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The Existence of Endophytic Actinobacteria from *Rhododendron zoelleri* Revealed by Culture-Dependent and Culture-Independent Approaches

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ABSTRACT

Endophytic actinobacteria from medicinal plant may play a significant role in producing bioactive compounds. The information regarding their diversity is an important. *Rhododendrons* are traditionally used for treating human disorders. One of the selected *Rhododendron* used in this study was *R. zoelleri* from Papua origin, which has been conserved and grown in Cibodas Botanical Garden, West Java, Indonesia. The aim of this study was to assess the existence of endophytic actinobacteria from *R. zoelleri* based on a culture-dependent and their community structure based on a culture-independent approach. Culturable actinobacteria were isolated and cultured on HV medium. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) targeting the metagenomic 16S rRNA was used to analyse the structure of the actinobacterial community. Six culturable endophytic actinobacteria (200 cfu/g fresh weight) from *R. zoelleri* were successfully isolated, three isolates from leaf, and the other isolates were obtained from stem. The six culturable isolates were RZP 1.3, RZP 1.1, RZP 2.2, RZPB 1.1, RZPB 7.1, RZPB 4.1. Based on their morphological characteristics, the endophytes have *Streptomyces* characters. The existence of *Streptomyces* spp. were also confirmed with molecular analysis based on 16S rRNA gene. The phylogenetic analysis based on 16S rRNA gene to the reference strains available in EzTaxon-e database showed that six isolates were closely related to *S. djakartensis* strains of NBRC 15409^T(99.19%), *S. tritolerans* strains of DAS 165T(99.90%), *S. coelicoflavus* strains of NBRC 15399T(99.59). However, they showed differences in morphological characteristics as compared with the reference strains. The metagenomic analysis of the DGGE profile based on 16S rRNA gene showed the community structure of endophytic actinobacteria from *R. zoelleri* which was represented by 13 DGGE bands. The bands were closely related to *Agromyces, Gordonia, Microbacterium, Micromonospora, Propionibacterium, Saccharomonospora, Streptomyces* which have 93.18%-100% similarity. Based on the data, it showed diversity of endophytic actinobacteria from *R. zoelleri* which may be further assess for their novelty and bioprospecting.

1. Introduction

Rhododendron zoelleri is a species of subgenus vireya, grouped in family of Ericacea, *R. zoelleri* grow in mountain forests with a height of 2000 above sea level (Argent 2006), origin from Papua, and has been collected in Cibodas Botanical Garden as a national plant conservation area in Indonesia. Species collection of *Rhododendrons* in Cibodas Botanical Gardens include *R. sessilifolium*, *Rhododendron* sp., *R. multicollor*, *R. javanicum*, *R. zoelleri*, each comes from Sumatera, Bengkulu, Jambi, Java, and Papua, respectively, and *R. hybrid* (*R. javanicum* and *R. sessilifolium*). *Rhododendron* has long been used as a traditional medicine, for example *R. brachycarpum* was used to treat diabetes and hypertension in Korea (Choi *et al.* 2012). *R. groenlandicum* was used to treat diabetes in Canada (Leduc *et al.* 2006). *R. tomentosum* was also used for the treatment of diabetes in China, Korea and Japan (Tam *et al.* 2011). *Rhododendron* was reported to produce bioactive compounds e.g. flavonoid and terpenoid (Qiang *et al.* 2011). The bioactive compounds

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from *Rhododendron* are presumably related to the presence and diversity of endophytic microbes residing in the plant tissue (Tan and Zou 2001), without causing disease in host plant (Schulz *et al.* 1993). Endophytic actinobacteria was known as a wide range producer of bioactive compounds (Zhao *et al.* 2011). Endophytic actinobacteria may have a significant contribution to the production of bioactive compounds in medicinal plants (Pujiyanto *et al.* 2012). Actinobacteria is a gram-positive bacteria within the high G+C content more than 55% (Emley and Stackebrandt 1994).

The information regarding diversity of endophytic actinobacteria from *R. zoelleri* has not been reported. The work is very important to explore endophytic actinobacteria *R. zoelleri*, as source of a germ plasm which can be used for further characterization on their potential utilization. The diversity of culturable actinobacteria can be obtained by cultivating actinobacteria on isolation medium under laboratory conditions, and the metagenomic approach which provide diversity based on genomic data. The genomic data may give more insight information regarding endophytic actinobacteria from *R. zoelleri,* since more than 99% microbes in the environtment can not be cultivated in a medium (unculturable) (Kimura 2006). The aim of this study was to investigate the diversity of culturable and unculturable endophytic actinobacteria from *R. zoelleri* using combination of a culture-dependent by cultivation in HV medium and a culture-independent approach by PCR-DGGE based on 16S rRNA gene.

2. Materials and Methods

2.1. Materials

R. zoelleri as the plant samples were originally collected from Papua in 2009 and now grown in Cibodas Botanical Garden, Indonesia. The samples were selected from healthy plants.

2.2. Methods

2.2.1. Isolation and Morphological Characterisation of Culturable Actinobacteria

Culturable endophytic actinobacteria was isolated and cultured on Humic Acid Vitamin B Agar (HV Agar) (Hayakawa and Nonomura 1987). About 1 g of sample of leave and stem were washed with sterile distilled water then surfaces sterilized by soaking in 70% alcohol for 1 min, folowed by 1% hypochlorite solution for 5 min, and 70% alcohol for 1 min, and finally rinsed with sterile distilled water. Then the sterilized samples were crushed and serial dilutions up to 10^{-3} were carried out. Each of suspension (0.1 mL) of sample was taken from 10^{-1}, $10^{-2,}$ and 10^{-3} dilutions, spread on HV Agar, and then incubated at room temperature for 4 to 8 weeks (Coombs and Franco 2003). Actinobacterial colonies were purified using International *Streptomyces* Project 2 (ISP 2). The colonies were morphologically characterized based on macroscopic (pigmentation, colour of colony and growth in four different media, i.e. ISP 2, ISP 3, ISP 4, Yeast Starch Agar (YSA)) character, and microscopic (aerial hypha type) observations using an Olympus Optilab microscope at 400x magnification.

2.2.2. Genomic DNA Isolation from Culturable Actinobacteria

Isolation of actinobacterial genomic DNA was performed according to the Geneaid Genomic DNA Mini Kit protocol (Blood/Cultured Cell). DNA concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.2.3. DNA Extraction from *R. zoelleri* Tissues

About 0.1 g of leave and stem of *R. zoelleri* were crushed into powder in a liquid nitrogen, then the total DNA was extracted according to protocol by Genomic DNA Mini Kit, Plant (Geneaid, Shijr, TPE, TW). The purity and the quantity of the extract were measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA).

2.2.4. The 16S rRNA Gene Amplification of Culturable and Unculturable Endophytic Actinobacteria

Genomic DNA was amplified by polymerase chain reaction (PCR) using the T1-thermocycler (Biometra, Goettingen, Germany). Genomic DNA was used as the template for amplification using two 16S rRNA gene primers specific for actinobacteria, i.e. 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16Sact1114R (5'-GAGTTGACCCCGGCRGT-3') (Martina *et al.* 2008). The 25 µL reaction volume consisted of 12.5 µL GoTaq Green Master Mix 2×, 2.5 µL of each primer (10 pmol), 2.5 µL template DNA (100 ng/µL) and 7.5 µL nuclease free water. PCR condition were as follows: 2 min pre-denaturation at 95°C, 30 sec denaturation at 95°C, 45 sec annealing at 55°C, 1 min elongation at 72°C, 5 min post elongation at 72°C, and 15 min cooling at 4°C PCR was performed over 35 cycles and the products electrophorized on 1% agarose gel. A single DNA band per sample was observed in the G:BOX gel documentation system (Syngene, Frederick, MD, USA) to observe the ±1087 bp target DNA band. The product of first phase PCR was used as template DNA for the second phase PCR using the universal primer for bacteria, i.e. P338-GC (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3')and P518R (5'-ATTAC CGCGGCTGCTGG-3') (Overeas *et al.* 1997). A PCR reaction volume of 25 µL consisted of 12.5 µL GoTaq Green Master Mix 2× (Promega, Madison, WI, USA), 1.25 µL of each primer (10 pmol), 1.25 µL of the product of first phase PCR, and 9 µL nuclease free water. PCR for the 16S rRNA gene was conducted over 30 cycles that consisted of 5 min pre-denaturation at 94°C, 30 sec denaturation at 92°C, 30 sec

annealing at 58°C, 1 min elongation at 72°C, and 5 min post elongation at 72°C. The PCR products (5 µL) were run on 1% agarose gel for 45 min at 80 V. The electrophoresis results were visualized after Ethidium Bromide (EtBr) staining and 180 bp bands were observed on the G:BOX gel documentation system (Syngene, Frederick, MD, USA).

2.2.5. DGGE Analysis of the 16S rRNA Gene

The PCR product of 16S rRNA gene was run on to a 0.75 mm vertical gel containing 8% (w/v) polyacrylamide [acrylamide-bisacrylamide (37.5: 1)] in 1× Tris-acetate-EDTA (TAE). The denaturant gradient concentrations used were 30% and 70% (100% denaturant in accordance with 7M urea and 40% formamide). Electrophoresis was conducted for 6 h at 60°C, 150 V using the D Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The electrophoresis gel was stained with EtBr for 30 min, followed by rinsing with 500 mL of 1× TAE buffer before observing the bands in the G:BOX gel documentation system (Syngene, Frederick, MD, USA). DGGE band was analyzed by using CLIQS 1D Pro software to estimate the total bands. Separated bands were cut using a sterile scalpel and placed into micro tubes containing 100 µL ddH_2O. The products of DGGE were incubated at 4°C overnight, and then incubated again at 60°C for 2 h, following the conditions and primers used in the second phase PCR but without the GC-clamp (Muyzer and Uitterlinden 1993).

2.2.6. Diversity and Abundance Analysis

Relatif abundance of endophytic actinobacterial community was analysed based on the volume of DGGE bands using CLIQS 1D Pro, to calculate the Operation Taxonomic Unit (OTU). Diversity index was analysed by Shannon-Wiener index (H') using past 3 software with formula:

$$H' = -\sum (pi \log pi)$$

H'= diversity index, pi = ratio of one individuals number to the total number of samples in the plot (n/N) (Hill *et al.* 2003).

2.2.7. Bioinformatic Analysis and Construction of Phylogenetic Tree

The PCR products of the 16S rRNA gene of culturable and unculturable endophytic actinobacteria *R. zoelleri* were sequenced following standard protocols of DNA sequencing (ABI PRISM 3100). The sequencing results were compared to those of reference strains available in the EzTaxon (https://www.ezbiocloud.net/). Phylogenetic analysis was conducted using MEGA 6 software, and the phylogenetic tree was constructed using a 1000 replication-bootstrap analysis and the neighbour-joining method (Tamura *et al.* 2011).

3. Results

3.1. Diversity of Culturable Endophytic Actinobacteria

Total of 6 isolates (200 cfu/g fresh weight) of endophytic actinobacteria were successfully isolated from *R. zoelleri*, 3 isolates from leaves and stems respectivelly. Isolation using HV agar medium and purified using ISP 2 medium. The collected endophytic actinobacteria showed to vary based on morphological characteristics (Figure 1 and Table 1).

The 6 isolates (200 cfu/g fresh weight) endophytic actinobacteria were grown in ISP 2 medium, showed morphological colony diversity, and based on this morphological characteristics of substrate mycelia color, aerial mycelia, medium pigmentation, and spore chain morphology. These morhological characters close to the groupped of *Streptomyces* (Shirling and Gottlieb 1966). The vary of hypa type based on spore chain of the isolate were retinaculum-apertum for RZP 1.3 and RZP 1.1, spira for RZP 2.2, RZPB 1.1, and RZPB 7.1, biverticillus-spira for RZPB 4.1 (Figure 1 and Table 1).

Morphological characteristics of endophytic actinobacteria from *R. zoelleri* were known by growing isolates on four different media (ISP 2, ISP 3, ISP 4, YSA). All isolates have abundant growth on ISP 2, ISP 3, ISP 4 and YSA medium, but RZPB 7.1 showed moderate growth in four media. The aerial mycelium had wide range of colors on different media (ISP 2, ISP 3, ISP

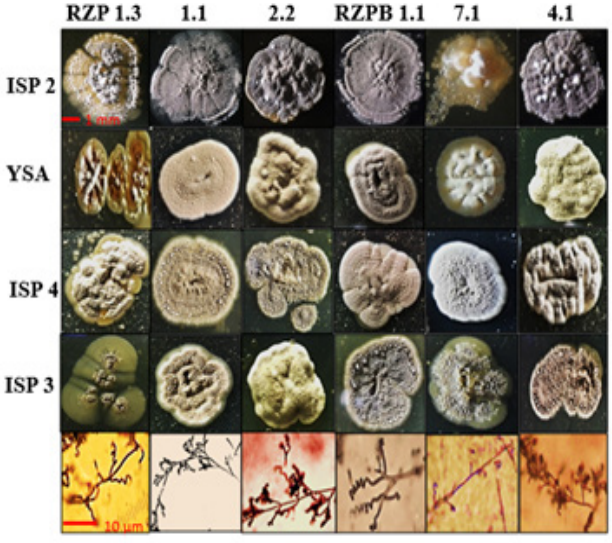

Figure 1. Morphological characteristics (macroscopic and microscopic) of culturable endophytic actinobacteria for 14 days incubation, microscopic using light microscope (Olympus) 400x magnification

Table 1. Morphological characteristics of culturable endophytic actinobacteria on diferent medium for 14 days incubation

Medium	Isolate code	Aerial mycelium	Substrat mycelium	Soluble pigment	Growth	Spore chain
YMA	RZP 1.3	Brownish gray	Brownish gray	Chocolate	Abundant	Retinaculum-apertum
	RZP 1.1	Gray	Yellow	-	Abundant	Retinaculum-apertum
	RZP 2.2	Gray	Yellow	-	Abundant	Spira
	RZPB 1.1	Gray	Yellow	-	Abundant	Spira
	RZPB 7.1	White	Brownish white	-	Moderate	Spira
	RZPB 4.1	Gray	Yellow	-	Abundant	Biverticillus-Spira
YSA	RZP 1.3	Yellowish brown	Yellowish brown	-	Abundant	Retinaculum-apertum
	RZP 1.1	Light brown	Yellow	Chocolate	Abundant	Retinaculum-apertum
	RZP 2.2	Light brown	Yellow	-	Abundant	Spira
	RZPB 1.1	Gray	Yellow		Abundant	Spira
	RZPB 7.1	White	Brownish white	-	Moderate	Spira
	RZPB 4.1	Greenish Chocolate	Yellow		Abundant	Biverticillus-Spira
ISA	RZP 1.3	Light brown	Brownish white	Chocolate	Abundant	Retinaculum-apertum
	RZP 1.1	Light brown	Brownish white	-	Abundant	Retinaculum-apertum
	RZP 2.2	Light brown	Brownish white	-	Abundant	Spira
	RZPB 1.1	Light brown	Brownish white	-	Abundant	Spira
	RZPB 7.1	White	Brownish white	-	Moderate	Spira
	RZPB 4.1	Light brown	Brownish white	-	Abundant	Biverticillus-Spira
OA	RZP 1.3	Brownish gray	Brownish white	-	Abundant	Retinaculum-apertum
	RZP 1.1	Chocolate	Gray	-	Abundant	Retinaculum-apertum
	RZP 2.2	Greenish Chocolate	Gray	-	Abundant	Spira
	RZPB 1.1	Gray	Gray	-	Abundant	Spira
	RZPB 7.1	Gray	Brownish yellow	-	Moderate	Spira
	RZPB 4.1	Dark brown	Gray	-	Abundant	Biverticillus-Spira

RZP = *Rododendron zoelleri* Leaves Papua; RZPB = *Rododendron zoelleri* Stems Papua

4, YSA), e.g. brownish gray, gray, white, yellowish-brown, light brown, greenish chocolate, light brown, chocolate, dark brown, meanwhile substrate mycelium showed tohave yellowish brown, yellow, white brownish, gray, brownish yellow. The RZP 1.3 showed brown pigmentation on ISP 2, ISP 4, YSA media but not on ISP 3 medium, while the other isolates were not found to produce pigmentation in the tested media.

3.2. Identification of Culturable Endophytic Actinobacteria Isolates Based on 16S rRNA Gene

The 16S rRNA gene from the genomic DNA of six culturable isolates were successfuly amplified using 27F and 16Sact1114R primers with ± 1087 bp DNA fragment as the target. The result of nucleotide sequencing of 16S rRNA gene was aligned between the forward and reverse sequences, then corrected by the primer. All isolates have homology sequences with the genus of *Streptomyces* (Table 2).

The partial sequence (± 1087 bp) of 16S rRNA gene and phylogenetic analyses (Figure 2; Table 2) confirmed that RZP 1.1, RZP 2.2, RZPB 1.7, RZPB 4.1 closely related with *S. tritolerans* strain DAS 165^T and showed high similarity by 99.90%. *Streptomyces tritolerans* was isolated from dryland in India, and has been known for its potency as a producer of antibiotic compounds (Mangamuri *et al.* 2014). These isolates (RZP 1.1, RZP 2.2, RZPB 1.7, RZPB 4.1) have similarity with *S. tritolerans* strain DAS 165^T on both substrate and aerial mycelia, e.g. gray and yellow but have different spore chain e.g. retinaculum-apertum (RZP 1.1, RZP 2.2), spira (RZPB 1.7), retinaculum-apertum (RZPB 4.1) while *S. tritolerans* strain DAS 165^T had straigh-flexous (Syed *et al.* 2007).

The RZP 1.3 was closely related with *S. djakartensis* strains of NBRC 15409^T (99.19%), as the type strain and not yet reported for its potency. RZP 1.3 had retinaculum-apertum of spore chain (Table 2). RZPB 1.1 showed to have similarity to *S. coelicoflavus* strain NBRC 15399^T (99.59%) which was isolated from crude oil contaminated soil (Ray *et al.* 2013). *Streptomyces*

Table 2. Comparison of 16S rRNA gene sequencing identification of culture-dependent endophytic actinobacteria with EzTaxon-e database

Isolate code	Species	Strain	Similarity (%)	Acession Number
RZP 1.3	*S. djakartensis*	NBRC 15409^T	99.19	AB184657
	S. tuirus	NBRC 15617^T	99.09	AB184690
	S. fumanus	NBRC13042^T	99.09	AB184273
RZP 1.1	*S. tritolerans*	DAS 165^T	99.90	DQ345779
RZP 2.2	*S. tendae strain*	ATCC 19812^T	99.90	D63873
RZPB 4.1	*S. rubrogriseus*	LMG 20318^T	99.90	AJ781373
RZPB 7.1	*S. fragillis*	NRRL 2424^T	99.70	AY999917
RZPB 1.1	*S. coelicoflavus*	NBRC 15399^T	99.59	AB1846650
	S. chillikensis	RC 1830^T	99.39	JN050256

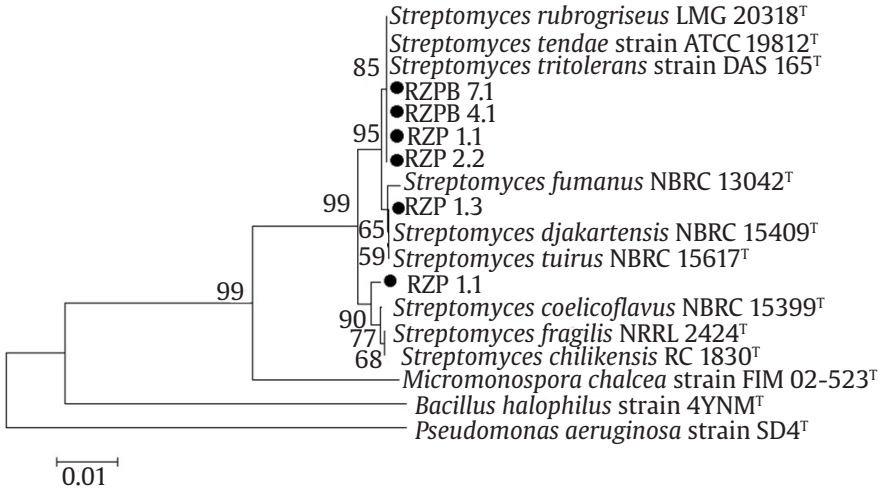

Figure 2. Phylogenetic tree of 16S rRNA gene of culture-dependent endophytic actinobacteria constructed using MEGA 6 software

Table 3. Similarity of 16S rRNA gene sequence of culture-independent endophytic actinobacteria to the data base available in EzTaxon-e

Band	Closest species	Strain	Similarity (%)	Acession Number
1	*Agromyces insulae*	CFH S0483T	100	KP232919
	Microbacterium esteraromaticum	DSM 8609T	99.44	Y17231
2,6	*Microbacterium pseudoresistens*	CC-005209T	100	FJ865214
	Microbacterium marinilacus	YM11-607T	100	AB286020
3,4,5,7	*Microbacterium testaceum*	DSM 20166T	100	X77445
	Microbacterium imperiale	DSM 20530T	100	X77442
8	*Propionibacterium namnetense*	NTS31307302T	96.05	LWHO01000015
	Cultibacterium acnes	DSM 1897T	96.05	AWZZ01000008
9	*Streptomyces lushanensis*	NRRL B_24994T	98.87	MAUD01000416
	Streptomyces spongiicola	HNM0071T	98.87	KT327868
10	*Saccharomonospora viridis*	DSM 43017T	93.18	CP001683
	Nocardia pseudovanccinii	NRRL B-24154T	93.18	CLG_48649
11,13	*Gordonia kroppenstedtii*	NP8-5T	99.43	AM883151
	Mycobacterium pyrenivorans	DSM 44605T	99.43	AJ431371
12	*Micromonospora rifamycinica*	AM105T	96.05	LRMV01000349
	Actinopolymorpha cephalotaxi	CPCC 202808T	96.05	jgi.1085066

coelicoflavus has been known for its potency as a producer of ramnolipid biosurfactants (Kalyani *et al.* 2014). RZPB 1.1 had similarity to *S. coelicoflavus* strain NBRC 15399T with spiral of spore chain, with aerial mycelia brown to gray, and yellow to brownish for substrate mycelia (Table 2). Meanwhile, *S. coelicoflavus* strain NBRC 15399T has greenish white substrate mycelium and light brown to dark brown (Ray *et al.* 2013). Non actinobacteria, *Bacillus halophilus* strain 4YNM (Gram +) and *Pseudomonas aeruginosa* strain SD4 (Gram -) were used as the outer group of the phylogenetic tree.

3.3. Diversity of Endophytic Actinobacteria Based on a Culture-Independent Approach

The genomic DNA from leaves and stems of *R. zoelleri* was successfully amplified with the 27F and 16Sact1114R as the spesific primers for actinobacteria. The PCR product of 16S rRNA gene of actinobacteria was re-amplified using nested PCR. The nested PCR was carried out by two phases of PCR, the first phase showed to have arround ± 1087 bp 16S rRNA gene target. The amplicon was used as a template in the second phase of PCR, to obtain the ± 180 bp 16S rRNA gene target as V3 region. The 16S rRNA

gene amplicons were separated using denaturing gradient gel electrophoresis (DGGE) (Figure 5a). There were 13 separate dominant bands in DGGE gel from stems and leaves *R. zoelleri samples which were* successfully isolated by gel cutting. The bands have various sequence homology with *Streptomyces* and Non-*Streptomyces* from rare-actinobacteria i.e *Agromyces, Gordonia, Microbacterium, Micromonospora, Propionibacterium, Saccharomonospora, Streptomyces* (Table 3).

The relative abundance of culture-independent endophytic actinobacterial community from stems and leaves of *R. zoelleri* represented by OTU value can be seen in (Figure 3). One DGGE band represented one OTU or one endophytic actinobacterial community. Table 4 shows the alpha diversity value of stems and leaves, with 9 and 8 OTU, respectively. The OTU 1, 4, 6, and 8 were found in both stem and leaves. The abundance of OTU 1, 4, 6, 8 in stems and leaves were (4,6%-17.5%), (7.9%-15.6%), (12.2%), (16.1%-49.9%), respectively. OTU 1 similar to *Agromyces insulae* CFH S0483^T. Meanwhile, OTU 4 and 6 have similarty to *Microbacterium genus,* i.e. *M. testaceum* DSM 20166T, *M. pseudoresistens* strain CC-005209T, respectively. OTU 8 has similarity with *Propionibacterium namnetense* NTS31307302^T. The value of gamma diversity can be interpreted as the number of different communities in each sample. There were 13 of the gamma diversity value found in both stems and leaves. It can be indicated that there were 13 different endophytic actinobacterial communities found in both stems and leaves of *R. zoelleri.*

Shannon Wiener's (H') index diversity analysis was used to estimate the diversity of the endophytic actinobacterial community in stems and leaves of *R. zoelleri.* Figure 4 shows that the value of Shannon-Wienner (H') index of endophytic actinobacterial community in stems and leaves ranged from 1.539-2.116. The stems show to have higher diversity index than leaves. Category of diversity in stems and leaves of the *R. zoelleri* is still moderate with the range of Shannon index between 1.5> H> 3.5 (Bibi and Ali 2013).

There were 13 bands obtained based on DGGE profile of 16S rRNA gene of actinobacteria *R. zoelleri* (Figure 5a). Phylogenetic analysis based on the 16S rRNA gene (Figure 5b) showed that band 1 belonging to genus of *Agromyces*, and showing the highest similarity to the *A. insulae* CFH S0483^T (100%) which was isolated from soil sample of Catba island,Vietnam (Huang *et al.* 2016). Bands 2 and 6 closed to the genus of *Microbacterium* especially to the *M. pseudoresistens* strain CC-005209 (100%), from *Agaricus blazei* mushroom stalk (Young *et al.* 2010). Bands 3, 4, 5, and 7 were afilliated with the genus of *Microbacterium*, displayed the highest similarity to *M. testaceum* DSM 20166T (100%). Band 8 showing low similarities to the genus *Propionibacterium*, especially with *P. namnetense* NTS31307302^T (96.05%).

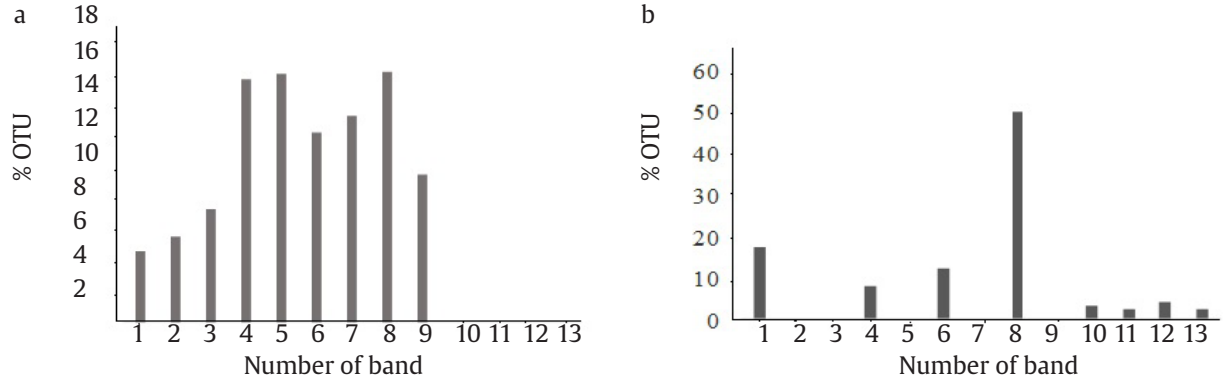

Figure 3. Abundance of endophytes base on OTU found in *R. zoelleri* tissue, a. stems, b. leaves

Table 4. Abundance of actinobacterial endophyte community found in *R. zoelleri* analysed based on OTU value

Sample	Alpha diversity	% OTU	Similar OTU	Gamma diversity
Stems	9	1 (4.6%), 2(5.5%), 3(7.3%), 4(15.6%), 5(15.9%), 6(12.2%), 7(13.32%), 8(16.1), 9(9.5)	1,4,6,8	13
Leaves	8	1(17.5%), 4(7.9%), 6(12.2%), 8(49.9%), 10(3.4%),11(2.2%),12(4.3%), 13(2.6%)	1,4,6,8	

Band 9 from the genus *Streptomyces* was closely related to *S. lushanensis* strain of NRRL B_24994^T (98.87%). *S. lushanensis* strain of NRRL B_24994^T was isolated from soil sample of Lushan Mountain, China (Zang *et al.* 2015). Band 10 belongs to the genus of *Saccharomonospora*, with low similarity to the S. *viridis* DSM 43017^T (93.18%). Band 12 had closely related to the genus of *Micromonospora,* especially with *M. rifamycinica AM105^T* (96.05%). *Micromonospora rifamycinica AM105^T* was isolated from mangrove sediment samples in shouth China Sea (Huang *et al.* 2008). Bands 11 and 13 from the genus of *Gordonia*, was closely related to *G. kroppenstedtii* strain NP8-5 (99.43%), isolated from a polluted steam in Gumil, Korea (Kim *et al.* 2009).

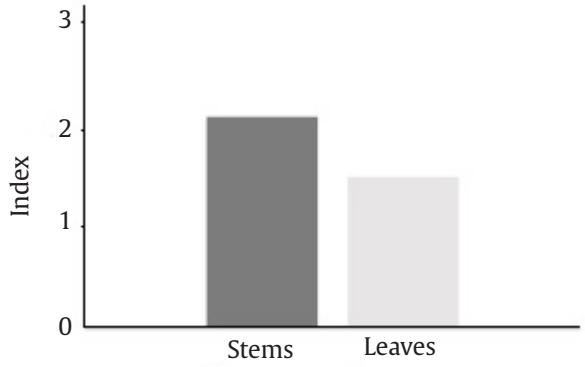

Figure 4. Alpha diversity (Shannon-Wienner) of endophytic actinobacterial community found in *R. zoelleri*

4. Discussion

4.1. Culturable Endophytic Actinobacteria by a Culture-Dependent Approach

This is the first report on the existence of endophytic actinobacteria found in *R. zoelleri* leaves and stems. Based on culture-dependent approach, there were six isolates of endophytic actinobacteria found. Their morhological characters indicated that all isolates close to the groupped of *Streptomyces* (Shirling and Gottlieb 1966). The six isolates (RZP 1.3, RZP 1.1, RZP 2.2, RZPB 1.1, RZPB 7.1, RZPB 4.1) had vary of mycelia substrate, mycelia aerial, and media pigmentation on ISP 2, ISP 3, ISP 4, YSA medium. According to Reddy *et al.* (2011), the same isolates showed vary in colors of mycelia substrate, mycelia aerial, media pigmentation, when grown on different medium. The isolate of RZP 1.3, RZP 1.1, RZP 2.2, RZPB 1.1, RZPB 7.1, RZPB 4.1 indicated as *Streptomyces*, based on their morphological characters.

Under a culture-dependent approach, the dominance of *Streptomyces* may be caused by HV medium used during cultivation, which may be more preference for *Streptomyces* group to grow compared to the non-*Streptomyces* group. The existence of culturable endophytic actinobacteria *R. zoelleri* can be explored further using various growth media. Humic Acid-Vitamin Agar was a selective medium for growth of large number of actinobacteria. HV agar containing of humic acid used as sole source of energy and nitrogen. In addition, humic acid initiated the occurrence of sporulation (Hayakawa and Nonomura 1987). The growth

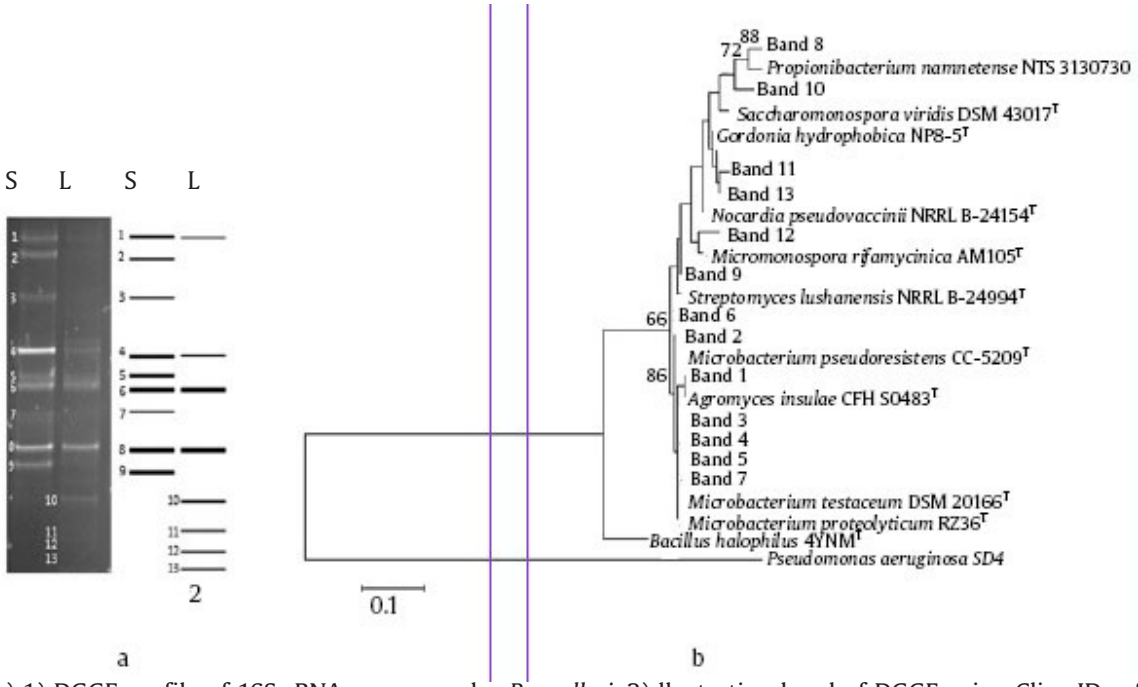

Figure 5. a) 1) DGGE profile of 16S rRNA gene samples *R. zoelleri*, 2) llustration band of DGGE using Cliqs ID software, stems (S), Leaves (L). b) Phylogenetic tree based on DDGE separation of 16S rRNA gene of culture-independent endophytic actinobacteria, was constructed using MEGA 6 software

development of *Streptomyces* can also be influenced by pH and temperature (Oskay 2009).

Phylogenetic analysis based on 16S rRNA gene partial sequence indicated that six isolates were in one cluster (*Streptomyces*) (Figure 2) and showed high similarity ranging from 99.09-99.90%. The highest similarity of the 16S rRNA gene sequence to the comparable sequence, still can be characterized further for the possibility to find novel species by considering different and unique characters of isolate. It can be further examined using the polyphasic taxonomic approach. Lisdiyanti *et al.* (2010) reported three novel spesies from six Indonesia strains with the highest simililarity to the other known strain in the genus of *Actinokineospora* ranging from 97-99.4%. Other report from Otoguro *et al.* (2011) showed that two isolates that proposed as novel species in the genus of *Actinophytocola,* have 98.1-98.2% similarity to the reference strain.

4.2. Communities of Endophytic Actnobacteria by a Culture-Independent Approach

Based on direct observation and considering the position of the band of DGGE (band with the same positions taken one) there were 13 separate dominant bands. The analysis of total of separated band using *software Cliqs 1D*, detected 9 bands from stems and 8 bands from leaves. Endophytic actinobacteria which reside in stems found to be slightly more diverse than leaves. Qin *et al.* (2012) reported that by metagenomic analysis, there were 240 clones of endophytic actinobacteria from stems and 213 clones from leaves. It seems likely that endophytic actinobacteria have preference and can reside in spesific tissues.

The 13 OTUs obtained from stems and leaves indicate that *R. zoelleri* may have 13 different endophytic actinobacterial communities which reside in stem and leave tissues. Based on reference data available in the gene bank, the 13 OTUs have similarity with the genus of *Agromyces, Gordonia, Microbacterium, Micromonospora, Propionibacterium, Saccharomonospora, Streptomyces*. There were 4 OTUs that always exist in stems and leaves of *R. zoelleri* i.e. OTU 1, OTU 4, OTU 6, and OTU 8, and based on reference data in gene bank, these 4 OTUs similar with *Agromyces insulae, Microbacterium testaceum, M. pseudoresistens, Propionibacterium namnetense,* respectively. There were diferences in endophytic actinobacteria diversity observed between culturable and culture-independent approaches. The culturable approach found only one genus of actinobacteria i.e. *Streptomyces.* Meanwhile, both *Streptomyces* and non *Streptomyces* were observed using culture-independent approach. Based on the Shannon (H') index, the diversity of actinobacteria in stems and leaves of *R. zoelleri* was in the medium category (1.539-2.116). Stem has the highest diversity index (1.539), while leave has the lowest diversity index (2.116). Plant tissue is considered as a very selective habitat, not all bacterial communities can survive in all plant tissue. According to Passari *et al.* (2015), endophytic actinobacteria could colonize different spesific tissues of host plants, indicated their diversity amongst different tissues.

Phylogenetic analysis showed that 13 DNA bands of culture-independent endophytic actinobacteria were affiliated with both *Streptomyces* and Non-*Streptomyces* from rare-actinobacteria namely *Agromyces, Gordonia, Microbacterium, Micromonospora, Propionibacterium, Saccharomonospora, Streptomyces* with 93.18-100% similarity compare to the reference strain in EzTaxon-e. *Agromyces, Microbacterium, Micromonospora, Propionibacterium* were observed to be predominat in stems and leaves of *R. zoelleri*. Tian *et al.* (2007) reported that *S. gailaeus was detected as* high abundance in rice tissues. It was discovered in rice stems and roots, and some endophytic actinobacteria populations correlated with host plant tissues.

4.3. Comparison between Community of Endophytic Actinobacteria by Culture-Dependent and Culture-Independent Approaches

There were diversity diferences between culturable actinobacteria and the culture-independent endophytic actinobacteria from *R. zoelleri*. Culture-dependent approach , that was conducted by isolating and cultivating endophytic actinobacteria in HV agar medium, recovered isolates from single genus i.e. *Streptomyces*. Whereas the culture-independent, as metagenomic approach, amplifying 16S rRNA gene of actinobacteria and using DGGE method, found both *Streptomyces* and Non- *Streptomyces* from rare-actinobacteria, There are 7 genera obtained i.e. *Agromyces, Gordonia, Microbacterium, Micromonospora, Propionibacterium, Saccharomonospora, Streptomyces.*

Based on the data comparison, the culture-independent approach using DGGE, give more information about community profile of endophytic actinobacteria than the culture-dependent approach. Combining both of culture-dependent and culture-independent approaches can be a good strategy to study the existence of actinobacterial community who reside in their host plant tissue of *R. zoelleri*.

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