CHAPTER V

DISCUSSION

5.1 Isolation of root nodule bacterial isolates

The use of seven soybean cultivars as "trapping hosts" was to ensure that all the soybean rhizobia present in the soil samples were isolated. Figures 4.5-4.19 indicated that all the 105 root nodule isolates were soybean rhizobia because PCR products were obtained when RPO1 was used as the primer in PCR fringerprinting. RPO1 anneals at the conserved 20 mer sequence of the promoter of *nifHDK* operons of fast- growing *Rhizobium tropici* and *R. meliloti*. In addition, all the 67 isolated strains were found to nodulate soybean cultivar CM60 used in Leonard jar experiments. (Results not shown).

When one RPO1 PCR product fragment was obtained, it was likely that the RPO1 primer had annealed to the promoter of *nifHDK* operon which encoded the enzyme nitrogenase. However, when more than one copy of PCR product was obtained (Figures 4.20-4.25), it might mean either there was more than one copy of *nifHDK* or RPO1 might anneal to other areas that were not the promoter of *nifHDK*. In 2006 Ly and Chansa-ngavej also obtained more than one RPO1 PCR product for soybean rhizobia isolated from three subdistricts in Wiangsa district, Nan province. Search on GIB (Genome Information Broker) website revealed only one *nifH* gene in *Bradyrhizobium japonicum* USDA110. Therefore, in the case where more than one RPO1 PCR product was observed, it was likely that the primer RPO1 annealed to other regions as well as the promoter region of *nifH* in *B. japonicum*.

The numbers of CRL-7 PCR products in some strains were not as expected .It was expected there would be more GC rich areas in soybean rhizobia genome which would reflect in the high numbers of CRL-7 PCR product fragments. The strains obtained from this research will be used to find if there is a statistically significant correlation between an ability to tolerate heat and the numbers of CRL-7 PCR product

fragments. In 2006, Ly and Chansa-ngavej reported that 4 heat-tolerant strains of soybean rhizobia (could not grow at 40 °C) isolated from three subdistricts in Wiangsa dictrict, Nan province contained 15 or more CRL-7 PCR product fragments and 9 strains of non heat tolerant strains (could not grow above 37 °C) contained less than 15 CRL-7 PCR product fragments. Soybean rhizobial strains obtained in this research will be used in the study to find if there is a statistically significant correlation between the number of CRL-7 PCR product fragments and an ability to tolerate heat. If a positive correlation is established CRL-7 PCR fingerprints can be used in large-scale screening of heat-lolerant strains to select high nitrogen fixing, heat-tolerant strains for development into soybean biofertilizers.

Growth curves of root nodule bacterial isolates indicated that 11 isolates were fast-growers and the remaining 94 isolates were slow- growers. However, results of comparisons of identical PCR fingerprints revealing 9 strains were fast- growers and 57 strains were slow- growers. These results were in contrast to the reports by Camacho and Santamaua, (2002) and Dowdle et al. (1985) that in Huibei province and in Honshu county of the People' s Republic of China fast- growing soybean rhizobia were found more than slow- growing soybean rhizobia. Probably the temperate climate in the People' s Republic of China is more conducive to the growth of fast- growing soybean rhizobia.

Table 4.4 and Figures 4.5 - 4.19 indicated that identical RAPD-PCR fingerprints showed that 66 distinct strains were obtained from the 105 bacterial root nodules used in the study. The results indicated RAPD-PCR fingerprinting was a very sensitive method to distinguish strains. At present, 16S rDNA of the 66 strains are being isolated for RFLP fingerprinting. It is expected that RFLP results of 16S rDNA would provide data for grouping of the bacterial isolates into smaller numbers of strains. In addition, the 66 different strains will be used in the identication of soybean rhizobia to the species level.

5.2 Multiplex PCR

Figure 4.27 showed PCR products obtained from multiplex PCR when forward

and reverse primers of *nodD1* and *nodY* were used as the primers. When chromosomal DNAs of fast- growers were used as templates, three groups of soybean rhizobia were found based on three types of PCR products formed as shown in Table 5.1

Table 5.1 PCR products obtained in multiplex PCR reactions when chromosomal DNAs of fast- growing soybean rhizobia were used as templates and forward and reverse primers of *nodD1* and *nodY* were used as primers.

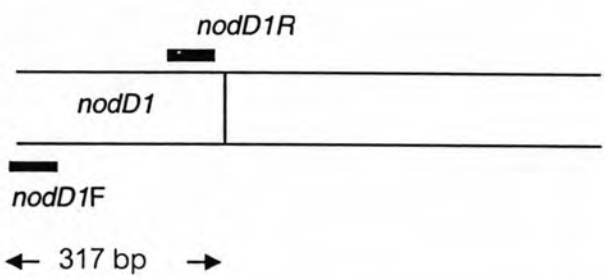
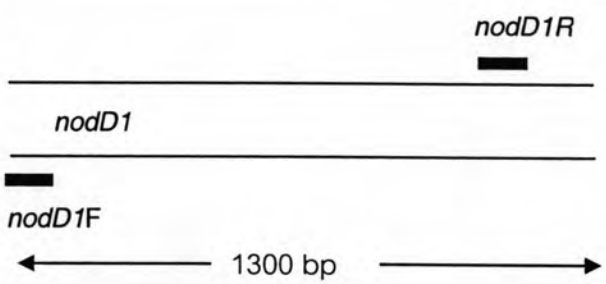
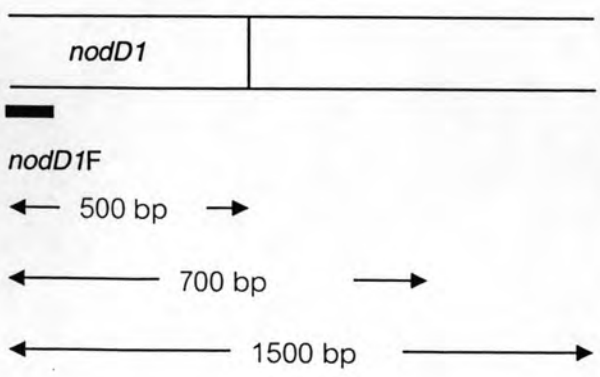
Group	Size (bp)	Amplification regions	Strains
1	317		D11,D301,D384
2	1300		D24,D87,D154, D279, D281, and D395
3	500 700 1500		D48

Figure 4.27 and Table 5.1 showed a 317 bp PCR product obtained in multiplex PCR reactions when DNA of the fast- growers D11, D301, D384 were each used as the template. Since multiplex PCR reactions also revealed slow- growing strains D291 and D481 yielded 317 bp fragments, the 317 bp fragments of D11 and D345 were sent for sequencing at the BioService Unit, Thailand. The sequences were given in Appendix E. Alignment of the sequences revealed *nodD1* sequence of the fast- grower D11 was identical to that of the representative of the slow- growers (D345) as shown in Figure 5.1. The results indicated that the developed multiplex PCR could not be used to distinguish certain groups of soybean rhizobia (fast- growing D11, D301, D384) and slow- growing soybean rhizobia (D291 and D481), because the 317 bp fragments formed in multiplex PCR reactions were found to have identical nucleotide sequence. In order to detect if the five strains are fast- or slow- growing soybean rhizobia, it is necessary to carry out separate PCR reactions using DNA of each of the five strains as target DNA. If no PCR product is obtained when *nodY* is used as the primers, or 1300 bp or a combination of 500, 700, 1300 bp is obtained when *nodD1* is used as the primer, the target DNA belongs to a fast- growing soybean rhizobium. On the other band, if 340 bp PCR product is obtained when *nodY* is used as the primer, and 317 bp PCR product is obtained when nodD1 is used as the primer, the target DNA belongs to a slow- growing soybean rhizobium.

Multiplex PCR of DNA from each of the fast- growing strains D24, D87, D154, D279 and D395 yielded multiplex PCR products of 1300 bp. The products could have arisen from the annealing of and extension from primers *nodD*$_1$F and *nodD*$_1$R as shown in Table 5.1. Multiplex PCR results when DNA of fast-grower D48 was used in multiplex PCR showed three PCR products of 500 bp, 700 bp, and 1300 bp which could have been obtained by annealing of primer as shown in Table 5.1. At present, sequencing of the 500 bp, 700 bp, and 1300 bp are being carried out to confirm the findings discussed above.

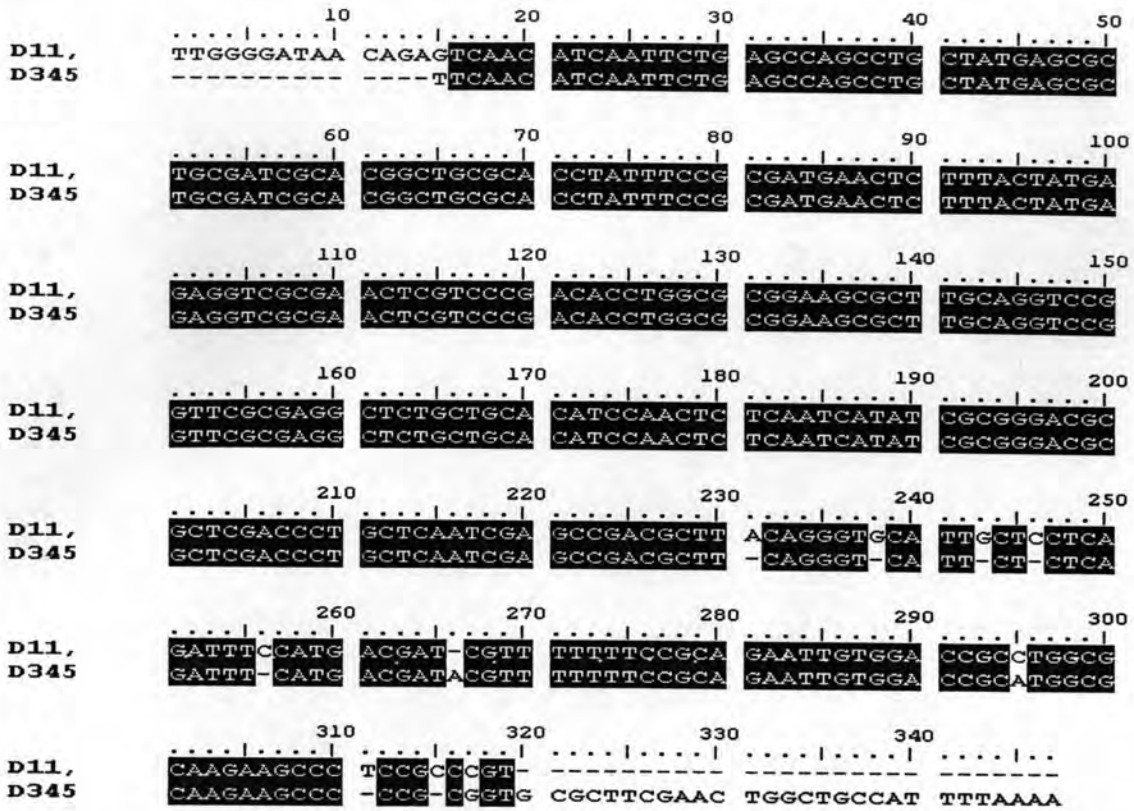
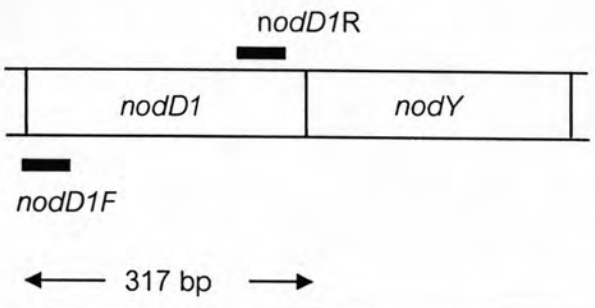
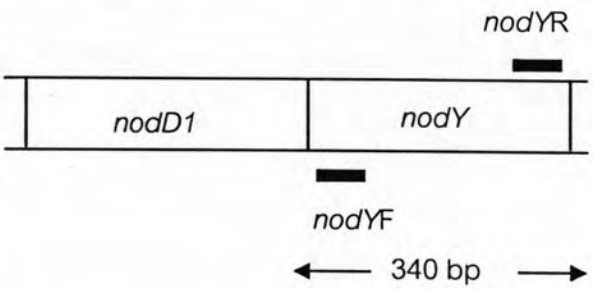
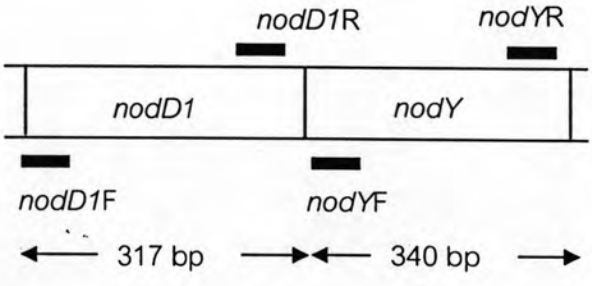
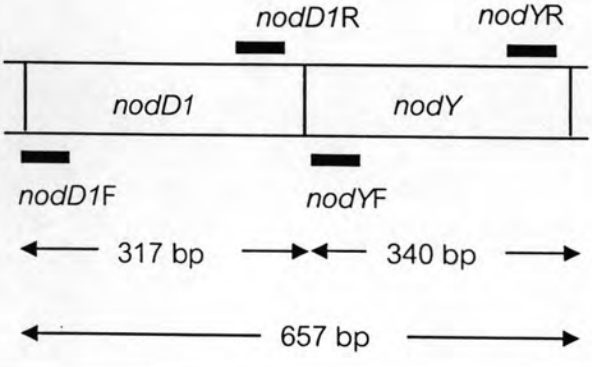
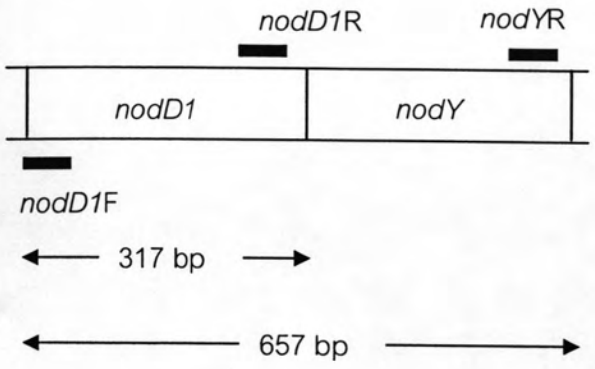
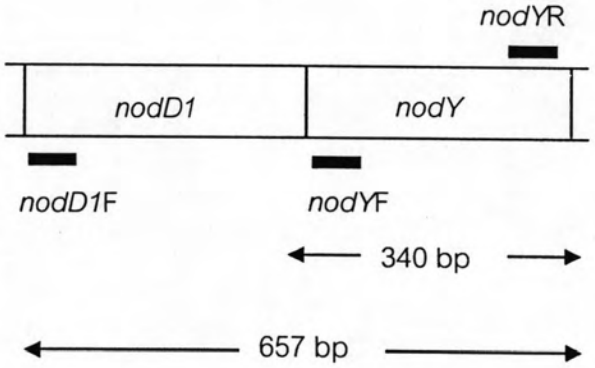


Figure 5.1 Alignment of sequences of of 317 bp fragments obtained from multiplex PCR reactions when primers *nodD1*F, *nodD1*R, *nodY*F, *nodY*R were used as primers.

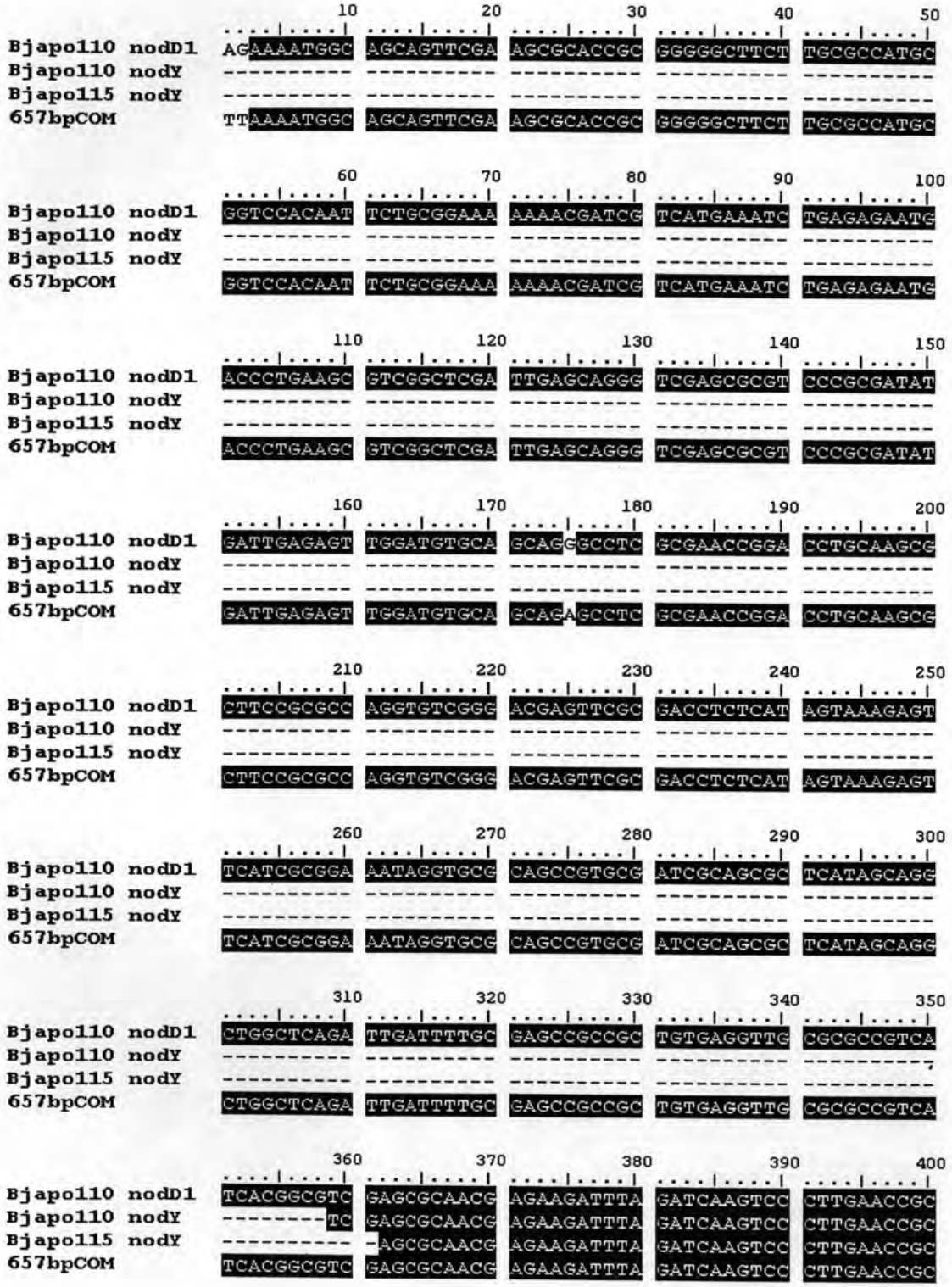
Template DNA was from fast- growing strain D11 and slow- growing strain D345. Figure 4.27 showed PCR products obtained from multiplex PCR when forward and reverse primers of *nodD1* and *nodY* were used as the primers. When chromosomal DNAs of slow- growers were used as templates, six groups of soybean rhizobia with different types of PCR products were obtained as shown in Table 5.2.

Table 5.2 PCR products obtained in multiplex PCR reactions when chromosomal DNAs of slow- growing soybean rhizobia were used as templates and forward and reverse primers of *nodD1* and *nodY* were used as primers.

Group	Size (bp)	Amplification regions	Strains
1	317		D291, D481 (2 strains)
2	340		D37, D57, D83, D103, D176, D195,D263, D404, D490 (9 strains)
3	317 340		D217, D467 (2 strains)
4	317 340 657		D464 (1strain)

Group	Size (bp)	Amplification regions	Strains
5	317 657		D97, D182, D286, D345, D373, D408 (6 strains)
6	340 657		D20, D43, D54, D64, D66, D71, D92, D106, D120, D121, D128, D132, D147, D165, D169, D188, D200, D203, D221, D213, D232, D243, D250, D267, D273, D306, D361, D366, D416, D423, D430, D447, D455, D459, D494, D509, D521 (37 strains)

The majority of the slow- growing soybean rhizobia belonged to group 6. Sequences of representative 317 bp, and 657 bp from strain D345 were shown in Appendix E. Alignment of the 657 bp from sequences indicated that the 657 bp fragment was *nodD1-nodY* as shown in the alignment results in Figure 5.2.



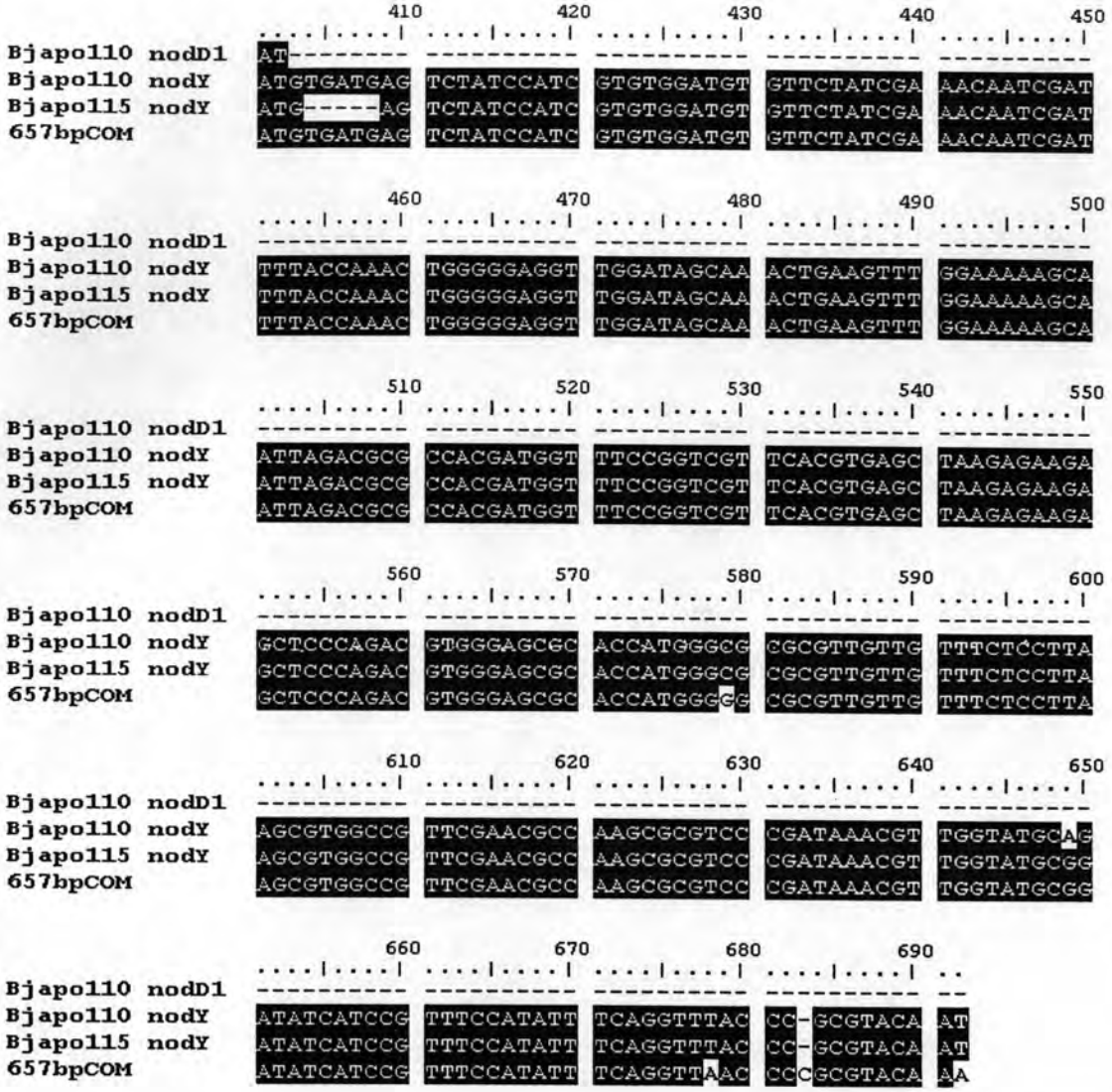
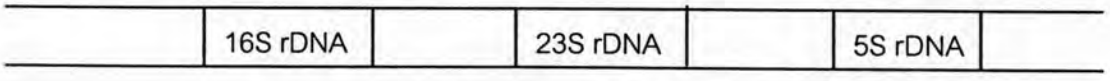


Figure 5.2 Alignment of sequence of 657 bp fragment obtained from multiplex PCR reactions when primers *nodD1*F, *nodD1*R, *nodY*F, *nodY*R were used. Template DNA was from slow- growing strain D345 . Sequences of *nodD1* and *nodY* for comparison were downloaded from Genbank.

Possible explanation for the observed PCR products from the multiplex PCR when DNAs from slow- growing soybean rhizobia were used as target DNAs may be in the fact that the connecting region between *nodD1* and *nodY*, where nucleotide sequence of *nodD1* ends and that of *nodY* begins , constitutes topology upon which it is difficult for *nodD1*R and *nodY*F primers to anneal. It is interesting to note that this connecting DNA region is close to the overlapping promoter regions of *nodD1* and *nodY* (Wang and Stacey, 1991). Upon binding of NodD1- flavonoid complexes, this overlapping promoter region is thought to bend to allow activation of transcription of either *nodD1* or *nodYABC* as shown in Figure 1.1. Therefore, it is likely that this DNA junction may be relatively difficult for primers to anneal. This speculation is supported by the finding that only two out of the six groups of PCR products were obtained due to the annealing of both *nodD1*R and *nodY*F primers to the target DNA (Groups 3 and 4, Table 5.2). The other four groups of PCR products (Groups 1, 2, 5, and 6, Table 5.2) were obtained because only one of the two primers (*nodD1*R, *nodY*F) annealed to the target DNAs due to the difficulty of both primers to simultaneously anneal to the DNA junction between *nodD1* and *nodY* as described above.

5.3 Possibility of designing a new set of primers and discovering a new gene.

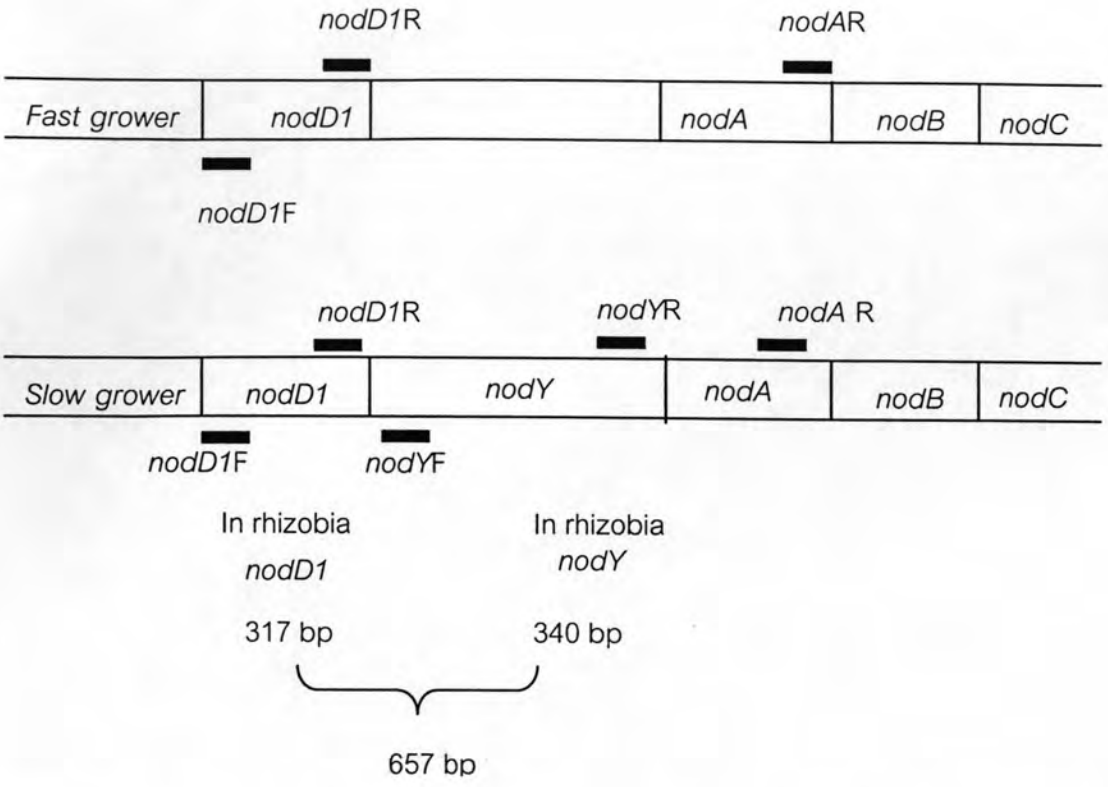
In an *rrn* operon :



ITS 1 ITS 2

. ITS = Internally Transcribed Spacer Region.

Consensus sequences of ITS are often used to design primers for RAPD-PCR. Sometimes ITS spacer regions are used for RFLP analysis . By analogy, it might be possible to use the spacer region between *nodD1* and *nodA* of the fast- growers to determine genetic variations in fast- growing soybean rhizobia and to detect fast- and slow growing soybean rhizobia in multiplex PCR reactions.



One strategy to determine nucleotide sequence variations in the spacer regions between *nodD1* and *nodA* in fast-growing soybean rhizobia is to obtain PCR fragments when *nodD1*F and *nodA*R are used as the primers. Comparisons of nucleotide sequences of fragments containing spacers between *nodD1* and *nodA* from slow-growers and those between *nodD1* and *nodA* from fast- growers could provide new genetic information which can be used in the further design of primers to overcome the limitation of the multiplex PCR developed in this study which could not be used to distinguish the fast- growing strains D11, D301, D384 and the slow- growing strains D291 and D481 which yielded identical 317 bp PCR fragments. The results might also lead to the discovery of a gene in the spacer regions of fast- growing soybean rhizobia. It is interesting to note that Scott (1986) reported *nodK* in place of *nodY* in the same operon as *nodABC* in the non-legume symbiont *Bradyrhizobium* sp. (*Parasponia*). Therefore, it is plausible that future research as outlined above might lead to the discovery of an open reading frame which might lead to the discovery of a new gene in the spacer region between *nodD1* and *nodA* in fast-growing soybean rhizobia.