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Original Article

COMMUNITY STRUCTURES OF ENDOPHYTIC ACTINOBACTERIA FROM MEDICINAL PLANT *CENTELLA ASIATICA* L. URBAN-BASED ON METAGENOMIC APPROACH

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ABSTRACT

Objective: This study aimed to assess the community structure of actinobacteria in rhizosphere and endophyte of a medicinal plant, *Centella asiatica*, based on a metagenomic approach using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) of 16S rRNA gene.

Methods: Total genomic DNA was extracted from the rhizosphere and plant tissue followed by PCR amplification of actinobacterial 16S rRNA gene using nested PCR. The community structure of actinobacteria was analyzed using the DGGE techniques on polyacrylamide gels. PCR products of excised bands result from polyacrylamide gel were sequenced and analyzed by bioinformatics software to construct a phylogenetic tree.

Results: The results of separation in DGGE gel showed 16 major bands from rhizosphere and plant tissue. The bands distribution pattern showed that the community of actinobacteria in the plant tissue was slightly more diverse than rhizosphere, although it is not significantly different based on Shannon-Wiener analysis. The BLAST. N analysis showed that 7 bands related to *Streptomycetaceae* (83-100%), 5 bands related to *Micromonosporaceae* (99-100%), 1 bands related to *Gordoniaceae* (99%) and 3 bands still belonged to unculturable (87-99%). There were 6 genera under those 3 families, i.e. *Streptomyces, Micromonospora*, *Verrucosispora*, *Actinoplanes*, *Couchioplanes*, and *Gordonia.* The percentage of strain similarity comparison to the database showed that there were 4 bands with<97% maximum identity which may be related to novel endophytic actinobacteria in *C. asiatica*.

Conclusion: Diversity of endophytic actinobacteria based on a metagenomic approach using 16S rRNA gene-targeted PCR-DGGE analysis was found associated with *C. asiatica*. Several of them may have potency as novel actinobacteria and can be further explored for their medicinal function.

Keywords: *Centella asiatica*, PCR-DGGE, Endophytic Actinobacteria, Metagenomic, 16S rRNA

INTRODUCTION

Centella asiatica (synonym: *Hydrocotyle asiatica* L.), belonging to the family of *Mackinlayaceae* is native to most of the countries of Asia, including Indonesia. It has been claimed that *C. asiatica* has various physiological effects and traditionally used for various diseases, including wound healing, diabetes, cancer, alzheimer, hypertension, improve memory ability, and many others [1]. It is used in traditional and alternative medicine due to the wide spectrum of pharmacological activities associated with these secondary metabolites. The biological function of *C. asiatica* may be associated with the presence and diversity of endophytic microbes including actinobacteria in the plant tissue. Endophytic microbes have the ability to produce bioactive compounds or secondary metabolites similar to its host plant. This ability may be due to genetic transfer within the evolution of host plant to the endophytic microbes [2]. Endophytic microbes such as actinobacteria from *C. asiatica* has not been widely studied. Endophytic actinobacteria originated from *Tinospora crispa* are known as the producer of secondary metabolites which can function as inhibitor alpha-glucosidase [3, 4].

It has been reported that 9 species of endophytic bacteria isolated from *C. Asiatica* leaves had antioxidant and antibacterial activity [6]. The diversity of endophytic actinobacteria of *C. asiatica* has not been reported. The knowledge of the genetic diversity of endophytic actinobacteria in *C. asiatica* is important as basic information to explore the function of endophytic actinobacteria from this plant. The biodiversity of microbes in nature is enormous, but about 99% of microbes still cannot be cultured, so there is not enough data on microbial diversity as well as their potencies [7]. Identification through culture-dependent method has been widely used to analyze a small portion of the total microbes. Moreover, some slow-growing actinobacteria are also difficult to be cultured [5]. Thus, the technology to explore a large number of endophytic actinobacteria that cannot be cultured is needed. Analysis of actinobacteria diversity using meta genomic approach is a relatively new method in the study of microbial communities that is based on analysis of DNA taken directly from the environment (without culturing step) [8]. An application of PCR-DGGE (*Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis*) to examine the diversity of actinobacteria in nature has been previously used. In this technique, every DNA that appear may represent a distinct actinobacteria species [9]. This study aimed to assess the diversity of actinobacteria in the rhizosphere and endophytic of *C. asiatica* through metagenomic approach using 16S rRNA gene targeted PCR-DGGE analysis.

MATERIALS AND METHODS

Collection and surface-sterilization of plant samples

Samples of plant *C. asiatica* were collected from the rice field's area, located at Situ Gede village, Bogor, West Java, Indonesia. Samples were taken from the rhizosphere soil and plant parts such as roots, stolons, and leaves. The surface-sterilization process of the plant tissue samples was done according to Coombs and Franco [10], with modification. The parts of plants that have been cut were soaked in 70% alcohol for 1 min, sodium hypochlorite (NaOCl) 1% for 5 min, 70% alcohol for 1 min, and rinsed in sterile distilled water for 3 times. A total of 0.1 ml of the last rinse water was inoculated to HV media (Humic Acid–Vitamin B Agar) and incubated for 1 mo, as a negative control to test the effectivity of the surface-sterilization samples.

Genomic DNA extraction from rhizosphere soil and plant tissues

Total genomic DNA from the rhizosphere samples was extracted according to the protocol using Power Soil® DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) with modification (increase the vortex time by an additional 15-20 min). While the DNA from

plant tissue (root, stolon, and leaf) was extracted using the Genomic DNA Mini Kit, Plant (Geneaid, Shijr, TPE, TW). The concentration and purity of the DNA were evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

PCR Amplification of 16S rRNA gene

The DNA amplification was performed using PCR by T1-thermocycler (Biometra, Goettingen, Germany). The 16S rRNA genes of actinobacteria were amplified using actinobacteria-specific primers by two steps of PCR. The first PCR was carried out with the forward primer 27F and the reverse primer 16Sact1114R [11] (table 1). Total 25 µl of reaction mix contained; 12.5 µl of GoTaq Green Master Mix 2× (Promega, Madison, WI, USA), 0.25 µl of each primer (60 pmol), 4 µl of DNA template (~100 ng μl^{-1}), and 8 µl nuclease free water. The amplification of DNA using PCR was done with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing for 45 s at 65°C (0.5 °C decrease per cycle for the first 20 cycles, finally down to 55°C), extension at 72 °C for 2 min, and final extension at 72 °C for 7 min.

The second PCR was carried out with the forward primer 338F with GC clamp and the reverse primer 518R [12] (table 1). For the second PCR, 1 µl of the first PCR product was used as a template. The 50 µl reaction mix contained 25 µl of GoTaq Green Master Mix 2× (Promega, Madison, WI, USA), 0.5 µl of each primer (100 pmol), 1 µl of DNA template, and 23.5 µl nuclease free water. The PCR conditions used were: initial denaturation step at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 92 °C for 1 min, annealing at 60°C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 7 min. The PCR product was visualized by using G: BOX gel documentation (Syngene, Frederick, MD, USA) on 1% of agarose gel electrophoresis (0.25 g agarose+25 ml 1× TAE buffer).

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

Primers	Sequences (5'-3')	Reference
27F	AGAGTTTGATCCTGGCTCAG	[11]
16Sact1114R	GAGTTGACCCCGGCRGT	[11]
P338F	ACTCCTACGGGAGGCAGCAG	[12]
P518R	ATTACCGCGGCTGCTGG	[12]
GC clamp*	CGCCCGCCGCGCGCGGCGGGCGGGCGGGGGCACGGGGGG	[12]

*GCclamp, sequences were added at the 5 'end primer P338F.

DGGE analysis of 16S rRNA gene

DGGE analysis was carried out using D-Code system from Bio-Rad Laboratories. The PCR product (35 µl; 30 µl DNA+5 µl *Loading Dye*) was loaded onto vertical gel containing 8% (w/v) polyacrylamide gel (acrylamide-bis acrylamide, 37.5:1), 16×16 cm size, 1 mm thick, in 1× TAE buffer (Tris–acetate-EDTA). The denaturing gradient contained a linear denaturing gradient from 30 to 70% [100% denaturant corresponded to 7 M urea and 40% (v/v) deionized formamide]. Electrophoresis was carried out at a constant voltage of 150 V and 60 °C, for 5-6 h. After electrophoresis, the gels were stained for 60 min with 30 ml of SYBR Safe (Molecular Probes, Invitrogen, Carlsbad, CA, USA) in 270 ml of TAE buffer in dark conditions. The gel DGGE result was analyzed using 1D Phoretix software (Total Lab) to estimate the total bands that appeared on polyacrylamide gel scanned by G: BOX gel documentation (Syngene, Frederick, MD, USA). Bands were excised from the polyacrylamide gel a using sterile scalpel and put into microtube containing 100 µl ddH_2O. The microtube was incubated at 4 °C overnight and 60 °C for 2 h. Each supernatant (10 µl, ~50 ng μl^{-1}) was used as the PCR template with the primers P338F (without GC-clamp) and P518R. The PCR condition was similar to PCR step 2.

Statistical analysis of DGGE profile was conducted by using alpha and beta diversity. The diversity within each sample was analyzed using an alpha diversity (Shannon-Wiener/*He*). The similarity of band pattern between the samples was also analyzed using beta diversity (*Dice similarity coefficient/SD*). The statistical analysis was performed using PAST Software based on the estimation analysis of band intensity using Phoretix 1D software (data not shown). The index was calculated as follows:

$$He = \sum_{i-1}^{S} Pi \ln pi$$

$$SD = 2Nc/(Na + Nb)$$

Where, S is the total number of bands in each lane; pi is the ratio of the intensity of a single band of the total intensity of all bands within the same lane; Na is the number of bands detected in the sample a; Nb is the number of bands detected in sample b; Nc is the number of bands detected in both samples [13].

16S rRNA gene sequencing, bioinformatics analysis, and phylogenetic tree construction

The PCR products from DGGE fragments were sequenced using DNA sequence (ABI PRISM 3100) in First Base Co. Sequencing results were analyzed using BLAST (*Basic Local Alignment Search Tool*) from NCBI. Phylogenetic analysis was conducted from BLAST result using the neighbor-joining method with bootstrap 1000× in 5.00 MEGA (*Molecular Evolutionary Genetics Analysis*) software.

RESULTS

PCR-DGGE profile of actinobacteria based on 16SrRNA gene

Analysis of the 16S rRNA gene of actinobacteria was carried out by 2 steps of PCR using two sets of specific primers for actinobacteria detection. The first primer set was 27F and 16Sact1114R with product size ~1087 bp. ThePCR product was used as a DNA template for the second PCR. The second primer set was P338F and P518R with product size ~180 bp (fig 1).

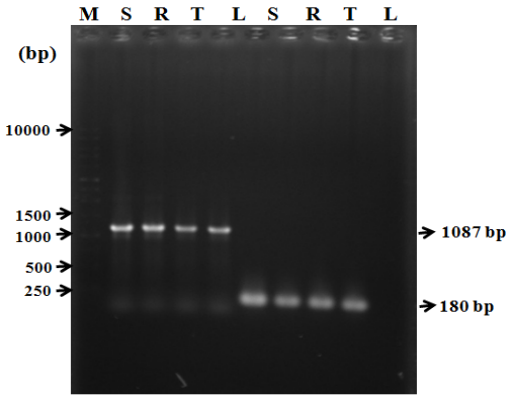

Fig. 1: PCR amplification of 16S rRNA gene from rhizosphere and plant tissues of *C. asiatica*, the first steps (left: ~1087 bp) and second steps (right: ~180 bp), (M): marker 1 Kb; (S): rhizosphere; (R): roots; (T): stolons; (L): leaves

The PCR product of 16S rRNA gene was electrophoresed with a DGGE method to analyze the diversity or endophytic actinobacteria communities. Each separated bands on a polyacrylamide gel has the same length of DNA fragments (~180 bp) but with different base sequences. A total of 16 major bands were excised from the polyacrylamide gel (fig 2A). Bands distribution pattern showed the various communities of actinobacteria in the sample. Interpretation from Phoretix 1D of the DGGE profile showed that the community of

actinobacteria in the plant tissue was slightly more diverse than those of rhizosphere.

There were 16 bands in the leaves, 14 bands in stolons, 12 bands in the roots, and 10 bands in the rhizosphere (fig 2B). This data was also supported by an index value of alpha diversity (Shannon-Wiener/*He*) (table 2).

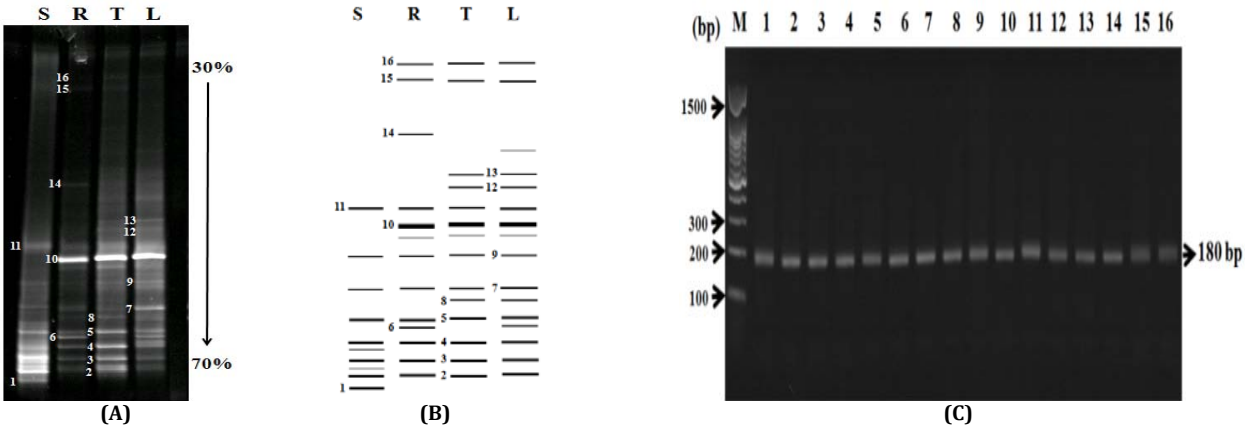

Fig. 2: A): DGGE band profile of the PCR products of 16S rRNA gene from rhizosphere and plant tissues of *C. asiatica*, B): Illustration of DGGE bands using 1D Phoretix software showing 1-16 excised bands, C): Re-amplification DGGE bands, (S): rhizosphere; (R): roots; (T): stolons; (L): leaves

Diversity analysis of actinobacteria based on 16S rRNA gene

In this study, Shannon-Wiener (H*e*) analysis were used to estimate the microbial diversity in each sample based on the result of band intensity analysis using Phoretix 1D software.

The high index value indicated the highest diversity of species (bands) within a sample. Although, H*e* index value was not significantly different (2.176-2.57) in each sample, but the actinobacteria diversity was higher in the plant tissues compared within the rhizosphere samples (table 2).

Table 2: Alpha diversity (Shannon-Wiener) of rhizosphere and endophytic actinobacteria community

Index value	Rhizosphere	Plant tissue (*C. asiatica*)		
		Roots	Stolons	Leaves
Shannon H*e*	2.176	2.261	2.406	2.570

The dice similarity coefficient (SD) analysis was used to describe the similarity of species (band) composition between different types of samples. SD index value approach to 1 indicated high similarity of the structure composition between the compared samples. The result of endophytic actinobacteria (in the leaves, stolons, and roots) showed the community structure among the samples shared relatively high similarity with SD index value 0.786-0.933. The highest index value was found in samples of leaves with stolons (0.933). While, the community structure in the rhizosphere and plant tissues (leaves, stolons, and roots) shared relatively low similarity with SD index value 0.538-0.636 (table 3). This data was also supported by the cluster analysis using binary data (fig 3).

Table 3: Beta diversity (Dice similarity coefficient) of rhizosphere and endophytic actinobacteria community

Samples	Rhizosphere	Roots	Stolons	Leaves
Rhizosphere				
Roots	0.636			
Stolons	0.583	0.846		
Leaves	0.538	0.786	0.933	

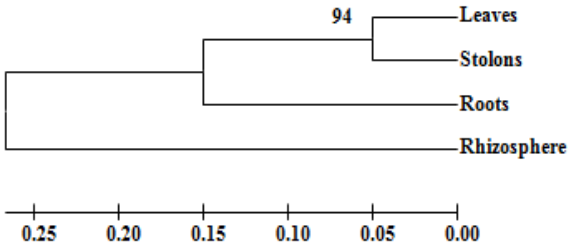

Fig. 3: Cluster of actinobacterial community similarity in rhizosphere and plant tissues of *C. Asiatica*

Cluster analysis was done to see the closeness relationship among the samples. The cluster analysis based on the bands distribution patterns showed that the community of actinobacteria in the rhizosphere and endophytic have similarities <75%. Endophytic actinobacterial community patterns on the leaves have 95% similarity with stolons, and both are different from 10% with roots (fig 3A). This result was also confirmed by the results of the dice similarity coefficient (SD) analysis (table 3).

Phylogenetic tree of actinobacteria based on 16SrRNA gene

Sixteen bands were excised from the polyacrylamide gels, reamplified, and sequenced. BLAST. N analyses revealed that 16 bands (83%-100% of maximum identity) were closely related to 3

families of actinobacteria. A total of 7 bands were related to *Streptomycetaceae*, 5 bands related to *Micromonosporaceae*, and 1 band related to *Gordoniaceae*. Meanwhile, three other bands, namely bands 6, 10, and 13 still belonged to unculturable species. There were 6 genera under those families, consist of *Streptomyces*, *Micromonospora*, *Verrucosispora*, *Actinoplanes*, *Couchioplanes*, and *Gordonia.* The study found that *Streptomyces* species was dominant in all samples. The BLAST. N analysis also showed that bands 6, 12, 13, and 16 have similar identity lower than 97% of the total sequence ~180 bp, this may indicate a novel species. Details of the sequence identities and their similarity percentages were given in table 4. Phylogenetic analysis showed that the whole sequence of the bands has a good consistency with their affiliation and separated from their out-group *Pseudomonas aeruginosa* RJ 16 (Gram-negative bacteria) (fig 4).

Table 4: Percent similarity of the sequences of 16S rRNA gene from rhizosphere soil and endophytic actinobacteria originated from *C. asiatica*

Family	Bands	References strain (GenBank)	Query cover (%)	Similarity (%)	Range	Accession no.
Gordoniaceae	1	*Gordonia otitidis* IFM 10032	100	99	328-490	NR_040988.1
Micromonosporaceae	2	*Verrucosispora wenchangensis* 234402	99	100	336-497	NR_117920.1
	3	*Couchioplanes caeruleus* SCC 1014	99	100	305-466	NR_037054.2
	11	*Micromonospora schwarzwaldensis* HKI0641	99	99	319-480	NR_118561.1
	7, 15	*Actinoplanes brasiliensis* IFO13938	99	100	319-480	NR_115628.1
Streptomycetaceae	4	*Streptomyces graminilatus* JL-6	100	100	321-483	NR_125579.1
	5	*Streptomyces rapamycinicus* ATCC 29253	100	99	290-452	NR_044199.1
	8	*Streptomyces acidiscabies* ATCC 49003	100	99	325-487	NR_116534.1
	9	*Streptomyces acidiscabies*ATCC 49003	100	100	325-487	NR_116534.1
	12	*Streptomyces* sp. SPMA 134	100	83	255-418	HQ_340166.1
	14	*Streptomyces acidiscabies* ATCC 49003	100	98	325-487	NR_116534.1
	16	*Streptomyces atacamensis* C60	98	94	314-476	NR_108859.1
Unculturable	6	Uncultured bacterium isolate DGGE gel band sxvpb21	100	87	19-180	KC_961605.1
	10	Uncultured bacterium clone B03_294	100	99	252-411	KM_498329.1
	13	Uncultured bacterium clone 7N227hH82	100	96	252-411	KJ_853606.1

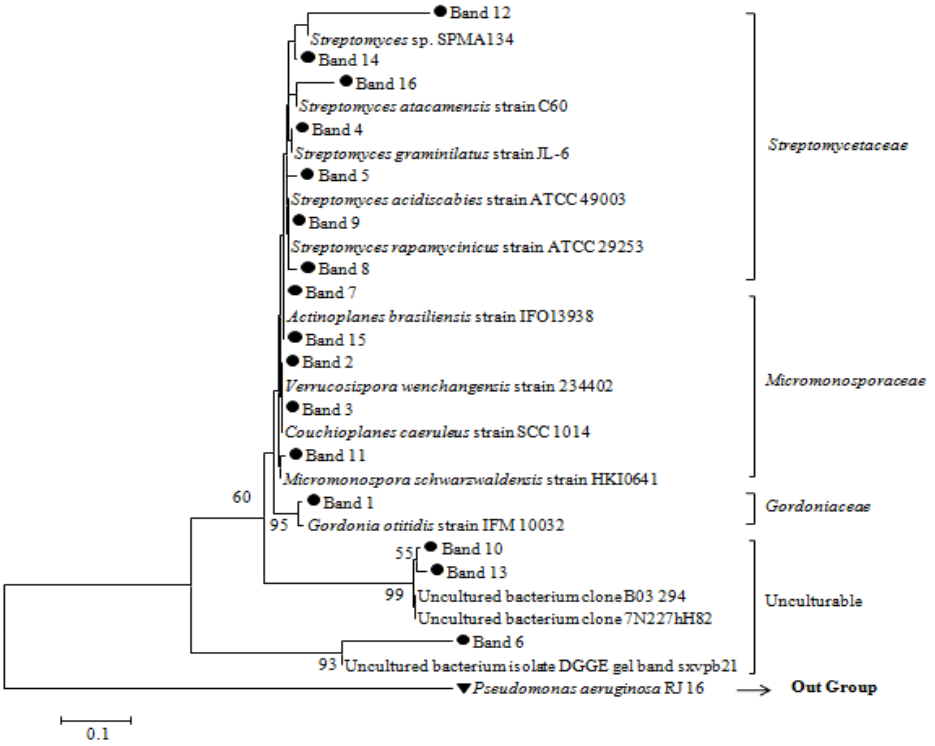

Fig. 4: Phylogenetic tree of actinobacteria based on 16S rRNA gene of DGGE product

Abundance of actinobacteria in rhizosphere soil and endophytic *C. asiatica* based on 16S rRNA gene targeted PCR-DGGE

One species were found only in the rhizosphere with 99% of similarity with *Gordonia otitidis* IFM10032 (fig 5A). The DGGE results were also obtained 8 species that were only found as an endophyte in *C. asiatica*, one species was only found in the roots (fig 5B), namely *S. acidiscabies* ATCC 4900346. Three other endophytic species were only found in the leaves and stolons i.e. uncultured bacterium clone 7N227hH82, *Streptomyces* sp. SPMA 134 and *S. acidiscabies* ATCC 4900346. Bands similar to *S. atacamensis* C60, Uncultured bacterium sxvpb21, and uncultured bacterium clone B03_294 were found as endophytic in the leaves, stolons and roots of *C. asiatica*.

Seven species from *Micromonosporaceae* and *Streptomycetaceae* can be found on the rhizospere and as endophytic (fig 5A and 5B), such as *C. caeruleus* SCC 1014, *M. schwarzwaldensis* HKI0641, *V. wenchangensis* 234402T, *A. brasiliensis* IFO13938, *S. graminilatus* JL6, *S. acidiscabies* ATCC 49003 and *S. rapamycinicus* ATCC 29253.

(A) **(B)**

Fig. 5: Abundance of actinobacteria community in, A): rhizosphere (S) and endophytic (E), B): Endophytic actinobacteria in, (T): stolons; (R): roots; (L): leaves

DISCUSSION

The application of molecular techniques to detect and identify microorganisms using certain molecular markers, such as 16S rRNA or its encoding gene, is now more frequently used to explore the microbial diversity and to analyze the structure of microbial communities [14]. The 16S rRNA gene-targetedPCR product of actinobacteria in rhizosphere soil and plant tissue of *C. asiatica* was analyzed using 2 sets of specific primer for the detection of actinobacteria [11]. Primer 27F was designed to amplify all domains of bacteria and 16Sact1114R was designed from 202 actinobacteria with a 1.3% false result. While primer of P338F and P518R were designed to amplify all V3 region of bacteria [12]. These primers have been used by Primanita *et al.* [15] to study the genetic diversity of endophytic actinobacteria from the medicinal plant of *T. crispa* by PCR-DGGE.

The bands distribution pattern on DGGE gel showed that the community of actinobacteria in the plant tissue was slightly more diverse than those of rhizosphere soil (fig 2A). Higher colonization of endophytic microbes in the leaves could be due to the position of *C. asiatica* leaves that was low and close to the soil. This condition facilitates the penetration and the colonization of endophytes in the leaf tissue [16]. Endophytic microbes move to the host plant through chemotaxis mechanisms, electrotaxis, or direct contact, and penetration into the plant tissue through wounds, stomata, lenticels, and the root zone [2]. Competition among microbial communities might influence to low diversity of actinobacteria in the soil. Population and diversity of microbial endophytes are influenced by various factors such as environmental and soil conditions, location, type of plant, age of the plant, and the type of plant organ [2]. Similar results were also reported by Primanita *et al.* [15] that the abundance of endophytic actinobacteria on the medicinal plant *T. crispa* showed greater diversity than those of the rhizosphere soil (non-endophyte).

The study shows that several similar endophytic actinobacteria are found in different plant organs (fig 5B). Endophytic microbes can migrate to other plant organs through the intracellular and vascular system [17]. Distribution of endophyte within plants depends on the combination of the ability to colonize and the allocation of plant resources. In addition, different plant tissues can also harbor compositionally distinct endophytic communities [18].

The dominant community and the intensity of each band indicate their relative abundance [5]. In our study, the band number 10 (99% similarity with Uncultured bacterium clone B03_294) has the highest abundance and found in all parts of plant tissue (leaves, roots, and stolons) (fig 2A). This band might be representing the endophytic actinobacteria, which are able to establish an association with their host plant, i.e. C. asiatica. This community cannot be found in the rhizosphere which may be due to strong competition with other rhizospheric microbes. Unculturable microbes have a great potential as a source of new bioactive compounds that were important in many fields [7].

Phylogenetic analysis showed that the three families, i.e. *Gordoniaceae, Streptomycetatceae* and *Micromonosporaceae*. The community of *Gordoniaceae* family found only in the rhizosphere samples. *Gordoniaceae* appears to be widely distributed in nature, and strains have been isolated from environments such as soil, mangrove rhizosphere, and oil-producing wells, as well as from clinical samples [19]. In the medical field, the genera *Gordonia* was known to degrade steroid compounds such as cholesterol [20]. The second family is *Streptomycetaceae.* Several species of *Streptomyces* are commonly found in the rhizosphere as well as endophytes. Some researchers also showed similar results in which *Streptomyces* can be found in the stem, soil and roots of several plants [17]. *Streptomyces* are ubiquitous microorganisms living mostly in the soil and environments. These members have a wide range of metabolic abilities and potential applications in the production of bioactive compounds which are important in pharmaceutical industries [21]. Endophytic *Streptomyces* spp. have been isolated from anti-diabetic medicinal plants and they can function as an enzyme inhibitor. *Streptomyces* sp. isolated from *T. crispa* has the capability to produce an inhibitor of alpha-glucosidase [3, 4]. While *S. longisporoflavus* which was isolated from *Leucas ciliata* is known to produce inhibitor alpha-amylase [22].

DGGE profiles found in this study suggest that the community of the family *Micromonosporaceae* can be found in all samples, both rhizosphere soil and plant tissue of *C. asiatica*. Furthermore, *Micromonospora* is widespread in nature, including soil and have recently been known that the genus is able to form associations with plants, as an endophyte in the roots of rice plant [17]. In the medical field, *Micromonospora* endophyte was known to produce many antibiotics, such as anthraquinone, and lupinacidins A and B (antitumor) [23]. *M. schwarzwaldensis* HKI0641 isolated from soil samples in the Black Forest, Germany was also known to produce antibiotics telomycin [24]. Genera *Verrucosispora* (*V. wenchangensis* 234402T) has been isolated from mangrove land in Wenchang, China [25], and previously no reported as endophytes. The genera are being the focus of interest as they are the source of new bioactive compounds, such as proximicins compounds that can function as antibacterial and antitumor [26]. The diversity of endophytic actinobacteria from medicinal plants and their bioactivities exploitation such as for pharmaceutical potency has been extensively reviewed [26]. Our study adds new information regarding the diversity of endophytic actinobacteria in *C. asiatica.*

CONCLUSION

The community structure of actinobacteria in rhizosphere sample was correlated with that in the plant tissues such as leaves, stolons and roots of *C. asiatica*. The results open up the opportunity for further exploration on the novel species of endophytic actinobacteria with medical potency originated from *C. asiatica*.

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CONFLICT OF INTERESTS

Declared none

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