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Original research article

Metagenomic of Actinomycetes Based on 16S rRNA and *nif*H Genes in Soil and Roots of Four Indonesian Rice Cultivars Using PCR-DGGE

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ABSTRACT

The research was conducted to study the metagenomic of actinomycetes based on 16S ribosomal RNA (rRNA) and bacterial *nif*H genes in soil and roots of four rice cultivars. The denaturing gradient gel electrophoresis profile based on 16S rRNA gene showed that the diversity of actinomycetes in roots was higher than soil samples. The profile also showed that the diversity of actinomycetes was similar in four varieties of rice plant and three types of agroecosystem. The profile was partially sequenced and compared to GenBank database indicating their identity with closely related microbes. The blast results showed that 17 bands were closely related ranging from 93% to 100% of maximum identity with five genera of actinomycetes, which is *Geodermatophilus, Actinokineospora, Actinoplanes, Streptomyces* and *Kocuria*. Our study found that *Streptomyces* species in soil and roots of rice plants were more varied than other genera, with a dominance of *Streptomyces alboniger* and *Streptomyces acidiscabies* in almost all the samples. Bacterial community analyses based on *nif*H gene denaturing gradient gel electrophoresis showed that diversity of bacteria in soils which have *nif*H gene was higher than that in rice plant roots. The profile also showed that the diversity of those bacteria was similar in four varieties of rice plant and three types of agroecosystem. Five bands were closely related with *nif*H gene from uncultured bacterium clone J50, uncultured bacterium clone clod-38, and uncultured bacterium clone BG2.37 with maximum identity 99%, 98%, and 92%, respectively. The diversity analysis based on 16S rRNA gene differed from *nif*H gene and may not correlate with each other. The findings indicated the diversity of actinomycetes and several bacterial genomes analyzed here have an ability to fix nitrogen in soil and roots of rice plant.

1. Introduction

Actinomycetes are very important microbes as they have an ability to produce various metabolite compounds. Among 22,500 biological active compounds that are produced by microbes, 45% are produced by actinomycetes, 38% by fungi, and 17% by unicellular bacteria (Berdy 2005). Actinomycetes that have been isolated from soil, rizosphere, and phylosphere showed their various biological functions such as antimicrobes and plant growth promoters (Correa *et al.* 2010; Sangmane *et al.* 2009). Sangmane *et al.* 2009 had cultured *Streptomyces* sp. which controlled the powdery mildew disease caused by *Oidium* sp. in sweet pea (*Pisum sativum*). Correa *et al.* (2010) also proved that some actinomycetes which have been isolated from the rhizosphere of white clover (*Trifolium repens*) are capable of solubilizing phosphate, producing siderophores, and fixing nitrogen. Nitrogen fixing microorganisms are one of the important soil components due to their ability to enhance available nitrogen for plant (Perez *et al.* 2014). Microorganisms are able to provide nitrogen by reducing atmospheric nitrogen (N_2) to ammonium which is catalyzed by nitrogenase enzyme (Zehr *et al.* 2003). Microorganisms that have the nitrogenase enzyme can be among the members of archaea and bacteria, including actinomycetes (Zehr *et al.* 2003). The N_2 fixation ability by actinomycetes that were identified to be the member of the genus *Micromonospora* was based on their ability to reduce acetylene into ethylene which is known as an indicator of nitrogenase activity (Villegas *et al.* 1997). Previous study proved that some endophytic actinomycetes had the ability to stimulate the growth of rice plants through their ability to fix nitrogen (Pratyasto 2012) and to produce

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indole acetic acid (Yusepi 2011). Hastuti *et al.* (2012a) reported that endophytic actinomycetes are also capable of producing siderophores, HCN, chitinase, and can solubilize phosphate as well as can inhibit *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) that caused leaf blight disease in rice plants.

In Indonesia, rice crops as agriculture land have several different types of agroecosystems. Rice cultivars such as Ciherang (CHR) and International Rice-64 (IR64) are usually planted in irrigated rice fields, Inpara 2 (INR) is grown in swampy tidal land, whereas Situ Patenggang (STP) is grown in the dry land (Suprihatno *et al.* 2009). Tian *et al.* (2007) stated that *Streptomyces* spp. and *Nocardiodies* sp. were isolated from rice plants and *Streptomyces* spp. was the most frequently isolated genus from stems and roots of rice plant from Guandong Province, South China. Previous study on culturable endophytic actinomycetes had reported that the highest population dynamics found in vegetative stage and roots of rice plant, and based on morphological identification, the strain member of the genus *Streptomyces* was found to be dominant (Jelita 2012). Furthermore, Sari *et al.* (2014) reported that 7 isolates of culturable endophytic *Streptomyces* that have the capability to fix nitrogen in rice plant were isolated. The influence or effect of agroecosystems on actinomycetes diversity in rhizosphere and roots of Indonesian rice plants has also been unexplored. Furthermore, the information of bacteria associated with rice plant which harbors *nif*H gene is not yet available. The culturable microbial diversity studied is predicted only 1% of the total populations, because the other 99% still can not be cultured on artificial media (Sekiguchi 2006). Thus, the diversity of whole microbial populations can be explored using metagenomic approach.

Metagenomic approach is able to analyze the endophytic actinomycetes which can not be cultured in the laboratory. This method involves the extraction of DNA directly from environmental samples. The study in stems and roots of China's rice plant showed that the population of actinomycetes was more diversed when studied using molecular approach than with conventional cultivation method (Tian *et al.* 2007). Microbial diversity can be identified with phylogenetic markers such as 16S ribosomal RNA (rRNA) and *rec*A genes based on the total DNA that has been isolated (Stein *et al.* 1996). Denaturing gradient gel electrophoresis (DGGE) is one of molecular-based techniques used to analyze the bacterial diversity. Other techniques which can also be used are terminal restriction fragment length polymorphism, amplified ribosomal DNA restriction analysis (ARDRA) (Marsh *et al.* 2000) and pyrosequencing (Fakruddin *et al.* 2013). Genetic diversity based on 16S rRNA and *nif*H genes can be analyzed using the DGGE technique. The principle of DGGE technique is based on the separation of genes that have the same size of DNA amplified by PCR but have different base sequences composition (Fischer & Lerman 1983). The DGGE technique has been successfully used to determine the diversity of complex microbial communities (Muyzer & Smalla 1998). Here, we reported the diversity analysis using metagenomic approach based on DGGE technique to determine the diversity of endophytic actinomycetes and to detect the present of *nif*H gene which indicates the ability of bacteria to fix nitrogen in soil and root tissues of rice plants.

2. Materials and Methods

2.1. Sample collection

Soils and roots of four rice cultivars (CHR, IR64, STP and INR) were selected from 3 types of agroecosystem (irrigated rice, swampy tidal, and dry) of rice plants in Bogor, West Java Province, Indonesia. Soils were collected from rice field rizhosphere and roots were collected from healthy rice plant at vegetative stage (30-day-old). The samples were transported to the laboratory immediately after collection and stored at 4 °C.

2.2. DNA extraction and quantification

Total DNA of soil (0.5 g) and rice root (0.5 g) materials was extracted following a protocol as described in Power Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) and Genomic DNA Mini Kit, Plant (Geneaid, Shijr, TPE, TW), respectively, with modification. The concentration and purity of the DNA were evaluated with a Nano drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The range of pure DNA was 1.8—2.0 (A 260/280) (Sambrook & Russell 2001).

2.3. Polymerase chain reaction (PCR) amplification of 16S rRNA and *nif*H genes

The DNA amplification was performed using PCR by T1-thermocycler (Biometra, Goettingen, Germany). Analysis of 16S rRNA gene of actinomycetes was conducted using two steps of PCR. The first PCR was done using specific primer of actinomycetes 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Bruce *et al.* 1992) and 16Sact1114R (5'-GAGTTGACCCCGGCRGT-3') (Martina *et al.* 2008). PCR was performed in a total volume of 25 μL, which contained 1× of GoTaq Green Master Mix 2X (Promega, Madison, WI, USA), 60 pmol of each primer, ~100 ng/μL DNA template, and nuclease free water. The PCR conditions were done using method of Zhang *et al.* (2013). The PCR products (~1087 bp) were separated using electrophorator on 1% (w/v) agarose gel and visualized using G:BOX gel documentation (Syngene, Frederick, MD, USA). The second amplification was done using universal bacterial primer P338F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and P518R (5'-ATTACCGCGGCTGCTGG-3') (Overeas *et al.* 1997). PCR was performed in a total volume of 50 μL, which contained 25 μL of GoTaq Green Master Mix 2X (Promega, Madison, WI, USA), 100 pmol of each primer, ~100 ng/μL of first PCR product, and nuclease free water. The PCR condition was conducted using the method of Edenborn & Sexstone (2007). The amount of PCR product in the sample was determined by agarose gel electrophoresis (1 % w/v) and visualized by using G:BOX gel documentation (Syngene). Analysis of *nif*H gene was conducted as follows: ~100 ng/μL of DNA template of soil and roots of rice plant was added to the amplification mixture that contained 25 μL of GoTaq Green Master Mix 2X (Promega), 10 pmol of primer PoL-F GC (5'- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGTGCGAYCCSAARGCBGACTC-3'), 10 pmol of primer Pol-R (5'- ATSGCCATCATYTCRCCGGA -3') (Poly *et al.* 2001), and nuclease free water. The amplification was done with an initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of amplification at 95 °C for 1 minute denaturation, 55 °C annealing for 1 minute, and 72 °C extension for 1 minute with a final extension step at 72 °C for 7 minutes. The PCR products (~360 bp) were separated using electrophorator on 1% (w/v) agarose gel and visualized using G:BOX gel documentation (Syngene).

2.4. DGGE analysis of 16S rRNA and *nif*H genes

PCR product of 16S rRNA and *nif*H genes (±30 μL) were loaded on to 1 mm vertical gel containing 8% (w/v) polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) in 1x Tris-acetate-EDTA (TAE). Linear gradient of 30%—70% denaturant (100% denaturant corresponding to 7M urea and 40% deionized formamide) was used to separate the PCR products of 16S rRNA and *nif*H genes. Electrophoresis was carried out at 60 °C and 150 V, for 5 hours (16S rRNA genes) and 6 hours (*nif*H genes) using the D Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Gel was stained for 60 min with 30 mL of SYBR Safe (Molecular Probes, Invitrogen, Carlsbad, CA, USA) in 270 mL of TAE buffer in dark conditions. Gel was rinsed with 500 mL of TAE buffer and scanned by G:BOX gel documentation (Syngene). DGGE result was analyzed using 1D Phoretix software (Total Lab) to estimate the total bands

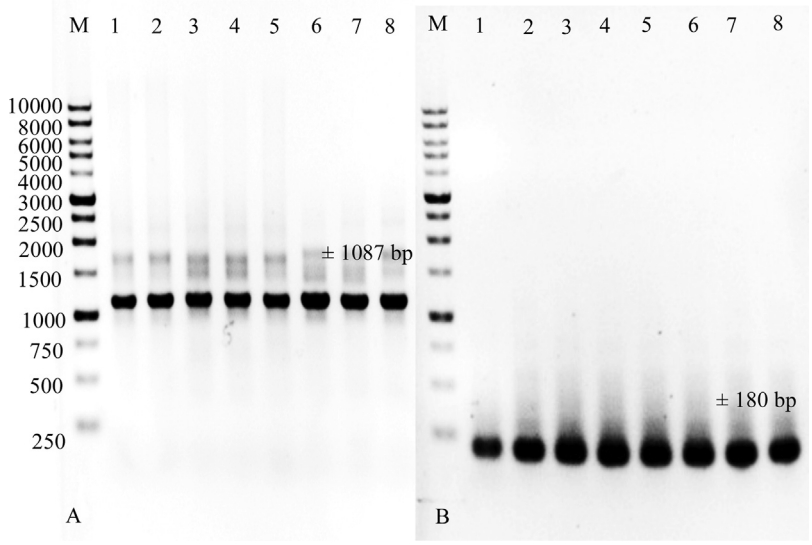

Figure 1. PCR amplification of 16S ribosomal RNA gene from soil and rice plant roots. (A) The first step PCR product size of 1087 bp using primer 27F and 16Sact1114R. (B) The second step PCR product size of 180 bp using primer p338F (with gc clamp) and p518R. Marker 1 Kb; lane 1 to 8: S-CHR, R-CHR, S-IR64, R-IR64, S-STP, R-STP, S-INR, and R-INR. CHR = Ciherang; INR = Inpara 2; IR64 = International Rice-64; PCR = polymerase chain reaction; STP = Situ Patenggang.

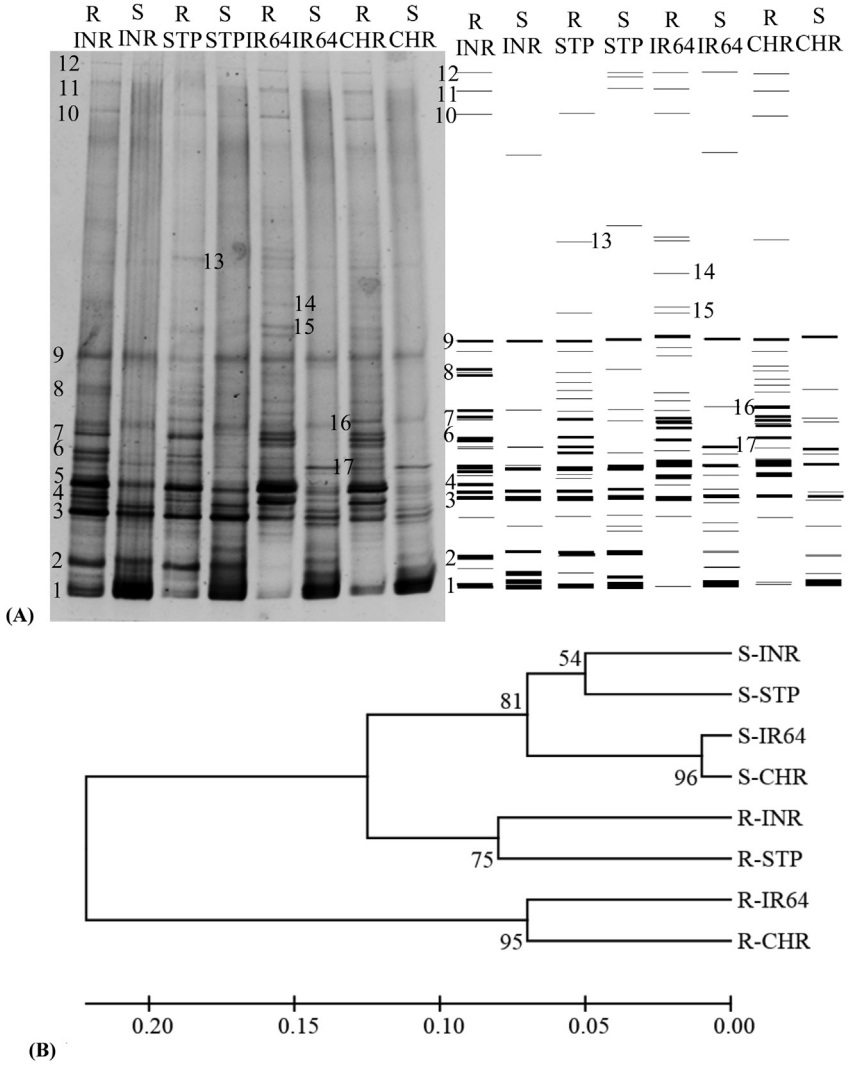

Figure 2. (A) DGGE band profile of the PCR products of 16S ribosomal RNA gene from soil and rice plant roots (left). Illustration of DGGE band using 1D Phoretix software (right) showing 1–17 excised bands. (B) Cluster analysis of actinomycetes community similarity in soils and roots of rice plant. CHR = Ciherang; DGGE = denaturing gradient gel electrophoresis; INR = Inpara 2; IR64 = International Rice-64; PCR = polymerase chain reaction; STP = Situ Patenggang.

Table 1. Percent similarity of 16S rRNA sequences of DGGE band from soil and roots of rice plant

Band	References strain (GenBank)	% Similarity	E-value	Accession number
1	*Kocuria rhizophila* DC2201	96%	4e-76	NR_74786.1
2	*Actinokineospora diospyrosa* NRRL B-24047	100%	1e-90	NR_024962.2
3	*Streptomyces alboniger* DSM 40043	100%	1e-90	NR_043228.2
	Streptomyces parvulus NBRC 13193	100%	1e-90	NR_041119.2
4	*Streptomyces chiangmaiensis* TA4-1	98%	1e-80	NR_113180.1
5	*Streptomyces acidiscabies* RL-110	99%	7e-89	NR_025866.1
	Streptomyces alboniger DSM 40043	99%	3e-87	NR_043228.2
	Streptomyces parvulus NBRC 13193	99%	3e-87	NR_041119.2
6	*Geodermatophilus terrae* PB261	93%	7e-69	NR_109441.1
7	*Kocuria aegyptia* YIM 70003	100%	1e-90	NR_043511.1
	Kocuria himachalensis K07-05	100%	1e-90	NR_043323.1
8	*Streptomyces alboniger* DSM 40043	99%	3e-87	NR_043228.2
	Streptomyces parvulus NBRC 13193	99%	3e-87	NR_041119.2
9	*Kocuria aegyptia* YIM 70003	100%	1e-90	NR_043511.1
	Kocuria himachalensis K07-05	100%	1e-90	NR_043323.1
10	*Streptomyces acidiscabies* RL-110	99%	9e-88	NR_025866.1
11	*Streptomyces acidiscabies* RL-110	99%	7e-89	NR_025866.1
	Streptomyces alboniger DSM 40043	99%	3e-87	NR_043228.2
12	*Streptomyces rapamycinicus* ATCC 29253	100%	2e-89	NR_044199.1
13	*Kocuria aegyptia* YIM 70003	99%	2e-88	NR_043511.1
	Kocuria himachalensis K07-05	99%	2e-88	NR_043323.1
14	*Kocuria aegyptia* YIM 70003	99%	7e-89	NR_043511.1
	Kocuria himachalensis K07-05	99%	7e-89	NR_043323.1
15	*Actinoplanes friuliensis* HAG 010964	98%	2e-83	NR_104746.1
16	*Kocuria aegyptia* YIM 70003	100%	5e-90	NR_043511.1
	Kocuria himachalensis K07-05	100%	5e-90	NR_043323.1
17	*Geodermatophilus normandii* CF 5/3	99%	5e-69	NR_108879.1

DGGE = denaturing gradient gel electrophoresis; rRNA, ribosomal RNA.

that appeared on polyacrylamide gel. Single appeared band was excised using sterile scalpel and put into microtube containing 100 µL ddH_2O. The microtube was incubated at 4 °C overnight and 60 °C for 2 hours (Coelho *et al.* 2009; Perez *et al.* 2014). Ten microliters (~50 ng/µL) of template was used for re-PCR using primer without GC-clamp. The condition of re-PCR was the same as that for previous PCR condition.

2.5. 16S rRNA and *nif*H genes sequencing, bioinformatics analysis and phylogenetic tree construction

PCR products of DGGE bands were sequenced according to standard protocols using a DNA sequencer (ABI PRISM 3100) in First Base Sequencing Services Company. The results of 16S rRNA and *nif*H genes sequence were compared to the available database at GenBank by using the BLAST software (blastn) on National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed using neighbor joining tree method in MEGA 5.2 software (Tamura *et al.* 2011).

3. Results

3.1. PCR amplification, DGGE and phylogenetic profile of actinomycetes based on 16S rRNA genes.

The 16S rRNA gene PCR product of actinomycetes in soils and roots of rice plants was analyzed using nested PCR technique with two sets of primer. The first sets of primer produced a product size of 1087 bp (Figure 1A), whereas the second sets of primer produced a product size of 180 bp (Figure 1B). Separation of those PCR products using DGGE showed that actinomycetes community pattern varied in each sample. Actinomycetes community in roots was more diverse than that of soil samples based on the distribution pattern of bands seen on polyacrylamide gel (Figure 2A). Similarity pattern analysis using binary data (Figure 2B) showed that actinomycetes community in soil of CHR had similarities with soil of IR64. Meanwhile, soil of STP and INR had similar community pattern but their cluster separated with soil sample of CHR and IR64 which indicates that they have different community patterns with soil sample of CHR and IR64. The same pattern was also shown in the roots; community actinomycetes in root of CHR have similarities with IR64. Roots sample of STP and INR had similar community pattern but their cluster separated with roots sample of CHR and IR64, which indicates that they have different community pattern with soil sample of CHR and IR64.

Sequence of the 17 bands was compared with 16S rRNA gene sequences in the GenBank database (Table 1). Band 1 was closely related with 96% maximum identity on *Kocuria rhizophila* DC2201, band 2 had 100% of maximum identity of similarity with *Actinokineospora diospyrosa* NRRL B-24047. Band 3 and 8 closely related with *Streptomyces alboniger* DSM 40043 and *Streptomyces parvulus* NBRC 13193 with 100% and 99% maximum identity, respectively. Band 4 had 98% sequence similarity with *Streptomyces chiangmaiensis* TA4-1. Band 5, 10, and 11 closely related with *Streptomyces acidiscabies* RL-110, *Streptomyces alboniger* DSM 40043, and *Streptomyces parvulus* NBRC 13193 with 99% maximum identity.

Table 2. Percent similarity of *nif*H sequences of DGGE band from soil and roots of rice plant

Band	References strain (GenBank)	% Similarity	E-value	Accession number
1	Uncultured bacterium clone J50 (*nif*H) gene	99%	7e-163	AM746604.1
2	Uncultured bacterium clone J50 (*nif*H) gene	99%	2e-159	AM746604.1
3	Uncultured bacterium clone J50 (*nif*H) gene	99%	3e-161	AM746604.1
4	Uncultured bacterium clone clod-38 (*nif*H) gene	98%	2e-172	JX268394.1
5	Uncultured bacterium clone BG2.37 (*nif*H) gene	92%	3e-132	JX079654.1

DGGE = denaturing gradient gel electrophoresis.

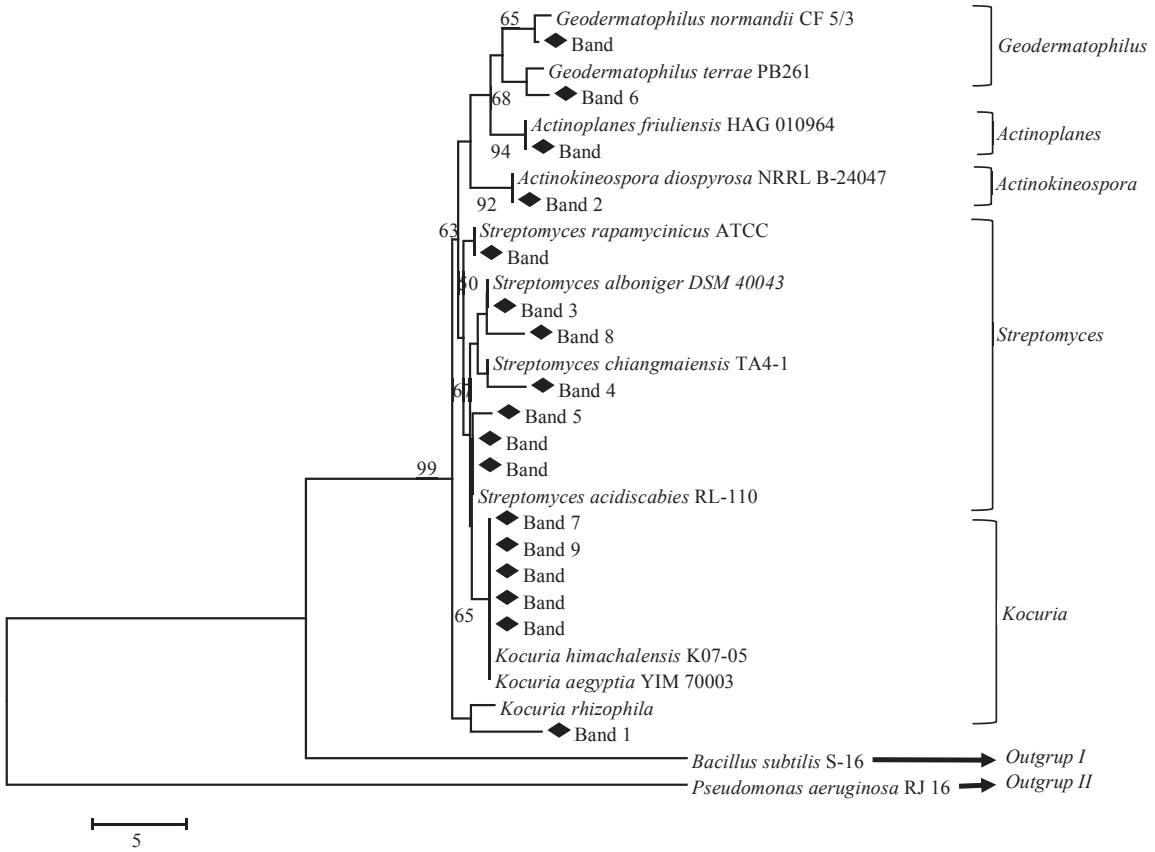

Figure 3. The closest sequence match of known phylogenetic affiliation with actinomycetes 16S ribosomal RNA gene band sequences recovered from the DGGE gel. Species or strain names are followed by their GenBank accession numbers. DGGE bands detected in this study are given in ◆ symbol. The numbers shown next to each bifurcation are bootstrap percent values based on 1000 pseudoreplications. The values below 50 are not shown. The scale indicates substitution per sites. DGGE = denaturing gradient gel electrophoresis.

Band 6 had 93% sequence similarity with *Geodermatophilus terrae* PB261. Band 7, 9, 13, 14, and 16 had 100%, 100%, 99%, 99%, and 100% of maximum identity of similarity with *Kocuria aegyptia* YIM 70003 and *Kocuria himachalensis* K07-05, respectively. Band 12 had 99% sequence similarity with *Streptomyces rapamycinicus* ATCC 29253. Band 15 closely related with *Actinoplanes friuliensis* HAG 010964 with 98% of maximum identity. Band 17 had 99% sequence similarity with *Geodermatophilus normandii* CF 5/3 (Table 2).

The 17 sequences affiliated with five genera of actinomycetes (Figure 3), such as *Geodermatophilus, Actinokineospora, Actinoplanes, Streptomyces* and *Kocuria*. The genus of *Actinokineospora* and *Actinoplanes* was only consisted of one species that is *Actinokineospora diospyrosa* NRRL B-24047 and *Actinoplanes friuliensis* HAG 010964, respectively. The genus of *Geodermatophilus* was consisted of two species that is *Geodermatophilus terrae* PB261 and *Geodermatophilus normandii* CF 5/3. The genus of *Kocuria* was consisted of three species that is *Kocuria rhizophila* DC2201, *Kocuria aegyptia* YIM 70003, and *Kocuria himachalensis* K07-05. *Streptomyces* genus was consisted of four species that is *Streptomyces alboniger* DSM 40043, *Streptomyces chiangmaiensis* TA4-1, *Streptomyces acidiscabies* RL-110, and *Streptomyces rapamycinicus* ATCC 29253. Bands similar to *Geodermatophilus terrae* PB261 were only detected in roots samples. Bands similar to *Actinoplanes friuliensis* HAG 010964 were detected in roots of IR64 and STP. Bands similar to *Streptomyces rapamycinicus* ATCC 29253 were found in roots of CHR, IR64, INR, soil of IR64 and STP. Bands similar to *Kocuria rhizophila* DC2201, *Streptomyces alboniger* DSM 40043, *Streptomyces acidiscabies* RL-110, *Kocuria aegyptia* YIM 70003 and *Kocuria himachalensis* K07-05 were detected in all samples both in soil and roots of rice plants. This result indicated that different types of agroecosytem and cultivars of rice plant did not give any significant effect to diversity of actinomycetes, but only give dominance effect

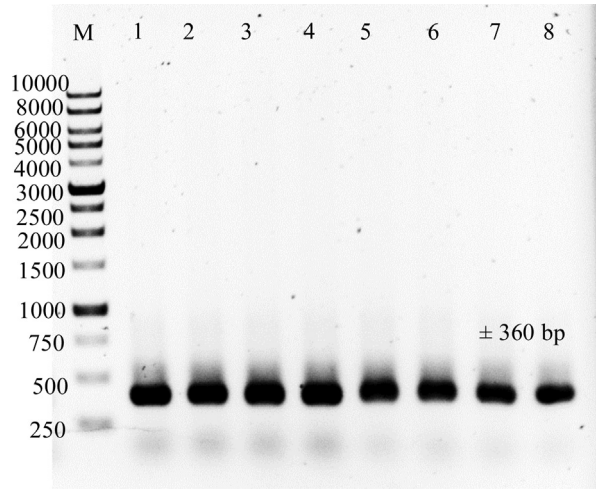

Figure 4. PCR amplification of *nif*H gene from soil and roots rice plant (~360 bp) using primer polF (with GC-clamp) and polR. Marker 1 Kb; lanes 1–8: S-CHR, R-CHR, S-IR64, R-IR64, S-STP, R-STP, S-INR, and R-INR. CHR = Ciherang; INR = Inpara 2; IR64 = International Rice-64; PCR = polymerase chain reaction; STP = Situ Patenggang.

to each sample. The dominance of actinomycetes species showed from the thickness of the band seen in polyacrilamide gel. This result showed that the community of *Streptomyces* spp. in soil and roots of rice cultivar plant in West Java, Indonesia was more varied than other genera (Figure 3).

3.2. PCR amplification, DGGE and phylogenetic profile of actinomycetes based on *nif*H Genes

The PCR products of *nif*H gene from soil and rice plants roots with the size of fragment DNA ~360 bp (Figure 4) were separated using polyacrylamide gel containing 40%–65% denaturant. Based on DGGE result, it showed that community structure of nitrogen fixing bacteria in soil samples were more varied than that of the root samples (Figure 5A). Cluster analysis of community showed that the community of nitrogen fixing bacteria in soil samples was different with roots sample (Figure 5B). Sequence analysis result showed that bands 1, 2, and 3 had 99% similarity with uncultured bacterium clone J50 based on maximum identity from GenBank database. Band 4 was closely related with uncultured bacterium clone clod-38 with 98% of maximum identity, and band 5 was closely related with uncultured bacterium clone BG2.37 with 92% of maximum identity.

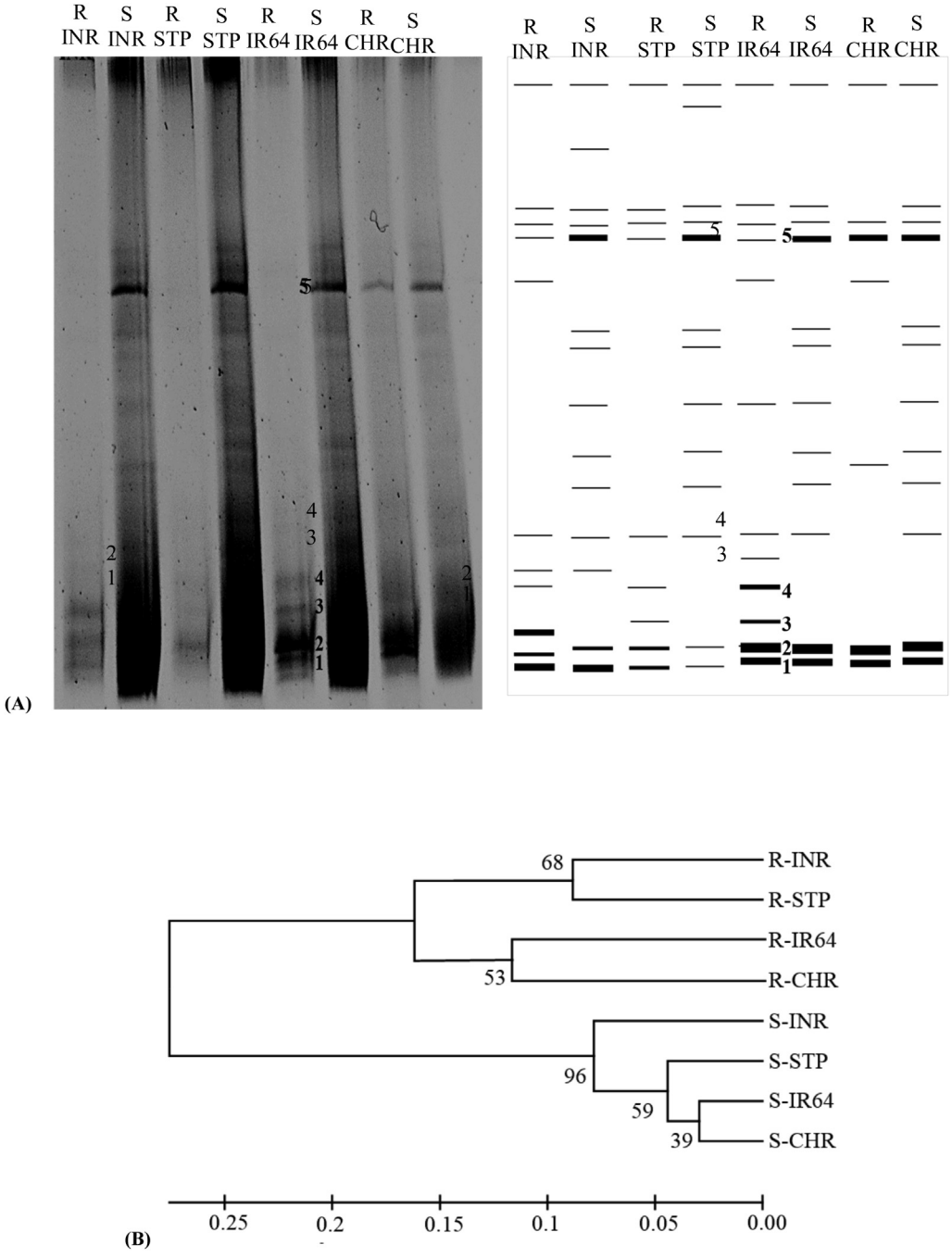

Figure 5. (A) DGGE band profile of the PCR products of *nif*H genes from soils and roots of rice plant (*left*). DGGE band illustration by 1D Phoretix software (*right*). 1-5 bands were excised. (B) Cluster analysis of community similarity based on *nif*H genes in soils and roots of rice plant. CHR = Ciherang; DGGE = denaturing gradient gel electrophoresis; INR = Inpara 2; IR64 = International Rice-64; PCR = polymerase chain reaction; STP = Situ Patenggang.

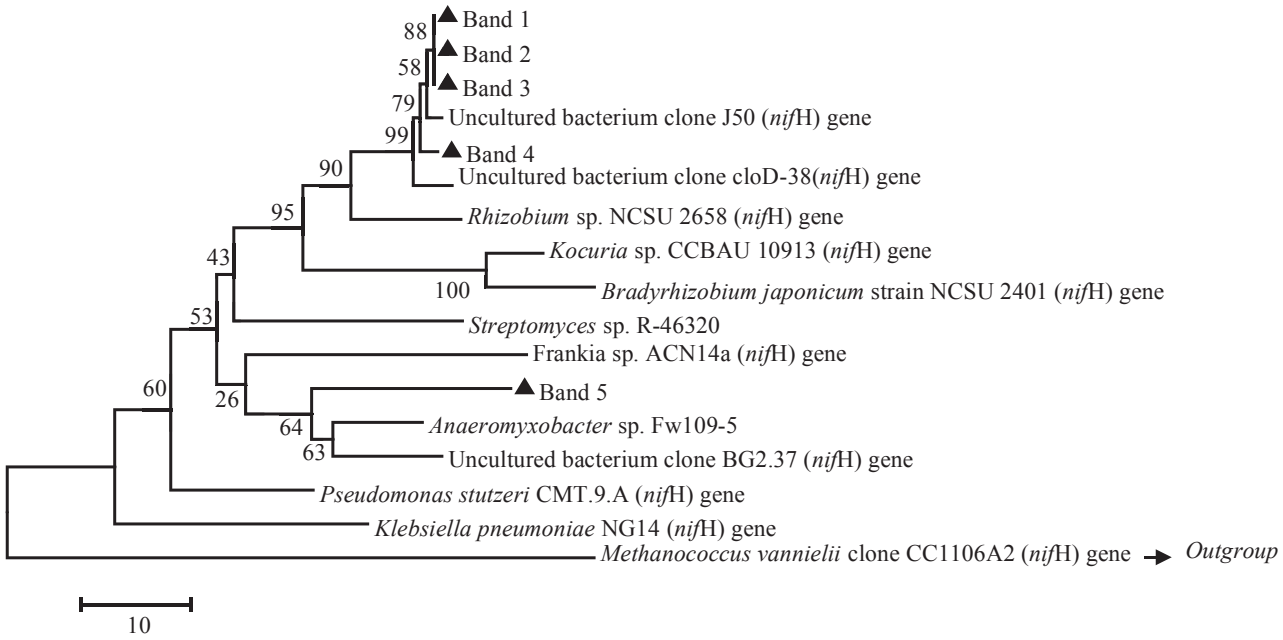

Figure 6. The closest sequence match of known phylogenetic affiliation with *nif*H gene band sequences recovered from the DGGE gel. Species or strain names are followed by their GenBank accession numbers. DGGE bands detected in this study are given in ▲ symbol. The numbers shown next to each bifurcation are bootstrap percent values based on 1000 pseudoreplications. The scale indicates substitution per sites. DGGE = denaturing gradient gel electrophoresis.

Phylogenetic tree profile of nitrogen fixing bacteria based on *nif*H gene showed that bands 1, 2, 3, 4, and 5 clustered together with uncultured bacteria. However, when the results were compared with common nitrogen fixing bacteria, such as strain member of the genus *Anaeromyxobacter, Frankia, Kocuria, Rhizobium, Streptomyces,* strain member of the species *Bradyrhizobium japonicum, Pseudomonas stutzeri,* and *Klebsiella pneumonia*, they showed closely related biological function (Figure 6). The *nif*H gene sequence of bands 1, 2, 3, and 4 clustered with the strain member of the genus *Rhizobium* and band 5 was related with the strain member of the genus *Anaeromyxobacter*.

4. Discussion

In this study, molecular approach based on 16S rRNA and *nif*H genes was used to analyze actinomycetes and bacteria which have the ability to fix nitrogen in soil and roots of 4 cultivars of rice plants from West Java based on PCR-DGGE. Analysis of actinomycetes 16S rRNA genes were conducted using two sets of primer, first sets of primer consist of primer 27F which was designed to amplify all domains of bacteria (Bruce *et al.* 1992) and 16Sact1114R was designed from 202 actinomycetes with 1.3% false result; thus this set of primer is classified as specific primer for the detection of actinomycetes (Martina *et al.* 2008). Second sets of primer were P338F and P518R, which were designed to amplify all v3 regions of bacteria (Overeas *et al.* 1997). The 16S rRNA based PCR-DGGE fingerprinting demonstrated that community structure variations of actinomycetes in soils and roots were quite similar, but only few species which could be found in specific samples such as *G. terrae* PB261, *A. friuliensis* HAG 010964 and *S. rapamycinicus* ATCC 29253. However, this result indicated that different types of agroecosytem and cultivars of rice plant did not give significant effect on diversity of actinomycetes but only gave effect on richness of each actinomycetes on each sample based on 16S rRNA gene analysis using 180 bp sequence comparison. The richness of actinomycetes species is shown from the thickness of the band seen in polyacrylamide gel (Nimnoi *et al.* 2010; Nubel *et al.* 1996). Priyadharsini and Dhanasekaran (2015) also stated that environmental factors such as the type of agroecosystem are the major factors affecting dominance, diversity, richness and evenness of microbe.

Streptomyces is the most frequently isolated genus member from rice stems and roots from China rice plant, both based on cultivated and uncultivated approach (Tian *et al.* 2007). The research on Australian wheat showed that *Streptomyces* spp. were the most widely distributed actinomycetes genus among the cultures isolated from wheat roots, with a dominance of *S. caviscabies* and *S. galilaeus* isolates (Coombs & Franco 2003). Previous study using cultivated approach of endophytic actinomycetes from Indonesian rice plant showed that seven isolates belonging to the genus of *Streptomyces* can be isolated (Sari *et al.* 2014). This study also found that the *Streptomyces* species community in soil and roots of rice cultivar plant in West Java, Indonesia were more varied than other genera, with dominant species in almost all of samples were *S. alboniger* and *S. acidiscabies*.

The 16S rRNA gene sequences which had similarity less than 97% indicate possibility to be member of novel species (Stackebrandt & Goebel 1994). Bands 1 and 6 are strongly suggested to be members of novel species because they had <97% similarity with *K. rhizophila* DC2201 (96%) and *G. terrae* PB261 (93%). Based on DGGE profile, it showed that several bands which closely affiliated with *K. rhizophila* DC2201, *S. alboniger* DSM 40043, *S. acidiscabies* RL-110, *K. aegyptia* YIM 70003 and *K. himachalensis* K07-05 were detected in every sample with different agroecosystem.

Many studies have shown that plants can get benefit from association with actinomycetes. *K. rhizophila* DC2201 which was isolated from the rhizoplane of narrow-leaved cattail (*Typha angustifolia*) known to have an ability to produce siderophore and can suppress the growth of rice pathogen fungi, strain member of the genus *Sclerotium* (Chaiharn *et al.* 2009). The siderophores produced by *S. acidiscabies* RL-110 promoted the growth of cowpea (*Vigna unguiculata* L.) under nickel contamination. Siderophores played a dual role of enhancing iron and preventing

uptake of toxic metals (nickel) for plants (Dimpkpa *et al.* 2008). *S. alboniger* DSM 40043 had an ability to produce phytases which initiates the stepwise dephosphorylation of phytate [myoinositol (1, 2, 3, 4, 5, 6) hexakisphosphate], the most abundant inositol phosphate in nature (Konietzny & Greiner 2002). Other actinomycetes such as *S. chiangmaiensis* TA4-1, *G. terrae* PB261 and *G. normandii* CF 5/3 were found in rice plant but their effect on rice were unknown because they were considered as new species which was recently published (Jin *et al.* 2013; Montero-Calasanz *et al.* 2013; Promnuan *et al.* 2013). Previous study on biocontrol mechanisms of selected *Streptomyces* spp. isolates showed that endophytic actinomycetes inhibited the growth of *Xoo*. These isolates were able to produce chitinase, phosphatase, and siderophore as biocontrol characteristics. The application of these isolates in rice field showed that they can suppress natural infection of *Xoo* during dry and wet seasons (Hastuti *et al.* 2012a; Hastuti *et al.* 2012b).

DGGE fingerprinting based on *nif*H gene demonstrated that diversity of nitrogen fixing bacteria in soils were higher than roots in four cultivars of rice plant. Meanwhile, the pattern of diversity in all soils seemed to be closely related to each other, and similar result was also found in roots. This result indicates that different types of agroecosystem and cultivars of rice plant give no significant effect on the diversity of nitrogen fixing bacteria in soils and roots of rice plant. The *nif*H gene sequence of bands 1, 2, 3, and 4 clustered with the strain member of the genus *Rhizobium*, which is known to function as plant growth promoter by enhancing the content of nitrogen, phosphorus, and potassium in rice field (Hussain *et al.* 2009), and band 5 was related with strain member of the genus *Anaeromyxobacter* that was known to have part in nitrogenase enzyme complex (*nif*H gene) which could reduce free nitrogen to ammonium (Pereira *et al.* 2013), and it has potential roles in the Fe (III) reduction and degradation of possible contaminant in rice field (Zhu *et al.* 2011). However, in this study, the band of *nif*H gene was not found from actinomycetes because it has an indication that 16S rRNA and *nif*H genes analyses may not be correlated to each other. Besides that, the primer which amplified the *nif*H gene sequences was designed for all bacteria. Previous study on three culturable rice endophytic *Streptomyces* also showed that 16S rRNA and *nif*H genes analyses may not be correlated to each other because they have *nif*H gene sequences which were closely related with *Herbaspirillum* sp. (Sari *et al.* 2014). Gaby and Buckley (2014) stated that the genetic divergence of *nif*H and 16S rRNA genes was not well correlated to define microbial species. The metagenomic study of actinomycetes based on 16S rRNA and bacterial *nif*H genes in soil and roots of four rice cultivars clearly reveal the genetic diversity of rice endophytic actinomycetes.

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