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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.

Abstrak

Filogeni *Nostoc* Asal Tanah Persawahan Indonesia berdasarkan Sekuen Parsial Gen 16S rRNA. Untuk memperoleh koleksi kekayaan hayati *Nostoc* Indonesia dilakukan isolasi mikroflora tanah dari beberapa lahan persawahan di wilayah di Jawa, Bali, dan Sulawesi Selatan. Isolat-isolat yang tumbuh cepat diidentifikasi secara morfologi dan molekuler mengunakan sekuen parsial dari gen 16S rRNA. Walaupun hubungan kekerabatan antar isolat belum sepenuhnya dapat dijelaskan, pohon filogeni yang dihasilkan dari analisis sekuen mendukung identifikasi secara morfologi bahwa isolat-isolat yang diteliti berbeda jenis. Uji coba 6 strain *Nostoc*, dalam bentuk inokulum tunggal, sebagai sumber nitrogen untuk padi dilakukan. Sebanyak 2 g biomasa basah dari masing-masing strain *Nostoc* diinokulasi ke dalam pot-pot yang telah berisi 3 tanaman padi. Percobaan dilakukan di rumah kaca selama 115 hari. Secara statistik (ANOVA; $\alpha = 0.05$) hanya strain GIA13a yang mempengaruhi panjang akar dan jumlah bulir padi bernas.

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 broken with a mortar and collected into Eppendorf tubes. The tubes containing culture samples were kept in a refrigerator overnight. The tubes were then boiled 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: CYA359F (5`-GGGGAATCTTCCGCAATGGG-3`), CYA781R (5`-GACTACAGGGGTATCTAATCC-3`), 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 1510R (5'-GGTTACCTTGTTACGACTT-3'), pA (5'-GAGTTTG ATCCTGGCTCAG-3') and 16S545R (5'-ATTCCGGA TAACGCTTGC-3') [12-15]. The PCR reactions were performed in a 12, 5 µl mixture, using puretaq ready-to-go PCR beads from GE Healthcare. The PCR was performed under the following conditions: 3 minutes at 94 ºC, followed by 35 cycles of 30 seconds at 95 °C, 15 seconds at 55 °C, and 60 seconds at 72 °C. PCR products were visualied 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 fulfiled 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 phycobiliprotein 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.

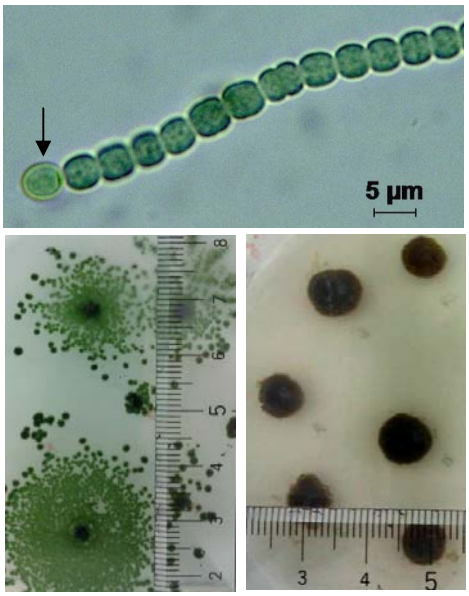

Figure 1. Profile of *Nostoc*. A. Filaments of Strain *Nostoc* TAK23 Shows Many Vegetative Cells and Terminal Heterocysts (Arrow). B. Irregular-shaped Colony of Strain TAK23. C. Spherical-shaped Colony of Strain TAB7d

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

Acession 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'

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. veruucosum* 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

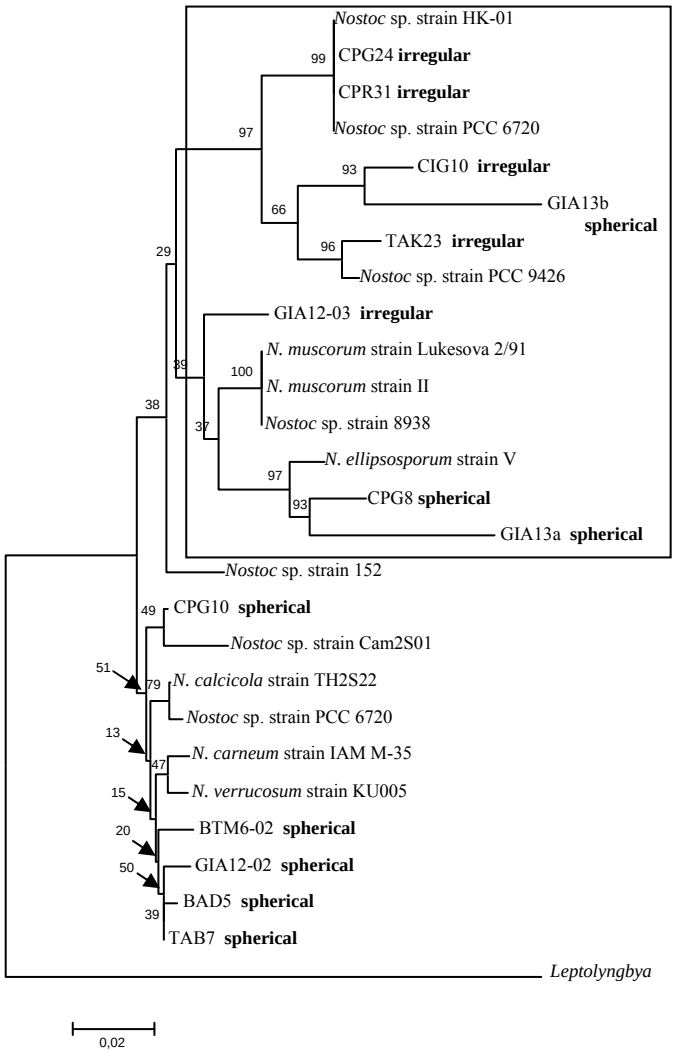

Figure 2. Phylogenetic Tree of *Nostoc* Inferred from 16S rRNA Gene

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

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 prooved by [19]. *Nostoc* mainly live as symbions in intrasellular or intersellular root epiderms. 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* occurence 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	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	Strains					
			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 exture of the treated paddy is crumbled/granulated, while the control soil is clumped. Occording 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.

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