Actinokineospora baliensis sp. nov., *Actinokineospora cibodasensis* sp. nov. and *Actinokineospora cianjurensis* sp. nov., isolated from soil and plant litter

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Six actinomycete strains isolated from soil and plant-litter samples in Indonesia were studied for their taxonomic position by using a polyphasic approach. Phylogenetically, all the strains were located in the broad cluster of the genus *Actinokineospora*. Chemotaxonomic data [cell-wall diamino acid, meso-diaminopimelic acid; cell-wall peptidoglycan, type III (A1 γ); major sugars, galactose and arabinose; major menaquinone, MK-9(H₄); major fatty acid, iso-C_{16:0}; major phospholipid, phosphatidylethanolamine] supported the affiliation of all six strains to the genus *Actinokineospora*. The results of DNA–DNA hybridization with DNA from type strains of *Actinokineospora* species with validly published names revealed three DNA–DNA relatedness groups. Group I (ID03-0561^T) showed low relatedness to the other strains studied. The three strains in group II (ID03-0784^T, ID03-0808 and ID03-0809) formed a group with high relatedness (98–100 %) and showed low relatedness to the other strains studied. The two strains in group III (ID03-0810^T and ID03-0813) showed 58–68 % relatedness to *Actinokineospora terrae* NBRC 15668^T and showed low relatedness (2–24 %) to the other strains studied. The description of three novel species is proposed: *Actinokineospora baliensis* sp. nov., for the single strain in group I (type strain ID03-0561^T = BTCC B-554^T = NBRC 104211^T), *Actinokineospora cibodasensis* sp. nov., for the strains in group II (type strain ID03-0784^T = BTCC B-555^T = NBRC 104212^T), and *Actinokineospora cianjurensis* sp. nov., for the strains in group III (type strain ID03-0810^T = BTCC B-558^T = NBRC 105526^T).

The genus *Actinokineospora* was proposed by Hasegawa (1988) for motile, arthrospore-bearing actinomycetes. Recently, Labeda *et al.* (2010) emended the description of the genus to accommodate species that have not been observed to produce motile spores, and transferred

Amycolatopsis fastidiosa to the genus as *Actinokineospora fastidiosa*. At the time of writing, the genus contains eight species: *Actinokineospora riparia* (the type species), *Actinokineospora inagensis*, *Actinokineospora globicatena*, *Actinokineospora terrae*, *Actinokineospora diospyrosa*, *Actinokineospora auranticolor*, *Actinokineospora enzanensis* and *Actinokineospora fastidiosa* (Hasegawa, 1988; Tamura *et al.*, 1995; Otoguro *et al.*, 2001b; Labeda *et al.*, 2010). These actinomycetes have meso-diaminopimelic acid as a cell-wall diamino acid, galactose and arabinose as diagnostic whole-cell sugars, MK-9(H₄) as the predominant menaquinone, phospholipid type II, iso-C_{16:0} fatty acid as

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains ID03-0561^T, ID03-0784^T and ID03-0810^T are AB447488, AB447489 and AB473945, respectively.

Minimum-evolution and maximum-parsimony 16S rRNA gene sequence-based trees are available as supplementary material with the online version of this paper.

the predominant fatty acid and DNA G + C contents of 69–70 mol%.

During the course of a study on the diversity of actinomycetes in Indonesia, six actinomycete isolates belonging to the genus *Actinokineospora* were found from soil and leaf-litter samples in Eka Karya Botanical Garden, Bali, and Cibodas Botanical Garden, West Java, in 2003. These isolates were isolated by the rehydration and centrifugation method for the selective isolation of motile rare actinomycetes, as described by Hayakawa *et al.* (2000) and Otoguro *et al.* (2001a). Humic acid-vitamin (HV) agar supplemented with 50 µg cycloheximide ml⁻¹ and 20 mg nalidixic acid ml⁻¹ was used as the isolation medium (Hayakawa & Nonomura, 1987, 1989). Details of the isolation of the six strains are given in Table 1.

PCR amplification and sequencing of the 16S rRNA gene were performed as described previously (Tamura & Hatano, 2001) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. Phylogenetic analysis of 16S rRNA gene sequences was performed using the software MEGA version 4 (Tamura *et al.*, 2007) after multiple alignment of data by using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Kluge & Farris, 1969) methods. Bootstrap analysis was used to evaluate the tree topology by performing 1000 resamplings (Felsenstein, 1985) (Fig. 1). The 16S rRNA gene sequences of the Indonesian strains were continuous stretches of 1465–1477 bp. The 16S rRNA gene sequence analysis revealed that the isolates fell in the cluster of the genus *Actinokineospora*. Sequence similarity calculations after neighbour-joining analysis indicated that the similarity to other known type strains in the genus *Actinokineospora* was 97.2–99.4 %. Strains ID03-784^T, ID03-808 and ID03-809 showed 100 % sequence similarity, as did strains ID03-810^T and ID03-813. The closest relatives of the strains were as follows: for ID03-561^T, the type strain of *Actinokineospora diospyrosa* (99.4 %

similarity); for ID03-784^T, ID03-808 and ID03-809, the type strain of *Actinokineospora auranticolor* (98.2 %); and for ID03-810^T and ID03-813, the type strain of *Actinokineospora terrae* (99.4 %).

Cell morphology was observed on YS and HV agar by phase-contrast microscopy and scanning electron microscopy (JEOL model JSM-5400). Samples for scanning electron microscopy were prepared as described by Tamura *et al.* (1995). Morphological observations revealed the presence of aerial mycelium with spore chains. The spores were rod-shaped and were formed by fragmentation of the hyphae (arthrospores); the spores had smooth surfaces, as revealed by scanning electron microscopy. Cultural characteristics were recorded after 14 days of incubation at 28 °C on ISP media 2–7, Bennett's agar, nutrient agar and water agar. The colonies were light yellow to brown. All of the isolates exhibited good growth on all of the media tested except peptone-yeast extract-iron agar (ISP medium 6) and water agar. None of the isolates produced a soluble brown pigment with the exception of ID03-810^T and ID03-813 on Bennett's agar and tyrosine agar (ISP medium 7). Motility was observed by light microscopy using cells grown for 7–10 days at 28 °C on ISP 2 agar.

Results of chemotaxonomic analysis are given in the species descriptions. Freeze-dried cells for chemotaxonomic analyses were grown in yeast extract/glucose broth (10 g yeast extract and 10 g D-glucose per litre distilled water, pH 7.0) on a rotary shaker at 28 °C. Analyses of isomers of *meso*-diaminopimelic acid, whole-cell sugar patterns, menaquinones, cellular fatty acids and phospholipids were performed as described previously (Tamura *et al.*, 1995). The chemotaxonomic properties support affiliation of the six Indonesian isolates to the family *Actinosynnemataceae* (Labeda & Kroppenstedt, 2000), in which all isolates have the cell-wall type IV/A of Lechevalier & Lechevalier (1970) with *meso*-diaminopimelic acid and have galactose and arabinose as the whole-cell sugars, MK-9 (H₄) as the major menaquinone and phospholipid type II of Lechevalier *et al.* (1981), with significant amounts of phosphatidylethanolamine and the absence of phosphatidylglycerol, phosphatidylcholine and an unidentified phosphorus-free aminolipid. The major cellular fatty acids of ID03-0561^T were iso-C_{16:0} (29.1 %), iso-C_{15:0} (17.1 %) and iso-C_{17:0} (16.7 %), with small amounts of iso-C_{16:0} 2-OH, iso-C_{15:0} 2-OH and anteiso-C_{17:0}; for the other Indonesian isolates, the major fatty acids were iso-C_{16:0} (22.4–44.2 %), iso-C_{15:0} (9.8–18.8 %) and iso-C_{16:0} 2-OH (10.8–14.3 %).

Genomic DNA for determination of G + C content and DNA–DNA hybridization was extracted by the method of Saito & Miura (1963). The genomic DNA G + C content was determined by enzymic hydrolysis of DNA followed by reversed-phase HPLC as described by Tamura *et al.* (1994). DNA–DNA hybridization was performed by the fluorometric method in microdilution wells with photobiotin as described by Ezaki *et al.* (1989). Results of G + C content determination and DNA–DNA hybridization are given in

Table 1. Indonesian actinomycetes included in this study

All strains were isolated by the rehydration and centrifugation method (Hayakawa *et al.*, 2000).

Strain	Location	Source
Group 1		
ID03-0561 ^T	Eka Karya Botanical Garden, Bali	Soil
Group 2		
ID03-0784 ^T	Cibodas Botanical Garden, West Java	Leaf litter
ID03-0808	Cibodas Botanical Garden, West Java	Leaf litter
ID03-0809	Cibodas Botanical Garden, West Java	Leaf litter
Group 3		
ID03-0810 ^T	Cibodas Botanical Garden, West Java	Leaf litter
ID03-0813	Cibodas Botanical Garden, West Java	Leaf litter

Fig. 1. 16S rRNA gene sequence dendrogram reconstructed by the neighbour-joining method using the software MEGA version 4 (Tamura *et al.*, 2007) displaying the relatedness of the novel strains and other members of the genus *Actinokineospora*. The sequence of *Pseudonocardia thermophila* IMSNU 20112^T was used as the outgroup. Bar, 0.005 substitutions per nucleotide position. Asterisks indicate branches of the tree that were also recovered using the minimum-evolution and maximum-parsimony methods (these trees are available as Supplementary Figs S1 and S2 in IJSEM Online).

Table 2. The range of G + C content of the isolates was 70.2–71.9 mol%. DNA–DNA hybridization revealed that the six Indonesian isolates were divided into three DNA–DNA relatedness groups. Group I contained a single isolate (ID03-0561^T), group II contained three isolates (ID03-0784^T, ID03-0808 and ID03-0809) and group III contained two isolates (ID03-0810^T and ID03-0813). Group I showed low relatedness to the other Indonesian strains and the reference strains used. Strains of group II showed high relatedness to each other (98–100 %) and low relatedness to the other tested strains. The strains of group III showed 58–68 % relatedness to *Actinokineospora terrae* NBRC 15668^T and low relatedness (2–24 %) to the other reference strains.

The results of phenotypic characterization, performed as described previously (Seino *et al.*, 1985; Shirling & Gottlieb, 1966; Otoguro *et al.*, 2001b), are given in the species descriptions. The isolates used for phenotypic tests

were grown in yeast extract/glucose broth medium as described for chemotaxonomic analysis and resuspended in distilled water. DNA–DNA relatedness groups I–III could be distinguished from the type strains of other species of the genus *Actinokineospora* by using a combination of phenotypic properties. Strain ID03-561^T in group I was positive for utilization of mannose and sucrose and negative for utilization of arabinose, galactose, fructose and rhamnose. The isolates in group II were positive for utilization of galactose, mannose, fructose, sucrose and maltose and negative for utilization of arabinose and rhamnose as sole carbon sources. The two isolates in group III were distinguished from *Actinokineospora terrae* by being positive for utilization of galactose and negative for utilization of arabinose and rhamnose.

It is clear from the genotypic, chemotaxonomic and phenotypic data that the six Indonesian strains represent three novel species in the genus *Actinokineospora*. The

Table 2. DNA base composition and DNA–DNA hybridization of *Actinokineospora* strains

Strain	G + C content (mol%)	DNA–DNA hybridization (%) with labelled DNA from strain:									
		1	2	3	4	5	6	7	8	9	10
1. ID03-0561 ^T	71.4	(100)	12	13	16	27	10	24	37	17	2
2. ID03-0784 ^T	71.3	12	(100)	ND	14	ND	6	15	17	17	14
ID03-0808	71.9	15	93	ND	17	ND	11	23	35	32	9
3. ID03-0809	71.5	13	94	(100)	15	ND	ND	52	ND	2	ND
4. ID03-0810 ^T	70.2	25	23	12	(100)	ND	3	26	25	58	16
ID03-0813	70.3	31	10	13	91	ND	9	ND	ND	65	25
5. <i>A. riparia</i> NBRC 14541 ^T	ND	7	7	7	8	(100)	19	25	2	7	38
6. <i>A. inagensis</i> NBRC 15663 ^T	ND	ND	20	ND	ND	ND	(100)	27	30	25	30
7. <i>A. globicatena</i> NBRC 15664 ^T	ND	26	24	ND	ND	28	1	(100)	13	26	32
8. <i>A. diospyrosa</i> NBRC 15665 ^T	ND	20	7	9	12	16	9	20	(100)	24	39
9. <i>A. terrae</i> NBRC 15668 ^T	ND	29	15	19	68	20	4	30	10	(100)	20
10. <i>A. enzaensis</i> NBRC 16517 ^T	ND	13	13	ND	ND	27	17	33	4	24	(100)
<i>A. auranticolor</i> NBRC 16518 ^T	ND	6	5	8	8	17	12	16	5	10	2

ND, Not determined.

names *Actinokineospora baliensis* sp. nov., *Actinokineospora cibodasensis* sp. nov. and *Actinokineospora cianjurenensis* sp. nov. are proposed for DNA–DNA hybridization groups I–III.

Description of *Actinokineospora baliensis* sp. nov.

Actinokineospora baliensis (ba.li.en'sis. N.L. fem. adj. *baliensis* pertaining to Bali, Indonesia, from where the type strain was isolated).

Morphological, chemotaxonomic and general characteristics are as given for the genus by Hasegawa (1988) and Labeda *et al.* (2010). Vegetative mycelium is pale yellow. When formed, aerial mycelium is white. Aerial mycelium produces rod-shaped arthrospores (diameter 1.5–2.0 µm) with smooth surfaces that exhibit motility when suspended in sterile distilled water. Good growth at 25–28 °C. Grows well on ISP media 2, 3, 5 and 7, Bennett's agar and nutrient agar; does not grow on ISP media 4 or 6 or water agar. Grows in the presence of 1 % NaCl, but not in the presence of 3, 5 or 7 % NaCl. Reduces nitrate to nitrite. Hydrolyses aesculin, urea and gelatin. Utilizes glucose, trehalose, mannose, sucrose and maltose as carbon sources, but not arabinose, galactose, rhamnose, lactose, raffinose, sorbitol, xylose, cellobiose, melibiose, *meso*-erythritol, inositol, mannitol, ribitol, dulcitol or xylitol. Degrades xanthine and elastin but not testosterone, gelatin or calcium malate. Peptonizes milk. iso-C_{16:0}, iso-C_{15:0} and iso-C_{17:0} are the major cellular fatty acids. The G + C content of the DNA of the type strain is 71.4 mol%.

The type strain, ID03-0561^T (=BTCC B-554^T =NBRC 104211^T), was isolated from soil under a *Manglietia glauca* tree in Eka Karya Botanical Garden, Bali, Indonesia.

Description of *Actinokineospora cibodasensis* sp. nov.

Actinokineospora cibodasensis (ci.bo.da.sen'sis. N.L. fem. adj. *cibodasensis* pertaining to Cibodas, West Java, Indonesia, from where the first strains were isolated).

Morphological, chemotaxonomic and general characteristics are as given for the genus by Hasegawa (1988) and Labeda *et al.* (2010). Vegetative mycelium is yellow to tan. When formed, aerial mycelium is white. Aerial mycelium produces rod-shaped arthrospores (diameter 1.5–1.8 µm) which have smooth surfaces and exhibit motility when suspended in sterile distilled water. Good growth at 25–28 °C. Grows well on ISP media 2, 3, 4, 5 and 7, Bennett's agar and nutrient agar; does not grow on ISP medium 6 or water agar. Grows in the presence of 1 % NaCl, but not 3, 5 or 7 % NaCl. Reduces nitrate to nitrite. Hydrolyses aesculin, urea and gelatin. Utilizes glucose, trehalose, galactose, mannose, fructose, sucrose and maltose, but not arabinose, rhamnose, lactose, raffinose, sorbitol, xylose, cellobiose, melibiose, *meso*-erythritol, inositol, mannitol, ribitol, dulcitol or xylitol. Degrades xanthine, elastin and

testosterone, but not gelatin or calcium malate. Does not peptonize milk. iso-C_{16:0}, iso-C_{15:0} and iso-C_{16:0} 2-OH are the major cellular fatty acids. The G + C content of the DNA of the type strain is 71.3 mol%.

The type strain, ID03-0748^T (=BTCC B-555^T =NBRC 104212^T), was isolated from a leaf-litter sample from Cibodas Botanical Garden, West Java, Indonesia. Strains ID03-0808 and ID03-0809, from the same source, are also representatives of the species.

Description of *Actinokineospora cianjurenensis* sp. nov.

Actinokineospora cianjurenensis (ci.an.jur.en'sis. N.L. fem. adj. *cianjurenensis* pertaining to Cianjur, West Java, Indonesia, from where the first strains were isolated).

Morphological, chemotaxonomic and general characteristics are as given for the genus by Hasegawa (1988) and Labeda *et al.* (2010). Vegetative mycelium is yellow to brown. When formed, aerial mycelium is white. Aerial mycelium produces rod-shaped arthrospores (diameter 1.5–2.0 µm) which have smooth surfaces and exhibit motility when suspended in sterile distilled water. Good growth at 25–28 °C. Grows well on ISP media 2, 3, 4, 5 and 7, Bennett's agar and nutrient agar, but not on ISP medium 6 or water agar. Grows in the presence of 1 % NaCl, but not 3, 5 or 7 % NaCl. Reduces nitrate to nitrite. Hydrolyses aesculin, urea and gelatin. Utilizes glucose, trehalose, galactose, mannose, fructose, sucrose and maltose, but not arabinose, rhamnose, lactose, raffinose, sorbitol, xylose, cellobiose, melibiose, *meso*-erythritol, inositol, mannitol, ribitol, dulcitol or xylitol. Degrades xanthine, elastin and testosterone, but not gelatin or calcium malate. Peptonizes milk. iso-C_{16:0}, iso-C_{15:0} and iso-C_{16:0} 2-OH are the major cellular fatty acids. The G + C content of the DNA of the type strain is 70.2 mol%.

The type strain, ID03-0810^T (=BTCC B-558^T =NBRC 105526^T), was isolated from a leaf-litter sample from Cibodas Botanical Garden, West Java, Indonesia. Strain ID03-0813, isolated from the same source, is a second representative of the species.

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