Ecological Study of Rare-Actinomycetes in Soils and Leaf-Litters

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Abstract

A total of 268 strains of Actinomycetes were isolated from soil and leaf-litter samples collected in Jambi (Sumatera) and Cibinong (West Java) using three selective isolation methods. All the isolates were identified by morphological characteristic and by analysis of 16S rDNA sequence. On the basis of their morphology and 16S rDNA sequence, 164 isolates were belonged to the Streptomyces Group and 104 isolates were belonged to the Rare-Actinomycetes (Non-Streptomyces) Group. Furthermore, 40% of isolates in Streptomyces Group and 62% of isolates in Non-Streptomyces Group are supposed to be new taxa. It is indicated the richness of Actinomycetes in these two area. The genus *Streptomyces* is the most abundant in soil samples, occupying 60-75% of all isolates; while the genus *Actinoplanes* is mainly found in leaf-litter samples (70%). The use of selective isolation media is important to elucidate the microbial diversity.

Key words: Actinomycetes, microbial diversity

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Introduction

Actinomycetes are a group of gram-positive bacteria that have high G+C contents. In general, actinomycetes could be divided into two group, (i) Streptomyces group which include only the genus *Streptomyces* and *Kitasatospora*; and (ii) non-Streptomyces group or so-called rare actinomycetes which include the genus *Actinomadura, Kutsneria, Microbispora, Microtetraspora, Nonomuraea, Saccharomonospora, Streptosporangium, Thermobifida, Actinoplanes, Actinokineospora, Actinosynnema, Catenuloplanes, Cryptosporangium, Dactylosporangium, Geodermatophilus, Kineosporia* and *Sporichihya*.

Habitat of actinomycetes are common and ubiquitous in natural substrates, such as soils, where they usually play a significant role in the degradation of the more recalcitrant, naturally occurring organic polymers (Williams et al. 1984). Many actinomycetes are commercially important, either in the production of antibiotics and other bioactive secondary metabolites, or in useful biological processes (Okami and Hotta, 1988). Therefore, the isolation and subsequent characterization of these organisms from diverse habitats is important not only to understand their role in natural ecosystems but is also of value for establishing novel strains with pharmaceutical and industrial applications.

Since the discovery of the antibiotic actinomycin in 1940 and streptomycin in 1944 by Waksman and co workers, much attention has been focused on the genus *Streptomyces*, the most abundant and recoverable actinomycete occurring in soil. Currently, the number of species of this genus is more than 400 species. Although the genus *Streptomyces* still continue to be a major source of isolation to search bioactive compounds, the rate of discovery of new metabolites from these ubiquitous species has declined.

In order to find less-known or new taxa of actinomycetes as well as to reduce the re-isolation of strains producing known bioactive compounds, the adoption of reliable methodologies for isolating actinomycetes have been developed and applied by using the information generated through microbial physiology and ecology (Goodfellow and Williams, 1986; Okami and Hotta 1988; Bull et al., 2000). Selective isolation methods to recover rare actinomycetes are then to be the

major targets in the search for novel antibiotics from actinomycetes (Lazzarini et al., 2000).

Additionally, to obtain new strains likely to produce novel metabolites, examination of samples from diverse habitats as well as the ones inhabiting at unexplored yet environments is also necessary. It is undoubted that the selection of novel bioactive producing microorganisms from nature requires a sound microbial taxonomical knowledge and fuller understanding of microbial ecology and physiology as means for revealing novelty (Goodfellow and Williams, 1986).

This is the aim of this study to isolate actinomycetes, mainly rare actinomycetes, from Indonesian soils and litters by selective isolation methods, to identify them by morphological characteristics and 16S rRNA gene sequencing, and primary characterize the bioactive compounds by bioassay from isolated strains.

Materials and Methods

Materials sampling sites, and samples treatment. Thirty-one soil and 20 leaf-litter collected from Bukit Sari Botanical Garden, Jambi, Sumatera and Cibinong, West Java, were used as samples. Soil samples, as well as leaf-litter samples previously ground with a blender, were sieved and air-dried, then actinomycetes were isolated.

Isolation method. Dry Heating (DH) method (Nomomura & Ohara, 1969) and SDS-Yeast Extract (SY) method were employed to isolate actinomycetes from soil samples; while Rehydration-Centrifugation (RC) method (Hayakawa *et al*., 2000) were employed to isolate actinomycetes from leaf-litter samples. Humic acid-vitamin (HV) agar supplemented with nalidixic acid and trimethoprim (Hayakawa & Nonomura, 1987) were used as isolation medium and Yeast Extract and Starch (YS) agar were used for maintaining of isolates. All plates were incubated at 30°C for 2-3 weeks.

Morphological characterization.
Actinomycetes were examined by eye and by using a light microscope and tentatively identified up to group rank based on morphological criteria.

16S rDNA sequence analysis. Genomic DNA was extracted as described by Saito and Miura (1963) and amplification of 16S ribosomal DNA (rDNA) genes was PCR (Saiki et al., 1988) mediated using TaKaRa Taq polymerase (Takara Shuzo, Kyoto, Japan) and the primer pairs, 9F (5`-GAGTTTGATCCTGGCTCAG) and 1541R (5`-AAGGAGGTGATCCAGCC). Amplified 16S rDNA (1.5 kb) was purified and directly sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA) as specified by the manufacturer. The sequencing primers were: 9F (5`-GAGTTTGATCCTGGCTCAG), 515F (5`-GTGCCAGCAGCCGCCGCGGT), 536R (5`-GTATTACCGCGGCTGCTG), 785F (5`-GGATTAGATACCCTGGTAGTC), 802R (5`-TACCAGGGTATCTAATCC), 1099F (5`-GCAACGAGCGCAACCC), 1115R (5`-AGGGTTGCGCTCGTTG), and 1541R (5`-AAGGAGGTGATCCAGCC). An Applied Biosystems PRISM 310 Genetic Analyser performed electrophoresis of the sequencing reaction mixtures. The 16S rDNA sequences determined in this study were manually aligned with the published sequences of reference strains available from the EMBL/GenBank/DDBJ databases. The CLUSTAL W software package (Thompson et al., 1994) generated evolutionary distances (the Knuc value of Kimura 1980) and similarity values. A phylogenetic tree was constructed by neighbour-joining (Saitou and Nei, 1987) from Knuc values. The topology of the phylogenetic tree was evaluated by bootstrap re-sampling as described by Felsenstein (1985) with 1000 replicates.

Bioassay. The ability to inhibit the growth of Gram-positive and Gram-negative bacteria, and yeasts was observed using a paper disc method. The tested bacteria were *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Escherichia coli*. The yeasts examined were *Candida albicans* and *Saccharomyces cerevisiae*. Zones of inhibition around the colonies were recorded after 24 h at 30°C.

Results and Discussion

The number of isolates. Totally, the number of isolates obtained in this study is 268 isolates, which is 153 strains from Jambi

(Sumatera) sampling site and 115 strains were isolated from Cibinong (West Java) sampling site. The number of isolates is shown in Table 1. The population of actinomycetes in soil samples from Jambi (Sumatera) was not so abundant as actinomycetes in soil samples from Cibinong (West Java). In 5 soil samples from Jambi (Sumatera), actinomycetes were absent. Dry-Heating (DH) and SDS-Yeast extract (SY) method were employed to isolate actinomycetes from soil samples because these two methods is effective to reduce the growth of Streptomyces Groups that is abundant in soil samples. The Rehydration-Centrifugation (RC) method was employed to isolates actinomycetes from leaf-litter samples because this method could significantly promote liberation of motile zoospores bearing actinomycetes that abundant in plant- or leaf-litter samples.

Morphology and sequence analysis of isolates.

All the isolates were identified by morphological observation and by sequencing of 16S rDNA. On the basis of their morphology, 164 isolates were belonged to the Streptomyces Group and 104 isolates were belonged to the Rare-Actinomycetes (Non-Streptomyces) Group. The morphological differences of Streptomyces and Rare-actinomycetes Group are as follows; Streptomyces Group is fast growth actinomycetes, with aerial mycelium, and usually do not has motile of zoospore. Non-Streptomyces Group (Rare Actinomycetes) has small colony, slow growth, usually no aerial mycelium, and has motile of zoospore.

On the basis of 16S rDNA analysis, 138 isolates that showed a high blast value (>99%) to the nearest known species were tentatively identified until the species level; while, 130 isolates which showed blast value less that 98% to the nearest known species were identified at the genus level. This is also indicated that almost 50% of isolates (<98% of blast value to the nearest known species) are supposed to be new taxa of actinomycetes

Diversity of Actinomycetes.

The diversity of Actinomycetes at the genus level is shown in Table 3. On the basis of 16S rDNA, eleven, eight, and ten genera of Actinomycetes were found in Jambi soil samples, Jambi leaf-litter samples, and Cibinong soil samples, respectively. The genus Streptomyces *is the most abundant in soil samples, occupying 60-75% of all isolates; while the genus* Actinoplanes *is mainly found in leaf-litter samples (70%).*

Ecology of Rare-Actinomycetes in soil and leaf-litter samples.

The compilation of rare-actinomycetes in soil and leaf-litter samples is shown in Table 4. In a soil sample, only one or two rare-actinomycetes were isolated. In a leaf-litter sample, at least 1 to 11 colonies of rare-actinomycetes were isolated. This is indicated the small number of rare-actinomycetes in soil sample compare to litter sample.

Table 1. The number of isolates from soil and leaf-litter samples

Sampling site	Sample	Number of sample	Isolation method	Number of isolate
Jambi, Sumatera	Soil	21	DH	17
			SY	69
	Litter	20	RC	67
Cibinong, West Java	Soil	10	DH	58
			SY	57
Total				268

Table 2. The number of isolates based on blast value

Sampling site	Group	Number of	Blast value		
		isolate	>99%	>98%	>97%
			(Identified strains)	(Supposed to be new taxa)	
Jambi, Sumatera	Streptomyces	72	42	16	14
	Non-Streptomyces	81	26	23	32
Cibinong, West Java	Streptomyces	92	57	14	21
	Non-Streptomyces	23	13	8	2
Total		268	138	61	69

Table 3. Diversity of actinomycetes

Genus	Number of isolate by isolation method			Total
	DH	SY	RC	
Jambi soil				
Actinobispora		1		1
Actinomadura		2		2
Actinoplanes	1	3		4
Dactylosporangium	3	1		4
Kitasatospora		16		16
Microbispora	1	1		2
Micromonospora	1			1
Nocardia		2		2
Planotetraspora		1		1
Streptacidophilus		1		1
Streptomyces	11	41		52
Total	**17**	**69**	**0**	**86**
Jambi litter				
Actinobispora			1	1
Actinoplanes			47	47
Actinosynnema			1	1
Dactylosporangium			1	1
Kineosporia			5	5
Micromonospora			7	7
Streptomyces			4	4
Streptosporangium			1	1
Total	**0**	**0**	**67**	**67**
Cibinong soil				
Dactylosporangium		1		1
Kineosporia	1	1		2
Kitasatosporia	3	2		5
Micromonospora	3	6		9
Nocardia		5		5
Nonomuraea		1		1
Pseudonocardia		2		2
Saccharopolyspora	1	1		2
Streptacidophilus	1			1
Streptomyces	49	38		87
Total	**58**	**57**	**0**	**115**

Table 4. Ecology of Rare-Actinomycetes in soil and leaf-litter samples I

Sample number	Number of isolates	Name of genera	
JS-02	1	*Nocardia*	
JS-03	1	*Micromonospora*	
JS-04	1	*Actinobispora*	
JS-05	1	*Actinoplanes*	
JS-06	2	*Actinoplanes*	*Streptacidophilus*
JS-07	2	*Actinoplanes*	*Dactylosporangium*
JS-08	1	*Dactylosporangium*	
JS-11	1	*Microbispora*	
JS-12	1	*Actinomadura*	
JS-14	1	*Microbispora*	
JS-15	1	*Actinomadura*	
JS-16	2	*Dactylosporangium*	*Planotetraspora*
JS-17	2	*Dactylosporangium*	*Nocardia*
JS-21	1	*Actinoplanes*	

JL-01	1	*Actinoplanes*	*Kineosporia*	
JL-02	3	*Actinoplanes*	*Micromonospora*	
JL-04	3	*Actinoplanes*	*Dactylosporangium*	
JL-05	8	*Actinoplanes*	*Micromonospora*	*Actinosynnema*
JL-07	6	*Actinoplanes*	*Micromonospora*	
JL-08	1	*Streptosporangium*		
JL-09	8	*Actinoplanes*	*Micromonospora*	*Kineosporia*
JL-10	11	*Actinoplanes*		
JL-12	2	*Actinoplanes*		
JL-14	1	*Actinoplanes*		
JL-15	7	*Actinoplanes*	*Micromonospora*	
JL-16	7	*Kineosporia*	*Actinoplanes*	
JL-18	3	*Actinoplanes*		
JL-19	3	*Actinoplanes*	*Micromonospora*	*Actinobispora*
JL-20	2	*Actinoplanes*		
CS-01	1	*Pseudonocardia*		
CS-02	1	*Pseudonocardia*		
CS-03	3	*Dactylosporangium*	*Nocardia*	
CS-04	8	*Micromonospora*	*Nocardia*	*Saccharopolyspora*
CS-05	2	*Saccharopolyspora*	*Nocardia*	
CS-06	4	*Micromonospora*	*Kineosporia*	
CS-07	3	*Micromonospora*	*Kineosporia*	
CS-10	2	*Nonomuraea*		

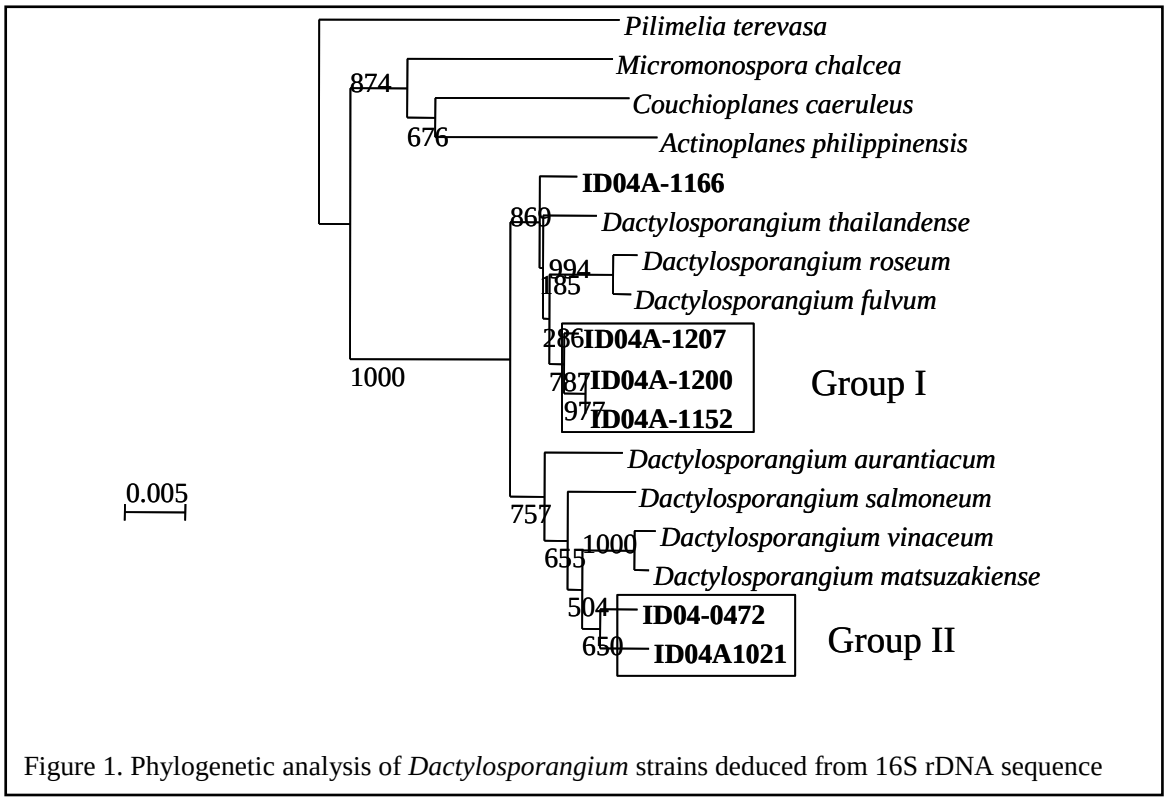

Figure 1. Phylogenetic analysis of *Dactylosporangium* strains deduced from 16S rDNA sequence

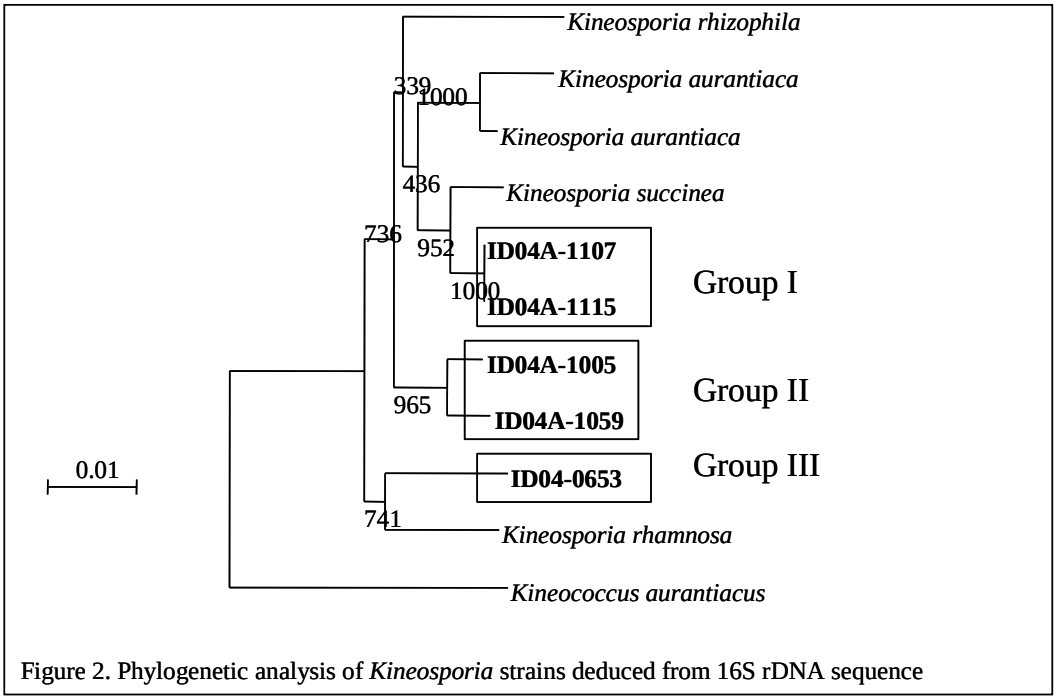

Figure 2. Phylogenetic analysis of *Kineosporia* strains deduced from 16S rDNA sequence

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References

Bull, A. T., Ward, A. C., and Goodfellow, M. (2000). Search and discovery strategies for biotechnology: the paradigm shift. Microbiology and Molecular Biology Reviews, 64: 573-606.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Hayakawa, M. and Nonomura, H. (1987) Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. Journal of Fermentation Technology 65: 501-509.

Hayakawa, M., Otoguro, M., Takeuchi, S., Yamazaki, T., and Iimura, Y. (2000). Application of a method incorporating differential centrifugation for selective isolation of motile actinomycetes in soil and plant litter. Antonie van Leeuwenhoek, 78, 171-185.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, 16: 111-120.

Lazzarini, A., Cavaletti, L., Toppo, G., aand Marinelli, F. (2000). Rare genera of actinomycetes as potential sources of new antibiotics. Antonie van Leeuwenhoek, 78, 399-405.

Nomomura and Ohara. (1969). Distribution of actinomycetes in soil. Journal of Fermentation Technology, 47, 701-709.

Okami, Y. and Hotta, Y. (1988). Search and discovery of new antibiotics. In Actinomycetes in Biotechnology pp. 33-67. London; Academic Press.

Saiki, R.K., Gelfand, D.H., Stoffe, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239, 487-491.

Saito, H. and Miura, K. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochimica et Biophysica Acta, 72: 619-629.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673-4680.

Williams, S.T., Locci, R., Beswick, A., Kurtbo»ke, D.I., Kuznetsov,Williams, S. T., Lanning, S. and Wellington, E. M. H. (1984). Ecology of actinomycetes. In the biology of actinomycetes. Pp. 481-528.