligned data matrices, the sequences of K326 and PVH03 cultivars (imported Virginia cultivar-group) were always different from others of Burley, Turkish and local cultivar-groups. The nucleotide differences between Virginia and other cultivar-groups were found in both of the base substitutions and the number of insertions-or-deletions (indels) in each region.

The aligned sequences of *pet*A-*psb*J region amplified from the extracted DNA of 18 fresh-leaf tobacco samples (Figure 4.13) were 753 bp in length and showed three nucleotide substitutions and two indels. These two indels were a small insertion (3 bp) at the aligned positions 744-746 and a large deletion (20 bp) at the sites 509-528 of K326 and PVH03 Virginia cultivars.

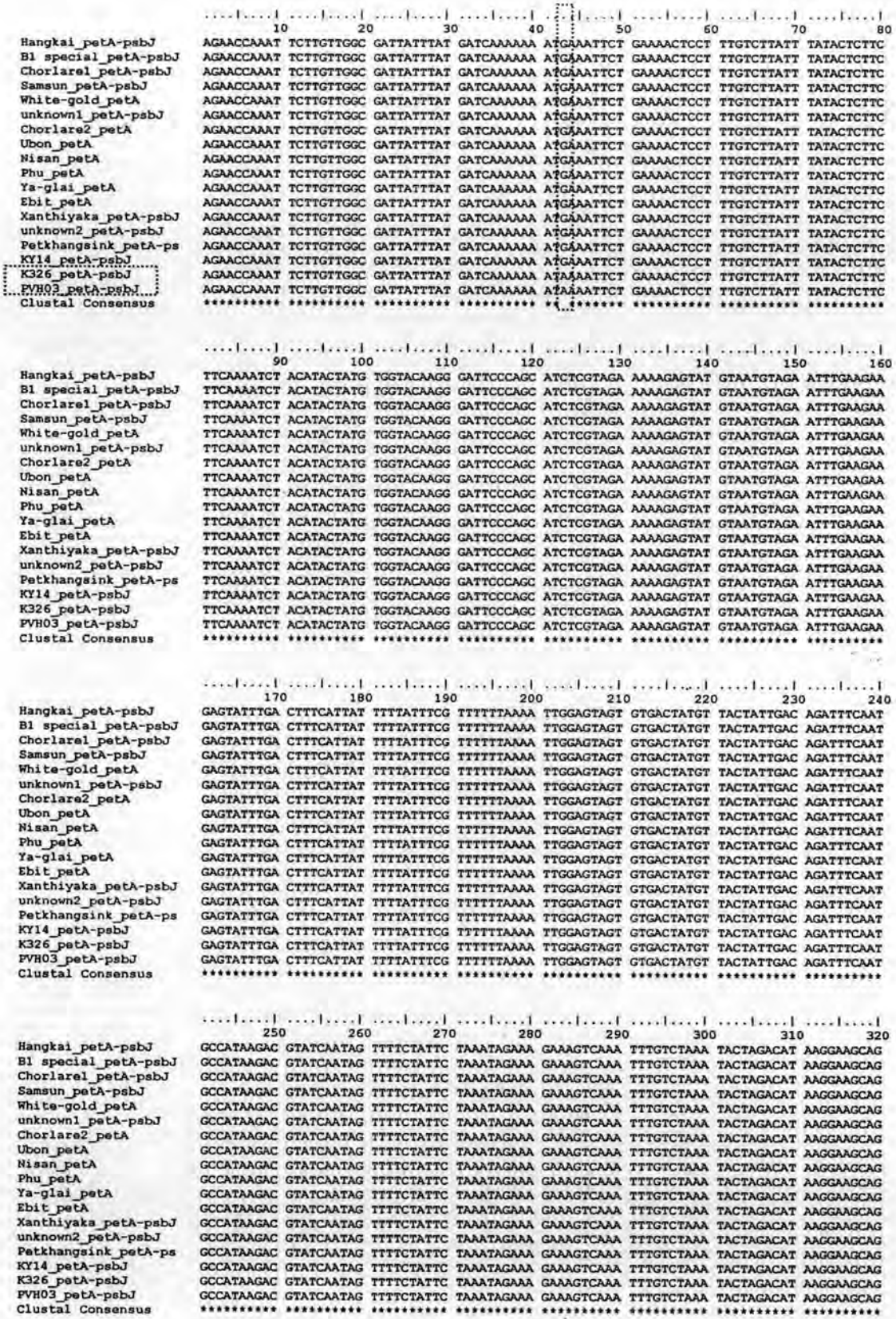

Figure 4.13 A 753 bp nucleotide data matrix of *petA-psbJ* region from fresh-leaf samples of total 18 tobacco cultivars. A gap symbol (-) indicates an insertion or a deletion at the site.

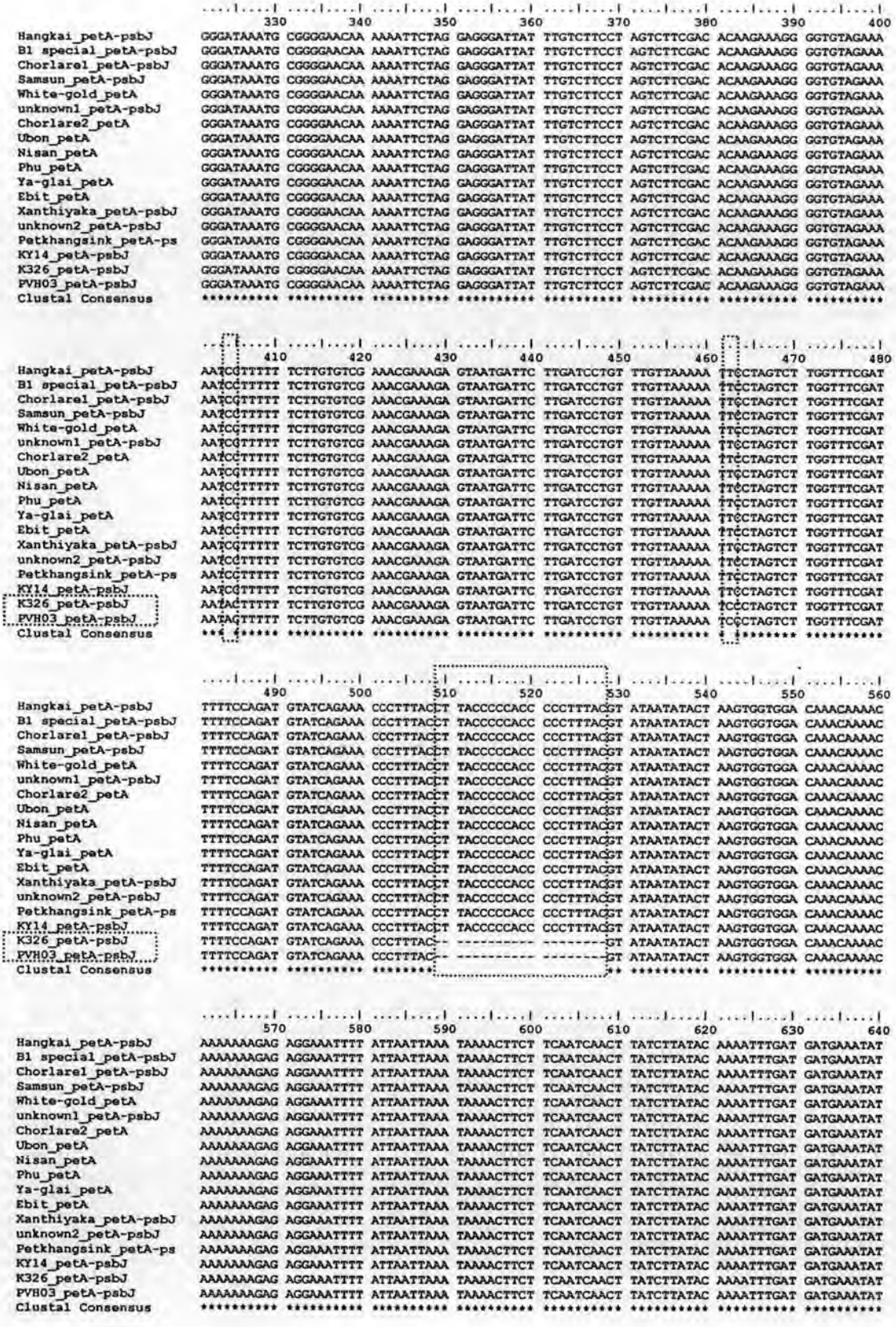

Figure 4.13 (continued)

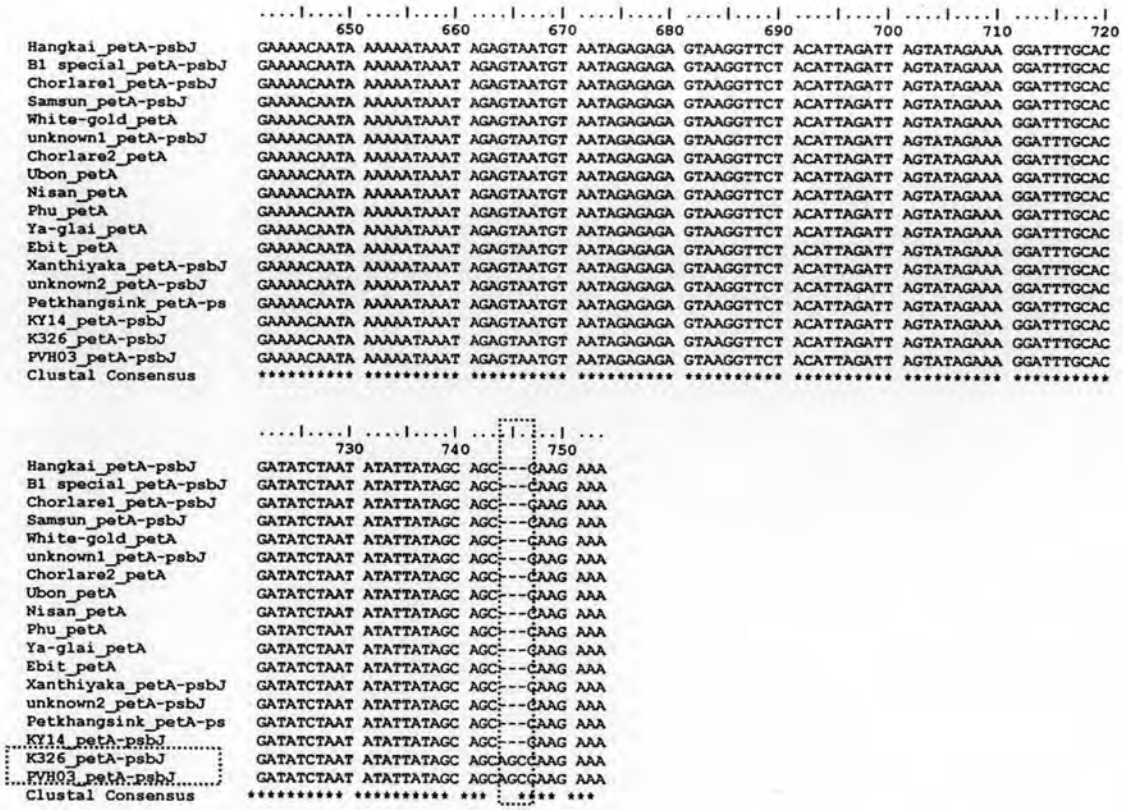

Figure 4.13 (continued)

For *ndh*C-*trn*V sequences from 23 fresh-leaf samples, their 685-bp aligned matrix (Figure 4.14) was also able to separate Virginia cultivars (K326 and PVH03) from the others with totally three base substitutions and two medium insertions (7 and 8 bp) at 359-365 and 388-395 aligned sites, respectively. In addition, a 731-bp aligned sequence matrix of *psb*D-*trn*T region from 18 fresh-leaf samples showed six nucleotide substitutions and only 1-bp insertion at the site 342 which also distinguished K326 and PVH03 cultivars from the other 16 cultivars (Figure 4.15).

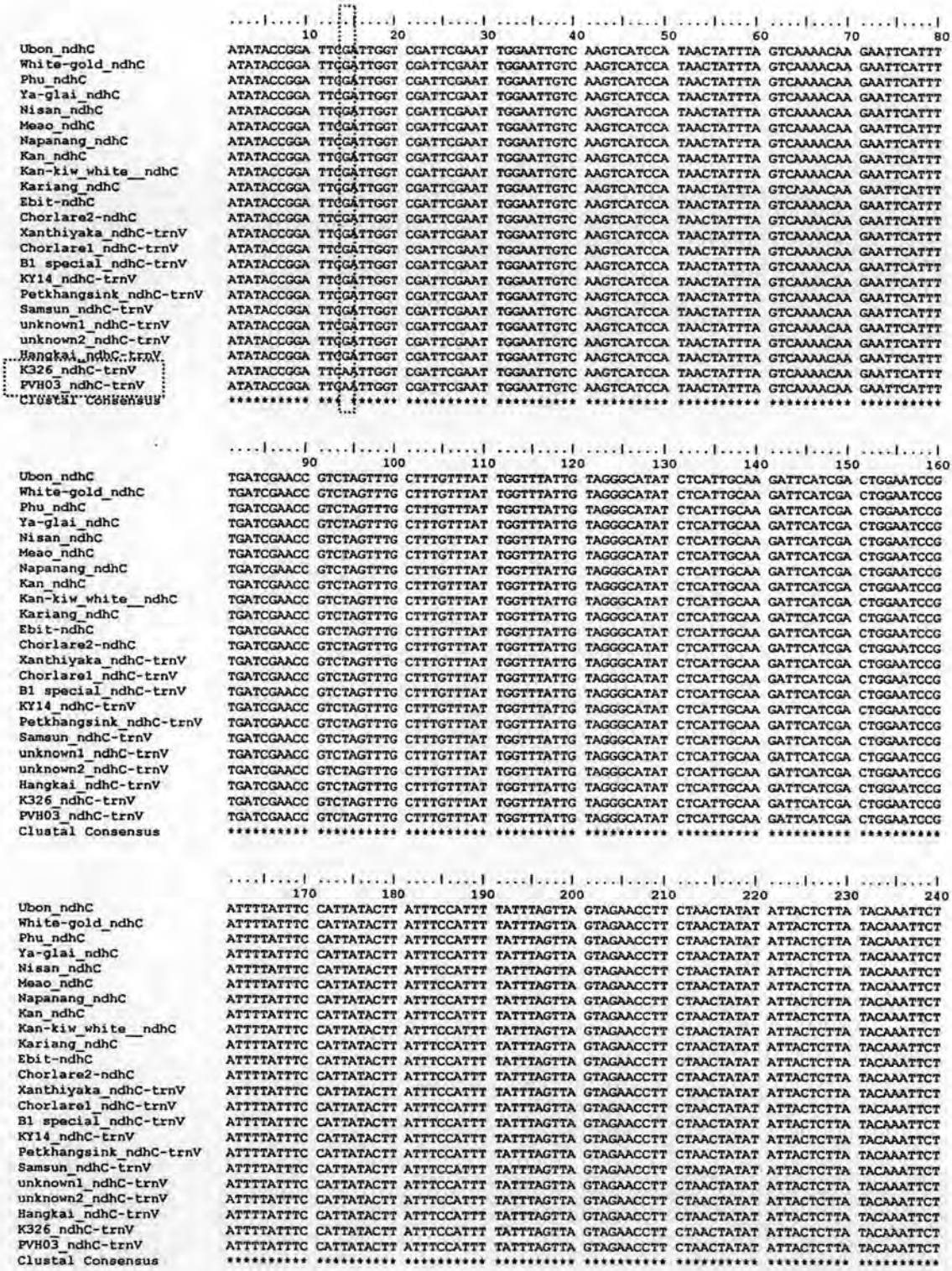

Figure 4.14 A 685 bp nucleotide data matrix of *ndh*C-*trn*V region from fresh-leaf samples of total 23 tobacco cultivars. A gap symbol (-) indicates an insertion or a deletion at the site.

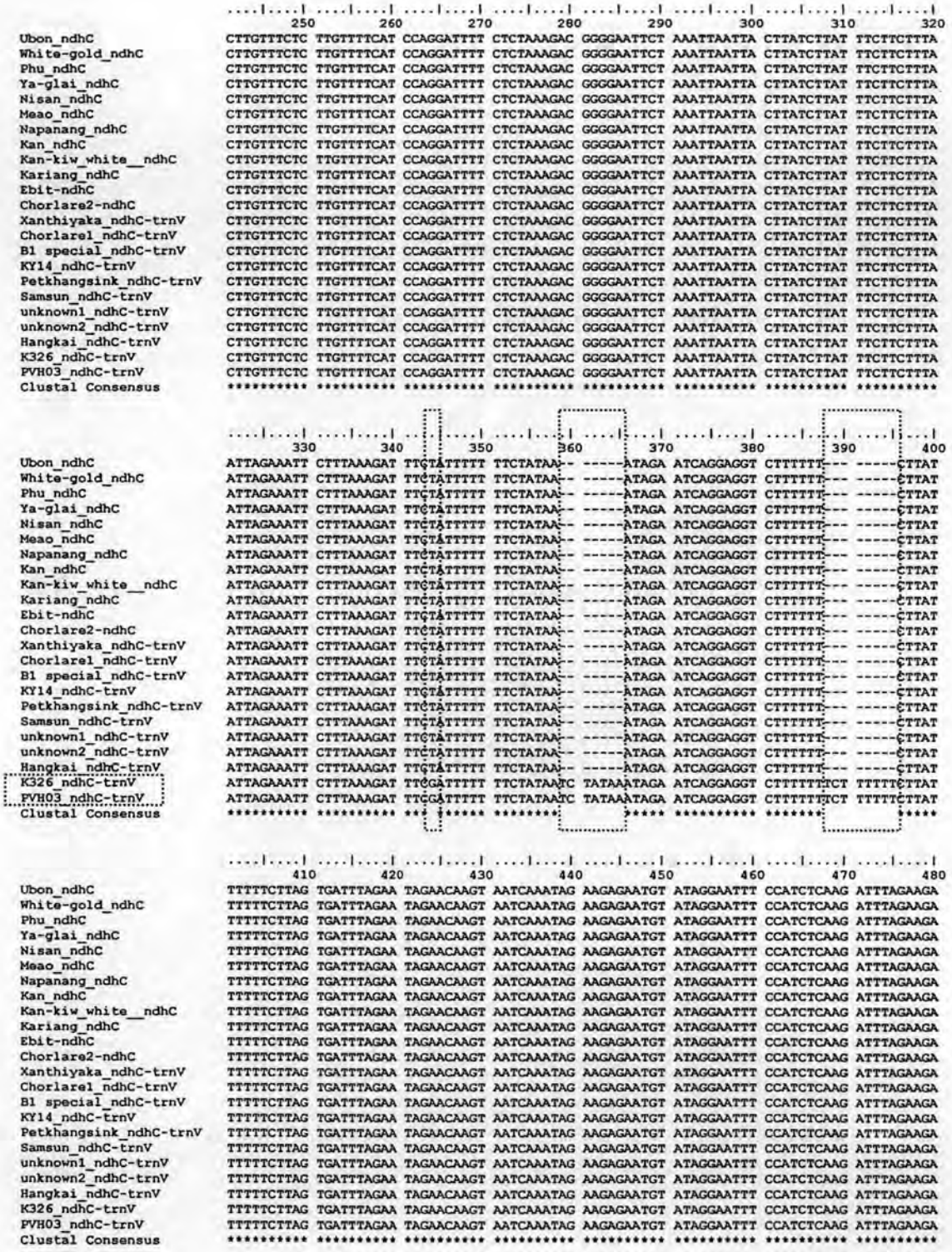

Figure 4.14 (continued)

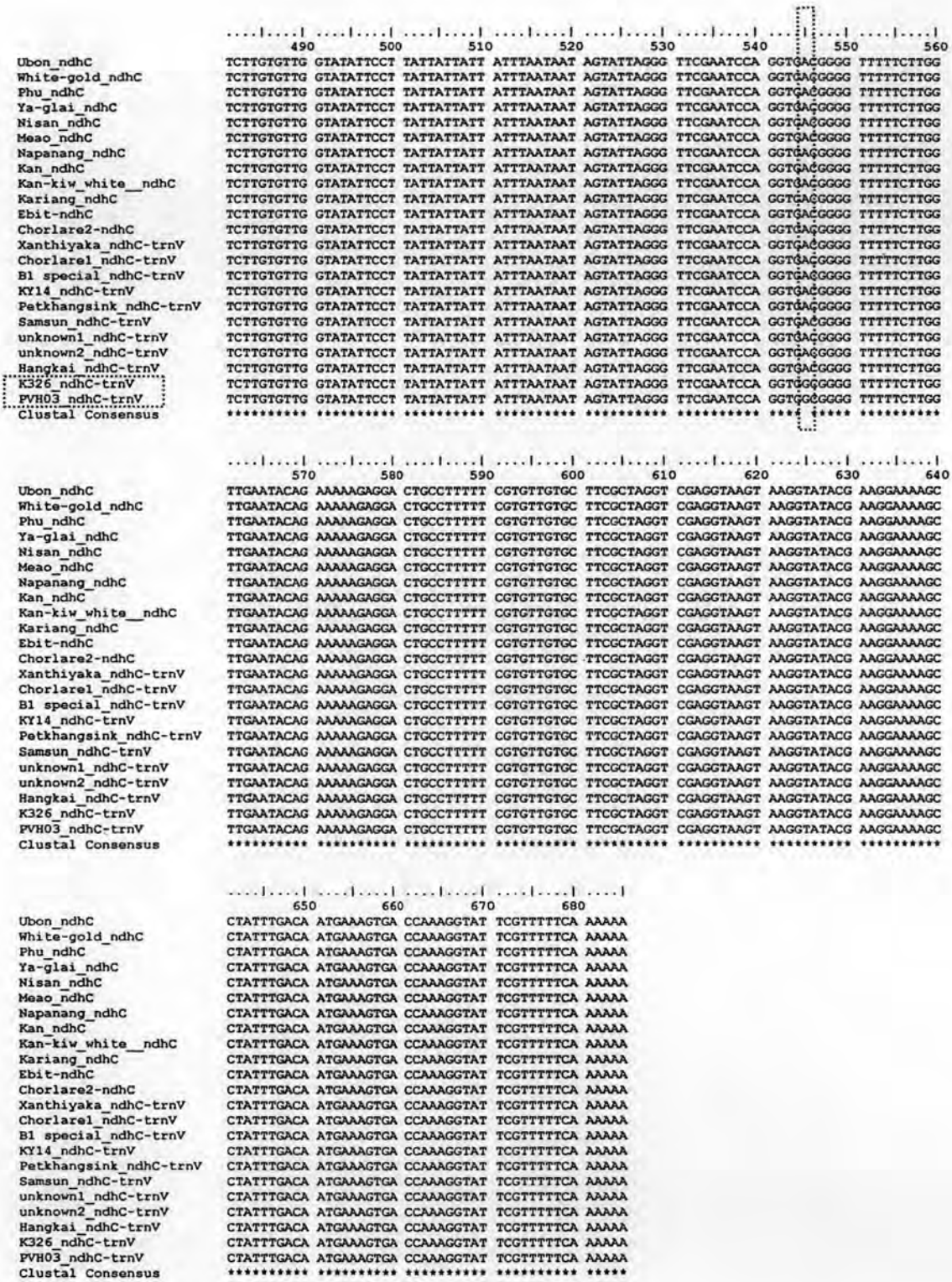

Figure 4.14 (continued)

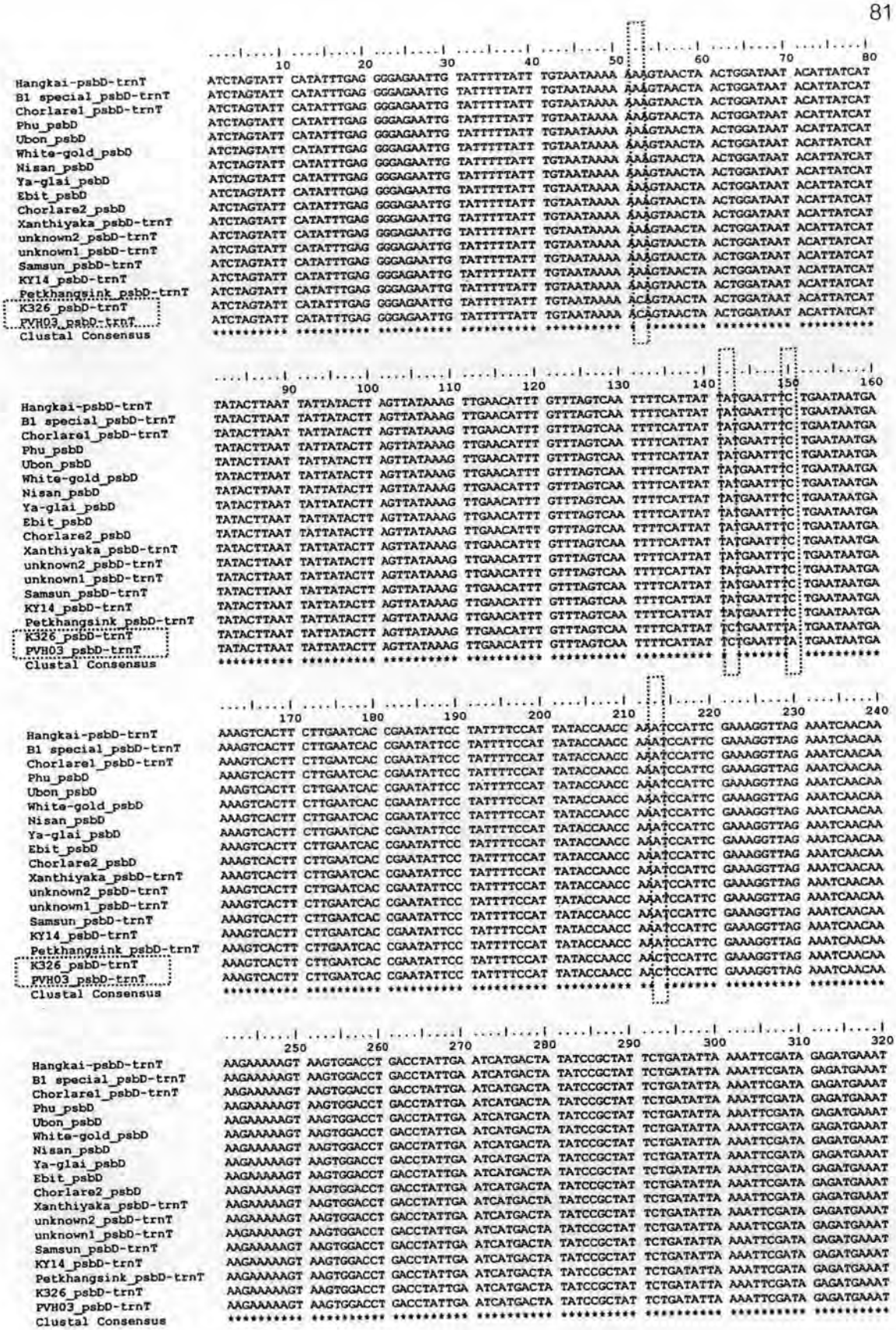

Figure 4.15 A 731 bp nucleotide data matrix of *psb*D-*trn*T region from fresh-leaf samples of total 18 tobacco cultivars. A gap symbol (-) indicates an insertion or a deletion at the site.

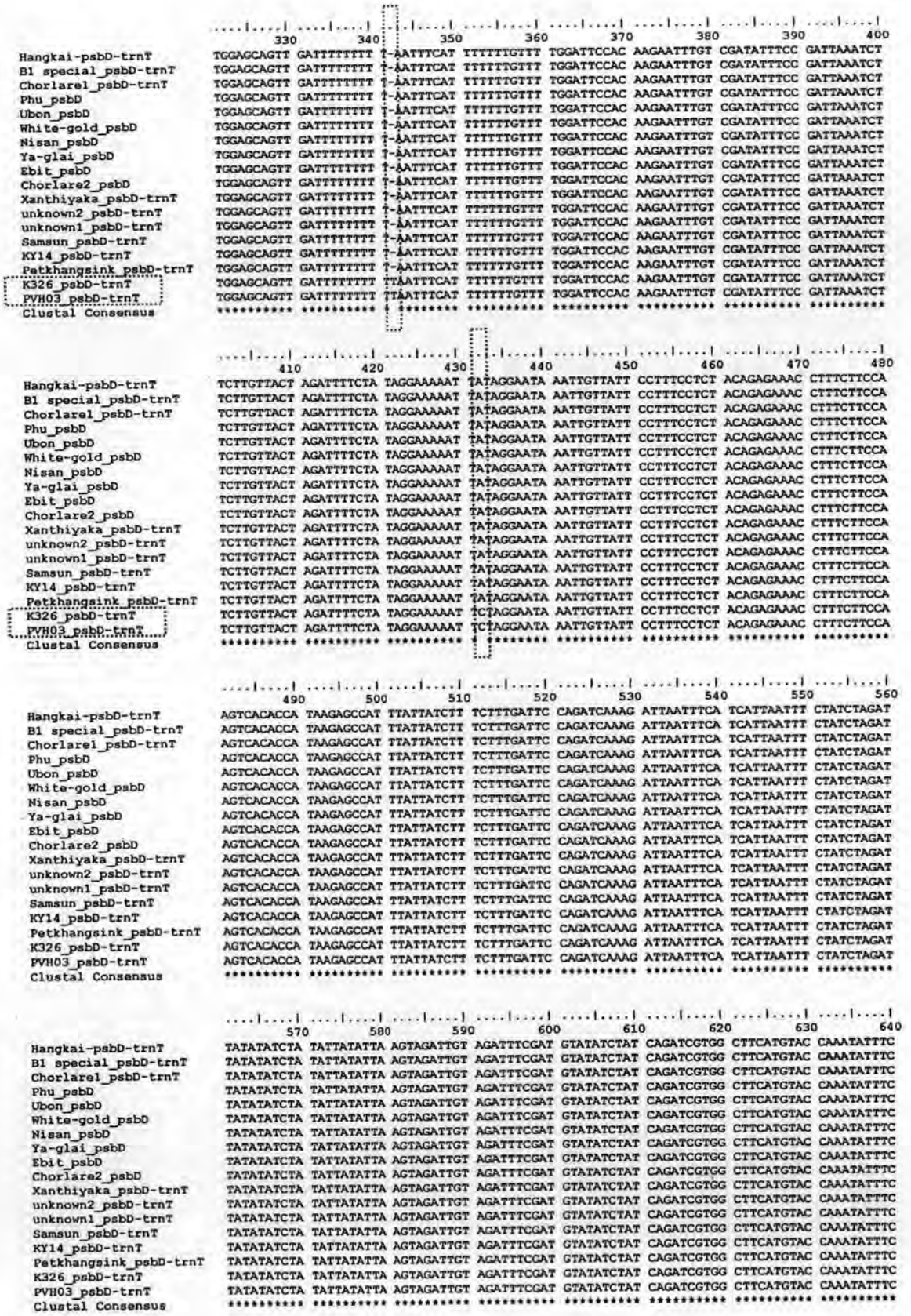

Figure 4.15 (continued)

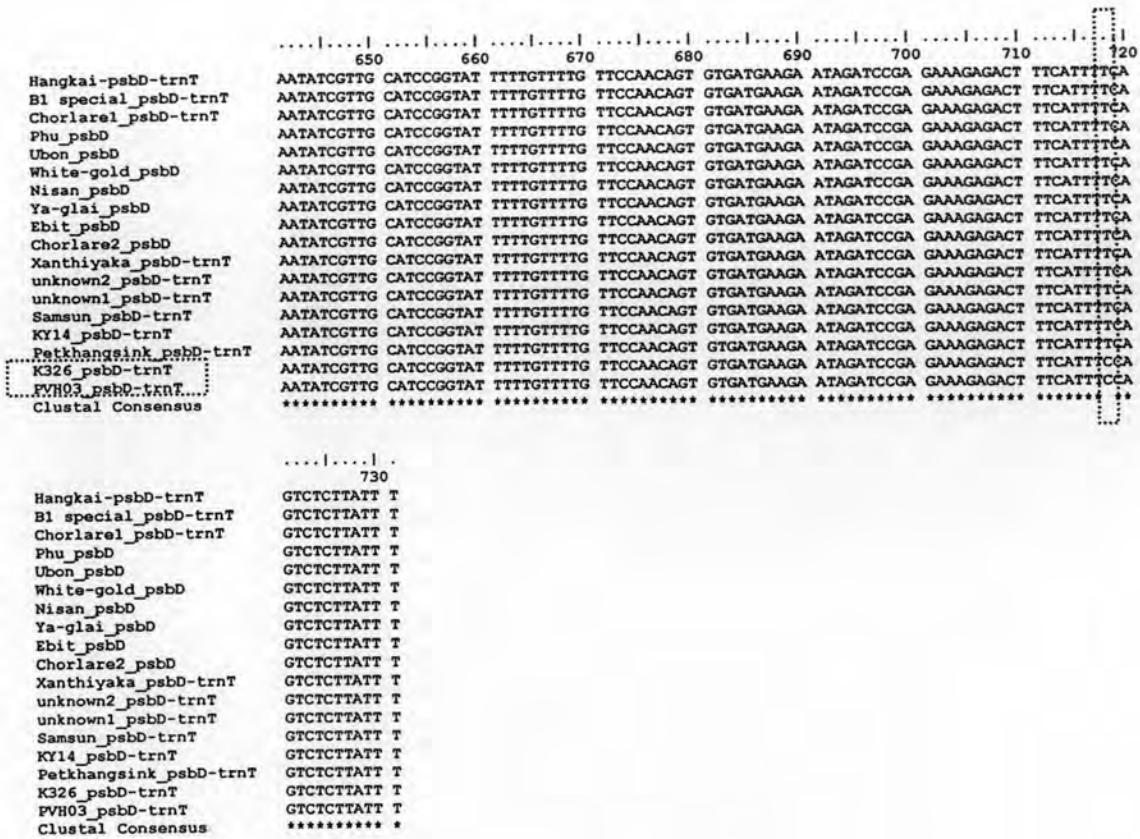

Figure 4.15 (continued)

From the alignment of *atp*I-*atp*H region of the genomic DNA extracted from 18 fresh-leaf tobacco samples (Figure 4.16), the aligned sequence length was 811 bp and the alignment revealed three base substitutions and one medium insertion (9 bp) at the positions 716-724. This alignment also distinguished K326 and PVH03 Virginia cultivars from the others. About the sequences of *ndh*F-*rpl*32 region (Figure 4.17), the aligned data matrix of this region was 769 bp long and totally showed three nucleotide substitutions and two medium insertions (6 and 13 bp) at the sites 708-713 and 673-685, respectively, which again separated K326 Virginia cultivar from the other four.

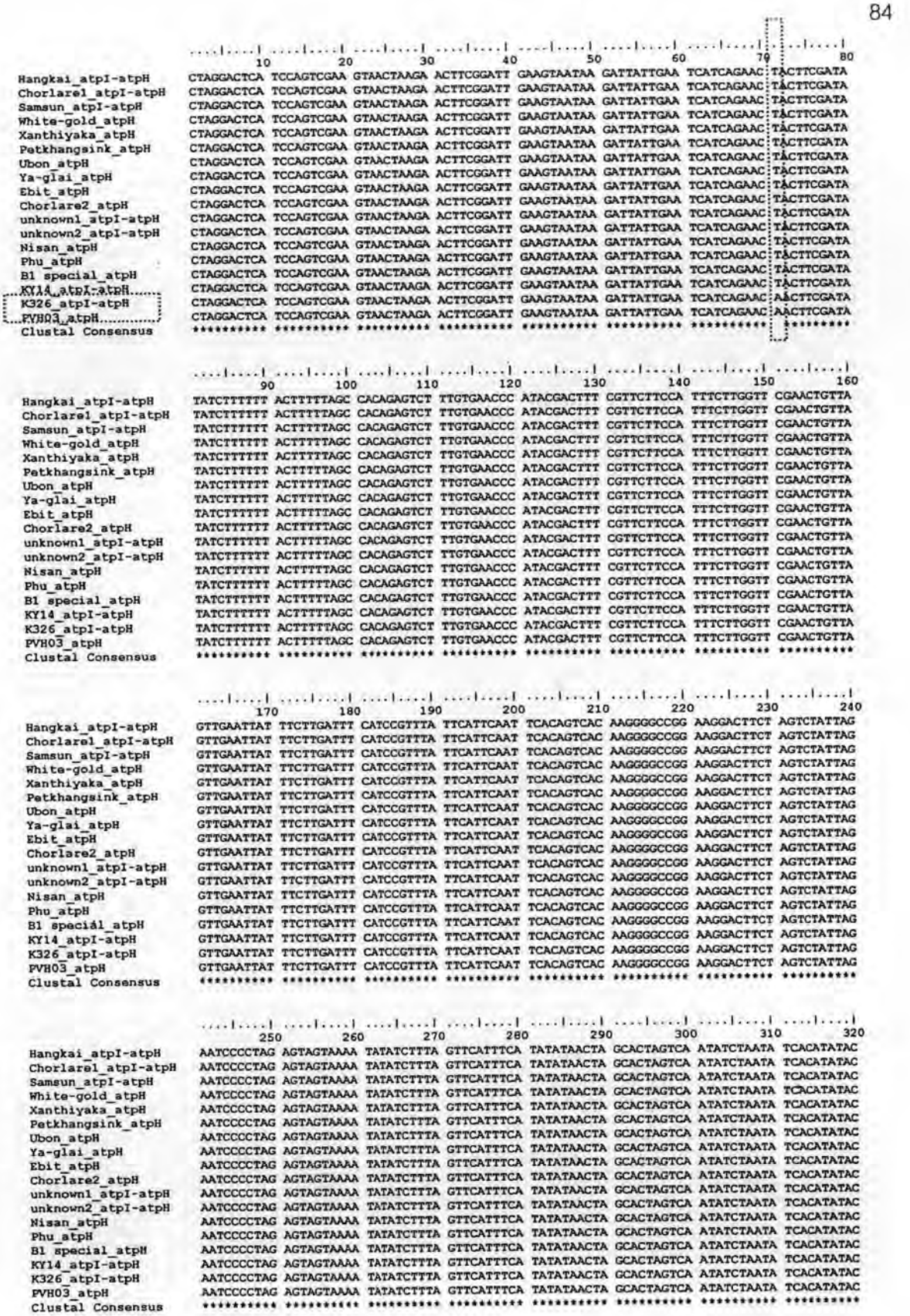

Figure 4.16 A 811 bp nucleotide data matrix of *atp*I-*atp*H region from fresh-leaf samples of total 18 tobacco cultivars. A gap symbol (-) indicates an insertion or a deletion at the site.

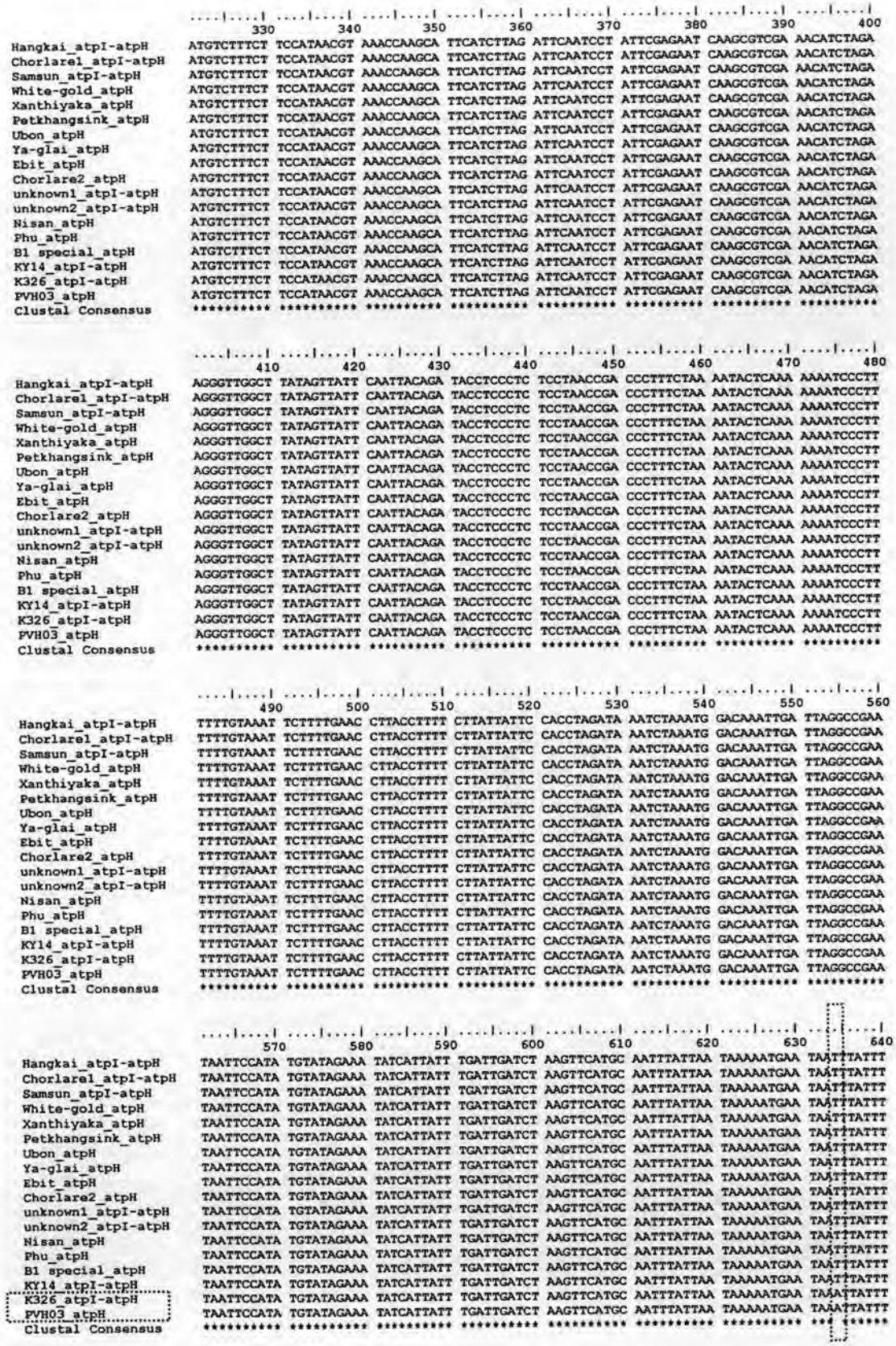

Figure 4.16 (continued)

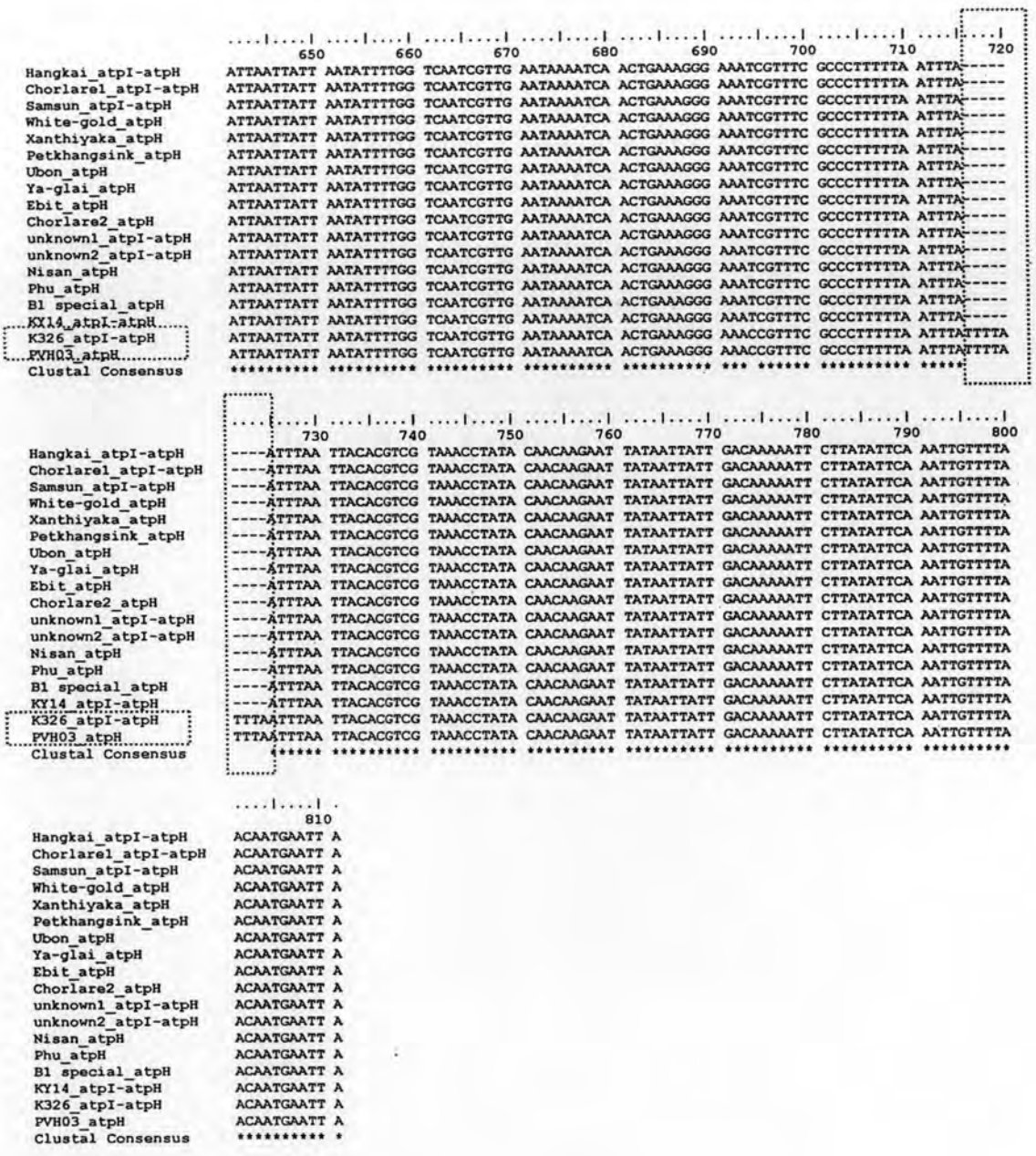

Figure 4.16 (continued)

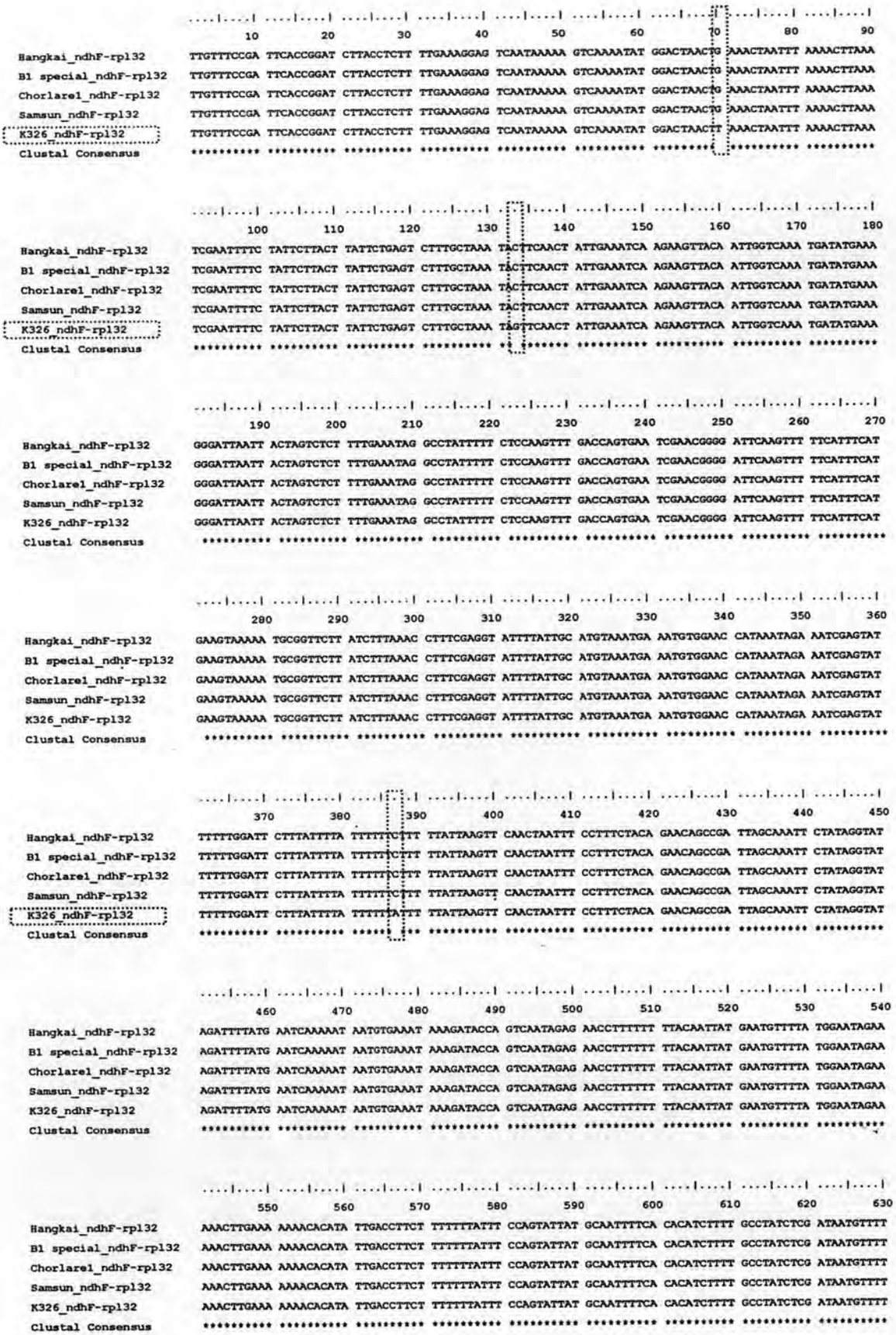

Figure 4.17 A 769 bp nucleotide data matrix of *ndh*F-*rpl*32 region from fresh-leaf samples of total five tobacco cultivars. A gap symbol (-) indicates an insertion or a deletion at the site.

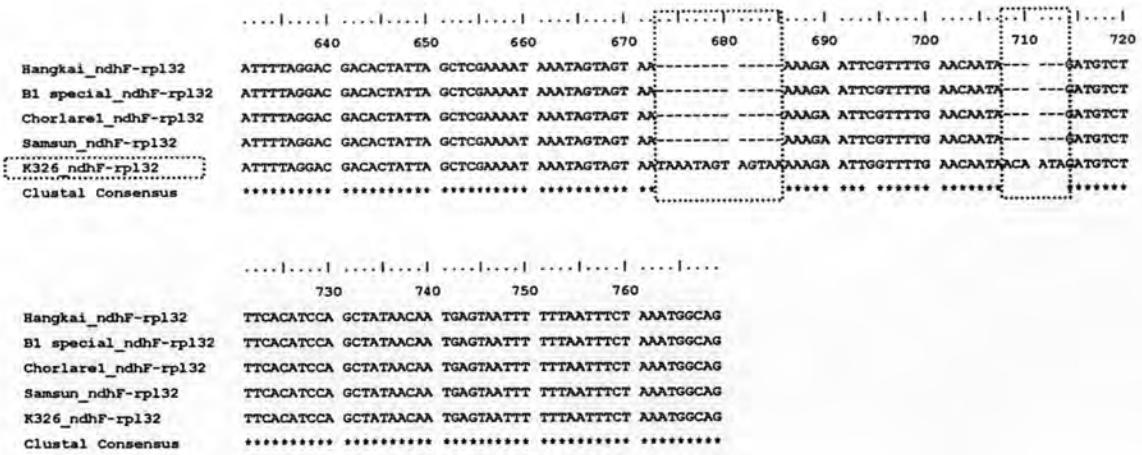

Figure 4.17 (continued)

For the last region, *rpl*32-*trn*L, the aligned sequences from 23 fresh-leaf tobacco samples (Figure 4.18) were 716 bp in length. Interestingly, not only this alignment could distinguish Virginia cultivar-group (K326 and PVH03 cultivars) from the other cultivars with nine nucleotide substitutions, but it also separated four local cultivars (Petkhangsink, Ubon Ratchathani, Kan and Hangkai) with a large 66 bp insertion at the 170-235 aligned sequence sites.

Therefore, this *rpl*32-*trn*L region was the best DNA target to amplify for genetic relationship analysis among all 43 fresh-leaf tobacco samples collected in this study. The alignment result of *rpl*32-*trn*L region of total 43 DNA samples was shown in Figure 4.19. It could separate six imported tobacco cultivars (K326, PVH03, PV09, HB01, HBO04P and TN97) from the others with totally nine base substitutions. Moreover, this alignment also distinguished five local cultivars (Petmakhuea, Petkhangsink, Ubon Ratchathani, Kan and Hangkai) from the others with a very large 66 bp insertion as expected.

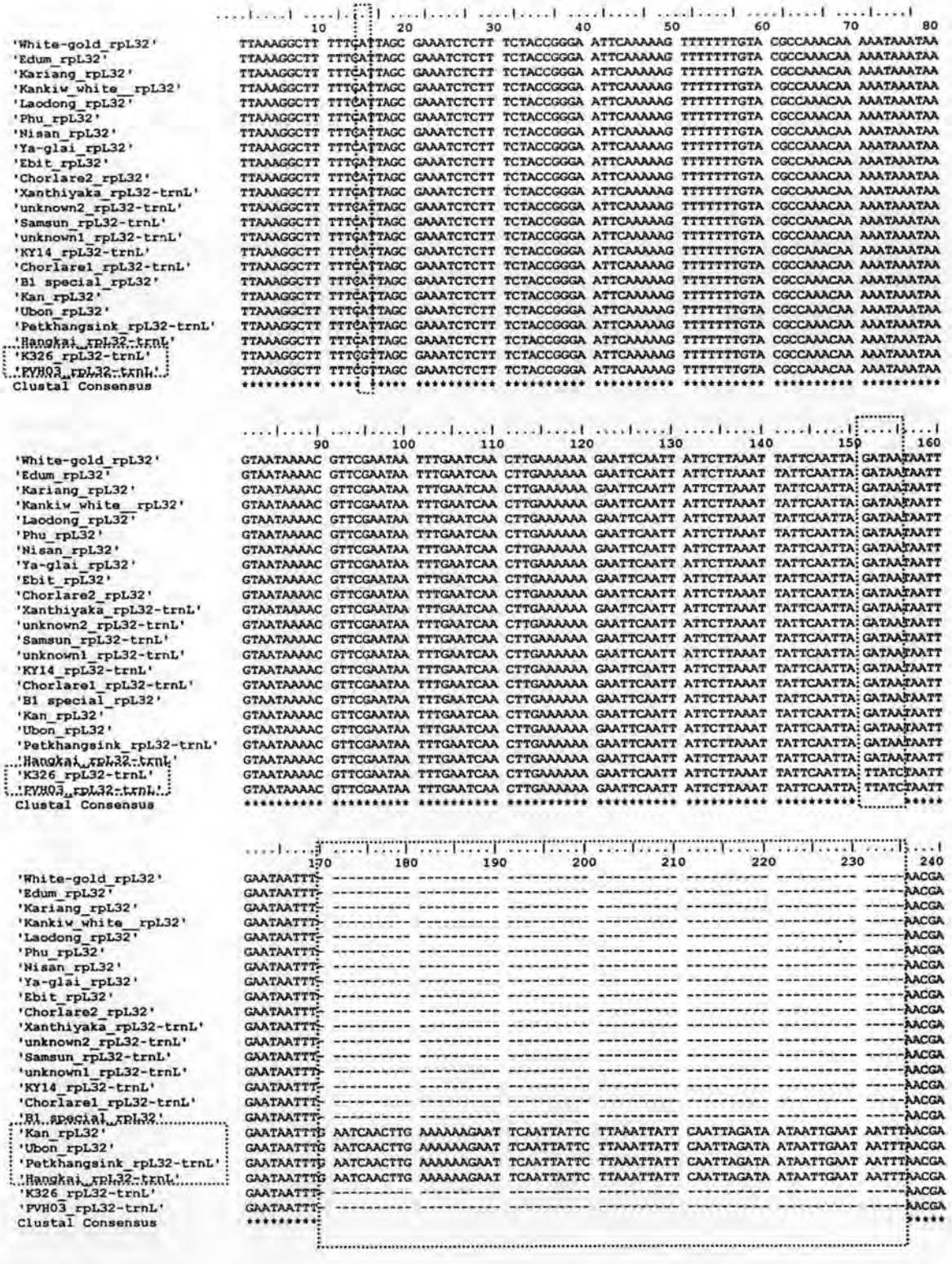

Figure 4.18 A 716 bp nucleotide data matrix of *rpl*32-*trn*L region from fresh-leaf samples of total 23 tobacco cultivars. A gap symbol (-) indicates an insertion or a deletion at the site.

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                                    250        260        270        280        290        300        310        320
'White-gold_rpL32'                  TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Edum_rpL32'                        TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Kariang_rpL32'                     TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Kankiw_white__rpL32'               TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Laodong_rpL32'                     TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Phu_rpL32'                         TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Nisan_rpL32'                       TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Ya-glai_rpL32'                     TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Ebit_rpL32'                        TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Chorlare2_rpL32'                   TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Xanthiyaka_rpL32-trnL'             TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'unknown2_rpL32-trnL'               TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Samsun_rpL32-trnL'                 TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'unknown1_rpL32-trnL'               TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'KY14_rpL32-trnL'                   TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Chorlare1_rpL32-trnL'              TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'B1 special_rpL32'                  TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Kan_rpL32'                         TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Ubon_rpL32'                        TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Petkhangsink_rpL32-trnL'           TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'Hangkai_rpL32-trnL'                TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTCGCTTC GCTTTTCTGA TTGATAGATA
'K326_rpL32-trnL'                   TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTGGCTTC GCTTTTCTGA TTGATAGATA
'PVH03_rpL32-trnL'                  TTTCCCTTTC ATATTTGATA TTGATTAGCT CACCAATCAA TACGTAATGG AACTGGCTTC GCTTTTCTGA TTGATAGATA
Clustal Consensus                   ********** ********** ********** ********** ********** **** ***** ********** **********

                                    330        340        350        360        370        380        390        400
'White-gold_rpL32'                  AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Edum_rpL32'                        AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Kariang_rpL32'                     AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
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'Phu_rpL32'                         AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Nisan_rpL32'                       AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Ya-glai_rpL32'                     AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Ebit_rpL32'                        AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Chorlare2_rpL32'                   AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Xanthiyaka_rpL32-trnL'             AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'unknown2_rpL32-trnL'               AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Samsun_rpL32-trnL'                 AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'unknown1_rpL32-trnL'               AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'KY14_rpL32-trnL'                   AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Chorlare1_rpL32-trnL'              AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'B1 special_rpL32'                  AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Kan_rpL32'                         AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Ubon_rpL32'                        AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Petkhangsink_rpL32-trnL'           AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Hangkai_rpL32-trnL'                AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'K326_rpL32-trnL'                   AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'PVH03_rpL32-trnL'                  AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
Clustal Consensus                   ********** ********** ********** ********** ********** ********** ********** **********

                                    410        420        430        440        450        460        470        480
'White-gold_rpL32'                  AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Edum_rpL32'                        AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Kariang_rpL32'                     AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
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'Phu_rpL32'                         AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
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'Ebit_rpL32'                        AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Chorlare2_rpL32'                   AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Xanthiyaka_rpL32-trnL'             AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'unknown2_rpL32-trnL'               AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Samsun_rpL32-trnL'                 AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'unknown1_rpL32-trnL'               AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'KY14_rpL32-trnL'                   AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
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'Hangkai_rpL32-trnL'                AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'K326_rpL32-trnL'                   AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'PVH03_rpL32-trnL'                  AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
Clustal Consensus                   ********** ********** ********** ********** ********** ********** ********** **********
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Figure 4.18 (continued)

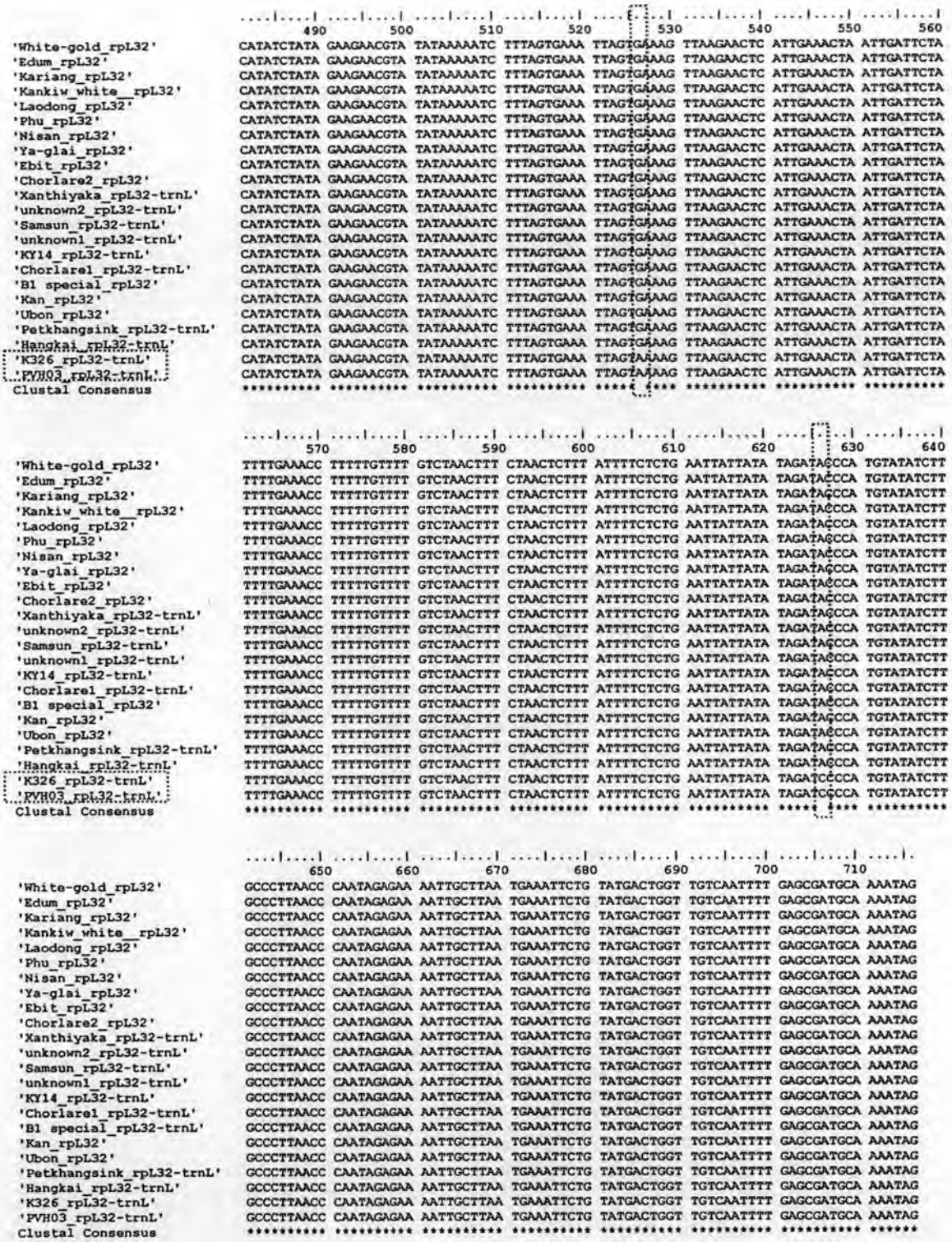

Figure 4.18 (continued)

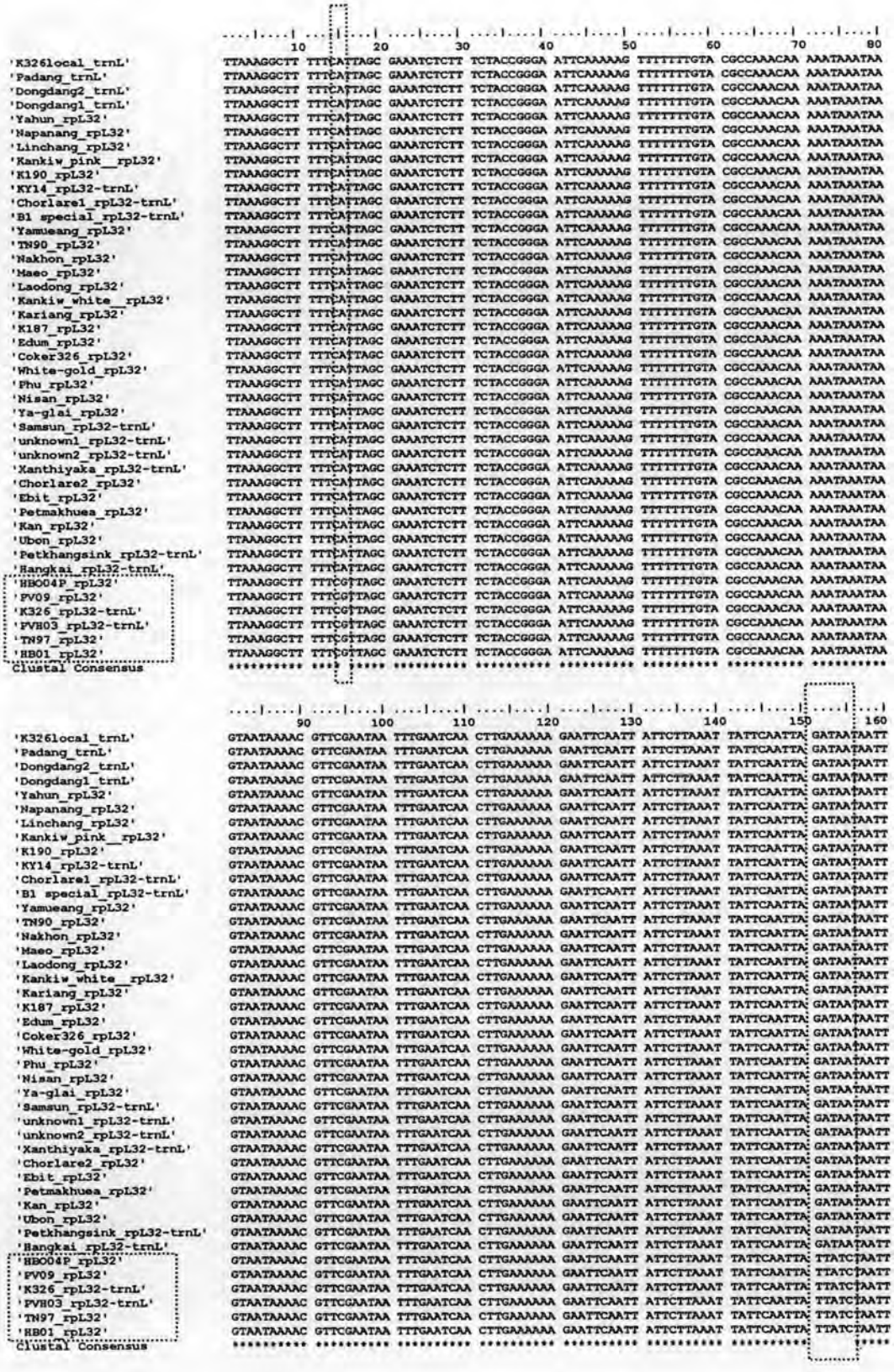

Figure 4.19 A 716 bp nucleotide data matrix of *rpl*32-*trn*L region from all fresh-leaf samples of total 43 tobacco cultivars. A gap symbol (-) indicates an insertion or a deletion at the site.

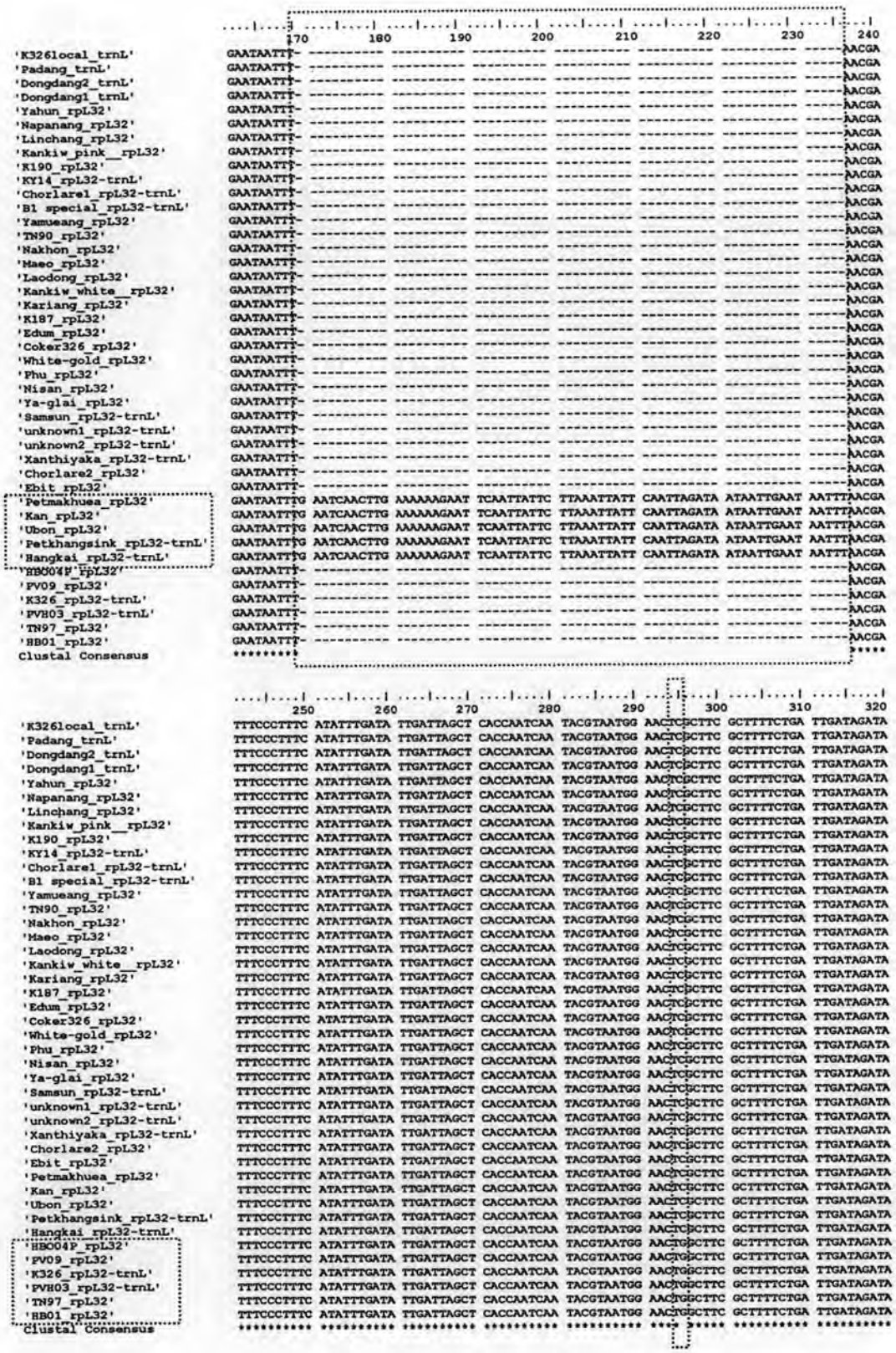

Figure 4.19 (continued)

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'Padang_trnL'             AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Dongdang2_trnL'          AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Dongdang1_trnL'          AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Yahun_rpL32'             AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Napanang_rpL32'          AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Linchang_rpL32'          AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Kankiw_pink__rpL32'      AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'K190_rpL32'              AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'KY14_rpL32-trnL'         AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Chorlare1_rpL32-trnL'    AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'B1 special_rpL32-trnL'   AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Yamueang_rpL32'          AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'TN90_rpL32'              AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Nakhon_rpL32'            AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Maeo_rpL32'              AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Laodong_rpL32'           AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
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'Edum_rpL32'              AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Coker326_rpL32'          AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'White-gold_rpL32'        AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Phu_rpL32'               AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Nisan_rpL32'             AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Ya-glai_rpL32'           AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Samsun_rpL32-trnL'       AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'unknown1_rpL32-trnL'     AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'unknown2_rpL32-trnL'     AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Xanthiyaka_rpL32-trnL'   AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Chorlare2_rpL32'         AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
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'Kan_rpL32'               AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
'Ubon_rpL32'              AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
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'Hangkai_rpL32-trnL'      AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
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'K326_rpL32-trnL'         AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
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'HB01_rpL32'              AAATAATAGA ATTAGGAAAT CCTCTATTTA CTGAATAATA ACTTTTTTGT TGACAAAAGA GTAAACATCA TTTCTATTCC
Clustal Consensus         ********** ********** ********** ********** ********** ********** ********** **********

                                  410       420       430       440       450       460       470       480
'K326local_trnL'          AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Padang_trnL'             AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Dongdang2_trnL'          AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Dongdang1_trnL'          AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Yahun_rpL32'             AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
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'B1 special_rpL32-trnL'   AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
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'Coker326_rpL32'          AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'White-gold_rpL32'        AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Phu_rpL32'               AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Nisan_rpL32'             AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Ya-glai_rpL32'           AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Samsun_rpL32-trnL'       AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'unknown1_rpL32-trnL'     AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'unknown2_rpL32-trnL'     AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Xanthiyaka_rpL32-trnL'   AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Chorlare2_rpL32'         AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Ebit_rpL32'              AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Petmakhuea_rpL32'        AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Kan_rpL32'               AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Ubon_rpL32'              AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Petkhangsink_rpL32-trnL' AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'Hangkai_rpL32-trnL'      AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'HBO04P_rpL32'            AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'PV09_rpL32'              AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'K326_rpL32-trnL'         AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'PVH03_rpL32-trnL'        AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'TN97_rpL32'              AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
'HB01_rpL32'              AAGGTGGGGA GTTTCATTTT CCCCATCGAC CTATTTGCAG AATTCCATTA AAAAAAAATT CTATATTTCC ATTCTATTTC
Clustal Consensus         ********** ********** ********** ********** ********** ********** ********** **********
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Figure 4.19 (continued)

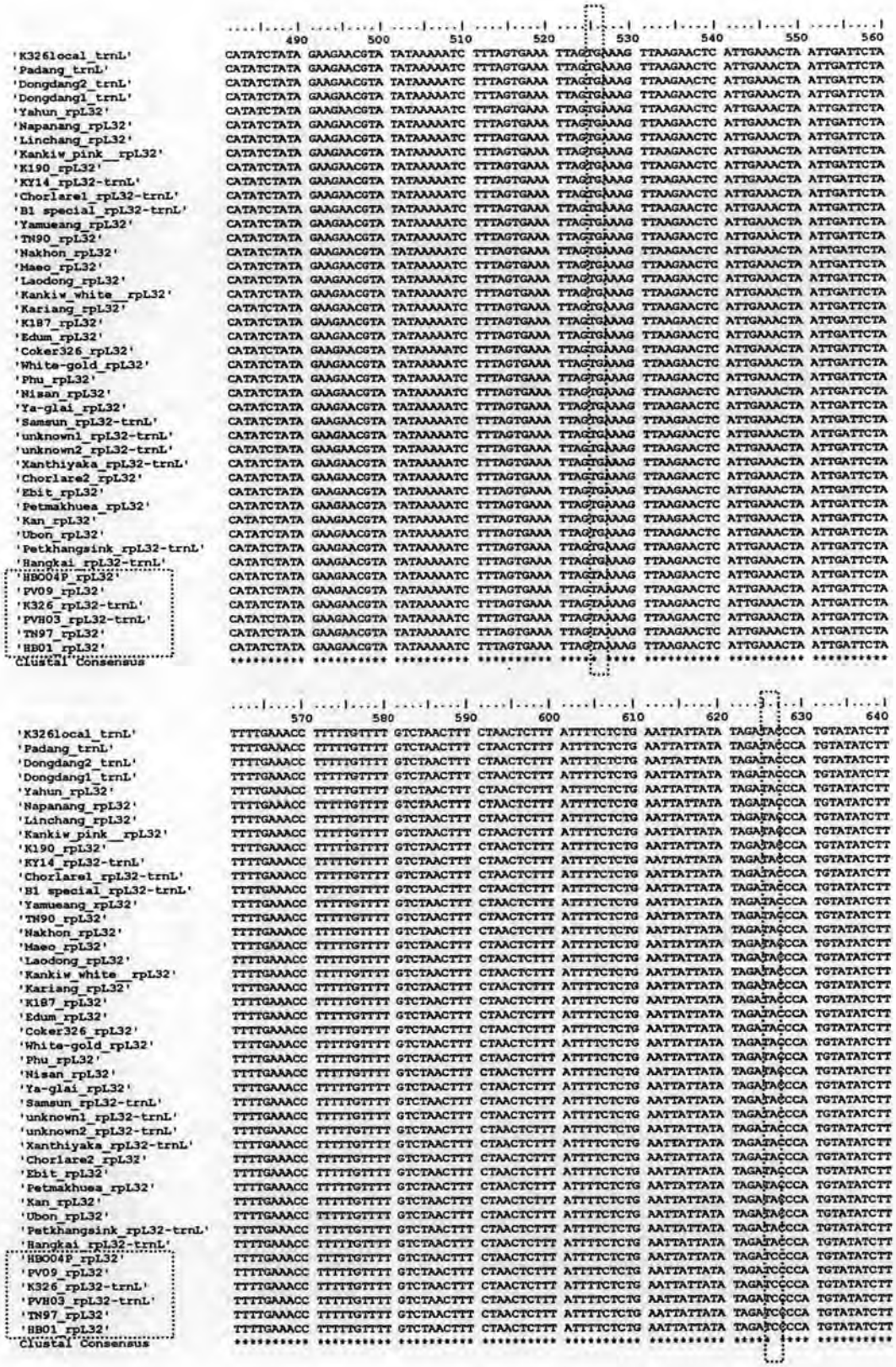

Figure 4.19 (continued)

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'Dongdang2_trnL'          GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Dongdang1_trnL'          GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Yahun_rpL32'             GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Napanang_rpL32'          GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Linchang_rpL32'          GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Kankiw_pink__rpL32'      GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'K190_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'KY14_rpL32-trnL'         GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Chorlare1_rpL32-trnL'    GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'B1 special_rpL32-trnL'   GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Yamueang_rpL32'          GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'TN90_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Nakhon_rpL32'            GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Maeo_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Laodong_rpL32'           GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Kankiw_white__rpL32'     GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Kariang_rpL32'           GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'K187_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Edum_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Coker326_rpL32'          GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'White-gold_rpL32'        GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Phu_rpL32'               GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Nisan_rpL32'             GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Ya-glai_rpL32'           GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Samsun_rpL32-trnL'       GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'unknown1_rpL32-trnL'     GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'unknown2_rpL32-trnL'     GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Xanthiyaka_rpL32-trnL'   GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Chorlare2_rpL32'         GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Ebit_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Petmakhuea_rpL32'        GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Kan_rpL32'               GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Ubon_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Petkhangsink_rpL32-trnL' GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'Hangkai_rpL32-trnL'      GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'HB004P_rpL32'            GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'PV09_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'K326_rpL32-trnL'         GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'PVH03_rpL32-trnL'        GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'TN97_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
'HB01_rpL32'              GCCCTTAACC CAATAGAGAA AATTGCTTAA TGAAATTCTG TATGACTGGT TGTCAATTTT GAGCGATGCA AAATAG
Clustal Consensus         ********** ********** ********** ********** ********** ********** ********** ******
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Figure 4.19 (continued)

4.2.2 Amplification of *rpl*32-*trn*L region from cured leaves and roll-your-own tobacco samples

From the extracted genomic DNA of 24 cured-leaf samples and three roll-your-own tobaccos, 19 tobacco DNA samples could be amplified with primers of the selected *rpl*32-*trn*L region. These 19 DNA samples consisted of ten imported cultivars and seven local cultivars (lanes 3, 5, 8, 10 and 11 in Figure 4.20, for example) and two roll-your-own tobacco DNA (examples in Figure 4.21). Although the *rpl*32-*trn*L amplification reactions of the eight failed tobacco samples (Kariang, White gold, Phu, E-lueang, Hangkai, Yamueang and Laodong local tobacco cultivars and another Maew roll-your-own tobacco) were redone, they still could not give any amplified products. The PCR products of 19 successfully amplified DNA samples were estimated to be around 1200 bp in length.

Only the PCR products of seven local tobacco cultivars (E-dum, Ya-glai, K326 local, Kan-kiw dok-khao, Kan, Bai-tung and Bai-lai) and two amplifiable roll-your-own tobaccos (Mae-somsong red-package and Mae-somsong white-package) were brought to sequencing. This was because there were enough *rpl*32-*trn*L sequences of imported cultivars (14 sequences) for the genetic relationship analysis and only some more data of local cultivars and roll-your-own tobaccos were needed since it may help differentiating local and imported cultivars.

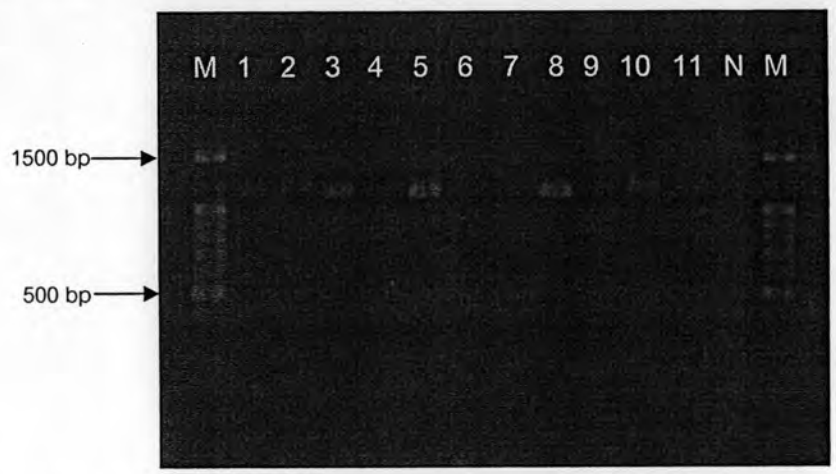

Figure 4.20 PCR products of *rpl*32-*trn*L region from cured-leaf samples of 11 local tobacco cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-11 = E-dum, Kan, Kan-kiw dok-khao, Kariang, Ya-glai, White gold, Phu, K326 local, E-lueang, Bai-lai and Bai-tung cultivars, respectively and lane N = negative control)

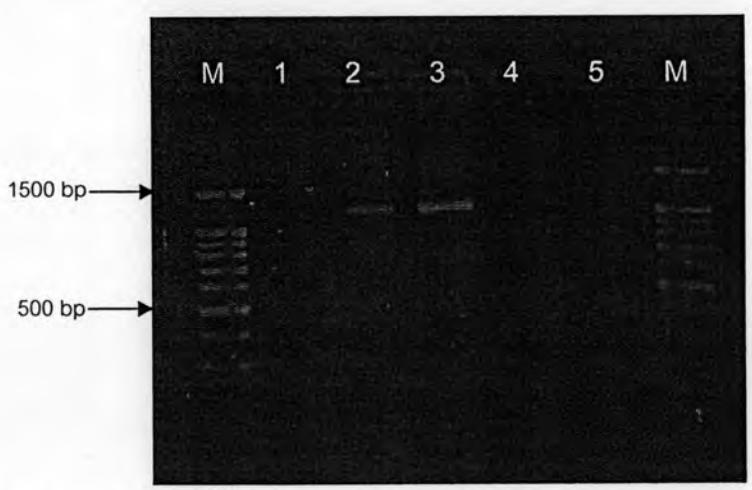

Figure 4.21 PCR products of *rpl*32-*trn*L region from three roll-your-own tobaccos and two cured-leaf samples of local tobacco cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-3 = roll-your-own tobacco: Maew, Mae-somsong red-package and Mae-somsong white-package and no. 4-5 = cured-leaf samples: Ya-glai and E-lueang cultivars, respectively).

Almost all *rpl*32-*trn*L sequences of seven PCR products from cured-leaf samples were clear with low noise signals. However, only the PCR product of Mae-somsong (white package) roll-your-own tobacco could give a clear nucleotide sequence whereas the sequence of Mae-somsong (red package) had high noise signals all along the length of sequence. Though repeatedly analysed, this failed sequencing were still persist and result could not be used further.

Totally 51 *rpl*32-*trn*L sequences amplified from 43 fresh-leaves, seven cured-leaves and one roll-your-own tobacco samples were aligned together and resulted in a 716 bp aligned sequence matrix (Figure 4.22). This newly aligned matrix revealed that six imported cultivars (K326, PVH03, PV09, HB01, HBO04P and TN97) were distinguished from the other tobacco samples with nine base substitutions. Moreover, eight DNA samples of seven local tobacco cultivars (Hangkai, Kan, Petmakhuea, Ubon Ratchathani, Petkhangsink, Baitung (cured leaf), Bailai (cured leaf)) and that of Maesomsong roll-your-own tobacco were uniquely separated from the other cultivars with a large 66 bp insertion. Consequently, these sequence results were further used for the genetic relationship analysis of tobacco cultivars.

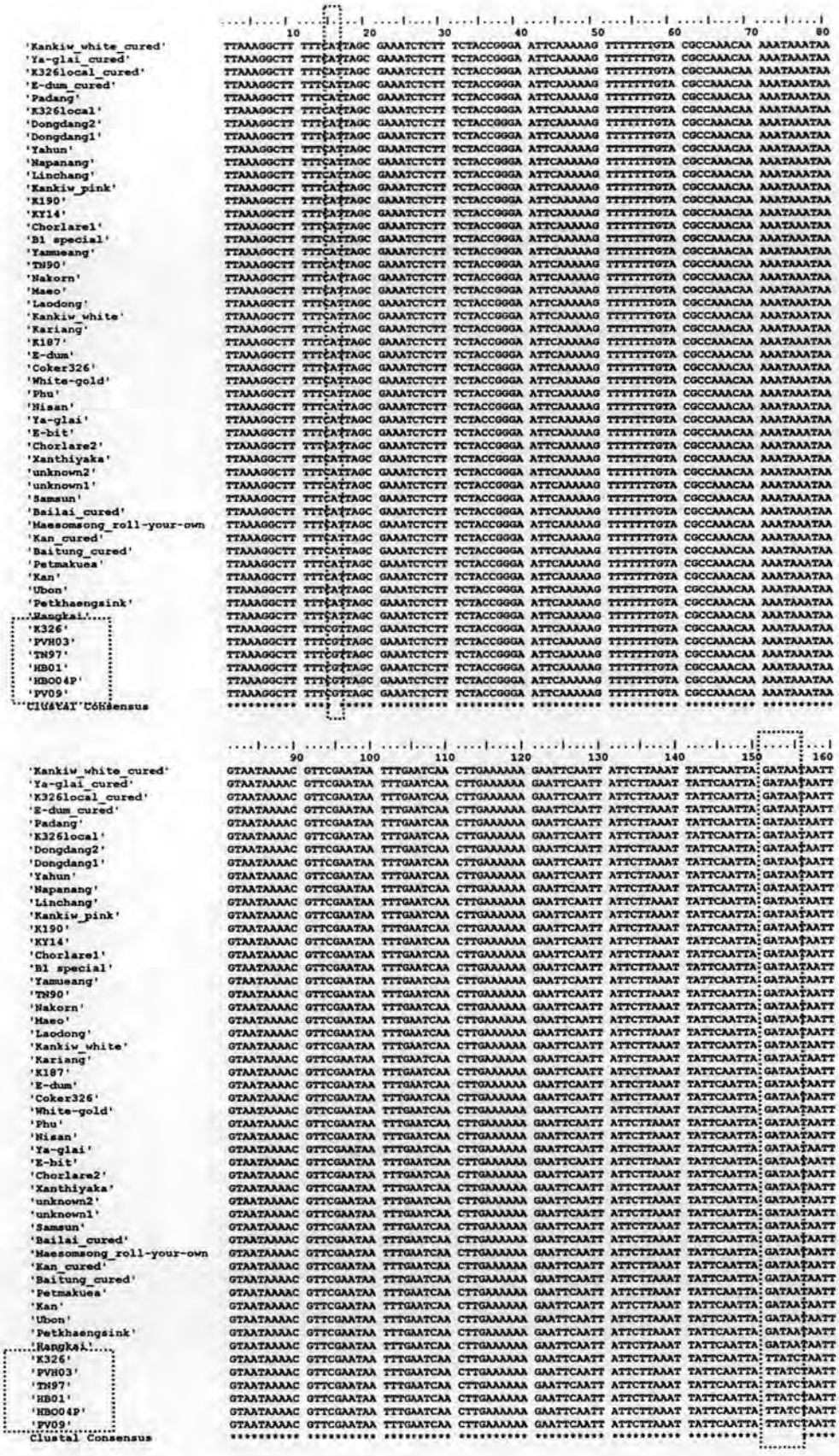

Figure 4.22 A 716 bp nucleotide data matrix of *rpl*32-*trn*L region from total 51 fresh-leaf, cured-leaf or roll-your-own tobacco samples. A gap symbol (-) indicates an insertion or a deletion at the site.

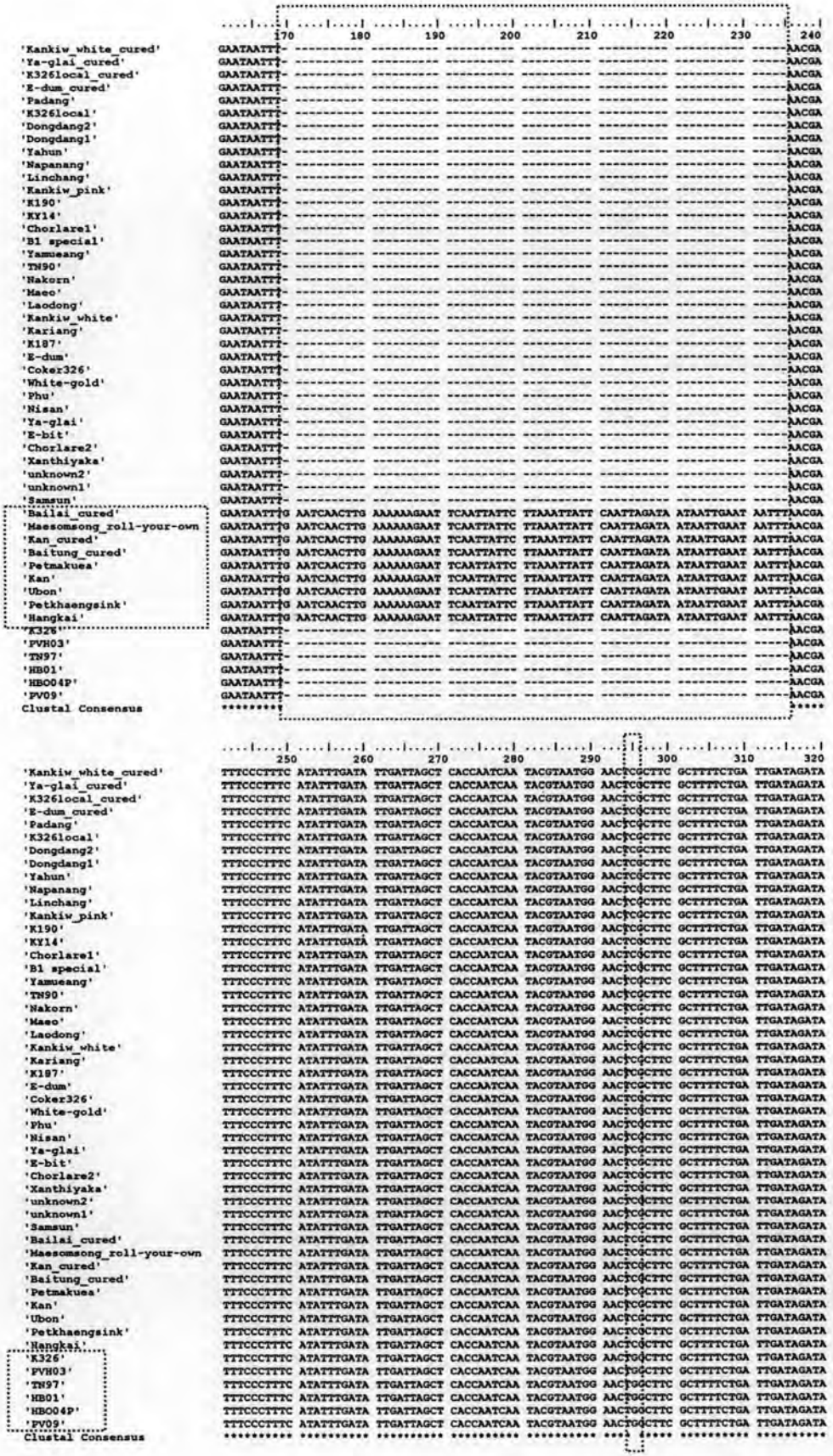

Figure 4.22 (continued)

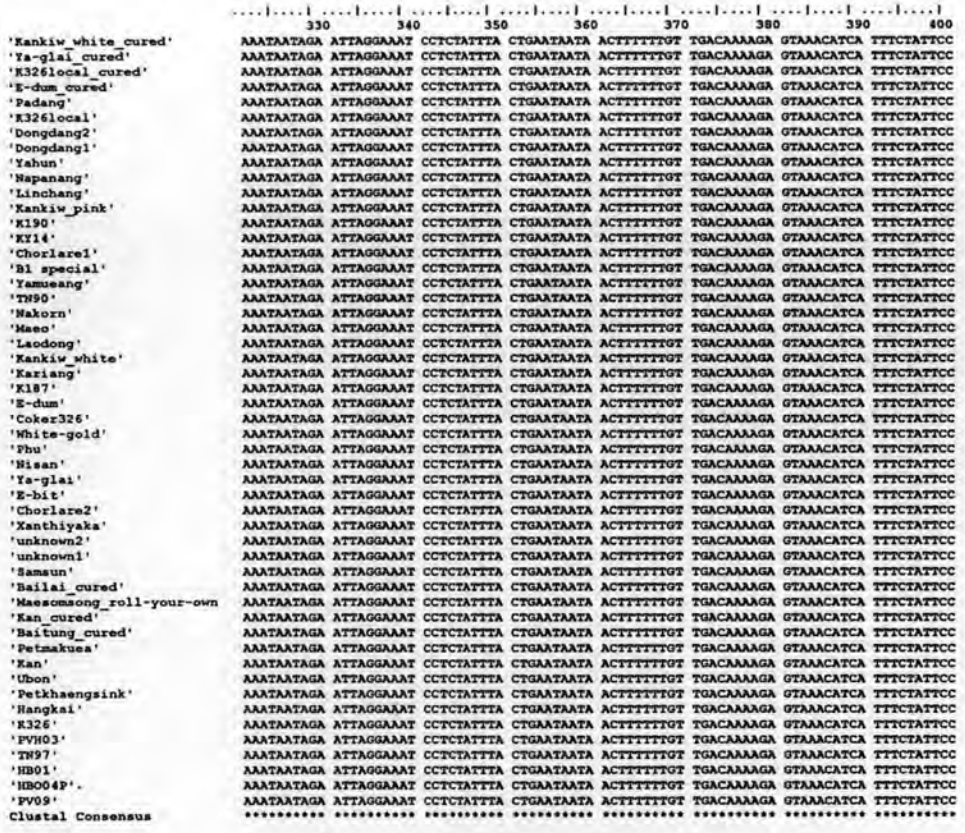

Figure 4.22 (continued)

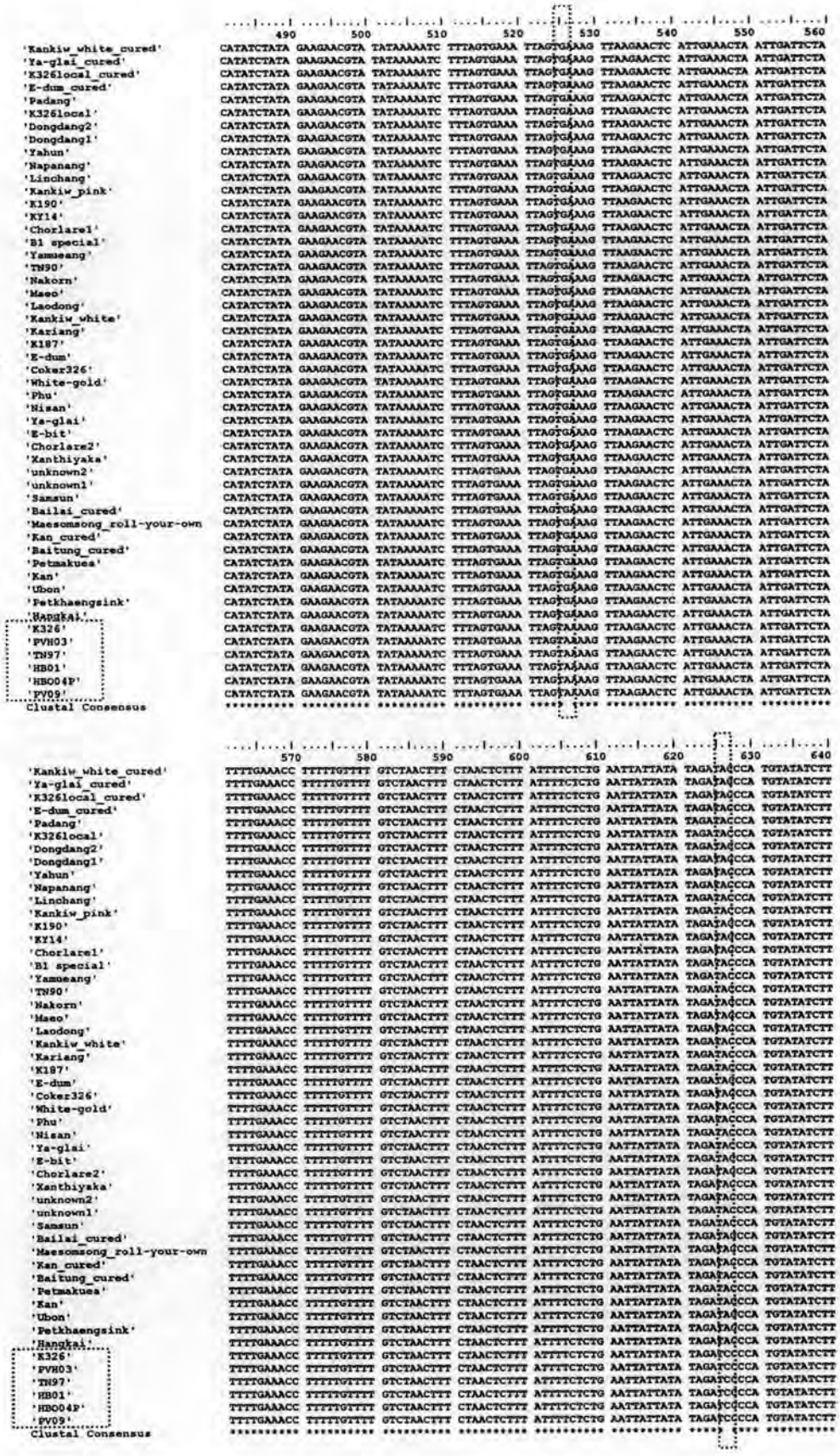

Figure 4.22 (continued)

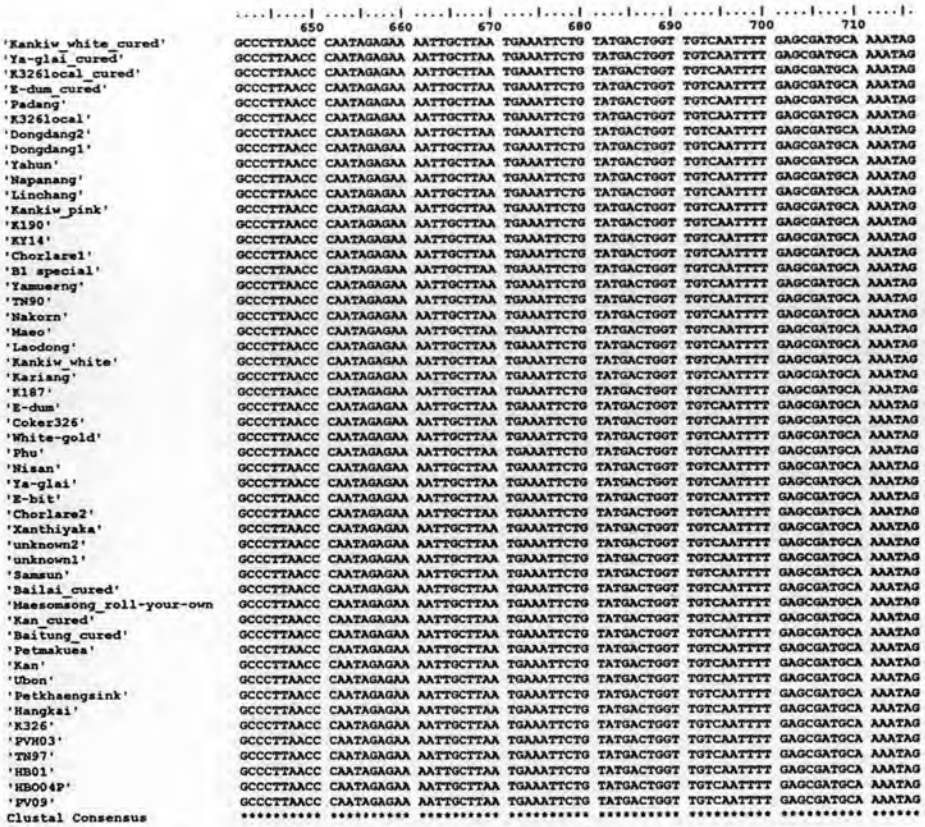

Figure 4.22 (continued)

4.3 Genetic relationship analysis of tobacco cultivars in Thailand

From the aligned sequence results of the six noncoding chloroplast DNA (cpDNA) regions (*pet*A-*psb*J, *ndh*C-*trn*V, *psb*D-*trn*T, *atp*I-*atp*H, *ndh*F-*rpl*32 and *rpl*32-*trn*L), the degrees of polymorphism in nucleotide sequence characteristics among different tobacco cultivars were calculated and shown in Table 4.2. The nucleotide polymorphisms of the six regions were found in both of the sequence lengths (from 1 to 66 bp differences) and the amounts of base substitutions (from 0.37% to 1.26%). The sequence polymorphism of *rpl*32-*trn*L region was very high and had the highest value of a variability percentage and a number of potentially informative characters (PICs): 10.47% and 75 PICs, respectively. The PIC value and the percentage of variability of *pet*A-*psb*J region were moderately high with 26 PICs and 3.45%, respectively, while the DNA sequence polymorphism of *ndh*F-*rpl*32 region was also moderately high, showing 23 PICs and 2.99% variability.

The DNA sequences of *ndh*C-*trn*V region revealed its polymorphism to be 18 PICs and 2.63% variability whereas twelve PICs and 1.48% variability were estimated from the nucleotide polymorphism in *atp*I-*atp*H region. The last region, *psbD-trnT*, had the lowest degree of DNA polymorphism of both the PIC value and the percentage of variability which were only 7 PICs and 1% variability, respectively.

Table 4.2 Degrees of polymorphism in nucleotide sequence characteristics of the six selected chloroplast noncoding regions.

Region	Base substitution	Indel	% variability	PIC
*rpl*32-*trn*L	9 bp (1.26%)	66 bp	10.47	75
*pet*A-*psb*J	3 bp (0.40%)	20 bp, 3 bp	3.45	26
*ndh*F-*rpl*32	4 bp (0.52%)	13 bp, 6 bp	2.99	23
*ndh*C-*trn*V	3 bp (0.44%)	8 bp, 7 bp	2.63	18
*atp*I-*atp*H	3 bp (0.37%)	9 bp	1.48	12
*psb*D-*trn*T	6 bp (0.82%)	1 bp	1.0	7

A phylogenetic tree analysis was performed to study genetic relationships between these 50 tobacco samples of local and imported cultivars and roll-your-own tobacco. The Neighbour-Joining (NJ) phylogram of *rpl*32-*trn*L sequence data revealed three clusters of tobacco cultivars having bootstrap values higher than 50% (Figure 4.23). The Cluster I consisted of almost all local cultivars (26 cultivars: 22 from fresh leaves and 4 from cured leaves), three Burley cultivars (B1 special, TN90 and KY14), all two Turkish cultivars (Samsun and Xanthiyaka), some Virginia cultivars (Coker 326, K187 and K190) and two samples of unknown cultivars. Cluster II clearly represented a special grouping of the other nine samples of seven local cultivars (Hangkai, Kan (both samples from fresh and cured leaves), Petmakhuea, Ubon Ratchathani, Petkhangsink, Bai-tung (cured leaf), Bai-lai (cured leaf) and Mae-somsong (roll-your-own tobacco)). This cluster II was supported with 63% bootstrap value. The last grouping, Cluster III, included the other three Virginia cultivars (PVH03, PV09 and K326) and three Burley (TN97, HB01 and HBO04P). This cluster was considered as having very strongly close relationship with 100% bootstrap supporting value.

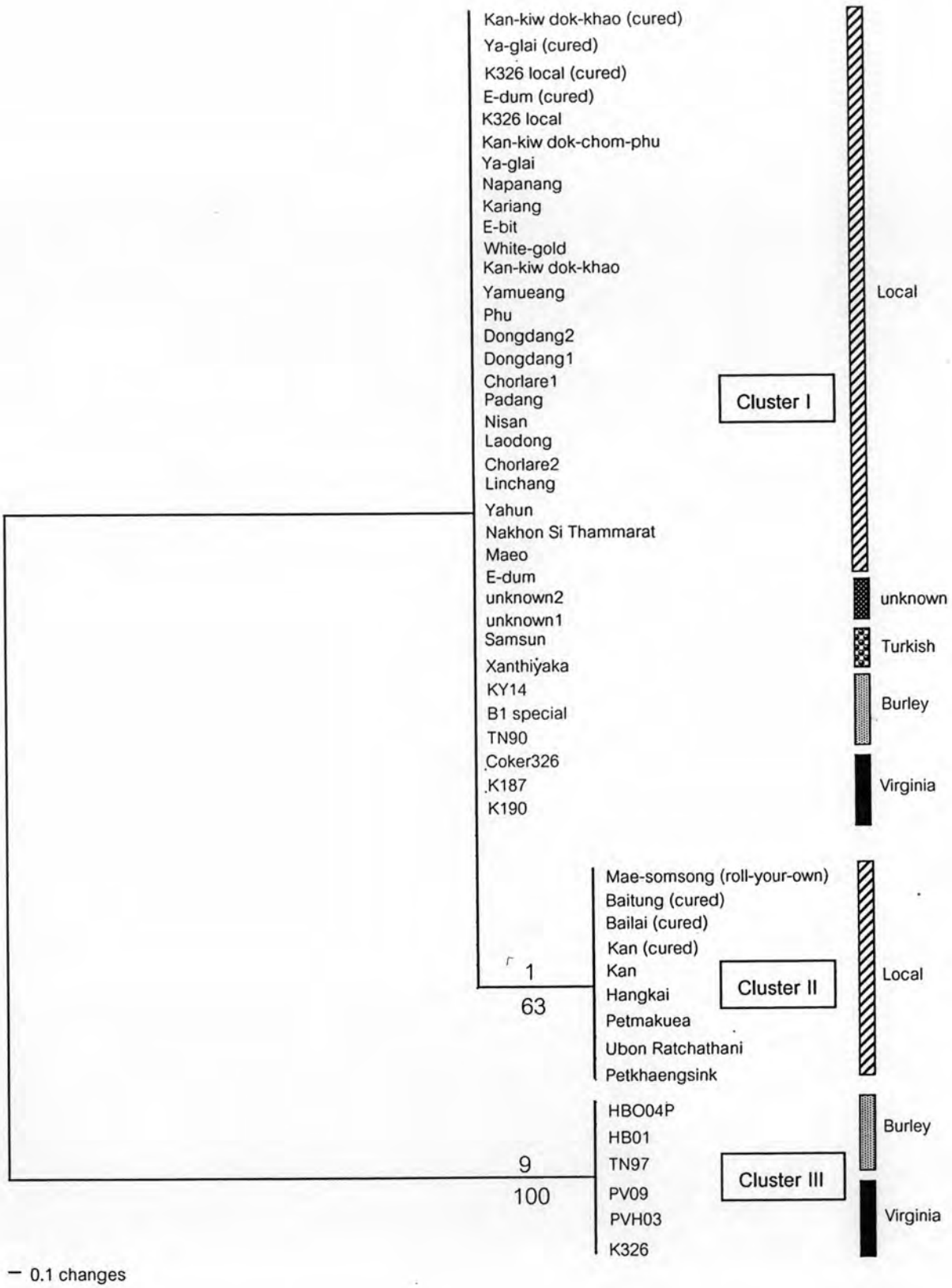

Figure 4.23 NJ tree of *rpl*32-*trn*L sequence data from 51 tobacco samples (including fresh-leaf, cured-leaf and roll-your-own tobacco samples). Branch lengths (with 1-gap included) are shown above each branch while bootstrap-supporting values (%) are shown below.

4.4 Preliminary experiment for multiplex PCR

4.4.1 *rpl*32-*trn*L molecular marker to differentiate Virginia and local cultivars.

The *rpl*32-*trn*L region was tested for its molecular-marker efficiency to distinguish Virginia imported cultivars and the special local cultivar-group from the others. First, the sequence result of the *rpl*32-*trn*L region amplified from 1:1 mixed DNA between K326 (Virginia) and Chorlae1 (local) cultivars showed combined electropherogram signals (or intra-individual sequences) of K326 and Chorlae1 cultivars at the sequence sites approximately 190 to 200 bp (Figure 4.24). This finding agreed with the previous sequence alignment (Figure 4.22) which showed five base substitutions of the K326 cultivar at the aligned sites 151-155 bp (also see Figure 4.25).

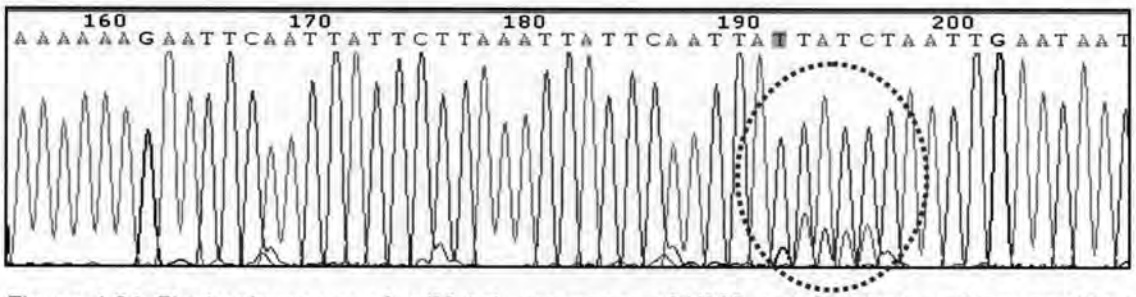

Figure 4.24 Electropherogram of *rpl*32-*trn*L sequences of K326 and Chorlae1 cultivars amplified together within a single reaction. (Four-coloured peaks represent four nucleotides: blue = cytosine (C), red = thymine (T), green = adenine (A) and cyan = guanine (G), respectively).

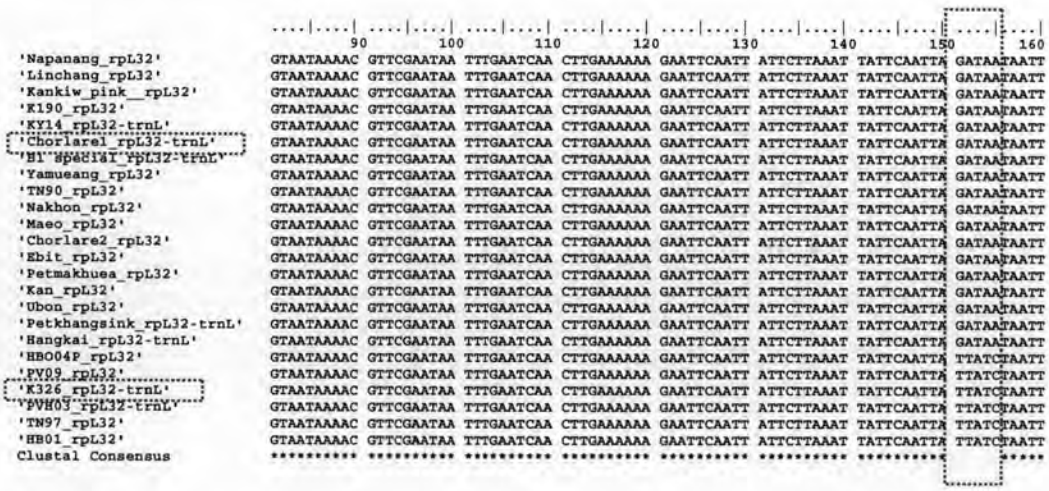

Figure 4.25 One part of the alignment of 43 *rpl*32-*trn*L sequences showed five base substitutions of K326 Virginia cultivar different from Chorlae1 local cultivar.

Another *rpl*32-*trn*L PCR reaction of 1:1 mixed genomic DNA between K326 Virginia cultivar and Hangkai special-local cultivar revealed that this region could also distinguish K326 cultivar from Hangkai cultivar. The electropherogram result of this experiment indicated two types of combined sequence signals. First one was the 5 bp combined signals occurred at the electropherogram sequence sites 199 to 203. The other type of the combined sequence signals which were unreadable appeared continuously from the position 218 bp and so on (Figure 4.26). These two types of the combined sequence signals congruence with the previous alignment (Figure 4.22) which showed five base substitutions at the aligned position 151 to 155 bp and a large 66 bp insertion of the five special-local cultivars at the sites 170-235 (Figure 4.27).

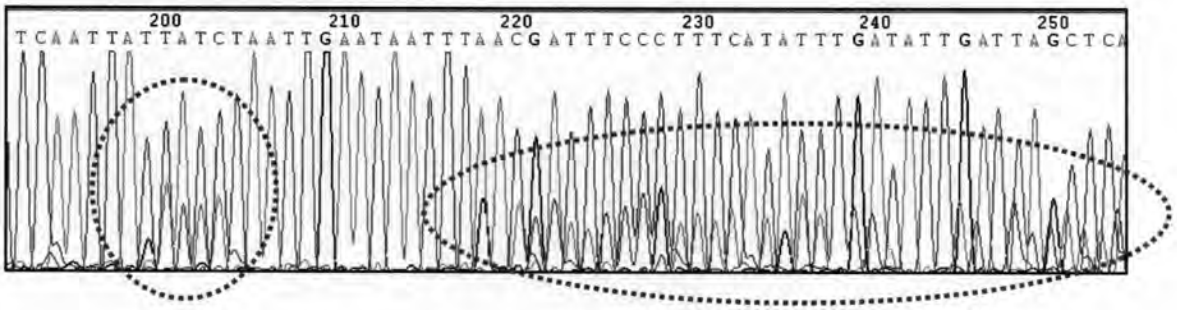

Figure 4.26 Electropherogram of *rpl*32-*trn*L sequence of K326 and Hangkai cultivars amplified together within a single reaction. (Four-coloured peaks represent from nucleotides: blue = cytosine (C), red = thymine (T), green = adenine (A) and cyan = guanine (G), respectively).

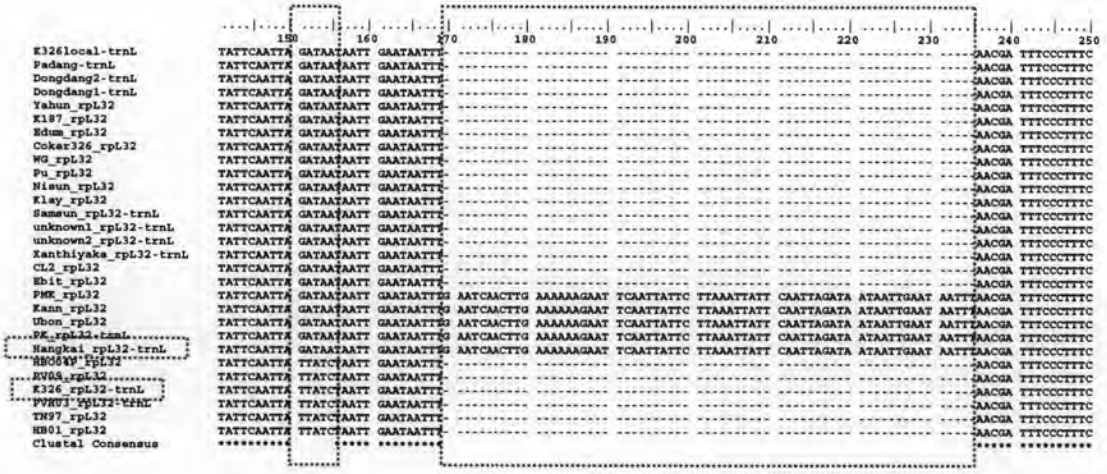

Figure 4.27 One part of the alignment of 43 *rpl*32-*trn*L sequences showed five base substitutions of K326 Virginia cultivar and a large 66 bp insertion of Hangkai special-local cultivar different from the other.

To test the simplicity of utilising *rpl*32-*trn*L marker on a simple gel electrophoresis instead of direct sequencing, the PCR products of both previous reactions were compared on 1.8% agarose gel electrophoresis (Figure 4.28). Although the combined *rpl*32-*trn*L regions amplified from the mixed DNA of K326 (Virginia) and Chorlare1 (common-local) cultivars appeared as only one PCR band (lane 1 in Figure 4.28), the other reaction which amplified the mixed K326 (Virginia) and Hangkai (special-local) DNA could show two distinguish bands on the agarose gel (lane 2 in Figure 4.28). This two *rpl*32-*trn*L fragments differing in size were then tested across other cultivar groups. Figure 4.29 showed the agarose-gel electrophoresis comparison between the *rpl32-trnL* markers of five representation tobacco cultivars and the mixture between K326 and Hangkai cultivars. The comparison result confirmed that special-local cultivars (represented by Hangkai cultivar) could give a unique PCR band different from those of other tobacco cultivars (lanes 5-6 of Figure 4.29).

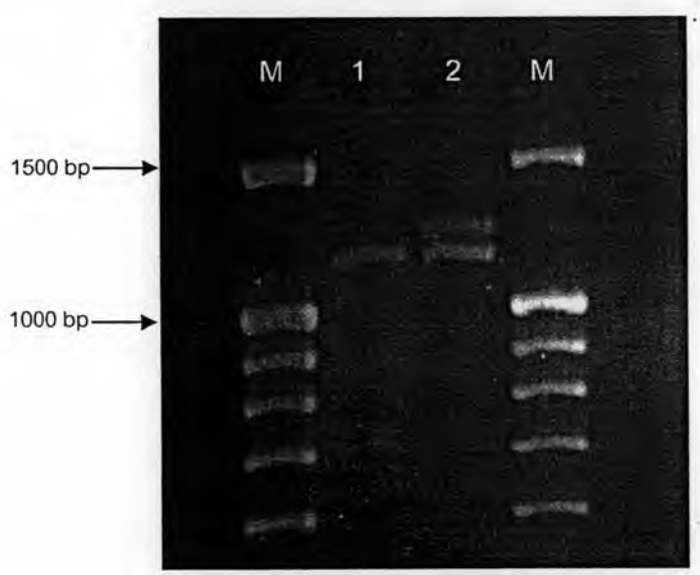

Figure 4.28 PCR products of *rpl*32-*trn*L region amplified from mixed genomic DNA of different tobacco cultivars. (Lane M = 1.5 kb + 100 bp DNA marker, no. 1 = K326 (Virginia) mixed with Chorlae1 (local) and no. 2 = K326 mixed with Hangkai (special local)).

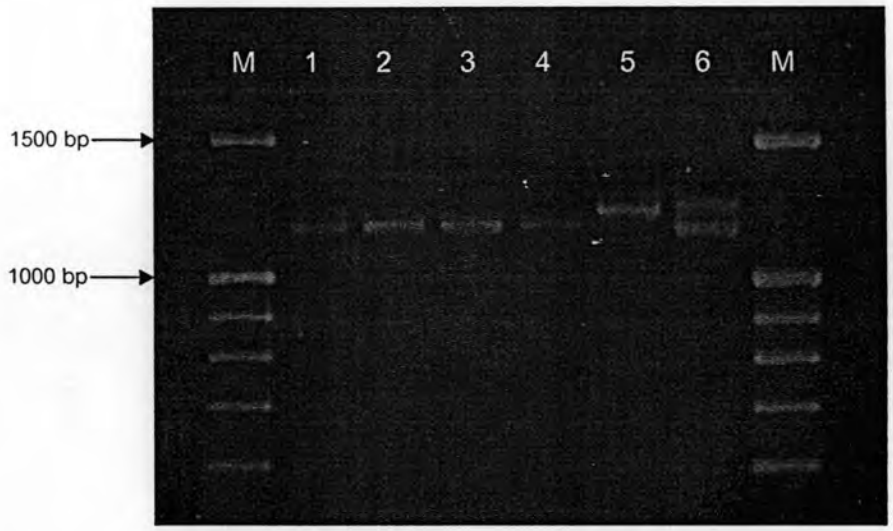

Figure 4.29 PCR products of *rpl*32-*trn*L region of five tobacco cultivars compared with a mixed reaction of local and imported cultivars (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-6 = K326 (Virginia), B1 special (Burley), Samsun (Turkish), Chorlae1 (local), HangKai (special local) and K326 mixed with Hangkai cultivars, respectively).

To improve the resolution of agarose gel electrophoresis for *rpl*32-*trn*L marker, two high-resolution agarose gels, Nusieve 3:1 and MetaPhor, were introduced to this experiment. The PCR band patterns produced from these two high-resolution gels were mostly the same as previously found from a normal agarose-gel electrophoresis, but presenting as sharper and clearer bands (the gel results not shown here). However, such high-resolution gels were difficult to prepare, especially MetaPhor which its solidified gel was too much softer than other gels, and the PCR fragments mobilised through them much slower than through the normal gel. Therefore, because of its low cost and easiness to prepare, a 1.8% normal agarose gel was still suitable for using in the *rpl*32-*trn*L band separation.

4.4.2 Combined *rpl*32-*trn*L and *ndh*F-*rpl*32 markers to differentiate Virginia and local cultivars.

Since the sequence alignments of other chloroplast noncoding regions also revealed indel differences between Virginia cultivar-group and other cultivars, there was an opportunity to perform a multiplex PCR amplification using more than one suitable primer-pair in a single PCR reaction. Three primer pairs (*pet*A-*psb*J, *ndh*C-3′*trn*V$^{(UAC)}$x2 and *ndh*F-*rpl*32R) were compared with *rpl*32F-*trn*L$^{(UAG)}$ primers by amplifying a 1:1:1 mixed genomic DNA of K326, B1 special and Hangkai cultivars. The amplification of these four primers could generate two different PCR bands (lanes 1-4 in Figure 4.30). One of the two bands of *pet*A-*psb*J region was of K326 cultivar and the other larger band was of both B1 special and Hangkai (lane 2 in Figure 4.30). The larger band of *ndh*C-*trn*V and *ndh*F-*rpl*32 regions was of K326 cultivar and the other smaller band was of both B1 special and Hangkai (lanes 3-4 in Figure 4.30).

From the testing results above, *ndh*F-*rpl*32 region was selected to be amplified together with *rpl*32-*trn*L as a multiplex PCR reaction. That was because both of the PCR products of *ndh*F-*rpl*32 primers were not in the same range of the lengths of *rpl*32-*trn*L fragments (lanes 1 and 4 in Figure 4.30). The multiplex PCR reaction using *rpl*32F-*trn*L$^{(UAG)}$ and *ndh*F-*rpl*32R primer pairs was successfully performed with the mixed DNA of K326 (Virginia) and Hangkai (special-local). This result showed four PCR bands (lane 3 in Figure 4.31) compared with the two PCR bands of each single PCR reaction (lanes 1 and 2 in Figure 4.31). Notably, the two different *ndh*F-*rpl*32 region bands were so close to each other, not separating well like the two bands of the *rpl*32-*trn*L fragments (lane 3 in Figure 4.31).

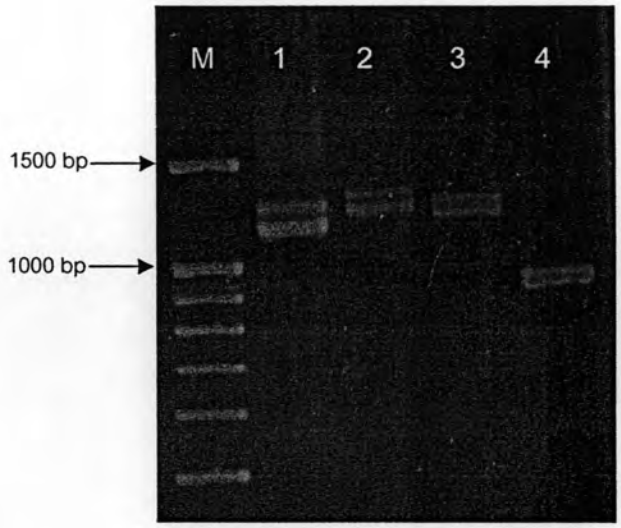

Figure 4.30 PCR products of four different regions amplified from mixed DNA of K326 (Virginia), B1 special (Burley) and Hangkai (special-local) cultivars. (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-4 = *rpl*32-*trn*L, *pet*A-*psb*J, *ndh*C-*trn*V and *ndh*F-*rpl*32 regions, respectively).

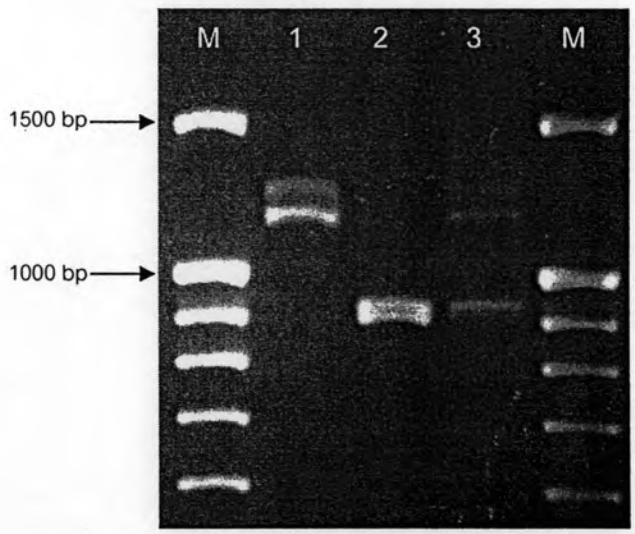

Figure 4.31 PCR products of two different regions amplified from mixed DNA of K326 (Virginia) and Hangkai (special-local). (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-3 = *rpl*32-*trn*L, *ndh*F-*rpl*32 and *rpl*32-*trn*L+ *ndh*F-*rpl*32, respectively).