IV. RESULTS

1. Amplification of *C.trachomatis* DNA by PCR

1.1 Amplification of the MOMP gene

From 34 *C.trachomatis* positive samples, chosen for this study, 20 were positive by the Gen Probe DNA hybridization test, 5 by PCR assay and 9 by culture technique. Those samples were amplified for the MOMP sequence by PCR assay using FLA-FLS primers. All samples produced a clear single band of approximate 1,200 bp on 1% agarose gel electrophoresis (Fig.4).

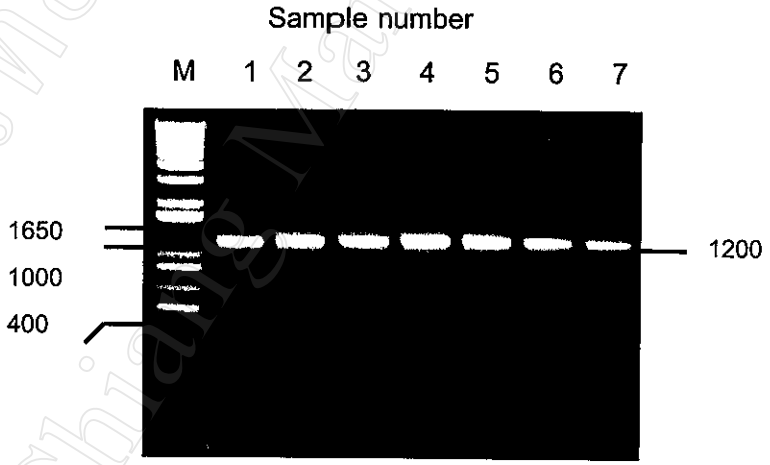


Figure 4. The amplification of the *C.trachomatis* MOMP gene from clinical samples by PCR.

Lane M = DNA marker

1.2 Amplification of the VD4 - MOMP gene

All 34 positive samples were amplified for the VD4 – MOMP gene. The PCR amplification using Nest 2 and Nest 4 primers was performed. The amplified product was shown on 1% agarose gel electrophoresis as a clear single band of 350 bp (Fig.5). Then, the products were used for further RFLP analysis.

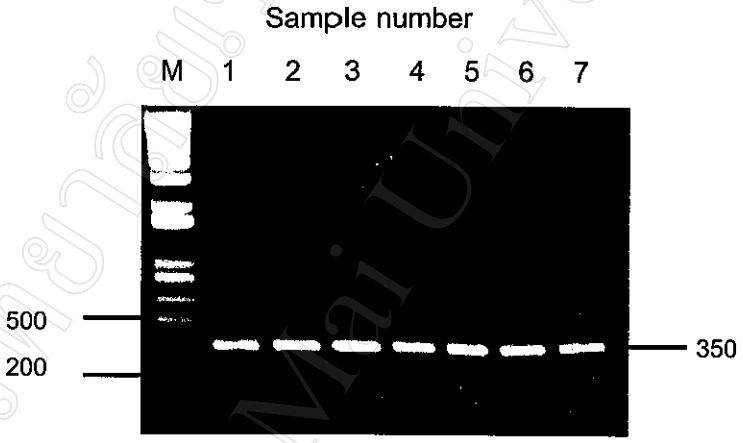


Figure 5. The amplification of the *C.trachomatis* VD–4 MOMP gene from clinical samples by PCR.

Lane M = DNA marker

2. PCR Cloning of the MOMP gene

The PCR amplified MOMP sequences from 34 samples were cloned into the pGEM® -T Easy vector plasmid (Promega corporation, USA). The vectors were commercially prepared by cutting with *Eco*RV and adding a 3' terminal thymidine to both ends. These 3'- T overhang vectors were hybridized with the protruded adenine (A) at the 3' terminal of the inserted fragment and then ligated by the action of T4 ligase. These plasmids were transformed to *E.coli* and the transformed colonies were selected by using the PCR. The pGEM® -T Easy vector plasmid had a molecular weight of approximately 3,000 bp. After insertion of the MOMP gene, it became a circular plasmid of approximately 4,200 bp (Fig.6). These recombinant *E.coli* were stored at –70 °C for nucleotide sequencing

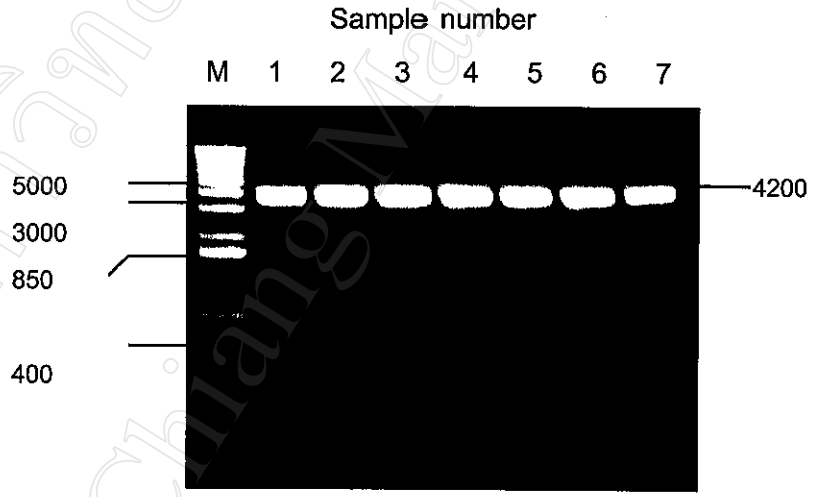
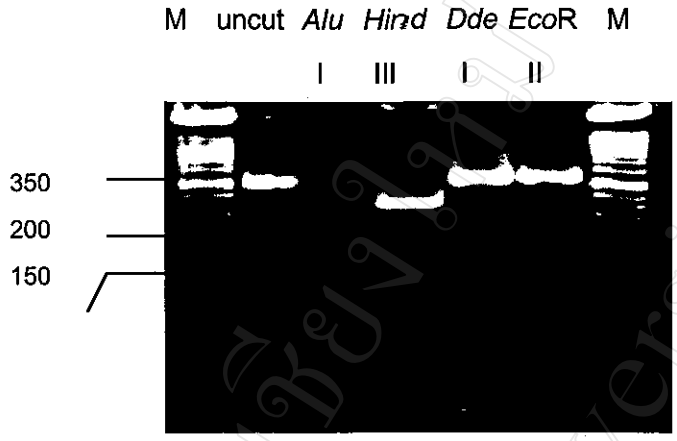


Figure 6. The recombinant plasmid containing the MOMP gene from clinical samples.
Lane M = DNA marker

3. Genotyping of *C.trachomatis* by RFLP analysis

The RFLP analysis was carried out for the genotyping of *C.trachomatis*. The VD4 region, flanked by Nest 2 and Nest 4 primers, was amplified and then subjected to the restriction digestion with *Alu*I, *Hind*III, *Dde*I and *Eco*RII. The digested fragments were visualized on 6% polyacrylamide gel electrophoresis. The profile of the RFLP was analyzed by a comparison with the reference strains of *C.trachomatis* (Table 1). Among the 34 samples analyzed, genotype F was identified predominantly in 9 (26.5%) followed by genotype D/Da/L_1, which was found in 8 (23.5%). Genotypes K, E, H/Ia/J and G were found in 6 (17.6 %), 5 (14.7 %), 4 (11.8 %) and 2 (5.9%) respectively. Since the nucleotide sequence of the VD4 region of genotype D/Da/L1 was different in only a few bases, it was not in the recognition site of the restriction endonuclease used. This made it impossible to distinguish between these genotypes by the RFLP. It also happened among the genotype groups of H, Ia and J that exhibited an identical RFLP pattern of VD4 sequences. In this study, genotypes A, B, Ba, C, I, L1, L2, L2a and L3 were not detected. The overall genotype distribution is summarized in Table 2. The RFLP patterns of genotypes identified are shown in Figure 7-10.

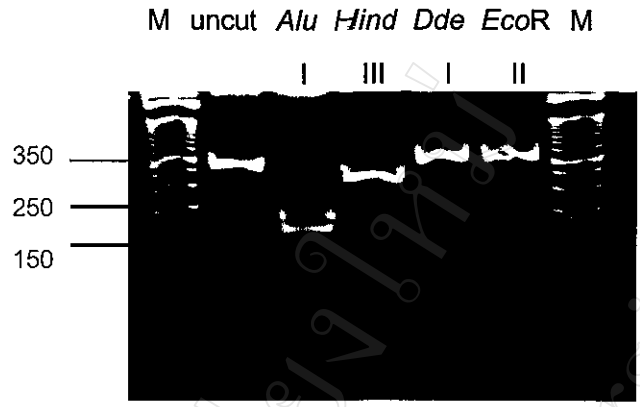
(A)



(B)

Figure 7. Illustration of the PCR-RFLP patterns of D/Da/L1 serotypes (A) and E serotype (B) after digestion by *Alu*I, *Hind*III, *Dde*I and *Eco*RII.
Lane M = DNA marker Uncut = No restriction enzyme

(A)



(B)

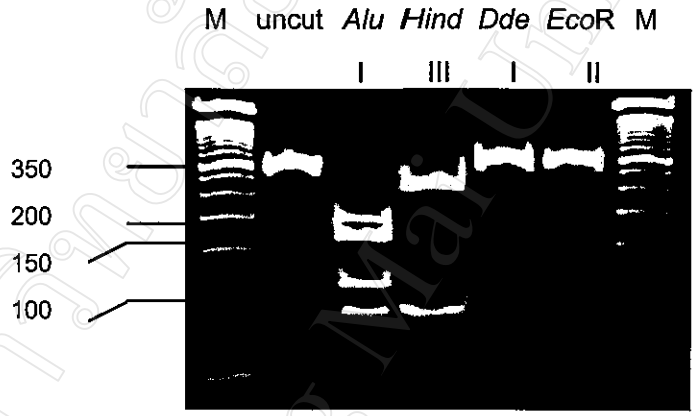
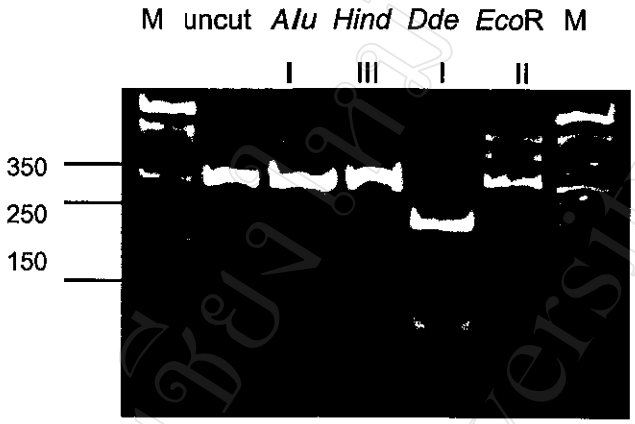


Figure 8. Illustration of the PCR-RFLP patterns of F serotype (A) and G serotype (B) after digestion by *Alu*I, *Hind*III, *Dde*I and *Eco*RII.

Lane M = DNA marker Uncut = No restriction enzyme

(A)



(B)

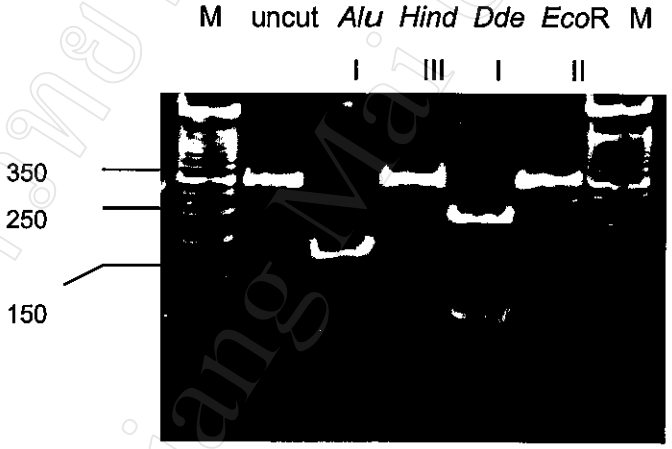


Figure 9. Illustration of the PCR-RFLP patterns of H/Ia/J serotypes (A) and K serotype (B) after digestion by *Alu*I, *Hind*III, *Dde*I and *Eco*RII.

Lane M = DNA marker Uncut = No restriction enzyme

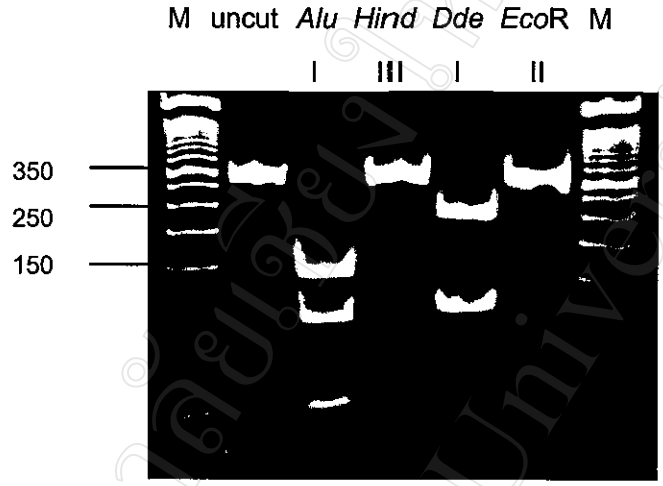


Figure 10. Illustration of the PCR-RFLP pattern of serotype L2 control after digestion by *Alu*I, *Hind*III, *Dde*I and *Eco*RII.

Lane M = DNA marker Uncut = No restriction enzyme

Table 2. Genotype distribution of *C. trachomatis* determined by VD4 PCR–RFLP

Serogroup	RFLP genotype	No. of samples n=34	Percentage (%)
B complex	D/Da/L1	8	23.5
	E	5	14.7
Intermediate	F	9	26.5
	G	2	5.9
C complex	H/Ia/J	4	11.8
	K	6	17.6

4. Genotyping of *C.trachomatis* by nucleotide sequence analysis

As shown in several studies, there were some drawbacks in the RFLP technique for genotyping. For example, it was not reliable in identifying mixed infection. It also could not differentiate the type variants, especially those that occurred outside the recognition sequence. In addition, as shown in Table 2 of this study, the VD4-RFLP alone cannot completely differentiate all of the *C. trachomatis* into an individual type. For example, those groups of D/Da/L1 and H/Ia/J genotypes that have an identical VD4 sequence . To ensure that the genotypes were classified correctly from the RFLP patterns, and to differentiate those groups of genotypes into individual types, the nucleotide sequencing of the entire MOMP gene was determined.

The MOMP gene of *C. trachomatis* from all samples was sequenced in both directions by using primers, FLA and FLS. The FLS primer was used to sequence forward through the MOMP gene, while the FLA primer was used to sequence backwards. The electrophoregrams of nucleotide sequences of the MOMP gene of the samples are shown in Figure 11 and 12. In these figures, the four different colored peaks indicate the fluorescent intensity of a particular dye that was linked to the specific ddNTP involved in the termination of the primer extension reaction. The green, red, black and blue color are linked to ddATP, ddTTP, ddGTP, and ddCTP, respectively. The 3-terminal base of each terminated oligonucleotide was identified by the fluorescence liberated from the gel, then detected and recorded by the device. The data were analyzed by ABI 310 data collection version 3.0 and ABI 310 DNA sequencing version 2.2 computor programs.

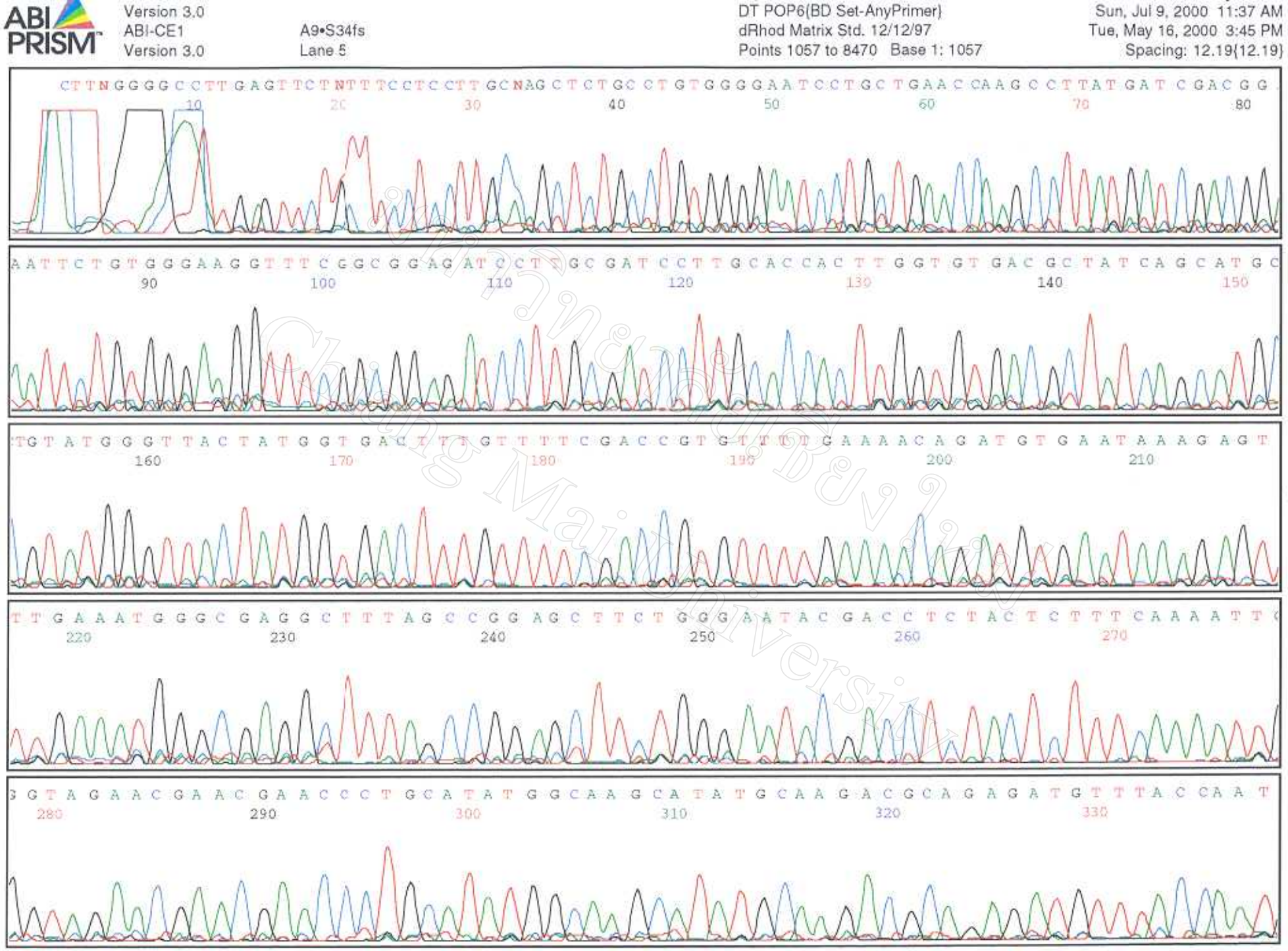
The forward and backward sequences were assembled by using the Autoassembler ™ 1.4.0 (Perkin-Elmer, Applied Biosystem, USA). Then, the whole nucleotide sequence was compared to the prototype sequences obtained from the Gen

Bank by using the same program. Figure 13 shows the nucleotide sequence comparison between samples and the reference prototypes.

The genotypes of *C. trachomatis* determined by the nucleotide sequencing of the entire MOMP gene were in complete agreement with the RFLP analysis (Table 3). There were 8 samples shown as genotype D, 5 as genotype E, 9 as genotype F, 2 as genotype G, 3 as genotype H, 1 as genotype J, and 6 as genotype K . Genotype F and D were still the most prevalent genotypes and accounted for 50% of those identified in this study (Table 3). Among the 8 samples identified by RFLP patterns, a group of genotypes D/Da/L1 all were genotype D after the sequence analysis. However, the comparison of VD1-VD4 nucleotide sequences between the identified D genotypes and the D/UW-3 prototype showed that seven of eight samples were not really identical to the prototype, and they were called a D variant in this study. Only one of the D genotypes contained a nucleotide sequence identical to the D/IC-cal-8 reference strain described by Sayada *et al.* (43). It was also found that three of five E genotypes were E variants as they contained a nucleotide sequence in VD1 that differed from the E Bour prototype. In addition, one of nine samples identified as the F genotype by using the RFLP technique, had a nucleotide sequence in VD1, which was different from that in the F/IC-cal-13 prototype. Thus, that sample was identified as the F variant. All G genotypes determined by the RFLP technique contained nucleotides in VD2 and VD4, which were different from those in the G/UW-57 prototype. Among 4 samples identified by RFLP patterns as genotypes H/Ia/J, one was identified as genotype J and 3 as genotype H by nucleotide sequencing. Only one from three H genotypes was identical to the prototype, while the other two were H variants, as their nucleotide sequences in VD1, VD2 and VD3 were different from their prototype. All K genotypes identified here have an identical nucleotide sequence, but they differ from the K/UW-13 prototype in the VD4 region. The comparison between the

genotype distribution, as determined by the RFLP, and nucleotide sequencing, is shown in Table 3. The conclusion of genotype distribution is also shown in Table 4.

Figure 11. The example of electrophoregram of nucleotide sequence in the MOMP gene using FLS primer


ABI PRISM
Version 3.0
ABI-CE1
Version 3.0
A9•S34fs
Lane 5
DT POP6(BD Set-AnyPrimer)
dRhod Matrix Std. 12/12/97
Points 1057 to 8470 Base 1: 1057
Sun, Jul 9, 2000 11:37 AM
Tue, May 16, 2000 3:45 PM
Spacing: 12.19(12.19)

ABI PRISM

Version 3.0
ABI-CE1
Version 3.0

A9•S34fs
Lane 5

DT POP6(BD Set-AnyPrimer)
dRhod Matrix Std. 12/12/97
Points 1057 to 8470 Base 1: 1057

Sun, Jul 9, 2000 11:37 AM
Tue, May 16, 2000 3:45 PM
Spacing: 12.19(12.19)

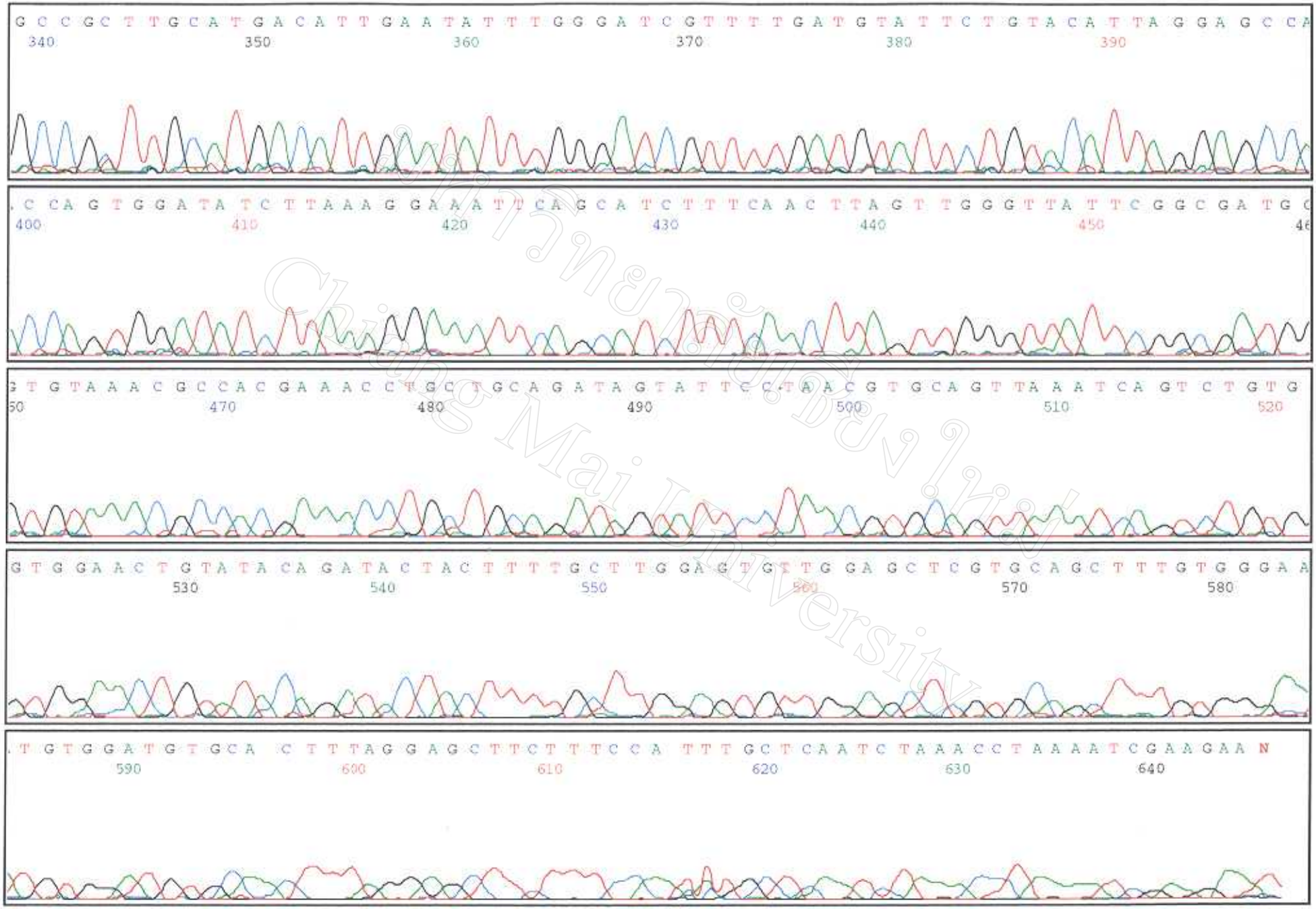


Figure 12. The example of electrophoregram of nucleotide sequence in the MOMP gene using FLA primer

ABI PRISM

Model 310
Version 3.0
ABI-CE1
Version 3.0

A7•S34fa

A7•S34fa
Lane 4

Signal G:270 A:316 T:426 C:248
DT POP6{BD Set-AnyPrimer}
dRhod Matrix Std. 12/12/97
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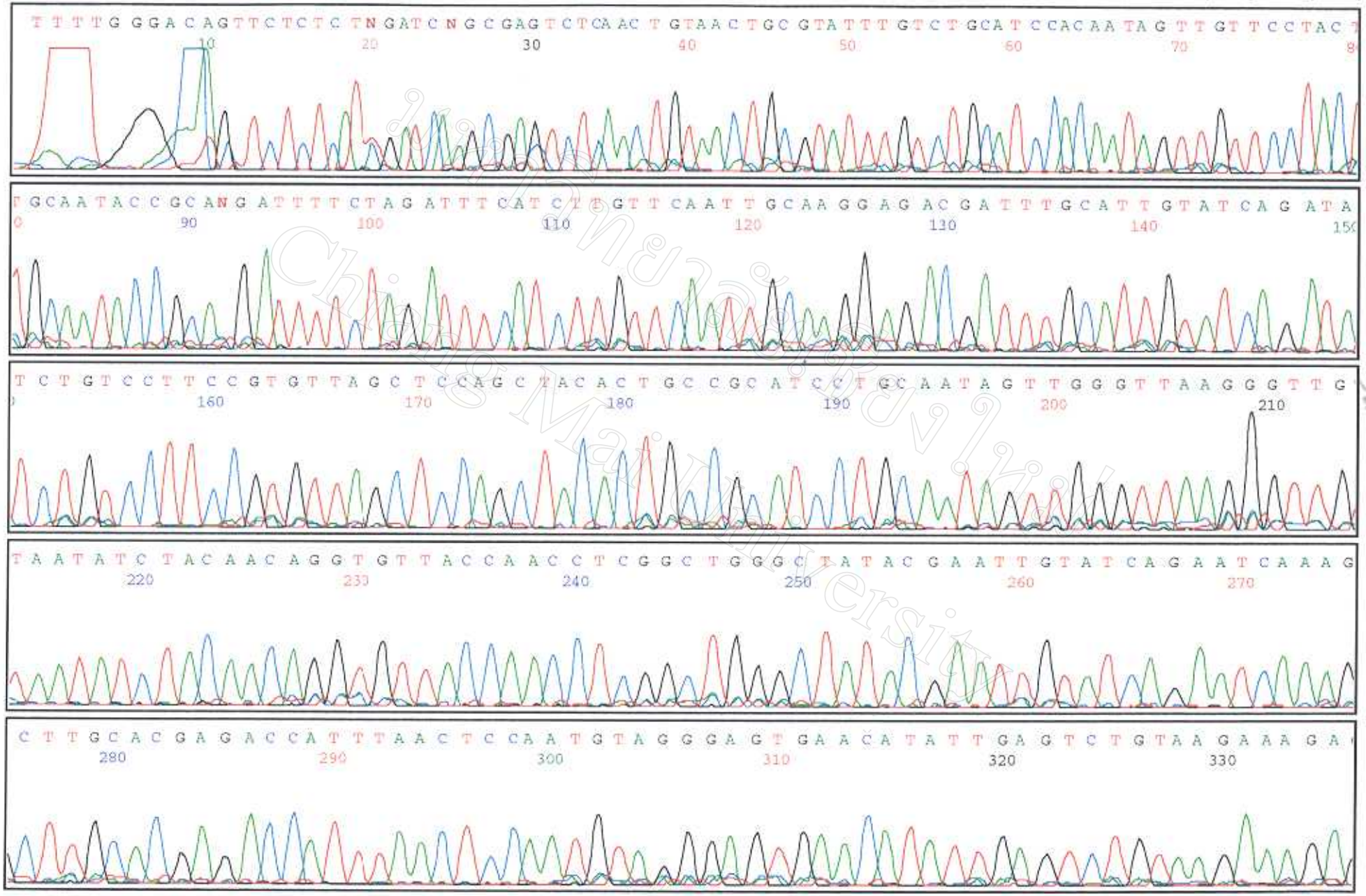
Spacing: 12.19{12.19}



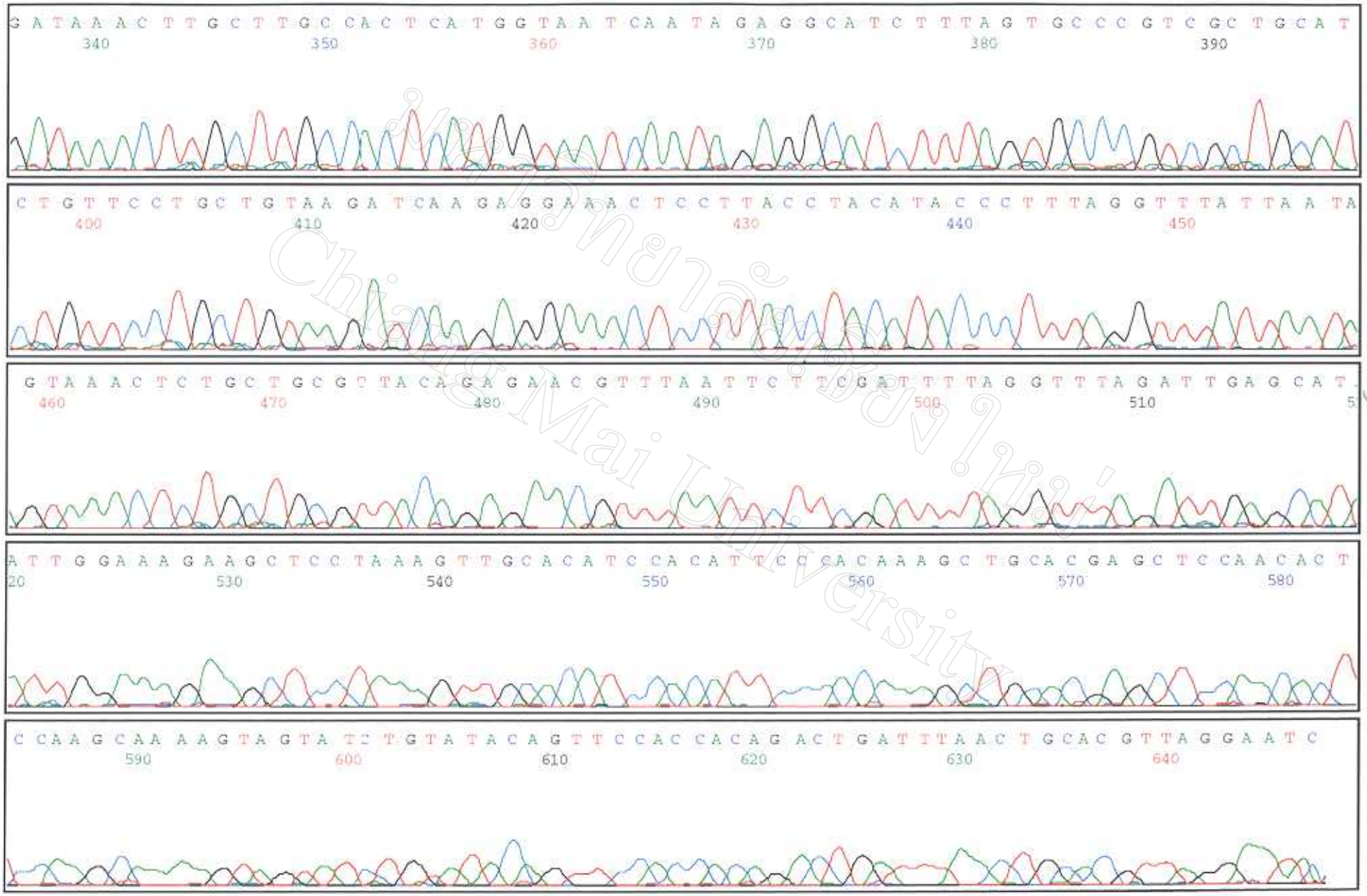
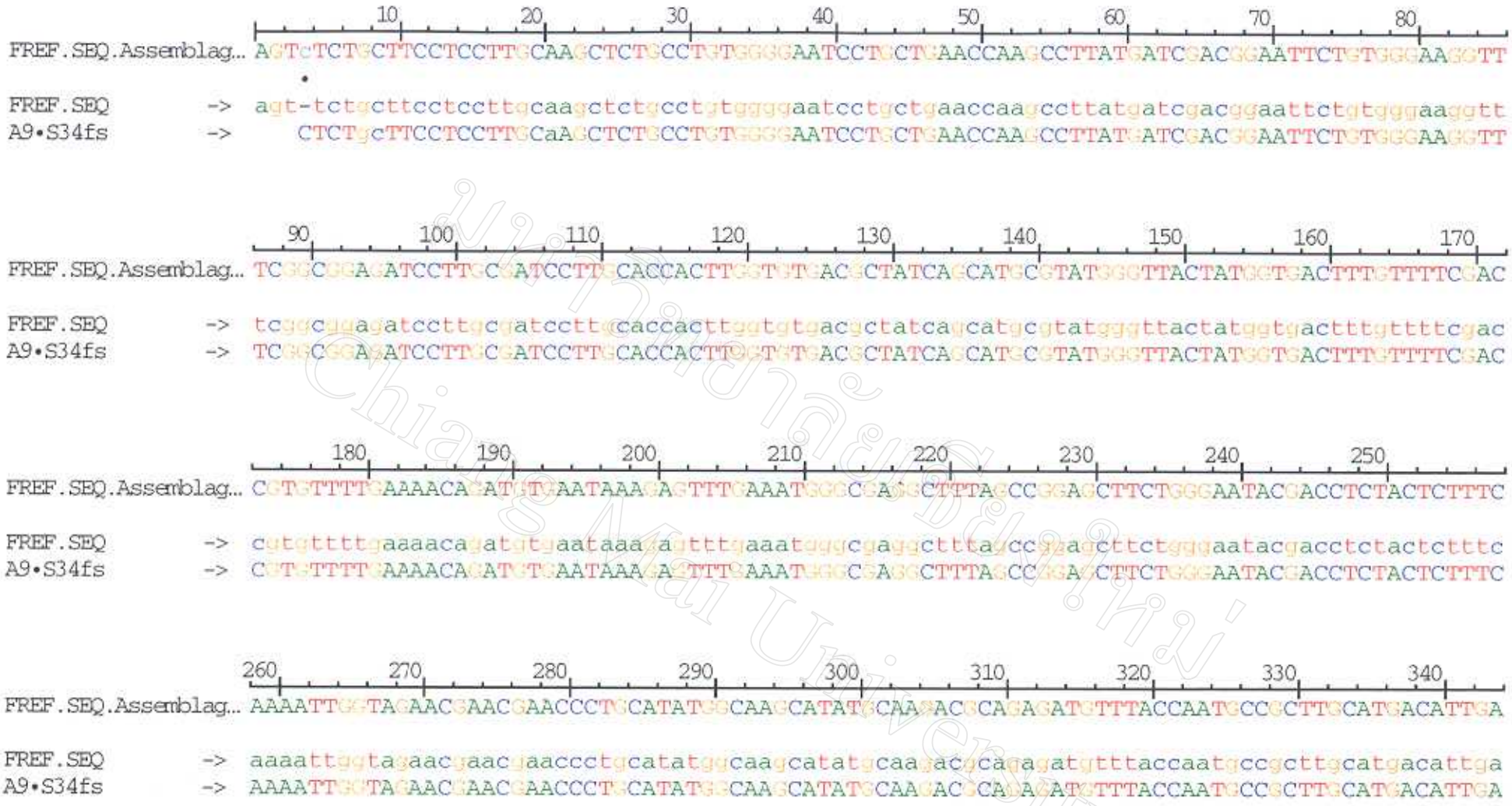
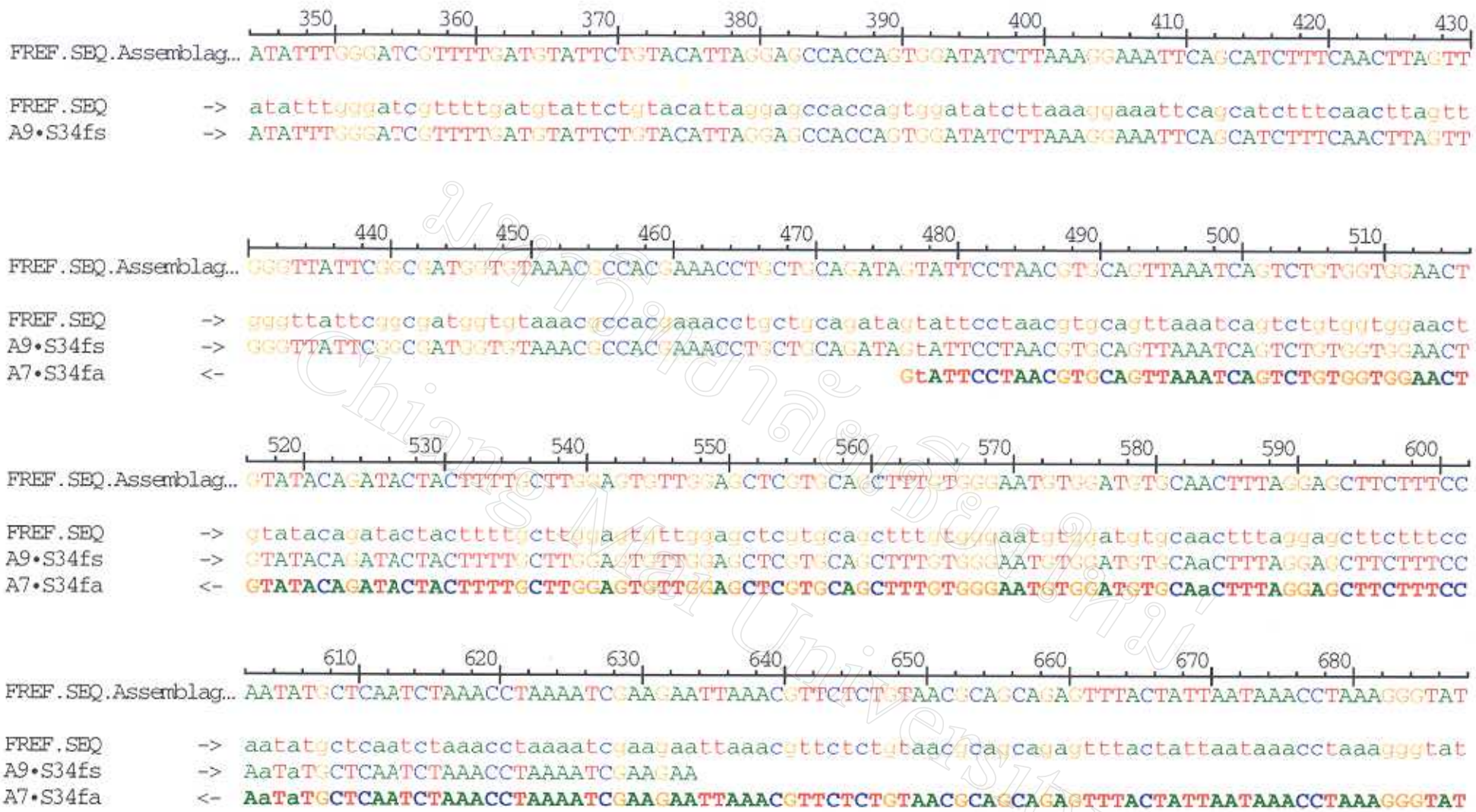


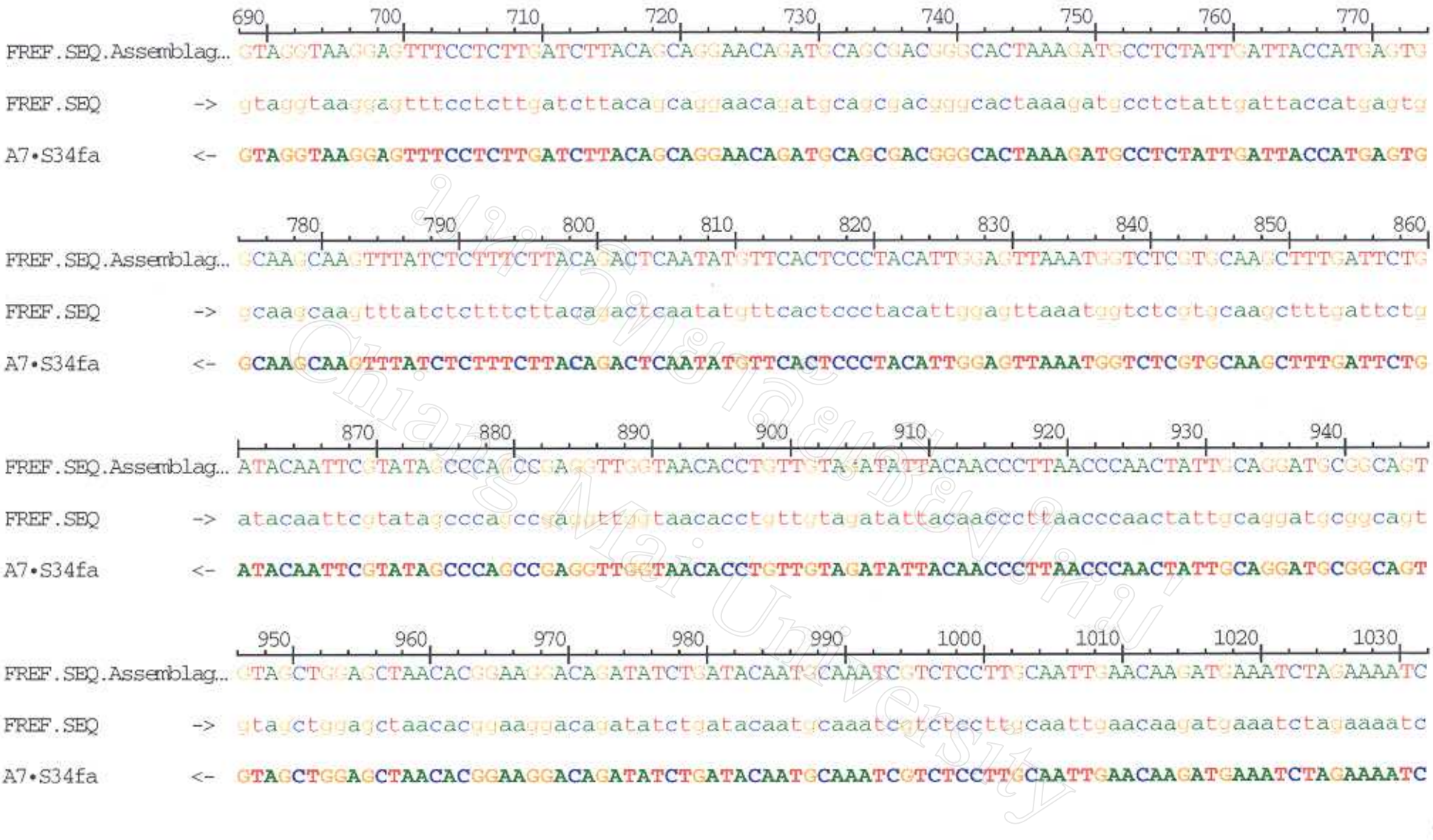
Figure 13. The example of sequences comparison between clinical isolate no. 34 and the F reference genotype.



Biosystems



Biosystems



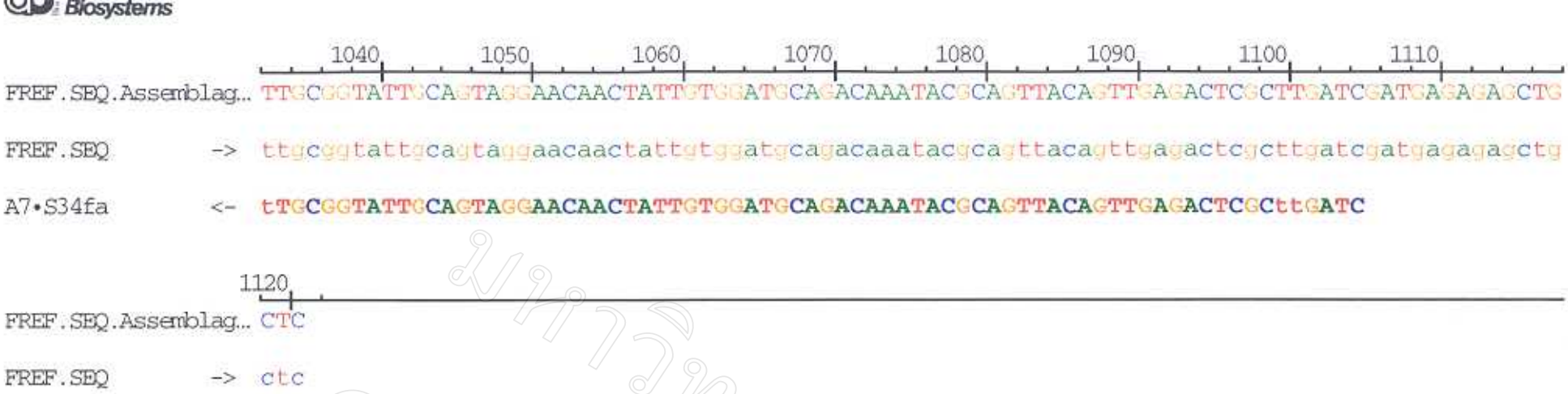


Table 3. Comparison of *C. trachomatis* genotypes determined by VD4 PCR-RFLP and nucleotide sequencing of the MOMP gene.

RFLP of the VD4-MOMP gene		Nucleotide sequencing of the MOMP gene	
Genotype	No. of samples n=34	Genotype	No. of samples n=34
D/Da/L1	8	D	8
E	5	E	5
F	9	F	9
G	2	G	2
H/Ia/J	4	H	3
		J	1
K	6	K	6

Table 4. The summary of *C. trachomatis* genotype distribution as determined by VD4 PCR–RFLP and nucleotide sequencing of the MOMP gene.

Serogroup	RFLP and nucleotide sequencing genotype	No. of sample n=34	Percentage (%)
B complex	D	8	23.5
	E	5	14.7
Intermediate	F	9	26.5
	G	2	5.9
C complex	H	3	8.8
	J	1	2.9
	K	6	17.6

5. Analysis of nucleotide sequence variation in the MOMP gene

Nucleotide sequence analysis of the MOMP gene in 34 *C. trachomatis* isolates revealed that 13 (38.2%) had a sequence identical to the prototypes, while, 21 (61.8%) had a differing degree of sequence variation from the prototypes. Those variations were distributed in all *C. trachomatis* genotypes except genotype J (Table 5 and Fig. 14-32). The nucleotide sequence variation was found mostly in the form of one or two nucleotide substitutions.

When compared to the D/ UW-3 prototype, 7 out of 8 genotype D had a sequence variation in the VD4 region. Only one nucleotide substitution was observed at the nucleotide position 979, and the guanine was substituted by an adenine. This observation was identical to those previously reported in Chiang Mai by Veeraseatakul (44). Furthermore, it was the same variation as that found by other studies such as those by Poole *et al.* (20), Sayada *et al.* (43) and Lampe *et al.*(45). This kind of transition gave rise to the changes in an amino acid sequence. In these cases, the threonine was substituted by an alanine. The comparison of nucleotide sequences in the variable domains of D variant in this study, other D variants reported elsewhere and the D/UW-3 prototype are shown in figures 14-17. These D variants were not detected by the RFLP analysis, since the mutation did not occur in the restriction sites. Three E variants found in this study had one nucleotide substitution in the VD1 region at nucleotide position 258. The cytosine was substituted by adenine. This substitution also resulted in the transition of the aspartic acid to glutamic acid. Only one of the F variants identified here had one nucleotide substitution at nucleotide position 289 in the VD1. Here, thymine was substituted by cytosine, which made the amino acid change from serine to proline. Among the two G variants, one had the nucleotide substitution in the VD2 at position 523. Thymine was substituted by cytosine, but this did not lead to any changes in amino acid sequence. In

addition, both G variants also had the nucleotide substitution in the VD4 at position 1003. The thymine was substituted by guanine, which resulted in the substitution of amino acid: serine to alanine. The nucleotide substitutions observed in these G genotypes were identical to Ga reported by Morre *et al.*(38) and G' reported by Poole *et al.*(20). Apart from the VDs, the nucleotide change was also observed in the preregion of VD1. The thymine was substituted by cytosine, but the amino acid remained the same. Among the two H variants, one had both nucleotide substitution and amino acid changes in the VD3 region. The nucleotide substitution, adenine to guanine, was observed at position 766 and the amino acids also changed from threonine to alanine. The other H variant had the nucleotide substitutions in both of VD1 and VD2. At nucleotide position 272 in the VD1, adenine was substituted by guanine, which was similar to observation made by Lampe *et al.* in 1995 (45). In the VD2 region, there were two nucleotide substitutions, one at position 501 and the other at 508. At position 501, thymine was substituted by adenine, which resulted in change of amino acid: aspartic acid to glutamic acid. While at position 508, adenine was substituted by guanine, which made the amino acid change from threonine to alanine. All six K variants had the same nucleotide substitution at position 973. The adenine was substituted here by guanine and the amino acid changed from alanine to threonine. The K variants observed here were identical to those previously reported from other countries (20, 29, 34, 43, 46).

Table 5. The summary of nucleotide sequence variation in the MOMP gene of *C. trachomatis* detected in this study

Nucleotide sequence variation of MOMP	Genotype	No. of samples N=34	Percentage (%)
Variant		21	61.8
	D	7	20.6
	E	3	8.8
	F	1	2.9
	G	2	5.9
	H	2	5.9
	K	6	17.7
Prototype		13	38.2
	D	1	2.9
	E	2	5.9
	F	8	23.5
	H	1	2.9
	J	1	2.9

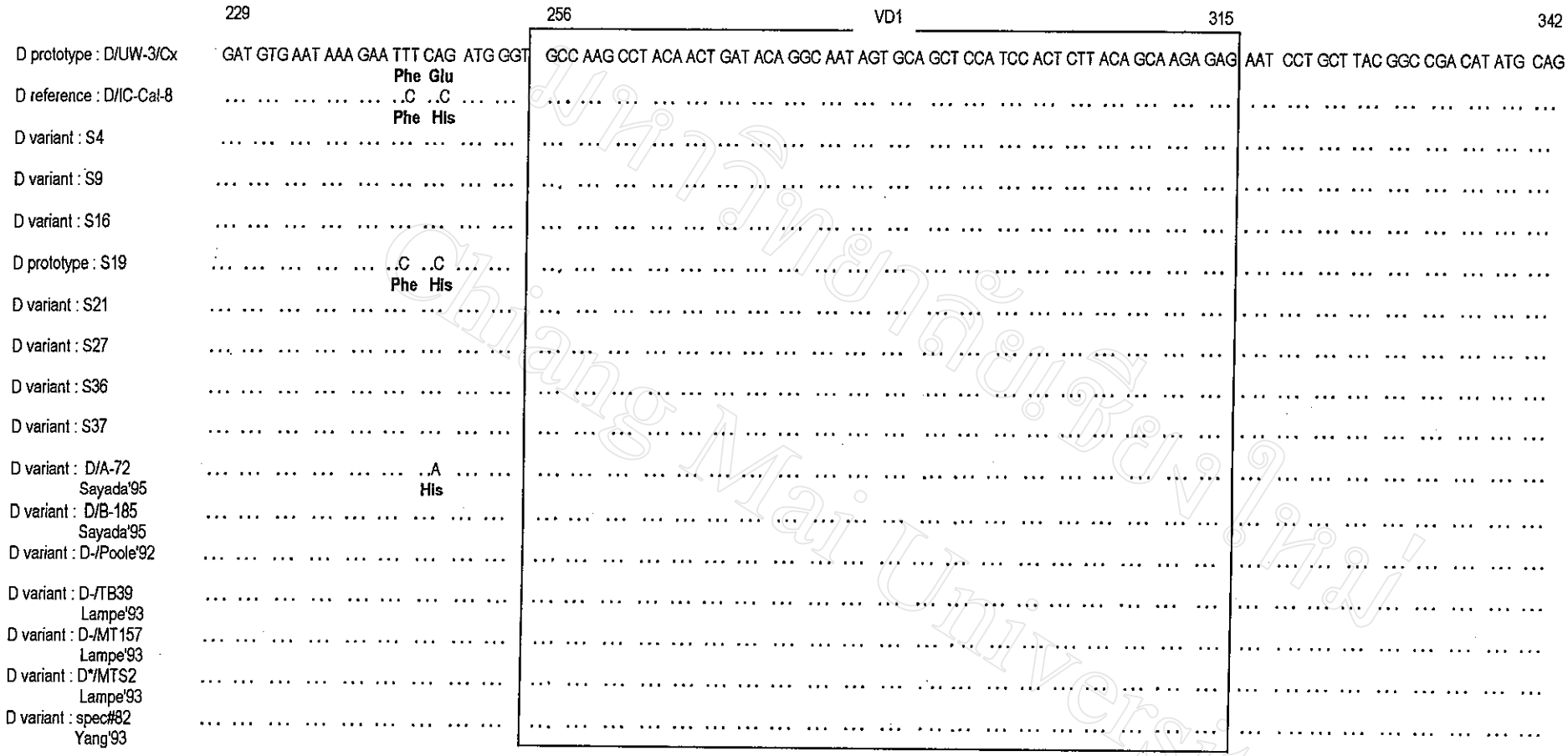


Figure 14. Nucleotide and amino acid sequences comparison of VD1-MOMP gene of the prototype D/UW-3/Cx, D/IC-Cal-8, D genotype found in this study and other D variants.

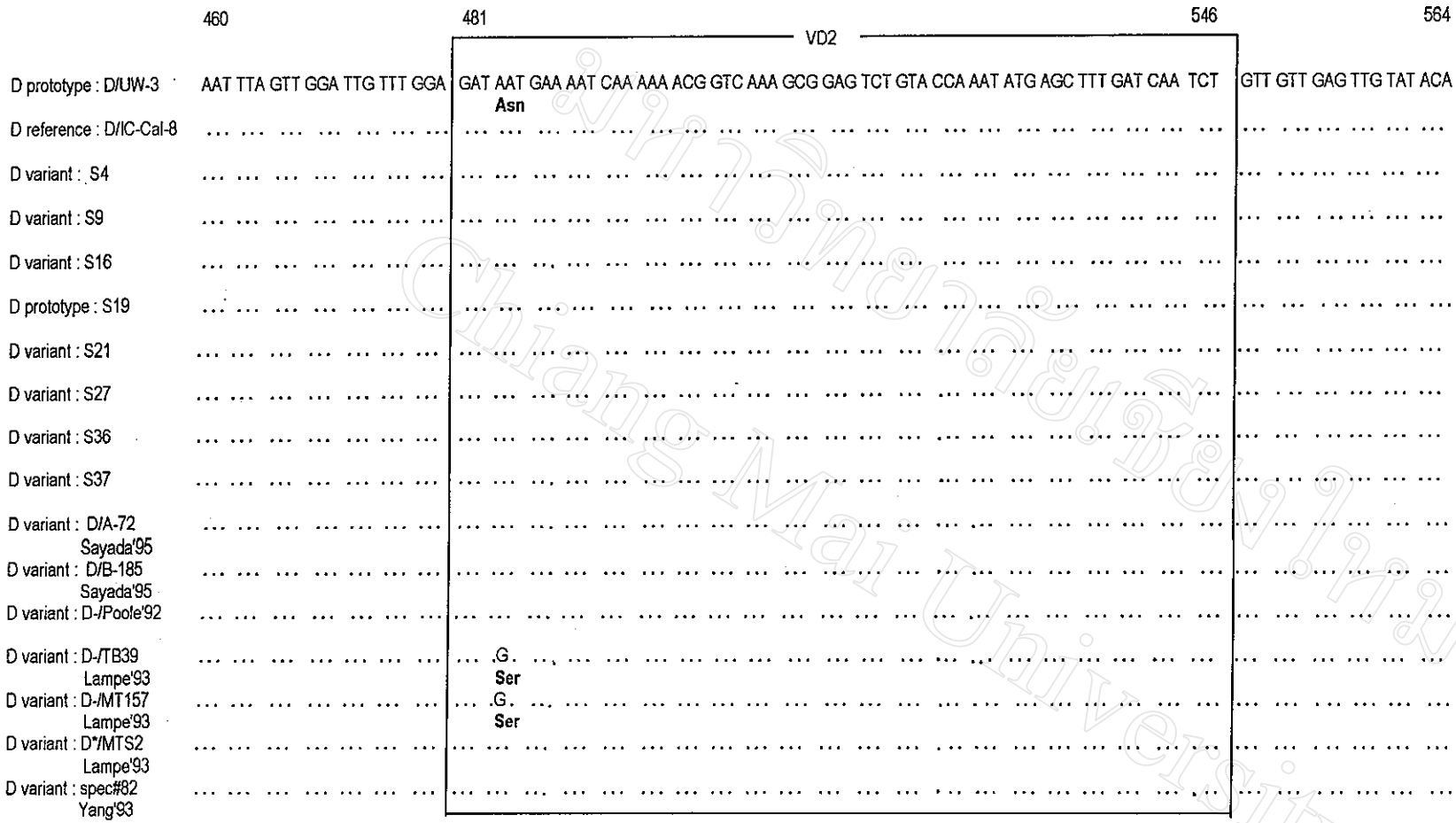


Figure 15. Nucleotide and amino acid sequences comparison of VD2-MOMP gene of the prototype D/UW-3/Cx, D/IC-Cal-8, D genotype found in this study and other D variants.

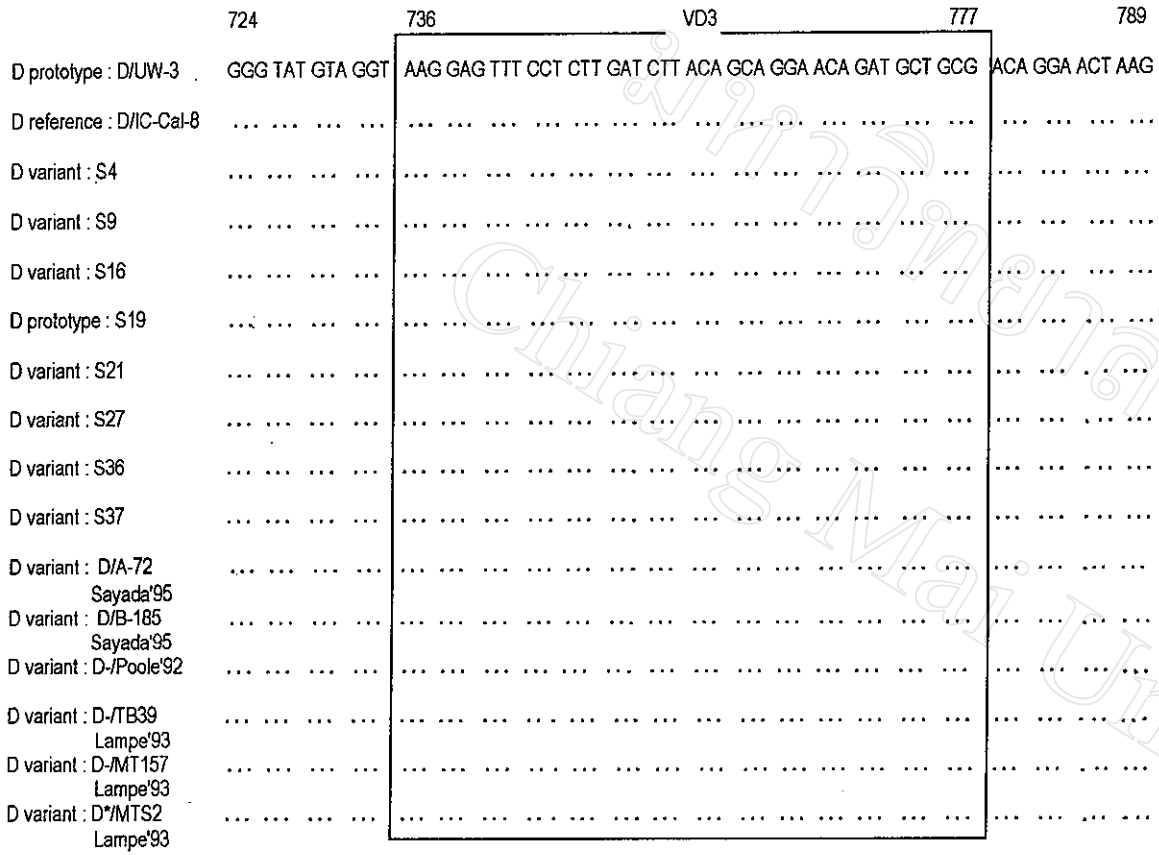


Figure 16. Nucleotide and amino acid sequences comparison of VD3-MOMP gene of the prototype D/UW-3/Cx, D/IC-Cal-8, D genotype found in this study and other D variants.

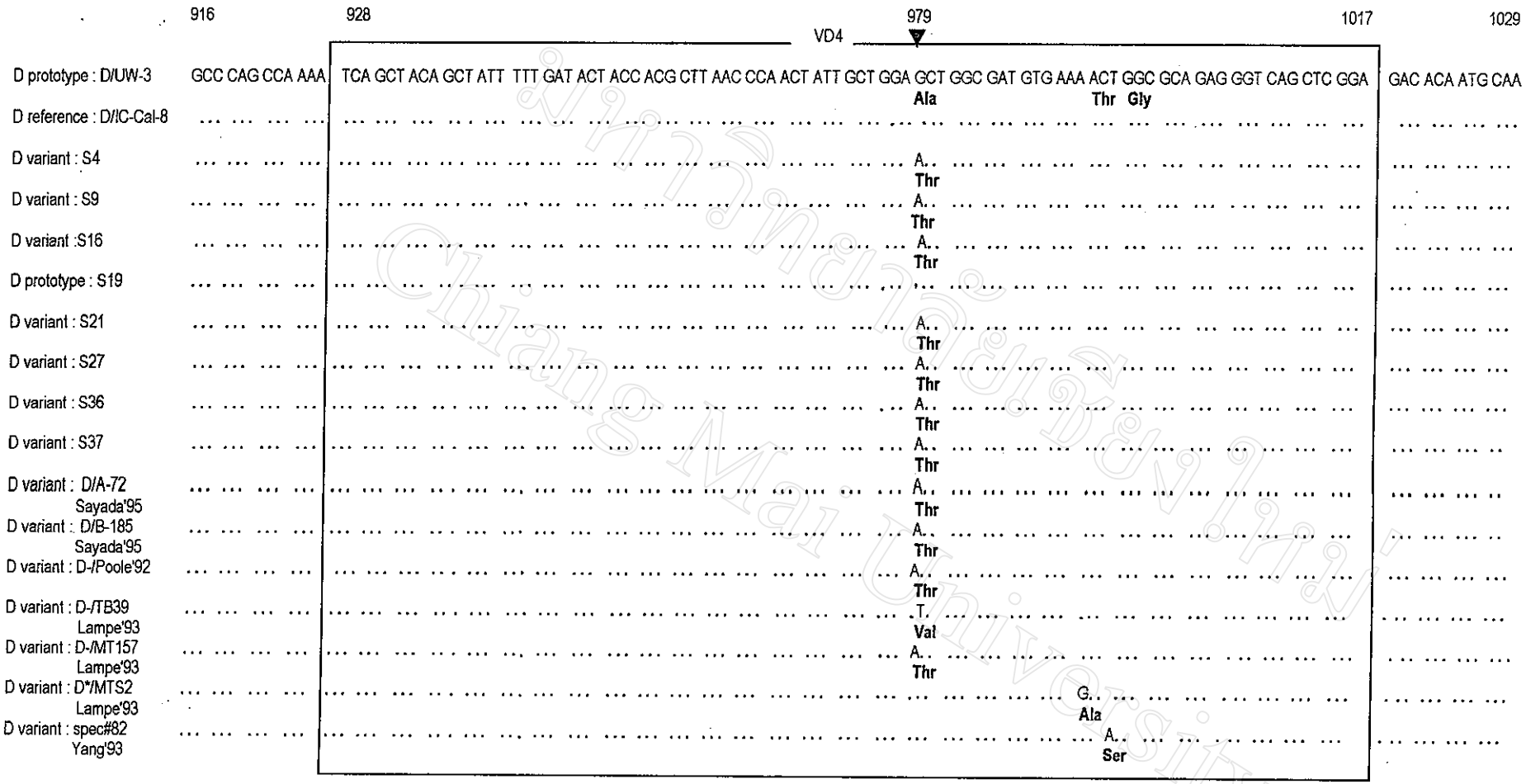


Figure 17. Nucleotide and amino acid sequences comparison of VD4-MOMP gene of the prototype D/UW-3/Cx, D/IC-Cal-8, D genotype found in this study and other D variants.

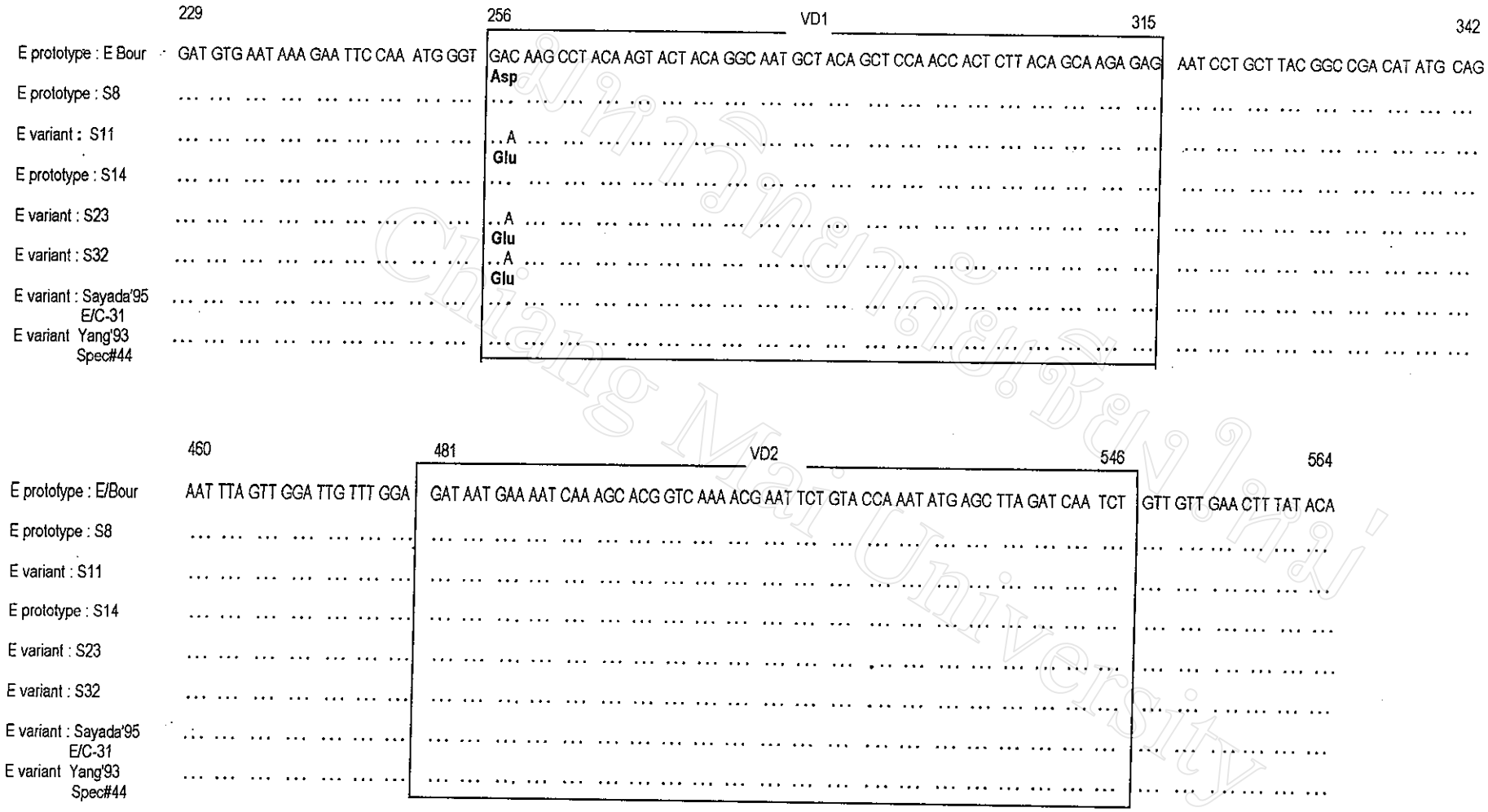


Figure 18. Nucleotide and amino acid sequences comparison of VD1-MOMP gene and VD2-MOMP gene of the prototype E/Bour, E genotype found in this study and other E variants.

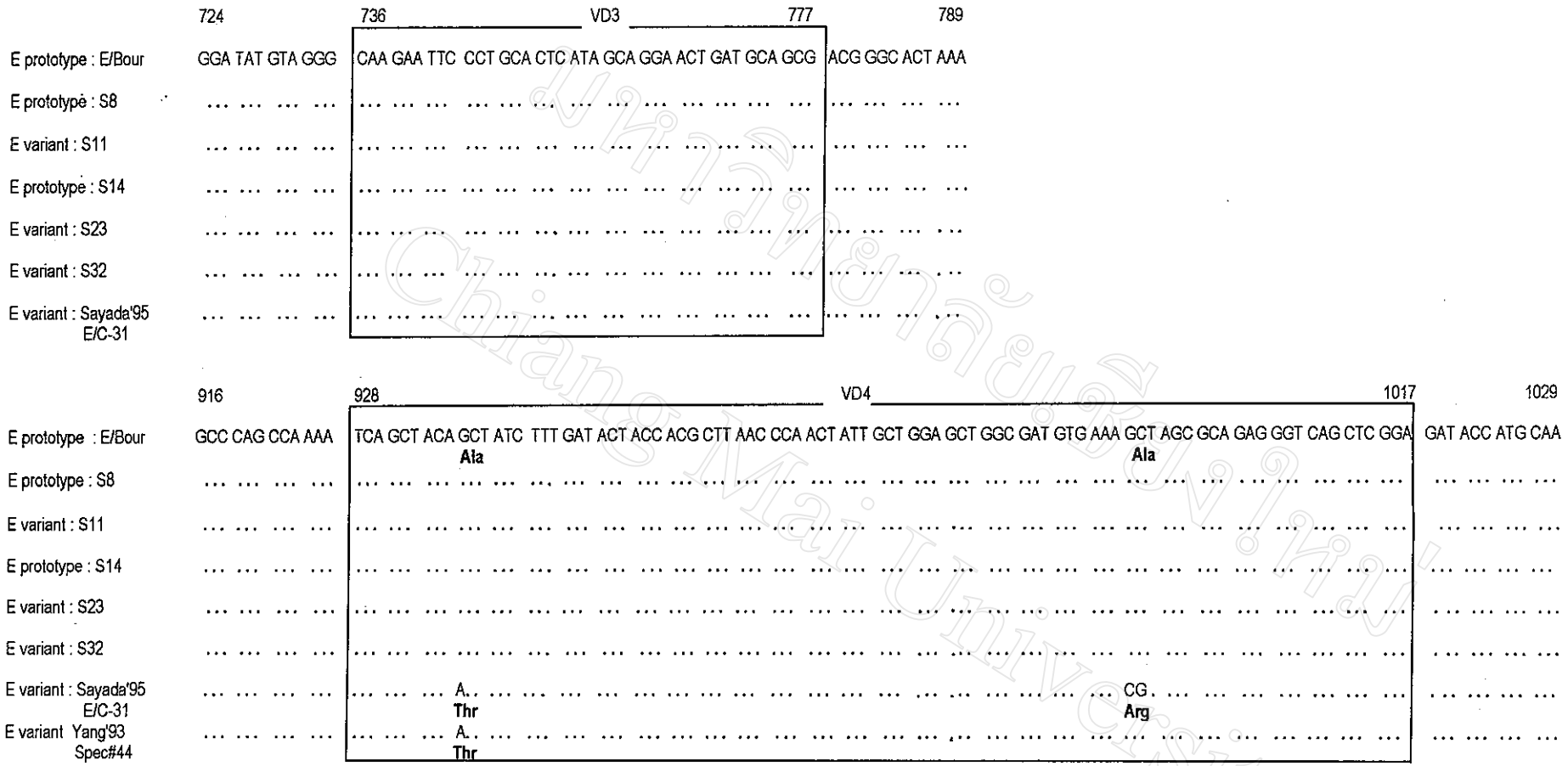


Figure 19. Nucleotide and amino acid sequences comparison of VD3-MOMP gene and VD4-MOMP gene of the prototype E/Bour , E genotype found in this study and other E variants.

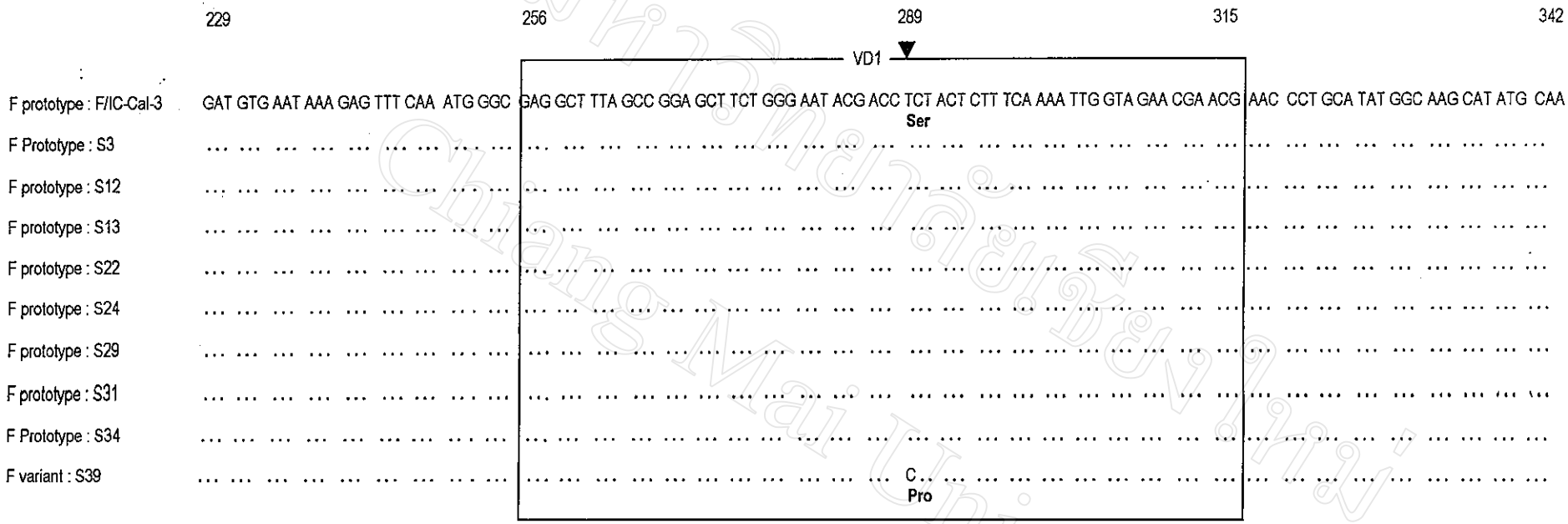


Figure 20. Nucleotide and amino acid sequences comparison of VD1-MOMP gene of the prototype F/IC-Cal-3 and F variant found in this study.

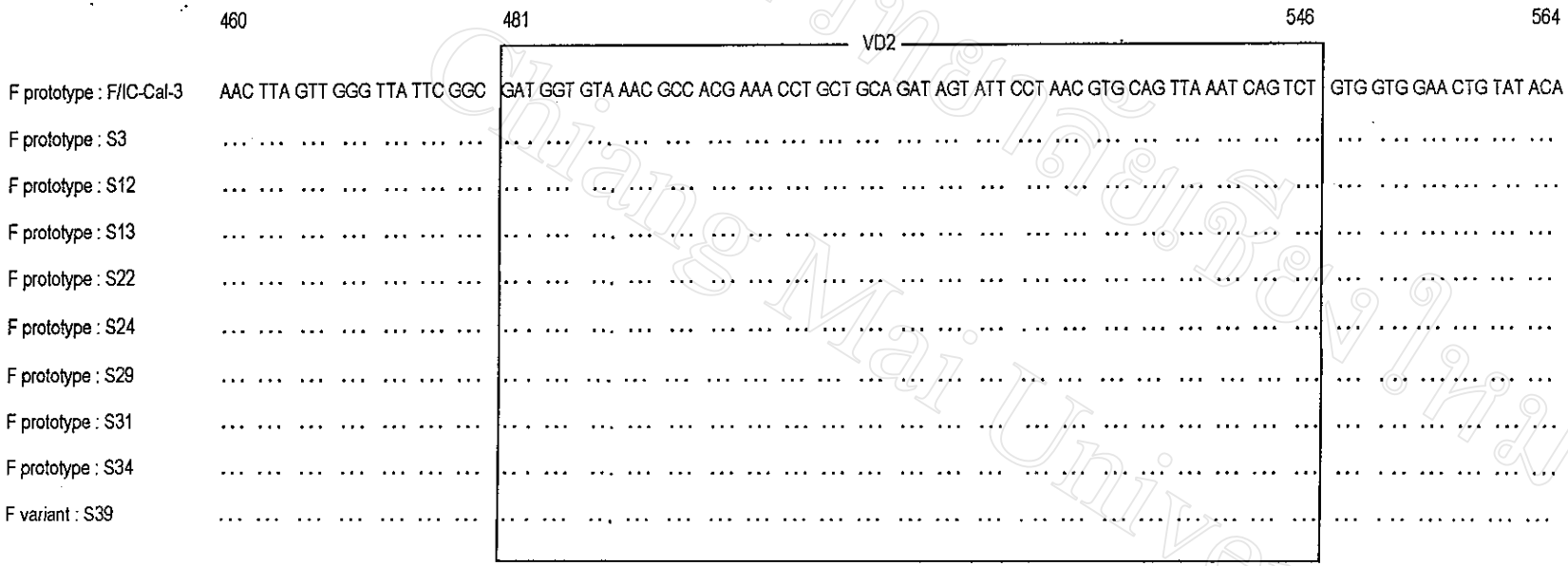


Figure 21. Nucleotide and amino acid sequences comparison of VD2-MOMP gene of the prototype F/IC-Cal-3 and F variant found in this study.

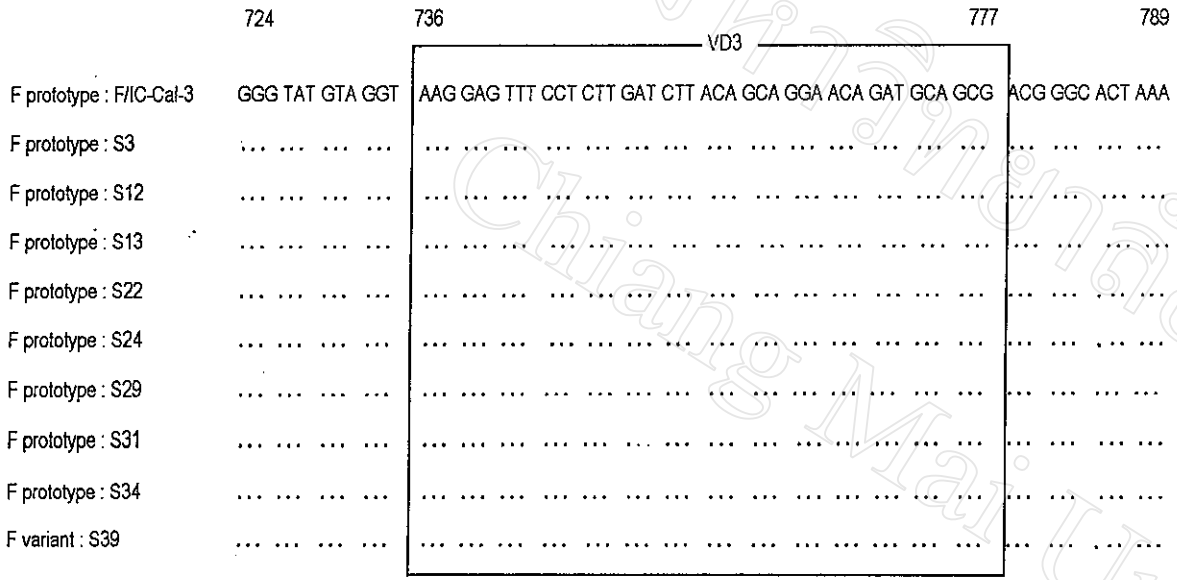


Figure 22. Nucleotide and amino acid sequences comparison of VD3-MOMP gene of the prototype F/IC-Cal-3 and F variant found in this study.

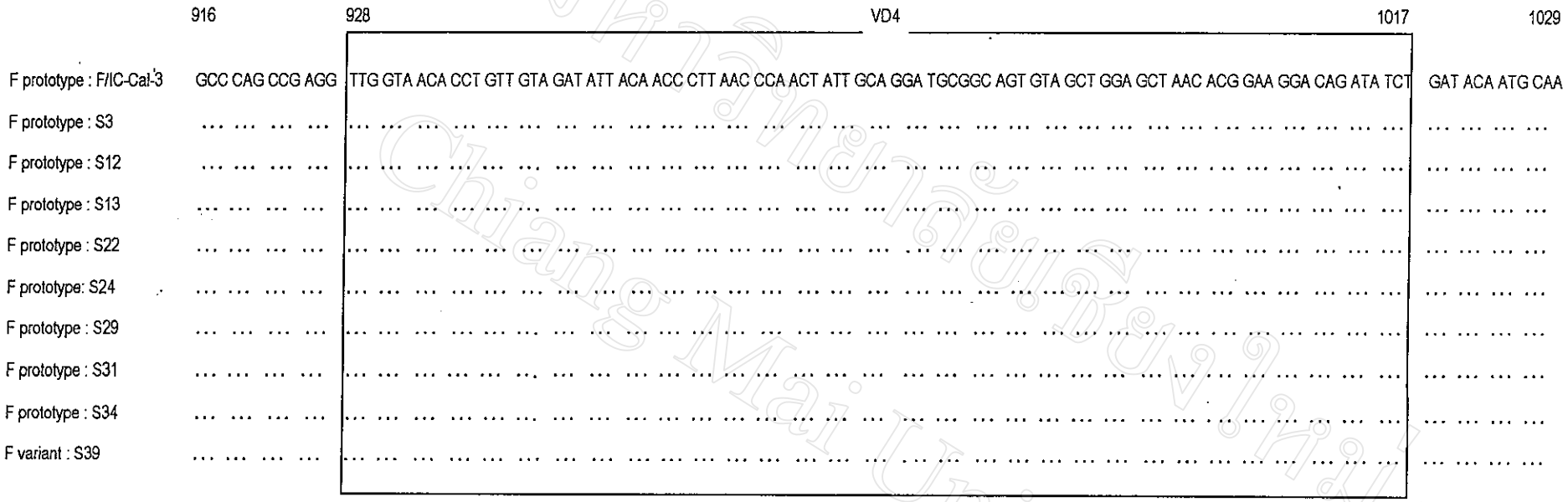


Figure 23. Nucleotide and amino acid sequences comparison of VD4-MOMP gene of the prototype F/IC-Cal-3 and F variant found in this study.

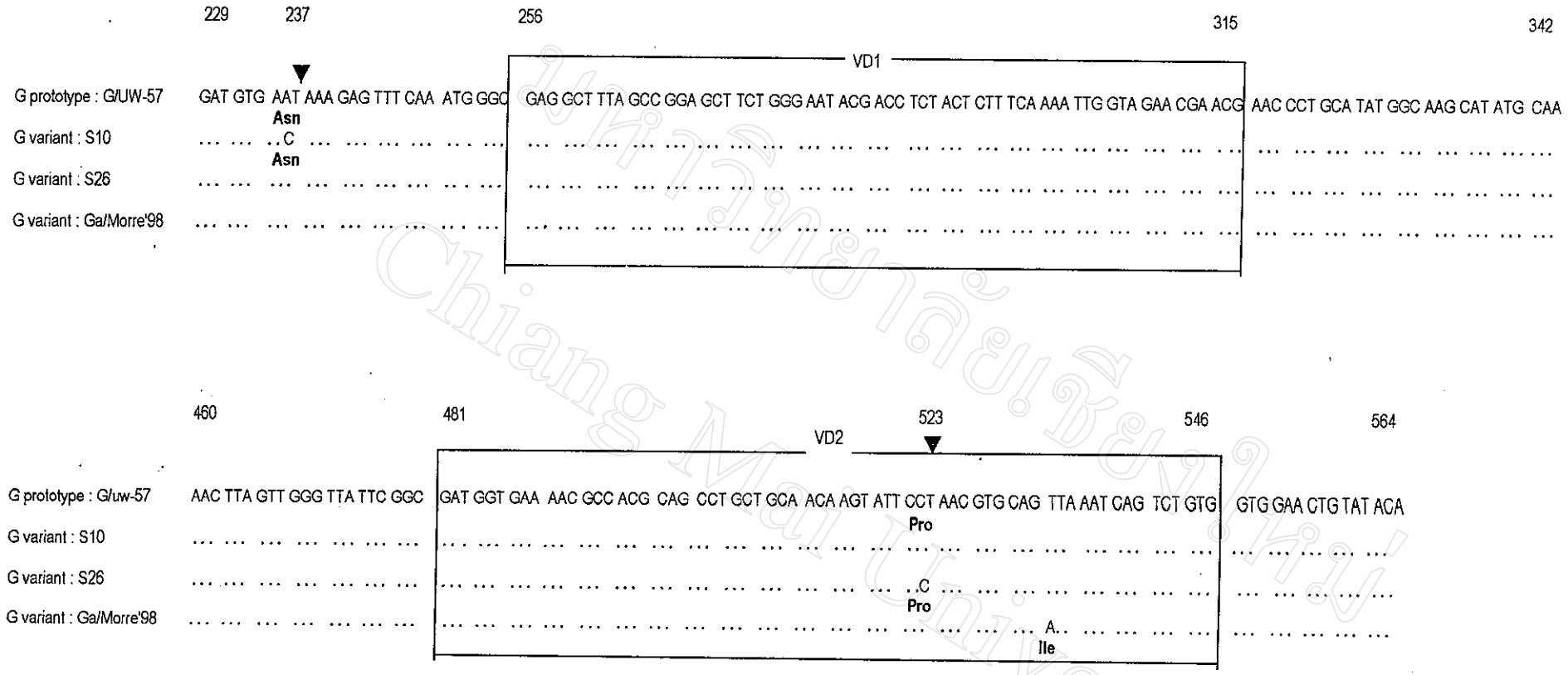


Figure 24. Nucleotide and amino acid sequences comparison of VD1-MOMP gene and VD2-MOMP gene of the prototype G/UW-57, G genotype found in this study and other G variant.

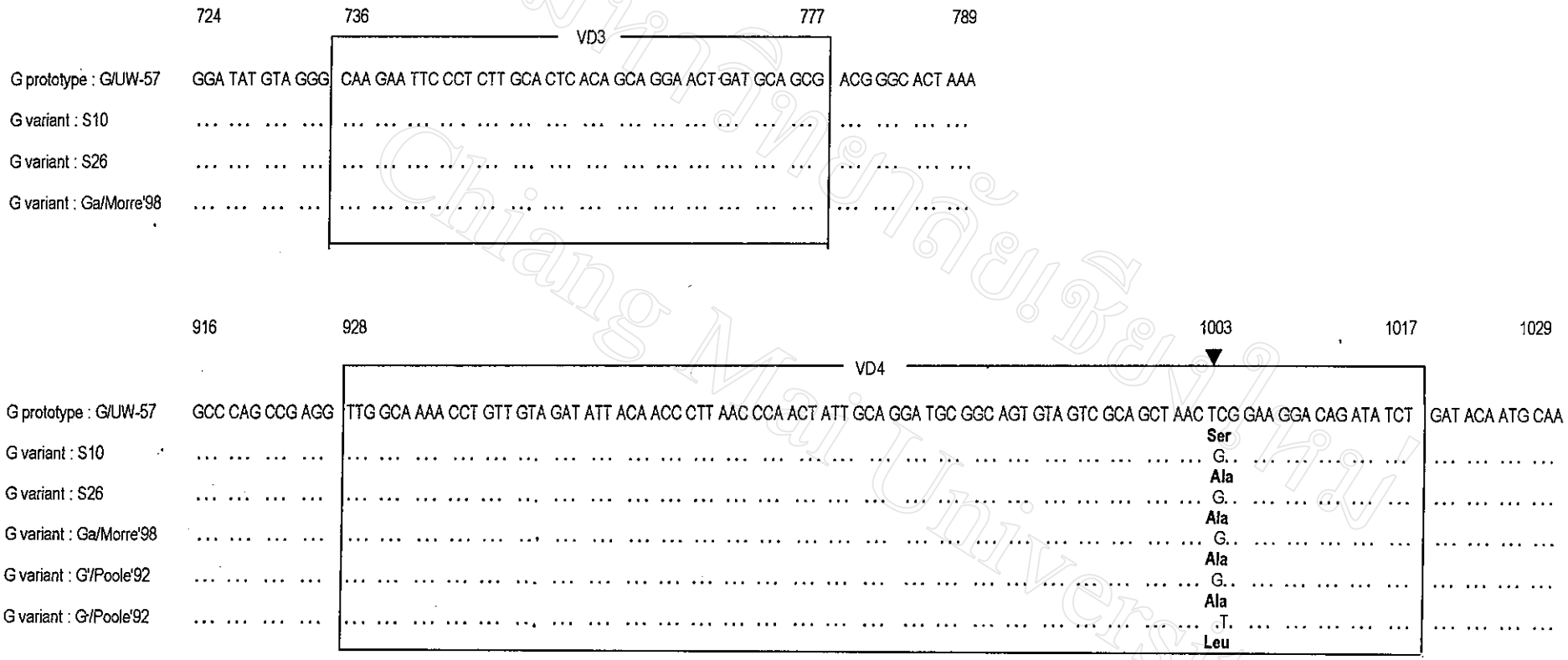


Figure 25. Nucleotide and amino acid sequence comparison of VD3-MOMPgene and VD4-MOMPgene of the prototype G/UW-57, G genotype found in this study and other G variants.

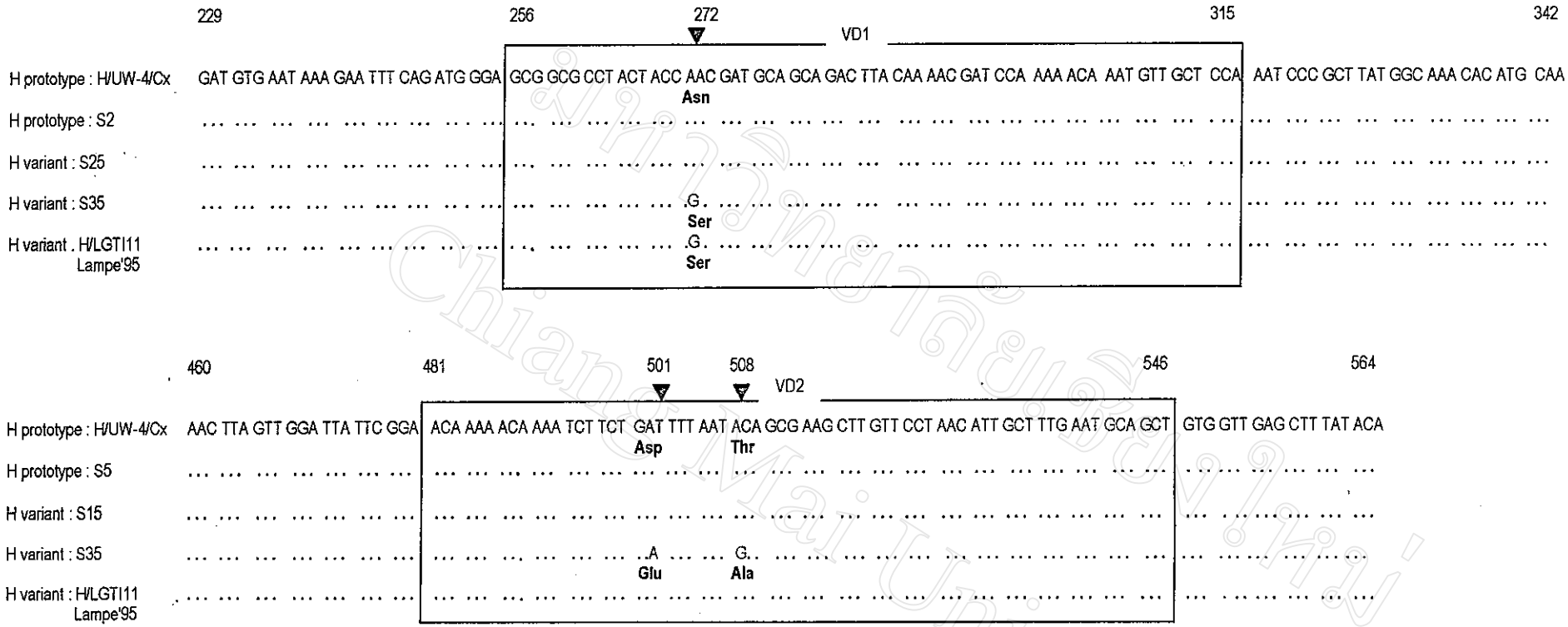


Figure 26. Nucleotide and amino acid sequences comparison of VD1-MOMP gene and VD2-MOMP gene of the prototype H/UW-4/Cx, H genotype found in this study and other H variant.

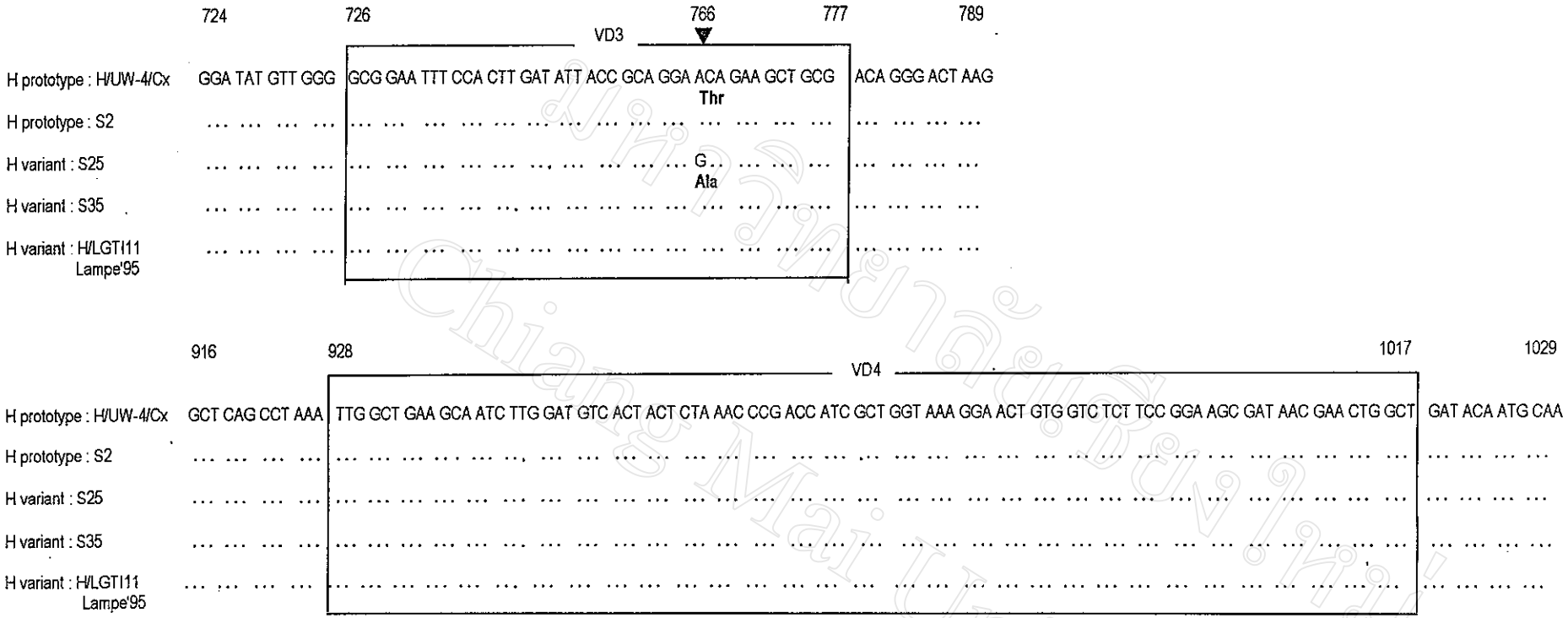


Figure 27. Nucleotide and amino acid sequences comparison of VD3-MOMPgene and VD4-MOMP gene of the prototype H/UW-4/Cx, H genotype found in this study and other H variant.

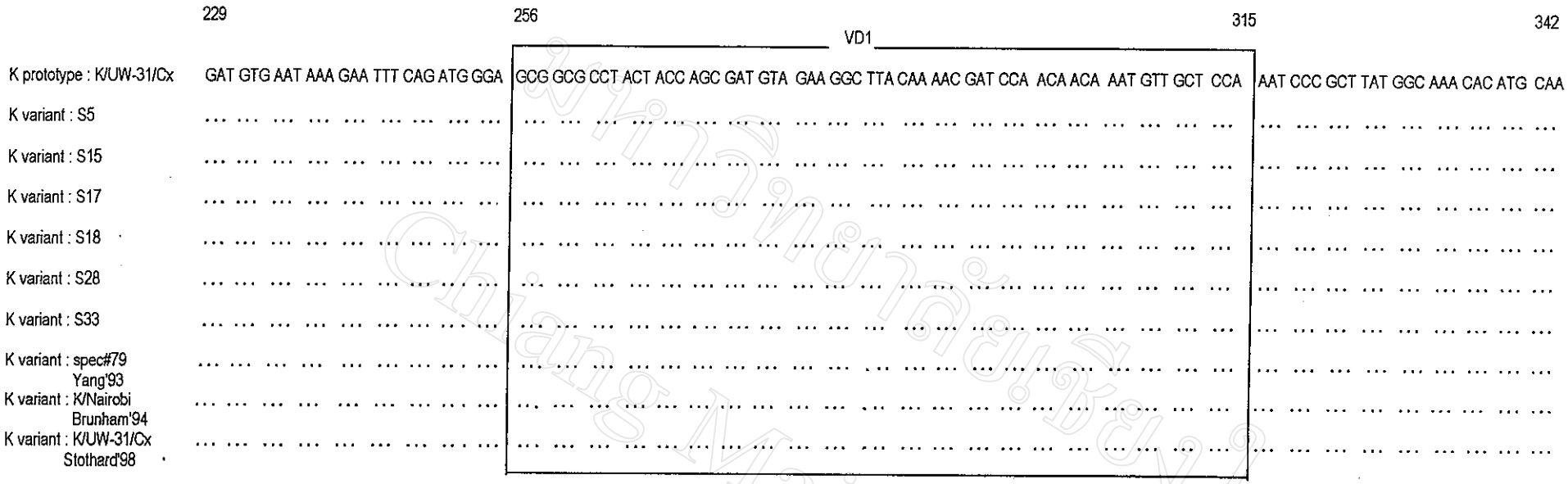


Figure 28. Nucleotide and amino acid sequences comparison of VD1-MOMP gene of the prototype K/UW-31/Cx, K genotype found in this study and other K variants.

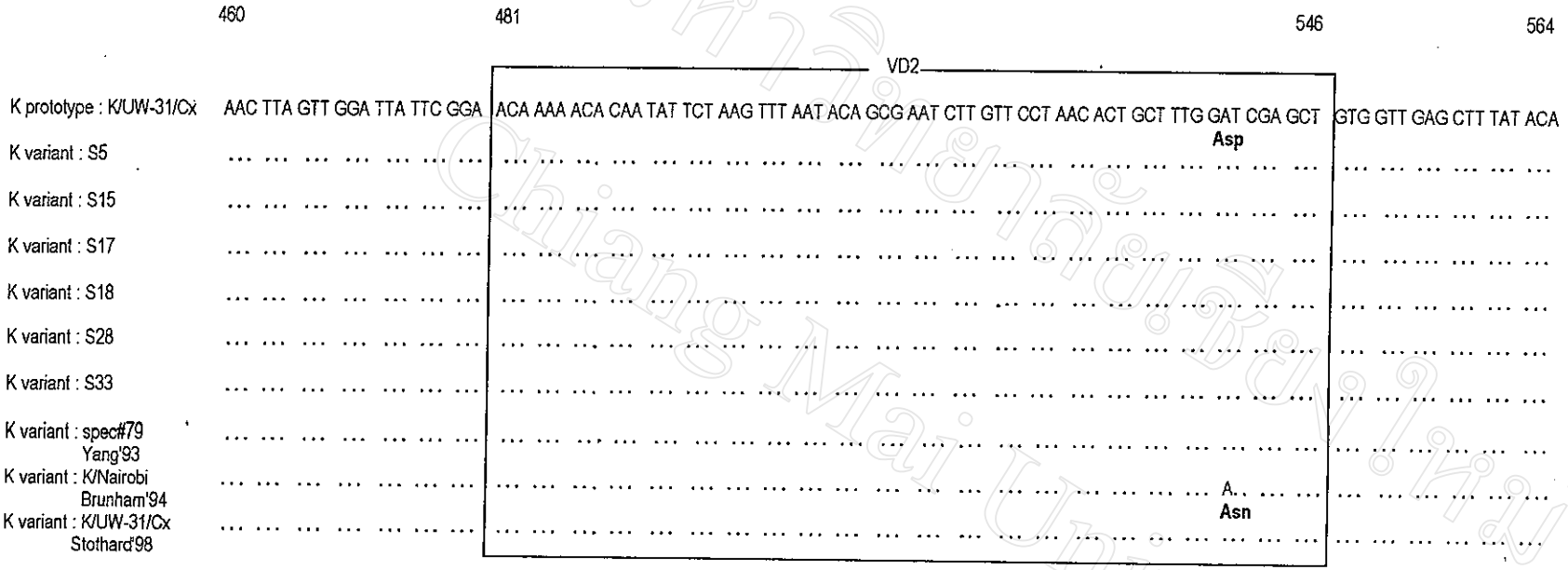


Figure 29. Nucleotide and amino acid sequences comparison of VD2-MOMP gene of the prototype K/UW-31/Cx, K genotype found in this study and other K variants.

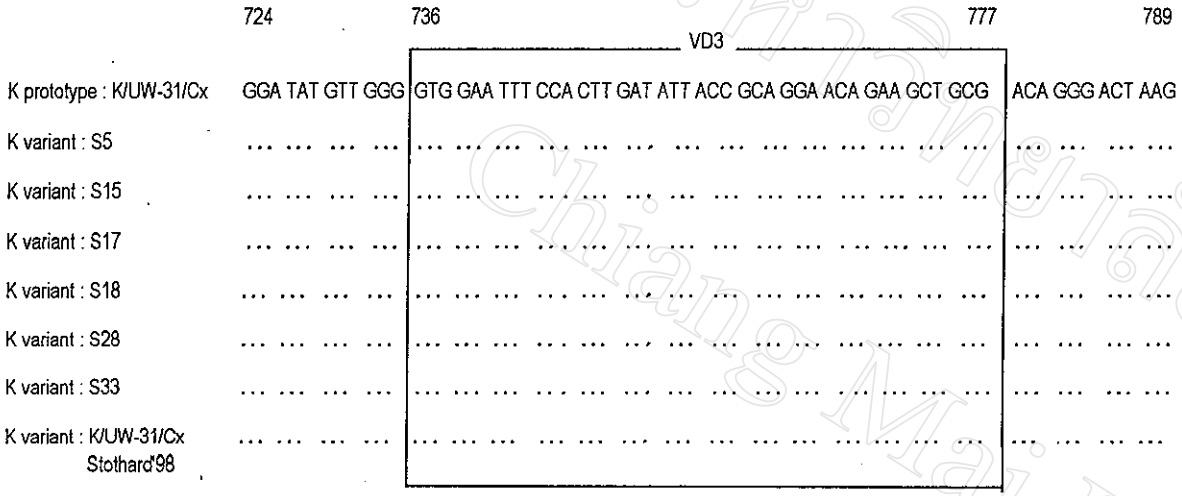


Figure 30. Nucleotide and amino acid sequences comparison of VD3-MOMP gene of the prototype K/UW-31/Cx, K genotype found in this study and other K variants.

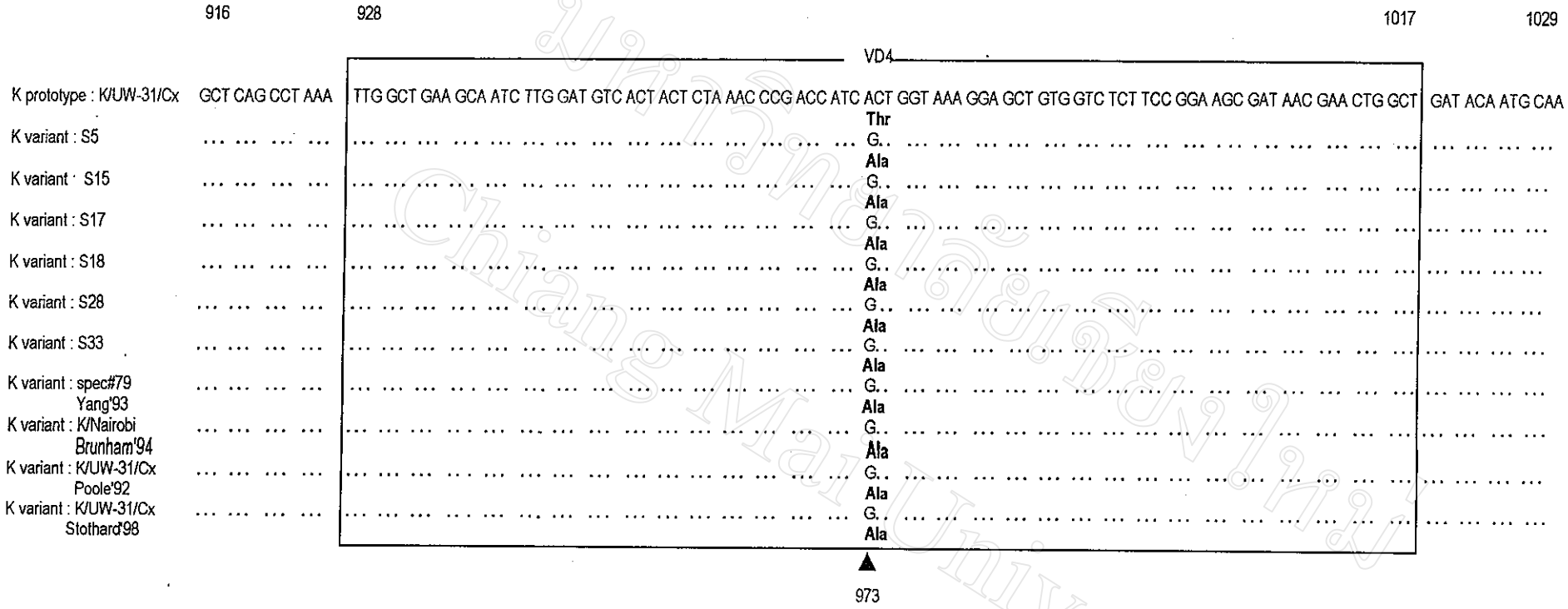


Figure 31. Nucleotide and amino acid sequences comparison of VD4-MOMP gene of the prototype K/UW-31/Cx, K genotype found in this study and other K variants.

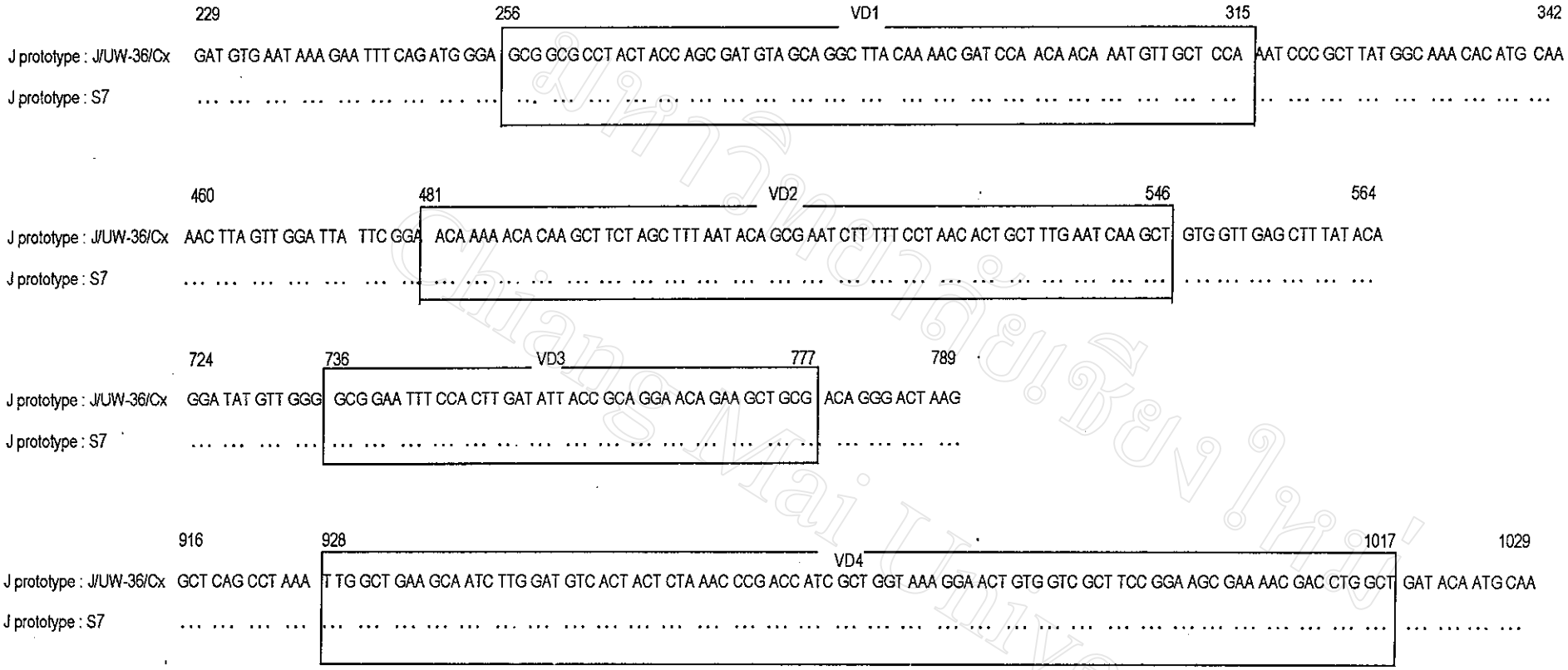


Figure 32. Nucleotide and amino acid sequences comparison of VD1-, VD 2-, VD3- and VD4-MOMP gene of the prototype J/UW-36/Cx and J genotype found in this study.

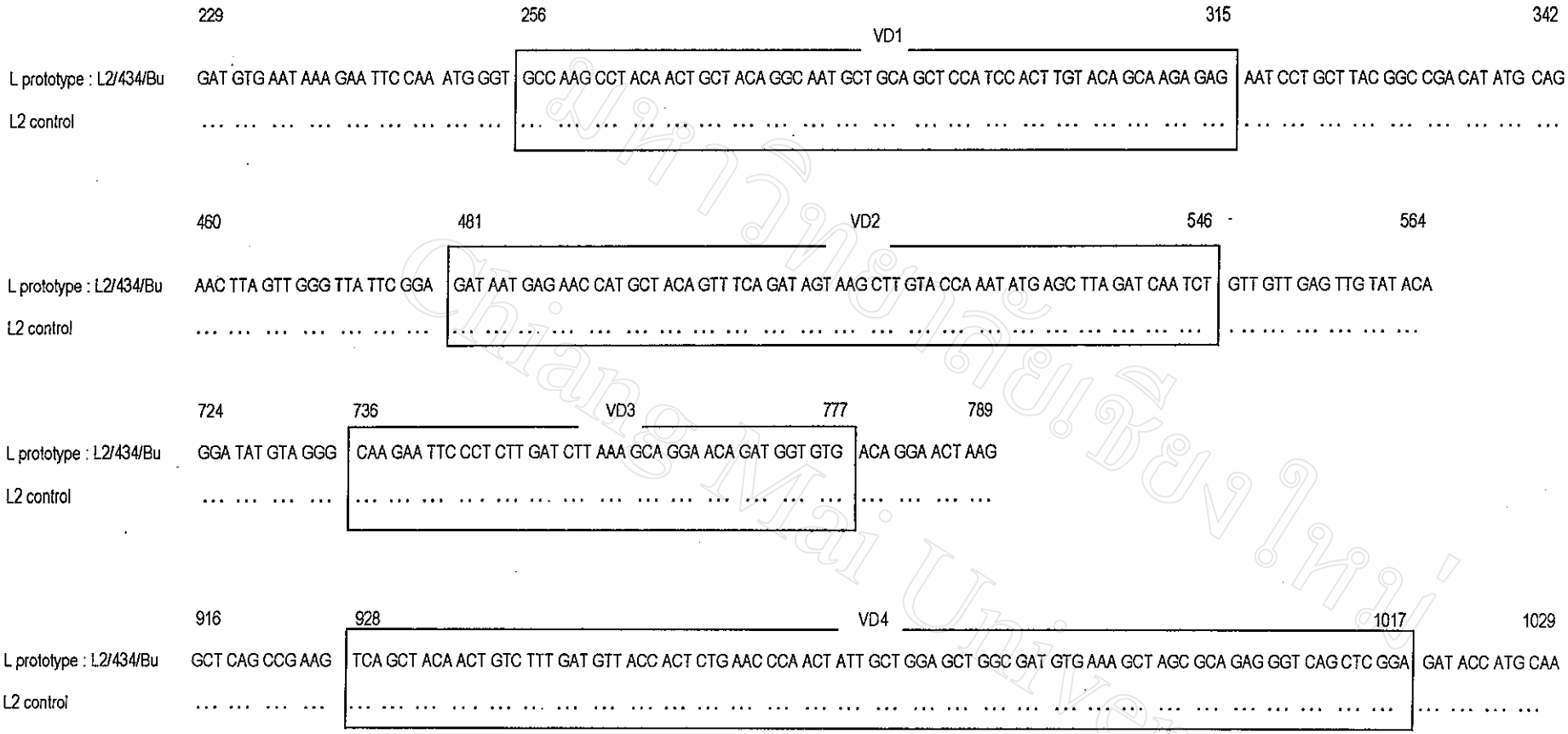


Figure 33. Nucleotide and amino acid sequences comparison of VD1-, VD 2-, VD3- and VD4-MOMP gene of the prototype L2/434/Bu and the L2 control used in this study.