Phylogeny of Indonesian Nostoc (Cyanobacteria) Isolated from Paddy Fields as Inferred from Partial Sequence of 16S rRNA Gene

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Abstract

In order to collect Indonesian Nostoc, isolation of soil microflora from several paddy fields in West Java, Bali, and South Celebes was carried out. Fast-growing isolates of Nostoc were selected to describe and perform molecular identification using partial sequences of 16S rRNA. The results showed that partial sequences of 16S rRNA could not resolve the phylogeny of the isolates. However, it supported the morphological studies that recognize isolates as different species of Nostoc. Potential use of Nostoc as a nitrogen source for paddy growth was carried out using six strains as single inoculums. A total biomass of 2 g (fresh weight) for each strain was inoculated, respectively, into the pot planted with three paddy plants. This experiment was conducted in the green house for 115 days. Statistical analyses (ANOVA; $\alpha = 0.05$) showed that of six strains tested in this study, only strain GIA13a had influence on the augmentation of root length and the total number of filled grains.

Keywords: biofertilizer, diversity, inoculum, Nostoc, paddy

1. Introduction

Cyanobacteria are prokaryotic organisms able to photosynthesize and fix atmospheric nitrogen (N$_2$). Efficient nitrogen fixing cyanobacteria are known to be a prominent component of the microbial population in tropical soil [1], especially in paddy fields, contributing significantly to the soil fertility. As an agricultural country, Indonesia is endowed with diverse organisms, particularly Nostoc, one of the cyanobacteria dominating in rice-fields. Like many cyanobacteria, the appearance of Nostoc in soil gives many benefits. Application of Nostoc in soil increases the organic carbon and nitrogen content of the surface soil and enhances plant growth [2]. Nostoc also improves the aggregation of top soil [3]. Chemical analyses showed that Nostoc caused significant increases in extracellular polysaccharide substances (EPS) and soil carbon content, which later increased the aggregation of the soil [4].

In order to collect Indonesian Nostoc, sampling and isolation of Nostoc from several paddy fields in Java, Bali, and Celebes were carried out in 2008. Isolated strains were categorized into two groups, based on the colony shaped on the agar medium: spherical-shaped and irregular shaped colonies. Morphologically, the genus Nostoc has high similarity with the genus
Anabaena, therefore strain identification was conducted using DNA sequencing.

Use of cyanobacteria as a biofertilizer in paddy fields has been reported in India, Nepal, and Chile, as well as in Indonesia [5-8]. Gurung and Prasad (2005) inoculated a mixture of Nostoc, Anabaena, Aulosira, and Tolypothrix to enhance rice productivity, while Pereira used Anabaena and Nostoc spp [6-7]. In Indonesia, Simanungkalit (2001) reported the application of organic fertilizer E-2001, which contained N. muscorum [8]. So far, cyanobacteria have been proved to have potential to support paddy growth. In this study, we have investigated the effect of our Nostoc collection for the vegetative and generative growth of paddy fields.

2. Methods

Samples. All of the Nostoc strains used in this study were collected and isolated by DH [9]. Strains CPG8, CPG10, CPG24, CPR31, BAD5, and CIG10 were collected from West Java. GIA12-02, GIA12-03, GIA13a, GIA13b, and TAB7d were collected from Bali. The strains BTM6-02 and TAK23 were collected from South Celebes. Based on colony shape, the strains were grouped into spherical-shaped colonies (CPG8, CPG10, BAD5, TAB7d, GIA12-02, GIA13a, GIA13b, BTM6-02) and irregular-shaped colonies (CPG24, CPR31, CIG10, CIM7, GIA12-03, BTM6-01, TAK23). Isolates were maintained in agar medium Blue Green 11 (BG-11) [10], but the nitrogen source was eliminated following the method of Jeong-Dong & Lee [11]. The culture was stored at 20-23°C room temperature and provided with a light intensity of 1200-1300 lux, with an L:D period of 14:10. Morphological observations of strains were done using a light microscope Olympus, with 400x magnification.

DNA Extraction and Polymerase Chain Reaction Amplification. The culture of Nostoc was maintained in BG-11 agar medium without nitrogen sources. Cells were harvested by taking a colony with sterile toothpaste during exponential growth. Cells were washed with a mortar and collected into Eppendorf tubes. The tubes containing culture samples were kept in a refrigerator overnight. The tubes were then boiled in a water bath for 30 minutes and centrifuged at 13,000 rpm for 15 minutes to collect the supernatant as a DNA template. Six primers were used to amplify the 16S rRNA gene: 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 1510R (5'-GGTTACCTTGTTACGACTT-3'), pA (5'-GAGTTTGATCCTGGCTCAG-3'), CYA359F (5`-GGGGAATCTTCCGCAATGGG-3`), Six primers were used to amplify the 16S rRNA gene: 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 1510R (5'-GGTTACCTTGTTACGACTT-3'), pA (5'-GAGTTTGATCCTGGCTCAG-3'), CYA359F (5`-GGGGAATCTTCCGCAATGGG-3`), and 16S545R (5'-ATTCCGGGA TAACGCTTGC-3`) [12-15]. The PCR reactions were performed in a 12.5 µl mixture, using puretag ready-to-go PCR beads from GE Healthcare. The PCR was performed under the following conditions: 3 minutes at 94 °C, followed by 35 cycles of 30 seconds at 95 °C, 15 seconds at 55 °C, and 60 seconds at 72 °C. PCR products were visualized by electrophoresis in a 1% agarose in a tris-EDTA buffer at 100 V for 25 minutes. Purified PCR products were directly used for cycle sequencing reactions. The PCR cycle-sequencing reaction started at 96 °C for 2 minutes, followed by 25 cycles of 10 minutes at 96 °C, 5 seconds at 50 °C and 60 seconds at 60 °C. The purified products of the cycle sequencing were sequenced by an automated DNA sequencer (310 genetic analyzer, Applied Biosystem, USA).

Phylogenetic analysis. The sequences of the 16S rRNA gene were aligned using ClustalX 1.83. The primer sequences were checked for homology to any other known sequences deposited in the available databases, using the BLAST. Data matrices from the sequence were analyzed using the neighbor-joining method with the Kimura Two-parameter model [16]. Confidence levels for the individual branches of the resulting tree were assessed by bootstrap analysis, using 1,000 bootstrap resamplings.

Nostoc Inoculation in Paddy Plants. This experiment was done at the green house at the Department Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia. Six fast-growing strains of Nostoc (CPG8, CPG24, BAD5, CIM7, GIA13a, TAB7d) were selected and each strain was used as a single inoculum for paddy growth. Biomasses for the inocula were obtained by culturing Nostoc in BG11 N-free agar medium, provided with 20-23 °C room temperature under a light intensity of 1200-1300 lux, with an L:D period of 14:10. The design experiment was the Full Random Method, with six treatments (paddy with Nostoc inoculation) and one control (paddy without Nostoc), each having six duplications. The soil used in this experiment was obtained from the garden around the greenhouse. Before application, the soil was sterilized with 1% formalin. Three (3) paddy plants were planted in a basket with a 25 cm mouth diameter. Nostoc biomass (fresh weight) for paddy plants was inoculated four times: 0.4 g at 15 days after plantation (dap), 0.4 g at 30 dap, 0.6 g at 45 dap, and 0.6 g at 60 dap. Requirements of phosphate and potassium minerals (0.5 and 0.25g respectively) for paddy plants were fulfilled at 10 dap. Observation of vegetative and generative growth was carried out for 115 days, until harvest time.

3. Results and Discussion

The genus Nostoc is characterized by a filamentous form consisting of vegetative and heterocyst cells that are located terminally or intercalary (Fig. 1A). A variety of morphological types and pigmentation was observed among the strains studied here. The majority of strains...
were green-brown in color (Fig. 1B-C). The color of a colony implies the presence of red phycobiliproteins and phycoerythrin, in addition to the blue phycocyanin [17]. Most of the vegetative cells were cylindrical in shape, while the heterocysts were spherical to ovoid. Because of the limited morphological traits available for strain identification, subjecting strains to DNA sequencing might reveal the strain’s identity.

The primer pair, designated CYA 359F and CYA 781R, were successfully amplified to 13 strains. However, the primers failed to amplify the DNA of the strains CIM7 and BTM6-01. Approximately 380-510 nucleotides of sequence were initially determined directly from PCR-amplified material by using the CYA359F-CYA781R primers set. Because the sequences were too short, we tried to make them longer by using pA—16S545R primers set to amplify other segments of the 16S rRNA gene. The final sequence length of 634-732 nucleotides was obtained and subjected to BLAST to find homology with other known sequences of *Nostoc* in GenBank (Table 1).

The phylogenetic tree, as inferred from the 16S rRNA gene, showed that those strains having an irregular-shaped colony were separated from strains having a spherical-shaped colony (Fig. 2). Although the grouping was not supported with strong bootstrap values, the inner clades of the irregular-shaped colony and the clades containing spherical-shaped colony strains of [CPG8/GIA13a] had high bootstrap values (93-99). Strain GIA13b was also an exception to the other spherical-shaped colony strains, because it was placed in one group, together with irregular-shaped strains. Strains CPG24 and CPR31, from Kasepuhan Village, West Java, showed a high degree of similarity with *Nostoc sp. strain HK-01* and *Nostoc sp. strain PCC 6720* (99 bootstrap value). Strains CIG10, GIA13b, and TAK23 were grouped together with *Nostoc sp. strain PCC 9426* (66 bootstrap value).

Contrary to clades containing irregular-shaped colonies, the clades of the spherical-shaped colony was unconvincing. Strains BTM6-02, GIA12-02, BAD5, and TAB7d did not show any relationship with *N. calcicola* strains TH2S22, *N. carneum* IAM M-35, or *N. verrucosum* KU005. Most clades have a weak bootstrap value. The addition of more samples to the phylogenetic tree may reveal the phylogenetic relationship among strains.

The branch of strains with an irregular shape appeared later on the tree topology, suggesting that the irregular-shape evolved from the spherical-shape lineage. If so, this explains why some strains having a spherical-shape colony (ex. CPG8 and GA13a) still remain similar to their ancestor, as found in the inner clades. Furthermore, the hypothesis conclude that the character of a colony seems to be genetically inherited and not the result of plastic morphology. However, this is not in agreement with the results of the study. The strains CPG8 and GIA13a showed a high homology sequence with *N. ellipsosporum* strain V. *Nostoc ellipsosporum*, was first described by Rabenhorst (1865). The colony of *N. ellipsosporum* is reddish brown with irregular-shaped [18], vegetative cells, and is 3.8-4.2 µm in width and 6-14 µm in length. The width and length of the heterocyst

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**Figure 1. Profile of *Nostoc*. A. Filaments of Strain *Nostoc TAK23* Shows Many Vegetative Cells and Terminal Heterocysts (Arrow). B. Irregular-shaped Colony of Strain TAK23. C. Spherical-shaped Colony of Strain TAB7d**

**Table 1. Strains of *Nostoc* from GenBank used for Phylogenetic Tree Reconstruction**

| Accession Number | Strain *Nostoc*          |
|------------------|--------------------------|
| AM711529.1       | *N. calcicola* strain TH2S22 |
| AB325906.1       | *N. carneum* strain IAM M-35 |
| AJ630450.1       | *N. ellipsosporum* strain V |
| AM711524.1       | *N. muscorum* strain Lukesova 2/91 |
| AJ630452.1       | *N. muscorum* strain II |
| AB494996.1       | *N. verrucosum* strain KU005 |
| AB085687.1       | *Nostoc* sp. strain HK-01 |
| DQ185240.1       | *Nostoc* sp. strain PCC 6720 |
| AM711538.1       | *Nostoc* sp. strain PCC 9426 |
| AY742454.1       | *Nostoc* sp. strain 8938 |
| AJ133161.1       | *Nostoc* sp. strain 152 |
| AM711543.1       | *Nostoc* sp. strain Cam2S01 |
| DQ185208.1       | *Nostoc* sp. strain Mollenhauer 1:1-088’ |

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doi: 10.7454/mss.v16i3.1483/Makara J. Sci. 16/3 (2012) 203-208
cells are 6-7 µm and 6-14 µm, respectively [18]. Morphological examination of strains CPG8 and GIA13a showed that the shape of the colony of the two strains is spherical. Although the color of the colony is same, the vegetative and heterocyst cells of strains CPG8 and GIA13a are smaller than those of *N. ellipsosporum* (Table 2). Inconsistent data are also visible between GIA13b (spherical) and CIG10 (irregular), which are placed in the same clade supported by a high bootstrap (93). Therefore, at present, it is not clear whether the colony profile can be considered as stable or plastic in character.

Overall, the 13 strains of *Nostoc* studied here showed a relationship with other *Nostoc* strains from many areas, as published in GenBank. One major problem with the reconstruction of the phylogenetic tree of *Nostoc* is the limited data of *Nostoc* sequences in the GenBank. Compared to the extensive morphological studies that resulted in the identification of the *Nostoc* species, sequence data is scarce. Many sequences end up with a strain code, not a species name. Inputting new sequences to the GenBank will help to reveal homology sequencing amongst *Nostoc* strains.

Six *Nostoc* strains were selected for the paddy growth experiment. These six strains showed fast growth, stability, and adapt better on medium. At the end of observation (115 days), paddy plants were measured for vegetative parameters and generative parameters (Table 3). Compared to the control, the vegetative growth of the paddy inoculated with *Nostoc* showed higher performance. The plants are generally taller and weighter, with the exception of BAD5, which showed low performance in the plant’s weight. The plants inoculated with strain GIA13a obtained the highest value of all vegetative parameters. However, generative parameter data were more variable. Plants inoculated

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**Figure 2. Phylogenetic Tree of *Nostoc* Inferred from 16S rRNA Gene**

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doi: 10.7454/mss.v16i3.1483/Makara J. Sci. 16/3 (2012) 203-208
with several strains showed lower performance than the control. For example, plants inoculated with strain CIM7 have a low number of filled grains. In paddy plantations, filled grains are important, because they are the final product of the plants. Of the six strains, the strain GIA13a again obtained the highest value for the number of filled grains and for the fresh weight of grains.

The association of *Nostoc* with the paddy root has been proved by [19]. *Nostoc* mainly live as symbions in intracellular or intercellular root epidermis. However, free-living *Nostoc* is also found in rhizospheres. In this case, ammonium produced by the process of N-fixation is absorbed by the root, together with water and other soil nutrients. Besides nitrogen provision, *Nostoc* occurrence in soil increases soil fertility by making the

### Table 2. Morphology of the 13 Strains *Nostoc* Used in this Study

| Strain | Colony color | Colony surface | Colony growth | Shape of veg cell | Veg cell size (μm) | Shape of het cell | Het cell size (μm) |
|--------|--------------|----------------|--------------|------------------|-------------------|------------------|-------------------|
| CPG8   | olive green  | granular       | spheric      | oval             | (2.5-6.25)x(2.5-5) | spheric-oval     | (5-8.75)x(5-6.25) |
| CPG10  | grass green  | smooth         | spheric      | spheric-cylindric | (2.5-5)x(2.5-3.75) | spheric-oval     | (3.75-7.5)x(3.75-5) |
| CPG24  | grass green  | smooth         | irregular    | spheric-cylindric | (2.5-5)x(2.5-3.75) | spheric-oval     | (2.5-6.25)x(2.5-5) |
| CPR31  | grass green  | smooth         | irregular    | oval-cylindric   | (2.5-5)x(2.5)     | oval             | (2.5-7.25)x(2.5-5) |
| BAD5   | olive green  | granular       | spheric      | spheric-cylindric | (2.5-6.25)x(2.5-5) | spheric-oval     | (5-6.25)x(2.5-6.5) |
| CIG10  | grass green  | smooth         | irregular    | spheric-cylindric | (2.5-5)x(2.5-5)   | oval             | (5-7.5)x(3.75-5)   |
| GIA12-02 | olive green | granular       | spheric      | oval-barrel shaped | (3.75-6.25)x(2.5-5) | spheric-oval     | (3.75-7.5)x(5-6.25) |
| GIA12-03 | olive green | granular       | irregular    | spheric-cylindric | (2.5-5)x(2.5-3.75) | spheric          | (7.5-10)x(5-6.25)  |
| GIA13a | olive green  | granular       | spheric      | oval-barrel shaped | (5-7.5)x(2.5-5)   | oval             | (7.5-11.25)x(5-6.25) |
| GIA13b | olive green  | smooth         | spheric      | spheric-cylindric | (2.5-5)x(2.5-3.75) | oval             | (7.5-10)x(5-6.25)  |
| TAB7d  | olive green  | granular       | spheric      | spheric-oval     | (3.75-5)x(2.5-3.75) | oval             | (5-7.5)x(5-6.25)   |
| BTM6-02 | olive green | granular       | spheric      | spheric-oval     | (2.5-6.25)x(2.5-5) | spheric-oval     | (7.5-10)x(6.3-7.5) |
| TAK23  | olive green  | smooth         | irregular    | spheric-cylindric | (2.5-6.25)x(2.5-5) | spheric-oval     | (5-6.25)x(2.5-5)   |

*Veg = vegetative, het = heterocyst*

### Table 3. Vegetative and Generative Growth of Plants at 115 Days

| No. Parameters | Control | CPG8 | CPG24 | CIM7 | TAB7d | GIA13a | BAD5 |
|----------------|---------|------|-------|------|-------|--------|------|
| Vegetative     |         |      |       |      |       |        |      |
| 1. Plant height (cm) | 98      | 99   | 102   | 109  | 100.2 | 107.3  | 99.8 |
| 2. Root Height (cm)  | 20      | 25   | 20    | 19   | 27    | 31     | 23   |
| 3. Fresh weight (g)  | 39.9    | 47.1 | 40.5  | 47.1 | 42.3  | 49.4   | 38.3 |
| 4. Dry weight (g)    | 12.5    | 13.1 | 11.3  | 12.7 | 12.9  | 13.5   | 11.0 |
| Generative          |         |      |       |      |       |        |      |
| 5. Number of filled-grains | 218     | 312.5| 347   | 107  | 263.5 | 388.3  | 315  |
| 6. Number of empty-grains | 146     | 96   | 53    | 107  | 45    | 57     | 60   |
| 7. Fresh weight of grains (g) | 89.1    | 98.8 | 95.6  | 67.0 | 81.8  | 107.7  | 91.1 |
| 8. Dry weight of grains (g) | 6.4     | 7.4  | 6.5   | 6.7  | 5.5   | 6.9    | 6.3  |
soil pores bigger [20]. Observation of soil texture in this study showed that the soil texture of the treated paddy is crumbled/granulated, while the control soil is clumped. According to Okuda & Yamaguci (1952), crumble also helps roots to penetrate the soil [cf. 21], thus enhancing the possibility of long roots and more hairs. As shown in this study, the roots of the paddy inoculated with Nostoc are longer than in the control, with the exception of strain CIM7.

Although the data in Table 3 show plant treatments had a higher performance than in the control, there were no statistically significant differences among treatments. Therefore, it was not clear which strains fared better. It might be that the total biomass (inoculum) given during the treatment was not sufficient to augment the nutrient supply of the paddy.

4. Conclusions

Partial sequencing of 16S rRNA determined in the present study could not resolve the phylogenetic relationship of the 13 strains of Indonesian Nostoc. However, the strains having an irregular-shaped colony might be genetically different from the strains having a spherical-shaped colony. Statistically, strain GIA13a was the only strain that had an influence on the augmentation of root length and the total number of filled grains.

Acknowledgements

We would like to thank the Directorate of Research and Community Engagement of University of Indonesia for supporting this research through the Riset Unggulan Universitas Indonesia (RUUI) Fund to DH under contract No. 212L/DRPM-UI/NI.4/2007 and 747T/DRPM-UI/NI.4/2009. We would also like to extend our gratitude to Ariyanti Oetari, Ph.D., Chairman (2007-2012) of the Center of Excellent Indigenous Biological Resources and Genome Studies (CoE IBR-GS), Fac. Mathematics and Natural Sciences University of Indonesia for facilitating the molecular works.

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doi: 10.7454/mss.v16i3.1483/Makara J. Sci. 16/3 (2012) 203-208