THE USE OF 16s RNA AND *NODC* GENE SEQUENCE IN RESOLVING THE PHYLOGENETIC RELATIONSHIP OF *Rhizobia* ASSOCIATED WITH *Paraserianthes falcataria* (L.) Nielsen PLANT

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ABSTRACT

Studies on genetic position of several isolates living symbiotically on *Paraserianthes falcataria* have been carried out using amplification and sequencing techniques of 16S rDNA and specific gene for nodulation (*nodC*). The "phylogenetic tree" constructed based on 165S rDNA showed that rhizobia growing symbiotically on *Paraserianthes falcataria* consisted of 3 groups. The first group, was fast-growing *rhizobia* which have close relationship to *Rhizobium tropicii*, the second and the third groups were slow-growing *rhizobia* which have close relationship to *Bradyrhizobium elkanii* and *B.japonicum*. However, the "phylogenetic tree" constructed on the basis of partial *nodC* gene indicated the existence of an independent group, since they did not show any significant degree of relationship with the existing groups. This was also supported by differences in physiological characteristics i.e Indole Acetic Acid production and salt tolerance of the isolates. These differences shows us that "direct sequencing" method of certain specific genes could give a more specific result that would display more clearly the degree of relationship at species level.

Keywords: *NodC gene*, 16S rRNA, *rhizobia, phylogenetic tree*

ABSTRAK

Studi mengenai posisi genetic dari beberapa isolat yang hidup bersimbiosa dengan tanaman *Paraserianthes falcataria* telah dilakukan dengan menggunakan tehnik amplifikasi dan sekuensing dari 16S rDNA dan gen spesifik untuk nodulasi (*nodC*). "Phylogenetic tree" berdasarkan sekuen 16S rDNA menunjukkan bahwa *rhizobia* yang bersimbiosa dengan tanaman *Paraserianthes falcataria* terdiri dari 3 kelompok. Kelompok pertama, *rhizobia* tumbuh cepat (fast-growing *rhizobia*) yang memiliki hubungan kekerabatan dengan *Rhizobium tropicii*, kelompok kedua dan ketiga merupakan kelompok *rhizobia* tumbuh lambat (slow-growing *rhizobia*) yang memiliki kekerabatan dengan *Bradyrhizobium elkanii* dan *B.japonicum*. Namun berdasarkan "phylogenetic tree" yang dibangun dari partial gene *nodC* terindikasi adanya kelompok yang independen, karena tidak memiliki nilai kekerabatan yang tinggi dengan kelompok yang sudah ada. Hal ini didukung pula oleh sifat-sifat fisiologi seperti kemampuan memproduksi IAA dan kemampuan tumbuh pada konsentrasi garam yang tinggi yang dimiliki oleh isolat-isolat tersebut. Perbedaan hasil ini menunjukkan bahwa metoda "direct sequencing" dari gen-gen khusus dapat memberikan hasil yang lebih spesifik untuk melihat tingkat kekerabatan pada tingkat spesies.

Kata kunci: Gen *NodC* , 16S rRNA, *rhizobia, phylogenetic tree*

INTRODUCTION

Since the conventional chracterization which was based solely on phenotypic characters and symbiotic performance of *rhizobia* has been regarded as incomplete and hence became less acceptable (Elkan, 1992), other method had been developed with various degree of resolution. Some methods such as multilocus enzyme electrophoresis (MLEE), total protein finger printing by SDS-PAGE, and the repetitive extragenic palindrom (REP) sequence has been increasingly and widely used in analysing the bacterial genome. The technique has become the focus of interest especially for those specializing in taxonomy.

Molecular characterization for *rhizobia* actually has been initiated a long time ago soon after DNA-DNA reassociation technique to discriminate homology groups in *Rhizobium japonicum* species was successfully applied (Hollis *et al.*, 1981) followed by the successful attempt to separate *B. elkanii* from *B. japonicum* (Kuykendall *et al.*, 1992). The technique was considered to be superior to other described methods (van Berkum *et al.*, 1996), however, this method requires a special set of equipment which may not be available in every laboratory and rather tedious preparatory works.

Analysis of small-subunit rRNA (16S rRNA) sequences was initiated after the invention of Polymerase Chain Reaction (PCR) apparatus (Saiki *et al.*, 1988) . Today 16S rRNA sequence analysis has become the most favourable technique for studying genetic relationship and has been regarded as the minimal standards for genetic characterization of *rhizobia* (Graham *et al.*, 1991). Some research works on *rhizobia* group related to classification and or taxonomic study usually used 16S rRNA technique. Based on 16S rRNA data, several new species in the *Rhizobium* group were proposed.

There are several reasons for the popularity of the technique compare to the other molecular techniques. Among others, due to the fact that over 3000 bacterial small subunit (SSU) sequences have been published (Maidak *et al.*, 1994), so that any new sequence created it can be immediately compared to the reference. However the technique also has its weakness i.e. it does not have the power to sort out the relationship between closely related species (Young and Haukka, 1996). It has also proven that the results of the SSU rRNA-based analysis often do not correlate with the DNA reassociation values. Each method is strong in those areas of relationships in which the others fail to reliably determine the relationship (Fox *et al.*, 1992; Stackebrandt *et al*, 1994). Yamamoto and Harayama (1998) suggested that to resolve the phylogenetic relationship of closely related organisms, it would be necessary to use other gene sequences, which provide a higher resolution than that SSU rRNA. In case of *rhizobia* group, some studies had been initiated by using the genes which involved in nodulation as well as nitrogen-fixing ability (Ueda *et al.*, 1995; Haukka *et al.*, 1998).

The purpose of this work is to study the possibility of using direct sequencing method of partial *nodC* gene for identification of *rhizobia* strains living symbiotically with leguminous-tree, *Paraserianthes falcataria*. The application of partial *nodC* gene for identification has its own advantage compare to the use of other common nod genes. This is due to the fact that all *rhizobia* contain only a single copy of *nodC* (Ueda *et al.*, 1995). In the present study phylogenetic analysis based on direct sequencing of partial *nodC* gene and 16S rDNA gene sequence were compared to reveal the exact phylogenetic position of the isolates.

MATERIALS AND METHODS

Study on the phylogenetic position assessed by 16S rDNA sequence

Bacterial strains and DNA isolation.

A number of 10 bacterial strains representing of fast-growing and slow-growing group were cultured on the medium with the composition exactly the same as that described in our earlier work (Seki *et al*, 1998). After cultivation, the

cell were collected and treated with 0.2 N HCl with a brief vortexing. The suspensions were then washed two times and centrifuged to collect the pellet. By this treatment most of extracellular polysaccharides, which usually abundantly produced by rhizobia strains, were removed. The cells were resuspended in Tris-EDTA (TE) buffer for DNA isolation.

Sequencing reaction was conducted by using Big Dye™ Terminator Cycle Sequencing Kit (PERKIN ELMER, Japan, Applied Biosystem) with three pairs of primer:

20F : 5'-GAGTTTGATCCTGGCTCAG-3'
1500R : 5'-TTACCTTGTTACGACTT-3'
520F : 5'-CAGCAGCCGCGGTAATA-3'
520R : 5'-TATTACCGCGGCTGCTG-3'
920F : 5'-AAACTCAAATGAATTGACGG-
3'920R : 5'-CCGTCAATTCATTTGAGTTT-3'

Table 1. List of the isolates used in this study

No	Isolate code	Host Plant	Geographic Origin/Source
1	PF1	*P. falcataria*	Medan, North Sumatra, Indonesia/Prana
2	PF2	*P. falcataria*	Medan, North Sumatra, Indonesia/Prana
3	PF3	*P. falcataria*	Medan, North Sumatra, Indonesia/Prana
4	PF16	*P. falcataria*	Sukabumi, West Java, Indonesia/Prana
5	PF20	*P. falcataria*	Cibinong, West Java, Indonesia/Prana
6	PF21	*P. falcataria*	Bandung, West Java, Indonesia/Prana
7	PF25	*P. falcataria*	Sukabumi, West Java, Indonesia/Prana
8	PF26	*P. falcataria*	Cibinong, West Java, Indonesia/Prana
9	PF31	*P. falcataria*	Central Java, Indonesia/Prana
10	PF40	*P. falcataria*	Cibinong, West Java, Indonesia/Prana

Chromosomal DNA was isolated from cell by using an Insta Gene Purification Matrix (BIORAD) according to the manufacture's protocol. The lysate was then treated with RNAse, followed by phenol-chloroform purification. DNA concentration was determined by using Beckman spectrophotometer Model DU640. The DNA solution was then used as a template for polymerase chain reaction (PCR).

Determination of 16S rDNA gene sequence

About 1321 bp of 16S rDNA gene was amplified by using a pair of primer 20F (5'-GAGTTTGATCCTGGCTCAG-3') and primer 1500R (5'-TTACCTTGTTACGACTT-3'). Reaction condition of PCR program consisted of 30 cycles: denaturation at 95°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute. The amplified rDNA fragment were then separated by agarose gel electrophoresis and purified by Qiaex II Gel Extraction Kit (QIAGEN-Germany).

Reaction for sequencing was set in 25 cycles under following condition: 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C followed by a final elongation step for 10 min at 4°C. The amplified rDNA fragment was denatured at 96°C for 5 min. before adding to the reaction mixture. Nucleotide sequences were determined by using the DNA sequencer ABI PRISM 310 Genetic Analyser.

Phylogenetic analysis

Nucleotide sequences were analysed by using GENETYX-MAX 10.0 program. The generated rDNA sequence and those reference strains obtained from the GenBank, EMBL and DDBJ data libraries were aligned by using the Clustal W ver.1.6 program (Thompson *et al.*, 1994). The phylogenetic tree was constructed from the evolutionary distance data applying the alogarithm of the neighbor-joining method (Saitou and Nei, 1987) to Knuc values (Kimura, 1980). To evaluate the robustness of branches

in the inferred tree, the bootstrap procedure (Felsentein, 1985) was used to sample the data.

Study on the phylogenetic position assessed by partial *nodC* gene sequences

Bacterial strains and DNA isolation.

Isolates were cultured in Tryptone-yeast (TY) broth media with composition as described by Somasegaran and Hoben (1985).

Chromosomal DNA was extracted from the cells by using the Isoplant DNA extraction Kit (NIPPON GENE, code no.314-02731) according to the manufacture's protocol. The lysates were then treated by RNAse, followed by phenol-chloroform extraction and ethanol precipitation. The crude DNA was then resuspended in TE (Tris-EDTA) buffer and subjected to PCR reaction for *nodC* gene amplification.

DNA amplification

To optimize the appearance of particular band, three kinds of PCR condition were conducted. Condition I was set in 2 steps. *First step* was set in 10 cycles at 94°C for 1 min, followed by another 94°C for 30 sec for denaturation, 30 sec at 37°C for annealing and 60°C for 2 min for the extension reaction. *The second step* was set for 35 cycles with the condition as followed: 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and another 72°C for 5 min to complete the elongation. Condition II was set exactly the same as condition I with an exception in annealing temperature 52°C instead of 57°C, and the reaction was conducted for 27 cycles instead of 35 cycles. Condition III was set at 94°C for 4 min (pre denaturation), followed by another 94°C for 1 min for denaturation step, 60°C for 1 min for annealing, 72°C for 1 min and another 72°C for 5 min to complete the elongation. The reaction was conducted in 35 cycles. The PCR mix solution for all conditions was consists of 10 ng of template DNA, 2.5 mM of MgCl2 solution, 0.2 mM of dNTPs mix solution (dATP, dTTP, dGTP and dCTP) 0.1 Unit of *Taq* DNA polymerase (Perkin Elmer), and 20 pmole of each primer. A pair of primer used in this study is: 251F (19 mers): 5'-AYGTIGTYGAYGWYGGWTC-3' and primer 566R (23 mers): 5'-AGCCARTACTCCATGTAGATCAA-3' (Ueda *et al.*, 1995).

The PCR product was then subjected to 1.5% of SEAKEM Agarose (special grade for DNA recovery) in 1x TAE (Tris-Acetic acid-EDTA) buffer. Fragment of DNA was visualizized in a horizontal Mupid elctrophoresis, 100 bp DNA ladder was used as a marker size. Band of interest (about 270 bp to 300 bp) was then purified directly from the gel by using Recochip sephaglass (Takara Recochip, cat. No 9039) under UV light followed by phenol-chloroform extraction and ethanol precipitation. Purified DNA resuspended in 10 ml sterilized distilled water and used for sequencing.

Determination of partial *nodC* gene sequence

PCR reaction for sequencing was conducted in 25 cycles with the condition as followed: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. PCR reaction mix contained of 30 ng of template DNA (final concentration), 8 ml of Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem), 50 pmole of forward primer (251F), and 6.4 pmole of reverse primer (566R). Total volume of the reaction mixture was 20 ml. PCR product was purified by using Centri-Sep Column (Princeton-Separation Inc. cat.no CS-900) to remove the Dye Terminators prior to sequencing. The purified DNA was then used for sequencing work. Sequencing was conducted by using ABIPRISM™37 DNA Sequencing System (PE Applied Biosystem). Nucleotide sequence of particular gene were analysed by using GENETYX MAC. 9.1

RESULTS AND DISCUSSION

A phylogenetic tree was successfully generated based on a comparison of the 1321 bp sequence of 16S rDNA gene of the 10 isolates and 16S rDNA gene sequence of all available *rhizobia* species and related bacteria. As shown in Figure 1, the *rhizobia* living

symbiotically with *Paraserianthes falcataria* plant could be divided into three main groups. Group 1, consist of three isolates, PF3, PF16 and PF25, which showed high degree of relationship with the reference strain *Bradyrhizobium elkanii* USDA 76. Group II, which had a close relationship with the reference strain *Bradyrhizobium japonicum* IAM 12608 and *B. japonicum* USDA 110. The reference strain belonging to *Blastobacter denitrificans* was also included in this group. This group is composed of two strains, PF21 and PF31. Group III composed of three isolates (PF1, PF26 and PF40) which felt into the same group with *Rhizobium tropicii* and *Agrobacterium rhizogenes*. Group I and Group II were classified as slow-growing *rhizobia*, meanwhile the isolates in Group III were classified as fast-growing *rhizobia* (Prana, 1997). This present study therefore supports the earlier investigator who stated that leguminous tree species could be infected by either one or both slow and fast-growing rhizobia (Zhang *et al.*, 1991).

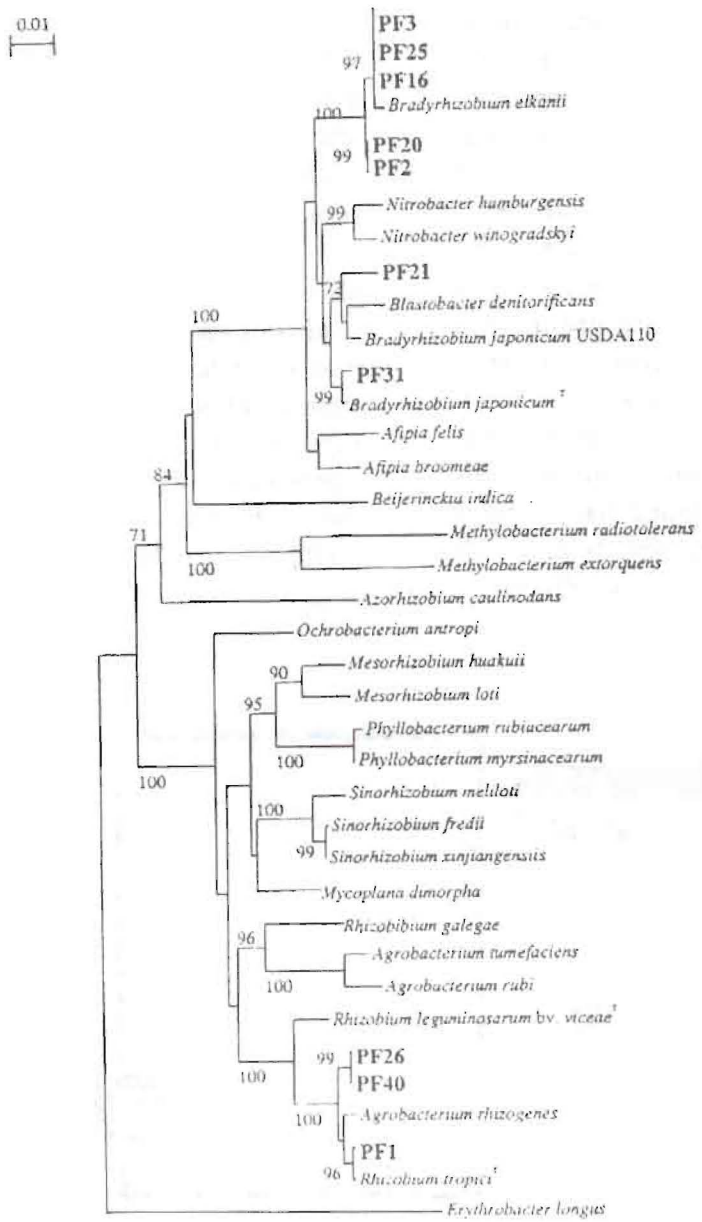

Figure 1. Phylogenetic relationship among isolates and related species based on 1321 bp of 16S rDNA gene sequence. Bootstrap supports higher than 70% are display.

Analysis of 16S rDNA is at present the most useful means to define relationship among *rhizobia* (Hernandez-Lucas *et al.*, 1995). But in some cases the phylogeny of 16S rDNA molecules does not correspond with that of phenotypic characters. The works of Dupuy *et al.* (1994) and Gao *et al.* (1994) who studied the diversity of tropical *rhizobia* found such inconsistencies.

The present study eventually found out an example, which could reflect inconsistencies. The result of study using 16S rDNA clearly showed that the relationship between isolate PF2 and *Bradyrhizobium elkanii* group quite strong as reflected by the high boostrap value. In contrast, this isolate did not have any capability to produce Indole-3 Acetic Acid (IAA) (Prana, 1997) which was said to be characteristic of *B. elkanii* species (Kuykendall *et al.*,1992; Fukuhara *et al.*, 1994). A similar case was also found in isolate PF20 which phylogenetically belongs to *Bradyrhizobium* group, but reasonably tolerant to high concentration of NaCl (preliminary study, data not shown). This is certainly not in agreement with the results of observation by some previous researchers in which was found that members of *Bradyrhizobium* were sensitive to salt concentration (Jordan, 1984; Zhang *et al.*,1991). The ability of *rhizobia* to grow on the media containing certain level of salt concentration was indeed quite important character. The character not only could be used in grouping the bacteria, but it also influences the process of nodule formation on the host plants (Lal and Khana, 1994).

Futhermore it was clearly shown from the figure that the high boostrap value was not only detected in the relationship between those two strains and *B.elkanii* group, the same situation also shown in the relationship between *Bradyrhizobium* group and the other generas, such as *Beijerinckia indica*. This figure lead us to the suggestion that 16S rDNA gene sequence is not suitable enough to show the precise relationship between two species, since the figure could not demonstrate which relationship is strong enough to be one group.

Considering that situation, another phylogenetic tree based on partial *nodC* gene sequences was constructed to resolve the precise position of those two strains. A number of four strains representatives for *B. elkanii* group, *B. japonicum* group, and isolate PF20 as representative for "*unclear*" strain were used in this study. About 300 bp fragment of partial *nodC* gene has been successfully amplified (Figure 2).

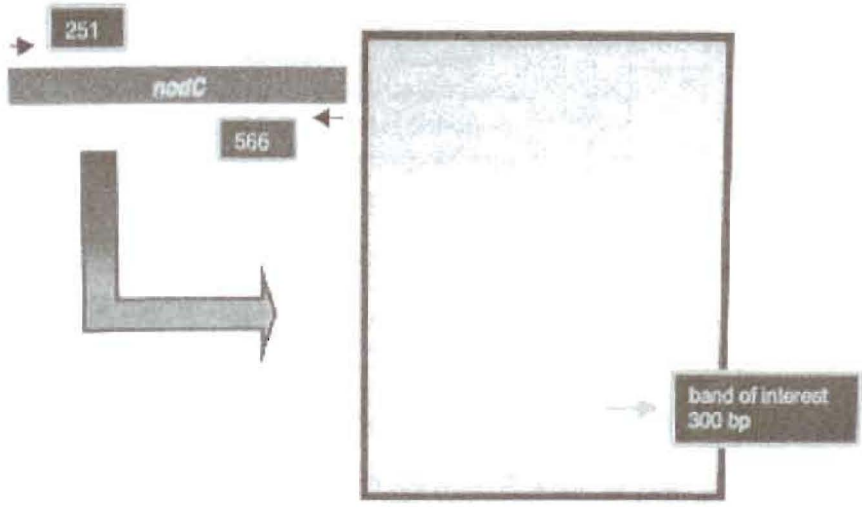

Figure 2. Determination of partial *nodC* gene of isolate PF20. 100bp DNA ladder was used as a size marker.

Based on the partial *nodC* gene sequences data obtained a phylogenetic tree was then constructed and the relationship among the isolates and between isolates and reference strains could be assessed. The position of those representative strains and related species on both phylogenetic trees was compared (Figure 3).

value 559. Therefore this recent study proposed the PF20 strain to be put into "independent group" separated from *B. elkanii* group. More detail studies should be done both on the phenotypic as well as on genotypic characters to elucidate this finding. This study also proved that direct sequencing of partial *nodC* gene could

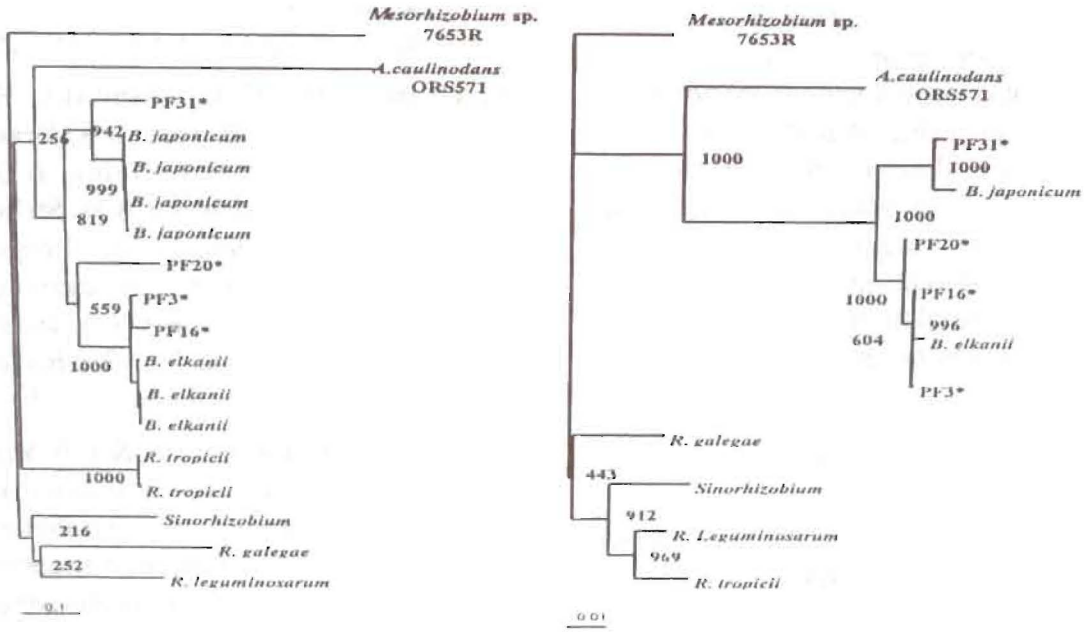

Figure 3. Phylogenetic trees of *Bradyrhizobium* strains and the related bacteria based on the nucleotide sequence of the partial *nod C* gene (a) and 16S rDNA (b).

By looking at the figure, it was clearly showed that the position of isolates was not completely the same as reflected in 16S rDNA phylogenetic tree. All the strains are belong to the genus *Bradyrhizobium* although they seemed to spread into three groups. Strain PF31 was closely related to *B. japonicum*, three other strains belonged to the same group with *B.elkanii*. While the position of strain PF20 which in the tree constructed by 16S rDNA has a strong relationship to *B.elkanii* (boostrap value 1000), actually has a separated position from *B.elkanii* group as reflected by the boostrap

be used for the grouping of *rhizobia* group. It also could be noted that the best condition for *nodC* amplification was condition III, since the condition gives more clear resolution of amplified DNA compare to the others (figure not shown).

Recently using of special gene in phylogenetic analysis has become popular. Some previous studies proved that the resolution of the special gene sequence was superior to that of 16S rDNA (Suzuki *et al.*,2001; Jian *et al.*,2001). Therefore using this technique for taxonomic consideration at the species level is suggested.

CONCLUSION

Results showed hat *Paraserianthes falcataria* plant could be infected by both fast and slow-growing *rhizobia*. All fast-growing isolates showed a high degree of relationship with *Rhizobium tropicii*, meanwhile the slow-growing one consisted of two groups, some isolates belonging to *Bradyrhizobium elkanii* and the other one indicated to be placed in the group close to reference strains *Bradyrhizobium japonicum*. However based on another phylogenetic tree constructed based on direct sequencing of partial *nodC* gene showed a little bit different feature. On the *nodC* phylogenetic tree an independent strain could be detected. This recent finding proved that direct sequencing technique of partial *nodC* gene gave a better resolution therefore could be suggested for *rhizobia* grouping at the species level.

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