Diversity of Nitrogen Fixing Bacteria Based on nifH Gene in Rice Fields

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Abstract

Biological nitrogen fixation is an important process in changing N2 gas in the atmosphere into ammonium which performed by nitrogen fixing bacteria. The molecular analysis of nifH gene is commonly used and proved more accurate for detecting the diversity of nitrogen fixing bacteria contained in the rice field soil. The diversity of nitrogen-fixing bacteria based on nifH gene can be determined by molecular approaches, i.e. DGGE (Denaturant Gradient Gel Electrophoresis). This research were conducted to study the diversity of nitrogen fixing bacteria in rice fields soil and also to measure the plant growth. The plant growth parameters of rice showed the treatment which soaked by biofertilizer were better than control treatment and treatment which spread by biofertilizer. However, the diversity of nitrogen fixing bacteria was more vary in control treatment based on DGGE results. There were three of eight bands which always appears in every month. The sequencing results showed all sequences were classified as uncultured bacterium clone nitrogenase iron protein (nifH) gene, partial cds.

INTRODUCTION

Nitrogen is a macro element required by plants but the composition of 78% nitrogen in the atmosphere cannot be used directly by plants. Plants can only absorb soluble nitrogen in the soil by using root. Supplying the nitrogen in the soil can be done by fertilization or naturally with the help of microorganisms. Biological nitrogen fixation is an important process in nature which performed by nitrogen-fixing bacteria in changing free nitrogen gases in the atmosphere into ammonium. Ammonium is an important nitrogen source in the ecosystem. Nitrogen fixing bacteria is able to live freely (free living) and can also live in symbiosis with plants. Biological nitrogen fixation is limited to prokaryotes while there is no report for eukaryotes as nitrogen fixer.

The ability of nitrogen fixing bacteria to convert nitrogen in the atmosphere into ammonium due to its nitrogenase enzyme. Nitrogenase enzyme consists of two subunits that dinitrogenase and dinitrogenase reductase. Dinitrogenase enzyme is encoded by nifD and nifK gene, while dinitrogenase reductase enzyme encoded by nifH gene. The diversity of nitrogen-fixing bacteria can be identified by molecular approaches. One of the molecular techniques that can be used is DGGE (Denaturant Gradient Gel Electrophoresis).

DGGE can distinguish species based on differences in the GC composition of the analyzed DNA sequences [10]. This is caused by the use of denaturant gradient gel used. The diversity of nitrogen-fixing bacteria studied by nifH gene. The use of nifH gene in DGGE is commonly used and proven to be more accurate for the detection of diversity nitroge fixing bacteria contained in the soil [21,4,5,11].

According to reports by Pingak [12], the use of bio-fertilizers in rice fields can increase rice productivity. Rice productivity can be supported by a various factors, such as nitrogen. Nitrogen element itself can be acquired from fertilization or nitrogen fixing bacteria. Research related to the relationship between the use of bio-fertilizer and rice productivity associated with the diversity of nitrogen fixing bacteria in rice fields has not been reported in Sukabumi. This research aimed to study the diversity of nitrogen-fixing bacteria in rice fields by DGGE technique.
MATERIAL AND METHODS

This study was conducted in June 2013 - May 2014. Field research was performed in rice fields at Cidahu village, Cidahu, Sukabumi, West Java. The analysis was performed at the Laboratory of Microbiology and Laboratory of Integrated, Department of Biology, Bogor Agricultural University. The treatment used in this study were control, soaked and spread. The control treatment using 300 kg/ha of NPK fertilizer. Soaked and spread treatment using 200 kg/ha of NPK fertilizer and biofertilizers. Rice clump in the soaked treatment was soaked with biofertilizer while in the spread treatment, biofertilizer was spread directly into rice fields.

Soil sampling:
Rice field soil in the control, soaked and spread treatment were taken by using a 10 mL syringe. 2 replications were used per treatment. Control treatment has an area of 150 m² while soak and spread plots have an area of 600 m². Soil sampling is done every 30 days during the rice planting namely 30 DAP (days after planting), 60 DAP and 90 DAP. A total of ± 1 kg of soil in each treatment was taken in the beginning and end of the growing season then sent and analyzed at the Soil Research Institute, Bogor to determine the physical and chemical characteristics of the soil.

Plant Growth Measurement:
Plant growth measurement was carried out by measuring the plant height and number of tillers. There were 10 clumps that had been used in this study. Measurement of wet weight and dry weight of the clump, roots and seeds are also carried out in the end of the planting.

DNA Extraction:
DNA extraction was done by using the Power Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA). Extraction was done according to the procedures of the company. The quality of DNA then checked using Nano Drop 2000 (Thermo Scientific, Wilmington, DE, USA).

Polymerase Chain Reaction:
DNA was amplified by using PolF / PolR primer sets [13]. 40 GC sequences were attached to the end of the forward primer. PCR was performed by using KAPA Hot Start ReadyMix (KAPA Biosystems, Wilmington, MA, USA). Each PCR reaction contained 12.5 µL KAPA Hotstart ReadyMix, 1.25 µL forward primer (0.5 uM), 1.25 µL reverse primer (0.5 uM), 3 µL template (~100 ng) and 7 µL nuclease free water. PCR was performed using T-Gradient Thermocycler (Biometra GmbH, Goettingen, Germany). The PCR thermal cycling conditions were as follows: initial denaturation at 95 °C for 1 min; 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and elongation at 72 °C for 15 s; and a final elongation step at72 °C for 5 min. Products were run at 1.5 % agarose gel for checked correct size, and stored at -20 °C until analyzed on DGGE.

DGGE Analysis:
DGGE was performed using the D Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The DGGE gels were prepared and run under the following conditions: 25 µL of the PCR product was loaded onto 1 mm thick 8% (w/v) polyacrylamide gels [acrylamide–bisacrylamide (37.5:1)] in 1× TAE buffer (20 mM Tris, 20 mM acetic acid, and 1 mM EDTA) using urea as the denaturing agent with a denaturing gradient from 35% to 60% (100% denaturant corresponded to 7M urea and 40% (v/v) deionized formamide). Electrophoresis was performed at 60 °C and 130 V for 6 h. After electrophoresis, the gel was stained for 60 min in SYBR Safe (Invitrogen-Molecular Probes, Carlsbad, CA, USA) and visualized by G:BOX (Syngene, Frederick, MD, USA). Bands appearing on the gels were excised and transferred to micro tubes containing 50 µL ddH2O. The bands were incubated at 4 °C overnight. Band analyzed using Phoretix 1D (Total Lab) to estimate total band that appeared.

Sequencing and Phylogenetic Tree Construction:
Supernatant from each band was checked using Nano Drop 2000 to determine the quantity of DNA from excised band. A 10 µL (~50 ng) supernatant was re-amplified under the PCR conditions described above using non GC primer. The PCR products were sent to 1stBASE Malaysia. Sequencing results will be analyzed using BLAST program with NCBI database and then the phylogenetic tree created using MEGA 5.2 [17].

RESULT AND DISCUSSION

Soil Characteristics:
Soil in the control treatment contained of 10% sand, 43% silt and 47% clay. Soil in the soaked treatment contained of 16% sand, 33% silt and 51% clay. Soil on spread treatment contained of 18% sand, 38% silt and...
48% clay. Soil in the control treatment, soak and spread was the same pH range, ie pH 5. Carbon content in the control treatment, soak and spread is 1.97%, 1.81%, and 1.91%, respectively. Nitrogen content of the control treatment, soak and spread is 0.17%, 0.17% and 0.14%, respectively. C/N ratio of each treatment was 12, 11 and 14, respectively. Based on the criteria of the USDA [19], the soil in the control treatment classified as Silty Clay, while soaked treatment classified as Clay and spread treatment classified as Clay. Based on the criteria of BALITANAH [2], those soils was classified as acidic soil (pH 4.5 - 5.5) with carbon and nitrogen was low while the C/N ratio of the three soil was classified as moderate. According AGRISNET [1], rice plants can grow well in Clay soil, Silty Clay and Silty Clay Loam. The optimum pH condition which required to grow rice well ranged 5-7. So, the soil characteristics in this study fairly well for rice planting.

**Rice growth:**

Height measurement results indicated that rice plants at soaked treatment showed maximum height than other treatments. Control treatment occupied the lowest position among others. The number of tillers in soaked treatment also showed compared to the other two treatments. Spread treatment place at second place followed by control treatment (Figure 1). Soaked treatment had the highest wet weight and dry weight compared to the other treatments in all parameters measured (Figure 2). These results were supported with reports [12] that the soaked treatment resulted in the best growth response than the other two treatments.

**Fig. 1:** Height and number of tillers in the control, soaked and spread treatment.

**Fig. 2:** Weight of wet (a) and dry (b) of the clump, roots and grain of rice in the control, soaked and spread treatment.
DNA Extraction:
DNA was extracted from soil showed various results ranged around 24 ~ 28 ng/µL. The purity of the DNA obtained from the extraction is quite good with an average A260/A280 parameter ranges 1.9 (Table 1). Recommended value of A260/A280 ratio is ranged 1.8 - 2.0 [14]. Good quality of DNA template was important for downstream process like PCR.

DNA Amplification:
Nine samples were successfully amplified by PolF-GC/PolR primer with fragment size obtained as expected, around 360 bp (Fig 3). PolF / PolR primer was designed to amplify \(nifH\) genes possessed by nitrogen fixing bacteria and has been shown to successfully amplify 19 important species of nitrogen-fixing bacteria such as Azospirillum, Agrobacterium, Burkholderia, Pseudomonas, Rhizobium, Streptomyces, and Xanthomonas [13].

Table 1: The DNA concentration using Power Soil DNA Isolation Kit.

<table>
<thead>
<tr>
<th>No</th>
<th>Treatment</th>
<th>DNA Concentration (ng/µL)</th>
<th>A260/A280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control, 30 DAP</td>
<td>26</td>
<td>1.96</td>
</tr>
<tr>
<td>2</td>
<td>Soaked, 30 DAP</td>
<td>24.4</td>
<td>1.97</td>
</tr>
<tr>
<td>3</td>
<td>Spread, 30 DAP</td>
<td>24.5</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>Control, 60 DAP</td>
<td>28</td>
<td>1.94</td>
</tr>
<tr>
<td>5</td>
<td>Soaked, 60 DAP</td>
<td>24.4</td>
<td>1.95</td>
</tr>
<tr>
<td>6</td>
<td>Spread, 60 DAP</td>
<td>27.9</td>
<td>1.98</td>
</tr>
<tr>
<td>7</td>
<td>Control, 90 DAP</td>
<td>27.4</td>
<td>1.99</td>
</tr>
<tr>
<td>8</td>
<td>Soaked, 90 DAP</td>
<td>26.6</td>
<td>1.92</td>
</tr>
<tr>
<td>9</td>
<td>Spread, 90 DAP</td>
<td>27.2</td>
<td>1.98</td>
</tr>
</tbody>
</table>

Fig. 3: The \(nifH\) gene amplification result with PolF-GC/PolR primer on 1.5% agarose gel. Wells from left to right: 100 bp marker, (a) control 30 DAP, (b) soaked 30 DAP, (c) spread 30 DAP, (d) control 60 DAP, (e) soaked 60 DAP, (f) spread 60 DAP, (g) control 90 DAP, (h) soaked 90 DAP, (i) spread 90 DAP.

DGGE Profile:
DGGE results showed all analyzed sample were separated into several separate bands. Each separate bands represent one of its own species. The results showed there were three bands that always appeared in the treatment every month. Control treatment showed the highest number of bands with different variations of each month. Soaked treatment tended to be stable every month with 4 bands. Spread treatment showed increasing number of bands from 4 bands in first month into 6 bands in the third month (Figure 4).

The control treatment showed the highest diversity compared than other treatments. It was probably because there was no biofertilizer application then there is no dominance effect at this treatment. Biofertilizer contain metanotrophic bacterial isolates capable of enhancing the growth of rice plants. However, the introduction of biofertilizers decreased nitrogen-fixing bacteria communities. Even though the highest diversity of nitrogen-fixing bacteria was obtained in the control treatment but it had little role to encourage the growth of rice plants. It can be shown by the less growth of rice plants in control treatment than soaked and spread treatment. There were three reason to explain this phenomenon. First, the diversity of nitrogen-fixing bacteria found in the control treatment may be high but not necessarily active. The activity \(nifH\) genes can be more accurately known when it analyzed by RNA approach [11]. Second, bacterial isolates were used as a biological fertilizer could encourage the growth of rice because it has been tested according to Pingak [12]. Third, some bacterial isolates metanotroph used as a biological fertilizer also has the capability of nitrogen fixation [3].
Fig. 4: DGGE profile analysis of \textit{ni}f\textit{H} gene. Left: Photo of G: BOX and Right: Interpretation of Phoretix 1D software. The number showed the bands which cut to be reamplified. Wells from left to right: (a) control 30 DAP, (b) soaked 30 DAP, (c) spread 30 DAP, (d) control 60 DAP, (e) soaked 60 DAP, (f) spread 60 DAP, (g) control 90 DAP, (h) soaked 90 DAP, (i) spread 90 DAP.

Sequencing and Phylogenetic Tree:

BLAST results showed that all sequences obtained were uncultured bacteria dinitrogenase (\textit{ni}f\textit{H}) gene from various bacteria (Table 2). This indicated that the sequences obtained belong to unculturable bacteria. It also showed that the sequence found was relatively new as a percentage identity of the result of the blast sequence databases under 95% of the ~ 360bp sequences of bases. Band 1, 6, and 8 were shown on DGGE gel on each treatment every month. The three of bands were suspected to dominant bacteria in rice field due to appear in each treatment in every month. The results showed that band 1 had closeness to \textit{Uncultured bacterium clone MDE\textunderscore amb\textunderscore 35f2 dinitrogenase reductase (ni}f\textit{H}) gene, partial cds for 89%. Band 6 had closeness to \textit{Uncultured bacterium clone cloA\textunderscore 42 nitrogenase iron protein (ni}f\textit{H}) gene, partial cds for 88% and band 8 had closeness to \textit{Uncultured bacterium clone Sipa\textunderscore L24 nitrogenase iron protein (ni}f\textit{H}) gene, partial cds for 92%.

Table 2: Results of BLAST sequence of the \textit{ni}f\textit{H} gene

<table>
<thead>
<tr>
<th>Band</th>
<th>Description</th>
<th>Query Cover</th>
<th>E Value</th>
<th>Base/Base</th>
<th>Identity</th>
<th>Acession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uncultured bacterium clone MDE\textunderscore amb\textunderscore 35f2 dinitrogenase reductase (ni}f\textit{H}) gene, partial cds</td>
<td>100%</td>
<td>5e-114</td>
<td>308/348</td>
<td>89%</td>
<td>KF846889.1</td>
</tr>
<tr>
<td>2</td>
<td>Uncultured bacterium clone Sipa\textunderscore 40 nitrogenase iron protein (ni}f\textit{H}) gene, partial cds</td>
<td>97%</td>
<td>5e-134</td>
<td>309/331</td>
<td>93%</td>
<td>JX268437.1</td>
</tr>
<tr>
<td>3</td>
<td>Uncultured bacterium clone Sipa\textunderscore 36 nitrogenase iron protein (ni}f\textit{H}) gene, partial cds</td>
<td>99%</td>
<td>5e-149</td>
<td>328/347</td>
<td>95%</td>
<td>JX268435.1</td>
</tr>
<tr>
<td>4</td>
<td>Uncultured bacterium clone Sipa\textunderscore 34 nitrogenase iron protein (ni}f\textit{H}) gene, partial cds</td>
<td>97%</td>
<td>3e-126</td>
<td>305/332</td>
<td>92%</td>
<td>JX268433.1</td>
</tr>
<tr>
<td>5</td>
<td>Uncultured bacterium clone JSR8\textunderscore 2 dinitrogenase reductase (ni}f\textit{H}) gene partial cds</td>
<td>98%</td>
<td>9e-107</td>
<td>292/330</td>
<td>88%</td>
<td>HM750439.1</td>
</tr>
<tr>
<td>6</td>
<td>Uncultured bacterium clone cloA\textunderscore 42 nitrogenase iron protein (ni}f\textit{H}) gene, partial cds</td>
<td>97%</td>
<td>2e-102</td>
<td>288/328</td>
<td>88%</td>
<td>JX268272.1</td>
</tr>
<tr>
<td>7</td>
<td>Uncultured bacterium clone BG2.3 dinitrogenase iron protein (ni}f\textit{H}) gene, partial cds</td>
<td>97%</td>
<td>3e-116</td>
<td>295/327</td>
<td>90%</td>
<td>JX079620.1</td>
</tr>
<tr>
<td>8</td>
<td>Uncultured bacterium clone Sipa\textunderscore L24 nitrogenase iron protein (ni}f\textit{H}) gene, partial cds</td>
<td>100%</td>
<td>5e-139</td>
<td>331/360</td>
<td>92%</td>
<td>KF032172.1</td>
</tr>
</tbody>
</table>

From the phylogeny tree (Fig. 5) it can be seen that the bands 1, 6 and 8 had closeness to \textit{Pseudomonas stutzeri}, \textit{Anaeromyxobacter} sp. \textit{Halorhodospira halophila} with compared restricted to ~ 360 bp range only. \textit{Pseudomonas stutzeri} is a bacteria which has broad habitat and relatively resistant to changes in environmental conditions [9]. This ability makes \textit{P. stutzeri} very tolerant of changes in management and planting methods on a depressing place. Perez found 20 DGGE sequences related to \textit{P. stutzeri} on agricultural land cultivated organically and conventionally [11]. Diallo also found \textit{P. stutzeri} of several sequences which were found...
predominantly in the soil [7]. H. halophila was classified into gamma proteobacteria, purple sulphur bacteria, Gram-negative and phototroph. H. halophila is belong to potential nitrogen fixing bacteria and dispersed quite widely. Some research and studies have shown that H. halophila has nifH gene and capable of fixing nitrogen [18] [4] [16]. Anaeromyxobacter sp. is belong to the delta proteobacteria bacteria, rod-shaped, Gram-negative, capable of forming spores, and natural habitat in soil [15]. Anaeromyxobacter sp. also capable of fixing atmospheric nitrogen and from several studies has been shown that it has nifH gene [8] [16] [20].

![Phylogenetic tree of 8 nifH gene sequences obtained from DGGE analysis](image)

**Fig. 5:** Phylogenetic tree of 8 nifH gene sequences obtained from DGGE analysis. Phylogeny tree was constructed with the Neighbour Joining method with 1000X bootstrap value.

Eight bands were successfully sequenced didn’t have closeness with metanotroph bacteria which was used as biofertilizer and added to the rice fields. Some metanotroph bacteria that added as a biofertilizer has been tested and analyzed the ability of nitrogen fixation [3]. However, this study found no sequence which similar to metanotroph bacteria. It was because the different primer used in this research instead to recognize the metanotroph bacteria. PolF/PolR primer designed to amplify the nitrogen fixing bacteria in widely coverage while the primers used by Bintarti [3] was a special primer designed by Dedysh [6] for metanotroph bacteria that had ability of nitrogen fixation. The target amplicons from two primer also different. PolF/PolR primer had a target of 360 bp, while primers designed Dedysh had a target of 453 bp [6].

**Conclusion:**

The diversity of nitrogen-fixing bacteria obtained from rice fields Cicurug village, Sukabumi tend to vary. Control treatment had the most diversity in comparison with the soaked and spread treatment. Sequencing results showed that all the bands were belong into uncultured bacteria dinitrogenase (nifH) gene.

**REFERENCES**


