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Rapid Discrimination of Potato Scab-Causing *Streptomyces* Species Based on the RNase P RNA Gene Sequences

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Scab disease significantly damages potatoes and other root crops. Some *Streptomyces* species have been reported as potato-scab pathogens. Identification of the phytopathogenic *Streptomyces* is mainly done on the basis of the 16S rRNA gene, but use of this gene has some limitations for discriminating these strains because they share high similarities of 16S rRNA gene sequences. We tested the RNase P RNA (*rnpB*) gene as a taxonomic marker to clarify the relationship among closely related scab-causing *Streptomyces* strains. The *rnpB* genes were analyzed for 41 strains including 9 isolates from Jeju, Korea. There were 4 highly variable regions including nucleotide gaps in the *rnpB* genes. Interspecies similarity of the *rnpB* gene in tested *Streptomyces* strains was lower than about 97%, while the intraspecies similarity was higher than about 98%. Phylogenetic analysis demonstrated that the *rnpB* tree has similar topology to the 16S rRNA gene tree, but produces a more divergent phyletic lineage. These results revealed that the *rnpB* gene could be used as a powerful taxonomic tool for rapid differentiation of closely related *Streptomyces* species. In addition, it was also suggested that the variable regions marked as α, β, γ, and δ in the *rnpB* gene could be useful markers for the detection of specific *Streptomyces* species.

***Keywords*:** *Streptomyces*, potato scab, 16S rRNA, *rnpB*, phylogenetic analysis

The genus *Streptomyces*, which encompasses the largest number of known species (581 species with validly published names, *List of Prokaryotic names with Standing in Nomenclature*) in *Bacteria*, is recognized for producing many useful materials such as biologically important secondary metabolites and antibiotics, but a few of these species are known as plant pathogens (Loria *et al.*, 2006). The representative plant disease caused by *Streptomyces* strain infection is scab disease of potatoes (*Solanum tuberosum* L.), which is prevalent in potato-growing areas in the world and produces economically significant yield losses. It has been reported that this disease is generally caused by several *Streptomyces* species including *S. acidiscabies* (Lambert and Loria, 1989a), *S. scabiei* (Lambert and Loria, 1989b; Truper and De'clari, 1997), *S. turgidiscabies* (Miyajima *et al.*, 1998), 3 French strains (*S. europaeiscabiei*, *S. reticuliscabiei*, and *S. stelliscabiei*), and 3 Korean strains (*S. luridiscabiei*, *S. niveiscabiei*, and *S. puniciscabiei*) (Bouchek-Mechiche *et al.*, 2000; Park *et al.*, 2003a). The genetic diversity of these scab-causing *Streptomyces* species was analyzed mainly on the basis of 16S rRNA genes, which are used as the "gold standard" in bacterial taxonomy. In previous studies, phylogenetic analysis based on 16S rRNA gene sequences showed that *S. acidiscabies*, *S. stelliscabiei*, and 3 Korean strains (*S. luridiscabiei*, *S. niveiscabiei*, and *S. puniciscabiei*) were separated into 5 different clades in the phylogenetic tree (Park *et al.*, 2003b; Song *et al.*, 2004). In contrast, *S. reticuliscabiei* was clustered into the same clade with *S. turgidiscabies*, while *S. scabiei* formed a robust phyletic line with *S. europaeiscabiei* (Song *et al.*, 2004). We also found that use of the 16S rRNA gene has some limitations for the analysis of closely related species and that 16S-23S rRNA ITS region was also not useful for analyzing the intra- and interspecies relationships of *Streptomyces* species (Song *et al.*, 2004). Many studies have recently been carried out to evaluate alternative markers, notably single or multiple housekeeping genes such as *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB* to differentiate closely related *Streptomyces* species that share high sequence similarity of 16S rRNA gene (Mun *et al.*, 2007; Guo *et al.*, 2008; St-Onge *et al.*, 2008; Rong *et al.*, 2009; Labeda, 2010; Rong and Huang, 2010).

The RNase P RNA (*rnpB*) gene encoding the RNA subunit of RNase P, an endoribonuclease P that forms the mature 5' ends of the tRNAs is essential and ubiquitous in *Bacteria* (Pace and Brown, 1995). RNase P RNAs, unlike rRNAs or other highly conserved RNAs, are relatively variable in the secondary structure, which evolves by substitution outside of the conserved structural core. These structural variations consist primarily of the insertion or deletion of discrete helical elements and play no role in the RNA structure or function (Haas and Brown, 1998). Earlier studies revealed that this gene could be a useful marker for inferring the phylogenetic relationships between related organisms (Yoon and Park, 2000; Schon *et al.*, 2002; Tapp *et al.*, 2003; Rubin *et al.*, 2005; Westling *et al.*, 2008), but it has never been adopted for the phylogenetic analysis of *Streptomyces* species. In this study, we tested the *rnpB* gene to differentiate closely related scab-causing *Streptomyces* species. For this aim, the *rnpB* genes from 41 streptomycetes that are related to potato-scab disease were

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sequenced, analyzed, and compared with 16S rRNA genes to evaluate the *rnpB* gene as a taxonomic marker in scab-causing *Streptomyces* species.

Materials and Methods

Bacterial strains and culture conditions

The 41 strains used in this study are listed in Table 1. Nine strains were isolated from scab lesion of field-grown potato tubers from different geographic locations in Jeju, Korea. All strains were grown in shaking flasks containing GYM (DSMZ - Medium 65) at 28°C for 3 days. They were preserved at -70°C as spore and hyphae suspensions in 15% (v/v) glycerol.

Polymerase chain reaction (PCR) amplification and sequencing of the RNase P RNA (*rnpB*) gene

Forty strains were the subject of *rnpB* gene sequencing using PCR reaction and cloning experiments. Extraction of genomic DNA and

Table 1. The list of length of RNase P RNA gene sequences of isolates and potato scab causing or related *Streptomyces* spp. used in this study

Species name	Strain*	Length of *rnpB* gene	Source and Origin	Pathogenicity‡
S. acidiscabies	ATCC 49003^T	411	*Solanum tuberosum*, USA	+
S. acidiscabies	DSM 41669	411	*Solanum tuberosum*, USA	+
S. acidiscabies	DSM 41670	411	*Solanum tuberosum*, USA	+
S. acidiscabies	DSM 41671	411	*Solanum tuberosum*, USA	+
S. bikiniensis		408		NC
S. bottropensis	DSM 40262^T	426	Soil	NC
S. diastatochromogenes	DSM 40449^T	416		NC
S. europaeiscabiei	CFBP 4497^T	422	*Solanum tuberosum*, France	+
S. eurythermus	KCCM 12267^T	410	Soil, Angola	NC
S. griseofuscus	IFO 12870^T	415	Soil, Japan	NC
S. griseus	ATCC 10137	418		NC
S. luridiscabiei	KACC 20252 ^{T}	420	*Solanum tuberosum*, Korea	+
S. neyagawaensis	KCCM 12304^T	429	Soil, Japan	NC
S. niveiscabiei	KACC 20254 ^{T}	412	*Solanum tuberosum*, Korea	+
S. puniciscabiei	KACC 20253 ^{T}	412	*Solanum tuberosum*, Korea	+
S. reticuliscabiei	CFBP 4531^T	428	*Solanum tuberosum*, France	+
S. sampsonii	DSM 40394^T	411	*Solanum tuberosum*	-
S. stelliscabiei	CFBP 4521^T	426	*Solanum tuberosum*, France	+
S. turgidiscabies	ATCC 700248^T	427	*Solanum tuberosum*, Japan	+
S. scabiei	DSM 40611	409	USA	weak
S. scabiei	DSM 40778	418	*Solanum tuberosum*, USA	NC
S. scabiei	DSM 40961	422	*Solanum tuberosum*, Italy	NC
S. scabiei	DSM 40962	418	*Solanum tuberosum*, USA	NC
S. scabiei	DSM 40995	422	*Solanum tuberosum*, USA	NC
S. scabiei	DSM 41005	409	USA	weak
S. scabiei	DSM 41114	409	USA	NC
S. scabiei	DSM 41658^T	418	*Solanum tuberosum*, USA	+
S. scabiei	DSM 41659	422	*Solanum tuberosum*, USA	+
S. scabiei	DSM 41660	418	*Solanum tuberosum*, USA	+
Streptomyces sp.	ADA1	419	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	ADB1	411	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	ASO2	418	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	DBB1	419	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	DSG1	411	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	HJA3	418	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	KJA2	420	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	KJO61	427	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	SSA4	419	*Solanum tuberosum*, Korea	NC
Streptomyces sp.	DSM 41745	427	*Solanum tuberosum*, Finland	+
Streptomyces sp.	DSM 41746	427	*Solanum tuberosum*, Finland	+
Streptomyces sp.	DSM 41747	418	*Solanum tuberosum*, Finland	+

* ATCC, American Type Culture Collection, Manssas, VA, USA; CFBP, Collection Francaise des Bacteries Phytopathogenes, INRA, Beaucouze´, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IFO, Institute for Fermentation, Culture Collection of Micro-organisms, Osaka, Japan; KACC, Korean Agricultural Culture Collection, Suwon, Republic of Korea; KCCM, Korean Culture Centre of Microorganisms, Seoul, Republic of Korea;. T, type strain

‡ Pathogenicity: data obtained from references (Bouchek-Mechiche *et al.*, 2000; Lambert and Loria, 1989a; Lambert and Loria, 1989b; Miyajima *et al.*, 1998; Park *et al.*, 2003b) and the DSM catalogue. NC, not known or confirmed

PCR amplification were conducted as described previously (Song *et al.*, 2004). Primers for the *rnpB* gene were designed from the sequences of *S. bikiniensis* (GenBank accession no. M64290.1) and *S. lividans* (GenBank accession no. M64552). The PCR products were amplified using primers SrnpF15 (5′-ACGGCAGACGAGCCG-3′) (primer positions 1-15 [the *rnpB* gene sequence numbering of *S. bikiniensis*]) and SrnpR392 (5′-GCAGACGAGTCGGGCTG-3′) (primer positions 408-392 [the *rnpB* gene sequence numbering of *S. bikiniensis*]).

PCR reaction conditions were as follows: 4 min at 94°C and then 25 cycles of 1 min at 94°C, 1 min at 70°C and 2 min at 72°C. Cloning and sequencing of the resultant PCR products were carried out using methods described previously (Song *et al.*, 2001).

Phylogenetic analyses of 16S rRNA and *rnpB* gene sequences
Sequences of the 16S rRNA and the *rnpB* genes of the 41 strains were aligned using CLUSTAL W software (Thompson *et al.*, 1994) prior to calculation of the nucleotide similarity values. Phylogenetic trees of the data sets were inferred using 2 tree-making algorithms, neighbor-joining and maximum parsimony, taken from the MEGA version 4.0 software package (Tamura *et al.*, 2007) with the option of complete deletion of gaps; the evolutionary distance tool of the Kimura-2-parameter model (Kimura, 1980) was used to generate an evolutionary distance matrix. Relationship stability was assessed by performing a bootstrap test based on 1,000 resamplings.

Results and Discussion

PCR amplification and sequencing of the RNase P RNA (*rnpB*) gene

The PCR products of the *rnpB* genes obtained in this study were confirmed to be single bands by agarose gel electrophoresis. PCR fragments cloned into pGEM-T Easy Vector (Promega) were sequenced using the plasmid primers, T7 and SP6. The *rnpB* gene sequences (409-429) were determined for 40 strains including the 9 isolates from Jeju, Korea. The lengths of the *rnpB* gene sequences of 41 strains including 1 strain obtained from GenBank are listed in Table 1.

Sequence analyses of 16S rRNA and *rnpB* gene

Regarding sequence alignment using the CLUSTAL W software (Thompson *et al.*, 1994), it was found that the *rnpB* gene sequences of all strains used in this study were relatively well conserved but had highly variable nucleotide gap-containing regions compared to the 16S rRNA gene sequences (Fig. 1). The regions corresponding to nucleotides 1-30nt, 48-138nt, 155-240nt, 261-349nt, and 377-408nt (the *S. bikiniensis rnpB* gene sequence positions) were relatively conserved, whereas the 4 regions between these nucleotides, 32-47nt (α), 139-154nt (β), 241-260nt (γ), and 350-376nt (δ), were highly variable and contained nucleotide gaps. These variable regions were similar to those of LL-2,6-diaminopimelic acid-containing actinomycetes (Yoon and Park, 2000) but different from those of the genus *Saccharomonospora* (Cho *et al.*, 1998). It is thought that these 4 highly variable regions can be used as a diagnostic marker for the detection of specific *Streptomyces* strains as in Manome *et al*. (2008) and Schlatter *et al.* (2010).

Shown in Table 1, the intraspecies sequence lengths of the *Streptomyces* spp. analyzed in this study were almost the same, such as the 4 strains (411nt) of *S. acidiscabies*. Closely related *Streptomyces* species also had the same or similar sequence lengths to those of *S. bottropensis* DSM 40262^T (426nt) and *S. stelliscabiei* CFBP 4521^T (426nt) or *S. griseus* ATCC 10137 (418nt) and *S. luridiscabiei* (420nt). The *S. scabiei* strain has 418nt, but some strains classified as *S. scabiei* had different sequence lengths, such as 409nt and 422nt. It is inferred that 6 strains (carrying 409nt or 422nt) among 10 strains could be different species. The similarity levels (84.5-100%) of the *rnpB* gene sequences of all strains used in this study were lower than those (94.7-100%) of the 16S rRNA gene sequences but almost higher than those (35-100%) of the 16S-23S rRNA ITS region sequences reported previously by Song *et al.* (2004). The interspecies similarities of *Streptomyces* spp. based on the *rnpB* gene sequences were lower than about 97%, while the intraspecies similarities were higher than about 98%, although some *Streptomyces* strains were out of range. Therefore, the *rnpB* gene is considered to be more useful than the 16S rRNA gene and 16S-23S rRNA ITS region for differentiating and discriminating closely related strains.

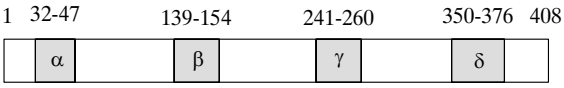

Fig. 1. The four variable regions on RNase P RNA gene correspond to nucleotides 32-47, 139-154, 241-260, and 350-376 and they are marked as α, β, γ, and δ, respectively.

Phylogenetic analyses of the 16S rRNA and *rnpB* genes

The 16S rRNA gene sequences (1424-1428nt) and *rnpB* gene sequences (408-429nt) of the 41 strains were analyzed to elucidate the phylogenetic relationship of scab-related *Streptomyces* species and isolates. The phylogenetic tree based on the *rnpB* gene sequences showed similar tree topology to that of the 16S rRNA gene sequences (Fig. 2). In the results of phylogenetic analysis, cluster I, the Diastatochromogenes group, encompassed the type strains of *S. bottropensis*, *S. diastatochromogenes*, *S. europaeiscabiei*, *S. scabiei*, *S. stelliscabiei*, and 5 isolates (ADA1, ASO2, DBB1, HJA3, and SSA4). In cluster I, type strains of *S. bottropensis*, *S. diastatochromogenes*, and *S. stelliscabiei* formed distinct phyletic lineages in both the 16S rRNA and *rnpB* gene trees. The type strain of *S. scabiei* shared a clade supported by a bootstrap value of 100% in the 16S rRNA gene tree with the type strain of *S. europaeiscabiei*, which has only a single nucleotide difference in the 16S rRNA gene sequence as described previously (Song *et al.*, 2004). They were, however, diverged perfectly in the *rnpB* gene tree with low similarity (90.7%) in *rnpB* gene sequences. This result showed that *rnpB* gene analyses could demarcate 2 species that were undifferentiated in the 16S rRNA gene tree. It is also clarified that three *S. scabiei* strains (DSM 40961, DSM 40995, and DSM 41659) may be reclassified as *S. europaeiscabiei* according to the above sequence length. The other three *S. scabiei* strains (DSM 40778, DSM 40962, and DSM 41660) and 5 Korean isolates (ADA1, ASO2, DBB1, HJA3, and SSA4), which fell within a single phyletic lineage with the type strain of *S. scabiei* in the 16S rRNA gene tree, showed high genetic diversity in the *rnpB* gene tree but were merged into a clade supported by a bootstrap value of 91%. Comparing the previous result with that of the *rpoB* gene by Mun *et al*.

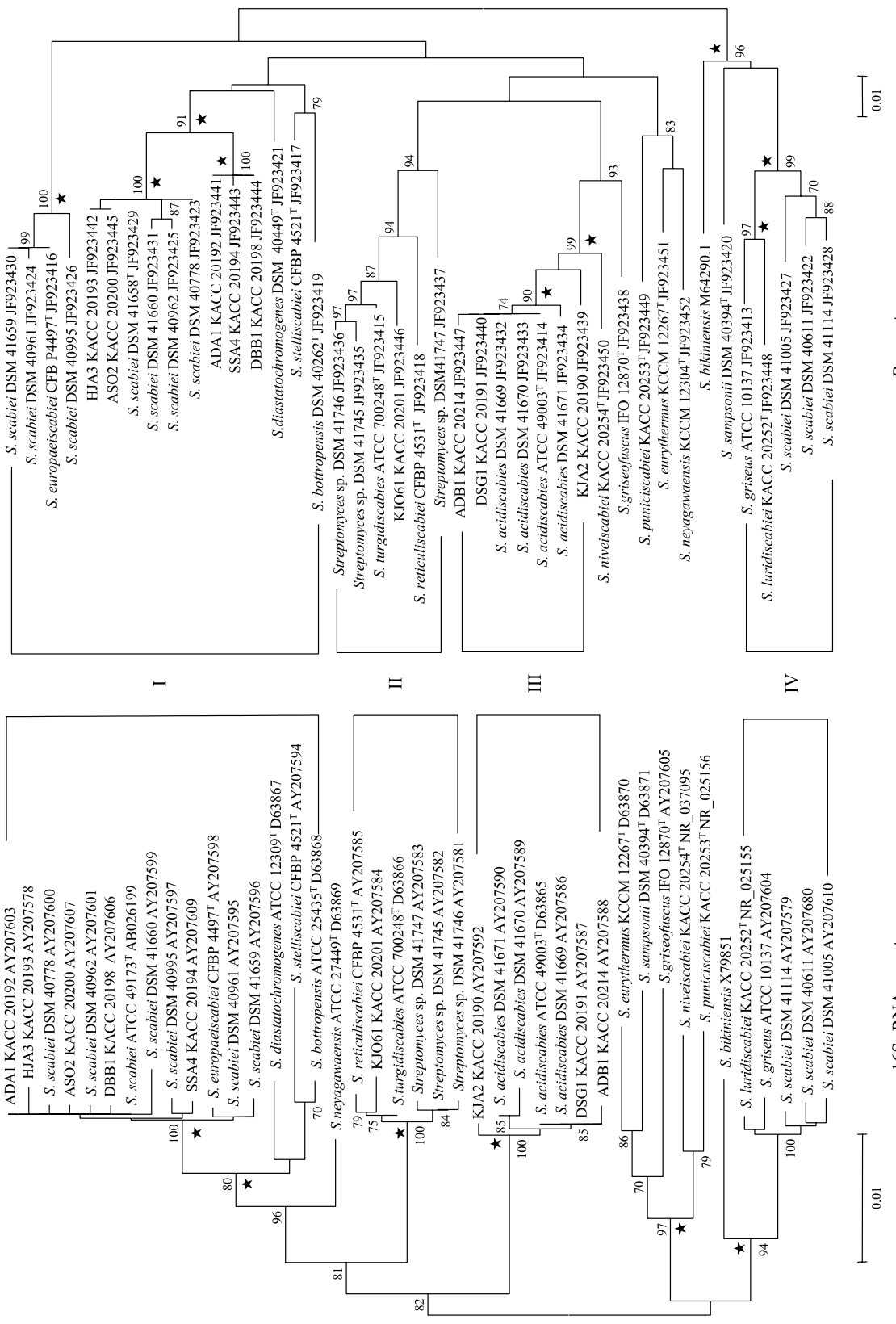

Fig. 2. Neighbor-joining trees of 41 strains of scab-causing and related *Streptomyces* species on the basis of the 16S rRNA (A) and RNase P RNA (B) gene sequences, respectively. Numbers at the branching points are the percentage of bootstrap values from 1,000 replicates (only values higher than 70% are shown). The asterisks label branches that were also recovered in the maximum parsimony tree. The scale bar indicates 0.01 nucleotide substitutions per nucleotide position.

(2007) in the Diastatochromogenes group, it was observed that the similarity in topology between the *rnpB* gene tree and the 16S rRNA gene tree is higher than that of the topology between the 16S rRNA gene tree and the *rpoB* gene tree. This result revealed that *rnpB* gene sequence analysis could be a more powerful specific marker for discriminating *S. scabiei* than *rpoB* gene sequence analysis. In the tree concatenated with the 16S rRNA and *rnpB* genes, the Diastatochromogenes group formed the same phylogenetic cluster as that of the 16S rRNA gene tree but showed more distinct phyletic lineages than did the 16S rRNA gene tree (data not shown).

In cluster II, the Turgidiscabies group encompassed the type strains of *S. turgidiscabies* and *S. reticuliscabiei,* and the 4 strains DSM 41745, DSM 41746, DSM 41747, and KJO61. These strains showed high similarities (99.5-99.9%) in the 16S rRNA gene analysis, were too close to discriminate clearly, and formed 2 phyletic lineages supported by a bootstrap value of 94% in the *rnpB* gene tree. It is shown that the type strain of *S. reticuliscabiei* was differentiated from the type strain of *S. turgidiscabies* with relatively low sequence similarity (96.7%) in the *rnpB* sequence, particularly a 12-nucleotide difference at the δ region. This result concurred with the report of Labeda (2010) that supported the proposal of Bouchek-Mechiche *et al*. (2006) to maintain them as distinct species names, *S. reticuliscabiei* and *S. turgidiscabies*. The 3 strains KJO61, DSM 41745, and DSM 41746 were able to be classified as *S. turgidiscabies*, but the other strain DSM 41747 was not resolved clearly because it had low sequence similarity (96.9% and 97.1%) with DSM 41745 and DSM 41746, respectively. It is thought that DSM 41747 formed a unique lineage due to its unique 9-nucleotide gap believed to be deletion mutation at the γ region but not the δ region, a key region that could demarcate *S. turgidiscabies* and *S. reticuliscabiei* in the *rnpB* gene sequence. It is suggested that this strain should be reanalyzed to confirm its taxonomic position using another housekeeping gene such as the *atpD* or *trpB* gene, as shown in the study by Labeda (2010).

In cluster III, the Acidiscabies group encompassed *S. acidiscabies* and 3 isolates (ADB1, DSG1, and KJA2) like the 16S rRNA gene tree and comprised a stable monophyletic lineage supported by a bootstrap value of 99% with intraspecies sequence similarity (97.8-100%). It was also indicated that the type strain of *S. acidiscabies* is very close to the type strain of *S. niveiscabiei*, unlike the 16S rRNA gene tree, and that these 2 strains have the same distinct gap in the α region of the *rnpB* gene sequence but the 2 species clearly diverge as reported previously by Labeda (2010).

In cluster IV, the Griseus group contained *S. griseus* ATCC 10137, *S. luridiscabiei* KACC 20252^T, and 3 strains of *S. scabiei* (DSM 40661, DSM 41005, and DSM 41114). In this cluster, *S. luridiscabiei* KACC 20252^T shaped a deep forked branch supported by a bootstrap value of 97% and a sequence similarity of 95.8%, unlike the 16S rRNA gene tree which had a bootstrap value of 23% and a sequence similarity of 99.6% with *S. griseus* ATCC 10137. This result was agreed with that of Guo *et al*. (2008) in which the phylogenetic relationship of the *S. griseus* 16S rRNA gene clade was analyzed using 6-gene concatenated sequences. As shown in the result of the *rnpB* gene tree, which showed better discrimination than did the 16S rRNA gene tree, 3 strains of *S. scabiei* (DSM 40661, 41005, and 41114) showed clear cleavage supported by a bootstrap value of 99% and low sequence similarity (94.6, 95.8, and 95.1%, respectively) to *S. griseus* ATCC 10137 in the *rnpB* gene tree. It was reconfirmed that these strains (DSM 40661, DSM 41005 and DSM 41114) were not *S. scabiei* and should be reclassified into the Griseus group as described previously by Song *et al*. (2004). The other *Streptomyces* strains, type strains of *S. eurythermus*, *S. griseofuscus*, *S. neyagawaensis*, *S. puniciscabiei*, *S. sampsonii*, and *S. bikiniensis* were branched with respective phyletic lineage in the *rnpB* gene tree.

The 16S rRNA gene was insufficient to be used for relationship analysis between closely related *Streptomyces* species, and the 16S-23S rRNA ITS region had too variable a length and sequence for the inference of a relationship with *Streptomyces* species (Song *et al.*, 2004). Multilocus sequence analysis using several housekeeping genes constructed a well-supported phylogenetic tree, but it is time consuming and labor-intensive. In this study, we showed that the *rnpB* gene has intermediate length and level of sequence variations compared to the 16S rRNA gene and the 16S-23S rRNA ITS region. We therefore propose that the *rnpB* gene could be used as a very suitable marker for the rapid discrimination between closely related scab-causing *Streptomyces* species, particularly the relationship between *S. scabiei* and *S. europaeiscabiei*. In addition, the variable regions marked as α, β, γ, and δ on the RNase P RNA gene would be useful in the making of the specific PCR primers for the detection of specific *Streptomyces* species.

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