CHAPTER IV

RESULTS

1. Pulsed field gradient gel electrophoresis

The DNA bands separated by PFGE were identified by their positions in the gel (size) and were referred to as chromosomes, though the genetic content of the DNA in the bands has not been significantly characterized. Following the preceding work as described in Chapter II, the chromosomes in this dissertation were also numbered from the smallest to the largest.

1.1 Pulsaphore electrophoresis

Initially, attempts were made to resolve the chromosomal DNA from P. falciparum by using Pulsaphore electrophoresis (LKB) with pointed electrode array. This experimental eletric field geometry has been called the double inhomogeneous configuration (Smith et al., 1987) or OFAGE (Carle and Olson, 1984) The chromosomal DNA under this square array tended to migrate in a curve manner, thus limitting the number of samples that could be applied and preventing comparison of the chromosome sizes(figure 10.1). Subsequently, the same equipment with a new hexagonal electrode array was used. Up to 9 bands were shown to run in a straight lane but only the four smallest

chromosomes were clearly seen (figure 10.2 and 10.3). This uniform mobility facilitated quantitative comparison of the bands from different tracks on the same gel and, of course, from different gels since a size standard were loaded on each gel.

1.2 CHEF-DR II eletrophoresis

With the hexagonal configuration of electrode and improved condition i.e. pulse time, at least 11 chromosomal DNA bands were ultimately obtained by CHEF-DR II (Bio-Rad) electrophoresis (figure 10.4 - 10.11).

The relationship between mobilities and molecular sizes of chromosome-sized markers, S. cerevisiae, are presented in figure 11. Consequently, a rough assessment of the chromosomal DNA sizes of all P. falciparum was obtained by comparing the relative mobilities of parasite chromosomes (figure 12) to those of yeast chromosomes.

A reference clone, T9/94, was loaded on to every gel. The band which migrated farthest from the slot was the smallest chromosome designated chromosome 1 and the chromosomes were numbered consecutively from smallest to largest. Thus, the band which migrated the most slowly from the slot (the largest chromosome) in this study was denoted chromosome 11.

Some chromosomal DNA may not be resolved with this running conditions but be fused into a single bunch. The negative films from all of the gels were, therefore, scanned by

densitometer to observe locations of the chromosomal bands, (only a part of the data is shown). Eleven peaks displayed were congruent with the 11 chromosomal bands mentioned above (figure 13). In figure 13b ten peaks are shown; it was likely that, however, the fifth peak should correspond to 2 bands (i.e. chromosome 5 and 6). This is because all clones from the same parent T9/94 tested here demonstrated 2 chromosomes at that size range.This was consistent with the densitometric tracing of T9/94 S300/300 clone (figure 13d). Chromosomal DNA bands called chromosome 6 revealed 2 peaks; this suggested that 11 bands proposed in figure 12 should be a minimal estimation of the total chromosome numbers.

It is evident that there was a chromosome size polymorphism among the clones as shown in the following results.

2. Chromosome Size Polymorphism

Among the parasite clones stemmed from a single isolate, chromosome size variation was demonstrated. In this study 12 P. falciparum clones from 4 isolates, T9, K31, 27, and F85 (i.e. T9/94 ; K31 CB2,...,K31 CB7 ; B3,..., C6 ; F85 VO3,..., F85 VO15) were analysed for chromosome polymorphism.

(1) For isolate K31, 4 clones (CB2, CB5, CB6, and CB7) were examined (figure 10.5 and 10.10). It was found that polymorphism was evident in 3 of them (CB2, CB5, CB6) whilst the

karyotypes of K31 CB7 and K31 CB6 were similar. The degree of differences was not so great. Chromosome 7 of K31 CB2 was larger than that of the other three clones of K31. In contrast, chromosome 4 - 9 of K31 CB5 was somewhat smaller than those from the other clones of K31.

For isolate 27, three clones menifested chromosome variations within their group (figure 10.2, 10.7, and 10.8). The sizes of chromosomes 1,10 and 11 were about 600, 3900, and 4100 kb respectively. Chromosome 2 of isolate 27 and clone C5 were similar whereas that of B3 and C6 were smaller. A substantial distinction was shown by the third chromosome of the clone B3; its size was about 1050 kb which was as large as the other Chinese clones and isolates. In addition, the ninth chromosome of B3 was the largest when compared to chromosome 9 all clones studied here. With regard to polymorphism within this group, karyotypes of C5 clone and its parent isolate designated 27 were alike but different from the other 2 clones.

For isolate F85, four clones were also shown chromosome size variations (figure 10.7 and 10.8). No 2 identical clones were obtained.

For isolate T9, only one clone (T9/94) was investigated and its chromosomal profile was different from that of clones from isolate K31, 27, or F85.

(2) Four additional subclones of T9/94 (RC1, RC2, RC3, and RC4) were also analysed. Negligible differences were

revealed among these subclones (figure 10.5, 10.6, 10.10, and 10.11). Compared to the parent T9/94, chromosome 7 of both T9/94 RC2 and T9/94 RC3 were slightly different, though they were in the range of seventh chromosome of T9/94. The first chromosome of T9/94 RC1 was rather smaller than that of T9/94. All chromosomes of T9/94 RC4 were similar to the parent clone.

(3) Another 12 clones derived from T9/94 were also studied. These clones were come from in vitro mutagenesis of T9/94 followed by pyrimethamine selection. All of them possess the pyrimethamine susceptibility that changed from sensitive to intermediate resistant.

It was found that a mutant clone which was mutagenized by EMS -T9/94 S300/300- showed that chromosome 9 was larger than the T9/94 clone (figure 10.6 and 10.10).

Although having the same pyrimethamine MIC value as T9/94 S300/300, karyotypes of three mutant clones denominated T9/94 (M1-1) a1, T9/94 (M1-1) a2, and T9/94 (M1-1) a6 were identical to the parent T9/94. Additionally, T9/94 (M1-1) a9 which has MIC value two times less than the three clones mentioned above, also showed the same chromosome pattern as the parent clone (figure 10.3 and 10.9).

Another group of mutant clones named T9/94 (M1-1) b1, T9/94 (M1-1) b3, T9/94 (M1-1) b5, T9/94 (M1-1) b6, T9/94 (M1-1) b9, T9/94 (M1-1) b10 , and T9/94 (M1-1) b14 which were mutagenized by MNNG exhibited more or less the same karyotypes except T9/94

(M1-1) b6 and T9/94 (M1-1) b14. Chromosome 9 of all these clones were similar to each other but different than that of T9/94 (figure 10.4, 10.5, 10.6, 10.10, and 10.11).

(4) Comparing polymorphism among P. falciparum isolates, karyotype of clones from 4 isolates were exhibited chromosome size variations.

One isolate and 3 clones from China menifested chromosome variations compared to all clones from Vietnam and Thailand. The mobility of chromosomes 1,10 and 11 of all Chinese strains was faster than all clones studied here.

Compared to chromosome 2 of Thai clones, that of isolate 27 and clone C5 were similar whereas that of B3 and C6 were smaller. Chromosome 2 of F85 VO3 and F85 VO15 were larger than all clones tested here.

A significant distinction was shown by the third chromosome of the clone B3; its size was about 1050 kb which was as large as the second chromosome of Thai clones.

Another variation was shown by the seventh chromosome of isolate 27 which was like the corresponding chromosome of T9/94 RC3 and K31 CB2 as well as F85 VO3.

Moreover, the chromosome 9 of B3 was the largest when compared to ninth chromosome all clones studied here. Chromosome 9 of four sensitive clones (called K31 CB2, K31 CB5, K31 CB6, and K31 CB7) from isolate K31 were unlike chromosome 9 of T9/94 from isolate T9 (figure 10.5 and 10.10).

Four clones from Vietnam were demonstrated the largest chromosome 10 and 11 when compared to those of all clones. Both chromosomes of 1 isolate and 3 clones from China were smallest when compared to those of all clones.

3. Chromosome 4 polymorphism

Taken chromosome 4 of 1 isolate and 28 clones of P. falciparum into consideration, size polymorphism was also shown.

The fourth chromosomes of T9/94 and its 4 subclones (RC1, RC2, RC3, and RC4) revealed the same size. Similar observation was found in 4 clones from in vitro mutagenesis of T9/94 followed by pyrimethamine selection [T9/94 (M1-1) a1, a2, a6, and a9] although their pyrimethamine susceptibilities changed from sensitive to intermediate resistant.

A karyotype of T9/94 S300/300 ,having the same pyrimethamine MIC value as T9/94 (M1-1) a1, a2, and a6 , was also identical to the parent T9/94.

Although being the same intermediate resistant to pyrimethamine as T9/94 (M1-1) a1, a2, a6 and a9, the fourth chromosome of 5 clones from in vitro MNNG mutagenesis of T9 /94 followed by pyrimethamine selection [T9/94 (M1-1) b3, b5, b6, b10, and b14] were slightly larger than that of the parent T9/94.In contrast, T9/94 (M1-1) b1 and b9 demonstrated the same chromosome 4 size as in T9/94. Nonetheless, T9/94 (M1-1) b6

and T9/94 (M1-1) b14 no longer contained the T9/94 chromosome 4. It was apparent that the fourth chromosomes of both clones were larger than that of parent and the other mutant clones. In T9/94 (M1-1) b6 there wasa tendency to fuse with chromosome 5 and in T9/94 (M1-1) b14 chromosome 4 was proximal to chromosome 5.

Sizes of the fourth chromosome of 4 pyr^{-} clones from isolate K31 exhibited a polymorphism. Compared to that of T9/94, chromosome 4 of K31 CB2 was larger whilst that of K31CB5 was smaller and those of K31 CB6 and K31 CB7 were the same.

For 1 isolate and 3 clones from China which had a pyrimethamine MIC value of 1×10^{-6}, chromosome 4-sized polymorphism was also shown. Chromosome 4 of C5 clone were the largest in this study.

Chromosome 4 of 3 clones from Vietnam which were the most resistant to pyrimethamine in this study, showed the same positions in the running gels while that of one clone (F85 VO3) was larger.

To reassure chromosome 4 localization, the gels were blotted to the Hybond-N membranes. Chromosomal DNA on these filters were then hybridized with DHFR-TS probe. Unfortunately, the blotting procedure was unsuccessful because of an inherent problem of the membrane (see Appendix III). Nevertheless, one autoradiogram from the gel in figure 10.7 showed the hybridization pattern of DHFR probe in fourth chromosome (figure 14).

4. Dot blot analysis

To test the possibility that DHFR gene could be amplified, thus causing overproduction of the enzyme in the parasite, dot blot analysis were used.

Measured amounts of the DNA from both the T9/94 (pyrimethamine-sensitive clone) and TM4 C8.2 (pyrimathamine-intermediate resistant clone) were spotted onto Hybond-C membrane and probed with the DHFR-TS fragment. In addition, T9/94 (M1-1) b3 and TM4 C8.2/4.1/10.1 which were the mutants of 2 clones mentioned above were also tested. As a control for quantity of total DNA from each parasite loaded in this experiment, the duplicated set of dots-blot membrane was probed with β-tubulin gene, which would not be expected to vary in the clone studied here. If the gene in the pyr^{r} clone had been amplified, more DHFR copies in its DNA would have been anticipated to see than in the DNA of the pyr^{s} clone.

Dot-blot autoradiograms prepared with genomic DNA of 6 samples are shown in figure 15. Quantitative data for comparisons were obtained by using a liquid scintillation counter (Table 3). No significant difference in the amount of DNA corresponding to the DHFR genes of T9/94 and of TM4 C8.2 was found.

The amount of DNA in the DHFR-TS gene in clones T9/94 and T9/94/M1-1(b3) which differ in the amount of DHFR-TS enzyme

produced (Thaithong, S., personal communication), therefore,were also compared. The ratio of DNA from DHFR gene :β-tubulin gene is rather similar when T9/94 (0.31" and 0.39) and T9/94 (M1-1) b3 (0.33" and 0.42) are used as source of DHFR . Compared to the copy number of DHFR gene in T9/94, that in T9/94 (M1-1) b3 had a mean value of 1.07 (1.06" and 1.08). Thus, it is likely that there was no amplification when the mutation from T9/94 to T9/94 (M1-1) b3 took place.

Furthermore, comparison of DNA from TM 4 C8.2 to TM 4 C8.2/4.1/10.1 indicates here also there was no amplification. The proportion of DHFR copy number in TM4 C8.2/4.1/10.1 clone compared to TM4 C8.2 clone was 0.96. Likewise, it is indicated that there was no amplification when the mutation from TM4 C8.2/4.1/10.1 to TM4 C8.2 occurred.

Figure 10 PFGE analysis of chromosomal DNA from 1 isolate and 28 clones of P. falciparum. For comparison, S. cerevisiae was used. In figure 10.1 the DNA was resolved by Pulsaphor (LKB) apparatus with pointed electrode array while in figure 10.2 and 10.3 the DNA was separated by the same apparatus but with hexagonal electrode array. For figure 10.4-10.11, DNA was fractionated by CHEF (Bio-Rad) apparatus.

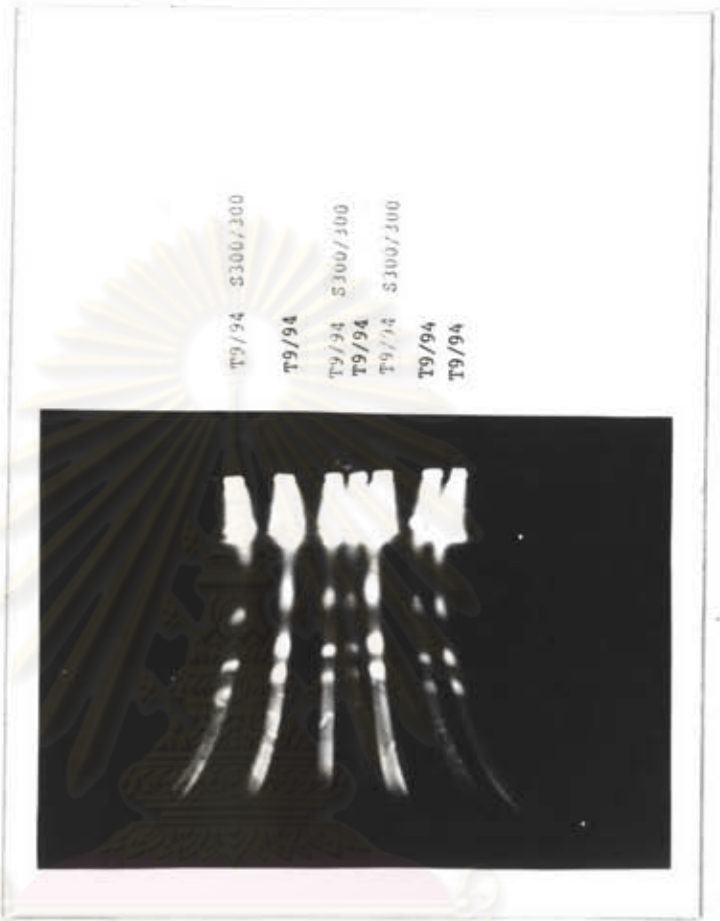

Figure 10.1 Chromosome patterns of T9/94 and T9/94 S300/300. Running conditions were 330 volt for 24 hr at 16° c with pulse time of 80 sec. Comparison between chromosome DNA bands was not allowed due to curvature of the tracks.

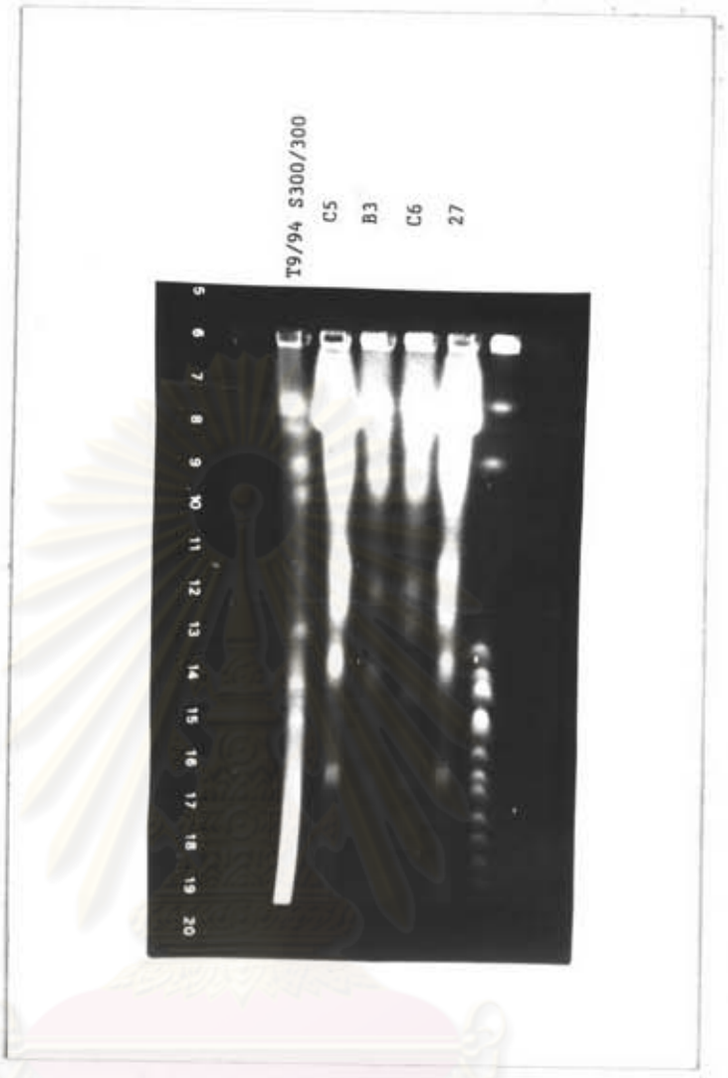

Figure 10.2 Chromosome patterns of one Chinese isolate and its 3 clones as well as T9/94 S300/300. Running conditions were 150 volt for 72 hr. 30 min at 11°c with pulse time of 200 sec. The first bands of DNA of the Chinese isolate and clones here show no vatiation.

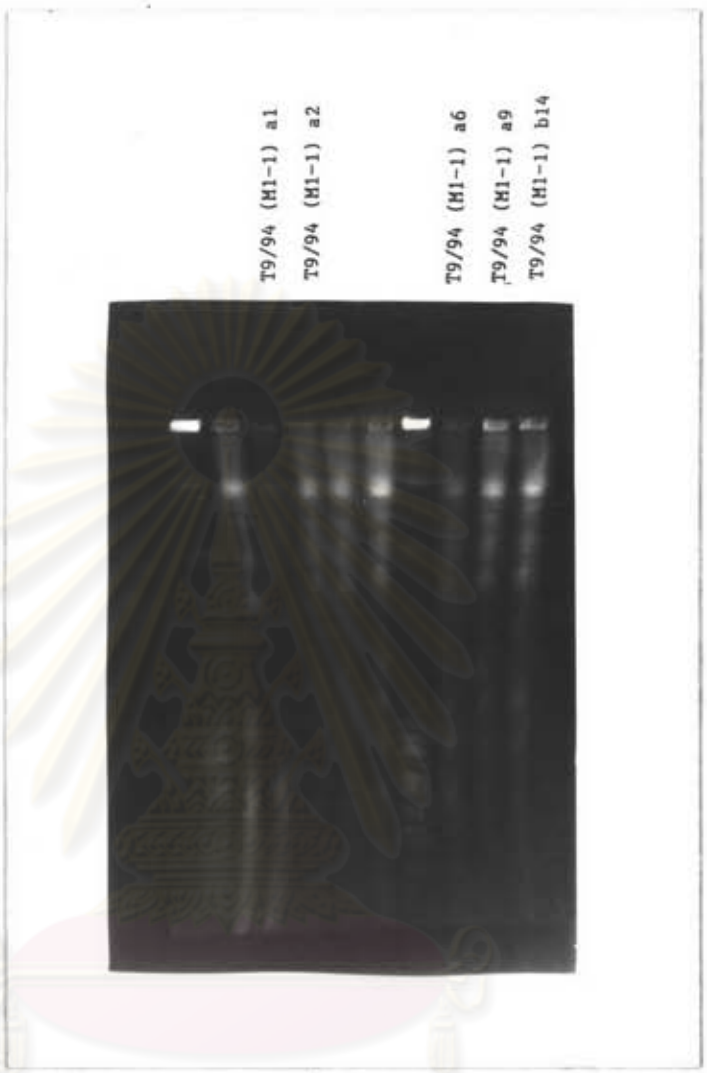

Figure 10.3 Chromosome patterns of T9/94 (M1-1) a1, a2, a6, a9 and T9/94 (M1-1) b14 . Running conditions were 150 volt for 71 hr at 12°c with pulse time of 200 sec. The first bands of all the mutant clones which disappeared from the gel in figure 10.9 here are all alike.

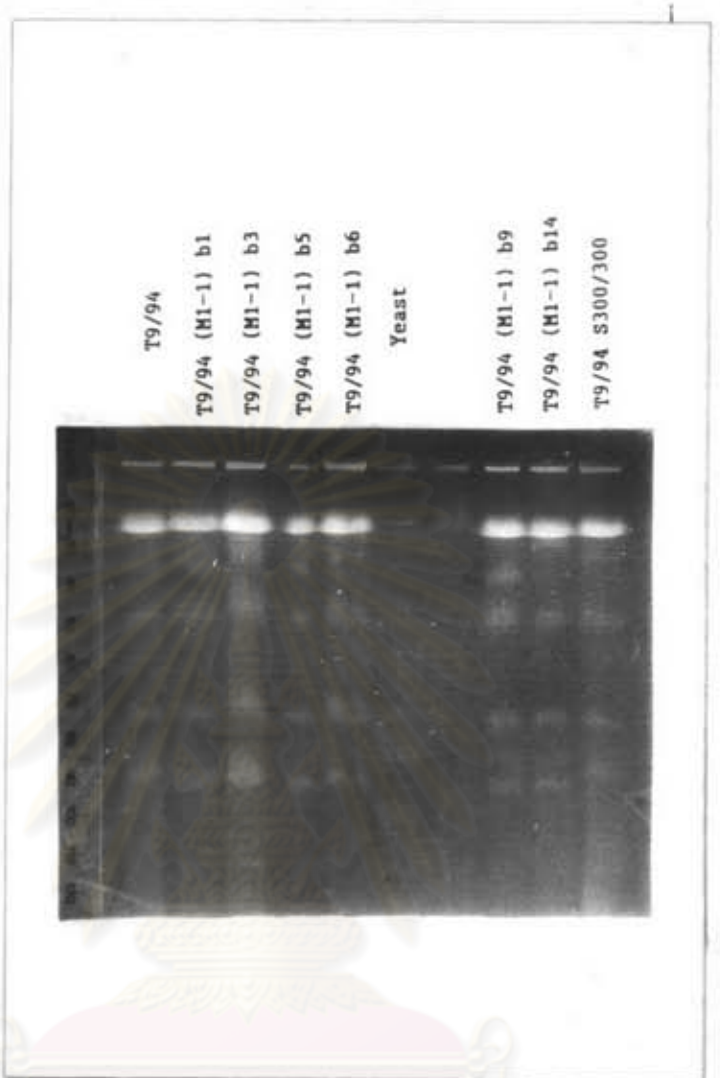

Figure 10.4 Chromosome patterns of parent T9/94 and its mutant clones. Electrophoresis was used for 120 hr with a pulse time of 240 sec. A 10-well comb was used and each block of sample was cut in a longitudinal section. Only the first 4 bands can be seen.

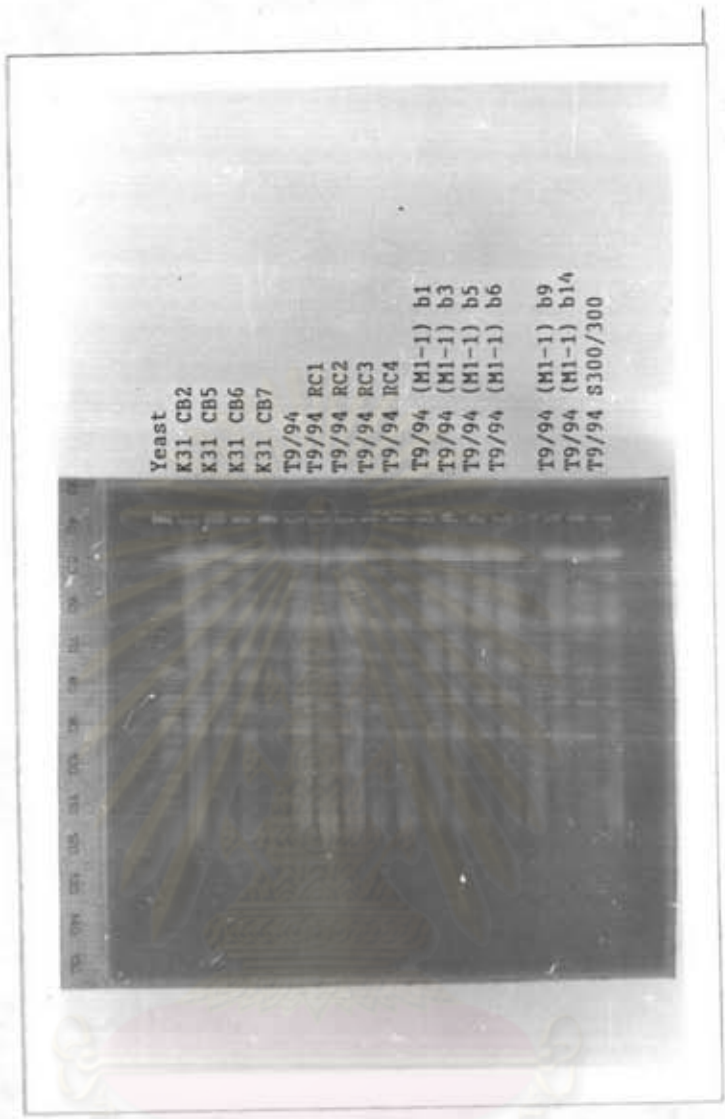

Figure 10.5 Chromosome patterns of a T9/94 and its derivatives compared to 4 subclones of K31. The pulse time was ramped from 180 to 468 sec and the PFGE was run for 72 hr to showing the 4 smallest well-resolved bands and intermediate-size chromosomal bands.

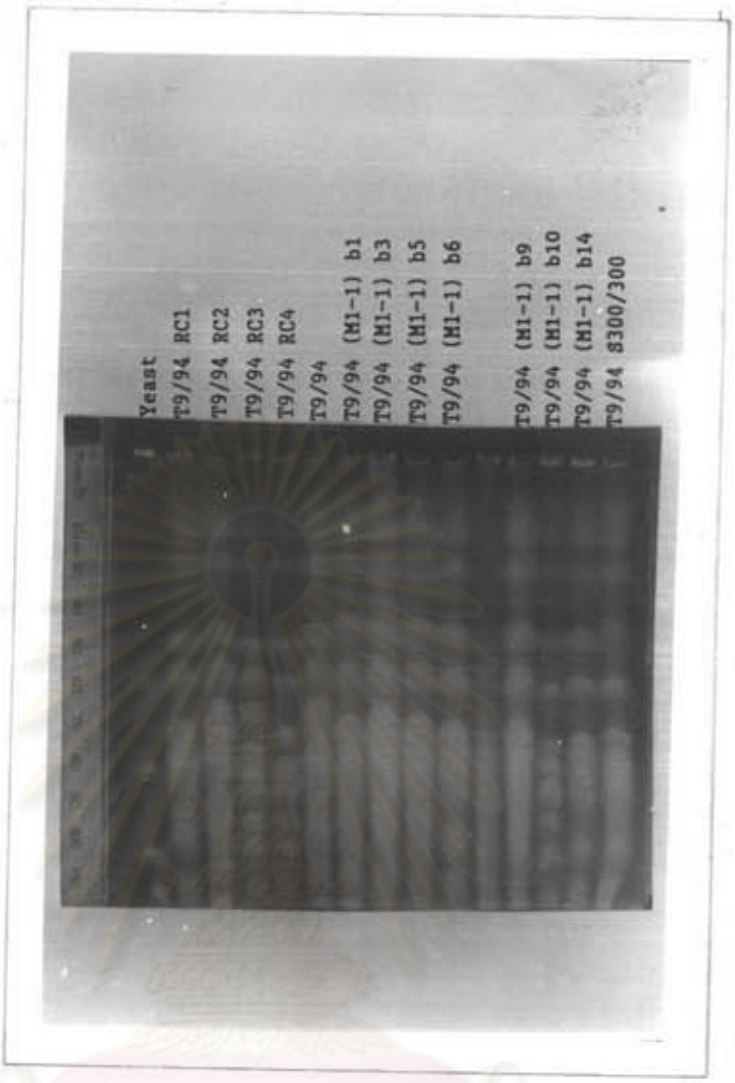

Figure 10.6 Chromosome patterns of parent T9/94 clone, its subclone and mutant clones (Table 1). Running time was 144 hr with pulse time ramped from 180-900 sec. Most of the bands are fused due to overloading of DNA samples.

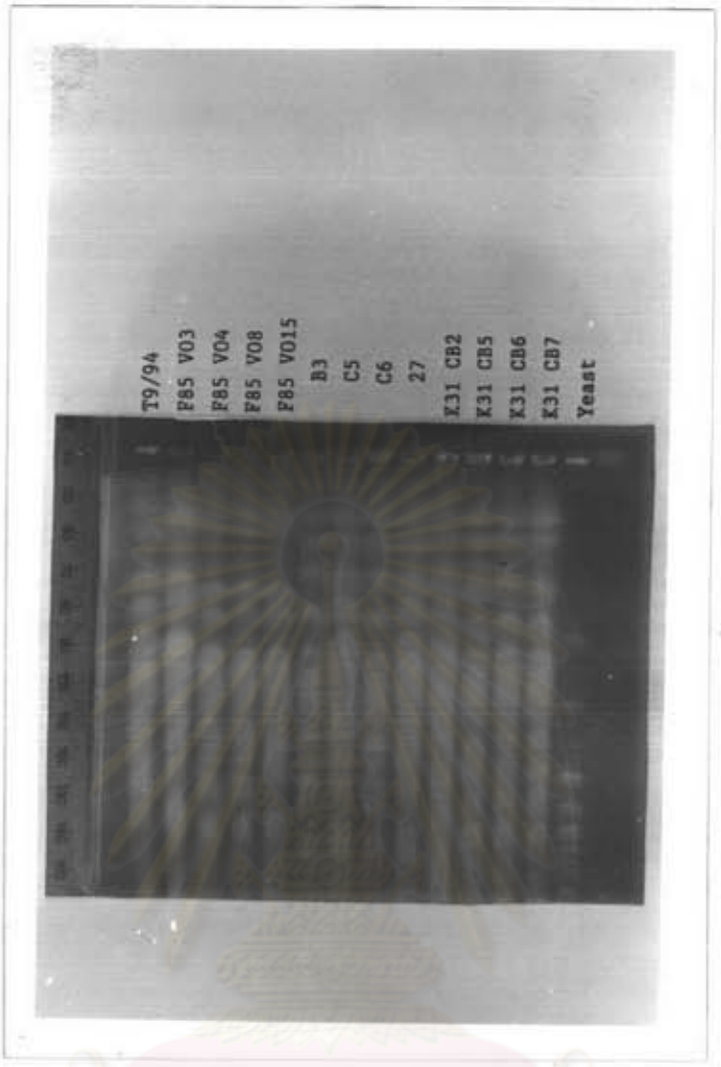

Figure 10.7 Chromosome patterns of Thai, Chinese and Vietnamese clones (Table 1). Running time was 120 hr of the same pulse time as figure 10.6.

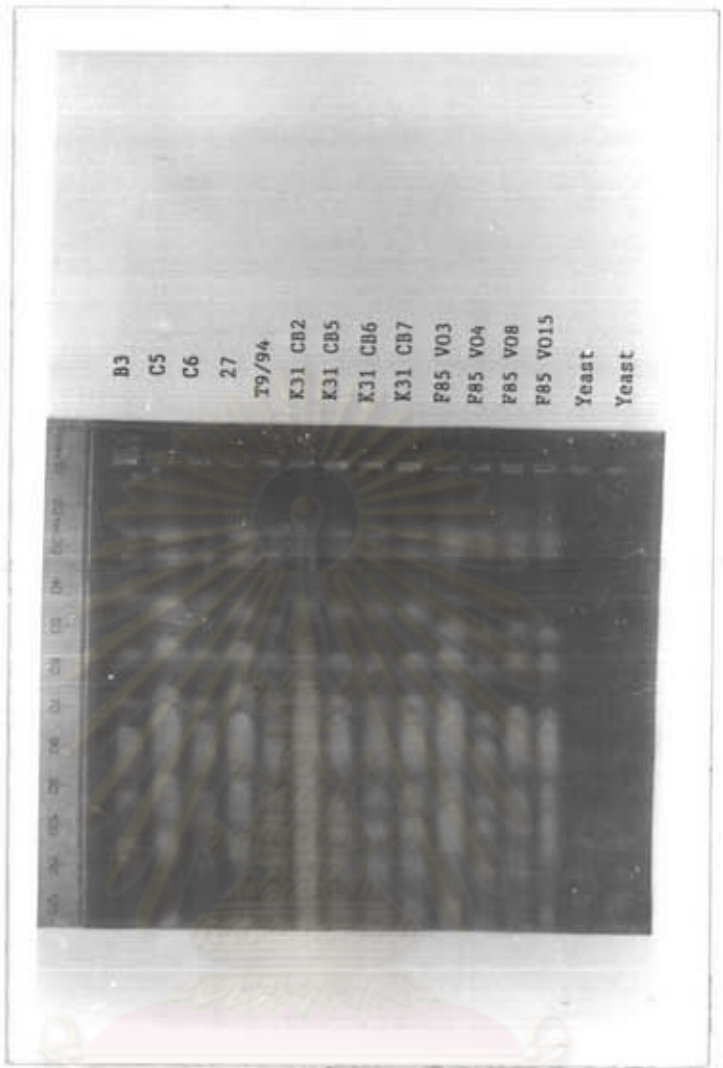

Figure 10.8 Chromosome patterns of the same samples as in figure 10.7 with a running time of 144 hr but pulse time was the same as in figure 10.6.

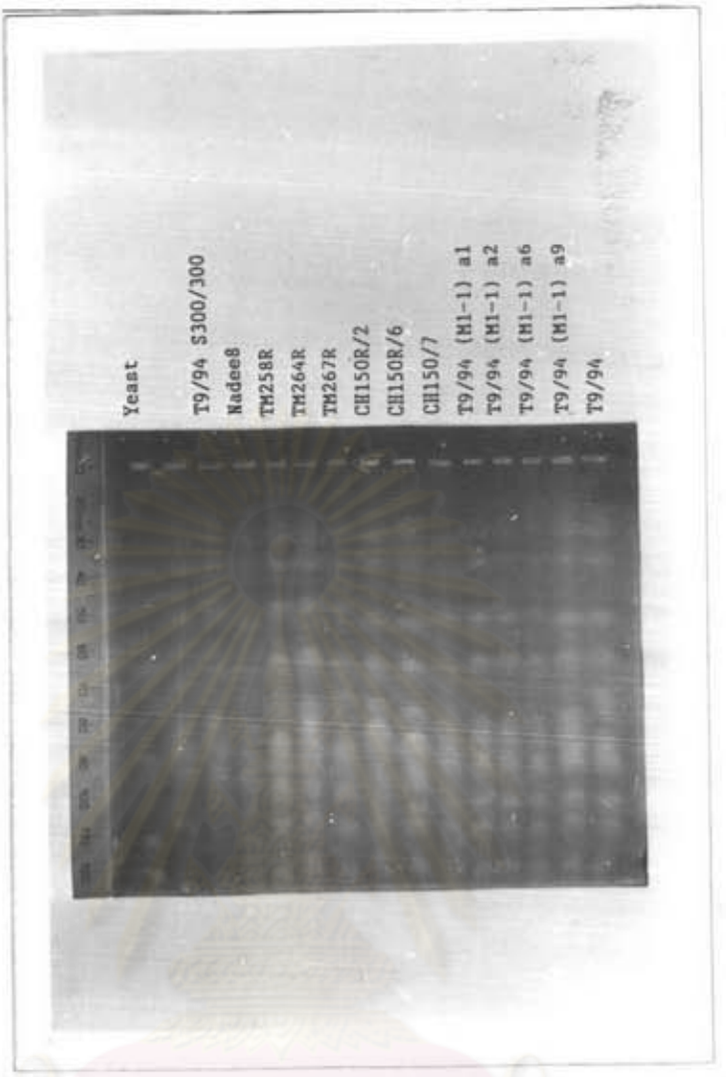

Figure 10.9 Chromosome patterns of parent T9/94 clone and its 4 mutant clones denoted T9/94 (M1-1) a1, a2, a6, a9. Running conditions were identical to those of the gel in figure 10.6.

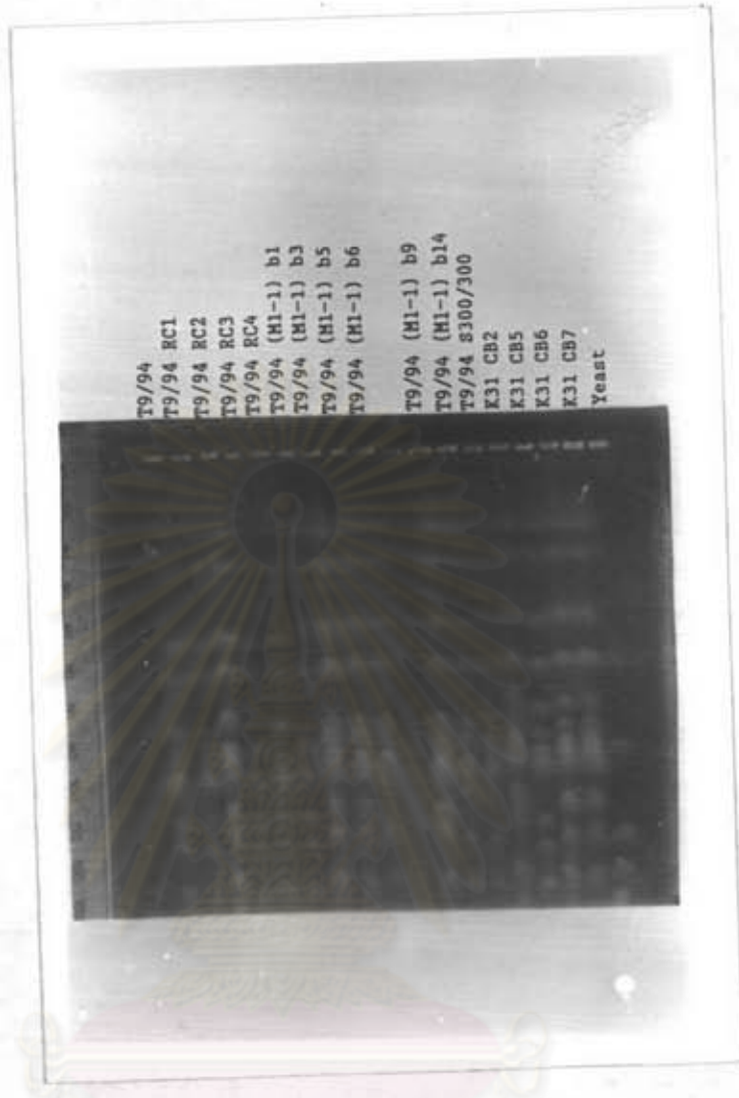

Figure 10.10 Chromosome patterns of a duplicate set of DNA samples as shown in figure 10.5. PFGE conditions were the same as described in figure 10.6.

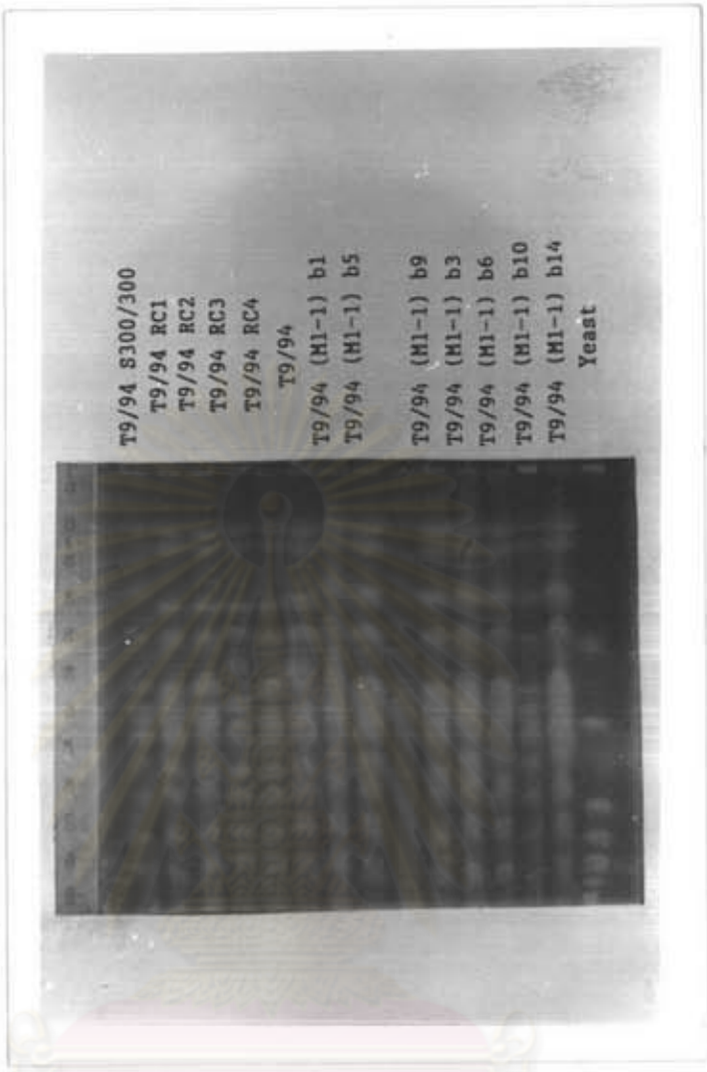

Figure 10.11 Chromosome patterns of parent T9/94 clone and its derivatives. A smaller piece of each sample blocks was loaded resulting in better resolution than the gel in figure 10.6. On this gel the first chromosome was not lost because the running time was reduced to 137 hr.

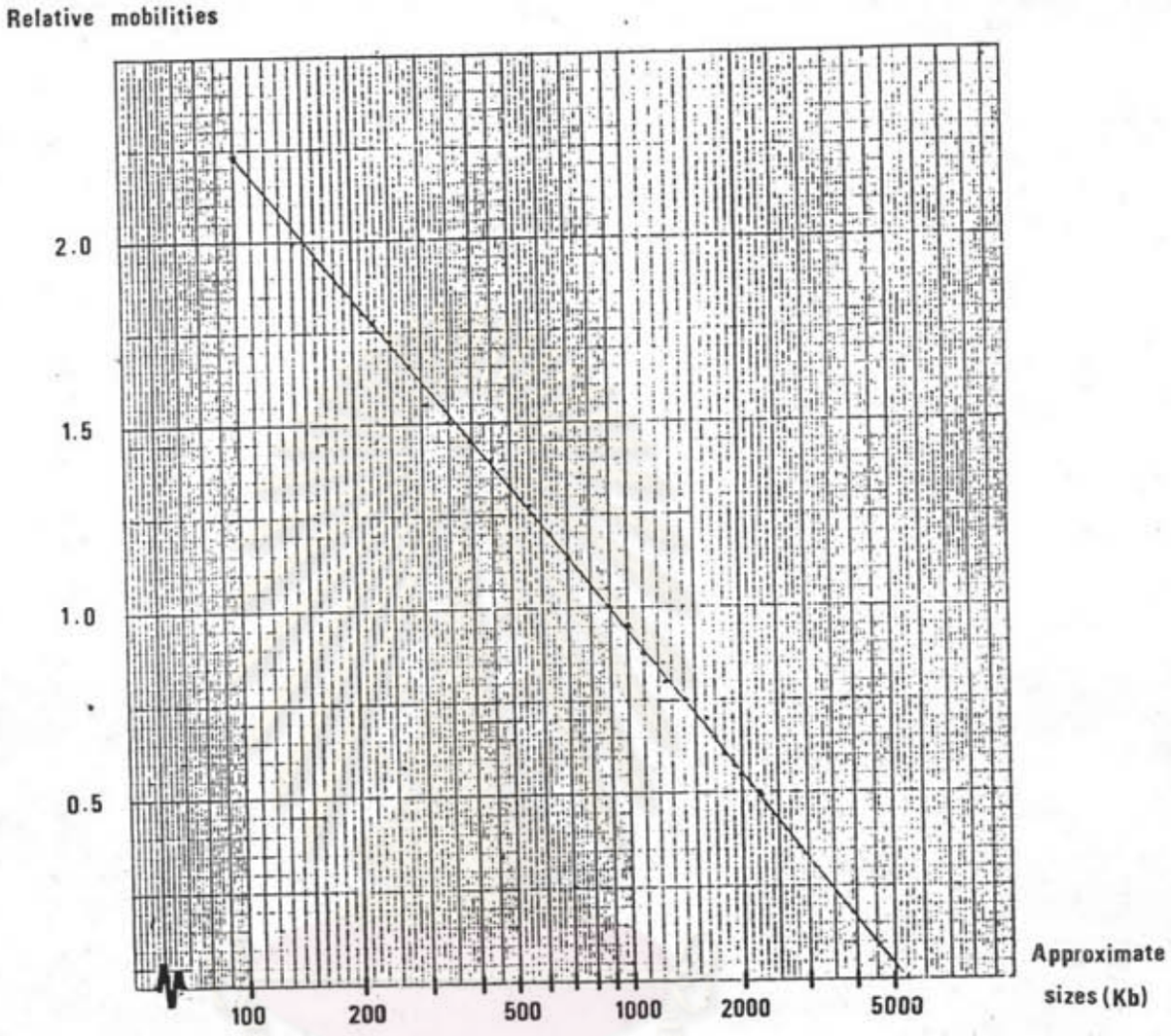

Figure 11 Plot of relative mobilities versus molecular sizes of chromosome marker, S. cerevisiae. Each point represents the mean value of the yeast chromosome size.

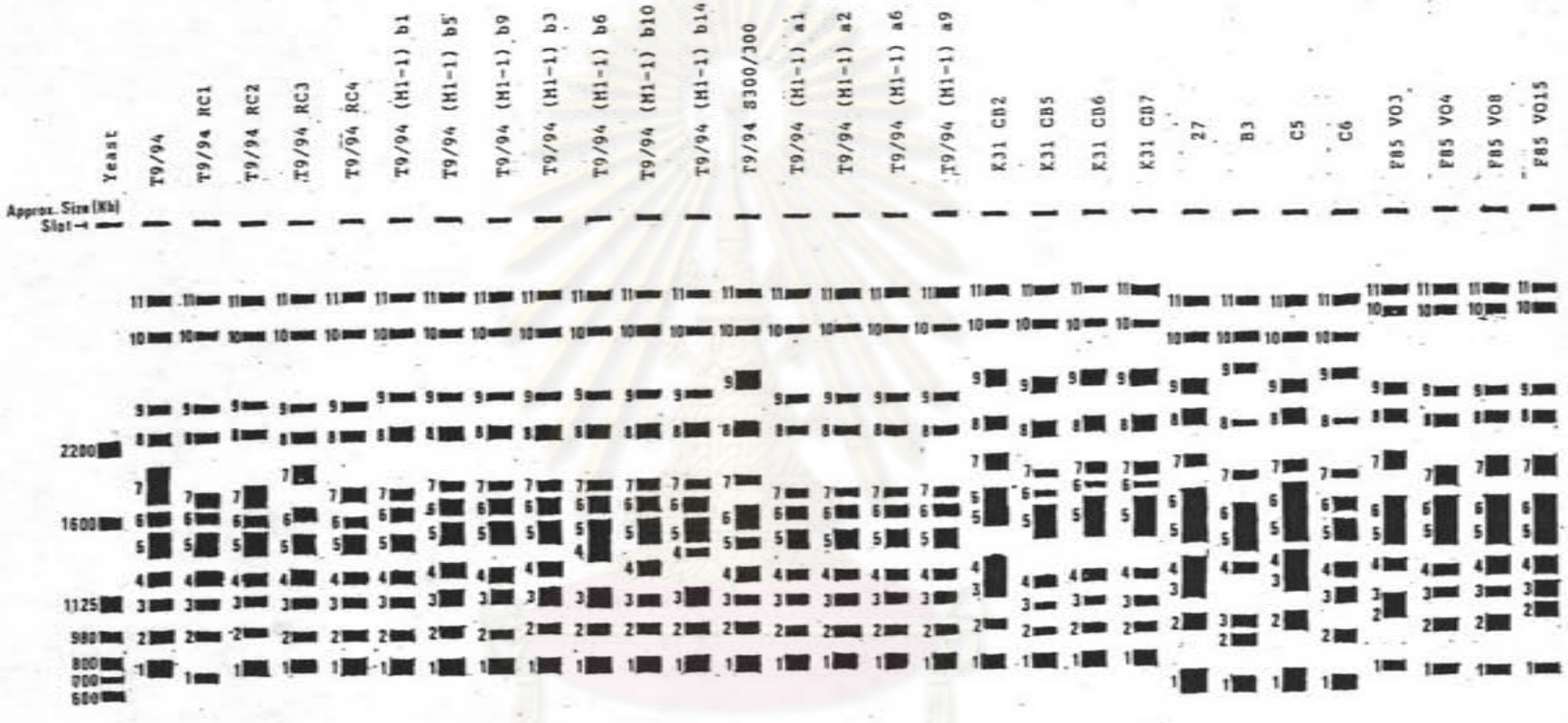

Figure 12 A schematic montage of the karyotypes from various gels as shown in figure 10. S. cerevisiae chromosomes were used as molecular weight markers and T9/94 clone was taken as the reference for P. falciparum chromosome patterns. Spacing between the bands are representative of the relative mobilities .

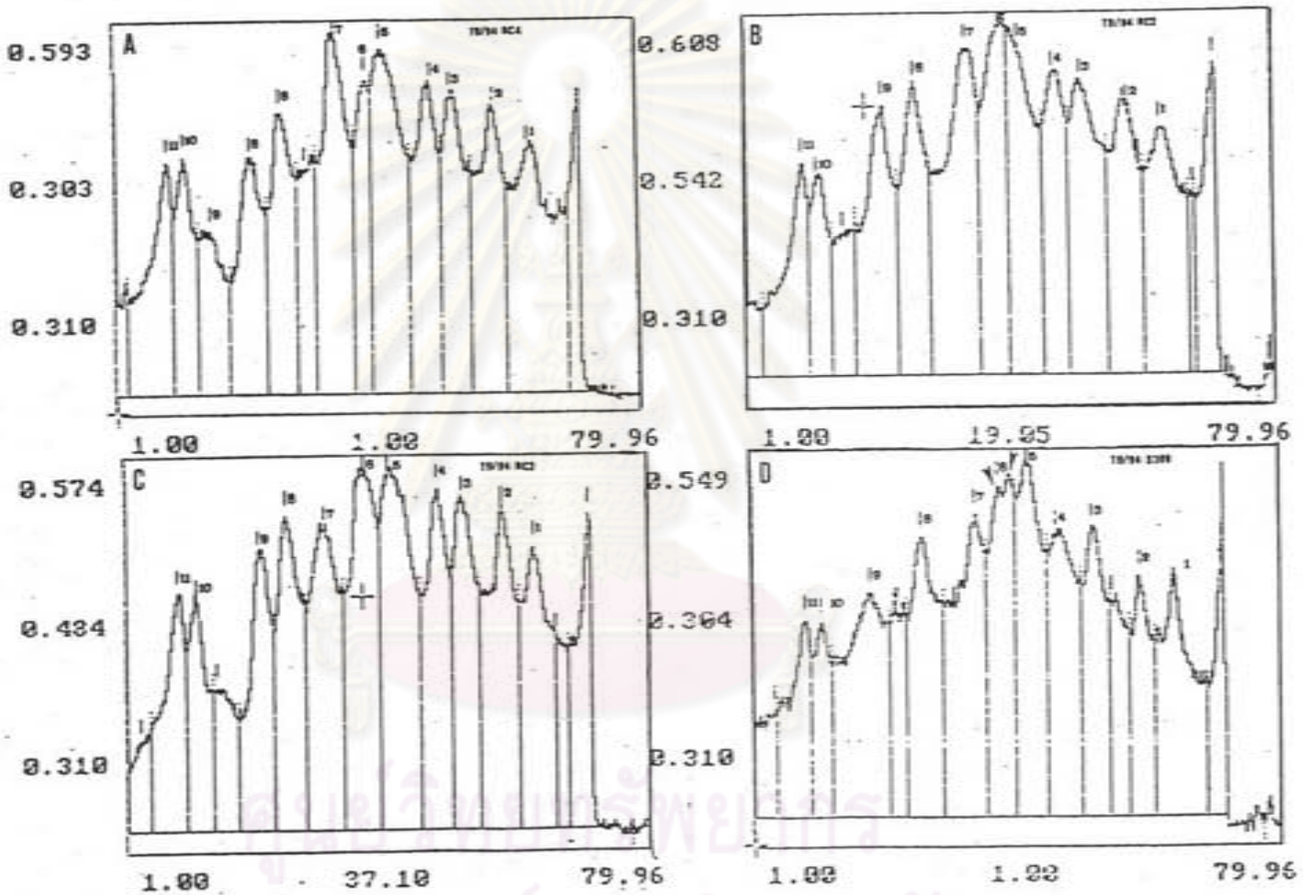

Figure 13 Densitometric tracings of the chromosomal DNA from 4 clone of P. falciparum.

At least 14 chromosomes was observed.

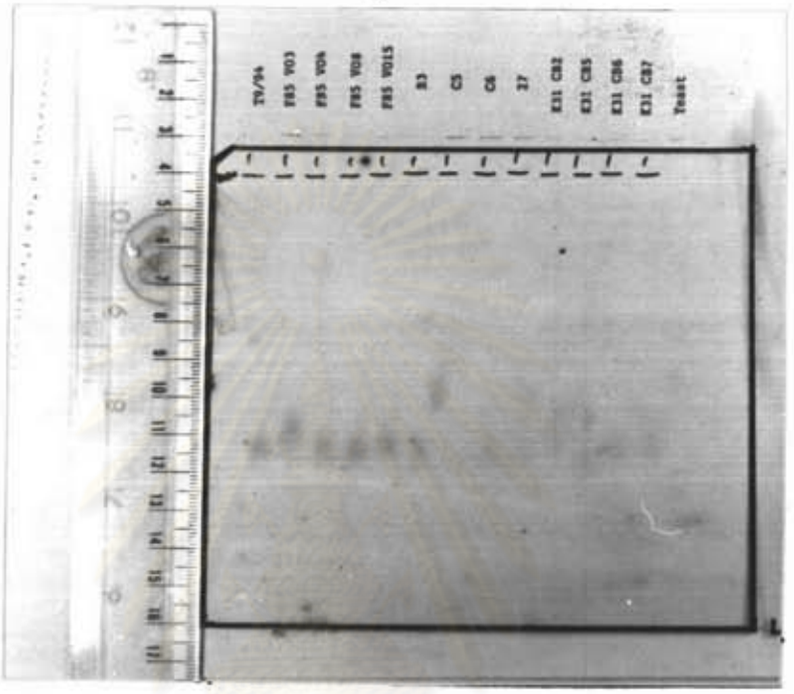

Figure 14 Southern blot of the gel in figure 10.7 hybridized with radiolabelled DHFR - TS probe. This gene was found in chromosome 4 of the parasites.

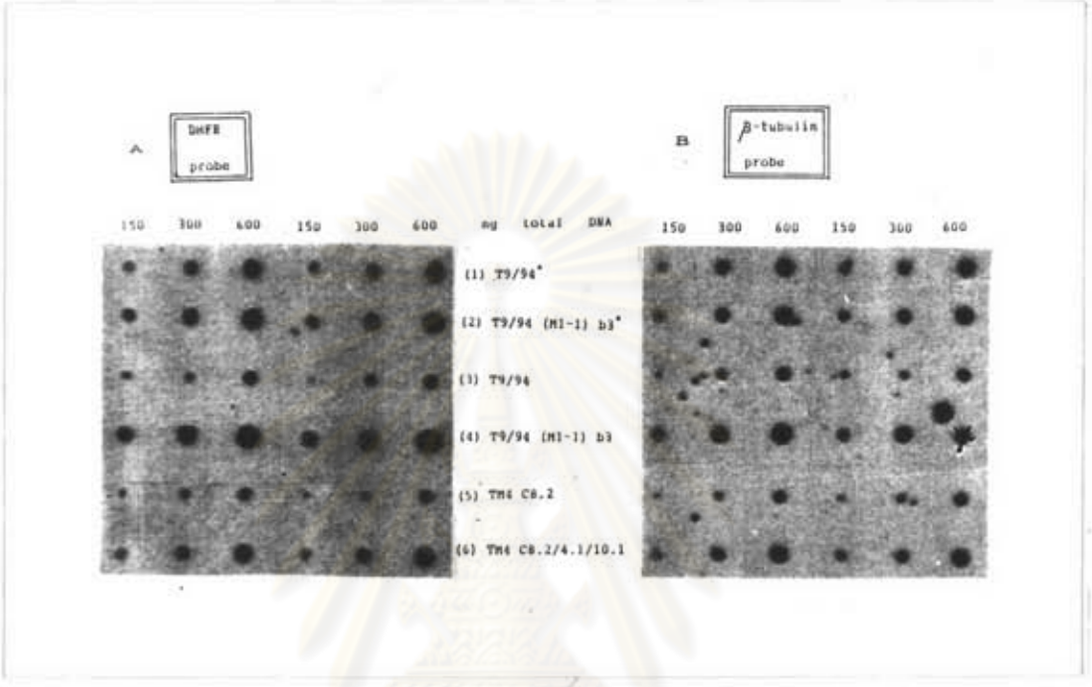

Figure 15 Dot blot analysis. DHFR-TS gene content of 6 samples of P. falciparum clones which have different MIC values for pyrimethamine (Table 2) was compared. (a) DNA on the experimental filter was hybridized to [α-^{32}p] labelled DHFR probe. (b) DNA on the control filter was hybridized to [α-^{32}p] labelled β-tubulin probe.

Table 3. Liquid scintillation data showing the radioactivity from dot blot of P. falciparum DNA

Clones	DHFR	β-tubulin	DHFR/ β-tubulin
T9/94*	250.06	807.75	0.31
T9/94 M1-1 (b3)*	285.33	860.79	0.33
T9/94	146.76	373.36	0.39
T9/94 M1-1 (b3)	177.85	418.89	0.42
TM4 C8.2	287.17	543.17	0.53
TM4 C8.2/4.1/10.1	159.84	310.93	0.51

* Two DNA samples was purified by centrifugation with ethidium bromide and CsCl solutionbefore used.