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Genus Diversity of Actinomycetes in Cibinong Science Center, West Java, Indonesia

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Actinomycetes are microorganisms that play important role to support human health and known as soil microorganisms. The aim of the research was to describe genus diversity of actinomycetes in Cibinong Science Center (CSC), West Java. Samples for isolation were soil and plant litters. The samples were air dried and ground. We employed isolation methods: dry heat (DH), sodium dodecyl sulphates-yeast extract (SDS-YE), rehydration and centrifugation (RC), and oil separation (OS). A total of 263 isolates of actinomycetes were isolated in CSC, in 2004-2006. Totally 58, 144, 50, and 11 isolates were isolated under each isolation methods, respectively. All isolates were identified using the 16S rRNA gene sequencing method. The results showed that the isolates were belonged to the family *Kineosporiaceae, Micromonosporaceae, Nocardiaceae, Pseudonocardiaceae, Streptomycetaceae, Streptosporangiaceae, Mycobacteriaceae, Nocardioidaceae, Nocardiopsaceae,* and *Thermomonosporaceae.* There were 23 genera under those families. Homology value of the isolates based on BLAST search using 16S rRNA gene sequence data as queries showed that 136, 91, 30, and 6 isolates were ≥99, 98, 97, and ≤ 96%, respectively, compared to the known sequence in data base. The later 6 isolates were interesting for further identification leading to new taxa. Recognized species of *Streptomyces* genera under the member of the S*treptomycetaceae* were dominant among other isolates.

Keywords: 16S rRNA gene sequencing, actinomycetes, diversity, Indonesia

Actinomycetes merupakan mikroorganisma tanah yang mempunyai peran pada bidang kesehatan. Oleh karena itu, upaya pencarian species actinomycetes baru banyak dilakukan. Tujuan penelitian ini adalah melihat keanekargaman actinomycetes tingkat genus di Cibinong Science Center (CSC), Jawa Barat. Sampel untuk isolasi actinomycetes adalah tanah dan serasah. Metode isolasi yang digunakan adalah *dry heat* (DH), sodium dodesil sulfat-yeast extract (SDS-YE), *rehydration and centrifugation* (RC), dan *oil separation* (OS). Sebanyak 263 isolat actinomycetes telah diperoleh dari CSC pada tahun 2004-2006. Dari jumlah tersebut 58 dari metode DH, 144 isolat dari SDS-YE, 50 dari RC dan 11 dari OS isolat. Identifikasi isolat dilakukan menggunakan metoda sekuen gen 16S rRNA. Hasilnya menunjukkan adanya famili *Kineosporiaceae, Micromonosporaceae, Nocardiaceae, Pseudonocardiaceae, Streptomycetaceae, Streptosporangiaceae, Mycobacteriaceae, Nocardioidaceae, Nocardiopsaceae,* dan *Thermomonosporaceae.* Dari famili tersebut diperoleh 23 genus. Nilai kesesuaian isolat berdasarkan analisis BLAST menggunakan sekuen 16S gen rRNA sebagai *queri* dibandingkan dengan species yang terdaftar pada *data base* menunjukkan bahwa 131, 91, 30, dan 6 isolat masing-masing mempunyai kesamaan sebesar ≤99, 98, 97, dan ≤96%. Enam isolat dengan kesesuaian ≤96% sangat menarik untuk diungkap sebagai taxa baru. Pada penelitian ini, diperoleh isolat terbanyak dari genus *Streptomyces*.

Kata kunci: actinomycetes, Indonesia, keragaman, sekuensing gen 16S rRNA

Microorganisms play important role in our activities, although some have pathogenic properties but some have positive role to support human life. Actinomycetes are microorganisms that play important role especially in pharmaceutical industry to support human health. They are potential as a novel source for the discovery of new bioactive compounds. Activities that focused on discovery of new bioactive compound

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for new products of antibiotics are carried out in some parts of the world recently. Different ecological niches showed many interesting new taxa of actinomycetes and soil has been reported as the natural habitat of actinomycetes. Results of isolation of actinomycetes differed depend on condition of soil and vegetation above the soil (Li *et al*. 2006; Jiang *et al*. 2008) or mangrove ecosystem (Tamura *et al*. 2005; Tamura *et al*. 2006).

Indonesia is located in an equatorial area and most of the area is covered by its tropical rain forests. Tropical rain forests has been known to be the most biologically diverse ecosystems for plants, animals and microorganims on earth. Compared to that of plants and animals, information on diversity microorganisms is very limited. So far, there is no report on diversity of actinomycetes from Indonesia. During the collaborative research on ecology and taxonomy of actinomycetes in Indonesia between Indonesia and Japan, we have collected thousands of actinomycetes isolates from several sites in Indonesia. Some interesting new taxa has been reported, including *Streptomyces baliensis* sp nov. from Bali (Otoguro *et al*. 2009), *Dietzia timorensis* from Timor (Yamamura *et al.* 2010); *Actinokineospora baliensis* sp. nov., *Actinokineospora cibodasensis* sp. nov., *Actinokineospora cianjurensis* sp. nov. from Bali and Cibodas Botanic Garden (Lisdiyanti *et al*. 2010); and *Actinophytocola timorensis* sp. nov. and *Actinophytocola coralina* sp. nov., from Kupang, East Nusa Tenggara (Otoguro *et al*. 2011). Cibinong Science Center (CSC), West Java, was selected as one site during the study. The aim of the research was to describe genus diversity of actinomycetes in CSC during the period of 2004-2006.

MATERIALS AND METHODS

Sample Collection and Preparation. Soil and plant litter samples were collected from different locations in CSC in the year of 2004, 2005, and 2006. Soil samples were taken by digging about 15 cm from the top soil and and put into plastic bag to keep their humidity while plant litter samples were kept in a paper bag. All samples were air dried for 5-7 d, then ground and sieved through a 2 mm sieve.

Isolation of Actinomycetes. Four different isolation methods for actinomycetes were employed with different target of isolates first Sodium dodecyl sulphate-yeast extract (SDS-YE) (Hayakawa and Nonomura 1989) for general actinomycetes. One g of a dried soil sample was suspended in 10 mL of water and stirred for 1 min using a thermo mixer. Then, 1 mL of this suspension was transferred to SDS-YE solution (0.05% of SDS and 6% of yeast extract were dissolved in 50 mM P-buffer pH 7.0). The suspension was heated at 40 °C for 20 min. SDS-YE solution was serially diluted and spread each 0.1 mL solutions to Humic acid-Vitamin (HV) medium (Hayakawa and Nonomura 1987). Second Rehydration-centrifugation (RC) (Hayakawa *et al*. 2000) for motile bearing arthrospore actinomycetes. Five hundred mg of dried soil sample were suspended in 10% of soil extract and P-buffer and kept at 28 °C for 1 h, to release the zoospores. Elimination of non-motile actinomyetes was carried out by centrifugation at 3000 g for 20 min. After centrifugation, the buffer was incubated at 28 °C for 30 min, to sediment the non-motile actinomycetes, while motile actinomycetes are swimming up in the supernatant. Supernatant was serially diluted and spread 0.1 mL solutions to HV medium. The third dry heat (DH)(Nonomura and Ohara 1969) for heat resistant and rare actinomycetes. One gram of air dried soil samples were put in a glass petri dish and heated in oven at 100-120 °C for 1 h to eliminate filamentous bacteria and *Streptomyces*. The sample (0.1 mL solutionswas) serially diluted and spread to HV medium. Fourth, oil separation (OS) as modification of Ishigami's water-hexane distribution method use of olive-oil instead of hexane (Ishigami *et al*. 2004) for lypolytic actinomycetes.This method is based on distribution with oil and water. Five hundred mg of soil sample were suspended in 5 mL of olive oil and mixed for 5 min. Then, 5 mL of water were added to oil and mixed again. Oil and water were distributed by centrifugation for 10 min. After centrifugation, 0.1 mL of oil was spread to HV medium with addition of Kabicidin (0.75 mg L^{-1}), Nalidixic acid (10 mg L^{-1}), and Chroltetracyclin (50 mg L^{-1}).

HV Medium Preparation. HV agar plates supplemented with kabicidin (0.75 mg L^{-1}) and nalidixic acid (10 mg L^{-1}) were prepared at least 4 d before used, for optimum absorption of inoculated samples. Diluted samples were inoculated and spread until dry. Incubation was done at room temperature for 5-20 d, with occasionally observation after 5 d. Plates were kept in a plastic bag and put in paper box.

Isolation and Selection of Actinomycetes. Colonies appeared around 5 d of incubation represent fast growing and the rest were slow growing actinomycetes. Colonies of interest were picked up using sterile woody tooth pick and inoculated to yeast

extract-starch (YS) agar plates. Incubation was carried out at room temperature. Morphological observation of colonies grown in YS agar was done under a microscope. Detection of pigment produced by the isolates was observed from both upper and lower sides of the plates. Selection of the isolates was based on their different morphological appearance.

Identification of Isolates Based on 16S rRNA Gene Sequencing. Selected isolates were subjected for sequence analysis of 16S rRNA gene. Genomic DNA of the isolates was isolated using DNA extraction kit (Promega, USA). The 16S rRNA gene of the isolates was amplified by PCR. PCR condition was as follows: pre denaturation 1.5 min at 96 °C; denaturation 10 sec at 96 °C, annealing 5 sec at 50 °C, elongation 4 min at 60 °C (denaturation, annealing and elongation were run for 25 cycles) and final extension 5 min at 72 °C. The PCR product was sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystem) according to the manufacturer's protocol. Cycle sequencing was performed using 6 primers, i.e. 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 515F (5'-GTGCCAGCAGCCGCGGT-3'), 1099F (5'-GCAACGAGCGCAACCC-3'), 536R (5'GTATTACCGCGGCTGCTG-3'), 1510R (5'-GGCTACCTTGTTACGA-3'), and 1541R (5'AAGGAGGTGATCCAGCC-3'). The 16S rRNA gene sequence data were aligned with published sequences of species of the related genus with validly published names available from EMBL/GenBank/DDBJ by using BLAST Search program (Altschul *et al.* 1990).

Phylogenetic Analysis. The neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum- parsimony (Fitch 1971) algorithms of the Clustal_X 1.8 program (Thompson *et al*. 1997) and MEGA version 3.1 (Kumar *et al*. 2004) were used for constructing a phylogenetic tree. The robustness for individual branches were estimated by bootstrapping withh 1000 replicates (Felsenstein 1985). Phylogenetic tree was constructed based on several genera within a family.

RESULTS

Isolation and Selection of Actinomycetes. CSC is one campus area of the Indonesian Institute of Sciences (LIPI) of about 190 Ha, consisting of buildings, roads, ponds and gardens with several vegetations. Characteristic of the area including altitude 161-170 m, temperature 29.9-33.7 °C, humidity 46.9-63% and pH of soil 6-6.5. Collection of samples in CSC was carried out almost in the same season between June and September each year. Although there is global climate change, the period of sample collection may represents end of dry season and beginning of rainy season in Indonesia. Different number of soil and plant litter samples from several rhizospheres and vegetations were collected. We used several isolation methods for soil samples and isolated many isolates compare to that of plant litter samples. Number of plant litter samples collected is also less than soil samples. Many colonies grew on HV medium from DH and SDS-YE isolation method and they appeared to be different each other so that we obtained many isolates from these isolation methods. Only RC method was used for isolation of actinomycetes from both soil and plant litter samples. There is no isolate obtained from both soil and plant litter by RC method in 2004, however we obtained many interested isolates by this method in 2005 and 2006. Actinomycetes isolated from plant litter by RC method did not grow well and difficult to purified, therefore no selected representative isolates in 2004 and 2006. We selected 6 isolates from plant litter by RC method in 2005. OS is a new modification method and was employed for soil samples (Table 1).

Selection of interest isolates of actinomycetes was based on morphological observation and selected as representative of those isolates with similar appearance. We tried to select as diverse as possible to describe the genus diversity of actinomycetes in CSC during the study.

Identification of Actinomycetes. We identified 119 isolates of actinomycetes and they belong to 10 genera and 6 families in 2004 (Table 2), there are more genera and families in 2005 (Table 3) and less genera and families in 2006 (Table 4). A total of 263 isolates represented of 10 genera and 10 families of actinomycetes were identified from this study (Table 5).

Phylogenetic Tree of Interesting Isolates. Identification of the interesting isolates and their position in the phylogenetic tree showed that most of the isolates were separated from the known species of actinomycetes. Among the families of actinomycetes present in CSC, family *Kineosporiaceae* showed a cluster of interesting isolates as candidates of new genera (Fig 1). We found a canditate of new taxa of *Verrucosispora* from CSC in 2005 (Fig 2).

DISCUSSION

We collected more soil samples than plant litter. So far actinomycetes were reported mostly isolated from the soil, although some actinomycetes were reported

Table 1 Actinomycetes isolated and selected during the study

Year	Source	Number of samples	Isolation method	Number of isolates	Number of selected isolates
2004	Soil	10	DH	149	58
			SDS -YE	232	61
			RC	0	0
	Plant litter	4	RC	0	0
2005	Soil	6	SDS -YE	78	57
			RC	45	28
			OS	14	11
	Plant litter	3	RC	21	9
2006	Soil	7	SDS -YE	74	32
	Plant litter	7	RC	64	16

Table 2 Genus diversity of actinomycetes isolated from CSC in 2004

No.	Family	No.	Genus	Source		Isolation method			BLAST result (%)				
				Soil	Total	DH	SDS	Total	≥99	98	97	≤96	Total
1.	*Kineosporiaceae*	1	*Kineosparia* spp.	1	1	1		1		1			1
2.	*Micromonosporacea*	2	*Dactylosporangium* spp.	1	1		1	1		1			1
		3	*Micromonospora* spp.	11	11	3	8	11	10	1			11
3.	*Nocardiacea*	4	*Nocardia* spp.	5	5		5	5	4	1			5
4.	*Pseudonocardiaceae*	5	*Pseudonocardia* spp.	2	2		2	2	1	1			2
		6	*Saccharopolyspora* spp.	2	2	1	1	2		2			2
5.	*Streptomycetaceae*	7	*Kitasatospora* spp.	3	3	3		3	1	2			3
		8	*Streptacidiphilus* spp.	1	1		1	1		1			1
		9	*Streptomyces* spp.	92	92	50	42	92	67	21	3	1	92
6.	*Steptosporangiaceae*	10	*Nonomuraea* spp.	1	1		1	1			1		1
	Total			119	119	58	61	119	83	31	4	1	119

from other habitats or substrates such as marine sediments, mangrove mud, composted pig manure and animal dung (Kurtböke 2000). Based on the isolation method used, we collected a huge number of *Streptomyces* from DH and SDS-YE methods. Actinomycetes are known to be heat resistant and able to stay in soil for a long period in dry soil. Heating of the soil at about 120 °C showed that *Streptomyces* are the most heat resistant actinomycetes. SDS-YE method based on SDS-YE solution containing 0.05% of SDS and 6% of yeast extract in 50 mM P-buffer and RC method which was developed for isolation of motile actinomycetes (Hayakawa *et al*. 2000) showed that those procedure is suitable for isolation of *Streptomyces*. Motile actinomycetes usually categorized as rare or non *Streptomyces*. However, we found many isolates of *Streptomyces* in the medium. They showed various morphological characteristics among others and interesting to be selected. Using OS method we selected 11 isolates dominated by isolates from *Streptomyces*, followed by *Mycobacterium* and *Actinomadura* genera. Naturally Streptomyces present abundantly in soil and can be isolated easily using all isolated methods used in this study. There are totally 93 from 186 isolates of selected Streptomyces and showed 99% or more BLAST similarity to the recognized species, this means the rest or a half number of the selected Streptomyces are not described yet. Under the family *Streptomycetaceae* we also selected 3 isolates of genera *Kitasatospora* and 1 isolate of genera *Streptacidiphilus*, with 98% BLAST similarity to the recognized species.

In general, selected actinomycetes from CSC in this study is dominated by isolates of *Streptomyces* that constituted about 70% of the total selected actinomycetes. The rest of the isolates belong to various families of actinomycetes (Table 5) . Isolates belong to single genera of family *Mycobacteriaceae, Nocardioidaceae,*

Table 3 Genus diversity of actinomycetes isolated from CSC in 2005

No	Family	No	Genus	Source			Isolation method				BLAST result (%)			
				Soil	Litter	Total	SDS	RC	OSS	Total	≥ 99	98	97	Total
1	*Kineosporiaceae*	1	*Cryptosporangium* spp.	1		1		1		1	1			1
		2	*Kineosporia* spp.	1	6	7		7		7	3	3	1	7
2	*Micromonosporacea*	3	*Actinoplanes* spp.	7	1	8		8		8	1	3	4	8
		4	*Dactylosporangium* spp.	4		4	2	2		4	4			4
		5	*Micromonospora* spp.	6		6	3	3		6	4	2		6
3	*Mycobacteriaceae*	6	*Mycobacterium* spp.	3		3			3	3	3			3
4	*Nocardiacea*	7	*Nocardia* spp.	4		4	3	1		4	3	1		4
5	*Nocardioidaceae*	8	*Kribbella*	2		2	2			2		2		2
6	*Pseudonocardiaceae*	9	*Amycolatopsis* spp.	1		1	1			1	1			1
7	*Streptomycetaceae*	10	*Streptomyces* spp.	60	1	61	41	13	7	61	19	31	11	61
8	*Streptosporangiaceae*	11	*Microbispora* spp.	1		1		1		1	1			1
		12	*Microtetraspora* spp.	1		1	1			1		1		1
		13	*Nonomuraea* spp.	2		2	2			2	1	1		2
9	*Thermomonosporaceae*	14	*Actinomadura* spp.	1		1			1	1			1	1
	Total			94	8	102	55	36	11	102	41	44	17	102

Table 4 Genus diversity of actinomycetes isolated from CSC in 2006

No	Family	No	Genus	Source	Isolation method			BLAST result (%)				
				Soil	SDS	RC	Total	≥ 99	98	97	≤96	Total
1	*Micromonosporacea*	1	*Actinoplanes* spp.	2		2	2	1	1			2
		2	*Verrucosispora* spp.	1		1	1				1	1
2	*Nocardiacea*	3	*Nocardia* spp.	1	1		1	1				1
		4	*Rhodococcus* spp.	1	1		1	1				1
3	*Nocardiopsaceae*	5	*Nocardiopsis* spp.	1	1		1	1				1
4	*Streptomycetaceae*	6	*Kitasatospora* spp.	1	1		1		1			1
		7	*Streptomyces* spp.	33	23	10	33	7	13	9	4	33
		8	*Planotetraspora* spp.	1	1		1		1			1
5	*Streptosporangiaceae*	9	*Streptosporangium* spp.	1		1	1	1				1
	Total			42	28	14	42	12	16	9	5	42

Nocardiopsaceae, and *Thermomonosporaceae* each appeared only one time during the course of the study. Good success of isolation is depend on variety of strategies to isolate rare and new taxa of actinomycetes. Four isolation methods used in this study have different isolates target.

Isolates belong to the genera *Kineosporia* family *Kineosporiaceae* are isolated from plant litter. It seems that their habitat is that kind of substrates and RC isolation method fit to get isolates of *Kineosporia*. Novel species *Kineosporia mesophila* was isolated from surface-sterilized stems of a pharmaceutical plant in China (Li *et al*. 2009) and *Kineosporia babensis* was isolated from plant litter in Vietnam (Sakiyama *et al.* 2009). The position of isolates of the family *Kineosporiaceae* selected in this study represented a line of descent distinct from previously described species of this family and further information are needed to describe the isolates (Fig 1).

There was only 1 species, *Verrucosispora gifhornensis* under the genus Verrucosispora reported previously (Rheims *et al.* 1998), however 5 other species were reported recently (Liao *et al*. 2009; Dai *et al*. 2010; Goodfellow *et al*. 2012; Xi *et al*. 2012; Xie *et al*. 2012). Isolate from CSC, *Verrucosispora* SP ID06-A0238 is separated from those known species (Fig 2),

Table 5 Genus diversity of actinomycetes from CSC in 2004-2006

No	Family	No	Genus	BLAST results (%)				
				≥99	98	97	≤96	Total
1.	*Kineo sporiaceae*	1	*Kineosporia* spp.	3	4	1		8
		2	*Cryptosporangium* spp.	1				1
2.	*Micromonosporacea*	3	*Dactylosporangium* spp.	4	1			5
		4	*Micromonospora* spp.	14	3			17
		5	*Actinoplanes* spp.	2	4	4		10
		6	*Verrucosispora* spp.				1	1
3.	*Mycobacteriaceae*	7	*Mycobacterium* spp.	3				3
4.	*Nocardiacea*	8	*Nocardia* spp.	8	2			10
		9	*Rhodococcus* spp.	1				1
5.	*Nocardioidaceae*	10	*Kribbella* spp.		2			2
6.	*Nocardiospase*	11	*Nocardiopsis* spp.	1				1
7.	*Pseudonocardiaceae*	12	*Pseudonocardia* spp.	1	1			2
		13	*Saccharopolyspora* spp.		2			2
		14	*Amycolatopsis* spp.	1				1
8	*Streptomycetaceae*	15	*Kitasatospora* spp.	1	3			4
		16	*Streptacidiphilus* spp.		1			1
		17	*Streptomyces* spp.	93	65	23	5	186
		18	*Planotetraspora* spp.		1			1
9.	*Streptosporangiaceae*	19	*Nonomuraea*	1	1	1		3
		20	*Microbispora* spp.	1				1
		21	*Microtetraspora* spp.		1			1
		22	*Streptosporangium* spp.	1				1
10.	*Thermomonosporaceae*	23	*Actinomadura* spp.			1		1
	Total			136	91	30	6	263

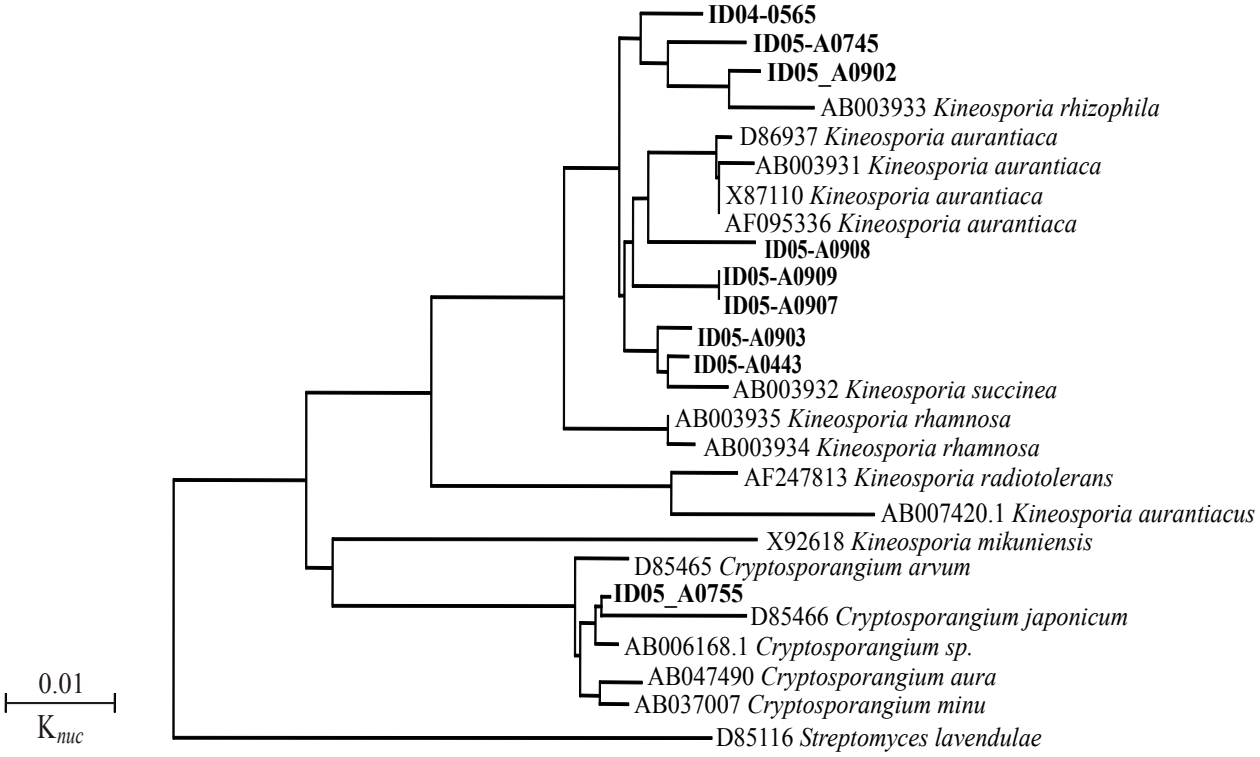

Fig 1 Phylogenetic position based on 16S rRNA sequences of several isolates under the *Kineosporia* genera from CSC. Bar, 1 substitution per 100 nucleotides.

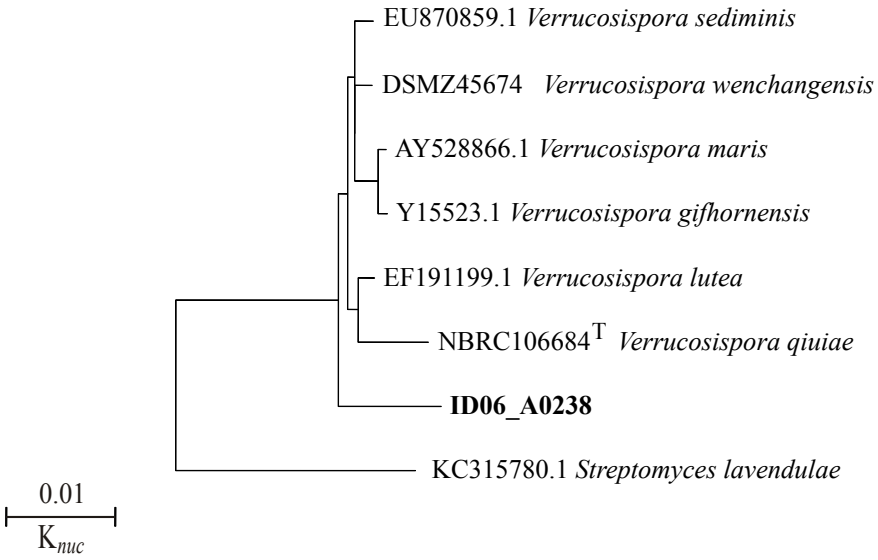

Fig 2 Phylogenetic position based on 16S rRNA sequence of isolate of *Verrucosispora* sp. ID06_A0238 from CSC. Bar, 1 substitution per 100 nucleotides.

therefore it is interesting candidate of new taxa and need further identification.

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