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DIVERSITY OF ACTINOMYCETES FROM EKA KARYA BOTANICAL GARDEN, BALI**

SHANTI RATNAKOMALA[1*], PUSPITA LISDIYANTI[1], NITA R. PRAYITNO[1], EVI TRIANA[2], YULIN LESTARI[3], RATIH D. HASTUTI[4], YANTYATI WIDYASTUTI[1], MISA OTOGURO[5], KATSUHIKO ANDO[5] and ENDANG SUKARA[1,4]

[1]*Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong 16911, Indonesia*
[2]*Research Center for Biology, Indonesian Institute of Sciences, Cibinong 16911, Indonesia*
[3]*Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Bogor 16680, Indonesia*
[4]*Soil Research Institute, Bogor 16002, Indonesia*
[5]*NITE Biological Resource Center (NBRC), 2-5-8 Kasuzakamatari, Kisarazu, Chiba, Japan*

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ABSTRACT

A total of 229 strains of actinomycetes were isolated and identified by full sequence of 16S rRNA gene analysis. Samples consisted of 18 soil and 20 leaf-litter were collected from Eka Karya Botanical Garden, Bali Island, Indonesia. Two isolation methods, i.e. SDS-Yeast Extract (SY) and Rehydration-Centrifugation (RC) were used in this study. Based on 16S rRNA gene analysis, isolated actinomycetes may be grouped into 28 genera. Based on molecular analysis of 16S rRNA gene similarities showed that isolated actinomycetes of Eka Karya Botanical Garden origin is diverse. Analysis on 144 isolates from soil samples, resulted in 24 genera and more than 87 species. *Streptomyces* is the most dominant genus where 65 isolates or 45% from isolated actinomycetes belong to this genus. It was followed by *Actinoplanes* (25 isolates = 17%). From leaf-litter samples, the total number of 85 isolates may be grouped into 9 genera and more than 41 species. The most dominated genus is *Actinoplanes* (42 isolates = 49%) followed by *Catenuloplanes* (16 isolates = 19%).

Keywords: 16S rRNA gene analysis, actinomycetes, biodiversity, Eka Karya Botanical Garden

INTRODUCTION

Actinomycetes are microorganisms belong to gram positive bacteria which are often saprophytic while some of them produce spores and mycelium. They play important roles in degrading and decomposing organic compounds in the soil. They may also produce secondary metabolites such as antibiotics, enzymes and other bioactive compounds for human welfare. Actinomycetes constitute a significant component of the microbial population in most soils and counted of over 1 million cells per gram of soil. Soil is, therefore, the most prolific source of this particular group. Soil represents the most intensively studied habitat of actinomycetes. Actinomycetes are also thought to be the most significant group in the degradation of relatively complex, recalcitrant polymers found naturally in plant litter and soil (Hopwood 2007; Baskaran *et al.* 2011).

The preliminary characterization of the actinomycetes isolates was colony appearance, observing of zoospore bearing isolates, and DAP analysis. DAP analysis itself is a biochemical analysis of cell walls to observe the DAP isomers of the cell wall of actinomycetes. Most of gram positive bacteria have lysine instead of DAP in their cell wall. Through this method, actinomycetes could be grouped into 3 groups. According to Miyadoh (2004), actinomycetes that have LL-DAP in their cell wall generally belong to genus *Streptomyces* and *Streptacidiphilus*; while those with *meso*-DAP type in their cell wall generally belong to the non-*Streptomyces* or so called rare actinomycetes. Actinomycetes that have both

* Corresponding author: shanti_ratna@ yahoo.com
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LL-DAP and meso-DAP usually belong to genus *Kitasatospora*. Therefore, the DAP analysis could differentiate those three types of actinomycetes.

Some actinomycetes share the same ability to release flagellated zoospores at a certain stage in their life cycle (Cross 1986). Current classification of motile-spored actinomycetes can identify at least six suborders containing zoosporic genera, including *Micromonosporinae, Micrococcinae, Frankinae, Pseudonocardinae, Kineosporiineae* and *Streptosporanginae* (Stackebrandt *et al*. 1997). These zoospores bearing actinomycetes have been associated with river, lake and fresh water, river sediments, desert soil (Garrity *et al*. 1996; Bredholt *et al.* 2008; Sibanda *et al*. 2010), decaying plant materials submerged in streams and cast up on lake shores (Kudo *et al.* 1998; Tamura *et al*. 2010), blades of grass inhabiting streams and soils (Hasegawa 1991). It has become increasingly apparent that motile actinomycetes can produce a variety of antibiotics and other bioactive metabolites or be used for biochemical conversion of complex compounds (Hasegawa 1991; Garrity *et al*. 1996; Khamna *et al*. 2010; Khanna *et al*. 2011).

According to Hayakawa *et al*. (2000), actinomycetes could be divided into two types based on spores produced by the non-motile and motile. Actinomycetes which bear non-motile spores that are not generally form flagella, for example, are *Streptomyces*, *Nocardia*, *Micromonospora*, and so on. *Actinoplanes* and *Catenuloplanes* are motile zoospores bearing actinomycetes and the zoospores can move. Several actinomycetes genera such as *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineosporia, Microbispora, Micromonospora* and *Nonomuraea* are often very difficult to isolate and cultivate due to their slow growth and those belong to the rare actinomycetes (Hayakawa 2008).

Eka Karya Botanical Garden, in Bedugul, Bali Island, Indonesia is a unique *ex situ* plant conservation site for plant species of high elevated eastern tropical rain forest of Indonesia, adjoining with the tropical forest of Batukahu nature reserve. This garden is located at 1,250-1,450 m above sea level, with area of 157.5 hectares (389 acres). Temperature is about 17-25 °C in daytime and is dropped to 10-15 °C at night with 70-90% humidity (Mukaromah & Suparta 2007). Based on the uniqueness of the above location, we studied about the diversity of actinomycetes in this location. This study was intended to be done as pioneer research in Eka Karya Botanical Garden. Several studies on diversity of actinomycetes were to be done in Indonesia, such as from Lombok Island (Lisdiyanti et al. 2012) and Cibinong Science Center (Widyastuti et al. 2013). To obtain new strains that can potentially produce new metabolites, it is still necessary to conduct exploration and examination of samples obtained from diverse habitats and environments. Few parts of the research had been orally presented in 2011 during Symposium on Recent Advances on Microbiological Researches and Its Application, conducted by Indonesian Society for Microbiology (PERMI) in Serpong.

MATERIALS AND METHODS

Sampling Methods

Soil samples were obtained from Eka Karya Botanical Garden located at 1,250-1,450 m above sea level, 115°9'0-58" E and 8°15-17'0-59" N, with 5-10 cm soil depth from soil surface. pH of the soil samples was between 6.0-6.5. The samples were immediately put into plastic bag. Decaying leaf-litter samples were collected from soil surface. The samples were immediately put into paper bag. All samples were air dried at room temperature for 1-2 weeks, ground using blender and filtered with 200 µm mesh filter paper.

SDS-Yeast Extract (SY) Isolation Method

SY isolation method was described by Widyastuti *et al*. (2013). A combination of 0.05% SDS (Sodium Dodecyl Sulphate) as a germicide to eliminate soil bacteria, 6% yeast extract as spore activating agents and heating at 40 °C for 20 minutes could also increases the recovery of actinomycetes from various soil samples. This method was used for isolating general actinomycetes.

Rehydration and Centrifugation (RC) Isolation Method

The RC isolation method was used for isolating motile actinomycetes. The sample is rehydrated by air-dried container in 10 mM phosphate buffer containing 10% soil extract, at

30 °C for 90 minutes, followed by centrifugation at 1,500 x g for 20 minutes (Hayakawa *et al*. 2000; Otoguro *et al.* 2001; Widyastuti *et al*. 2013).

Humic Acid with Vitamins (HV) Medium

HV medium was contained (in liter) 1 g humic acid, 0.02 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 1.71 g KCl, 0.05 g $MgSO_4.7H_2O$, 0.5 g $NaHPO_4$, 5 mL of vitamins solution, 50 mg cycloheximide, 18 g agar, pH 7.2. The composition of vitamins solution was 0.5 mg thiamine HCl, 0.5 mg riboflavin, 0.5 mg niacin, 0.5 mg pyridoxine HCl, 0.5 mg inositol, 0.5 mg Ca-panthotenate, 0.5 mg p-aminobenzoic acid and 0.25 mg biotin in 5 mL water and sterilized by 0.22 m filtration (Hayakawa & Nonomura 1987). This vitamin solution was added after autoclave sterilization.

Analysis of Diaminopimelic Acid (DAP)

Preliminary biochemical test performed is the DAP determination using a method of thin layer chromatography (TLC) on cellulose to separate isomers of DAP (Hasegawa *et al*. 1983). Three loops of the cells was put in screw cap plastic tube, added with three drops of 6N HCl, autoclaved at temperature of 121 °C, 1 atm for 15 minutes, and then applied to cellulose chromatography plate. TLC eluent solution used was mixture of methanol: water: 6N HCl: pyridine (80: 26: 4: 10 v/v), and eluted for 12 hours. After that, the spots were sprayed with ninhydrin solution (0.3 g ninhydrin in 100 mL of butanol + 3 mL of acetic acid), and heated at 100 °C for 3 minutes.

Preparation of Template DNA and PCR Amplification of 16S rRNA Gene

Chromosomal DNA was extracted as described by Saito & Miura (1963) from 14-day-old cell cultures grown on YG agar medium by using DNeasy Plant Maxi Kit (Qiagen). 16S rRNA gene replication reaction was performed using primer pair, 9F (forward: 5'-GAGTTTGATCCT-GGCTCAG-3' positions 9-27) and 1541R (reverse: 5'-AAGGAGGTGATCCAGCC-3' position 1541-1525) of *Escherichia coli* numbering system (Brosius *et al.* 1978). PCR amplification was performed used TaKaRa ex Taq with total volume of 50 L, consisting of 0.4 mM of each primer, 1 ng of DNA template, 2.5 mM of dNTP, 1 of TaKaRa PCR buffer, and 5U of Taq polymerase in final volume. PCR conditions was 95 °C for 3 minutes to denaturate the target DNA, then by 30 cycles at 95 °C for 3 seconds for denaturation again, 55 °C for 15 minutes for primer annealing, and 72 °C for 1 minute for primer extension, and subsequently, 1 cycle at 72 °C for 5 minutes to complete the process of amplification. PCR reaction was conducted using a GeneAmp PCR System 9700 (Applied Biosystem). PCR products were examined by electrophoresis on agarose 2%, to assure that the target DNA had been amplified. PCR products were then purified using the GFX-96 PCR Purification Kit (Amersham Pharmacia Biotech), with reference to the protocol.

16S rRNA Gene Sequencing

PCR products that had been purified were cycle sequenced using the BigDye Terminator sequence with Version 3.1 Cycle Sequencing Kit. This reaction used 6 primers to amplify 1,500 bp of 16S rRNA gene, which is 9F, 515F (5'-GTGCCAAGCAGCCGCGGT-3' position 515-531), 1099F (5'-GCAACGAGCGCAACCC-3' position 1099-1114), 536R (5'-GTATTACCGC-GGCTGCTTG-3' positions 536-519), 1115R (5'-AGGGTTGCGTCGTTG-3' position 1115-1100), and 1541R of *Escherichia coli* numbering system (Brosius *et al*. 1978). In total 10 L of reaction sequence containing 2.0 L of Big Dye Terminator premix, 1.0 L of 5 Big Dye sequencing buffer, 0.8 L of each primer (1 pmol/L), and 0.5 L of template DNA were synthesized of the chain by using a GeneAmp PCR System 9700 (Applied Biosystem) with the following conditions pre-denaturation at 96 °C for 1 minute, 45 cycles at a temperature of 96 °C for 10 seconds for denaturation, 50 °C for 5 seconds for primer annealing, and 60 °C for 90 seconds for primer extension, and subsequent to storage at 16 °C. The product was purified using Dyeex 96 Kit (Qiagen) and sequenced using ABI Prism 3700 (Applied Biosystem) DNA sequencer.

Sequence Data Analysis and Alignment Search

16S rDNA sequence was translated from the 16S rRNA gene by using ATGC Sequencing Analysis Software version 7.3 (ABI Prism) and corrected manually. Nucleotide sequence data of the isolates was searched the closest homology

with other strains in the 16S rRNA gene data base using BLAST (http://www.ncbi.nlm.nih. gov).

RESULTS AND DISCUSSION

From the total number of 38 samples consisted of 18 soil samples and 20 leaf-litter samples, 409 actinomycetes were isolated. A total of 229 isolates based on the colony appearance, were selected and used in this study. From 229 isolates, 144 were isolated from soil samples and 85 were isolated from leaf-litter samples. From soil samples, 60 and 84 actinomycetes were isolated by SY and RC isolation method, respectively; and 85 from leaf-litter samples were isolated by using RC isolation method (Table 1). The DAP analysis showed that within the actinomycetes isolated from soil source by SY isolation method, 18 isolates had LL-DAP, 31 isolates had *meso*/LL-*meso*/OH DAP, but the rest 11 isolates did not have DAP containing polymers. Actinomycetes isolated by RC isolation method showed that 24 isolates had LL-DAP, 48 isolates had *meso*-DAP/LL-*meso*/OH in their cell wall, and the rest 12 isolates did not have DAP containing polymers. From leaf-litter source using RC isolation method, 3 isolates had LL-DAP, 66 isolates had *meso* DAP/LL-*meso*/ OH on their cell wall, and 16 isolates did not have DAP containing polymers.

Table 1 Number of isolated and selected actinomycetes from Eka Karya Botanical Garden, Indonesia

Sampling site	Source	No. of samples	Isolation method	Selected isolates	DAP isomer		
					LL	M/LL-M/OH	ND
Eka Karya Botanical Garden	Soil	18	SY	60	18	31	11
		18	RC	84	24	48	12
	Leaf-litter	20	RC	85	3	66	16
		38		229	45	145	39

Table 2 Actinomycetes isolated from Eka Karya Botanical Garden, Bali, Indonesia, 2003

No	Suborder	No	Family	No	Genus	BLAST result				Total
						>99 %	98 %	97 %	<96 %	
1	Corynebacterineae	1	Nocardiaceae	1	*Nocardia*	2	5		2	9
				2	*Rhodococcus*				1	1
2	Frankineae	2	Cryptosporangiaceae	3	*Cryptosporangium*		1			1
		3	Kineosporiaceae	4	*Kineosporia**		7		3	10
3	Micrococcineae	4	Intrasporangiaceae	5	*Lapilicoccus*				1	1
		5	Promicromonosporaceae	6	*Promicromonospora*	1				1
4	Micromonosporineae	6	Micromonosporaceae	7	*Actinoplanes**	8	33	22	4	67
				8	*Catellatospora*		1			1
				9	*Catenuloplanes**		12	4		16
				10	*Dactylosporangium**		3			3
				11	*Krasilnikovia**			3		3
				12	*Micromonospora*	3		4	1	8
				13	*Verrucosispora*			1		1
5	Propionibacterineae	7	Nocardioidaceae	14	*Kribbella*	2				2
				15	*Nocardioides*	1			1	2
6	Pseudonocarnineae	8	Actinosynnemataceae	16	*Actinokineospora**		1			1
				17	*Saccharothrix*		1			1
		9	Pseudonocardiaceae	18	*Amycolatopsis*	1	2			3
				19	*Pseudonocardia*		3	1		4
				20	*Saccharomonospora*	1				1
7	Streptomycineae	10	Streptomycetaceae	21	*Kitasatospora*	5	2			7
				22	*Streptomyces*	42	24	4	3	73
8	Streptosporangineae	11	Nocardiopsaceae	23	*Nocardiopsis*	4				4
		12	Streptosporangiaceae	24	*Acrocarpospora*				1	1
				25	*Nonomuraea*	1	2	2		5
				26	*Streptosporangium*	1				1
		13	Thermomonosporaceae	27	*Actinocoralliia*	1				1
				28	*Actinomadura*		1			1
						73	98	41	17	229

Note: * = Zoospore-bearing actinomycetes

Table 3 Diversity of actinomycetes in soil samples

No	Suborder	No	Family	No	Genus	No of species	RC	SY	Total
1	Corynebacterineae	1	Nocardiaceae	1	*Nocardia*	4	1	8	9
				2	*Rhodococcus*	1		1	1
2	Frankineae	2	Kineosporiaceae	3	*Kineosporia**	1	1		1
3	Micrococcineae	3	Promicromonosporaceae	4	*Promicromonospora*	1	1		1
4	Micromonosporineae	4	Micromonosporaceae	5	*Actinoplanes**	12	25		25
				6	*Catellatospora*	1		1	1
				7	*Dactylosporangium**	1	3		3
				8	*Krasilnikovia**	1	3		3
				9	*Micromonospora*	1		2	2
				10	*Verrucosispora*	1	1		1
5	Propionibacterineae	5	Nocardioidaceae	11	*Kribbella*	2		2	2
				12	*Nocardioides*	2		2	2
6	Pseudonocarnineae	6	Actinosynnemataceae	13	*Actinokineospora*	1	1		1
				14	*Saccharothrix*	1	1		1
		7	Pseudonocardiaceae	15	*Amycolatopsis*	2	1	2	3
				16	*Pseudonocardia*	3	2	1	3
7	Streptomycineae	8	Streptomycetaceae	17	*Kitasatospora*	5	6	1	7
				18	*Streptomyces*	36	33	32	65
8	Streptosporangineae	9	Nocardiopsaceae	19	*Nocardiopsis*	4	2	2	4
		10	Streptosporangiaceae	20	*Acrocarpospora*	1	1		1
				21	*Nonomuraea*	3	2	3	5
				22	*Streptosporangium*	1		1	1
		11	Thermomonosporaceae	23	*Actinocoralliia*	1		1	1
				24	*Actinomadura*	1		1	1
						87	84	60	144

Note: * = zoospore bearing actinomycetes

Identification of 229 isolates based on 16S rRNA gene sequencing showed that the isolates belong to 8 suborders, 13 families and 28 genera of the class Actinomycetales (Table 2). The largest group of actinomycetes found belongs to genus *Streptomyces* (73 isolates). The second largest group belongs to genus *Actinoplanes* (67 isolates). The third largest group belong to genus *Catenuloplanes* (16 isolates). About 58 isolates (25%) may be new species or new genus, because it has <98% of 16S rRNA gene similarity compared to the known strains in the database.

SY isolation method and cultures incubation on HV agar plates containing nalidixic acid introduced by Hayakawa and Nonomura (1989) improved the possibilities of isolating actinomycetes while decreasing the number of bacterial colonies. This method proved to be an effective tool for isolating actinomycetes. RC isolation method described by Hayakawa *et al*. (2000) and Otoguro *et al*. (2001) was also found to be an effective tool for the isolation of zoospore from the genera of *Actinoplanes*, *Actinokineospora*, *Actinosynnema*, *Catenuloplanes, Dactylosporangium, Geodermatophylus* and *Kineosporia*. The phosphate buffer-soil extract solution significantly promoted liberation of motile zoospores from the source material, and the centrifugation eliminated Streptomyces and other non-motile actinomycetes. In general, actinomycetes isolated using SY method were dominated by many non-motile actinomycetes, while those isolated using the RC method were dominated by motile actinomycetes. RC is an isolation method developed for isolating motile zoospore (Hayakawa *et al*. 2000).

All 229 selected isolates were identified using molecular identification procedure based on full sequence of 16S rRNA gene (±1,500 bp). The isolates were further identified into genus and species level by BLAST and phylogenetic tree construction. Currently, actinomycetes consisted of 24 families, 80 genera and 500 species (Liu *et al*. 2009). In our study, we could identify 8 suborders, 13 families and 28 genera (Table 3). We predicted that there were more than 109 species. This is the first comprehensive study of actinomycetes conducted in Eka Karya Botanical Garden, Bali Island, Indonesia.

Diversity of Actinomycetes on Soil Samples

From soil samples, we obtained 144 isolates of actinomycetes that had been identified by 16S rRNA gene analysis and preserved well in liophilized form. The isolates contained 24 genera and more than 87 species. The most dominated genera in the soil samples was *Streptomyces* (65 isolates = 45%) and the next was *Actinoplanes* (25 isolates = 17%).

Based on the isolation methods, 15 genera (60 isolates) were successfully isolated by SY isolation method and 16 genera (84 isolates) were isolated by RC isolation method. Genera of *Nocardia*, *Rhodococcus*, *Catelatospora*, *Micromonospora*, *Kribbella*, *Nocardioides*, *Streptosporangium*, *Actinocoralia* and *Actinomadura* were easily isolated using SY isolation method; while RC method was useful for isolating genera *Actinoplanes*, *Krasilnikovia*, *Dactylosporangium*, *Verrucosispora*, *Actinokineospora* and *Saccharothrix*. Most of soil actinomycetes isolated by RC isolation method belong to zoospore bearing actinomycetes. By using different isolation method, the dominant species of actinomycetes isolated were also different. In this study, we proved that actinomycetes isolated using the RC method were dominated by groups of zoospore bearing actinomycetes. This result is similar to that described by Hayakawa *et al.* (2000) and Otoguro *et al*. (2001).

Several ecological factors that played a role in the distribution of genera actinomycetes included humus content and pH of the soil (Nonomura & Hayakawa 1988), climate may influence the specific type of soil-inhabiting actinomycetes (Hayakawa *et al*. 2010). Soil of Eka Karya Botanical Garden is a humus-rich soil with pH range from 6 to 6.5. This soil type is suitable for the growth of actinomycetes.

Some of actinomycetes are distributed in plant rhizosphere soils. Diverse plant species found in the garden should also support the growth of actinomycetes. Actinomycetes have been found to play an important role in rhizosphere soil (Suzuki *et al*. 2000; El-Tarabily & Sivasithamparam 2006). There is a possibility that these microorganisms can protect plant roots from plant pathogen and promote plant growth.

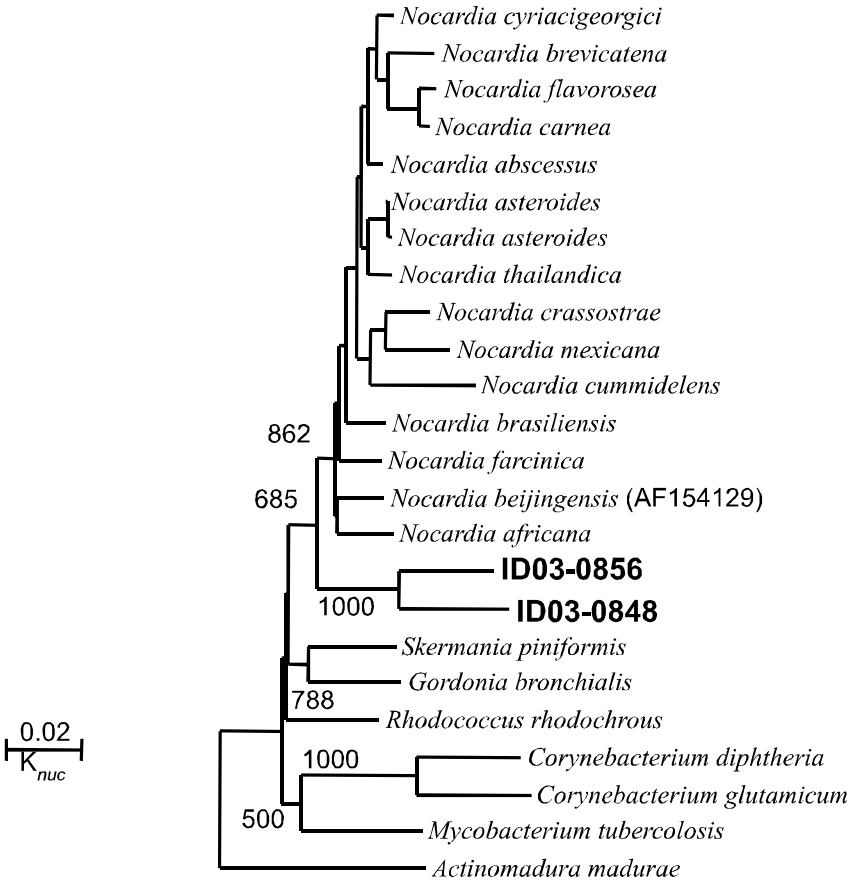

Figure 1 Phylogenetic position based on 16S rRNA sequences of several isolates under the *Nocardia* genera from Eka Karya Botanical Garden. Bar, 1 substitutions per 200 nucleotides

For plant root protection, the modes of action of actinomycetes include antibiosis, parasitism, the production of extracellular hydrolytic enzymes and competition for iron (Getha *et al*. 2005; Errakhi *et al*. 2007). SY isolation method was particularly successful for isolating common actinomycetes such as *Streptomyces* spp. In natural habitats, streptomycetes are common and are usually a major component of the total actinomycetes population. Kim (1984) reported that within population in the soil, actinomycetes are dominated by *Streptomyces* (95.43%).

Identification by molecular approach indicated that actinomycetes obtained from Eka Karya Botanical Garden should have potential value as a source to find new species or new genus. Based on the analysis of 16S rRNA gene, <97% sequence were in homology with the closest species on BLAST searching compared to the current database. New species and new genus among the strains studied were obvious. The16S rRNA gene sequence of strain ID03-0848 and ID03-0856 were aligned with those of the type species of the major *Nocardia* and other actinomycete lineages. The resulting phylogenetic tree is shown in Figure 1. Strain ID03-0848 and ID03-0856 formed a coherent clade within the *Nocardia* lineage, clearly distinguished from other described strains with highly bootstrap value. This was suspected to be new genus or new species in the *Nocardia* lineage.

Diversity of Actinomycetes on Leaf-litter Samples

Meanwhile, from the leaf-litter as a source material, we obtained 85 isolates of actinomycetes that had been identified by 16S rRNA gene analysis and preserved well in liophilized form. The isolates contained 9 genera (Table 4) and more than 41 species. The most dominated genus was *Actinoplanes* (42 isolates = 49%) and the next was *Catenuloplanes* (16 isolates = 19%) and *Kineosporia* (9 isolates = 10%). The same as in soil samples, most of the leaf-litter actinomycetes isolated by RC method belong to the zoospore bearing actinomycetes. This finding is in agreement with other reports which mentioned that actinomycetes belonging to genera *Actinoplanes, Catenuloplanes* and *Kineosporia* were frequently isolated from leaf-litter samples (Pagani & Parenti 1978; Kudo *et al*. 1998; Hayakawa *et al*. 2000; Ratnakomala *et al.* 2011). They showed very similar characteristics such as possession of motility, absence or rarity of hydrophobic aerial hyphae and formation of orange colonies, similar to the color of fallen leaves (Van Hop *et al.* 2011).

Xu *et al.* (1996) and Meliani *et al*. (2012) reported that there was a positive correlation between diversity of actinomycetes with vegetation. Land of primary forest has higher diversity of actinomycetes compared with land of secondary forest and agricultural land. On dry, barren and cold land, there are less actinomycetes found (Xu *et al.* 1996; Garrity *et al*. 1996). Search of new active compounds, especially from actinomycetes requires a large number of isolates. It would be more promising if sampling and isolation techniques are more specific (Lo *et al*. 2002). Therefore, it is essential to look for unique types of vegetation where the soil sample will be taken for finding new taxonomically important actinomycetes. It is also important to find

Table 4 Diversity of actinomycetes from leaf-litter samples

No	Suborder	No	Family	No	Genus	No of species	RC
1	Frankineae	1	Cryptosporangiaceae	1	*Cryptosporangium*	1	1
		2	Kineosporiaceae	2	*Kineosporia**	1	9
2	Micrococcineae	3	Intrasporangiaceae	3	*Lapilicoccus*	1	1
3	Micromonosporineae	4	Micromonosporaceae	4	*Actinoplanes**	21	42
				5	*Catenuloplanes**	2	16
				6	*Micromonospora*	6	6
4	Pseudonocarnineae	5	Pseudonocardiaceae	7	*Pseudonocardia*	1	1
				8	*Saccharomonospora*	1	1
5	Streptomycineae	6	Streptomycetaceae	9	*Streptomyces*	7	8
						41	85

Note: * = zoospore bearing actinomycetes

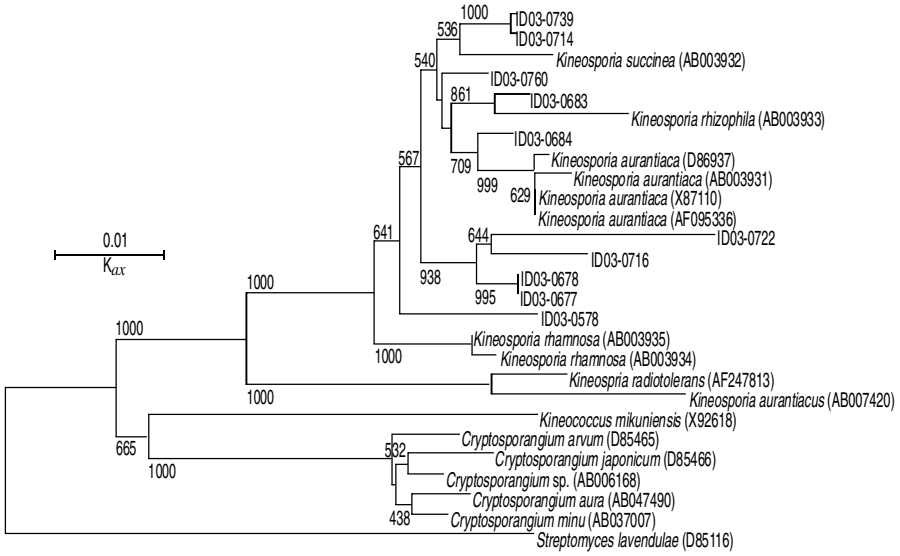

Figure 2 Phylogenetic position based on 16S rRNA sequences of several isolates under the *Kineosporia* genera from Eka Karya Botanical Garden. Bar, 1 substitution per 100 nucleotides

actinomycetes with new metabolic properties. There is a possibility to find a new actimomycete species for the production of new antibiotics or other secondary metabolites. These microbes will specifically generate new secondary metabolites which allow them to degrade toxic compounds from these plants (Park *et al.* 1999; Ho *et al*. 2000). Eka Karya Botanical Garden is one place for *ex situ* plant conservation of tropical forests in Indonesia. It is understood that high diversity of actinomycetes will likely to be found in such place.

Selection of proper method of isolation is crucial to obtain new actinomycetes species. It was obvious from our study that the use of RC method was significantly useful to isolate new species from leaf-litter samples, especially from genus *Kineosporia*. The 16S rRNA gene sequences of 10 strains (ID03-0578, ID03-0677, ID03-0678, ID03-0683, ID03-0684, ID03-0714, ID03-0716, ID03-0722, ID03-0739 and ID03-0760) were aligned with those of type species of the major *Kineosporia* and other actinomycete lineages. As shown in Figure 2, strain ID03-0739 and ID03-0714 were moderately related to the type strain *K. succinea* AB003932. Strain ID03-0683 and ID03-0760 were related to type strain *K. rhizophila* AB003933. Strain ID03-0684 was closely related to type strain *K. aurantiaca* D86937. Strain ID03-0677 and ID03-0678 shared the same branching position and formed a single clade with ID03-0716 and ID03-0722. These four strains were clearly distinguished from other described strains with highly bootstrap value. This was suspected to be new genus or new species in the *Kineosporia* lineage.

This study is significantly important to describe the diversity of actinomycetes in Indonesia. There are ample spaces to use isolated actinomycetes for the benefit of society. Further research on several important taxa including proposing new species or genus is mandatory. More data on phenotype, biochemical characterization, DNA-DNA hybridization and chemotaxonomic are required.

CONCLUSIONS

Selection of proper isolation method is crucial to obtain a new actinomycetes species. Using SY isolation method, this research was successfully isolated 2 new species of actinomycetes from Eka Karya Botanical Garden. This study is significantly important to describe the diversity of actinomycetes in Indonesia. There are ample space to use isolated actinomycetes for the benefit of society. Further research on some important taxa including for proposing new species or genus is mandatory. More data on phenotypic, biochemical characterization, DNA hybridization and chemotaxonomic data are required to describe the other actinomycetes candidates as new species.

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