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Short communication

Isolation and Molecular Identification of Endophytic Bacteria From Rambutan Fruits (*Nephelium lappaceum* L.) Cultivar Binjai

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ABSTRACT

Interactions between plants and endophytic bacteria are mutualistic. Plant provides nutrient for bacteria, and bacteria will protect the plant from pathogen, help the phytohormone synthesis and nitrogen fixation, and also increase absorption of minerals. These bacteria called plant growth-promoting bacteria. The aim for this study is to identify endophytic bacteria on rambutan (*Nephelium lappaceum* L.) cultivar Binjai with 16S rRNA. Sequencing results showed that the bacteria is derived from genus *Corynebacterium, Bacillus, Chryseobacterium, Staphylococcus* and *Curtobacterium,* which suspected play a role as plant growth-promoting bacteria.

1. Introduction

Endophytic bacteria are defined as bacteria that colonize healthy plant tissue without causing obvious symptoms or producing obvious injury to the host. Endophytic bacteria colonize a large number of plants, which include plant growth-promoting bacteria. Endophytic bacteria form associations with plants, at least in one phase in their life cycle. Endophytic bacteria normally live on intercellular spaces that contain carbohydrates, amino acids, and high amounts of inorganic nutrients (Bacon and Hinton, 2007).

To study the interaction between plant and bacteria, we can use cultivation and non-cultivation method. Cultivation method has some disadvantages, because the bacteria can be available for cultivation only if the metabolic and physiological needs can be produced *in vitro* (Nadkarni *et al.*, 2009). Non-cultivation method was relied on polymerase chain reaction (PCR) to amplify the 16S rDNA gene from metagenome sample from the plant (Andreote *et al.*, 2009). However non-cultivation method cannot produce bacteria culture to be used in agriculture improvement.

Tropical plants have a great diversity of endophytic microorganisms. The extent of diversity of endophytic bacteria proves that endophytic bacteria are able to live and associate with a variety of plants, both monocots and dicots. Research on the interaction of plants and bacteria can also be used in agricultural biotechnology, to improve growth and yields, or produce secondary metabolites, and biocontrol agents. Endophytic bacteria may also be used as biopesticide to prevent pathogen in plants. Some bacteria from genera *Bacillus*, for example, have the advantage of being biocontrol, because they are easy to cultivate, store, and distribute (Forchetti *et al.*, 2007). However, there is a lack of information on endophytes from tropical hosts. This study aimed to cultivate endophytic bacteria in rambutan (*Nephelium lappaceum* L.) cultivar Binjai using specific gene markers, 16S DNA gene marker.

2. Materials and Methods

In this study, rambutan cultivar Binjai was obtained from Balai Penelitian Buah, Kebun Percobaan Subang.

2.1. Isolation of bacteria from rambutan Fruit

The fruit was surface sterilized with 70% ethanol for 10 minutes, 2.5% sodium hypochlorite for 10 minutes, and 70% ethanol for 10 minutes, followed by three times rinses in sterile deionized water. One gram of rambutan endosperm were mixed with 1 mL 0.85% NaCl and then 0.1 mL suspension solution was taken and inoculated with spread method into the sugar agar plate (SAP) and then incubated for 2–7 days. SAP contained 4% of sucrose. Each colony was selected based on its morphology distinctions from mixed culture using four-way streak method. The cultures were incubated for 24 hours. This process was repeated until approximately 11

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times of subcultures to get a single pure colony. Gram staining was performed to ensure the purity of the colony.

2.2. Genome extraction and identification of bacteria using 16S rDNA gene

Single colony of bacteria was grown in liquid LB medium for 16–18 hours. Cultures were then centrifuged at approximately 15,000 *g* (14,000 rpm) for 1 minute and supernatant was discarded. Pellet was then resuspended in 750 mL lysis buffer (25 mm ethylenediaminetetraacetic acid, 50 mM Tris-Cl, and 0.5% sodium dodecyl sulfate), then added 750 mL of chloroform-isoamyl alcohol (24:1). The mixture was incubated for 10 minutes at −80°C and then centrifuged for 3 minutes at 14,000 rpm. Supernatant was taken and transferred to a new microtube, and then steps were repeated until a clear supernatant was obtained. Later, 1/10 volume of LiCl and 2.5 volumes of absolute ethanol were added and incubated at −20°C for 30 minutes. The samples were centrifuged for 3 minutes at 15,000 *g*. Supernatant was discarded and 200 mL of 70% ethanol was added. Samples were centrifuged for another 3 minutes at 15,000 *g*. The supernatant was discarded, and the samples were dried at room temperature. Then, sample was added with 50 mL TE buffer pH 8.0 and stored at −20°C. To perform molecular identification of bacteria, marker gene for 16S ribosomal DNA was used. The PCR used universal primers 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTA CGACTT) to amplify approximately 1500 bp of 16S rDNA gene (Lutzoni, 2013). PCR results were then visualized by electrophoresis and purified using a kit from GeneAid.

2.3. Data processing

Sequencing process was made at Macrogen Inc., Korea. The results are then processed and edited using BioEdit software. Sequencing results were compared with existing sequences using Basic Local Alignment Search Tool program on National Center for Biotechnology Information site (www.ncbi.nlm.nih.gov) to obtain the homology. Sequencesobtained from Basic Local Alignment Search Tool results and then analyzed using MEGA 5.2 software to determine the level of kinship. Construction of phylogenetic trees was created using character-based parameter models, maximum likelihood. The bootstrap method with 1000 replication was used to evaluate phylogenetic trees. Similarity value of each isolate was calculated manually using a scale that is produced by the software.

3. Results

Isolation of endophytic bacteria from rambutan fruit in SAP produces nine isolates which were selected based on morphological characteristics. Nine isolates were successfully amplified by PCR (Figure 1). Identification was made by using 16S rDNA gene which was barcoding gene to identify bacteria. Bioinformatics analysis grouped the endophytic bacteria into five genera, which are *Corynebacterium*, *Bacillus*, *Chryseobacterium*, *Staphylococcus*, and *Curtobacterium*.

Sp.1 isolate was gram-positive bacilli which form the yellow colony, nonmotile, circular shape, entire margin, and raised elevation. Sp.1 isolate showed 99% similarity to *Corynebacterium lipophiloflavum* (Figure 2). *Listeria innocua* was used as an outgroup which derived from the same family with *Corynebacterium*.

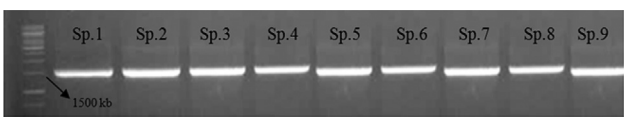

Figure 1. Electrophoresis gel stained by ethidium bromide of 16S rDNA gene polymerase chain reaction product.

Sp.5 isolate was gram-positive bacilli bacteria that had a pale yellow colony, motile, circular shape, filamentous margin, and convex elevation. Sp.5 isolate showed 97.2% similarity to *Bacillus pumilus* (Figure 3a). Sp.8 isolate was gram-positive bacilli bacteria that had a pale yellow colony, motile, rhizoid form, filamentous margin, and flat elevation. Sp.8 isolate showed 97.4% similarity to *B. safensis* (Figure 3B). Sp.9 isolate was gram-positive bacilli, with white and wrinkled surface colony, motile, circular shape, undulate margins and convex elevation. Sp.9 isolates showed 99% similarity to *B. tequilensis* (Figure 3C). *Alkalibacillus* was used as an outgroup which derived from the same family with *Bacillus*.

Sp.3 isolate was gram-negative bacilli bacteria that had white colony morphology, nonmotile, circular shape, undulate margin, and convex elevation. Sp.3 isolates showed 98.7% similarity to *Chryseobacterium hominis* (Figure 4). *Cloacibacterium normanense* was used as an outgroup species which derived from the same family with *Chryseobacterium*.

Sp.6 isolate was gram-negative cocci bacteria that had the white colony morphology, nonmotile, circular shape, entire margin, and convex elevation. Sp.6 isolate showed 98% similarity to *Staphylococcus haemolyticus* (Figure 5). *Salinococcus siamensis* was used as outgroup species derived from the same family with *Staphylococcus*.

Sp.7 isolate was gram-positive bacilli bacteria which have the morphological characteristics of a bright yellow color, nonmotile, circular shape, entire margin, and raised elevation. Sp.7 isolate showed 98% similarity to *Curtobacterium luteum* (Figure 6). *Cryobacterium mesophilum* was used as an outgroup species derived from different families with *Curtobacterium*.

Sp.2 isolate was gram-positive bacilli bacteria that had white colony morphology, nonmotile, circular shape, entire margin, and convex elevation. Sp.4 isolates are gram-negative cocci bacteria that had white colony morphology, nonmotile, circular shape, entire margin, and convex elevation. Construction of phylogenetic trees for sp.2 and Sp.4 isolates made those two become an outgroup among bacteria that have the highest similarity score with both isolates. Sp.2 becomes an outgroup of *Bacillus* bacteria (Figure 7A) and Sp.4 becomes an outgroup of *Staphylococcus* bacteria (Figure 7b). Based on this phylogenetic analysis, the Sp.2 and Sp.4 cannot be identified. Sp.2 and Sp.4 isolates are suspected as a new species or the data cannot be accessed yet at the National Center for Biotechnology Information.

4. Discussion

Corynebacterium was the largest genera in the phylum Actinobacteria. This bacteria had a habitat in soil, water, and can also be found in plants (Collins, 2004). *Corynebacterium* was found as endophytic bacteria in maize plant, potato tuber, root of lemon (*Citrus jambhiri*), root of beet (*Beta vulgaris*) (Chanway, 1998) and paddy (BPTPH, 2013). *Corynebacterium* produced natural biopesticide to control some pathogens, such as: *Xanthomonas campestris, Pseudomonas, Helminthosporium, Cercospora, Plasmodiophora brassicae,* and *Ralstonia solanacearum* (BPTPH, 2013).

Bacillus was an endophytic bacteria most commonly found in plant. *Bacillus* plays role as a biocontrol agent in plant and stimulates plant growth. *Bacillus* in sunflower (*Helianthus annuus*), serves as antipathogen because of the ability to inhibit the growth of specific pathogens. *Bacillus* was also able to induce the growth of plant by producing auxin and gibberellin, and able to adapt to drought (Forchetti *et al.*, 2007). On strawberry plant (Pereira *et al.*, 2012), some *Bacillus* species were able to produce IAA, siderophore, and proven to improve plant growth. Besides being able

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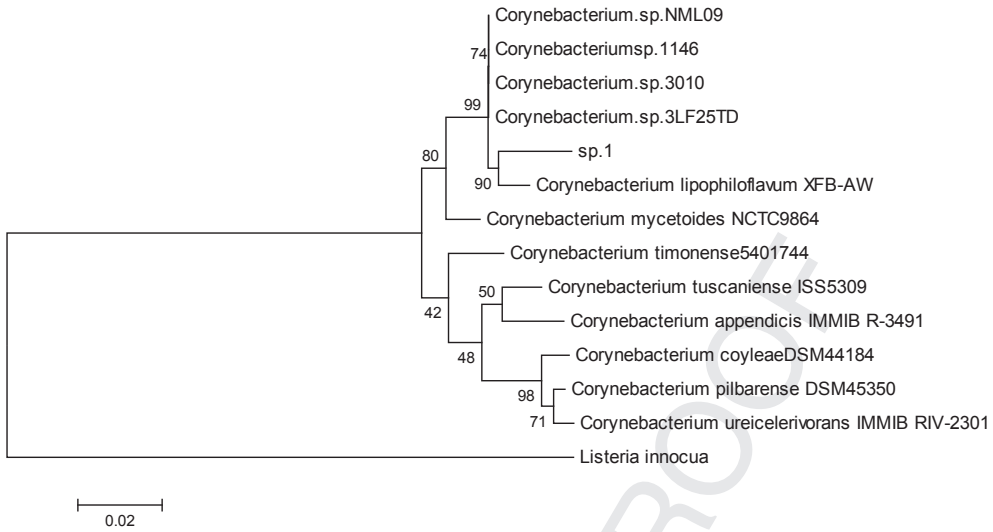

Figure 2. Construction of phylogenetic trees for Sp.1 isolates.

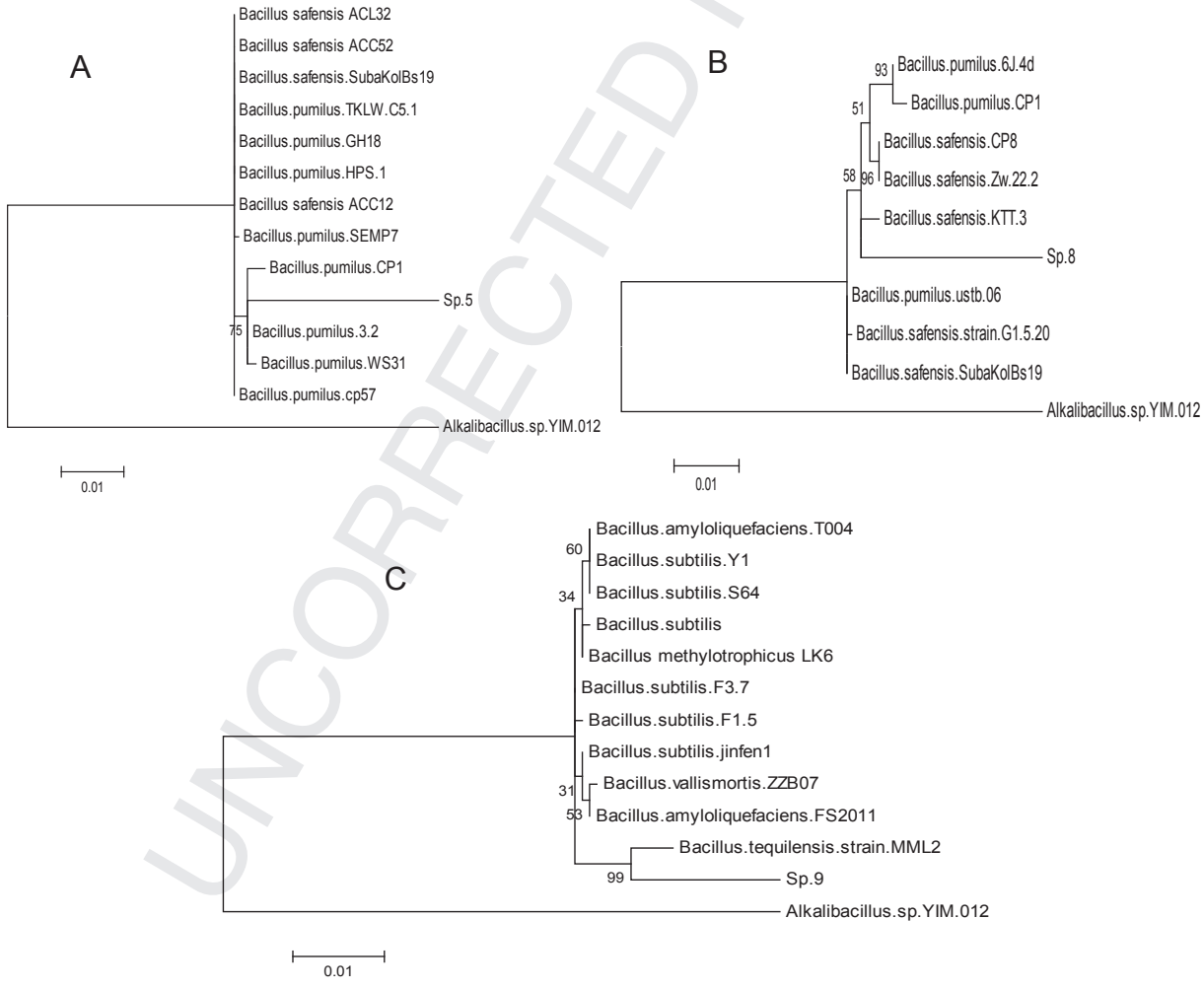

Figure 3. Construction of phylogenetic trees for (A) Sp.5, (B) Sp.8, (C) dan Sp.9 isolates.

to induce the growth of plants, IAA was also known to inhibit the growth of pathogens (Khare and Arora, 2010). *Bacillus* was found as endophytic bacteria inside the fruit of papaya (Krishnan *et al.*, 2012), coffee (Miguel *et al.*, 2013), strawberry (Pereira *et al.*, 2012), palm oil (Djafar *et al.*, 2010), paddy, maize, cucumber (*Cucumis sativis*), grapevine (*Vitis sp.*), hybrid spruce (*Picea glauca x Engelmannii*), pine (*Pinus contorta*), potato, and red clover (*Trifolium pratense*) (Chanway, 1998). *B. pumilus, B. safensis, and B.*

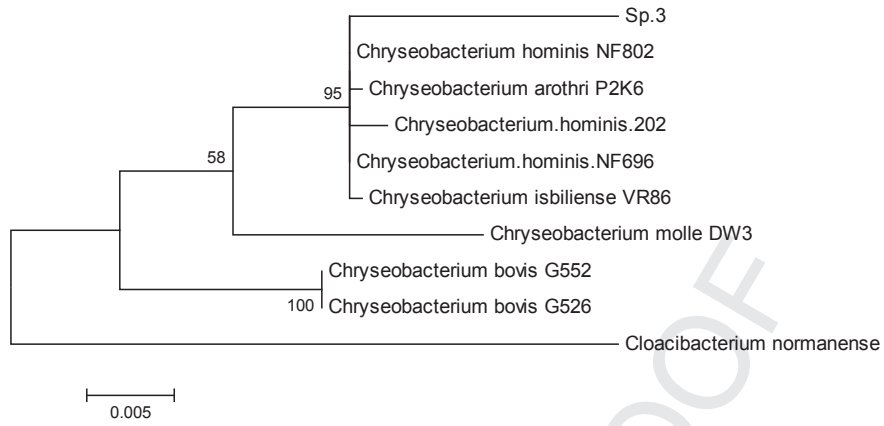

Figure 4. Construction of phylogenetic trees for Sp.3 isolates.

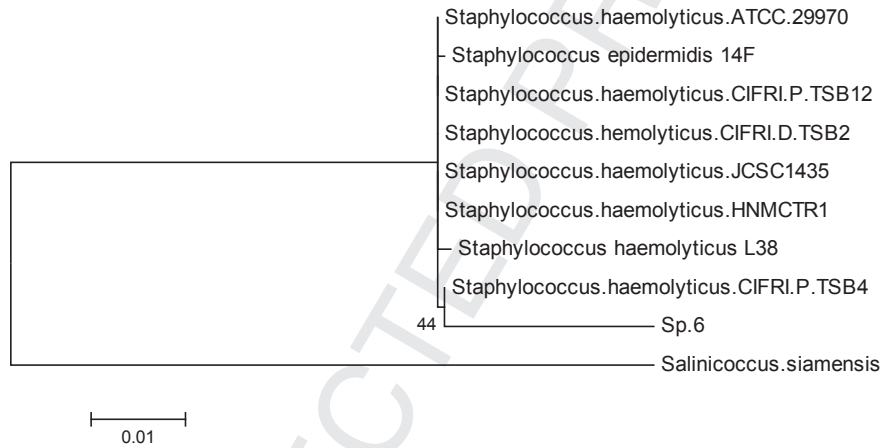

Figure 5. Construction of phylogenetic trees for Sp.6 isolates.

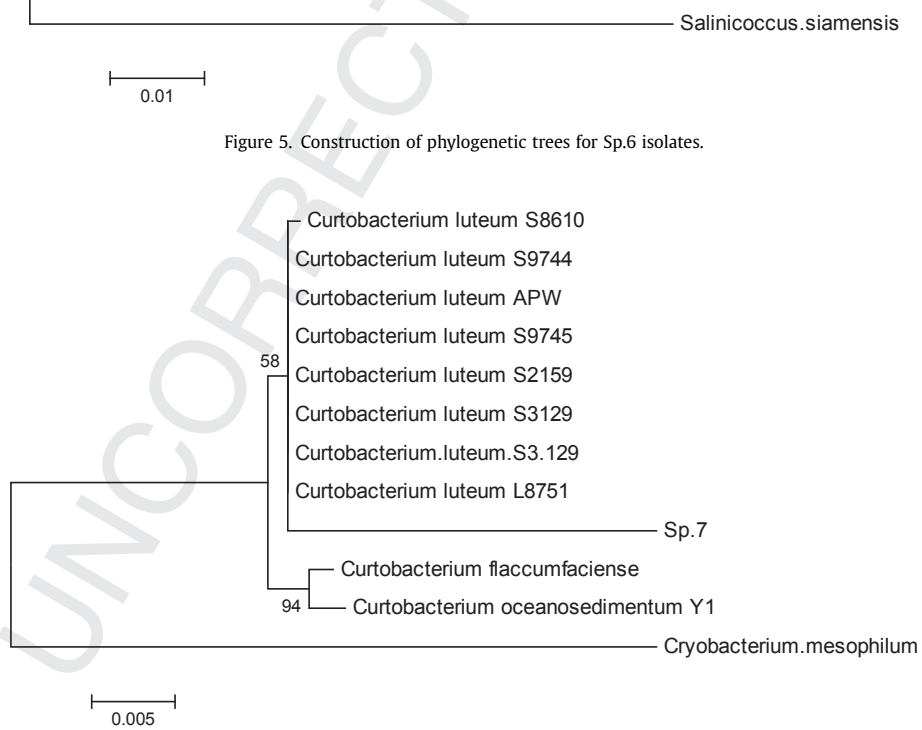

Figure 6. Construction of phylogenetic trees for Sp.7 isolates.

tequilensis were found on cacti in Brazil. Those bacteria played a role in the adaptation of plant under drought condition by produced exopolysaccharide, and become antipathogen with ability to produced cellulase enzymes (Kavamura *et al.*, 2013). *B. safensis* which was isolated from fermentation product *Hibiscus sabdariffa* in West Africa was also known to grow in medium with 10% NaCl concentration and the temperature reached 50°C (Agbobatinkpo *et al.*, 2013). *B. pumilus* which was isolated from vermicompost

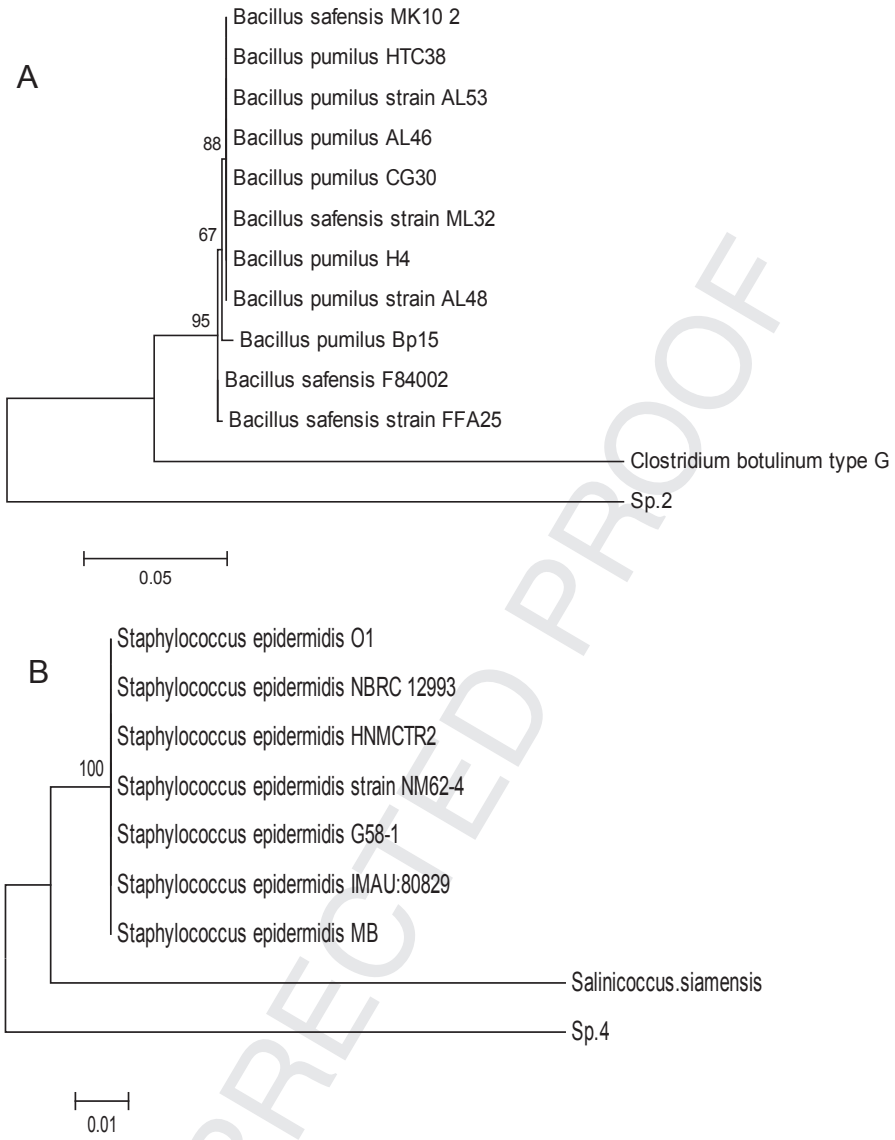

Figure 7. Construction of phylogenetic trees for (A) Sp.2 and (B) Sp.4 isolates.

fertilizer was able to produce the aminocyclopropane-1-carboxylate deaminase enzyme which degrades excess ethylene precursor, wherein the ethylene precursor in excess will inhibit plant growth. *B. pumilus* was also capable of dissolving phosphorus, producing siderophore, showing antifungal activity, and producing protease, cellulase, and xylanase. *B. tequilensis* which was isolated from vermicompost which also showed the same activity with *B. pumilus,* coupled with antibacterial activity and ability to produce the enzyme amylase (Jayakumar and Natarajan, 2013).

Chryseobacterium was the member of the phylum Bacteroidetes that had a habitat in water, soil, and can be associated with plant (Cho *et al*., 2010). *Chryseobacterium* found as endophytic bacteria in corn (Liu *et al*., 2012), paddy, coffee bean (Miguel *et al*., 2013), and cucumber (*Cucumis sativis*) (Chanway, 1998). *Chryseobacterium* isolated from vermicompost fertilizer was able to produced auxin for plant growth and aminocyclopropane-1-carboxylate deaminase which was able to degrade excess ethylene precursor, wherein the ethylene precursor in excess will inhibit the growth of plant. Besides, *Chryseobacterium* was also able to produce siderophore, protease, cellulase, amylase, xylanase, and show antifungal activity (Jayakumar and Natarajan, 2013).

Staphylococcus was a member of the phylum Firmicutes. This bacteria was endophytic bacteria in plant that are found in maize kernels (Liu *et al*., 2012), grapevine (*Vitis sp.*), and hybrid spruce (*Picea glauca x Engelmannii*) (Collins *et al*., 2004). *Staphylococcus* was also found as endophytic bacteria in phytoremediation plant, the poplar trees (Moore *et al*., 2012). *Staphylococcus* that was found in papaya mesocarp was able to produce amylase, cellulase, pectinase, and xylanase. Allegedly these bacteria played an important role as a provider of nutritional agent and had great potential in improving the post-fermentation product, such as an antioxidant (Krishnan *et al*., 2012).

Curtobacterium was a member of the phylum Actinobacteria. Some species of *Curtobacterium*, for example *Curtobacterium flaccumfaciens* has proven role as a biocontrol agent against pathogens by stimulated plant resistance system and antibiosis mechanism (Araûjo *et al*., 2001). *Curtobacterium* was found as endophytic bacteria on maize (Liu *et al*., 2012), soybean, strawberry (Pereira *et al*., 2012), *grapevine* (*Vitis sp.*), potato, and red clover (*Trifolium*

pratense) (Collins *et al*., 2004). *Curtobacterium luteum* was found as endophytic bacteria in phytoremediation plant, the poplar tree, but there was no enough information about role of these bacteria in plants (Moore *et al*., 2012).

It can be concluded from our results that endophytic bacteria isolated and identified from rambutan fruit were from genera *Corynebacterium, Bacillus, Chryseobacterium, Staphylococcus,* and *Curtobacterium*. These endophytic bacteria were suspected to have an antipathogenicity mechanism. Some endophytic bacteria from rambutan fruit is also thought to belong to a group of plant growth-promoting bacteria, which produce auxin and gibberellin growth hormone.

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