BIODIVERSITAS
Volume 19, Number 5, September 2018
Pages: 1743-1749

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d190521

TRFLP analysis for revealing the diversity of rice phyllosphere bacteria

SRI MARTINA WIRASWATI[1], ARIS TRI WAHYUDI[1,♥], IMAN RUSMANA[1], ABDJAD ASIH NAWANGSIH[2]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Jl. Agatis, Dramaga, Bogor 16680, West Java, Indonesia. Tel./fax. +62-251-8622833, ♥email: aristri2011@gmail.com

[2]Department of Plant Protection, Faculty of Agriculture, Institut Pertanian Bogor. Jl. Agatis, Dramaga, Bogor 16680, West Java, Indonesia

Manuscript received: 26 July 2018. Revision accepted: 28 August 2018.

Abstract. *Wiraswati SM, Wahyudi AT, Rusmana I, Nawangsih AA. 2018. TRFLP analysis for revealing the diversity of rice phyllosphere bacteria. Biodiversitas 19: 1743-1749.* Phyllosphere environment of rice plant is usually inhabited by diverse bacteria which mostly contribute beneficial effects to the plant fitness. TRFLP method is a rapid and straightforward method to determine the bacterial diversity of many environments, including rice phyllosphere environment. This study aimed to analyze rice phyllosphere bacterial diversity of healthy rice plant cultivar Ciherang obtained from Sukabumi, Jasinga, and Situgede. The bacterial genomes were amplified and digested with two restriction enzymes, i.e., *Msp*I and *Bst*UI. The bacterial diversity (H' index) and evenness (E index) were calculated from the peak value. From TRFs analysis, Betaproteobacteria and Pseudomonadales were dominantly found in nearly all samples with different relative abundance. In addition, Alphaproteobacteria and Gammaproteobacteria were also dominant in the several samples. The unique bacteria groups were inhabited in the sample from specific regions with certain growth phase. This finding informs us that the geographical factors might be more influent than the growth phase factor. Furthermore, the bacterial diversity and evenness of the metagenomic approach are higher than cultivation-dependent approach.

Keywords: Bacteria, phyllosphere, rice, TRFLP

INTRODUCTION

Phyllosphere environment is commonly occupied by diverse microbes such as bacteria, filamentous fungi, and yeast where bacteria are the most predominant (Lindow and Leveau 2002). The phyllosphere microbes mostly live as commensals on their host plant through their prosperity to increase plant fitness and function. They play an important role in decomposing natural substances as saprophytes, remediating of remnant pesticides and air pollutant, inducing plants health and development as biofertilizer, phytostimulator, and biopesticides against plant pathogen (Muller and Ruppel 2014). Studying the phyllosphere microbe diversity and behavior could facilitate biotechnology applications for combating plant diseases, increasing plant growth, preventing infection of the human pathogen in crop food, and handling volatile pollutant from the air (Vorholt 2012). The phyllosphere represents a habitat with great agricultural and environmental significance. Several evidence showed the importance of phyllosphere microbes for the fitness of natural plant populations and the quality as well as productivity of crops (Whips et al. 2008).

Rice is the most important crop in the world and staple food for 90% of the Indonesian population. However, rice can be attacked by several pathogens such as *Pyricularia oryzae* and *Xanthomonas oryzae* pv. *oryzae* as the major pathogens (Costa et al. 2006). The rice plant is habitat for diverse microorganisms that colonize aerial parts, tissues plant, root surface and area around the root (Knief et al. 2011). These various microorganisms have a significant role in increasing the health and growth of rice plant. The rice phyllosphere bacterial community is playing an important role in influencing the disease resistance of rice plant. Phyllosphere bacteria can promote plant growth, and both suppress and stimulate the colonization and infection of tissues by plant pathogens (Rasche et al. 2006). Several rice phyllosphere bacteria with antifungal and antibacterial activity against plant pathogen were successfully isolated from rice leaves. These become evidence that commensal bacteria dominantly inhabit phyllosphere environment. However, research on the diversity of rice phyllosphere bacteria is still rare, especially in Indonesia. Thus, it is essential to fill this gap by applying metagenomic analysis to reveal the diversity of the phyllosphere environment.

Terminal restriction fragment length polymorphism (T-RFLP) is one of high throughput microbial community analysis methods based on the use of 16S rDNA gene directed PCR process. There is no DNA sequencing process in TRLFP analysis. The combination of direct PCR from an environmental sample and restriction enzymes were the basic method of TRFLP. Direct PCR used fluorescently labeled primers on the 5'-end that would label 16S rDNA genes amplicon subsequently tracked. The labeled 16S rDNA gene amplicon is digested with one or more restriction enzymes that have four base-pairs recognition sites and the resulting labeled terminal restriction fragments (TRFs) are analyzed using an automatic DNA sequencer to determine the size and relative abundance of each TRF (Chauhan et al. 2011). The T-RFLP method is commonly used to analyze the microbial community because it is relatively simple, rapid and high reproducible. It can also be used to analyze large samples from several environments and microbial

community changing due to the current condition. Therefore, in this study, we use the T-RFLP method to determine the phyllosphere bacterial diversity of rice cultivar Ciherang from West Java, Indonesia.

MATERIALS AND METHODS

Sample collection

Healthy rice plants cultivar Ciherang were collected from rice field with blast symptoms at Jasinga, Situgede, and Sukabumi, West Java. The rice plants were collected at the vegetative and generative growth phase. The aerial parts of the plants were cut and immediately transferred for bacterial isolation.

Isolation of rice phyllosphere bacteria

The rice phyllosphere bacteria were isolated with serial dilutions method from Yadav et al. (2010). Ten grams of rice leaf from each region were transferred to 90 mL of saline buffer solution (NaCl 0.85%) and dislodged by shaking at 150 rpm for 1 hour at room temperature. The solution was diluted by the factor of 10^{-3}-10^{-7} and 100 µL solution aseptically transferred to Luria Bertani (LB) agar medium (1% NaCl, 1% Tryptone, 0.5% Yeast extract, 2% agar in 1 L aquadest). The rice phyllosphere bacteria were observed after three days of incubation for DNA extraction.

DNA extraction

The isolated rice phyllosphere bacteria were harvested by transferring 1 mL of distilled water to cultured bacteria. The bacteria solution from each sample was scraped and transferred to falcon tube and dried at 60°C of temperature. For DNA extraction, 0.25 gram of dried bacteria colonies were transferred to a microtube and extracted with Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.) based on the manufacturer's procedures. For metagenomic approach, the total bacterial genomes were directly extracted from rice leaf samples; 10 grams of leaf samples were transferred to a saline buffer solution (NaCl 0.85%) and dislodged by shaking at 150 rpm for 1 hours at room temperature. Bacteria on the leaf solution were pelleted by centrifugation at 10,000 rpm at 4°C for 15 minutes. The total DNA genomes from the bacteria pellet were also extracted with Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.). The bacterial genomes were then quantified with Nanodrop 1000 (*Thermo Scientific*, Wilmington, DE, USA).

Amplification and digestion of 16S rRNA gene

Fluorescein dye-labeled primer (5'-6 FAM) 27F (5'-AGAGTTTGATCCTGGCTCAG-‘3) and 1429R (5'-TACGGTTACCTTGTTACGACT-‘3) were constructed to amplify 16S rRNA gene from rice phyllosphere bacterial genome. PCR mixture contained 25 µL Go Taq Green Master Mix 1x (Promega®, USA), 1 µL (10 pmol. μL^{-1}) of each primer, 1 µL DNA template (~ 100 ng. μL^{-1}) and adjusted with 22 µl nuclease free water. The 16S rRNA gene amplification was conducted using the following reaction: predenaturation (94°C, 5 min), 30 cycles of denaturation (94°C, 45 sec), annealing (55°C, 45 sec), elongation (72°C, 45 sec) and post-elongation (72°C, 7 min). The PCR product was then visualized on 1% agarose under UV light and purified with Gel/PCR DNA Fragments Kit (Geneaid Biotech Ltd.) based on the manufacturer's procedures. The DNA purity was quantified before digested using restriction enzyme. Purified DNA was digested with *Msp*I and *Bst*UI (BioLabs, UK) restriction enzymes based on the manufacturing procedures. Afterward, DNA fragments digestion product was analyzed using Applied Biosystem Genetic Analyzer and interpreted using the GeneMapper® v 4.0 analysis software.

Data analysis

T-RFLPs and diversity analysis for each sample, the TRFs (terminal restriction fragments) peak size between 50 bp and 500 bp and peak high more than 1% from total peak high were further analyzed to determine the phylogenetic relationship. The TRFs from the same sample with differences size less than 0.5 bp digested with one restriction enzymes were grouped as one TRF (Zhang et al. 2008). The observed TRFs were identified by comparing each TRF with the Ribosomal Database Project (RDP) (R10 U27) 700,829 Good Quality (>1200 Bacteria) in MiCA III (Microbial Community Analysis) website (http://mica.ibest.uidaho.edu/digest.php) using Virtual digest (IsPaR) program. The TRFs with same size was assumed as same bacterial group. Furthermore, the richness of TRFs was analyzed by comparing the peak area of each TRF to total TRFs. The bacterial community diversity and evenness were analyzed using Shannon's index (H') and Pielou's evenness index (E) respectively, using the following formulation:

$H' = -\Sigma (Pi \times \log Pi)$, where $Pi = niN^{-1}$, ni is the peak area, and N is the sum of the total peak areas.

$E = H'/\ln (S)$, where S is the total number of TRFs.

RESULTS AND DISCUSSION

Digestion of 16S rRNA gene by restriction enzymes

TRFLP method was used to determine the bacterial diversity of rice phyllosphere environment from different regions and growth phase in West Java. One TRF represents one or several bacteria species from the samples. The amplified 16S rRNA genes were digested with restriction enzymes *Msp*I and *Bst*UI to obtain terminal restriction fragments. Digestion with restriction enzyme *Msp*I produced more TRFs than *Bst*UI, except the sample SKBV from Sukabumi (Table 1). A total of 29 TRFs were obtained from digestion with *Msp*I restriction enzyme, while 17 TRFs were obtained from digestion with *Bst*UI restriction enzyme.

A total of 29 TRFs were digested with *Msp*I restriction enzyme; 8 TRFs were found on metagenomic approach as well as the cultivation-dependent approach (Figure 1). Also, metagenomic approach analysis resulted in 12 TRFs, while cultivation-dependent approach resulted in 9 TRFs.

The number of TRFs from *Bst*UI digestion is less than that from *Msp*I digestion. A total of 7 of TRFs were found on both metagenomic and cultivation-dependent approach, while the metagenomic approach resulted in fewer TRFs than cultivation-dependent (Figure 1). Generally, the number of TRFs from metagenomic approach digested with *Msp*I restriction enzyme is higher than *Bst*UI. This finding indicates that the *Bst*UI restriction enzyme is less significant in the analysis of rice phyllosphere bacteria with metagenomic as well as cultivation-dependent approach.

Relative abundance analysis

The relative abundance represents the proportion of each TRF from each sample calculated based on the ratio between the peak area of each TRF to the total peak area. Histogram of relative abundance is constructed based on TRF data from *Msp*I and *Bst*UI digestion which had been analyzed using MiCA III website (Figure 2 and 3). The MiCA web-based tools were used to determine the affiliation between each TRF and bacteria group in MiCA database. One TRF usually represents one or more bacteria species or one bacteria group. The relative abundance histogram shows that *Msp*I restriction enzyme resulted in more diverse bacteria group than *Bst*UI restriction enzyme. Several bacteria groups such as Sphingobacteriia, Clostridia, and Deltaproteobacteria were not detected in the samples digested with *Bst*UI (Figure 3). In addition, the digestion with *Bst*UI resulted in only one TRF in several samples, which indicates that this restriction enzyme is less significant in the analyzing of the rice phyllosphere bacteria.

A total of 29 different TRFs were obtained from all samples digested with *Msp*I restriction enzyme. Afterward, the MiCA III analysis resulted in 15 different bacteria groups from the RDP database. This finding is more significant than *Bst*UI digestion, i.e., eight bacteria groups. From all bacteria groups, Betaproteobacteria is commonly found in all samples with diverse relative abundance. Also, the SKBV sample (cultivation-dependent) and STGG sample (metagenomic) were inhabited by the same bacteria group with different relative abundance (Figure 2). The Actinobacteria group is only found from JSNV and STGV samples with the metagenomic approach, while the Bacteroidetes group is only found from JSNG samples with the cultivation-dependent approach. This finding also indicates that the metagenomic approach generally shows more diverse bacteria groups than the cultivation-dependent approach.

Analysis of bacteria TRFs digested with MspI

The TRFs digested with *Msp*I restriction enzyme were used in the further analysis because it resulted in higher resolution than *Bst*UI. TRFLP analysis is approached by the metagenomic and cultivation-dependent method of DNA genome isolation. Bacterial diversity is analyzed by H' index whereas bacteria evenness is analyzed by E index. The TRFs obtained from metagenomic approach are higher and more diverse than those obtained from the cultivation-dependent approach. In addition, the sample SKB from vegetative growth phase showed the highest bacteria diversity of all samples. Furthermore, the bacteria evenness are very diverse among all samples (Table 2).

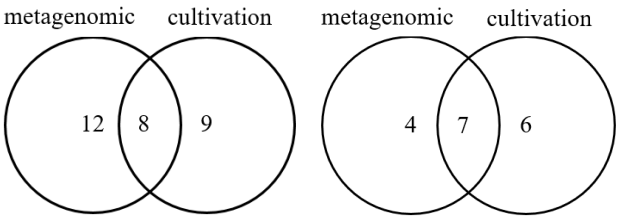

Figure 1. Comparison of total TRFs among metagenomic and cultivation-dependent approach from *Msp*I (left) and *Bst*UI digestion (right)

Table 1. The number of TRFs from rice phyllosphere bacteria communities that digested with two restriction enzymes, *Msp*I and *BstU*I

Rice plant Samples	Growth phase	Number of TRFs digested with restriction enzymes			
		*Msp*I		*Bst*UI	
		Metagenomic	Cultivation-dependent	Metagenomic	Cultivation-dependent
Sukabumi	Vegetative	7	2	1	5
	Generative	3	5	1	5
Jasinga	Vegetative	5	3	4	3
	Generative	6	4	6	3
Situgede	Vegetative	7	4	4	2
	Generative	2	3	1	1

Table 2. Comparison of the bacterial diversity (H') and evenness (E) from rice phyllosphere environment digested with *Msp*I restriction enzyme

Rice plant sample	Growth phase	Analysis approach			
		Metagenomic		Cultivation-dependent	
		H' index	E index	H' index	E index
Sukabumi	Vegetative	1.73	0.89	0.32	0.45
(SKB)	Generative	1.08	0.78	1.04	0.65
Jasinga	Vegetative	1.32	0.83	1.04	0.95
(JSN)	Generative	1.72	0.96	0.89	0.56
Situgede	Vegetative	1.65	0.85	1.23	0.77
(STG)	Generative	0.32	0.47	0.95	0.69

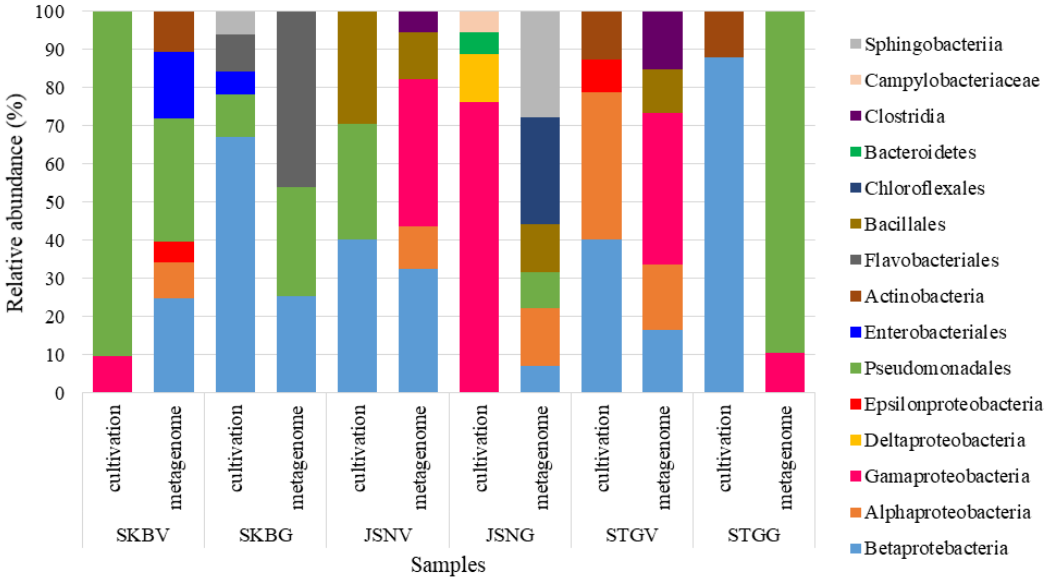

Figure 2. Relative abundance of rice phyllosphere bacteria communities with the metagenomic and cultivation-dependent approach. The 16S rRNA genes were digested with *Msp*I restriction enzyme

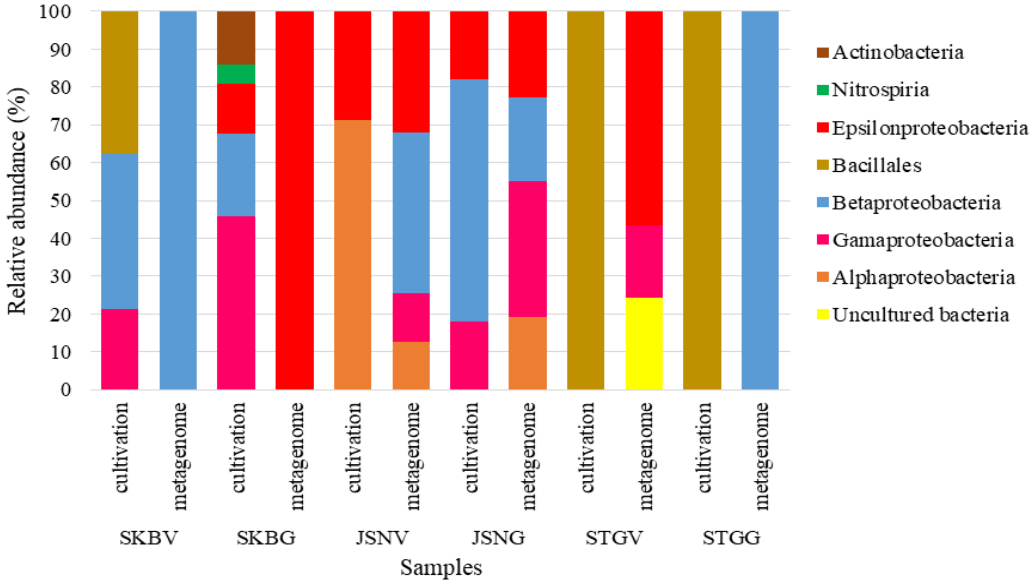

Figure 3. Relative abundance of rice phyllosphere bacteria communities with the metagenomic and cultivation-dependent approach. The 16S rRNA genes were digested with *Bst*UI restriction enzyme

Discussion

The variety of Ciherang has been known as cultivar susceptible to rice blast disease. This variety is commonly cultivated by several farmers in West Java, such as Sukabumi, Jasinga, and Situgede. These three regions are also known as blast disease-endemic areas in West Java. Healthy rice plants among infected rice plants from those three regions become an interesting phenomenon to explore. Therefore, rice phyllosphere bacterial diversity was analyzed from the rice leaves from Sukabumi, Jasinga, and Situgede, because this bacterial community is predicted as having an essential role in rice plants fitness. Knowledge of composition and diversity of rice phyllosphere bacteria is necessary for explaining the microbial mechanism inducing sustainable rice cultivation. The phyllosphere microbiology could be applied to the field of microbial ecology and contribute to more effective and environmentally friendly means of plant protection (Chaudhary et al. 2017). To describe the structure of rice phyllosphere bacteria taxa, the culture-independent method has often been used by researchers. In this study, rice phyllosphere bacterial diversity was analyzed using TRFLP

method with two restriction enzymes, i.e., *Msp*I and *Bst*UI. The type and number of restriction endonucleases are essential factors when an accurate representation of the microbial diversity is desired (Engebretson and Moyer 2003). Besides that, the use of more than one restriction enzymes can facilitate the resolution of a bacterial community (Liu et al. 1994). Engebretson and Moyer (2003) assessed 18 restriction enzymes and revealed that *Bst*UI, *Dde*I, *Sau96*I, and *Msp*I most often determined individual populations in their communities. Also, the *Bst*UI and *Msp*I restriction enzymes produced the highest number of TRFs and OTU in the range of 50-500 bp in length.

In this present work, the digestion with *Msp*I restriction enzyme produced more TRFs and OTU than digestion with *Bst*UI, i.e., 29 TRFs (15 OTU) and 17 TRFs (8 OTU), respectively (Table 1). Among 29 TRFs from *Msp*I digestion, there are 8 TRFs found from both metagenomic and cultivation-dependent approach (Figure 1). These TRFs have affiliations with Pseudomonadales, Betaproteobacteria, Bacillales, Actinobacteria, Epsilonproteobacteria, Gammaproteobacteria, and Sphingobacteriia. Besides that, a total of 7 TRFs from *Bst*UI digestion having associations with Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, and Epsilonproteobacteria were also found from the metagenomic and cultivation-dependent approach. Those bacteria groups were obtained from both *Msp*I and *Bst*UI digestion process. On the other hand, at least seven bacteria groups resulted from the digestion with *Msp*I, but not *Bst*UI, i.e., Sphingobacteriia, Campylobacteriaceae, Flavobacateriales, Bacteroidetes, Enterobacteriales, Pseudomonadales and Deltaproteobacteria (Figure 2). This finding indicates that the digestion with *Msp*I offers better resolution than *Bst*UI to determine the rice phyllosphere bacteria diversity. Therefore, the TRFs produced from *Msp*I restriction enzyme will be used for further analysis in this study.

The profiles of rice phyllosphere bacteria diversity from each sample were described in the histogram of relative abundance (Figure 2), in which each sample generally has a different profile of bacteria groups. Bacterial diversity profiles of rice plants from Sukabumi are different in the vegetative and generative growth phase. The Pseudomonadales group were found on vegetative and generative growth phase of rice plants. The relative abundance of Pseudomandales from the vegetative sample is higher than that from the generative sample. On the other hand, the relative abundance of Betaproteobacteria group from a generative sample is higher than that from the vegetative sample. This finding indicates that vegetative growth phase of rice plants from Sukabumi is dominantly inhabited by Pseudomanadales groups, while generative growth phase of rice plants is dominantly inhabited by Betaproteobacteria group. Also, Actinobacteria, Alphaproteobacteria, Gamaproteobacteria, and Epsilonproteobacteria were unique groups with low relative abundance only found in vegetative rice plants, while Flavobacteria and Sphingobacteriia group just found in generative rice plants (Figure 2).

The bacterial diversity from Jasinga also showed different profiles where the relative abundance of Betaproteobacteria on vegetative rice plants is higher than that of the generative rice plants. Meanwhile, the relative abundance of Gammaproteobacteria group on vegetative rice plants is lower than that of the generative rice plants. Different from Sukabumi, Pseudomonadales group was found in vegetative and generative rice plants from Jasinga with low relative abundance. Clostridia and Bacillales are unique groups just found on vegetative rice plants, while Campylobacteriaceae and Bacteroidetes are found in generative rice plants from Jasinga. Similar to Sukabumi, the generative rice plant from Situgede is dominantly inhabited by Betaproteobacteria. In this region, Pseudomandales group is only found from generative rice plants with high relative abundance. In addition, Epsilonproteobacteria, Clostridia, and Bacillales groups were found on vegetative rice plants with low relative abundance (Figure 2). Although those three regions have a different profile of phyllosphere bacterial diversity, Betaproteobacteria and Pseudomonadales were found from all areas. This finding indicates that the phyllosphere environment of rice variety Ciherang is generally harbored by Betaproteobacteria and Psedomonadales group with different abundance depending on the regions and growth phase of rice plants. In the previous study, Knief et al. (2011) revealed that several bacteria groups such as Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Actinobacteria mainly contributed to the bacterial community in the phyllosphere environment of rice variety IR-72. Bodenhausen et al. (2013) and Kembel et al. (2014) also showed that the phyllosphere environment is mainly inhabited by Alphaproteobacteria (e.g., *Methylobacterium* and *Sphingomonas*) and Gammaproteobacteria (e.g., *Pseudomonas*).

Several species from Betaproteobacteria and Pseudomonadales groups are known as biological control agents and plant growth promoters. *Nitrosomonas* sp. and *Burkholderia* sp. are two species from the Betaproteobacteria class having a beneficial effect on agriculture. *Nitrosomonas* sp. is known as nitrogen fixation bacteria that provide a nitrogen source for the host plant. Meanwhile, several species from *Burkholderia* sp. like *Burkholderia rhizoxinica* and *Burkholderia phytofirmans* PsJN have an important role in controlling *Rhizopus microsporus* (Martinez and Hertweck 2005) and increasing plants resistance to environmental stress (Compant et al. 2005). Also, *Pseudomonas fluorescens* has also been developed and commercially used by farmers to manage several plant pathogens such as *Pyricularia oryzae* and *Rhizoctonia solani* (Reddy and Reddy 2009). Based on the explanation above, this finding confirms that commensal bacteria inhabit the phyllosphere environment. This finding also confirms that healthy rice plants might be harbored by commensal phyllosphere bacteria increasing the rice plants fitness.

The unique bacteria groups were only found on certain samples such as Flavobacteriales and Enterobacteriales groups from SKBV and SKBG samples, Campylobactericeae, Bacteroidetes, Deltaproteobacteria

and Chloroflexales groups from JSNG sample. The relative abundance of those unique bacteria groups is interestingly lower than that of other groups, such as Betaproteobacteria and Pseudomonadales. Accordance to Knief et al. (2011), the relative abundance of Deltaproteobacteria and Chloroflexales groups on phyllosphere environment of rice variety IR-72 are 1.6% and 0.6%, respectively. Moreover, this result also confirms that geographical factors influence the bacteria community of rice phyllosphere environment. The rice variety of Ciherang that was planted in three different regions (Sukabumi, Jasinga, and Situgede) showed very different profiles of rice phyllosphere bacteria community. This had been proved by Finkel et al. (2011) who revealed that geographic factors are the factor significantly influencing epiphytic bacteria of *Tamarix* trees than plant species factors. Meanwhile, Knief et al. (2011) also declared that geographic factors play a more critical role than host species to determine epiphytic microbial composition.

In addition, the Epsilonproteobacteria and Clostridiales groups inhabit rice phyllosphere environment on the vegetative growth phase from three regions, while Sphingobacteria group only inhabits rice phyllosphere environment on the generative growth phase from Sukabumi and Jasinga. This result confirms that different growth phases of rice plant derived the shape of bacteria community in the phyllosphere environment. Lindow and Brandl (2003) state that bacterial population in young leaves comprises a higher number of taxa than old leaves. There are different morphological and physiological characters between vegetative and generative leaves of rice plants which also influence the bacteria community profiles. Costa et al. (2006) revealed that anatomical and physiological characteristics of rice leaf surface and its physiochemical environment properly affect the diversity and density of rice phyllosphere bacteria.

Species richness (the number of species within a community) and species evenness (the sizes of species populations within a community) are two essential parameters for defining community structure and diversity. In this present study, bacteria diversity of metagenomic approach is generally higher than cultivation-dependent approach. As explained before, the number of bacteria that could be cultured is only 1% of the total bacteria in the environment. Therefore, the metagenomic approach is an appropriate method for revealing the bacteria community in many environments. The highest bacteria diversity was found on sample SKB vegetative and JSN generative with a metagenomic approach, i.e., 1.73 and 1.72. The bacteria diversity and evenness are very fluctuating between metagenomic approach as well as cultivation-dependent approach. This finding also explains that the influence of geographical and environmental factors are more significant than the growth phase factor to the bacteria diversity and evenness. This present study provided information about the rice phyllosphere bacteria diversity from Indonesia and several factors that influence the diversity. Diverse bacteria that were found from rice phyllosphere of cultivar Ciherang might contribute to the plant fitness. As previously explained, the samples of healthy rice plant were obtained from rice field infected by blast disease.

We concluded that terminal restriction fragment length polymorphism is proved to be an effective method for revealing the rice phyllosphere bacteria from three regions of West Java, i.e., Sukabumi, Jasinga, and Situgede. The use of *the Msp*I restriction enzyme is more significant than *Bst*UI because *Msp*I generates more TRFs and OTU (operational taxonomical unit). This study has successfully revealed that the rice phyllosphere environment cultivar of Ciherang is dominantly inhabited by Betaproteobacteria, Pseudomonadales, Alphaproteobacteria and Gammaproteobacteria groups with diverse relative abundance. Furthermore, geographical factors are identified as having more influence than plant growth phase to the phyllosphere bacteria diversity from those three regions.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia through Research for Master program of Education Leading to Doctoral Degree for Excellent Graduate (PMDSU) in 2016 to Aris Tri Wahyudi. Therefore, authors thank for this funding to carry out this research.

REFERENCES

Bodenhausen N, Horton MW, Bergelson J. 2013. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. PLoS One e56329. DOI: 10.1371/journal.pone.0056329.

Chaudhary D, Kumar R, Sihag K, Rashmi, Kumari A. 2017. Phyllospheric microflora and its impact on plant growth: A review. Agric Rev 38 (1): 51-59.

Chauhan PS, Shagol CC, Yim W, Tipayno SC, Kim CG, Sa T. 2011. Use of terminal restriction length polymorphism (T-RFLP) analysis to evaluate uncultivable microbial community structure of soil. Korean J Soil Sci Fert 44 (1): 127-145.

Compant S, Reiter B, Sessitsch A. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Appl Environ Microbiol 71 (4): 1685-1693.

Costa DM, Pinto MTC, Geethanjalee HDN, Dissanayake N. 2006. Suppression of rice pathogens by phyllosphere associated microflora of different rice varieties in Sri Lanka. Trop Sci 46 (2): 97-104.

Engebretson JJ, Moyer CL. 2003. Fidelity of select restriction endonucleases in determining microbial diversity by Terminal Restriction Fragment Length Polymorphism. Appl Environ Microbiol 69 (8):4823-4829.

Finkel OM, Burch AY, Lindow SE, Post AF, Belkin S. 2011. Phyllosphere microbial communities of a salt excreting desert tree: geographical location determines population structure. Appl Environ Microbiol. **7:** 7647-7655.

Kembel SW, O'Connor TK, Arnold HK, Hubbell SP, Wright SJ, Green JL. 2014. Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. Natl Acad Sci 111**:**13715-13720.

Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, von Mering C, Vorholt JA. 2011. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J 1 (1)**:** 1-13.

Lindow SE, Brandl MT. 2003. Microbiology of the phyllosphere. Appl Environ Microbiol 69 (4): 1875-1883.

Lindow SE, Leveau JH. 2002. Phyllosphere microbiology. Curr Opin Biotechnol 13 (3): 238-243.

Liu W, Marsh TL, Cheng H, Forney LJ. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63 (11): 4516-4522.

Martinez PLP, Hertweck C. 2005. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437 (7060): 884-888.

Muller T, Ruppel S. 2014. Progress in cultivation-independent phyllosphere microbiology. FEMS Microbiol Ecol 87: 2-17.

Rasche F, Marco-Noales E, Velvis H, van Overbeek LS, Lopez MM, van Elsas JD, Sessitsch A. 2006. Structural characteristics and plant-beneficial effects of bacteria colonizing the shoots of field grown conventional and genetically modified T4-lysozyme producing potatoes. Plant Soil 289: 123-140.

Reddy PK, Reddy MS. 2009. Biochemical and PCR-RAPD characterization of *Pseudomonas fluorescens* produced antifungal compounds inhibit the rice fungal pathogens *in vitro*. J Pure Appl Microbiol 3 (1): 1-4.

Vorholt J.A. 2012. Microbial life in the phyllosphere. Natural Review Microbiol. 10: 828-840.

Whips JM, Hand P, Pink D, Bending GD. 2008. Phyllosphere microbiology with special reference to diversity and plant genotype. J Appl Microbiol 105: 1744-1755.

Yadav R.K.P., Karamanoli K., Vokou D. 2010. Estimating bacterial population on the phyllosphere by serial dilution plating and leaf imprint methods. Ecoprint Intl J Ecol 17: 47-52.

Zhang, R, Thiyagarajan V, Qian PY. 2008. Evaluation of terminal-restriction fragment length polymorphism analysis in contrasting marine environments. FEMS Microbiol Ecol 65: 169-178.