

Molecular characterization of Plant Growth Promoting Rhizobacteria using 16S rRNA sequences in the organic rice field of Sukorejo Village, Central Java, Indonesia

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Abstract. Santosa S, Sutarno, Purwanto E, Suranto, Sajidan. 2018. Molecular characterization of Plant Growth Promoting Rhizobacteria using 16S rRNA sequences in the organic rice field of Sukorejo Village, Central Java, Indonesia. *Biodiversitas* 19: 2157-2162. Plant Growth Promoting Rhizobacteria (PGPR) are rhizosphere bacteria that can be utilized to increase plant growth and suppress plant diseases. PGPR concentrate on the concept of sustainable agriculture due to their role as biostimulant by synthesizing and regulating the concentration of various phytohormones and as biofertilizers. It is important to determine the original PGPR found in the roots of rice plants. This study aimed to identify PGPR based on 16S rRNA sequences. The bacterial strain was isolated from the rhizospheric soil of IR 64 organic and inorganic rice fields in the region of Sragen District, Central Java, Indonesia. The bacterial isolates were grown on the Luria Bertani (LB) Agar Medium. There were 10 colonies obtained with different morphological variations, nine of those were from the organic rice field. Molecular characterization using 16R rRNA sequences suggested the identity the nine isolates were of *Pseudomonas aeruginosa* strain RI-98-1, *Stenotrophomonas maltophilia* strain S431, *Bacillus subtilis* strain CEB2, *Bacillus cereus* strain ATCC 14579 clone EA195, *S. maltophilia* strain 5517, *Exiguobacterium acetylicum* strain SSA-3, *Serratia nematodiphila* strain HC4, *Bacillus cereus* strain ANP221, and *Acinetobacter junii* strain M. pstv. 21.4 *Pseudomonas* and *Bacillus* produce phytohormones (auxins) and convert inorganic phosphate to organic and *Stenotrophomonas* can increase the growth of *Acinetobacter* and *Exiguobacterium* as phosphate solvents in the soil.

Keywords: 16S rRNA, organic rice field, Plant Growth Promoting Rhizobacteria

INTRODUCTION

There are two types of agricultural farming systems, namely the organic system and inorganic system. The organic farming system uses animal manure and compost as ingredients for fertilization on agricultural land. The agricultural system can be said to be organic if the minimum planting time is nine times planting or 3 years with organic fertilization. Biological properties contained in organic fertilizers can make food sources of microorganisms in the soil, such as fungi, bacteria, and other beneficial microorganisms (Hadisuwito 2008). Fertilization with biofertilizer is also able to improve soil fertility.

The composition in organic fertilizers can supply NPK nutrients and provide micronutrients, so it can prevent micronutrient defects in marginal soils caused by unbalanced chemical fertilization. Organic fertilizer in agriculture is usually packaged in the form of compost. Compost fertilizer is an organic material that undergoes a decomposition process by decomposing microorganisms. Compost has good physical properties, including being porous, holding water, and providing plant nutrients properly (Setyorini et al. 2006). Setyorini et al. (2006) state

that compost contains microorganisms (fungi, actinomycetes, bacteria, and algae). Soil microorganisms have an important role in phosphate solvent biofertilizers, such as *Bacillus* and *Pseudomonas* (Aarab et al. 2015). Soil microbes can increase plant growth as a growth booster (biostimulant) since it synthesizes and regulates growth regulating substances (phytohormones) such as IAA, gibberellin, cytokinin, and ethylene in roots and as a provider of nutrients (biofertilizer) by tethering N₂ and dissolving P nutrients bound in the soil (Yolanda et al. 2011).

Microbial diversity can be used as an indicator of soil fertility, such as *Celulomonas* and *Lactobacillus*. The microbes can produce a compound useful for plant fertility. One of the microbes in the soil is located near the root of a plant called rhizobacteria. These microorganisms are bacteria that live around the roots of plants. Rhizobacteria make the roots of plants as their source of life. Rhizobacteria can help all physiological processes in plants so that plants can grow well. Therefore, it is necessary to find and develop root-colonizing bacteria or potential rhizobacteria for plant fertilizers. Soil fertilizing microbes known as biofertilizers are soil fertilizing microbes which can improve the efficiency of fertilization, fertility, and soil quality.

Goenadi and Saraswati (1993) state that there are several groups of organic microbes which can dissolve nutrients in the soil. The first microbial group is P-solvent microbes. These microbes have high potential in dissolving bound P to be available P in the soil. Examples of these microbes include *Pseudomonas*, *Micrococcus*, *Bacillus*, *Flavobacterium*, *Penicillium*, *Sclerotium*, *Fusarium*, and *Aspergillus*.

Aarab et al. (2015) reported the results of their research that rhizobacteria capable of transforming organic phosphates into inorganic phosphates were *Aeromonas*, *Pseudomonas*, and *Enterobacter*. Rhizobacteria that produce growth promoters that can increase plant growth are often called plant growth-promoting rhizobacteria (PGPR). Plant growth-promoting rhizobacteria need to be developed due to their benefits. Characterization of the original PGPR population for organic rice fields is very important to conduct to find strains that can be used to increase the growth and sustainability of organic rice fields. This study aimed to identify bacterial strains from the rhizosphere of rice in organic rice field based on 16S rRNA sequences. The results of this study can be used to increase rice productivity, especially in the Sukorejo Village, Sragen District, Central Java, Indonesia.

MATERIALS AND METHODS

Study area

This study was conducted in the organic rice field in Sukorejo Village, Sambirejo Sub-district, Sragen District, Central Java Province, Indonesia (Figure 1). Sukorejo Village, a hilly area, is located at an altitude of 376 meters above sea level. The land in Sukorejo Village is mostly used for the agricultural sector, such as rice fields and plantations. Rice fields in Sukorejo village consist of two types, namely organic and inorganic rice fields. The area of the organic rice field in Sukorejo Village covers an area of

42.19 ha. The study site of organic rice field is located at latitude 7°30'46.8" S and 111°08'45.9" E. The molecular work was carried out at the Laboratory of Microbiology, Department of Biology Education, Faculty of Teacher Training and Education, Sebelas Maret University (UNS) Surakarta, Indonesia.

Isolation of PGPR from rhizopore

The bacterial strain was isolated from the rhizospheric soil of IR 64 organic and inorganic rice fields in the region of Sragen District, Indonesia. As much as 5 g of soil are diluted with sterile distilled water and dilution is carried out up to 10^{-9} and take 100 μ L of suspense extraction to spread on the Luria Bertani (LB) Agar Medium. Then, LB Agar Medium is incubated for 48 hours at a temperature of 28°C (room temperature). The number of colonies obtained is 10 bacterial colonies with different morphological variations. Bacterial colonies scratched by the quadrant to get a single colony. After obtaining a single colony, stored in 20% glycerol solution at -20°C.

DNA isolation

The culture of glycerol was grown using LB media and incubated 24 hours at 28°C (room temperature). Culture DNA was obtained using the lysozyme-SDS-phenol-chloroform method by Maniatis et al. (1982). DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol. The extracted DNA was treated with DNase-free RNase (Sigma Chemical Co. St. Louis. MO. USA) at a final concentration of 0.2 mg.mL⁻¹ at 37°C for 15 min, followed by second phenol-chloroform-isoamyl alcohol extraction and isopropanol precipitation. The DNA pellet was resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8), stored at -20°C, and used as template DNA in PCR to amplify the 16S rRNA for phylogenetic analysis.

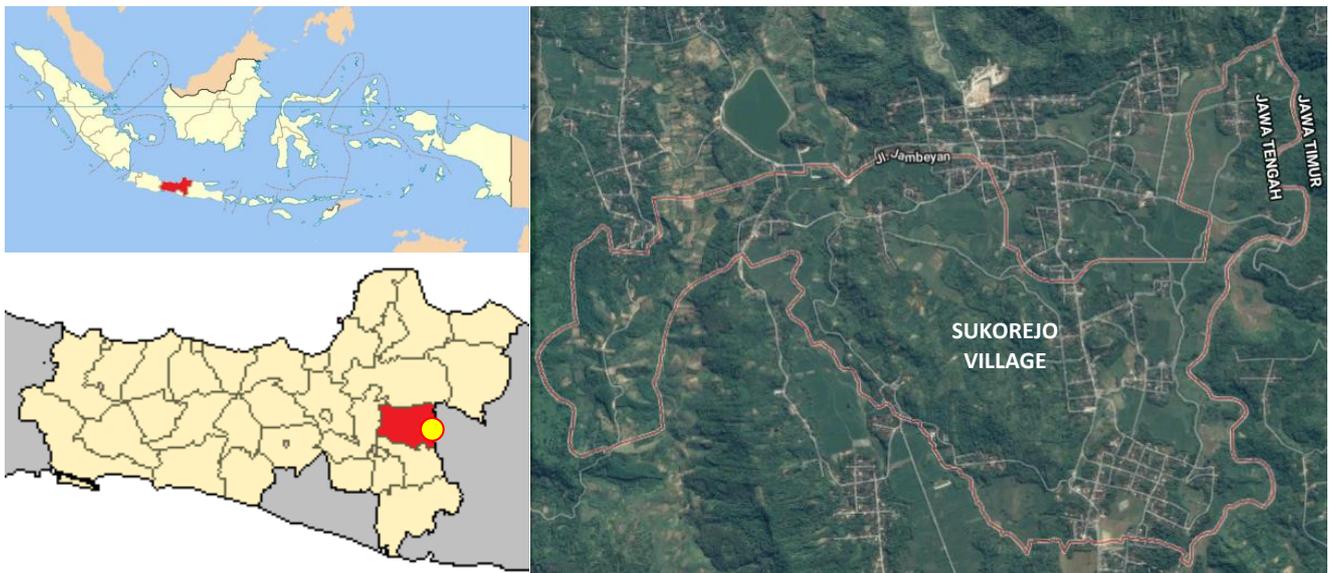


Figure 1. The map of study site in the organic rice field of Sukorejo Village, Sambirejo Sub-district, Sragen District, Central Java Province, Indonesia

PCR amplification and sequencing

Total genomic DNA was electrophoresis using 0.8% agarose gel and visualized by UV transilluminator after red gel staining. Amplification of the 16S rRNA gene was performed using a T1-Thermocycler PCR machine with 1387R primers pA (5'-GGGCGGWGTGTACAAGGC-3') and 63F primers pH (5'-CAGGCCTAACACATGCAAGTC-3') with a target fragment of \pm 1300 bp, then the total volume of PCR reaction used was 50 μ L, Primer was used for PCR amplification of 16S rRNA gene was 5' (Marccesi et al. 1998). The amplification was carried out in a 25 μ L volume. PCR amplifications were performed with 1 μ L (1 x Ex Taq Buffer), 5 μ L forward primer, 5 μ L reverse primer, 1 μ L DNA template, 13 μ L of nuclease-free water. The condition of PCR complied with the method of Marchesi et al. (1998). The temperature for PCR condition starts to pre-denaturation (94°C, 5 minutes), denaturation (94°C, 30 seconds), annealing (55°C, 45 seconds), elongation (72°C, 1 minute 30 seconds), post-elongation (72°C, 10 minutes), and cooling (4°C, 5 minutes). Denaturation phase, primer attachment, and elongation were conducted for 35 cycles. The PCR product was migrated on 1.5% of agarose gel by electrophoresis technique at 85 V for 40 minutes. The electrophoresis results were visualized using UV light after immersion using ethidium bromide dye for 10 minutes. The size of the 16S rRNA gene \pm 1300 bp. DNA sequencing was performed on the results of the amplification by Genetika Science Indonesia.

The nucleotide sequences of 16S rRNA gene were analyzed using BLAST online at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The results of BLAST showed the similarity between the nucleotide sequences of the 16S rRNA gene of the obtained isolates and the nucleotide sequences of the 16S rRNA gene in GenBank. Online alignment was done at <http://expasy.org/tools/> on each isolate which had a kinship with several bacteria which had 99% similarity of 16S rRNA gene. The phylogenetic tree was constructed on the aligned datasets using the neighbor-joining method (Saitou and Nei 1987) implemented in the program MEGA 4.0. (Tamura et al. 2007)

RESULTS AND DISCUSSION

Identify plant growth promoting rhizobacteria (PGPR)

The Query Cover (QC) for nine species of bacteria had a value in the range of 94% to 100% (Table 1). The E-

value of 0.0 indicated the number of alignments with a score equal to or higher than expected to occur in the database by chance. Therefore, the lower the E-value was, the more significant the score and the better quality of BLAST alignment search were. E-value was high in this study since the sequence of loci as the marker was very short. In this study, the 16S rRNA nuclear gene had a length of about less than 1300 base pairs (bp). Therefore, the search for similarities with the limited query sequence was performed. According to Claverie and Notredame (2003), DNA sequences have a high similarity if the Query Cover value approaches 100% and the E-value approaches 0.0. Based on the Query Cover (QC), the E-Value of 0.0, and similarity, showed that the 9 selected isolate strains in the organic rice field were *Pseudomonas aeruginosa* strain RI-98-1 with QC (100) and similarity (94%), *Stenotrophomonas maltophilia* strain S431 with QC (100) and similarity (99%), *Bacillus subtilis* strain CEB2 with QC (100) and similarity (99%), *Bacillus cereus* strain ATCC 14579 clone EA195 with QC (100) and similarity (98%), *S. maltophilia* strain 5517 with QC (100) and similarity (98%), *Exiguobacterium acetylicum* strain SSA-3 with QC (100) and similarity (99%), *Serratia nematodiphila* strain HC4 with QC (100) and similarity (99%), *B. cereus* strain ANP221 with QC (100) and similarity (99%), and *Acinetobacter junii* strain M. pstv. 21.4 with QC (99) and similarity (98%).

The 16S rRNA gene has a characteristic size of about 500 bases until 1550 bp. For the 16S rRNA used for sequencing measuring 1300 bp. Where in that area is a converse area. The use of 16S rRNA is often used in prokaryotic organisms rather than 23S rRNA because of its higher variation. In Eukaryote it uses 18S rRNA for identification. Therefore, identification of bacteria using 16S rRNA.

A phylogenetic relationship based on 16S rRNA nuclear gene

The construction of phylogenetic tree (Figure 2) described the phylogenetic relationship of the 9 species found, namely *P. aeruginosa* strain RI-98-1, *S. maltophilia* strain S431, *B. subtilis* strain CEB2, *B. cereus* strain ATCC 14579 clone EA195, *S. maltophilia* strain 5517, *E. acetylicum* strain SSA-3, *S. nematodiphila* strain HC4, *B. cereus* strain ANP221, and *A. junii* strain M. pstv. 21.4.

Table 1. The identity of 9 bacterial isolates found in the organic rice field based on 16S rRNA nuclear gene

Isolate code	Related species	Query cover (QC)	E-value	Similarity (%)	ACC number
A	<i>Pseudomonas aeruginosa</i> strain R1-98-1	100	0.0	94	JQ659534.1
B	<i>Stenotrophomonas maltophilia</i> strain S431	100	0.0	99	MF079262.1
C	<i>Bacillus subtilis</i> strain CEB2	100	0.0	99	MH220245.1
D	<i>Bacillus cereus</i> ATCC 14579 clone EA195	100	0.0	98	KY034413.1
E	<i>Stenotrophomonas maltophilia</i> strain 5517	100	0.0	98	HQ185398.1
F	<i>Exiguobacterium acetylicum</i> strain SSA-3	100	0.0	99	KY486010.1
G	<i>Serratia nematodiphila</i> strain HC4	100	0.0	99	KY357291.1
H	<i>Bacillus cereus</i> strain ANP221	100	0.0	99	KT074452.1
I	<i>Acinetobacter junii</i> strain M.pstv.21.4	99	0.0	98	KM108505.1

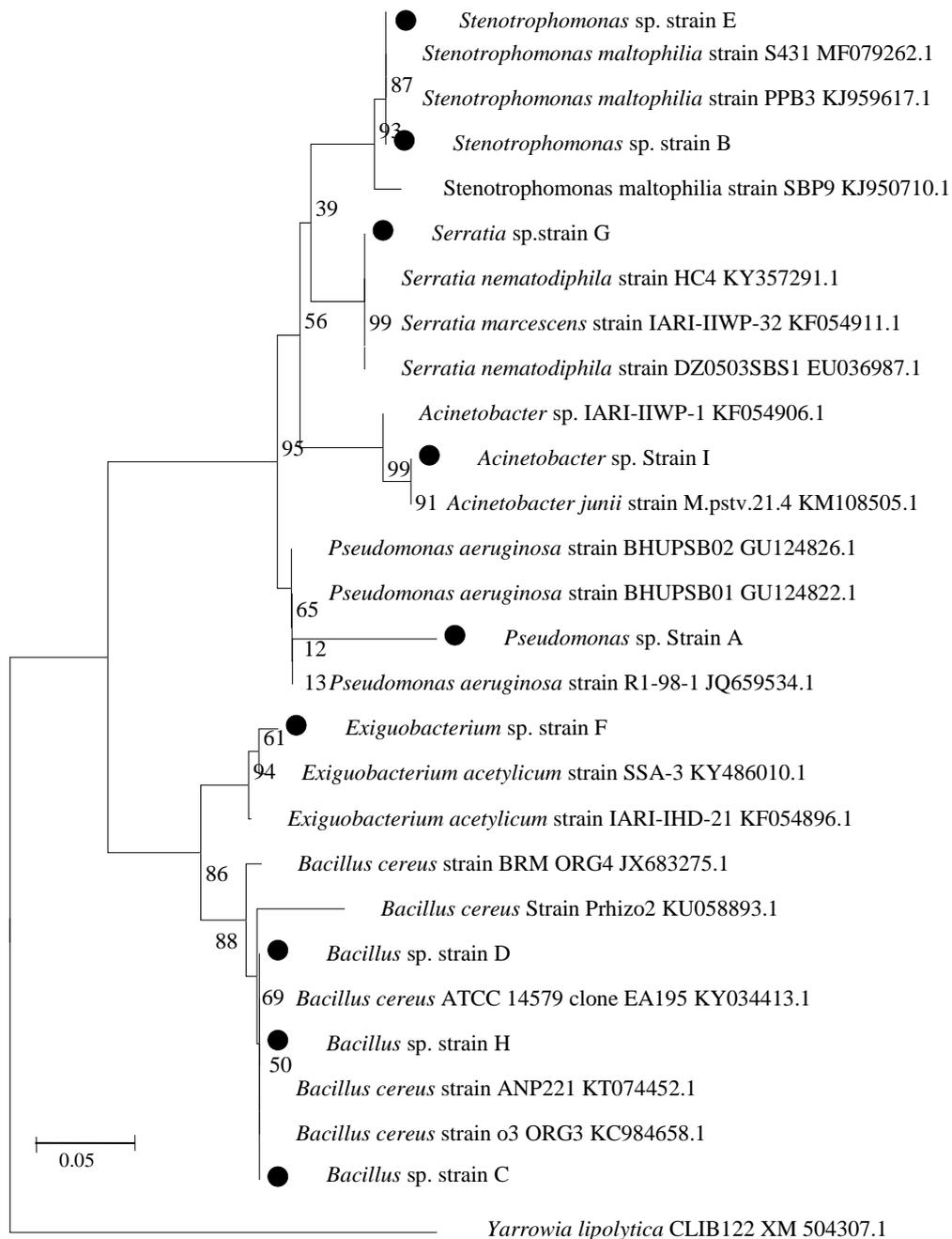


Figure 2. The phylogenetic tree based on sequencing of 16S rRNA gene using MEGA4 software (Tamura et al. 2007)

Figure 2 showed that *S. maltophilia* strain S431 MF079262.1 have similarity as the amount of 99% with *S. maltophilia* strain S431 MF079262.1, *S. maltophilia* strain PPB3 KJ959617.1, and *S. maltophilia* strain SBP9 KJ950710.1. *S. maltophilia* strain 5517 HQ185398.1 have similarity as the amount of 98% with *S. maltophilia* strain S431 MF079262.1, *S. maltophilia* strain PPB3 KJ959617.1, and *S. maltophilia* strain SBP9 KJ950710.1. *S. nematodiphila* strain HC4 KY357291.1 have similarity as the amount of 99% with *S. nematodiphila* strain HC4

KY357291.1, *Serratia marcescens* strain IARI-IIWP-32 KF054911.1, and *S. nematodiphila* strain DZ0503SBS1 EU036987.1. *A. junii* strain M.pstv.21.4 KM108505.1 has similarity as the amount of 98% with *Acinetobacter* sp. IARI-IIWP-1 KF054906.1. *P. aeruginosa* strain R1-98-1 JQ659534.1 have similarity as the amount of 94% with *P. aeruginosa* strain BHUPSB02 GU124826.1, *P. aeruginosa* strain BHUPSB01 GU124822.1, and *P. aeruginosa* strain R1-98-1 JQ659534.1. Five species include the Phylum Proteobacteria group (Verma et al. 2014).

Based on the results of phylogenetic can be grouped into six genera because of its very high proximity, i.e., (i). *Stenotrophomonas* sp. *S. maltophilia* strain S431 MF079262.1 and *S. maltophilia* strain 5517 HQ185398.1; (ii). *Serratia* sp.: *S. nematodiphila* strain HC4 KY357291.1; (iii). *Acinetobacter* sp.: *A. junii* strain M. pstv.21.4 KM108505.1; (iv). *Pseudomonas* sp.: *P. aeruginosa* strain R1-98-1 JQ659534.1; (v). *Bacillus* sp.: *B. cereus* strain ANP221 KT074452.1, *B. cereus* ATCC 14579 clone EA195 KY034413.1, and *B. subtilis* strain CEB2 MH220245.1; (vi). *Exiguobacterium* sp.: *E. acetylicum* strain SSA-3 KY486010.1.

Exiguobacterium acetylicum strain SSA-3 KY486010.1. have similarity as the amount of 99% with *E. acetylicum* strain SSA-3 KY486010.1 and *E. acetylicum* strain IARI-IHD-21 KF054896.1. *B. cereus* ATCC 14579 clone EA195 KY034413.1 have similarity as the amount of 98% with *B. cereus* Strain Prhizo2 KU058893.1, *B. cereus* strain ANP221 KT074452.1, and *B. cereus* strain o3 ORG3 KC984658.1. *B. cereus* strain ANP221 KT074452.1 have similarity as the amount of 99% with *B. cereus* Strain Prhizo2 KU058893.1, *B. cereus* strain ANP221 KT074452.1, and *B. cereus* strain o3 ORG3 KC984658.1. *B. subtilis* strain CEB2 MH220245.1 have similarity as the amount of 99% with *B. cereus* Strain Prhizo2 KU058893.1, *B. cereus* strain ANP221 KT074452.1, and *B. cereus* strain o3 ORG3 KC984658.1. Four species include the Phylum Firmicutes group (Verma et al. 2014).

Mahwish et al. (2015) reported that the genera *Bacillus* and *Pseudomonas* were the most dominant and most commonly found in various plant studies. Hayat et al. (2012) also reported that *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Serratia* sp. were very good PGPRs with PGP properties such as IAA production, phosphate dissolution, and N₂ fixation and were also used for crop production as bioinoculants. *Bacillus* sp. was also reported to increase grain yield (Çakmakçı et al. 2007), corn (Pal 1998) and legumes. Gholami et al. (2009) also stated that the *Pseudomonas* bacteria inoculated on the seeds of corn plants were able to increase the growth and productivity of corn through phytohormone synthesis.

Kishore et al. (2005) also revealed that many *Serratia* species had anti-fungal characteristics along with PGP properties and increased the growth and yield of legumes, corn, and sorghum. Research conducted by Rahni (2012) added that bacteria in the genera *Pseudomonas*, *Azotobacter*, *Bacillus*, and *Serratia* were able to increase the production of corn. This situation was due to the ability of PGPR to synthesize IAA phytohormones and ACC deaminase. Other causing factors were good nitrogen fixation ability, increased P nutrient availability, and siderophore.

Various researchers reported that under controlled conditions, root and seed inoculation with PGPRs increased root growth through PGP activity. Better root growth generally produces good shoots and grain. Plants can reduce damage caused by insects. Similar results were presented by Shaharoon et al. (2008) who reported an increase in nutrient absorption efficiency by PGPRs inoculation which resulted in increased root growth and

hence efficient absorption of nutrients by plants. Plants will be more resistant to drought, salinity, and toxins derived from metals and metals. Ashrafuzzaman et al. (2009) added that PGPR was able to induce IAA production. PGPR is also able to maintain plants against pathogens and pests. The use of PGPR can efficiently replace chemical fertilizers and pesticides for rice cultivation. Likewise, the use of PGPR to improve plant nutrition under sustainable agriculture has been reported by Karlidag et al. (2007).

In conclusion, 16S rRNA gene was successful in distinguishing all species within seven species, i.e. *S. maltophilia*, *A. junii*, *S. nematodiphila*, *Pseudomonas aeruginosa*, *E. acetylicum*, *B. cereus*, and *B. subtilis* in Indonesia organic rice field. In addition, the 16S rRNA gene was also successful in resolving phylogenetic relationships at the genus level, namely genus *Stenotrophomonas* sp., *Acinetobacter* sp., *Serratia* sp., *Pseudomonas* sp., *Exiguobacterium* sp., and *Bacillus* sp.

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