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The Bacterial Diversity Investigation in Oil Palm Plantation using Terminal Restriction Length Polymorphism

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Abstract. Oil palms ((*Elaeis guineensis* Jacq.), have been widely cultivated in Sumatera for over two planting generations and occupied lands with various soil characteristics. In order to investigate microbial diversity in various soil characteristics of oil palm plantation, a molecular analysis called Terminal Restriction Length Polymorphism (T-RFLP) was adopted. The aim of this research was to analyze the bacterial diversity within several oil palm plantations in Sumatera. DNA extraction was performed using soil DNA extraction kit and analysis was performed using fragment analysis following T-RFLP techniques. Data analysis was performed using MICA III public database. The result showed that soil samples were grouped into two clusters based on their chemical properties. Both *Hha*I and *Hinf*I digestions affected the number of culturable bacteria dominancy. *Hha*I was more suitable for revealing a possible culturable genera and species abundance. Dominant genera were detected in each cluster. Cluster-2, which consisted of peat soils, was dominated by *Clostridium*, *Burkholderia*, *Catenibacterium* and *Pasteuria*. As the diversity of microbes reveals soil fertility, the result showed that metagenomic approach could be used to predict soil fertility indicator, in order to improve agricultural practices for oil palm cultivation.

Keywords: Bacterial diversity, metagenome, oil palm (*Elaeis guineensis* Jacq.), T-RFLP.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.), is an important crop in Indonesia, as it provides employment and produces high-quality oil to be transformed into different products, from edible food to bio-fuel. At present, where land became a limiting factor, oil palm estate uses various available lands. Some plantations differ in productivity from others, due among other to soil biological factor, influenced by the richness and abundance of soil microbial community [1]. The soil is a complex system, rich in microbial diversity which forms an important ecosystem for nutrient and biogeochemical cycles [2].

The bacterial community has a major role in soil biotic components and is involved in ecosystem functions. Bacteria not only make up a large proportion of the biological diversity of the soil environment but also are a fundamental component of nutrient cycling and productivity [3]. Any change in land use and management, such as forest conversion for agriculture and fertilizer application, drive changes in the soil chemistry; for example, altering the pH, which leads to the loss of soil carbon and modifying the C/N ratio and the content of phosphorus and

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calcium [4]. Bacterial soil communities are in many ways a product of their chemical and physical environment [5] and therefore, can be affected by physicochemical changes to the soil. Understanding the existence and function of soil bacteria should be useful in plantation management practices [6].

In oil palm estate, soil analysis is essential to monitor soil condition in order to keep high plant productivity. Soil physique, chemical element and microbial community changes could contribute to soil fertility. Microorganisms have an important role in biogeochemical and organic cycles, plant health, soil structure and also soil fertility. Soil quality of oil palm plantation as micro-habitat and nutrient source can be estimated by evaluating and observing microbial community structure as soil bio-indicator.

A metagenomic approach using molecular technique can reveal both culturable and unculturable microorganisms. Some techniques that are widely used for investigation of microbial diversity are Terminal Restriction fragment Length Polymorphism (T-RFLP), Denaturating Gradient Gel Electrophoresis (DGGE) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) [7, 8]. T-RFLP has been widely used for its rapid and accurate production and data analysis. This technique is also effective for distinguishing the microbial community from a wide range of environmental samples. Some advantages of using T-RFLP compared to other techniques are its higher reproducibility, resolution and sensitivity [9].

The study was conducted in Sumatra Island, Indonesia. The study plots were located in an industrial plantation. The aim of the study was to investigate the diversity of bacterial communities according to metagenomic approach using the T-RFLP technique. The result is expected to provide information about soil biological fertility and serves as a preliminary indicator of soil fertility.

MATERIALS AND METHOD

Soil Sampling and Soil Property Analysis

The study was conducted in eight plantation sites (Fig. 1) in Sumatera, Indonesia with different soil characteristics and topographical features but same soil management practices and cropping systems. The sites were located in The North of Sumatera, Riau, Jambi, Bangka and Lampung provinces. Samples were collected from planting blocks, which represented each plantation area. Ten trees from a chosen block were selected and soil samples were taken from each sampling point. The sampling method followed the procedures of Soil Sampling for Microbiological Analysis issued by Indonesian Soil Research Institute [10]. All the plots chosen for the study were from flat terrain on hilltops. Sampling was carried out in 2013 and 2014 following experimental design applied by Carron *et al.* [11].

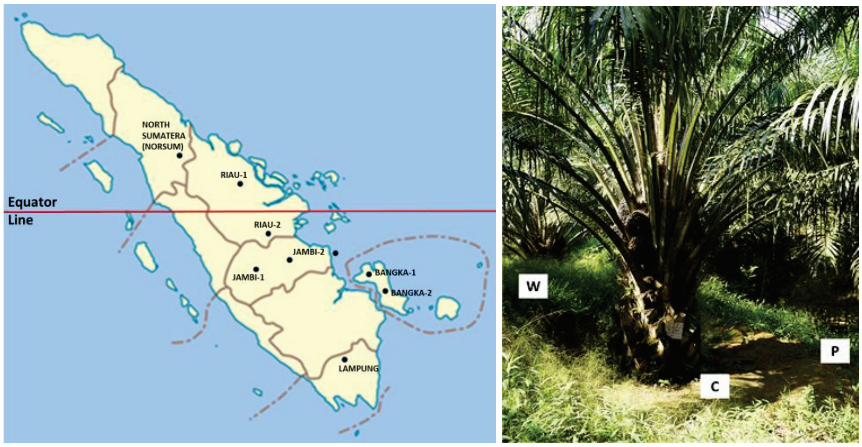

FIGURE 1. Sampling Area Location (a); Sampling zone around oil palm (b). C = circle, W= windrow and P = harvest path

Palm density in the industrial blocks is 136 tree · ha^{-1}. The trees were planted in staggered rows with equilateral triangular spacing. Three treatments were examined corresponding to three different zones around the palm tree, which represent spatial heterogeneity. Harvest path (P) is the zone compacted by the passage of plantation workers; however, the circle (C) is a circular zone with a radius of 1.8 m directly around the palm trunk, which is kept clean

to facilitate the collection of fruit bunches. Windrow (W) is the zone where pruned palm fronds are placed on the ground forming a U-shaped windrow around the tree during harvest. This zone is kept free from all disturbances during the entire cropping cycle and is mainly populated by weeds, such as grasses and *Nephrolepis* spp. These three zones were distinguished by the different amounts of organic and mineral fertilizers they received, as well as weeding and soil compaction. The plot was subject to conventional fertilization with mineral fertilizer application to zones P, C, or W every 6 mo.

Soil samples were taken from the top 20 cm of soil around each tree using soil cork-borer. For each site, approximately one kg of soil was collected from the circle, harvest path and windrow areas and thoroughly mixed to obtain a single representative composite sample of 200 g. Samples were sieved and air dried for 24 h at room temperature, then divided into two parts; the first part was subjected to an analytical process for soil chemical and nutrient properties characterization and the second was used for metagenome analysis. Both analyzes were performed in Bogor, Indonesia.

The following analyses were performed: total N and organic C (C org) by dry combustion; pH H_2O; total K; phosphorus: total P and available P (Bray 1); exchangeable cations Ca, Mg, K, Na and Cation Exchange Capacity (CEC) was measured following the extraction with ammonium acetate at pH 7; and with 1 N of potassium chloride. The base saturation was also calculated.

Genome Extraction

Bacterial community profile was revealed using metagenomic approach [12]. Genome DNA extraction was prepared using kit MoBio Powersoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). First, 0.25 g of sample was put in Power Beads tubes then homogenized for 10 min using vortex machine at maximum speed and 60 µL of the C1 solution was added to destroy the microbial cell, then centrifuged at 10 000 × *g* for 1 min at room temperature [(*g* = Relative Centrifugal Force (RCF)]. Next, 400 µL to 500 µL of supernatant was transferred into 2 mL tubes and added with C2 solution to fix humic acid, other organic and non-organic compounds. After homogenizing, incubation was done for 5 min at 4 °C, followed by centrifugation at 10 000 × *g* for 1 min. The supernatant (600 µL) was transferred into 2 mL tubes, then added by 200 µL of C3 solution, vortexed, incubated for 5 min at 4 °C, then centrifuged for 1 minute at 10 000 × g at room temperature. After repeating this procedure three times, 500 µL of the C5 solution was added and centrifuged at 10 000 × *g* for 30 s. After transferring the pellet, 50 µL of the C6 solution was added, then centrifuged again at 10 000 × *g* for 30 s at room temperature. Isolate genome could be amplified immediately or stored at -20 °C for further use. PCR amplification was carried out using labeled 8F* FAM primer (GAGTTTGATGGCTCAG) and 1510R (GGTTACCTTGTTACGACTT) for bacteria [13, 14].

Terminal Restriction Length Polymorphism (T-RFLP) analysis was used to monitor changes in the structure and composition of bacterial communities [15]. Fragments were generated using two restriction endonucleases: *Hha*I from *Haemophilus haemolyticus* and *Hinf*I from *Haemophilus influenzae* (New England Biolabs, Beverly, MA). The choice was based on the grouping consistency and susceptibility to noise [16]. Fluorescent fragments resulting from restriction enzymes digestion will be separated using capillary electrophoresis and quantified by sequencer [17] in the form of electropherogram peaks. These peaks were analyzed using MiCA III database (*http.mica.Ibest.idaho.edu*) to obtain bacterial community profile [18].

T-RFLP Establishment and Analysis

Quantitative and Qualitative Measurements of Genome

Quantitative measurement was done by measuring the absorbance of 1 µL of the C6 solution as blank. For sample measurement, 1 µL of the isolate was used. DNA concentration was measured in ng · μL^{-1} and DNA purity was indicated by $\lambda_{260}/\lambda_{280}$ nm ratio.

Qualitative measurement for visualization was carried out using electrophoresis. Agarose gel was prepared by dissolving 0.4 g of agarose in 40 mL of TAE buffer (pH 8.0) to obtain the concentration of 1 %. The gel was run at 100 V for 20 min to 30 min. Visualization was carried out using UV trans-illuminator Gel-Doc.

DNA Amplification

Amplification was done using KAPA 2G™ Robust PCR Kit. Master mix was prepared by mixing 10 µL of Buffer B; 5 µL of Enhancer; 1 µL of dNTP Mix; 0.24 µL of DNA Polymerase ($5U \cdot \mu L^{-1}$); primer 8F and 1510R each 2 µL; 1 µL of DNA and ddH2O to reach 25 µL of volume. PCR mix was then put in Thermal Cycler machine, which was set with the following conditions: pre-denaturation at 95 ^{o}C for 5 min, denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min, post extension at 72 °C for 5 min, with a total of 35 cycles.

PCR Product Purification

PCR product was mixed with Binding Buffer and silica 1:1 and filled into a purification column. The mixture was centrifuged 10 000 × *g* for 1 minute. Supernatant was discarded and 700 µL of Washing Buffer was added into the purification column, centrifuged at 10 000 × *g* for 1 min, then the supernatant was discarded. Purification Column was once again centrifuged at 10 000 × *g* for 1 min and then the pellet was transferred to a new tube, added with 50 µL of elution buffer, centrifuged at 10 000 × *g* for 1 min and the purified PCR product was kept at -20°C.

Genome Restriction

Fragments were generated using two restriction endonucleases *Hha*I from *Haemophilus haemolyticus* and *Hinf*I from *Haemophilus influenzae* (New England Biolabs, Beverly, MA). The choice was based on the grouping consistency and less susceptibility to noise [16]. Purified DNA product was digested using restriction enzyme *Hinf*I (G^ANTC). The mixture contained 12 µL of purified PCR product, 2 µL of buffer 10X; 0.1 µL of *Hinf*I; and ddH_2O for a total volume of 20 µL. The mixture was incubated at 37 °C for 15 min and then the temperature was raised to 80 °C for 20 min to stop the reaction. The restriction products were visualized using agarose gel electrophoresis.

Fragments Separation using Capillary Electrophoresis

First, 2 µL of DNA product was added with 2.5 µL of buffer and 0.5 µL of marker standard (GS-1000 ROX, PE Biosystems) after restriction to get a total volume of 5 µL. DNA was then denatured by heating at 95 °C for 2 min to 4 min then put into ice cubes for 5 min. The mixture was filled into 96-well plate and put into capillary electrophoresis in ABI automatic sequencer analyzer. Labeled fragment lengths were determined using GeneScan®. Fluorescent fragments resulting from restriction enzymes digestion will be separated using capillary electrophoresis and quantified by a sequencer [17] in the form of electropherogram peaks.

Fragments Analysis

Peaks or electropherograms were analyzed using online public MiCA III database http://mica.ibest.uidaho.edu/ MiCA III database (*http://mica.ibest.uidaho.edu/*) to obtain bacterial community profile [18]. The abundance data of each species amplified were grouped and analyzed statistically. The 10 most abundant culturable bacterial genera were selected. A literature study was conducted to analyze their potential role in plant growth and soil fertility.

Statistical Data Analysis

Cluster Analysis and Principal Component Analysis (PCA) was performed on soil properties data. Ward's Clustering was performed based on standardized variables according to Euclidean distance. PCA was used to determine the primary differences in soil chemical variables between sites. Characteristics of soil samples were described using simple descriptive statistics. Sampling sites were not statistically compared due to a time sequence, climatic factors and other unmeasured differences of environmental factor.

RESULTS AND DISCUSSION

Characteristics of Soil Samples

There was great variability of properties among soil samples (Table 1), even with the same agronomic practices. Soil from Riau-2 and Jambi-2 showed the highest concentrations of both organic Carbon and Nitrogen, with lower pH ranging from pH 2.90 to pH 3.78. High C content was due to the composition of the soil in these locations, which was dominated by peat. Their cation exchange capacity was also higher than the other soil samples. Bangka-1 had a pH scale of around pH 3 to pH 4 as the site was close to the copper mining area. The composition of sand, clay and silt varied and could not represent plantation soil characteristic in the area because the soil samples were part of rhizosphere around oil palm roots from 20 cm of depth. Soils that have been characterized belong to mineral, mineral to sandy and peat soils.

TABLE 1. Soil chemical and nutrient properties of samples collected from several oil palm plantations

Soil Properties [a]	North Sumatera	Riau		Jambi		Bangka		Lampung
	NORSUM	RIAU-1	RIAU-2	JAMBI-1	JAMBI-2	BANGKA-1	BANGKA-2	LAMPUNG
Sand (%)	44.4 ± 8.9	78.1 ± 8.3	*NA*	9.9 ± 7.5	88.7 ± 5.1	77.9 ± 6.2	82.1 ± 6.5	63.6 ± 41.5
Silt (%)	22.7 ± 6.1	12.4 ± 4.8	*NA*	22.3 ± 2.2	11.3 ± 5.1	20.4 ± 3.8	14.5 ± 4.5	13.6 ± 15.5
Clay (%)	32.9 ± 6.6	10.5 ± 5.7	*NA*	67.7 ± 6.9	**nd	4.5 ± 2.1	5.6 ± 4.4	22.8 ± 27.3
pH_{Water} [b]	4.35 - 4.98	4.01 - 4.59	2.90 - 3.78	3.95 - 4.51	3.25 - 3.56	3.31 - 3.97	4.00 - 4.72	4.57 - 5.56
$C_{Organic}$ (%)	1.56 ± 0.29	1.52 ± 0.61	38.77 ± 9.16	1.38 ± 0.03	31.42 ± 4.97	3.05 ± 1.06	2.77 ± 0.95	0.74 ± 0.47
N_{Total} (%)	0.15 ± 0.05	0.10 ± 0.04	0.71 ± 0.19	0.18 ± 0.03	0.51 ± 0.27	0.10 ± 0.04	0.19 ± 0.11	0.12 ± 0.06
P_{Total} (ppm*)	102 ± 23	32 ± 18	69 ± 16	102 ± 84	289 ± 263	38 ± 19	194 ± 139	84.3 ± 38.5
K_{Total} (ppm*)	655 ± 127	31 ± 13	112 ± 44	63 ± 23	663 ± 273	97 ± 71	142 ± 78	244 ± 201
$P_{Avaliable\text{-}BRAY}$ (ppm*)	5.4 ± 4.8	15.5 ± 24.9	5.4 ± 2.9	13.4 ± 10.3	47.1 ± 8.8	7.8 ± 4.8	17.1 ± 21.3	15.9 ± 11.3
CEC (me ·100 g^{-1})	6.9 ± 0.9	4.6 ± 1.2	17.5 ± 1.1	7.4 ± 0.9	26.5 ± 2.3	6.2 ± 2.3	8.6 ± 2.6	7.04 ± 3.63
Ca-EC (me/100g)	2.3 ± 0.7	0.3 ± 0.3	10.1 ± 4.4	1.4 ± 0.6	10.1 ± 4.4	0.8 ± 0.4	0.5 ± 0.4	2.07 ± 1.53
Mg-EC (me/100g)	360 ± 87	18 ± 15	549 ± 278	52.3 ± 14.6	416 ± 125	43 ± 24	41 ± 8	1.48 ± 1.08
K-EC (me/100g)	0.67 ± 0.15	0.04 ± 0.02	0.58 ± 0.61	0.09 ± 0.04	0.70 ± 0.76	0.17 ± 0.11	0.21 ± 0.14	0.57 ± 0.51
Na-EC (me/100g)	0.07 ± 0.05	0.03 ± 0.03	0.21 ± 0.05	0.07 ± 0.03	0.25 ± 0.06	0.05 ± 0.02	0.07 ± 0.04	0.08 ± 0.05
Base Saturation (%)	57.4 ± 11.3	9.9 ± 7.8	82.8 ± 33.4	27.6 ± 9.4	55.0 ± 21.1	20.9 ± 6.3	11.3 ± 4.8	60.3 ± 21.4
H-EC (me/100g)	0.56 ± 0.18	0.81 ± 0.27	4.95 ± 1.13	0.99 ± 0.26	6.18 ± 1.51	0.68 ± 0.18	0.70 ± 0.17	1.26 ± 0.85
Al-EC (me/100g)	0.57 ± 0.34	1.42 ± 0.38	0.26 ± 0.18	1.24 ± 0.41	0.59 ± 0.39	0.73 ± 0.52	1.53 ± 0.52	2.37 ± 1.10

Values are the mean of six to ten replicates ± standard deviation.
[b] Range of the minimum – maximum values of samples.
*ppm = parts per million equal 0.0001 % = mg · kg^{-1}
**nd is representing the non-detected value of the sample.
NA is representing not analyzed samples.

Sampling site clusters were constructed based on soil chemical characteristics data. Sampling sites were clustered into two main groups at a similarity level of 37 %. The first cluster, consisting of Jambi-2 and Riau-2, was separated from other plantation sites, which were grouped in the second cluster (Fig. 2). PCA analysis result confirmed that the two sites were separated, particularly due to their low pH and Al-Exchange Capacity. The first two PCA components of the soil chemical properties represented 67.9 % and 15.3 % of explained inertia. The first component separated sampling sites mainly according to the pH characters. Along with the clustering result, this component explained the characteristic of soils with and without peat. The second component separated sampling sites mainly according to the total of P and K content.

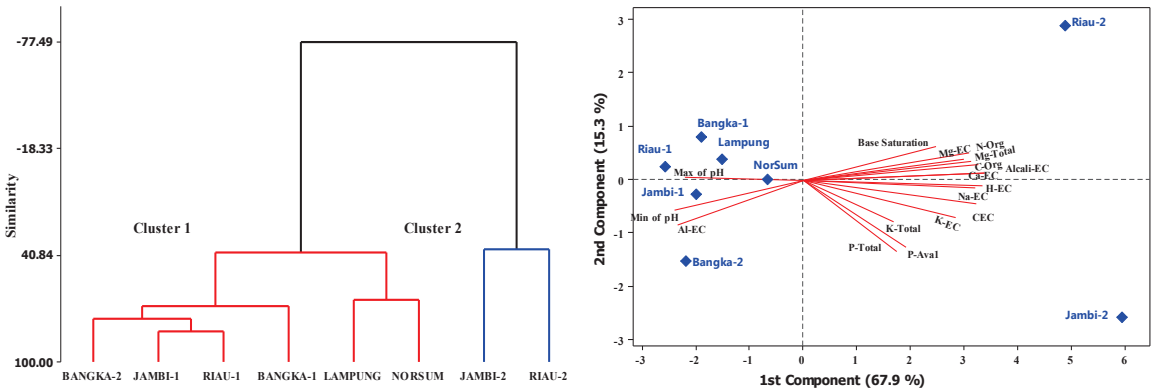

FIGURE 2. Ward's clustering of sampling sites (left). Biplot of two first principal components (right)

Genome Extraction and Fragment Restriction

The bacterial genome has been successfully isolated. Restriction fragments (TRF) obtained after digestion with *Hha*I and *Hinf*I endonucleases gave an average between three and nine fragments (Fig. 3). Each enzyme digested specific restriction site, but in general, they generated similar fragment number, except Jambi-2 and Riau-2 with high C content from peat composition. *Hha*I revealed more fragments than *Hinf*I; therefore, *Hha*I was preferrable for T-RFLP analysis using peat soil.

Abundance analysis which focused only on culturable bacteria showed similar profile given by the two enzymes, except North Sumatera sample in which *Hinf*I revealed more culturable bacteria than *Hha*I. The portion of culturable bacteria was between 7 % to 19 %. Bangka with its tin contaminated sandy soil and peat dominated Riau-2, were poorer in culturable bacteria. Severe habitat condition, such as low pH, will limit type and population that live in this area.

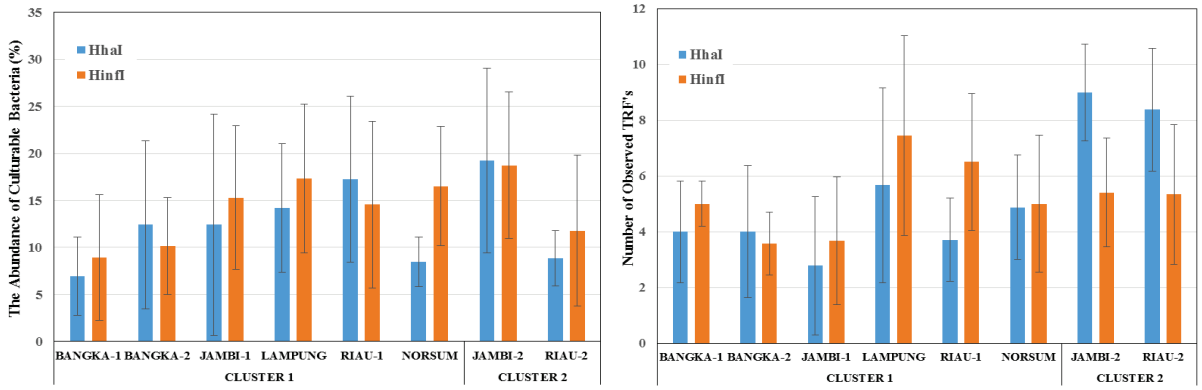

FIGURE 3. Number of observed terminal restriction fragments (TRF) after digestion by *Hha*I and *Hinf*I (left) and the abundance of cultured bacteria after capillary electrophoresis of TRF

*Hha*I was more suitable for revealing possible culturable genera and species abundance, as shown in Fig. 4. This enzyme revealed up to 60 culturable genera in Jambi-2, followed by Riau-2, Riau-1, Lampung and Bangka-1. In term of culturable species, the profile was similar to genera. The most species number was found in Jambi-2 and Riau-2. The poorest for both genera and species was Jambi-1. The probable reason was its location, which was a non-adaptive living condition for microorganisms.

The use of several restriction enzymes (RE) in T-RFLP technique was reported to be necessary to reveal more microbial diversity, to eliminate false positives and to avoid wrong conclusions due to the effect of any pseudo-terminal restriction fragments (TRFs) [16, 19]. The nonconsistency data indicates that conclusion based on T-RFLP generated from just one RE may be erroneous. Each peak in a T-RFLP profile generated from a complex bacterial

community is likely to represent more than one species. The use of different REs will group these species together in different peak combinations, means that each RE produces a different simplified representation of the community. DNA from different sample generates the same-sized TRF that originates from different sets of 16S rRNA genes. These sets can be expected to be distributed in different peaks using different RE. The use of *Hinf*I and *Hha*I were reported to generate significantly different RFLP patterns from one another. This could be due to random sequencing of clones which resulted in lower detection [19].

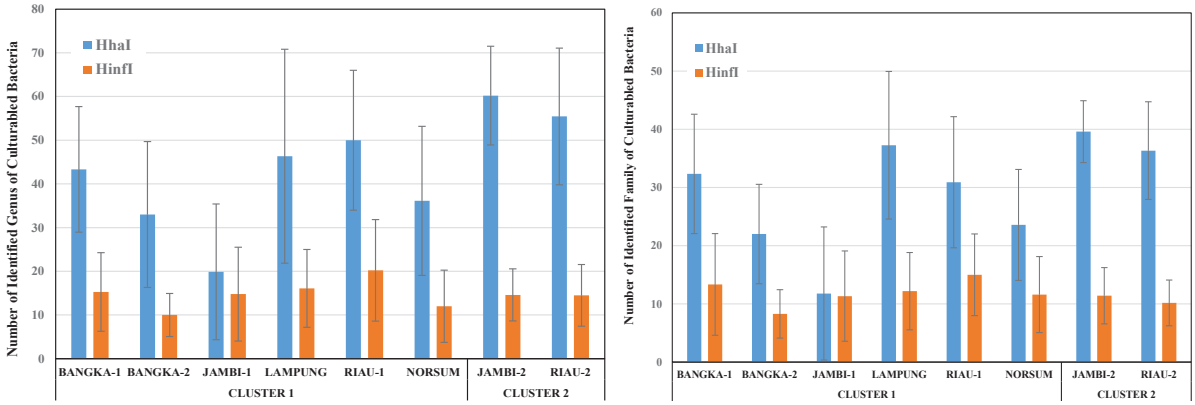

FIGURE 4. Number of identified family (left) and number of identified genus (right) of culturable bacteria in two clusters based on *Hha*I and *Hinf*I digestion

Ecological Function Analysis of Dominant Culturable Bacteria

For ecological function analysis, the investigation was carried out based on *Hha*I restriction fragments, as this enzyme revealed much more genera and species. The sample was extracted from rhizosphere, which is the zone of soil influenced by roots through the release of substrates called exudates that influenced microbial activity. Exudates are transported across the cellular membrane and secreted into the surrounding rhizosphere. The compositions of root exudates are affected by various environmental factors, including pH, soil type, oxygen status, light intensity, soil temperature, nutrient availability and the presence of microorganisms [20].

The result showed that the second cluster, which consisted of peat soil, was dominated by *Clostridium*, *Burkholderia*, *Catenibacterium* and *Pasteuria*. *Bukholderia* is known as biofilm producer [21] and also known as antifungal bacteria against *Ganoderma boninense*, a pathogen of oil palm; [22] whereas *Pasteuria* is soil nematode endophyte [22]. In contrary, *Streptomyces* was the most dominant genus in the first cluster (16.5 %; Fig. 5), which consisted of mineral and sand. In fact, it was only found in Bangka, which had more sandy texture compared to other sampling sites. Other bacteria present in this cluster are *Streptomyces*, which produces antibiotics; along with *Clostridium* and *Pseudomonas*, which are beneficial bacteria used as biofertilizer due to their phosphate solubilizing and nitrogen fixing activity [24].

Variation of detected genus or microbial community across the sampling site was affected by many factors, including soil types, history of land use and interaction between unmeasured biotic and abiotic factors [4, 25]. Soil bacterial community and relative abundance also dynamically change by time due to their interaction with those factors. The results presented are only a snapshot in time for profiling purposes. Therefore, it would be necessary to increase the frequency of sampling for generating more stable and accurate data representing a particular area of interest.

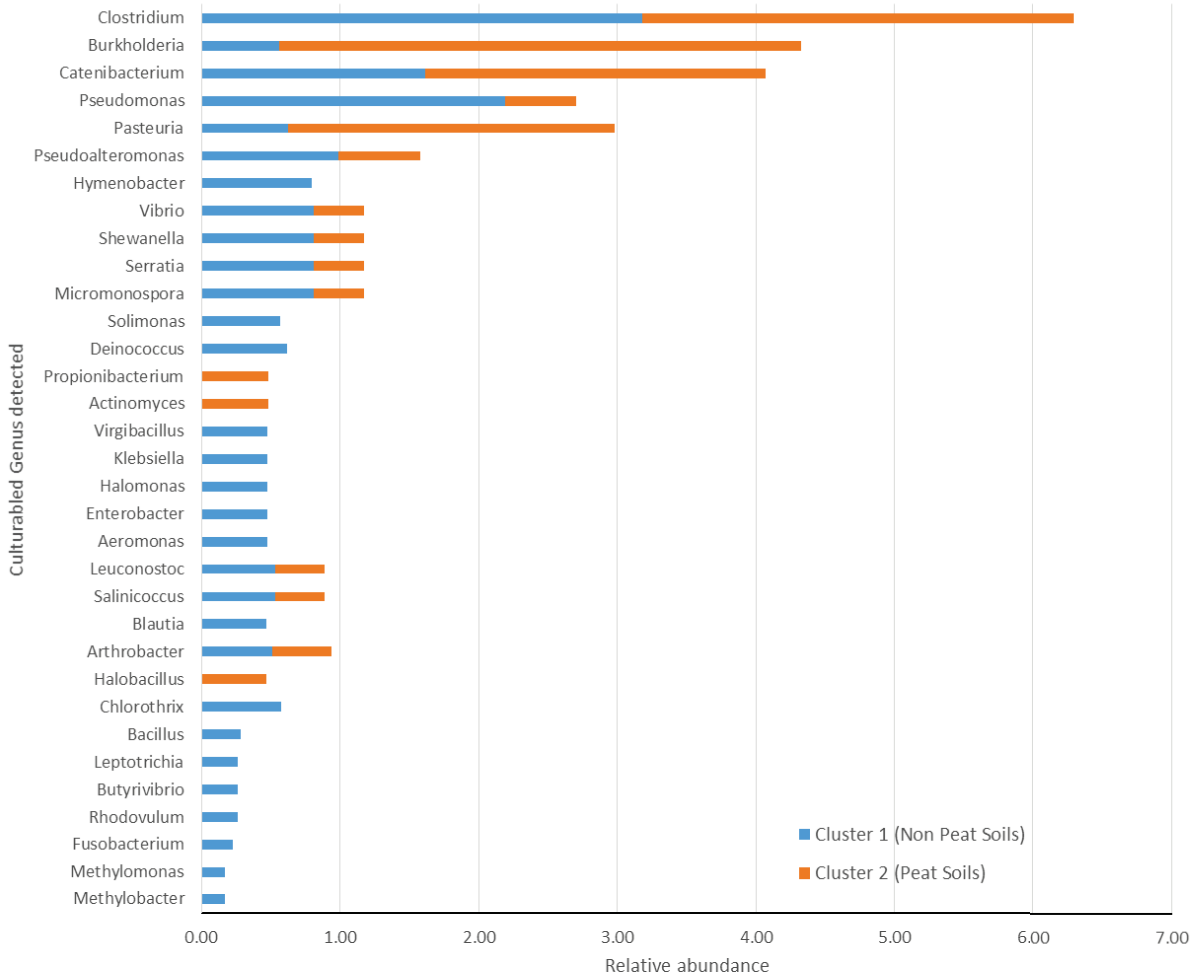

FIGURE 5. T-RFLP profiles of the bacterial 16S rRNA gene based on *HhaI* restriction enzyme. Median values of the relative abundance of ten dominant culturable genera within cluster are displayed

CONCLUSION

In conclusion, soil bacterial diversity among palm oil plantations has been investigated using the metagenomic approach from plantation sites with heterogeneous soil texture and characteristics. The result showed that all sampling areas could be grouped into two clusters. The diversity specific to soils was found both in bacterial genera/species and in their abundance. The two restriction enzymes are used, *Hha*I and *Hinf*I, affected the number of culturable bacteria dominancy. This study showed that bacterial community was affected by soil characteristics. Specific bacteria, such as *Halobacillus* and *Propionibacillus*, which lived in each soil type including peat soil, were influenced by micro soil environment where they lived and adapted to habitat conditions, such as anaerobic condition and high oxide-reduction activities.

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