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Comparative study on the diversity of endophytic actinobacteria communities from *Ficus deltoidea* using metagenomic and culture-dependent approaches□

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Abstract. *Janatiningrum I, Solihin DD, Meryandini A, Lestari Y. 2018. Comparative study on the diversity of endophytic actinobacteria communities from* Ficus deltoidea *using metagenomic and culture-dependent approaches. Biodiversitas 19: 1514-1520.* Actinobacteria endophytes of medicinal plants may play an essential role in producing a variety of critical bioactive compounds. However, the possible contribution of such actinobacteria to the pharmacological properties of traditional herbal remedies remains mostly unknown. For example, the diversity and attributes of actinobacteria endophytes in *Ficus deltoidea*, a small tree species that has long been used to treat diseases such as cancer, diabetes, and cardiovascular illnesses, have not been explored. Here, the actinobacteria endophyte community structure in *F. deltoidea* was investigated using both culture-dependent and metagenomics approaches. Based on morphological characteristics and 16S rRNA gene analysis, the dominant culturable actinobacteria isolates exhibited a close relationship with *Streptomyces*. The metagenomic technique using PCR-DGGE analysis of the 16S rRNA gene showed the presence of 11 OTUs in *F. deltoidea* tissue. Whereas the dominant culturable actinobacteria endophytes in *F. deltoidea* was *Streptomyces*, the metagenomic approach showed non-*Streptomyces*, particularly *Rhodococccus* and *Verrucosispora*, to be also important. Thus, results from both culture-dependent and metagenomic approaches provided useful indicators on the diversity and community structure of actinobacteria endophytes in *F. deltoidea.*

Keywords: Actinobacteria, Endophytes, *Ficus deltoidea*, Metagenomic, PCR-DGGE

INTRODUCTION

Ficus deltoidea, a medicinal plant native to Indonesia and other parts of Southeast Asia, is traditionally used as a treatment for some diseases such as cancer, diabetes, and cardiovascular illnesses (Abdulla et al. 2010; Akhir et al. 2011). However, it is still unclear as to whether the bioactive compounds reputedly responsible for its effects are produced by the plant itself or by microbes including endophytic actinobacteria. Microbial endophytes are essential for the survival of many plants. They are microbes that live in the plant tissues without causing substantive harm to their hosts which benefit from useful secondary metabolite compounds that the endophytes produce (Kado, 1992). Such microbial endophytes are beginning to attract the attention of researchers due to their ability to produce various compounds of pharmacological value. Hence, it is not inconceivable that the bioactive compounds believed to be present in *F. deltoidea* may be the result of its interaction with actinobacteria endophytes in the plant tissue.

While the study of endophytic actinobacteria diversity is necessary, the primary constraint in studying its diversity lies in the fact that the culturable microbes in this group are thought to constitute less than 1% of the total, with the remaining 99% being unculturable microbes (Sekiguchi, 2006). There is lack of data regarding the diversity and pharmacological potential of endophytic actinobacteria associated with *F. deltoidea*. It is therefore essential to seek strategies to uncover the diversity of both culturable and unculturable endophytic actinobacteria. Metagenomics is a widely applied approach for assessing endophytic microbial diversity since it can be performed without prior culturing of the microorganisms under investigation (Patrick and Handelsman, 2005). It is, hence, suitable for studying the diversity of endophytic actinobacteria from *F. deltoidea*. DGGE (Denaturing Gradient Gel Electrophoresis) is one of the most effective metagenomics techniques. In DGGE analysis, the DNA fragments with the same length but different nucleotide sequences are separated in polyacrylamide gels set in a linear denaturing gradient based on the differences in the mobility of the PCR-amplified DNA molecules (Muyzer and Smalla 1993).

The objective of the present study was to assess the diversity of endophytic actinobacteria from *F. deltoidea* using both the cultivation and metagenomic approaches. The use of these two different approaches allows for a more comprehensive acquisition of information on the actinobacteria endophytic community in *F. deltoidea*. This research presents the first description of the actinobacteria endophytic community in *F. deltoidea*.

MATERIALS AND METHODS

Sample collection and actinobacteria endophytes isolation

Endophytic actinobacteria were isolated from various parts (root, stem, leaf, and fruit) of the *F. deltoidea* plant in the Collection of the Medicinal Plants Garden of the Biopharmaca Research Center, Bogor Agricultural University in Bogor, Indonesia. Surface-sterilization of the plant tissue samples was performed according to Coombs and Franco (2003), with some modifications. Samples were soaked in 70% alcohol for 1 minute, then soaked in 1% sodium hypochlorite (NaOCl) for 5 minutes, then in 70% alcohol for 1 minute. The final step samples were rinsed using sterile distilled water 3 times. About 1 g of samples were crushed, and serial dilutions of up to 10^{-3} were carried out. A 100 µL sample of the suspension was then plated on Humic Acid Vitamin agar (HVA) medium containing 50 ppm of griseofulvin and 30 ppm nalidixic acid and incubated for 14 days at room temperature (25-28° C). Actinobacteria colonies that grew on the agar medium were purified on International Streptomyces Project (ISP) 2 medium.

Morphological characterization

Morphological identification of the isolated actinobacteria endophytes was carried out by plating them on three different media, *viz.* ISP 2, ISP 4, and Yeast Starch Agar (YSA). Identification was carried out based on mycelium substrate color, aerial mycelium color, and pigmentation using the RAL color chart. The bacterium microstructure was examined using a light microscope at a magnification of 4 × 10.

16S rRNA gene identification of culturable actinobacteria endophytes

Actinobacteria endophytes were identified through molecular analysis of the bacterium's 16S rRNA gene. The spores and mycelia of the actinobacteria endophytes were collected in 1.5 mL microtubes and extracted using the Geneaid Presto Mini gDNA Bacteria Kit according to the manufacturer's protocol. The concentration and purity of the DNA were quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Genomic DNA was amplified using the Polymerase Chain Reaction (PCR) with 16S-specific primers for actinobacteria, *viz.* 16Sact1114R and 27F (Table 1).

Table 1. Primers of 16S rRNA gene used for PCR amplification

Primer	Sequence (5'-3')	References
27F	AGAGTTTGATCCTGGCTCAG	Bruce et al. (1992)
16Sact1114R	GAGTTGACCCCGGCRGT	Martina et al. (2008)
P338F	ACTCCTACGGGAGGCAGCAG	Overeas et al. (1997)
P518R	ATTACCGCGGCTGCTGG	Overeas et al. (1997)
GC clamp	CGCCCGCCGCGCGCGGCGGG CGGGCGGGGGCACGGGGGG	Overeas et al. (1997)

The PCR conditions were as follows: Pre-denaturation at 94 °C for 5 minutes, 35 cycles of denaturation at 92 °C for 1 minute, annealing at 53 °C for 30 s, elongation at 72 °C for 30 s, and post-elongation at 72 °C for 3 minutes. Following gel electrophoresis and EtBr staining, the amplification results were visualized using a UV transilluminator. The PCR product was sent for sequencing to sequencing services company. The results obtained were analyzed using Seqtrace software. The actinobacteria were identified by entering the primary data on the EzBioCloud web. Phylogenetic tree was subsequently constructed using MEGA 7 software using a 1000 replication-bootstrap analysis and the neighbour-joining method (Saitou and Nei 1987).

Metagenomic analysis was using total genomic DNA extracted from plant tissue used to examine the genetic diversity of the actinobacteria endophytes. About 0.5 g of plant samples (root, stem, leaf or fruit) were crushed in liquid nitrogen. The DNA was extracted using the DNA Mini Plant Kit (Geneaid, Shijr, TPE, TW), following the manufacturer's instructions. After checking its concentration and purity, the DNA was amplified using nested PCR technique. The first PCR primers used were 27F and 16Sact1114R, while the second pair comprised the 338F primers with GC clamp and 518R primers (Table 1). The first PCR product was used as the template for the second PCR. The amplification results were visualized on a UV transilluminator following gel electrophoresis and EtBr staining.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE analysis was conducted using the Universal Code Mutation Detection System (Bio-Rad, Hercules, CA, USA). About 15 µL DNA sample and 8 µL loading dye were run on a 8% polyacrylamide gel (Acrylamide-Bisacrylamide [37.5: 1]) in 7 L TAE 1× using as denaturant urea adjusted to a 30-70% gradient (100% Urea 8.4 g, 8 mL formamide, 50 × TAE 0.4 mL, Acrylamide-Bisacrylamide 4 mL). The samples were migrated at 60°C, 150 volts for 5 hours (Ernawati et al. 2016). The DGGE gel was then stained with EtBr and visualized using a G: BOX (Syngene, Frederick, MD, USA). The separated DGGE bands were analyzed by using CLIQS 1D Pro software to estimate the total number of bands. The separated bands were cut using a sterile scalpel and placed into microtubes containing 100 µL of ddH_2O and incubated at 4°C overnight.

Sequencing, phylogenetic tree construction, and clustering analysis

DNA eluted from the DGGE bands were evaluated with Nanodrop 2000 to determine the concentration and purity of the DNA. About 50 µL (~ 50 ng) of the DNA was re-amplified using the 338F and 518R primers without GC Clamps, and the PCR product was sent for sequencing. The results were analyzed with Seqtrace software and the DNA identities established using EzBioCloud web. Phylogenetic analyzes were performed using MEGA 7 with a 1000 replication-bootstrap analysis and the neighbor-joining method (Saitou and Nei 1987).

Cluster analysis is used to compare similarities all samples based on DGGE band position. An analysis of the clustering of endophytic actinobacteria community from *F. deltoidea* using data binary and constructed UPGMA tree using MEGA 7 (Kumar *et al.* 2008)

Data analysis

The Shannon-Wienner diversity index was used to calculate the abundance of endophytic actinobacteria in *F. deltoidea* using past 3 software (Felsenstein, 1985). The DGGE bands were analyzed using the CLIQS 1D pro to determine the volumes operation taxonomic units (OTU) which indicate the abundance evenness of actinobacteria in each band representing an actinobacteria endophytic community. The calculation of the diversity index using the Shannon-Wiener index (H') was carried out (Hill et al. 2003).

The Simpson diversity index indices were used to calculate evenness of endophytic actinobacteria in *F.deltoidea.* Evenness is a measure of the relative abundance of the different communities making up the richness of a sample. The calculation of the dominance index using Simpson index (S) was carried out using pas 3 software (Simpson 1949).

RESULTS AND DISCUSSION

The diversity of culturable endophytic actinobacteria

A total of 40 actinobacteria endophytes were successfully isolated from *F. deltoidea*. Amongst them, 60%, 20%, 15 % and 5% of isolates were obtained from the leaf, stem, root, and fruit, respectively. There were four different strains based on the color of the cultures, i.e., pure white, silk grey, pebble grey, moss grey, and ivory. Nine isolates were selected for molecular identification. Based on morphological characteristics, the culturable endophytic actinobacteria from *F. deltoidea* in the present study appeared to be of the genus *Streptomyces* (Shirling and Gottlieb 1966). Sequence homology results using EzBioCloud showed that the nine isolates for which 16S rRNA molecular identification was performed bore similarity to the genus of *Streptomyces,* this being consistent with their morphological characterization (Table 2).

Table 2. The similarity of culturable actinobacteria endophytes to GeneBank references strains based on 16S rRNA sequences

No	Code	Species	Strain	Similarity (%)	Accession number
1	TBL2	*Streptomyces alboflavus*	NRRL B-2373(T)	98.76	JNXT01000131
2	TBL3	*Streptomyces tritolerans*	DAS 165(T)	99.63	DQ345779
3	TBL4	*Streptomyces alboflavus*	NRRL B-2373(T)	98.48	JNXT01000131
4	TBL24	*Streptomyces tendae*	ATCC 19812 (T)	99.91	D63873
5	TBL25	*Streptomyces tendae*	ATCC 19812(T)	99.62	D63873
6	TBL32	*Streptomyces galbus*	DSM 40089(T)	99.15	X79852
7	TBR2	*Streptomyces alboflavus*	NRRL B-2373(T)	98.69	JNXT01000131
8	TBR4	*Streptomyces alboflavus*	NRRL B-2373(T)	98.69	JNXT01000131
9	TBF2	*Streptomyces longwoodensis*	DSM 41677(T)	100	KQ948572

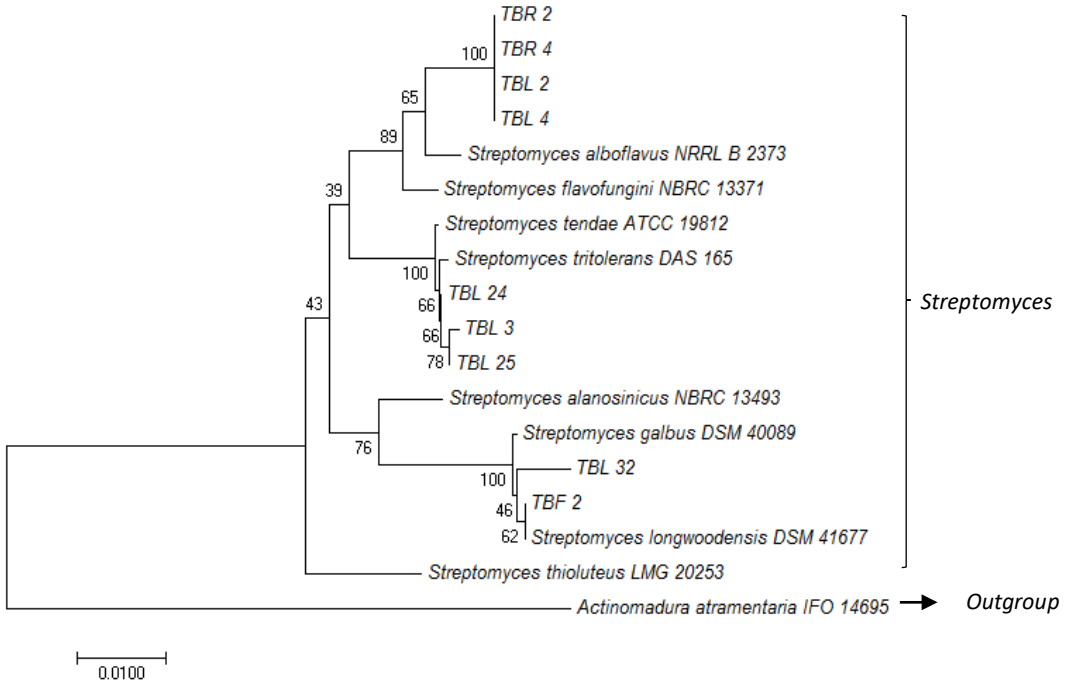

Figure 1. Neighbour-Joining tree of 16S rRNA gene of actinobacteria endophytes. Numbers at each nodes indicate the percentages of branch support of 1,000 bootstrap replicates. Bar 0.01 indicates nucleotides substitution per site.

An examination of the phylogenetic tree (Figure 1) showed that the strains TBL 2, TBL 4, TBR 2, and TBR 4 were similar to *Streptomyces alboflavus* strain NRRL B 2372 (98% similarity). TBF 2 had similarity with *S. longwoodensis* (100%), TBL 32 has homology with *S. galbus* (99%), while TBL 24, TBL 25, and TBL3 isolates had > 99% homology with *S. tendae* and *S. tritolerans*. *Actinomadura*, a non-*Streptomyces*, has been incorporated as an outgroup in the phylogenetic reconstruction.

The diversity of endophytic actinobacteria determined by the metagenomic approach

In the metagenomic approach, the community structure of endophytic actinobacteria was assessed using the PCR-DGGE technique. The amplified PCR product contained a total of 11 separated bands which were excised from the polyacrylamide gel (Figure 2A). The band distribution pattern indicated the presence of several communities of actinobacteria in the sample. The CLIQ 1D pro program was used to interpret the result of separated DGGE bands from the polyacrylamide gel (Figure 2B). The Shannon-Wienner (H') index that was used to appraise the actinobacteria endophyte community based on the 16S rRNA gene ranged from 1.191 to 2.094 for isolates from the different parts of the plant. The highest diversity index was found in the stem, while the lowest was found in the fruit. However, diversity in all four parts of the plants was still moderate as the indices fell within the Shannon index range of 1> H> 3.5 (Table 3). In this study, the Simpson index ranged from 0.65 to 0.87, indicating an evenness of *F. deltoidea* endophytic actinobacteria that was moderate to high in diversity.

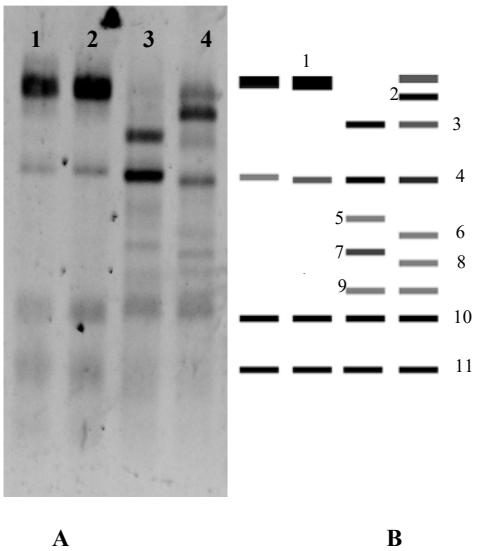

Figure 2. A. DGGE profile of actinobacteria endophytes from *F. deltoidea* based on 16S rRNA gene. B. Illustration of DGGE bands using 1D Phoretix software showing 1-11 excised bands. Line 1 (Fruit), 2 (Leaf), 3 (Root), and 4 (Stem)

Table 3. The alpha diversity indices of actinobacteria operational taxonomic units (OTUs) from samples root, stem, leaf, and fruit *F. Deltoidea*

Samples	No. of OTU	Shannon	Simpson
Root	7	1.82	0.82
Stem	9	2.09	0.87
Leaf	4	1.19	0.65
Fruit	4	1.31	0.71

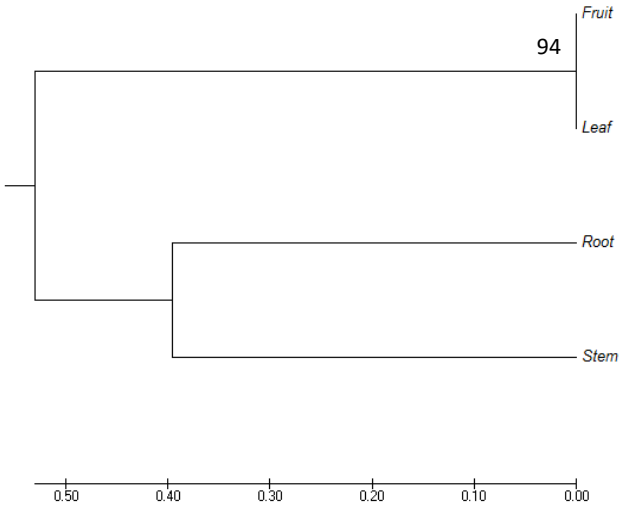

Figure 3. Clustering of similarity actinobacteria endophytes community found in *F. Deltoidea* using UPGMA. The scale below the dendrogram represents the evolutionary distance value.

Clustering analysis, which compared the similarity among the samples based on the position of the DGGE band, divided the plant samples into two main actinobacteria community structures. The cluster analysis based on the bands distribution patterns showed that the community of actinobacteria in the root and stem with endophytic actinobacteria in fruit and leaf have similarities <50%. Meanwhile, endophytic actinobacterial community patterns on the leaf and fruit have >95% similarity. Root and stem have 60% similarity to their respect community of endophytic actinobacteria. The pattern of stem endophytic actinobacteria community was closer to that of the root than of the population found in the leaf or the fruit (Figure3).

Of the 11 separated bands obtained from the DGGE approach, ten bands were successfully amplified and sequenced. The sequences were aligned to the reference data available in GenBank using EzBioCloud web (Table 4). Band 1 showed 100% similarity to an uncultured *actinobacterium clone.* Band 2 had 98.32% similarity to *Kineospora rhizophila* strain DSM 44908, while Band 3 had 99.44% homology with *Streptomyces jeddahensis* strain G25. Bands 4 and 7 showed similarities to *Streptomyces gamaensis* strain NEAU Gz11 (99.44%). Band 5 had 99.42% homology with *Streptomyces*

rapamysinnicus strain NRRL B 549. Band 6 displayed 98.87% identity with *Actinomadura algenensis* strain ACD1 while Band 8 had 97.79% similarity with *Intrasporangium calvum* strain DSM 40543. Band 10 showed 96.20% homology with *Verrucosispora gifhomensis* DSM 44337. The 11th band had a close relationship (99.88%) with *Rhodococcus kroppenstedtii.* Strain DSM 44908. The phylogenetic tree analysis presented in Figure 4 incorporates *Streptococcus mutans* as an outgroup. The metagenomic approach showed generally higher diversity due to the emergence of the *F. deltoidea* non-*Streptomyces* genera that were not culturable.

The OTU relative abundance chart lists the genera from highest to lowest abundance in different parts of the plant (Figure 5). It can be seen that the OTUs of 4, 10 and 11 occurred in every part of the plant examined. OTU 1 appeared in every part of the plant except the root, whereas OTU 3 and OTU 9 were found only in the root and stem. OTU 5 and 7 were found just in the stem, while OTU 2, 6, and 8 were only isolated from the roots (Figure 6).

Table 4. Percent similarity of the sequences of 16S rRNA gene from actinobacteria endophytes from *F. deltoidea* using PCR-DGGE.

Band code	Species	*Strain*	*Similarity (%)*	*Accession number*
1	Uncultured actinobacterium clone 3G1820-35	-	100	
2	*Kineospora rhizophila*	DSM 44908	98.32	FR749986
3	*Streptomyces jeddahensis*	G25	99.44	LOHS01000151
4	*Streptomyces gamaensis*	NEAU Gz11	99.44	KT963951
5	*Streptomyces rapamysinnicus*	NRRL B 5491	99.42	EF408733
6	*Actinomadura algenensis*	ACD1	98.87	KT259320
7	*Streptomyces gamaensis*	NEAU Gz11	99.44	KT963951
8	*Intrasporangium calvum*	DSM 40543	97.79	GCA000184685.1
9	Unidentified	-	-	-
10	*Verrucosispora gifhomensis*	DSM 44337	96.20	AF131630.1
11	*Rhodococcus kroppenstedtii*	DSM 44908	99.88	jgi.1107894

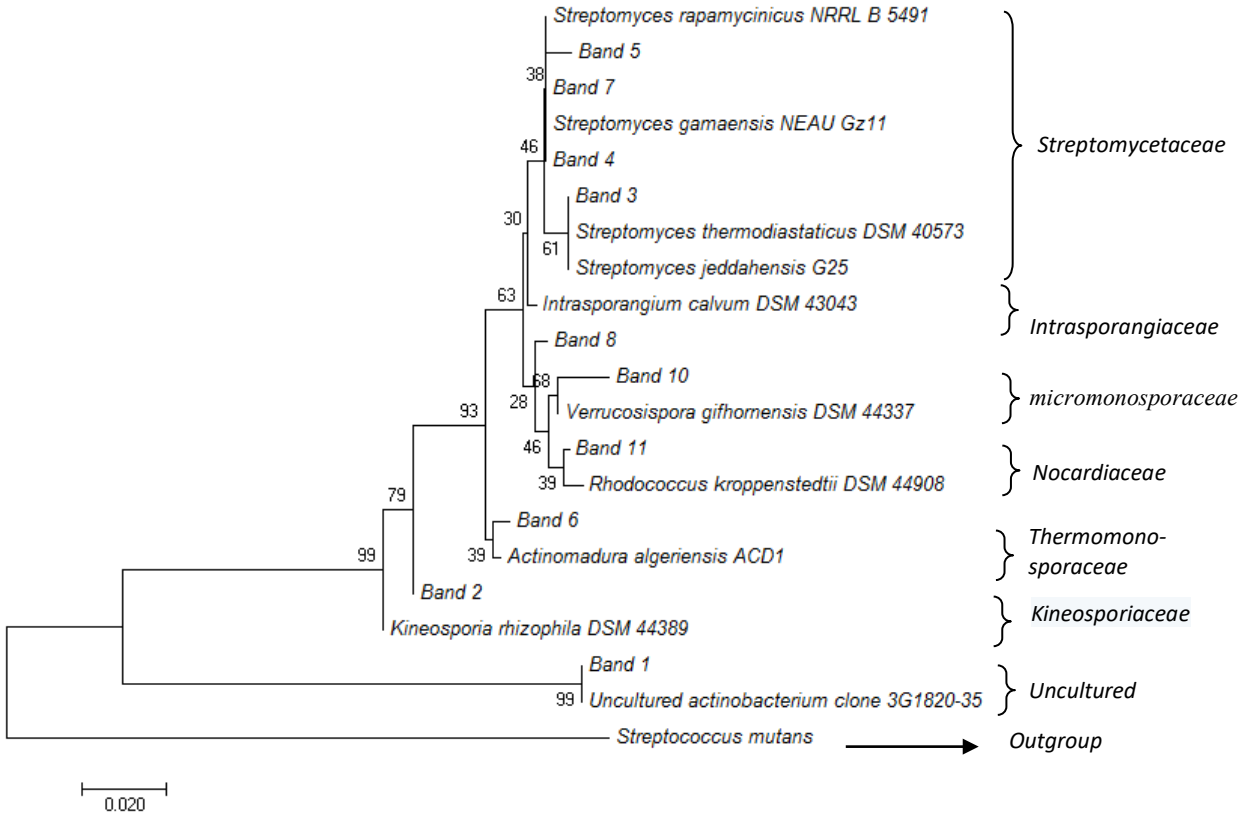

Figure 4. Neighbour joining tree community structure of actinobacteria endophytes *F. deltoidea* based on 16S rRNA using PCR-DGGE. Numbers at each nodes indicate the percentages of branch support of 1,000 bootstrap replicates. Bar 0.02 indicates nucleotides substitution per site

Discussion

The distribution of culturable actinobacteria endophytes in the *F. deltoidea* tissue was based on the number of colonies that grew on each part of the plant. The leaf was the part mostly colonized by actinobacteria endophytes. About 60%, 20%, 15%, and 5% of actinobacteria endophytes lived in the leaf, stem, root, and fruit tissue of *F. deltoidea,* respectively. The leaf of *F. deltoidea* is the plant part most widely used by local communities as traditional medicine to treat various illnesses because it contains bioactive compounds. It is possible that the bioactive compound production may be influenced by endophytic actinobacteria that live in plant tissues (Qin et al. 2011).

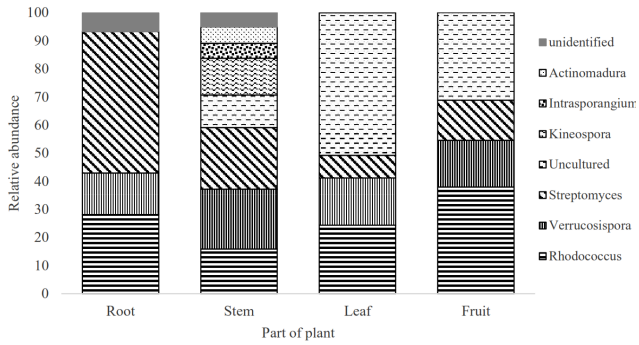

Figure 5. Abundances of different genera in actinobacteria in samples of root, stem, leaf, and fruit base on OTU. The same actinobacteria genera are displayed in the same pattern

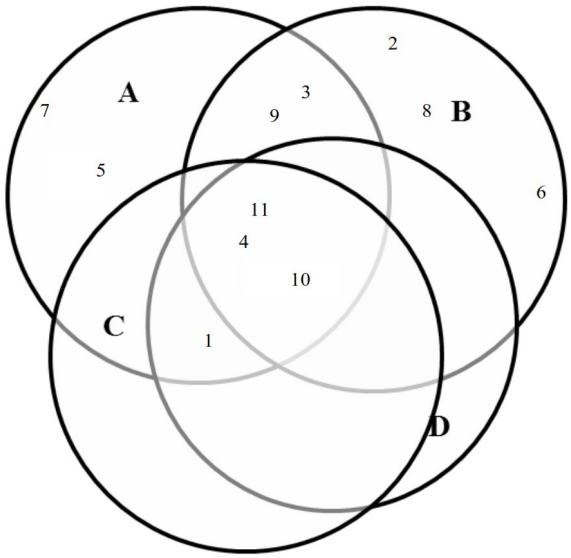

Figure 6. Venn diagrams of actinobacteria showing OTU in each f the samples; stem (A), root (B), Leaf (C), and Fruit (D) in F. deltoidea. The numbers indicate shared and unique OTUs in the four types of samples

In the present research, all isolates of actinobacteria endophytes that were cultured on HVA medium appeared to belong to the genus *Streptomyces* based on their morphological characters and their 16S rRNA genes. *Streptomyces* is the most abundant genus of the actinobacterium phylum. There are about 7630 secondary metabolite compounds produced by actinobacteria that are derived from *Streptomyces* (Qin et al. 2011). The original habitat, the soil may also influence the capability of *Streptomyces* especially its various endophytic species to produce a variety of secondary metabolites.

The metagenomic approach was used to investigate the diversity of microorganisms as a whole without invoking the culturing steps. PCR-DGGE is a simple but widely accepted metagenomic method to examine the diversity of a microbial community. The relative abundance of the OTUs based on the 16S rRNA gene was analyzed to illustrate the relationship between abundance and evenness of the actinobacteria endophytic community in each part of the plant. It aimed to determine the distribution of actinobacteria endophytes isolated from each part of the plant. In the present study, the number of actinobacteria communities, based on the OTU value of each sample, was dissimilar in different parts of the plant. The root and stem had nine and six OTUs respectively, the difference not being statistically different. It appeared that the root and stem had higher actinobacteria endophyte diversity compared with the leaf or fruit. Actinobacteria endophyte diversity also varied among individual plants.

The relatively high diversity of endophytic actinobacteria found in the stem and root may indicate that the endophytic actinobacteria colonization originated from the soil (Mahyarudin et al. 2015), with actinobacteria in the rhizosphere first colonizing the root, aided by the root chemotactic signals (Berendsen et al. 2012). The Shannon and Simpson indices were used to determine both diversity and evenness of the endophytic actinobacteria in *F. deltoidea* tissue. The Shannon-Wienner (H') diversity analysis was used to estimate the microbial diversity in each sample based on the volume of the DGGE band analyzed using the CLIQS 1D pro software. The diversity index of endophytic actinobacteria in the *F. deltoidea* was moderate, falling within the Shannon index range of 1> H> 3.5. The Simpson index was used to determine the level of dominance or evenness of a community. On a scale of D = 0 (no dominant species) to D =1 (dominant species prevailing), endophytic actinobacteria in the *F. deltoidea* that ranged from 0.65 to 0.87 indicated no predominance of any species and evenness of actinobacteria that were moderate to high in diversity.

The abundance of actinobacteria endophytes in *F. deltoidea* is attributed to six genera, viz. *Streptomyces, Verrucosispora, Rhodococcus, Kineospora*, *Intrasporangium* and *Actinomadura*, and one OTU having similarity with uncultured actinobacterium. There were 11 different actinobacteria endophytic communities (11 different OTUs), but not all the communities were evenly distributed throughout the plant. Amongst them, only three OTUs were present in all the parts of the plant, viz. OTU 4 (*Streptomyces*), OTU 10 (*Verrucosispora*), and OTU 11

(*Rhodococcus*). According to relative abundance, these three dominant genera might have essential roles in producing various kinds of bioactive compounds that exist in the *F. deltoidea* plants. Also, the presence of the same OTU in every part of the plant examined suggested that certain actinobacteria endophytes might have migrated throughout the plant. However, it is possible that only some endophytic actinobacteria could thrive in the distal tissues of the leaf and fruit, leaving the highest diversity found in plant tissues close to the soil. The endophytes could have originated from the surrounding environment of the plant, such as the rhizosphere or the phyllosphere. They might enter the plant tissue through stomata, lenticels or physical injuries or areas in which the lateral roots grow (Shimizu et al. 2011).

The cultivated endophytic actinobacteria had morphological characteristics similar to those of the genus of *Streptomyces*. This finding was consistent with the molecular identification based on the 16S rRNA gene, which also showed that the cultured isolates had close similarity with *Streptomyces* sp. On the other hand, based on the metagenomic approach using PCR-DGGE, 11 different communities of endophytic actinobacteria were isolated in the *F. deltoidea* tissue, four of which were *Streptomyces*. The other communities were of the non-*Streptomyces* groups, *viz. Verrucocispora, Kineospora, Rhodococcus*, *Intrasporangium,* and *Actinomadura.* Regarding abundance based on OTUs, *Verrucocispora, Streptomyces,* and *Rhodococcus* were prominent. The OTUs data from *F. deltoidea* put the *Streptomyces* community in second place after the *Rhodococcus* community. These data differed from the cultivation results, probably due to the differing culture requirements that might favor some actinobacteria over others when plated on a specific culture medium (Jiang et al. 2007; Sun et al. 2010). For example, the Humic Vitamin Agar (HVA) medium, a selective medium that suppresses the growth of Gram-negative bacteria, could favor *Streptomycetes*, fungi, and fast-growing bacteria. The composition of medium containing poor nutrients causes this medium to suit only bacteria that can adapt to the minimal nutrients such as *Streptomyces*. Moreover, actinobacteria colonies that were initially isolated on HVA were subsequently purified on International Streptomyces Project (ISP) 2 medium that favored the growth of *Streptomyces*. Hence, alternative culture media might be considered to isolate other culturable endophytic actinobacteria besides *Streptomyces* in future research. On the whole, nevertheless, the metagenomic approach can be said to be more useful in describing the diversity of endophytic actinobacteria existing in the *F. deltoidea* plant tissue.

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