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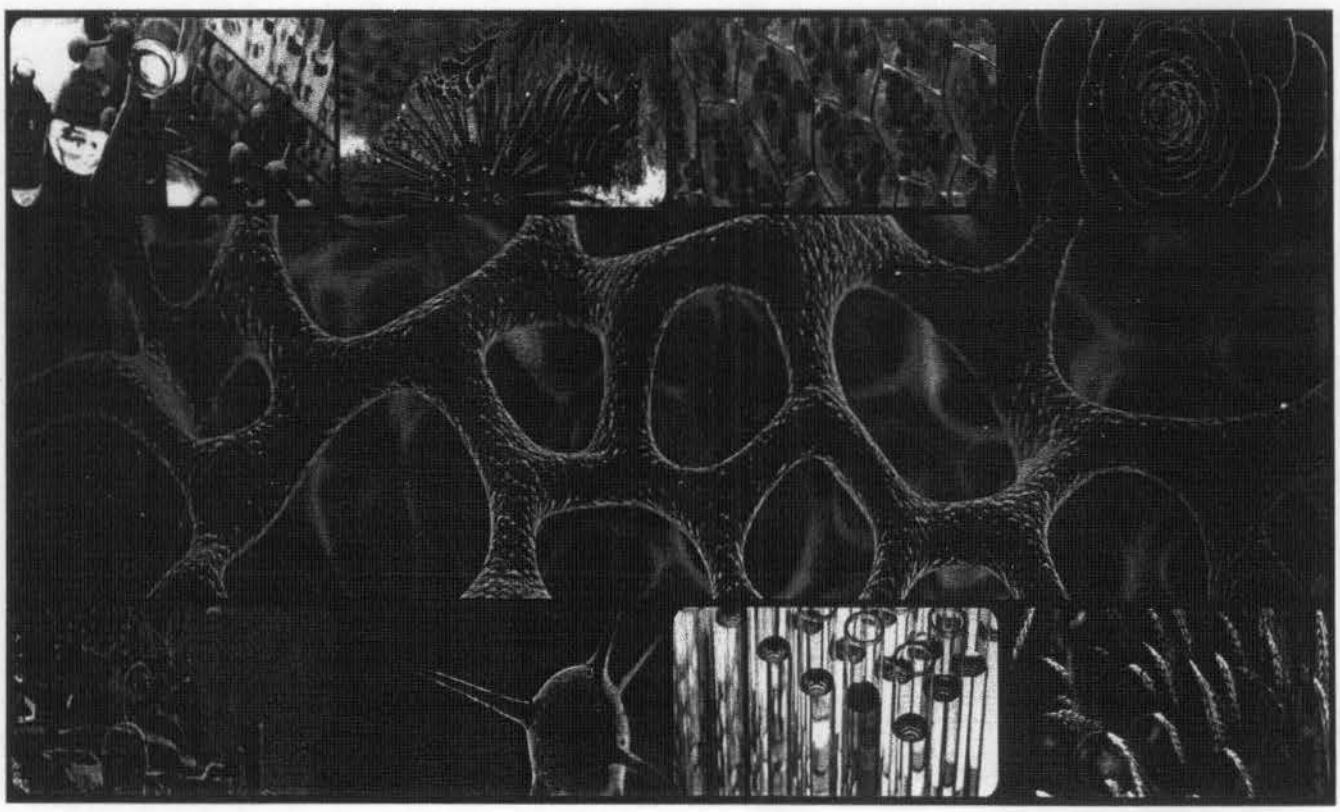

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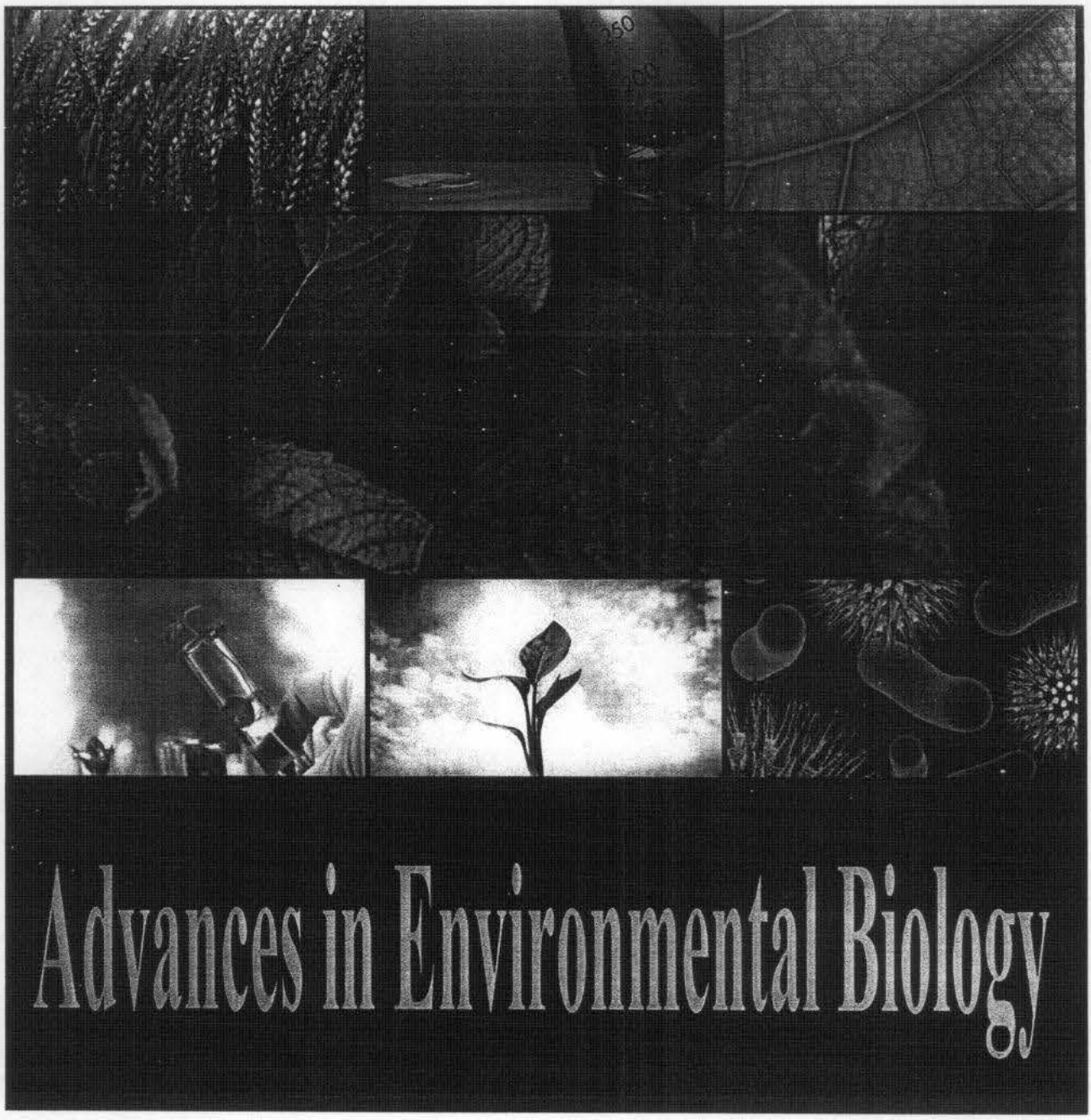


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# Identification of Endophytic Actinomycetes from Indonesian Rice Plant Based on 16S rRNA and *nifH* genes Analyses

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ABSTRACT

Indigenous actinomycetes are known to have high biodiversity and chance to acquire a novel species. Molecular identification and the role of rice endophytic actinomycetes need to be studied. The research aimed to identify endophytic actinomycetes from Indonesian rice plant based on 16S rRNA and *nifH* genes properties. DNA genome from the seven isolates of endophytic actinomycetes was isolated using Genomic DNA Mini Kit followed by PCR amplification of 16S rRNA and *nifH* genes. Indication of their nitrogen fixing activities was conducted based on their capability to grow in N-free medium, ammonia production, and presence of *nifH* gene. PCR products were sequenced and analyzed by bioinformatics software to construct phylogenetic tree. An analysis of 16S rRNA gene sequences demonstrated that the seven isolates are most closely related to *Streptomyces* spp. The 16S rRNA gene sequences of the six isolates were closed related with *S. albidoflavus*, *S. cavourensis* subsp. *cavourensis*, *S. annulatus*, and *S. fungoeasis* with ≥ 97% maximum identity, and another isolate was closed related with *S. misionensis* with 99% maximum identity. Based on *nifH* gene sequences analysis, three isolates of endophytic actinomycetes showed that they were closely related to *nifH* from *Herbaspirillum* sp., the similarity was 93 to 99%. Data derived from the phylogenetic tree with p-distance analysis which showed diversity of genetic distances between three isolates of endophytic actinomycetes compared with *Frankia* sp., *Rhizobium* sp., *L. ferrooxidans*, also *B. japonicum*, were about 18-28% and more than 52% when compared with *B. japonicum*. Those differences indicated diversity of rice endophytic actinomycetes *nifH* gene. Based on *in vitro* assay, IPBCC b.14.1530, IPBCC b.14.1531, and IPBCC b.14.1536 isolates were also capable to grow in N-free medium and produced 0.065 ppm, 0.014 ppm, and 0.076 ppm ammonia in N-free medium, respectively. The results indicated that the three isolates had promising role as a $N_2$ fixing bacteria on rice plant.



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## INTRODUCTION

Almost all vascular plant species examined to date were found to be associated with endophytic microbes, which may produce various bioactive compounds related to the host [25]. Endophytic microbes live in and colonize plant tissues during some periods and usually obtain nutrition and protection from the host plants [8]. These microbe are known as potential sources of natural products for agriculture, medicine, and industrial exploitation [22]. In agriculture, endophytic microbe is considered as agents to stimulate plant growth, for management of soil and plant health [4] including fixing $N_2$ [15]. Various kinds of microorganisms, including actinomycetes, fungi, and other bacteria, have been found inside plants and designated as endophytes [13].

Actinomycetes are Gram-positive bacteria with high G+C% and known to have high biodiversity and chance to acquire a novel species [14]. Several members of actinomycetes produce important secondary metabolites, including antibiotics, siderophore, enzyme, and plant growth-promoting substances which may contribute to their host plant by promoting growth and enhancing their ability of with standing the environmental stressing [10,17]. Actinomycetes play a vital role in the soil such as immobilization of nutrient, antibiosis, mineralization of organic matters, and production of plant promoters [2,20]. Endophytic actinomycetes are also well known as producer of various bioactive secondary metabolites which include antibiotics, antimicrobes, phytohormones, and enzymes inhibitor [8,12]. In addition, *Streptomyces* spp. from endophytic rice plant was reported to controll Bacterial Leaf Blight (BLB) disease during dry and wet season

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by 35 cycles [27] at denaturation 94°C for 1 min, annealing 50°C for 1 min, and extension 72 °C for 45 s with a final extension step at 72 °C for 5 min. The PCR products were separated on a 1.5% (wt/vol) agarose gel.

*16S rRNA and nifH genes sequencing, bioinformatics analysis and phylogenetic tree construction:*

The PCR product was directly sequenced using DNA sequencer (ABI PRISM 3100) in First Base Co. The 16S rRNA and *nif*H genes sequences data from each isolate were compared to the available database at GenBank by using the BLAST software (blastn) on National Center Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The 16S rRNA and *nif*H genes sequences were aligned and the phylogenetic tree was constructed using the MEGA 5.05 software [24], based on neighbor-joining tree (NJT) method and refers to the best model tree TN93+G (Tamura-Nei) for 16S rRNA analysis and T92 (Tamura-3 parameter) for *nif*H analysis, with bootstrap 1000x.

*In vitro Analysis of Nitrogen Fixing Activity:*

Nitrogen fixing activity was assayed by growing the culture in N-free medium (Biological $N_2$ fixation or BNF) agar, based on Phillips method [15]. Nitrogen fixing responses were also assayed based on ammonia produced in N-free medium using Penat method [6]. An analysis of nitrogen fixing activity, *B. japonicum* used as a positive control and *E. coli* used as a negative control.

*Results:*

*Morphological characteristics of rice endophytic actinomycetes:*

The seven isolates of rice endophytic actinomycetes showed various morphological colony (Fig. 1). The IPBCC.b.14.1531, IPBC.b.14.1532, IPBCC.b.14.1533, IPBCC.b.14.1534, IPBCC.b.14.1535, IPBCC.b.14.1536, and IPBCC.b.13.1530 isolates examined formed aerial hyphae in agar medium with various colour from white, less-brown, brown, until grey. The tested isolates were also produced various of spores chain type e.g. spirales (S), rectiflexibiles (RF), and retinaculiaperti (RA) (Fig. 1). These morphological observation indicated that most of the tested actinomycetes isolates belonged to *Streptomyces* spp.

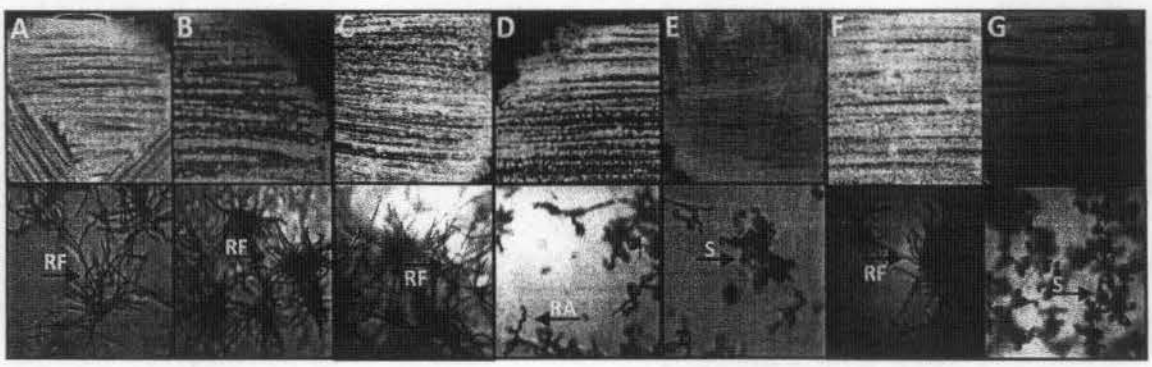


**Fig. 1:** Colony of endophytic *Streptomyces* spp. isolated from rice plant grown in YSA media, after 10 days incubation (above), and microscopic of spores chain type of endophytic *Streptomyces* spp., with 400x magnification (below). A= IPBCC.b.14.1531, B= IPBCC.b.14.1532, C= IPBCC.b.14.1533, D= IPBCC.b.14.1534, E= IPBCC.b.14.1535, F= IPBCC.b.14.1536, G= IPBCC.b.13.1530.

*Molecular identity of rice endophytic actinomycetes:*

The 16S rRNA gene of the seven isolates of rice endophytic actinomycetes with the expected size of fragment DNA ~ 1480 bp (Fig. 2) was compared with 16S rRNA gene sequences in the GenBank database. The IPBCC.b.14.1531(1320 bp), IPBCC.b.14.1532 (1424 bp), IPBCC.b.14.1533 (1398 bp), and IPBCC.b.14.1536 (1386 bp) were closed related with *S. albolongus* strain NBRC 13465 and *S. cavourensis* subsp. *cavourensis* strain NRRL 2740 with 94%, 92%, 94%, and 95% maximum identity, respectively. In addition, IPBCC.b.14.1534 (1478 bp) was closed related sequences with *S. anulatus* strain NBRC 12755 with 92% maximum identity, and IPBCC.b.14.1535 (1118 bp) was closed related sequences with *S. bungoensis* with 92% maximum identity. Whereas, IPBCC.b.13.1530 (1410 bp) was closed related sequences with *S. misionensis* strain NRRL B-3230 with 99% maximum identity (Table 1).

The phylogenetic dendogram was showed that IPBCC.b.14.1531, IPBC.b.14.1532, IPBCC.b.14.1533, IPBCC.b.14.1534, IPBCC.b.14.1535, and IPBCC.b.14.1536 were clustered together (cluster I) and were closely related with *S. albolongus*, *S. cavourensis* subsp. *cavourensis*, *S. anulatus*, and *S. bungoensis*. The IPBCC.b.13.1530 was separated from another and it clustered together (cluster II) with *S. misionensis*. Both of cluster I and cluster II were separated from outgroup cluster (*Micromonospora* sp. and *Pseudomonas*

sequences from *Herbaspirillum* sp. strain B501 with 95 to 99% maximum identity, 95 to 98% maximum identity with uncultured bacterium clone BN-A6, 94 to 99% maximum identity with uncultured bacterium clone IPA64, and 93 to 98% maximum identity with uncultured bacterium clone IPA100 (Table 2).

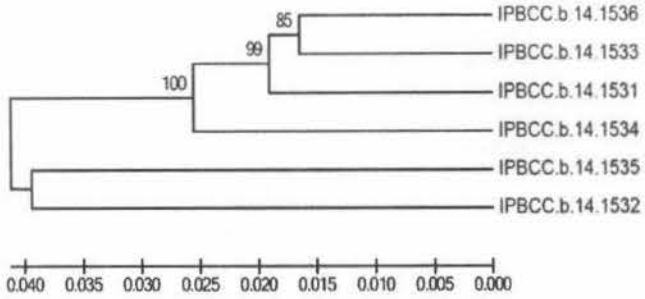


**Fig. 4:** Phylogenetic tree based on matrix of genetic distances (*p*-distance) among 16S rRNA gene sequences of six isolates of rice endophytic actinomycetes.

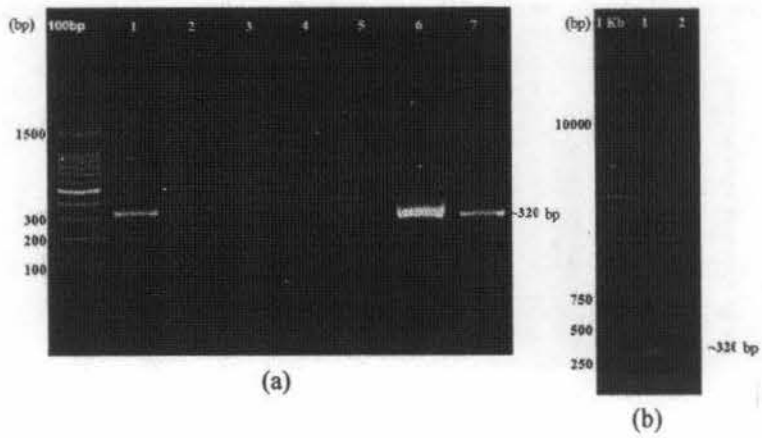


**Fig. 5:** PCR amplification of *nif*H gene from rice endophytic actinomycetes (~320 bp) using primer PolF and AQER. (Left): marker 100 bp, lane 1 to 7: IPBCC.b.14.1531,IPBCC.b.14.1532, IPBCC.b.14.1533, IPBCC.b.14.1534, IPBCC.b.14.1535,IPBCC.b.14.1536, and IPBCC.b.13.1530; (Right), marker 1 Kb, lane 1: *B.japonicum* as positive control, lane 2: *E.coli* as negative control.

**Table 2:** Percent similarity the sequences of a *nif*H gene from rice endophytic actinomycetes.

| References (GenBank) | % Similarity | | | Accession no. |
|---|---|---|---|---|
| | IPBCC.b.13.1530 | IPBCC.b.14.1531 | IPBCC.b.14.1536 | |
| *Herbaspirillum* sp. B501 *nif*H | 95 | 95 | 99 | AB196476.1 |
| *Uncultured bacterium clone* BN-A6 *nif*H | 95 | 95 | 98 | HQ335398.1 |
| *Uncultured bacterium clone* IPA64 *nif*H | 94 | 94 | 99 | EU048006.1 |
| *Uncultured bacterium clone* IPA100 *nif*H | 93 | 93 | 98 | EU048040.1 |

Based on phylogenetic tree of the *nif*H gene, the result showed that IPBCC.b.13.1530, IPBCC.b.14.1531, and IPBCC.b.14.1536 were clustered together with *Herbaspirillum* sp. (cluster I). But when compared with out-group cluster, the result clearly showed that the IPBCC.b.13.1530, IPBCC.b.14.1531, and IPBCC.b.14.1536 were separated from them (Fig. 6). An analysis of phylogenetic tree based on matrix genetic distances (*p*-distance), which indicate the diversity of genetic distances (336 nucleotide length) between three isolates and *Frankia* sp., *Rhizobium* sp., *L. ferrooxidans*, also *K. pneumonia* showed the sequence differences of *nif*H gene which were 18-28% and more than 59% when compared with *B. japonicum* (Fig. 6).

*Nitrogen fixing activities of rice endophytic actinomycetes:*

The data examined in this report showed that from the seven isolates of endophytic actinomycetes, IPBCC.b.13.1530, IPBCC.b.14.1531, and IPBCC.b.14.1536 were capable of fixing nitrogen. They produced ammonia which the concentration ranging from 0.065 ppm, 0.014 ppm, 0.076 ppm, respectively (Fig. 7). They

*Streptomyces* are generally quite useful for species identification and grouping of similar taxa, including spore colour and spore surface ornamentation [11]. Based on 16S rRNA gene analysis, reported here that IPBCC.b.14.1531, IPBCC.b.14.1532, IPBCC.b.14.1533, IPBCC.b.14.1534, IPBCC.b.14.1535, and IPBCC.b.14.1536, have an indication as a novel species with <97% maximum identity, E. value 0.0, and they were belong to *Streptomyces* spp. They clustered together with *S. albolongus*, *S. cavourensis* subsp. *cavourensis*, *S. anulatus*, and *S. bungoensis*. Based on *p*-distances analysis were known that the six isolates have a difference sequences of nucleotide. These internal comparation indicate a diversity of species among them. In other side, the IPBCC.b.13.1530 was closely related with *S. misionensis* strain NRRL B-3230 with 99% maximum identity. *S. misionensis* commonly produced aerial mass in grey series with spirales or retinaculiaperti spores chain type. This result indicated that based on morphological characteristics and phylogenetic analysis, IPBCC.b.13.1530 belong to *S. misionensis*.

This study is considered as the first work which use molecular data to show that endophytic actinomycetes isolated from rice plant varieties in Indonesia, have *nif*H gene sequences. In addition, the international information of *nif*H gene sequences in endophytic actinomycetes was still poorly studies. Actinobacteria, especially genus *Micromonospora* and *Thermonospora* were successfully isolated from roots of *Casuarina equisetifolia*, they have *nif*H gene sequences which were closely related with *Frankia* sp. [27]. An phylogenetic tree analysis in this report showed that the internal *nif*H gene of IPBCC.b.13.1530, IPBCC.b.14.1531, and IPBCC.b.14.1536 were closely related with *nif*H gene from *Herbaspirillum* sp. strain B501 (cluster I). In this cluster, the position of IPBCC.b.13.1530 was closer with IPBCC.b.14.1531 compared with IPBCC.b.14.1536. Both IPBCC.b.13.1530 and IPBCC.b.14.1531 have less than 97% sequences similarity compared with the available *nif*H gene sequences data from GenBank, meanwhile, IPBCC.b.14.1536 has more than 97% sequences similarity. These data indicated that IPBCC.b.13.1530 and IPBCC.b.14.1531 have more sequences diversity of their *nif*H gene. The presence of 3% nucleotide differences are indicate certain hyper-variable regions. The positions of the hyper-variable regions are taxon specific and need to be determined for novel organisms by sequences analysis of the complete molecule [21]. Different strain of endophytic *Streptomyces* may cause diversity of their *nif*H gene sequences, and that phenomenon may also be influenced by different varieties of rice cultivars. The diversity of genetic distances between three isolates (IPBCC.b.13.1530, IPBCC.b.14.1531, and IPBCC.b.14.1536) and *Frankia* sp., *Rhizobium* sp., *L. ferroxidans*, also *K. pneumonia* indicated high diversity of endophytic actinomycetes *nif*H gene (Fig.6). *Herbaspirillum* sp. isolated from wild rice that known capable of colonizing of roots and stems of rice plant, fixed nitrogen, also increased growth of rice plant [29]. Based on *in vitro* assayed, the three *Streptomyces* spp. (IPBCC.b.13.1530, IPBCC.b.14.1531, and IPBCC.b.14.1536) isolated from rice plant are potential to promote rice plant growth through their capability in producing ammonia and growth in N-free medium. Previously, IPBCC.b.13.1530 was reported to be able to fix nitrogen via their activity to grow in N-free medium, reduced acetylene, and produced ammonia. The capability to grow on an N-deficient medium, positive acetylene reduction and $^{15}N$ isotopic dilution assays, as well as possessing *nif*H gene gives strong support to the conclusion that the Actinobacteria fix atmospheric N to ammonia [27].

*Conclusion:*

Based on morphological characteristics and 16S rRNA gene analysis, the seven isolates of rice endophytic actinomycetes were belong to *Streptomyces* spp. The IPBCC.b.14.1531, IPBCC.b.14.1532, IPBCC.b.14.1533, IPBCC.b.14.1534, IPBCC.b.14.1535, and IPBCC.b.14.1536 have an indication as a novel species with <97% maximum identity, and they clustered together with *S. albolongus*, *S. cavourensis* subsp. *cavourensis*, *S. anulatus*, and *S. bungoensis*. While, the IPBCC.b.13.1530 was closely related with *S. misionensis* with 99% maximum identity. The internal *nif*H gene of IPBCC.b.13.1530, IPBCC.b.14.1531, and IPBCC.b.14.1536 were closely related with *nif*H gene from *Herbaspirillum* sp. Based on *in vitro* assayed and *nif*H gene analysis, the three *Streptomyces* spp. isolated from rice plant are potential to promote rice plant growth through their capability in fixing nitrogen.

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## REFERENCES

[1] Adegboye, M.F. and O.O. Babalola, 2012. Taxonomy and ecology of antibiotics producing actinomycetes. *Afr. J. Agr. Res.*, 7: 2255-2261. DOI: 10.5897/AJARX11.071.

[25] Tan, R.X. and W.X. Zou, 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.*, 18: 448-459. DOI: 10.1039/b100918o.

[26] Tian, X.L., L.X. Cao, H.M. Tan, Q.G. Zeng, Y.Y. Jia, W.Q. Han, S.N. Zhou, 2004. Study on the communities of endophytic fungi and endophytic actinomycetes from rice and their antipathogenic activities *in vitro*. *World J. Microbiol. Biotechnol.*, 20: 303-309.

[27] Valdes, M., N.O. Perez, P.E. los Santos, J.C. Mellado, J.J. Pena-Cabriales, P. Normand, A.M. Hirsch, 2005. Non-*Frankia* actinomycetes isolated from surface-sterilized roots of *Casuarina equisetifolia* fix nitrogen. *Appl. Environ. Microbiol.*, 71: 460-466. DOI: 10.1128/AEM.71.1.460-466.2005.

[28] Weisburg, W.G., S.M. Barns, D.A. Pelletier, D.J. Lane, 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, 173: 697-703.

[29] Zakria, M., J. Njoloma, Y. Saeki, S. Akao, 2007. Colonization and nitrogen-fixing ability of *Herbaspirillum* sp. strain B501 gfp1 and assessment of its growth-promoting ability in cultivaterice. *Microbes Environ.*, 22: 1-10.

[30] Zhi, X.Y., W.J. Li, E. Stackebrandt, 2009. An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class Actinobacteria, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. *Int. J. Syst. Evol. Microbiol.*, 59: 589-608.