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To cite this article: F Ningsih *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **457** 012015

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# Isolation and 16S rRNA gene sequences analysis of thermophilic *Actinobacteria* isolated from soil in Cisolok geothermal area, West Java, Indonesia

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**Abstract.** The thermophilic *Actinobacteria* are known not only as producers of pharmaceutically important bioactive compounds, but also commercially important enzymes, yet their diversity in geothermal area in Indonesia have been rarely reported. In our previous study, a new thermophilic *Actinobacteria* genus belonging to the family *Pseudonocardiaceae* has been isolated from soil in Cisolok geothermal area. This current study reports the taxonomy and findings of potentially new taxa of thermophilic *Actinobacteria*, based on phylogenetic analysis of 16S rRNA gene sequences. Twenty-five isolates of thermophilic *Actinobacteria* were isolated from soil samples and maintained using previously described methods. The 16S rRNA gene sequence-similarity search against all related species was performed using EzTaxon-e database. The sequences of 25 isolates showed similarity to member of family *Streptomycetaceae* (genus *Streptomyces*; 10 isolates), *Thermomonosporaceae* (genus *Actinomadura*; 3 isolates), *Streptosporangiaceae* (genus *Microbispora*; 6 isolates), *Micromonosporaceae* (genus *Micromonospora*; 2 isolates), *Pseudonocardiaceae* (genus *Amycolatopsis*; 3 isolates), and *Nocardiaceae* (genus *Nocardia*; 1 isolate). Fifteen out of 25 isolates are belong to non-*Streptomycetaceae* family, thus regarded as rare *Actinobacteria*. Nineteen out of 25 isolates showed 16S rRNA gene sequence-similarity values 97 – 99% to their closely related species, suggested the potential for findings novel taxa of thermophilic *Actinobacteria* from Cisolok geothermal area.

**Keywords:** Actinobacteria, thermophilic, Cisolok, geysers

## 1. Introduction

*Actinobacteria* is one of the most dominant phyla in the bacteria domain and has an ecologically significant role in biological processes such as biogeochemical cycles and bioremediation [1]. The



high abundance of actinobacterial species in various environments was recorded not only in normal but also in extreme environments defined by high temperatures, such as geothermal and volcanic areas, terrestrial hot springs, and geysers [2]. Thermophilic *Actinobacteria* grow well at quite high temperatures ranging from 40 to 80 °C. They are known not only as producers of pharmaceutically important bioactive compounds such as antibiotics and anti-inflammatory compounds, but also industrially and clinically important enzymes [1, 3].

The microbial ecology of *Actinobacteria* has been diversely reported, yet the ecological diversity, species distribution, and biogeography in hot springs are still poorly described. The difficulties in isolation process and maintenance in pure culture have caused thermophilic *Actinobacteria* been less explored [4]. Cisolok geothermal area in Sukabumi, West Java, is still less exploited as sources of thermophilic microorganisms, and thus a high-potential area for isolation of novel indigenous thermophilic *Actinobacteria*. The natural product screening from novel thermophilic *Actinobacteria* from unexplored and extreme environments will increase the possibility in finding novel secondary metabolites with great potential [5, 6].

In previous studies, Yokota *et al.* [7] successfully isolated new species of thermophilic bacterium namely *Paenibacillus cisolokensis* sp. nov., from litter of Cisolok geyser. A novel thermophilic *Actinobacteria* genus and species, *Gandjariella thermophila* gen. nov., sp. nov., belonging to the family *Pseudonocardiaceae* has also been isolated from soil forest in Cisolok geothermal area [8]. These studies indicated the high possibility of findings potentially new thermophilic *Actinobacteria* taxa in Cisolok geothermal area. This current study reports the taxonomy and findings of potentially new taxa of thermophilic *Actinobacteria* isolated from soil in geothermal area of Cisolok, based on phylogenetic analyses of 16S rRNA gene sequences.

## 2. Materials and methods

### 2.1 Isolation, purification, and preservation of thermophilic *Actinobacteria* isolates

Soil samples were collected from geothermal area of Cisolok (6°56'0.5"S, 106°27'13.4"E), West Java, Indonesia on September 2015. The samples were collected 20 cm under the soil surface from three different sampling locations: (1) soil around big geyser (6°57'221"S, 106°27'507"E); (2) soil around small geyser (6°57'189"S, 106°27'365"E); (3) soil forest which includes: (3.1) soil under *Gmelina* tree (6°57'482"S, 106°28'655"E), and (3.2) soil under Bamboo tree (6°57'482"S, 106°28'655"E). Soil samples from location 1, 2, and 3, are given code SL1-1 to 2, SL2-1 to 2, and SL3-1 to 2 respectively. Isolation of thermophilic *Actinobacteria* was performed using method as described by Yabe *et al.* [9] on Reasoner's 2A (Daigo, Nihon Seiyaku) agar medium with some modifications, incubated at 45 °C for 4 weeks, and purified several times on 1 % (w/v) International *Streptomyces* Project (ISP) 1 (tryptone 5 g, yeast extract 3 g L<sup>-1</sup>) agar medium [10] to obtain pure isolates. The pure isolates were grown on ISP 1 and ISP 3 (oatmeal 20 g, agar 18 g, trace salt solutions 1 ml L<sup>-1</sup>) agar media [10], incubated at 45 °C for 7 days, and maintained at room temperature as stock cultures. The pure isolates were also stored as agar block in 20 % (v/v) glycerol stock solutions at -80 °C, and preserved as lyophilized cells for long-term preservation [8]. All thermophilic *Actinobacteria* isolates were deposited at Universitas Indonesia Culture Collection (UICC), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia.

### 2.2 Bacterial genomic DNA isolation

Genomic DNA for PCR amplification of the 16S rRNA gene was prepared using the method as described by Yabe *et al.* [11]. The 16S rRNA gene of bacterial isolates was amplified using polymerase chain reaction (PCR) with universal eubacterial primers: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTGTTACGA-3'). The PCR amplification for the 16S rRNA gene were carried out according to the conditions of MyTaq™ Red Mix (Bioline) which includes initial denaturation at 95 °C for 3 min, followed by 35 cycles including denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s and extension at 72 °C for 1 min. The PCR

products were sequenced using 1st BASE DNA sequencing service (<http://base-asia.com/dna-sequencing-services>). The sequences of all type strains used in phylogenetic analyses were retrieved from the DDBJ/EMBL/GenBank databases.

### 2.3 Phylogenetic analyses

The partial sequence of the 16S rRNA gene from thermophilic *Actinobacteria* isolates was used for a sequence-similarity search against all related species in the database through EzTaxon-e (<https://www.ezbiocloud.net/>; Yoon *et al.* [12]), and for phylogenetic analyses. The sequences of 25 thermophilic *Actinobacteria* isolates were aligned with the sequences of type strains retrieved from the DDBJ/EMBL/GenBank databases and analyzed using MEGA v7.0.26 software package [13]. Phylogenetic trees were reconstructed based on nearly full-length 16S rRNA gene sequences of type strains of the closely related taxa to thermophilic *Actinobacteria* isolates using the neighbor-joining [14], minimum-evolution [15], and maximum-likelihood [16] methods in the MEGA v7.0.26 software package [13], with bootstrap values based on 1000 replications [17]. Kimura two-parameter method was used to compute the evolutionary distances of each phylogenetic tree [18].

## 3. Results and discussion

A total of 25 selected thermophilic *Actinobacteria* isolates were successfully obtained from soil in geothermal area of Cisolok. The number of isolates selected from soil samples around big geyser (location 1), small geyser (location 2), and forest near big geyser (location 3) are 4, 4, and 17 isolates, respectively. Isolation of the genomic DNA and amplification of the 16S rRNA gene from thermophilic *Actinobacteria* isolates were performed, then the PCR product samples were sequenced. The result of 16S rRNA gene sequence analysis is shown in table 1.

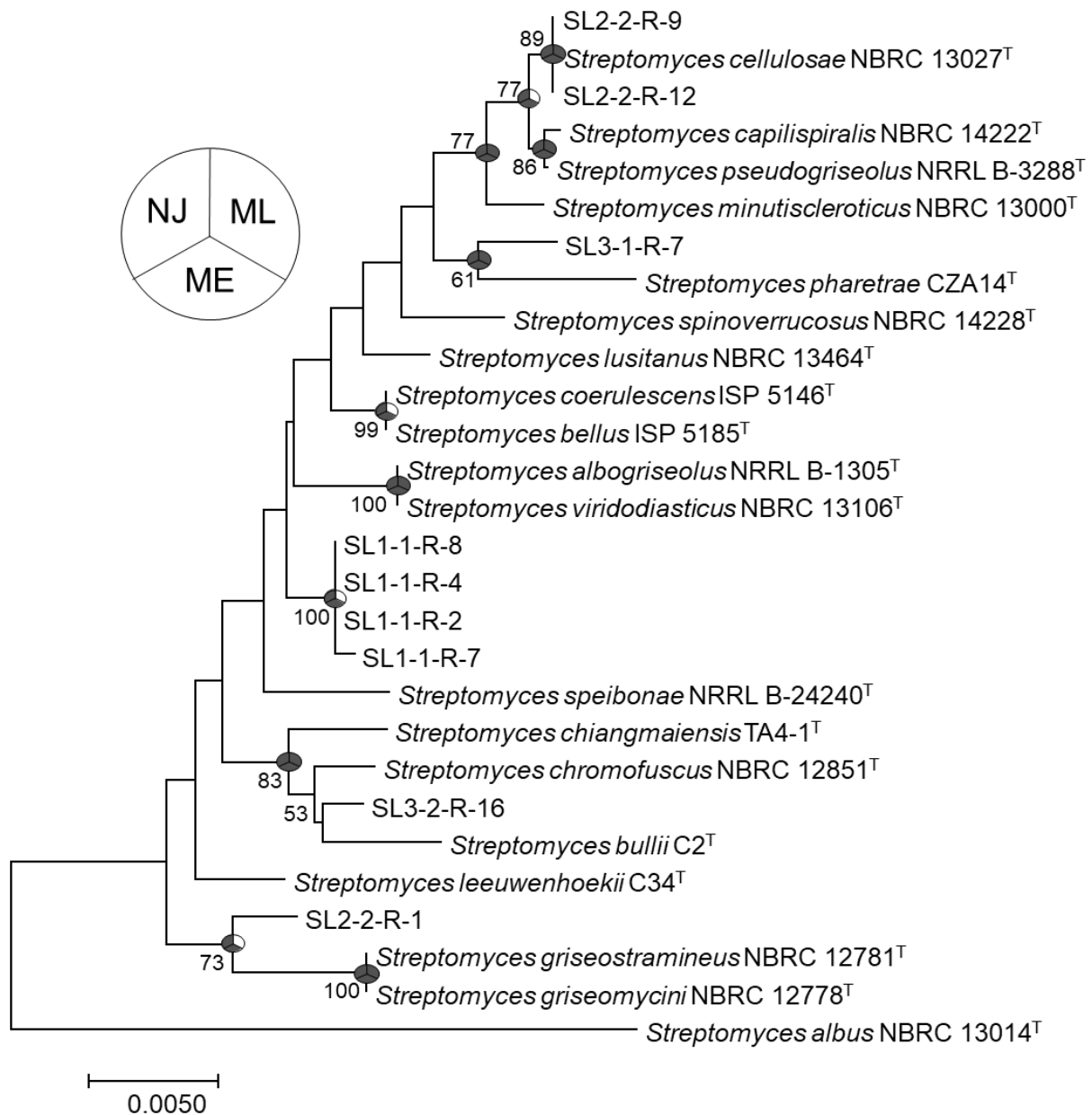
The sequences of 25 isolates showed similarities to member of families *Streptomycetaceae* (genus *Streptomyces*; 10 isolates), *Streptosporangiaceae* (genus *Microbispora*; 6 isolates), *Thermomonosporaceae* (genus *Actinomadura*; 3 isolates), *Pseudonocardiaceae* (genus *Amycolatopsis*; 3 isolates), *Micromonosporaceae* (genus *Micromonospora*; 2 isolates), and *Nocardiaceae* (genus *Nocardia*; 1 isolate). Similarity values of 19 out of 25 isolates showed 97 – 99% to their closely related species (table 1). The result showed that soil of Cisolok geothermal area is potential source for isolating thermophilic *Actinobacteria* with high possibility of new taxa. According to Panda *et al.* [3], based on phylogenetic tree analysis using the 16S rRNA gene sequences, the actinobacterial groups isolated from various hot springs were dominated by orders *Streptomycetales* (family *Streptomycetaceae*), *Micromonosporales* (family *Micromonosporaceae*), and *Streptosporangiales* (families *Thermomonosporaceae* and *Streptosporangiaceae*). Our results showed that four out of six *Actinobacteria* families are member of actinobacterial groups that are mostly found in various hot springs. Among 6 *Actinobacteria* families identified, 5 are regarded as rare *Actinobacteria* groups, except for family *Streptomycetaceae*. According to Tiwari and Gupta [6], rare *Actinobacteria* are usually defined as non-streptomycete strains of class *Actinobacteria*, with low frequency of isolation compare to the streptomycete strains using common methods. The isolation of rare *Actinobacteria*, thus becomes an important effort for the discovery of new secondary metabolites and development of clinically important antibiotics and enzymes [6].

Detailed phylogenetic analyses based on 16S rRNA gene sequences using neighbour-joining (NJ), minimum evolution (ME), and maximum-likelihood (ML) methods were performed for two groups of isolates belong to *Streptomycetaceae* (figure 1) and non-*Streptomycetaceae* (figure 2) families. The sequences of 25 isolates were aligned with the sequences of type strains retrieved from the DDBJ/EMBL/GenBank databases. The analyses involved 28 and 40 nucleotides sequences for *Streptomycetaceae* and non-*Streptomycetaceae* family groups, respectively. The 16S rRNA gene sequence of 10 isolates showed highest similarity to members of species in the family *Streptomycetaceae*: *Streptomyces coerulescens* ISP 5146<sup>T</sup> (isolates SL1-1-R-2, SL1-1-R-4, SL1-1-R-7, and SL1-1-R-8), *S. leeuwenhoekii* C34<sup>T</sup> (SL2-2-R-1), *S. cellulosa* NBRC 13027<sup>T</sup> (SL2-2-R-9 and

SL2-2-R-12), *S. minutiscleroticus* NBRC 13000<sup>T</sup> (SL3-1-R-7), *S. chiangmaiensis* TA4-1<sup>T</sup> (SL3-2-R-8), and *S. chromofuscus* NBRC 12851<sup>T</sup> (SL3-2-R-16), with similarity values of 97 to 100% (table 1).

**Table 1.** The 16S rRNA gene sequence analysis result of 25 thermophilic *Actinobacteria* isolates

No.	Code of Isolate	Sequence length (nt)	EzTaxon-e top hit taxon and strain	Homology (%)	Accession number
1	SL1-1-R-2	1349	<i>Streptomyces coerulescens</i> ISP 5146 <sup>T</sup>	1336/1348 (99.11)	AY999720
2	SL1-1-R-4	1353	<i>Streptomyces coerulescens</i> ISP 5146 <sup>T</sup>	1343/1352 (99.33)	AY999720
3	SL1-1-R-7	1315	<i>Streptomyces coerulescens</i> ISP 5146 <sup>T</sup>	1305/1314 (99.32)	AY999720
4	SL1-1-R-8	1329	<i>Streptomyces coerulescens</i> ISP 5146 <sup>T</sup>	1321/1329 (99.40)	AY999720
5	SL2-2-R-1	1341	<i>Streptomyces leeuwenhoekii</i> C34 <sup>T</sup>	1329/1340 (99.18)	LN831790
6	SL2-2-R-9	1485	<i>Streptomyces cellulosa</i> NBRC 13027 <sup>T</sup>	1449/1449 (100)	AB184265
7	SL2-2-R-12	1344	<i>Streptomyces cellulosa</i> NBRC 13027 <sup>T</sup>	1344/1344 (100)	AB184265
8	SL2-2-R-15	1344	<i>Nocardia farcinica</i> NCTC 11134 <sup>T</sup>	1344/1344 (100)	LN868938
9	SL3-1-R-1	1317	<i>Amycolatopsis methanolica</i> 239 <sup>T</sup>	1317/1317 (100)	AQUL01000001
10	SL3-1-R-3	1290	<i>Amycolatopsis methanolica</i> 239 <sup>T</sup>	1290/1290 (100)	AQUL01000001
11	SL3-1-R-7	1352	<i>Streptomyces minutiscleroticus</i> NBRC 13000 <sup>T</sup>	1341/1352 (99.19)	AB184249
12	SL3-1-R-13	1320	<i>Micromonospora globbae</i> WPS1-2 <sup>T</sup>	1319/1320 (99.92)	LC177396
13	SL3-1-R-14	1359	<i>Micromonospora costi</i> CS1-12 <sup>T</sup>	1349/1358 (99.34)	AB981048
14	SL3-1-R-16	1353	<i>Amycolatopsis methanolica</i> 239 <sup>T</sup>	1353/1353 (100)	AQUL01000001
15	SL3-2-R-1	1317	<i>Microbispora rosea</i> subsp. <i>rosea</i> ATCC 12950 <sup>T</sup>	1312/1317 (99.62)	FTNI01000083
16	SL3-2-R-2	1269	<i>Microbispora bryophytorum</i> NEAU-TX2-2 <sup>T</sup>	1229/1260 (97.54)	KF886293
17	SL3-2-R-5	1359	<i>Microbispora bryophytorum</i> NEAU-TX2-2 <sup>T</sup>	1348/1355 (99.48)	KF886293
18	SL3-2-R-8	1275	<i>Streptomyces chiangmaiensis</i> TA4-1 <sup>T</sup>	1225/1244 (97.67)	AB562507
19	SL3-2-R-11	1356	<i>Microbispora rosea</i> subsp. <i>rosea</i> ATCC 12950 <sup>T</sup>	(99.63)	FTNI01000083
20	SL3-2-R-12	1311	<i>Microbispora hainanensis</i> 211020 <sup>T</sup>	1302/1310 (99.39)	FJ261972
21	SL3-2-R-16	1341	<i>Streptomyces chromofuscus</i> NBRC 12851 <sup>T</sup>	1335/1341 (99.55)	AB184194
22	SL3-2-R-17	1343	<i>Actinomadura miaoliensis</i> BC 44T-5 <sup>T</sup>	1330/1341 (99.18)	EF116925
23	SL3-2-R-18	1343	<i>Microbispora hainanensis</i> 211020 <sup>T</sup>	1327/1342 (98.88)	FJ261972
24	SL3-2-R-37	1351	<i>Actinomadura miaoliensis</i> BC 44T-5 <sup>T</sup>	1338/1349 (99.18)	EF116925
25	SL3-2-R-48	1352	<i>Actinomadura barringtoniae</i> GKU 128 <sup>T</sup>	1344/1351 (99.48)	KF667497



**Figure 1.** Neighbour-joining tree showing phylogenetic relationship between isolates belong to *Streptomyces* genus and the type species of the genus *Streptomyces* in the family *Streptomycetaceae*, based on 16S rRNA gene partial sequences. *Streptomyces albus* NBRC 13014<sup>T</sup> was used as an outgroup (BBQG01000088). Bar 0.005 nucleotide substitutions per site.

In the neighbour-joining tree (figure 1), 4 isolates (SL1-1-R-2, SL1-1-R-4, SL1-1-R-7, and SL1-1-R-8) formed monophyletic cluster, while isolate SL2-2-R-1 formed clade with *S. griseostramineus* NBRC 12781<sup>T</sup> and *S. griseomycini* NBRC 13014<sup>T</sup> instead of *S. leeuwenhoekii* C34<sup>T</sup>, supported by NJ and ME trees with 100% and 73% bootstrap values, respectively. The same pattern also shown by isolate SL3-1-R-7 which formed monophyletic cluster with *S. pharetrae* CZA14<sup>T</sup>, instead of *S. minutiscleroticus* NBRC 13000<sup>T</sup> with 61% bootstrap values supported by NJ, ME, and ML trees.

Meanwhile, isolate SL3-2-R-16 formed clade with *S. bullii* C2<sup>T</sup>, *S. chromofuscus* NBRC 12851<sup>T</sup>, and *S. chiangmaiensis* TA4-1<sup>T</sup> supported by NJ, ME, and ML trees with 83% bootstrap value.

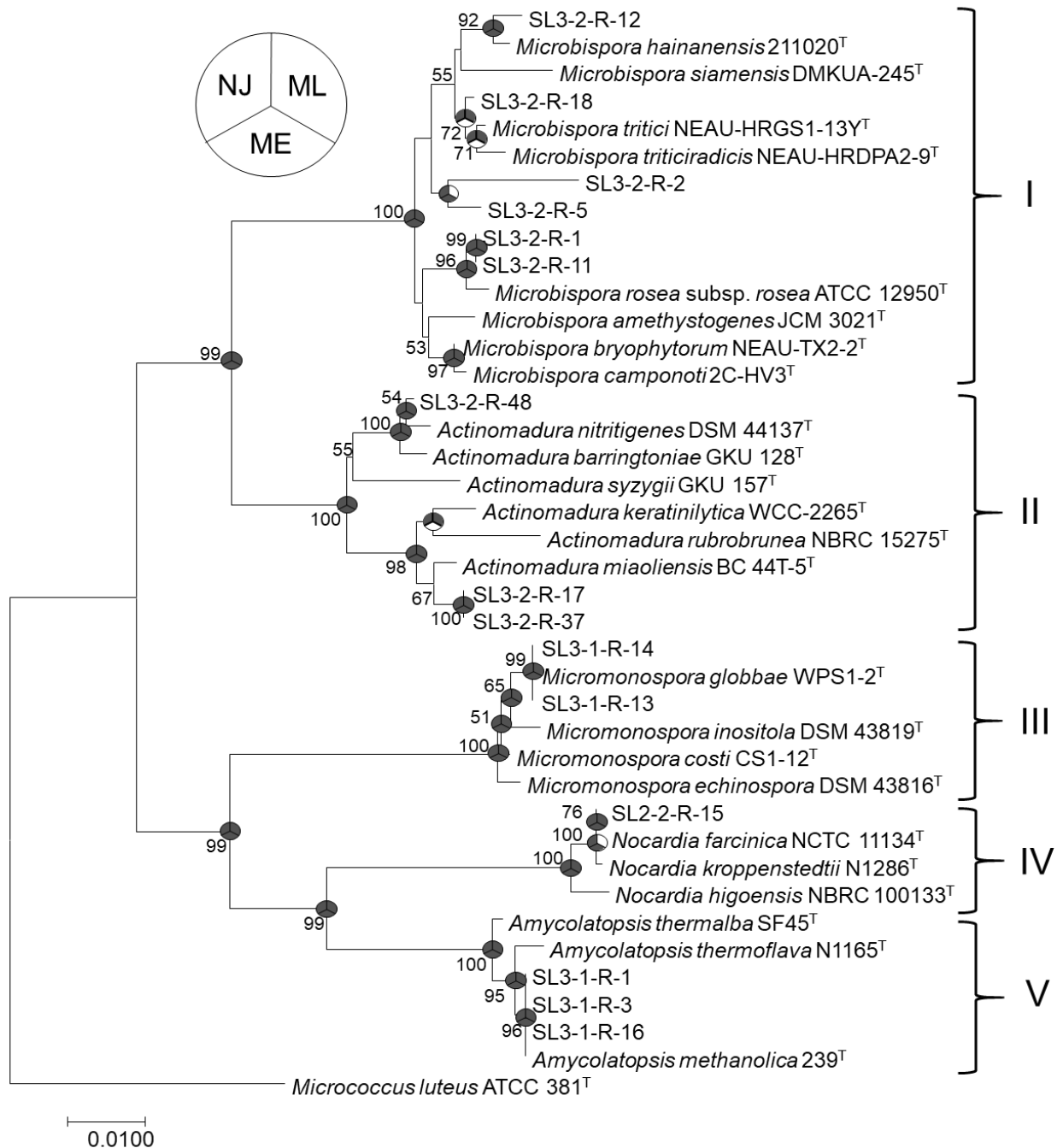
Waksman and Henrici [19] first proposed the genus *Streptomyces* on 1943, with *Streptomyces albus* as the type species. Until recently, there are more than 800 validly published *Streptomyces* species have been reported (<https://www.bacterio.net/streptomyces.html>) [20]. The *Streptomyces* species are widely distributed and abundant in soil and well-known as producers of ~39% of all microbial bioactive compounds [5]. The genus *Streptomyces* mainly includes mesophilic species with optimum temperature ranging from 25 to 35 °C [21]. However, some thermotolerant (able to grow at 45 °C), and few thermophilic (growth temperatures between 28–55 °C) streptomycetes are presence [21]. In this study, all *Streptomyces* isolates were able to grow at temperature of 45 °C, which suggested the possibility of thermophilic species. The isolates also have 97.67 – 99.55% sequence similarities to their most closely related species, which indicated high possibilities for finding new taxa in the family *Streptomycetaceae*.

Phylogenetic analyses of isolates belongs to non-*Streptomycetaceae* family showed that the isolates were situated in the clade of five rare *Actinobacteria* families: *Streptosporangiaceae*; *Thermomonosporaceae*; *Micromonosporaceae*; *Nocardiaceae*; and *Pseudonocardiaceae* with high bootstrap support (100%) (figure 2). Six isolates showed highest similarity to members of species in the family *Streptosporangiaceae*: *Microbispora rosea* subsp. *rosea* ATCC 12950<sup>T</sup> (SL3-2-R-1, 99.62% and SL3-2-R-11, 99.63%); *Microbispora bryophytorum* NEAU-TX2-2<sup>T</sup> (SL3-2-R-2, 97.54% and SL3-2-R-5, 99.48%); *Microbispora hainanensis* 211020<sup>T</sup> (SL3-2-R-12, 99.39% and SL3-2-R-18, 98.88%). Isolates SL3-2-R-2 and SL3-2-R-5 formed a monophyletic clade, supported by NJ and ME trees with less than 50% bootstrap value, however not located in the same clade with their highest similarity type strain *Microbispora bryophytorum* NEAU-TX2-2<sup>T</sup>. Isolate SL3-2-R-18 located in the same clade with *Microbispora triticiradicis* NEAU-HRDP2-9<sup>T</sup> and *M. tritici* NEAU-HRGS1-13Y<sup>T</sup> supported by NJ and ML trees with 72% bootstrap value, instead of with *Microbispora hainanensis* 211020<sup>T</sup>. Isolates SL3-2-R-2 and SL3-2-R-18 have lowest similarities amongst 6 isolates in family *Streptosporangiaceae*, 97.54% and 98.88% respectively, thus have high potential as new taxa. According to Janda and Abbott [22], the definition of species identification based on 16S rRNA gene sequence is that the same species has ≥99%, while different species showed <99% sequence similarities, respectively.

Three isolates are member of family *Thermomonosporaceae*, and closely related to *Actinomadura miaoliensis* BC 44T-5<sup>T</sup> (SL3-2-R-17 and SL3-2-R-37, 99.18%), and *A. barringtoniae* GKU 128<sup>T</sup> (SL3-2-R-48, 99.48%). Isolates SL3-2-R-17 and SL3-2-R-37 formed monophyletic cluster, and located in the same clade with *Actinomadura miaoliensis* BC 44T-5<sup>T</sup> with 100% and 67% bootstrap values, respectively. Meanwhile, isolate SL3-2-R-48 also formed monophyletic cluster with *Actinomadura nitritigenes* DSM 44137<sup>T</sup>, and located in the same clade with *A. barringtoniae* GKU 128<sup>T</sup> with 54% and 100% bootstrap values supported by NJ, ME, and ML trees. Trujillo and Goodfellow [23] reported that the *Actinomadura* species are mostly isolated from soil and act as decomposers.

Isolates SL3-1-R-13 and SL3-1-R-14, were located in the clade of the family *Micromonosporaceae* and most closely related to the type species *Micromonospora globbae* WPS1-2<sup>T</sup> (99.92%) and *M. costi* CS1-12<sup>T</sup> (99.34%), respectively. Meanwhile, phylogenetic analyses showed that both isolates were located in the same cluster only with *Micromonospora globbae* WPS1-2<sup>T</sup> supported by NJ, ME, and ML trees with 99% bootstrap value. The genus *Micromonospora* is known as sources of new secondary metabolites and novel biosynthetic gene clusters (BGCs) after streptomycetes [24]. Thus potentially new taxa from genus *Micromonospora* isolated from unique habitat will increase the possibilities of findings new bioactive compounds. Isolate SL2-2-R-15 showed highest similarity (100%) to the member of family *Nocardiaceae*: *Nocardia farcinica* NCTC 11134<sup>T</sup>, and formed monophyletic cluster with 76% bootstrap value supported by NJ, ME, and ML trees. Isolates SL3-1-R-1, SL3-1-R-3, and SL3-1-R-16 also showed 100% similarities and formed monophyletic clade with

member of family *Pseudonocardiaceae*, *Amycolatopsis methanolica* 239<sup>T</sup> with 96% bootstrap value supported by NJ, ME, and ML trees.



**Figure 2.** Phylogenetic relationship between rare *Actinobacteria* isolates belong to non-*Streptomycetaceae* families: I, *Streptosporangiaceae*; II, *Thermomonosporaceae*; III, *Micromonosporaceae*; IV, *Nocardiaceae*; V, *Pseudonocardiaceae*, and the representative type species from each family, based on nearly full-length 16S rRNA gene sequences. *Micrococcus luteus* ATCC 381<sup>T</sup> was used as an outgroup. Bar 0.0100 nucleotide substitutions per site.



The genera belongs to non-*Streptomycetaceae* family obtained from this study, e.g. *Actinomadura*, *Amycolatopsis*, *Microbispora*, and *Nocardia*, are regarded as rare actinomycetes and widely distributed in terrestrial and aquatic ecosystems [6]. They are regarded as important sources of novel secondary metabolites, however the information about their genetics and physiology are still rarely described [6]. As mentioned by Mohammadipناه and Wink [25], *Actinobacteria* genera including *Streptomyces*, *Micromonospora*, *Nocardia*, and *Amycolatopsis* have shown high bioactivity. Moreover, according to Carro *et. al.* [24], *Actinobacteria* harboring large genome size, e.g. *Streptomyces* strains, *Amycolatopsis*, and *Micromonospora*, indicated abundant in BGCs encoding biosynthetic pathways of certain secondary metabolites. Thus, soil in Cisolok geothermal area is a potential source for findings potentially novel *Actinobacteria* taxa with promising future applications.

Further 16S rRNA gene sequencing with primers 27F, 785F, 800R, and 1492R will be performed to get complete sequences of the thermophilic *Actinobacteria* isolates with 97 – 99% similarities and re-confirm their position in the phylogenetic tree analyses. The information regarding morphological, physiological, and chemotaxonomic properties will also be required to distinguish the potentially new taxa from related species in their respective families. The combination of phenotypic data and phylogenetic tree analyses will provide evidence to demonstrate the novelty of the potentially new taxa.

#### 4. Conclusion

Thermophilic *Actinobacteria* isolates have been successfully obtained from soil samples in Cisolok geothermal area, West Java, Indonesia. The 16S rRNA gene sequences of 25 isolates are belongs to 6 *Actinobacteria* families, including family *Streptomycetaceae*, *Nocardiaceae*, *Thermomonosporaceae*, *Streptosporangiaceae*, *Micromonosporaceae*, and *Pseudonocardiaceae*. Fifteen out of 25 isolates are belong to non-*Streptomycetaceae* family, thus regarded as rare *Actinobacteria*. Meanwhile, the 16S rRNA gene sequence-similarity values of 19 out of 25 isolates showed 97 – 99% to their closely related species, which suggested potentially new taxa. Complete 16S rRNA gene sequencing and detailed phylogenetic analysis from the potentially new taxa are required to clarify their identity within *Actinobacteria* families.

#### Acknowledgments

This work was funded by Hibah Tugas Akhir Doktor (TADok) 2019 research grant, contract no. NKB-0149/UN2.R3.1/HKP.05.00/2019 to W. S.; Hibah Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT), Direktorat Jenderal Pendidikan Tinggi (DIKTI) 2018, Kementerian Riset Teknologi dan Pendidikan Tinggi Republik Indonesia research grant, contract no. 387/UN2.R3.1/HKP.05.00/2018 to W. S.; and research grant from Tohoku University to S. Y.

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