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Research article

Exploration of core endophytic bacteria from different organs of diploid *Musa balbisiana* and triploid *Musa acuminata*

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Abstract

Musa balbisiana Colla cv. Kluthuk (BB) is extensively cultivated because it is relatively resistant to biotic and abiotic stresses, while there have been difficulties regarding the cultivation of the less-tolerant cultivar *Musa acuminata* cv. Ambon (AAA). Furthermore, the composition of banana genomes has been reported to play an important role in resistance. Even though their microbiome is involved also in plant adaptability and survival, no analysis has been performed to compare the bacterial composition and its role between resistant and susceptible cultivars. Hence, this study determined the core endophytic bacteria in the Kluthuk and Ambon cultivars growing in loamy sand (LM) and silt loam (SL) soil types. The banana organs (roots, corm and petiole) were subjected to 16S amplicon sequencing. The data obtained showed that the Proteobacteria, Actinobacteria, Firmicutes and Acidobacteria dominated all organs in both cultivars at the phyla level, while *Bacillus, Gaiella* and *Sphingomonas* dominated at the genus level. Interestingly, cv. Ambon had a lower number of core endophytic bacteria compared to cv. Kluthuk. *Pseudomonas* was discovered pre-eminently in the corms and petioles, followed by *Bacillus* in all organs of Kluthuk. Conversely, the same genera were depleted in Ambon, and instead, high levels of *Brevibacillus* and *Acinetobacter* were detected mainly in the roots. This was the first examination of the core endophytic bacterial distribution between these cultivars, which is also necessary for further banana resistance development through amendment of the bacteria.

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Introduction

Musa balbisiana Colla cv. Kluthuk is a wild ancestor of bananas, easily cultivated on different soil types such as loamy, clay, and sandy (Price, 1995). It has been reported as relatively resistant against not only to biotic but also to abiotic stress conditions (De Langhe et al., 2009; Santos et al., 2018) resulting in it being broadly distributed in Southeast Asia (Price, 1995; Davey et al., 2013). The genome "BB" composition of this cultivar is the fundamental factor contributing to its tolerance ability (Nakato et al., 2019). In contrast, *Musa acuminata* cv. Ambon with the genome "AAA" has less tolerance towards both kinds of stresses (Sutanto et al., 2011; Ravi et al., 2013). Tolerance ability is determined from the association between microbes and plants, known as phytomicrobiome (Andrade et al., 2014; Souza et al., 2016).

Bananas' phytomicrobiome composition is affected by cultivars, biotic interactions, soil conditions and organs of the plant. The major to minor diversity of the phyla Cyanobacteria, Proteobacteria, Firmicutes, Actinobacteria and Acidobacteria, respectively, are found in *Musa paradisiaca* cv. Nipah (Suhaimi et al., 2017), while the cultivar "Sukari Ndizi" is dominated by the Proteobacteria, Bacteriodetes and Actinobacteria (Kaushal et al., 2020a). *Bacillus, Acidobacteria, Pseudomonas, Xanthobacteraceae* and *Bryobacter* are the dominant bacterial genera found in *Musa acuminata* cv. Brazil, associated with Fusarium wilt disease (Köberl et al., 2017; Zhou et al., 2019). The abundance of the orders Alteromonadales and Burkholderiales has been discovered to be higher in banana roots compared to corms (Kaushal et al., 2020b). Thomas and Soly (2009) reported that the phyla Proteobacteria, Firmicutes and Actinobacteria dominate the growing shoot tips of *Musa* sp. cv. Grand Naine. Soil conditions such as the physical and chemical structure also affect phytomicrobiome diversity (Marcano et al., 2016).

To date, only a few studies have been conducted on the phytomicrobiome composition of each organ in the banana, more so in cv. Kluthuk. A corm is the main organ for banana regeneration and is effectively applied in plant propagation; however, it has also become an entry point of *Fusarium oxysporum* infection (Robinson and Saúco, 2010; Bhende and Kurien, 2015). The petiole is a bending organ between the leaf and pseudostem and has a unique characteristic in its internal structure. However, studies on phytomicrobiome diversity in corm have not been reported. Therefore, the current project conducted a comparative analysis of endophytic bacterial diversity between resistant (Kluthuk) and susceptible (Ambon) cultivars. This study aimed to determine the core endophytic bacteria of Kluthuk and Ambon being grown on loamy sand (LM) and silt loam (SL) soils.

Materials and Methods

Experimental design and sampling procedure

Musa balbisiana Colla cv. Kluthuk and *Musa acuminata* cv. Ambon were grown on LM and SL soils in Indonesia (specific codes can be found in Table S1). For the analysis of endophytic bacteria, the roots, corm and petiole were collected from 12 healthy suckers aged 4–5 mth of both cultivars. The banana rhizospheric soil within 10 cm depth was collected in a sterile plastic pouch and stored at 4°C in a cooling box. This was then screened on a 2-mesh sieve for physical and chemical properties. For metagenomic DNA extraction, 100 g of the rhizospheric soil was stored at -20°C (Schreiter et al., 2014).

Sterilization of banana plant organs was conducted according to Ngamau et al. (2012) with little modification. Briefly, plant organs were washed using running water and then soaked in a detergent solution for 30 min while shaking concurrently. Afterward, they were soaked in a fungicidal solution for another 30 min followed by immersion in 70% ethanol for 2 min. All samples were soaked in 1.5% sodium hypochlorite for 10 min while shaking. Finally, they were washed with distilled water five times and air-dried on tissues. The effectiveness of this sterilization procedure was confirmed from the last washed samples, spread on tryptone soy agar (TSA) medium, and incubated at room temperature for 48 hr. The absence of fungal and bacterial growth indicated the effectiveness of the sterilization; otherwise, the procedure has to be repeated (Sekhar and Thomas, 2015; Karthik et al., 2017).

Physical and chemical analysis of soil

Banana suckers of the Kluthuk and Ambon cultivars were collected on 27 January–27 February 2019 from two regions in Indonesia with have different soil types, soil properties and geographical conditions, as shown in Fig. 1 and Table S1.

Total DNA extraction

Total DNA from each sample was extracted using a FavorPrep™ Plant Genomic DNA Extraction Mini Kit according to the manufacturer's protocol. Total DNA of the soil rhizosphere was extracted using a ZymoBIOMICS™ DNA Miniprep Kit according to the manufacturer's protocol.

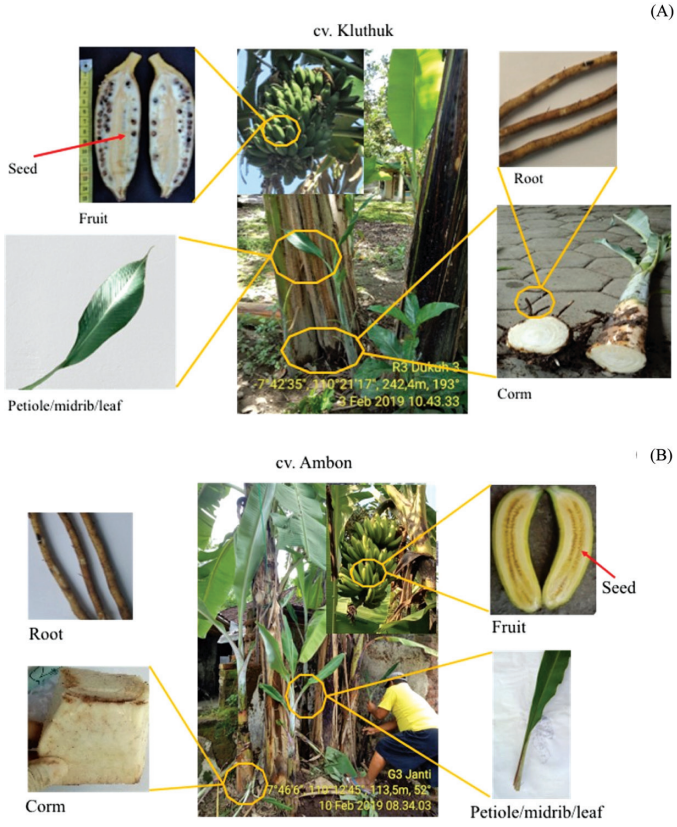

Fig. 1 Morphology of the two banana cultivars (cv.): (A) Kluthuk with a bigger sucker, whiter corm color and big seeds; (B) Ambon with a smaller sucker, brown color of corm and smaller seed

Endophytic bacteria analysis

Analysis of endophytic bacteria was carried out using Illumina HiSeq 2500 PE250 (Novogen, Korea) based on the V3–V4 hypervariable regions of 16S rDNA and amplified using Pro341F/Pro805R for prokaryotes, 341F/R806 for bacteria, and ARC344F/Arch806R for archaea primer sets (Takahashi et al., 2014). The amplicons were amplified using the 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') primer pair. All PCR reactions were conducted with Phusion High-Fidelity PCR master mix (New England Biolabs).

Quantification and qualification of polymerase chain reaction products

Same volumes of 1X loading buffer (contained SYBR green) were mixed with PCR products and run on 2% agarose gel electrophoresis for detection. Samples with a bright main strip in the range 400–450 bp were chosen for further experiments.

Sequencing library preparation

Sequencing libraries were generated using the NEBNext® UltraTM DNA Library Pre Kit for Illumina, following the manufacturer's recommendations and index codes were added. The library quality was assessed using a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina platform and 250 bp paired-end reads were generated.

Data analysis

The composite sample approach (Caporaso et al., 2011) was applied for sequencing analysis. The triplicate samples from each organ consisting of soil (S1, S2, S3), roots (R1, R2, R3), corms (C1, C2, C3) and petioles (P1, P2, P3) were combined. The data from each paired-end sequenced reading were combined using the FLASH software (Magoč and Salzberg, 2011) which then produced raw tags data which were filtered based on the QIIME (v1.7.0) software (Caporaso et al., 2010) to obtain clean (high-quality) tags (Bokulich et al., 2013). High quality tags were obtained by filtering the raw tags using the UCHIME algorithm and clustered into operational taxonomical units (OTU) using a cutoff percentage of bases with the quality score > 20 and error rate < 0.01 (Q20). The clean tags were compared to databases (Gold database) using the UCHIME algorithm (Edgar et al., 2011) to detect chimera sequences which were then removed to obtain effective tags (Haas et al., 2011). Sequenced data (effective tags) were then analyzed using the UPARSE software (Edgar, 2013). Sequences with a similarity ≥ 97% were grouped into the same OTU. Afterward, each OTU was compared with the SILVA 132 database (https://www.arb-silva.de/) to annotate species at each taxonomic rank (threshold: 0.8–1). The annotation results were then used to calculate the Shannon-Weiner index (relative abundance) and its effect on data distribution using principal coordinate analysis (PCoA). The Shannon-Weiner relative abundance and index were calculated using the R software (R Core Team, 2019) and the Minitab 19 software (Minitab, 2021) was used for the PCoA.

Results and Discussion

Soil type and endophytic bacterial diversity

The present study showed that there were no differences in the endophytic bacterial composition in the two banana

cultivars planted in the LM soil. However, there was a difference in the order taxonomic level between the two cultivars planted in SL soil wherein 135 OTUs was observed in Kluthuk but in Ambon 131 OTUs was detected (Table S3). It is interesting that there was similarity of the bacterial composition, at the genus level, between the SL and LM soils, such as *Bacillus, Gaiella, Sphingomonas, Varibacter* and *Streptomyces* (Fig. S2). Furthermore, the beta diversity number also indicated that the soil type did not significantly affect the endophytic bacterial diversity ($p > 0.05$ in Fig. S1). In fact, bacterial diversity is linearly affected by the ratio of soil cations, while bacterial abundance is significantly reduced by a decrease in the clay content (Faoro et al., 2010; Xue et al., 2018). In the present study, the potassium and phosphorous (P_2O_5 method) in the SL soil were much higher than in the LM soil (approximately 71 parts per million [ppm] and 53.5 ppm, respectively), as shown in Table S2. Therefore, difference in cation, at least for potassium and phosphorus, in the two cultivars was expected because these nutrients are among important nutrients for banana (Twyford and Walmsley, 1974; Mia et al., 2010).

Determinants of endophytic bacterial community in banana

The 97% DNA sequence similarity of 30,195 OTUs was used to obtain and combine endogenous community determinants from the 139,332 readings. The assumed contamination sequences classified as chloroplast and mitochondria were then excluded and the remaining 20,399 OTUs were distributed into 10,389 OTUs, 3,523 OTUs, 2,347 OTUs and 4,140 OTUs of the soil, roots, corms and petioles, either from cultivar Kluthuk or Ambon.

The Shannon-Weiner diversity index values used to compare the endophytic bacterial communities from each banana organ between cv. Kluthuk and cv. Ambon were significantly different. Cultivar Kluthuk was more diverse than Ambon, especially when grown on the LM soil (Table S4). The largest distribution of the endophytic bacteria was detected in petiole organs, indicating the banana variety affected bacterial diversity.

The unconstrained PCoA of UniFrac unweighted distance was used for structural isolation of the endophytic bacterial communities. The data showed high variation (81.92%) of endophytic bacteria communities among the samples. The distribution pattern corresponded to the sample organs and the root's endophytic bacteria were also discovered in the corms and petioles, suggesting that these organisms tended to spread from roots to corms. In addition, the composition of endophytic bacteria in the roots was found in petioles, as shown by the same quadrant between two samples of its (Fig. 2).

Kluthuk cultivar

At the phylum level, the Proteobacteria (46.48%) dominated the petiole of Kluthuk planted in the LM soil, followed by the Actinobacteria (19.04%), Acidobacteria (8.81%), Firmicutes (6.81%), Bacteriodetes (4.96%), Gemmatiomonadetes (4.02%), Chloroflexi (3.10%), Thaumarchaeota (2.33%), Nitrospirae (1.92%), Verrucomicrobia (1.07%) and Latescibacteres (0.45%), as shown in Fig. 3A. Conversely, there was little endophytic bacterial diversity detected for the Ambon cultivar, for either the SL or LM soil. Furthermore, the numbers of Bacteriodetes on the petiole of Ambon were lower than for Kluthuk.

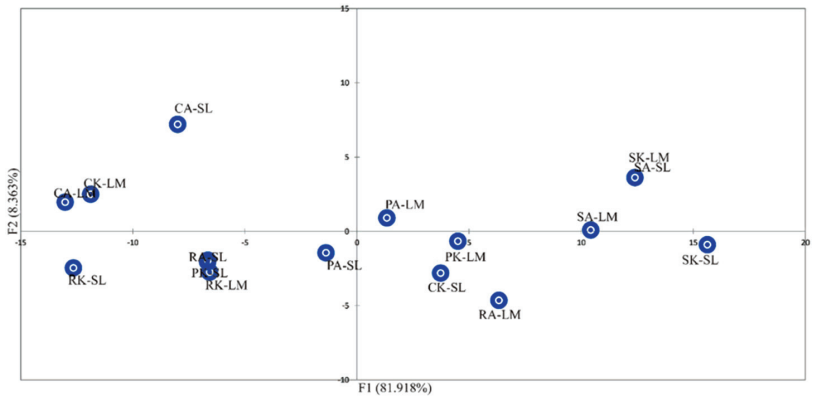

Fig. 2 Principal coordinate analysis plot based on unweighted UniFrac distance matrix indicating high variation of endophytic bacterial communities among samples (81.918% and 8.363% for F1 and F2, respectively), where codes are defined in Table S1

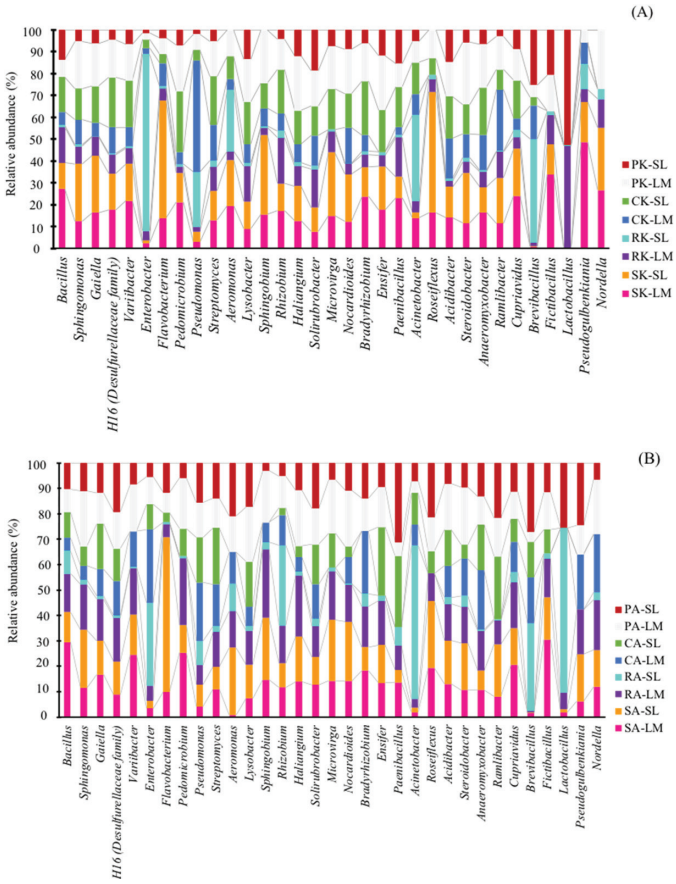

Fig. 3 Relative abundance in genus level of endophytic bacteria found in cv. Kluthuk (A) and Ambon (B), where legend codes are defined in Table S1

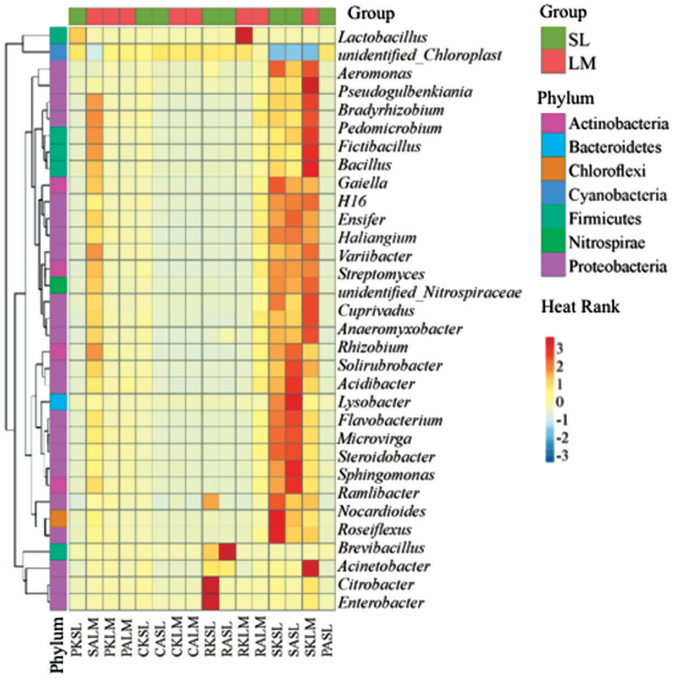

Fig. 4 Heatmap showing relative percentage of each bacterial genus and relative values for bacterial genus by color intensity, where SL = silt loam soil and LM = loamy sand soil and x axis label codes are defined in Table S1

Importantly, compared with the LM soil, the corms of Kluthuk in the SL soil had the largest difference in OTUs (about 27), as can be seen in Table S5 and Fig. 5A. The number of OTUs on roots of Kluthuk in the SL soil was lower than in the LM soil. Endophytic bacterial communities differed at the genus level between the roots, corms, petioles and the LM or SL soil types (Fig. S3A). *Bacillus* (33.2%), *Gaiella* (4.62%), and *Sphingomonas* (3.67%) were dominant in the Kluthuk cultivar when cultivated in the LM soil. Therefore, the variation trends of endophytic bacteria in the corms and petioles of both soil types were similar, such as *Bacillus*, *Sphingomonas*, and *Gaiella*. Roots in the LM soil were dominated by *Lactobacillus* (32.31%), *Bacillus* (20.26%) and *Gaiella* (2.44%), while *Enterobacter* (45.8%), *Pseudomonas* (12.51%) and *Brevibacillus* (5.55%) dominated in the SL soil. The proportions of Pseudomonas (25.50%) and *Bacillus* (19.87%) were the highest detected in the corms of the LM and SL soils, respectively. In addition, *Lactobacillus* (36.13%) accumulated in the roots and petioles of the Kluthuk cultivar only in the SL soil (Fig. 4). Compant et al. (2010) reported similar results, indicating that facultative root bacteria migrate into leaves or penetrate phloem and xylem tissues through plant transpiration.

Ambon cultivar

The bacterial biodiversity found in the Ambon roots isolated from the two soil types was lower than for Kluthuk. At the phylum level, about 16 OTUs and 30 OTUs were detected in the SL and LM soils, respectively (Table S5). The LM soil has been suggested as the appropriate soil for banana growth (Delvaux, 1995). In addition, the diversity in Ambon corms growing in the SL soil was higher than for the LM soil (about 15 OTUs and 10 OTUs, respectively). These data showed the diversity of endophytic bacteria inhabiting the SL soil was much higher than for the LM soil.

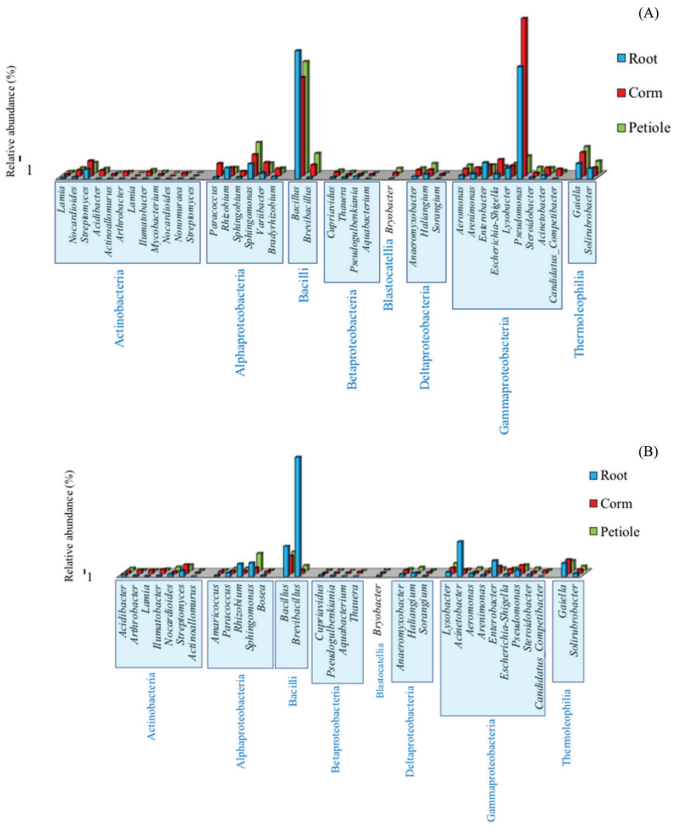

Fig. 5 Core bacteriome endophytic bacterial distribution in banana cultivars: (A) Kluthuk; (B) Ambon

There was also more variation detected on the Ambon organs on the different soil types compared to Kluthuk (Fig. 3B). *Acinetobacter* (9.59%) was the most common genus in the roots, while *Flavobacterium* (5.51%) and *Streptomyces* (4.67%) were only detected in the soil and corms, respectively, of the SL soil. Interestingly, *Enterobacter* was only detected in the roots of Ambon (Fig. 4). There was no difference in the variation in endobacteria occupying the petioles in both cultivars. *Bacillus* (9.50%) followed by the H16 family of *Desulfurellaceae* (5.51%), and *Gaiella* (4.79%) occupied the highest proportion of Ambon petioles (Fig. S3B).

The petiole is an aerial organ that is channeled from above and rounded beneath, while its tissues are composed of parenchyma and large aerenchyma air canals (Ennos et al., 2000). Bacteria in the environment have two entrances; first, they can use a petiole stoma, while alternatively, the ones that have accumulated in rain and air tend to easily enter through the channel (Vorholt, 2012; Frank et al., 2017). The accumulation of enteric Gammaproteobacteria in the pseudostem, which is bent directly into the petiole, has also been reported (Köberl et al., 2018).

Core endophytic bacteria in Kluthuk and Ambon cultivars

The population of bacteria shaping the phytomicrobiome is significantly influenced by plant cultivars (Jiang et al., 2017). The core endophytic bacteria are defined as the organisms that are consistently present in at least 90% of the plant samples and also have an abundance > 0.1% (Souza et al., 2016; Lemanceau et al., 2017). In Kluthuk, about 40 OTUs were shared in all organs and soil samples (Fig. S4A). In addition, *Pseudomonas* and *Bacillus* were the main contributors to the core endophytic bacteria of this cultivar (Fig. 5A). They were followed by *Sphingomonas, Paracoccus, Gaiella, Brevibacillus* and *Solirubrobacter*. Conversely, *Brevibacillus* was the main contributor in the Ambon cultivar, followed by *Acinetobacter, Bacillus* and *Gaiella* (Fig. 5B), while the abundance of *Pseudomonas, Bacillus*, and *Solirubrobacter* was depleted. In addition, Ambon only shared 34 OTUs from all sample organs (Fig. S4B). Furthermore, only 30 genera were identified, and the unidentified were excluded from the core. On the other hand, the abundance of *Pseudomonas, Bacillus, Solirubrobacter* and *Streptomyces* was higher in the Kluthuk cultivar (Fig. 5A) and *Pseudomonas* covered most of the corms. Gamez et al. (2019) stated the expression level of the gene encoding for DNA-binding transcription factors of *Pseudomonas fluorescent* involved in cell proliferation, stress response, and plant defense had been upregulated when it was inoculated on *Musa acuminata*. Therefore, it is conceivable that the interaction between *Pseudomonas* and Kluthuk helped to increase the plant's ability to adapt to a broad ecological environment. The highest colonization ability of *Pseudomonas* and *Acinetobacter* into plant roots and their production of indole-3-acetic acid promoted plant growth (Lin et al., 2018). These results, in line with the previous analysis, showed these genera were associated with many crops (White et al., 1996; Thomas and Soly, 2009; Miliute et al., 2015; Xue et al., 2015). *Brevibacillus laterosporus* has been isolated from the rhizosphere and reported to produce toxic compounds against a wide range of insect pests (Ormskirk et al., 2019). The richness of *Brevibacillus* was escalated and suppressed by *Fusarium* when bioorganic fertilizer was continuously applied on bananas (Fu et al., 2016). Köberl et al. (2017) suggested diversity of Gammaproteobacteria as a potential indicator of healthy bananas. A genus of *Arenimonas* was detected with high abundance in all organs of Kluthuk but not in Ambon. This genus synthesize homoserine from the methionine biosynthesis pathway and which is an important in inducing plant defense against biological stress environment (Schenk et al., 2014; Gamez et al., 2019).

The current study was the first to evaluate the core endophytic bacterial population distribution in a relatively resistant cultivar (Kluthuk) in which the population being much larger and having greater diversity than in the susceptible cultivar (Ambon). Furthermore, the presence of *Bacillus* and *Pseudomonas* suggested they played an important role in inducing resistance and stimulating the growth of the relatively resistant cultivar (Kluthuk). Therefore, this report should be valuable for further development of banana resistance through endophytic bacterial amendment based on clear results.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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