BACTERIAL COMMUNITY STRUCTURE IN BANANA RHIZOSPHERE OF ORANG ASLI FIELDS AND COMMERCIAL PLANTATIONS, AS REVEALED BY 16S rRNA GENE SEQUENCES

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INSTITUTE OF BIOLOGICAL SCIENCES
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Abstract

Bacteria play important roles in the soil ecosystem, and in the rhizosphere, their dynamic and complex nature could be either beneficial or deterimental to the plants. Banana grows well in the tropics and is popularly grown in Orang Asli (OA) (indigenous people) settlements. Bananas are also grown in commercial plantations. In traditional planting practices, the OA do not add pesticide nor fertiliser to their crops which are planted for self-sustenance mainly. On the other hand, fertiliser and pesticide are added to commercial banana plantations as a means to maximise yield. Rhizosphere soils were collected from several varieties of banana plants: "Pisang Siam", "Pisang Nangka" and "Pisang Nipah" in one OA field (Paya Mendoi, Pahang); and "Pisang Berangan" and "Pisang Nipah" in one commercial plantation (Parit Serong, Selangor). Rhizosphere from only "Pisang Nipah" was collected from another OA field (Kampung Pian, Pahang) and another commercial plantation (Mersing, Johor). The rhizosphere bacteria were analysed by clone library construction of the 16S rRNA gene. From the result of the clone libaries, *Acidobacteria*, *Proteobacteria* and *Actinobacteria* were found in all the soil. Within the OA fields, *Acidobacteria* was dominant in the rhizosphere of all of the 3 banana varieties in Paya Mendoi but not in Kampong Pian where there was more *Proteobacteria*. Conversely, *Bacteroidetes* (13.79%) and *Cyanobacteria* (0.86%) were found in Kampong Pian but little to none found in Paya Mendoi. Comparing the commercial plantations, the *Nitrospirae* (9.4% in Pisang Nipah; 0.6% in Pisang Berangan) and *Gemmatimonadetes* (1.3% in Pisang Nipah; 0.9% in Pisang Berangan) were found in Parit Serong but none

in Mersing. The overall bacterial communities in banana rhizosphere of OA fields and commercial plantations showed little differences between them where certain minor bacteria phyla such as *Nitrospirae*, *Bacteroidetes, Chloroflexi*, *Verrumicrobia*, *Gemmatimonadetes, Cyanobacteria* and uncultured bacteria were variable (found in some soil but not all). The commercial plantation in Parit Serong had higher nitrogen (N), phosphorus (P) and potassium (K) content compared to the OA banana fields, meanwhile, the N, P, K contents in the commercial plantation in Mersing were roughly similar to the OA fields, but calcium and sodium contents were higher in both the commercial plantations than the OA fields. No organochlorine and organophosphorus (common constituents of pesticides) was detected in the rhizosphere of both the OA fields and the commercial plantations. One possible reason is the chemicals could have degraded or washed off by the time the rhizosphere samples were collected. To sum up, the bacterial communities were roughly similar between banana varieties but different when comparing between the two OA fields and between the two commercial plantations. The rhizosphere within the same location showed a similar bacteria composition but different when compared between different locations. Meanwhile, the PAST analysis (Diversity Analysis Using Paleontological Statistics) supported the evidence of higher bacterial diversity in OA banana fields compared to the commercial plantations.

STRUKTUR KOMUNITI BAKTERIA DI DALAM RIZOSFERA POKOK PISANG DI LADANG ORANG ASLI DAN KOMERSIAL MENGIKUT URUTAN GEN 16S rRNA

Abstrak

Bakteria memainkan peranan penting di dalam ekosistem tanah dan rizosfera, sifat bakteria yang dinamik dan kompleks boleh menguntungkan atau memberi kesan negatif kepada tumbuh-tumbuhan. Pokok pisang boleh bertumbuh dengan baik di kawasan tropika dan ianya sangat popular untuk ditanam di penempatan Orang Asli (OA) dan ladang-ladang komersial. OA mengamalkan penanaman tradisional dan mereka tidak menambahkan racun perosak atau baja kepada tanaman mereka, terutamanya tanaman yang menghasilkan rezeki kepada mereka. Sebaliknya, ladang pisang komersial mengamalkan penambahan baja dan racun perosak untuk memaksimumkan hasil pertanian. Sampel tanah rizosfera dari OA (Paya Mendoi, Pahang) telah dikumpulkan daripada beberapa jenis pokok pisang seperti "Pisang Siam", "Pisang Nangka," dan "Pisang Nipah"; manakala sampel tanah rizosfera dari ladang komersial (Parit Serong, Selangor) dikumpulkan daripada pokok pisang jenis "Pisang Berangan" dan "Pisang Nipah". Sampel tanah rizosfera dikumpulkan dari ladang OA (Kampung Pian, Pahang) dan ladang komersial (Mersing, Johor) pula, hanyalah daripada pokok pisang jenis "Pisang Nipah". Rizosfera bakteria telah dianalisis dengan menggunakan klon gen 16S rRNA. *Acidobacteria*, *Proteobacteria* dan *Actinobacteria* ditemui di dalam semua tanah. Di dalam tanah rhizosfera OA, *Acidobacteria* merupakan bakteria dominan terutamanya di dalam 3 jenis pisang di Paya Mendoi tetapi tiada langsung dijumpai di Kampung Pian di mana terdapat lebih banyak *Proteobacteria* di dalam kawasan tersebut. *Bacteroidetes* pula boleh didapati di Kampung Pian tetapi sedikit atau tiada langsung dijumpai di dalam

Paya Mendoi. Di ladang komersial pula, *Nitrospirae* (9.4%, Pisang Nipah; 0.6%, Pisang Berangan) dan *Gemmatimonadetes* (0.9%, Pisang Berangan; 1.3%, Pisang Nipah) ditemui di Parit Serong tetapi tidak dijumpai di Mersing. Komuniti bakteria di antara ladang Pisang Nipah rizosfera OA dan perladangan komersial menunjukkan sedikit perbezaan di antara mereka di mana bakteria filum seperti *Nitrospirae, Bacteroidetes*, *Chloroflexi*, *Verrumicrobia*, *Gemmatimonadetes*, *Cyanobacteria* dan bakteria yang tidak boleh dikulturkan dijumpai tidak sekata di dalam tanah rhizosphere tersebut. Pisang rizosfera di ladang komersial, Parit Serong mempunyai kandungan nitrogen (N), fosforus (P) dan kalium (K) yang lebih tinggi berbanding dengan OA. Sementara itu, kandungan N, P, dan K dalam perladangan komersial di Mersing boleh dikatakan agak sama dengan OA, tetapi kandungan kalsium dan natrium adalah lebih tinggi dalam semua ladang komersial berbanding dengan OA. Tiada organoklorin dan organofosforus (kandungan yang biasa didapati di racun perosak) dikesan di dalam rizosfera OA dan ladang komersial. Salah satu sebab kandungan tersebut tidak dapat dikesan adalah mungkin disebabkan oleh degradasi atau kehilangan bahan kimia semasa sampel rizosfera telah dikumpulkan. Kesimpulannya, komuniti bakteria adalah agak sama di antara jenis-jenis pisang yang berlainan tetapi berbeza apabila dibandingkan di antara kedua-dua ladang OA dan di antara kedua-dua ladang komersial. Bukti-bukti tersebut menunjukkan terdapat komuniti bakteria yang sama dikesan berdasarkan sampel tanah rizosfera yang diambil pada kawasan yang sama. Tetapi, komuniti bakteria ini berbeza jika dibandingkan dengan komuniti bakteria dari sampel tanah rizosfera yang diambil dari kawasan yang berlainan. Analisis PAST (Diversity Analysis Using Paleontological Statistics) menyokong bukti bahawa lebih banyak jenis bakteria di dalam rizosfera OA daripada perladangan komersial.

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List of Symbols and Abbreviations

α	Alpha
~	Around
β	Beta
^{o}	Degree
≥	Greater than or equal to
µ	Micro
%	Percentage
®	Registered sign
™	Trademark sign
Abs	Absorbance
NH_3	Ammonia
NH_4^+	Ammonium ion
bp	Base-pair
BLAST	Basic Local Alignment Search Tool
BPB	Bromophenol Blue
Ca	Calcium
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
E.coli	*Escherichia coli*
C_2H_2OH	Ethanol

Etbr	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
CH_3COOH	Glacial acetic acid
$C_3H_5(OH)_3$	Glycerol
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
LB	Luria Bertani
LBA	Luria Bertani agar
MARDI	Malaysian Agricultural Research and Development Institute
Mg	Magnesium
$MgCl_2$	Magnesium chloride
N	Nitrogen
NCBI	National Center for Biotechnology Information
OA	Orang Asli
OD	Optical density
OTU	Operational taxonomic unit
P	Phosphorus
PAST	Paleontological statistics
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
K	Potassium
KCl	Potassium chloride

RFLP	Restriction fragment length polymorphisms
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
S	Sulphur
SOC	Super optimal broth with catabolite repression
TAE	Tris-acetic
Taq	*Thermus aquaticus*
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

CHAPTER 1 : INTRODUCTION

Banana (genus *Musa* in the family *Musaceae*) is a major food crop, ranking fourth among the most significant foodstuffs in the world, after rice, corn and milk (Shyam *et al*., 2011). It comes pre-packaged in its own yellow jacket with firm and creamy flesh, and is available year round. Banana trees are planted in large quantities in tropical and sub-tropical areas. The genus *Musa*, previously classified into five groups (*Ingentimusa*, *Australimusa*, *Callimusa*, *Musa* and *Rhodochlamys*), has now been re-classified into three groups, namely *Ingentimusa, Australimusa & Calimusa* and *Musa & Rhodochlamys* (Wong *et al*., 2002).

To aid the health and growth of banana plants in the commercial plantations, fertilisers and pesticides are applied to enrich the soils on which the banana plants are located. However, this is not the case with banana plants in the Orang Asli (OA) Settlement where no fertiliser and/or pesticide is used. In recent years, a number of researchers have reported their findings on the effects of fertiliser and pesticide applications to the soil microbial system. Broeckling *et al*. (2008) reported that fertiliser influenced soil microbial growth and activity. Beauregard *et al*. (2010) reported that repeated fertiliser applications to soil changed the soil microbial community since they changed the soil physical, chemical and biological properties. Ge *et al*. (2008) reported that fertilisation has had significant impacts on the soil micro-organisms, increasing their activites by altering their soil microbial biomass, nutrient content of the soil and degenerating the soil quality. Lazcano *et al*. (2012) and Peacock (2001) reported that fertilised soil encouraged microbial growth as well as stimulating changes of the microbial community by increasing the Gram-negative bacteria. However, other studies (Okano, 2004; Treseder, 2008) found that fertilisers had had little or no effect on the soil microbial diversity and activities. In light of the inconclusive findings cited above, this study has been undertaken to compare the rhizosphere bacterial communities in soils that

have been both disturbed and undisturbed by the addtion of fertiliser and pesticides to the soil.

Rhizosphere microbiology has received considerable attention in the last century on account of the importance of rhizosphere micro-organisms to plant growth and health (Rovira, 1991; Hirsch *et al*., 2013). Root exudates, mucilage, and sloughed-off root cells provide a nutritional source for microbial cell multiplication and colonization of root surfaces and adjacent soil (Hirsch *et al*., 2013). The rhizosphere is a dynamic niche containing complex microbial communities and microbial members may participate in a variety of beneficial and detrimental interactions with plants (Rovira, 1991). Beneficial interactions include the roles micro-organisms play in enhancing nutrient uptake by plants, stimulating plant growth by a variety of mechanisms and offering biological disease control. In contrast, major and minor pathogens can impair plant health and decrease productivity in agricultural and forested environments.

Studies of the diversity and ecology of micro-organisms in the natural environment by the molecular method have started since the mid-1980s (Head *et al*., 1998). The reason of practicing this new approach is due to limitations in the traditional use of culture-dependent method to study bacterial diversity since more than 90% of bacteria in nature cannot be cultivated (Moyer *et al*., 1993). This means that the bacterial diversity identified in a sample by using culture-dependent method cannot reveal the actual bacterial diversity of a sample. This may be due to the fastidious growth requirement of bacteria (Whitford *et al.,* 1998). Consequently scientists are now in favour of using the molecular method for their bacterial studies as this method allows identification of all bacteria in nature.

In this study, the molecular method is used to analyse bacterial communities in the rhizosphere of the banana plants in two types of operations, namely the OA

Settlements and the commercial plantations. Molecular method targets on 16S rRNA gene which is conserved in all bacteria, regardless of whether they are culturable or unculturable. Information thus gathered can be utilized in understanding the complexity of these bacteria in the soil as well as differentiating the bacterial activities in relation to the soil chemical compositions. Since there is to date very limited information on the microbial diversity in the banana rhizosphere in Malaysia (Tripathi *et al*., 2012), it is hoped that results from this study would increase the understanding of these microbial activities.

1.1 Objectives of the study

The objectives of the study are two-fold:

1. To assess the bacterial community in the rhizosphere of banana plants in the Orang Asli fields and the commercial plantations.
2. To determine whether there are similarities or differences in bacterial community and some chemical properties in the rhizosphere of banana plants in the Orang Asli fields and the commercial plantations.

CHAPTER 2 : LITERATURE REVIEW

2.1 Rhizosphere

The rhizosphere is the zone of soil surrounding the plant roots where the roots must compete not only with the invading root systems of neighboring plant species for space, water and mineral nutrients, but also with soil-borne bacteria, fungi, and insects feeding on an abundant source of organic materials. Thus, in the rhizosphere, there would be a lot of interactions between the organisms, ranging from root-root interactions to root-microbe interactions involving parasitic plants, pathogenic bacteria, mycorrhizal fungi, and nitrogen-fixing bacterial. In short, there may be an abundance of interactions in the soil where signaling of molecules are taking place, conjugating the roots and other organisms in their daily life. Furthermore, soil microbes are important components of the soil habitat as they control the nutritional cycle in order to maintain the soil fertility as well as the soundness of soil structure (Clegg & Murray, 2002).

2.2 Rhizosphere bacteria

Microbial communities are influenced by many factors such as food network associated with the microbial community and the presence of organic and inorganic nutrients. Hence, the soil surrounding the root system is very rich in microorganisms fueled by the supply of root exudates ultimately originating from the shoot through photosynthesis (Compant *et al.*, 2010). These microorganisms can change the soil characteristic surrounding the root to increase nitrogen and phosphorus availability and can enhance the uptake of nutrients by plants roots (Gaskins *et al*., 1985). Because of their positive benefits to plant growth, the rhizosphere organisms are very important for increasing agricultural yield (Hinsinger *et al*., 2009). Rhizobacteria are not the only organisms inhabiting close to root surfaces but others include fungi, nematodes and

protozoa. Some of these rhizobacteria cause diseases in plants while others stimulate plant growth (Doornbos *et al*., 2012) which are generally referred to as plant growth promoting rhizobacteria (PGPR) (Hayat *et al*., 2010).

Combining the technology of molecular biology and the analysis of phylogenetic information, it is possible to assess microbial community diversity in the soil habitat (Liao *et al.*, 2007). Various ecological diversity indices have been developed to study the diversity and richness of organisms in different ecological environments such as Alpha (α), Beta (β), Shannon-Weiner, and Simpson's index etc to name a few. (Oswald, 2007). Magurran (2004) cited that the definition of ecological diversity is the richness and variety of natural logical communities.

2.3 The effect of fertiliser on banana rhizosphere bacteria

The banana roots are adventitious and horizontally proliferate in the top soil, so, they cannot get water and nutrients from the deeper part of soil like other fruit crops. This root system prevents the production of the bananas in large scale under adverse tropical soil condition where these root systems are crucial for the support of the plant, water and nutrient acquisition and also in production of plant growth regulators by rhizosphere bacteria (Mia *et al.,* 2010). The banana fruit crop is cultivated widely in tropical areas where large amount of chemical fertilisers (NPK fertiliser) may commonly be applied. Commercial cultivation of banana needs inorganic fertilisers such as urea, therefore, organic fertilisers are seldom being used.

Research comparing organic and inorganic fertiliser provides compelling evidence that organic fertiliser boost soil health over the long term. Olajire *et al*. (2015) reported on the difference in soil structure crop quality between an organic and inorganic system. They found that soil in the organic system had higher fertiliser, and organic crops

had higher starch content than the inorganic system. In contrast, long term use of synthetic fertiliser depletes soil organisms of the organic matter they need. Eventually, these organisms disappear in soil dependent on inorganic fertiliser (Olajire *et al*., 2015).

As noted previously, research has shown that fertiliser will influence soil microbial growth and activity. Repeated fertiliser applications to soil can change the soil microbial community since they change the soil physical, chemical and biological properties (Beauregard *et al*., 2010). Some studies have documented that fertilisation has had significant impacts on the population, composition and function of soil microorganisms, thereby increasing the soil microorganisms' activities (Ge *et al*., 2008). However, other studies have found that fertilisers have had relatively little or no effect on the soil microbial diversity and activities (Okano *et al*., 2004).

2.4 Soil characteristic in Malaysia

There are three main geological types in Malaysia which are the granite residual, sedimentary residual soils and quaternary deposits soils. In Malaysia, the soft clay dominates the coastal, meanwhile, the rest of the area are covered by granite residual and sedimentary residual soils (Jamal *et al.,* 1997). More than three quarters of West Malaysia is covered by residual soil. The residual soil composite of sand, silt and clay with various features where they are influenced by the geological formation (Jamal *et al.*, 1997). These residual soils which are found at the top layer of the soil which have the highest concentration of organic matter, therefore, created a likable medium for microorganisms to grow. This rich soil microorganisms that were found in both OA and commercial plantations would be a good material to study bacterial diversity.

2.5 Traditional approach of studying bacterial communities

Pure culture technique is the traditional approach of studying bacterial communities. Due to the convenience of culturing, microbiologist ignored the challenge to identify and characterize the uncultured organisms. Instead, they focused on the discovery found in the readily cultured model organisms, leading to an explosion of knowledge in microbiology and genetics from the 1960s to the mid-1980s (Handelsman, 2004). Many studies that relied on this traditional technique are hampered by the fastidious growth requirement of bacteria and by problems associated with the phenotypic criteria used to define microbial taxa of those bacteria that can be cultured (Whitford *et al.*, 1998). The problem with this technique is further compounded by the fact that more than 90% of bacteria in nature cannot be cultivated (Moyer *et al.*, 1993).

The traditional approach aims at developing an artificial environment to mimic the natural environment for bacteria growth. However, this technique is of limited usefulness on account of insufficient knowledge to reproduce the natural conditions in the laboratory or to create viable synthetic conditions for all organisms (Alain & Querellou, 2009). In addition, although some microorganisms can be grown in the laboratory, such efforts may have been overlooked by the impatient researcher because obvious turbidity or colonies have not developed due to slow growth rates and poor abilities of the organisms to adapt to laboratory growth conditions. Longer incubation time is needed to increase the cultivation efficiency (Leadbetter, 2003).

As well, the traditional approach of isolating and classifying cultured bacterial strains may not be directly applicable as its classification is based on the phenotypic characteristics of bacterial strains, meaning that the classification is done according to observable characters of the cell such as morphology, physiological activities and cell component structure. The bacterial populations retrieved from such environmental

samples require a lot of isolation and maintenance procedures in culture (Brightwell *et al.*, 2006).

Although the traditional approach involves the culture of diluted sample on nutrient-rich media for bacteria isolation, the cultivated isolates may provide relative measures of community diversity but not accurate description of community diversity in situ (Dunbar *et al.*, 1999). Besides, any departure from the original environmental parameters during cultivation can affect the structure of bacterial community through the imposition of new selective conditions (Liu *et al.*, 1997).

2.6 Molecular way of studying bacterial communities

The drawbacks of traditional approach in the study of bacterial communities have led to the shift from the traditional culture method to the molecular method. Usage of the molecular method involving ribosomal RNA (rRNA) sequence-based analysis to characterize microbial populations has increased significantly since the mid-1980s. The modern era of microbial studies started with the analysis of nucleic acids recovered from the environment and isolated prokaryotic and eukaryotic cells. The pioneering work of Carl Woese which showed that rRNA genes can provide evolutionary chronometers (Woese, 1987) was followed by Pace and collaborators who created a new branch of microbial biology by directly analyzing the 5S rRNA and 16S rRNA gene sequences in the environment sample without culturing (Pace *et al.*, 1986). 16S rRNA gene is preferred to 5S rRNA gene in community analysis even though both of them are conserved in all bacteria in nature. This is because of the difficulty of physically separating 5S rRNA gene. Besides, the relatively small size of 5S rRNA provides only limited information content (Ward *et al.*, 1992). The 16S rRNA gene is a section of DNA found in all prokaryotes, including both bacteria and archaea. The 16S rRNA gene has emerged as a common

marker for bacteria identification for a number of reasons, including its presence in most all bacteria, its function remaining unchanged over time, and its sufficiently large size (1,500 bp) for informatics purposes (Janda & Abbott, 2007). The development of culture-independent techniques of molecular biology involving the use of 16S rRNA gene as a marker for bacteria identification has led to a much greater understanding and insight on the diversity and structure of natural bacterial communities (Sinclair *et al*., 2015).

In addition to providing a universal culture-independent means to assess bacterial diversity, 16S rRNA sequences have also aided in culturing efforts. Bacteria may be recalcitrant or difficult to be cultured because the lack of necessary symbionts, nutrients or surfaces, excess inhibitory compounds, incorrect combinations of temperature, pressure or atmospheric gas composition, accumulation of toxic waste products from their own metabolism and intrinsically slow growth rate and/or rapid dispersion from colonies (Simu & Hagstrom, 2004). By using the molecular approach, the needs of laborious efforts may be substantially reduced.

The application of 16S rRNA gene technique in environmental samples requires the development of methods enabling DNA or RNA to be extracted and amplified in the presence of inhibitory substances (O'Donnell & Görres, 1999). Direct amplification and analysis of 16S rRNA gene sequences have replaced cultivation as a way to compare the composition, richness, and structure of bacterial communities. Even though amplification of rRNA gene can distort the apparent structure of a community due to biases in cellular rRNA gene copy number, DNA extraction and PCR amplification, it still provides meaningful comparisons of bacterial communities (Dunbar *et al.*, 1999). However, as 16S rRNA gene sequencing has low phylogenetic power at the species level and poor discriminatory power for some genera, DNA relatedness studies are necessary to provide absolute resolution to these taxonomic problems (Janda & Abbott, 2007).

2.7 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a revolutionary biochemical technology developed by Kary Mullis in 1983. The PCR represents an equally momentous advance in the field of molecular biology, in particular in the analysis of DNA (Peake, 1989). Using this technique, a few copies of a piece of DNA can be amplified to generate thousands or even millions of copies of a particular DNA sequence. PCR is very different from gene cloning, in that PCR does not involve the use of living cells. As well, PCR does not induce selection problem because the desired gene is automatically selected as a result of the positions at which the primers anneal. The time required for PCR experiment is short, needing only a few hours in order to complete, compared to weeks in gene cloning. PCR is now a common technique used in medical and biological research for various applications. This technique requires the use of a thermal cycler which is a scientific instrument that performs repeated cycles of heating and cooling processes. The steps of PCR involve denaturation, annealing, and extension of DNA templates. In the denaturation step, the reaction is heated to 94°C at which temperature, hydrogen bonds that hold the double stranded DNA together are broken, causing the molecule to denature and yield single stranded DNA molecules. Annealing of the oligonucleotide primers to the single-stranded DNA template occurs at lower temperatures between at 50 and 60°C. At this temperature, the two strands of each molecule may rejoin, but most do not because the mixture contains a large excess of short DNA molecules, called oligonucleotide primers, which anneal to the DNA molecules at specific positions. At the extension step, the temperature is raised to 74°C which is the working temperature required for *Taq* DNA polymerase to perform optimally. The *Taq* DNA polymerase which is present in the PCR reaction mixture attaches to one end of each primer and synthesizes a new complementary strand of DNA, resulting in four strands of DNA instead of the two that was started with.

These denaturation-annealing-extension steps are repeated for 25 to 30 cycles to amplify the double–stranded DNA molecules to thousand or million copies (Brown, 2001).

Originally, the DNA polymerase used for PCR was extracted from *Escherichia coli (E.coli).* Although this enzyme may be used for a wide range of applications, it has had significant disadvantages in PCR because the reaction must be heated to denature the double-stranded DNA product after each cycle of synthesis. Consequently, the heating process inactivated the *E.coli* DNA polymerase and fresh aliquots of enzyme had to be added at the start of each cycle (Bartlett & Stirling, 2003). The introduction of a thermo-stable DNA polymerase isolated from thermophilic bacteria known as *Thermus aquaticus (Taq)* has replaced the use of *E.coli* DNA polymerase. Since this heat-resistant polymerase is relatively unaffected by the denaturation step, it does not need to be replenished at each cycle (Saiki *et al.*, 1988). This modification improves the overall performance of PCR, not only increasing the specificity, yield and sensitivity, but also amplifying the length of targets.

Nothwithstanding the improved efficiency of the PCR technique, there are a couple of drawbacks. Firstly, PCR cannot be used to isolate genes that have not been previously studied because sequences of the annealing sites for primers to attach must be known a priori. If the sequences of the annealing sites are unknown, appropriate primers cannot be made. Secondly, the length of DNA sequence that can be copied using PCR is limited. This poses a problem when lengths of genes copied by PCR are shorter than lengths of many genes, especially human genes (Brown, 2001). Because of these limitations, cloning is sometimes preferred in situations where PCR is not feasible.

2.8 Cloning

Cloning is essential especially when one wants to isolate pure samples of a gene. Cloning involves three basic steps, namely ligation, transformation and screening. During ligation, the DNA fragment or the gene of interest is inserted into a cloning vector (a small piece of DNA taken from a virus, a plasmid or the cell of a higher organism which can be stably maintained in an organism), and the foreign DNA fragment can be inserted into it for cloning purposes (Brown, 2001). The ligated product is then being transformed or taken up into a host cell, usually a competent *E. coli* cell. The transformed product is then spread on agar plates containing medium added with specific antibiotic and other selection chemicals. Within the host cell, the vector multiplies, producing numerous identical copies. When the host divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place. Following a large number of cell divisions, a colony or clone of identical host cells is produced. Each individual clone contains multiple copies of the genes of interest (Brown, 2001). The resultant clones may either consist of DNA inserted vector or original vector because of the presence of antibiotic resistance marker in the vector which allows the host cell containing the vector to multiply and grow in the specific antibiotic-added medium. Common antibiotics used in cloning are ampicillin, kanamycin, streptomycin and tetracycline (Brown, 2001). Modern cloning vectors not only include selectable antibiotic resistance genes, but also colour selection markers. For example, in the blue white screening, the agar medium used for plating is not only added with antibiotics, but also with isopropyl β-D-1-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal). Cells thus transformed with vectors containing recombinant DNA will produce white colonies while cells transformed with only the vector without recombinant DNA will grow into blue colonies.

The method of blue white screening in cloning is based on the principle of the β-galactosidase gene. β-galactosidase is a protein encoded by the *lac*Z gene of the *lac* operon. IPTG, a non-metabolizable inducer of the *lac* operon, is needed to switch on the expression of *lac*Z gene. The presence of an active β-galactosidase can be detected by X-gal within the agar plate. X-gal is a colourless chromogenic substrate which can be cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo (Aitken, 2012). If the host cell contains recombinant DNA inserted vector, it means that the *lac* operon in the vector has been disrupted. Consequently, no β-galactosidase will be formed, no X-gal will not have been cleaved and the resulting clone will appear as white in colour. In short, blue clones show that they may contain a vector with an uninterrupted *lac* operon, while white clones indicate the presence of a DNA insertion.

However, blue white screening has drawbacks as well. A white clone may not contain inserted DNA, probably the result of excision of the inserted DNA at some stage during the colony growth. This is a general problem that can arise with any cloning vector. In order to fix this problem, the white clones are usually subjected to second screening, usually via colony PCR to confirm the insertion of DNA (Brown, 2000).

2.9 Colony PCR

Colony PCR is used to screen colonies for correct insertion of desired products in vector. Primers are required to generate a PCR product of known size. Types of primers used in colony PCR are dependenton the vector used in cloning. Colony PCR which involves lysing the bacteria and amplifying a portion of the vector is a very promising screening technique to identify the insertion of gene interested (Brown, 2000). Any colonies which give rise to an amplification product of the expected size are likely to contain the correct DNA sequence.

3.0 Restriction fragment length polymorphisms (RFLP)

Restriction fragment length polymorphisms (RFLP) is a technique requiring the use of restriction enzyme to digest the DNA sample, and separating by length through agarose gel electrophoresis. RFLP can be used to trace inheritance patterns, identify specific mutations and other molecular genetic techniques. A fragment sizing analysis of RFLP patterns on PCR-amplified 16S rRNA genes was said to be a very promising tool for simple and rapid identification of methanogens (Hiraishi *et al*., 1995).

CHAPTER 3 : MATERIALS AND METHODS

The rhizosphere of banana plants in the OA settlements was collected in Paya Mendoi and Kampung Pian in the state of Pahang. The rhizosphere of banana plants in the commercial plantations was taken from Parit Serong, Selangor and Mersing, Johor. The genomic DNAs were extracted from all the sampling sites. The 16S rRNA gene fragment was amplified from the genomic DNA of each sample by the polymerase chain reaction (PCR). The PCR products were then pooled together for purification, followed by ligation using the pGEM®T Easy Vector System (Promega, U.S.A). Transformation of the plasmid into competent cell was done and plate spreading was performed for blue-white screening. Colony PCR was done by using T7 and SP6 primers to check for the correct insertion of DNA in each colony. The colony PCR products of the correctly inserted colonies were then subjected to restriction fragment length polymorphism (RFLP) analysis. Each operational taxonomic unit (OTU) that was determined represents one bacterial species. Several clones identified with different OTUs were grown and the plasmids were extracted and sent for sequencing. Phylogenetic analysis was done when the sequencing results were obtained from the sequencing company.

For this study, the molecular method was used to analyse bacterial communities in the rhizosphere of the banana plants in two types of cultivations, namely the OA settlements and the commercial plantations and to correlate the diversity with some environmental variables such as presence of nitrogen (N), phosophorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and sulphur (S). Molecular method targets on 16S rRNA gene which is conserved in all bacteria, regardless of whether they are culturable or unculturable.

3.1 Biochemicals and materials

All microcentrifuge tubes, micropipette tips, media, solutions, glassware, Falcon tubes and distilled water were steam sterilized at 121℃ for 20 minutes at 15 psi by using an autoclave machine. Upon completion of autoclaving, all materials with the exception of media and solutions were dried in the oven at 65℃. For biohazard waste, it was autoclaved at 121℃ for 30 minutes at 15 psi before being disposed.

All chemicals used are commercially available from the following sources: iNtRON Biotechnology Inc (South Korea), Invitrogen (USA) Promega (USA), MoBio Inc (Canada) and Qiagen (Germany).

A summary of the materials and biochemicals used in this study was provided in Tables 3.1-3.3.

Table 3.1: Materials, instruments and apparatus used

Material/Instrument/Apparatus	Source	Country
• 1.5 mL Centrifuge Tube	• Eppendof	• North America
• 10X Buffer Tango (RFLP)	• Thermo Scientific	• North America
• 2X Rapid Ligation Buffer	• Promega	• North America
• Aluminium Foil	• Diamond	• China
• Autoclave Machine	• Tomy	• North America
• Beakers	• Duran	• Germany
• Biophotometer	• Eppendof	• North America
• Buffer (PCR) (X5)	• Promega	• North America
• Buffer (PCR) (X10)	• Invitrogen	• North America
• Centrifuge	• Eppendof	• North America
• Conical Flasks	• Duran	• Germany
• DNA Ladder (1 kb and 100 bp)	• Promega	• North America
• dNTP Mix (10 mM)	• Invitrogen	• North America
• Electronic Balance	• AND	• North America
• Falcon Tubes	• Grenier Bio-One	• North America
• Freezer (-20°C)	• Acson	• North America
• Fridge (4°C)	• Biodis	• North America
• Gel Comb	• Bio-Rad	• North America
• Gel Electrophoresis Set	• Bio-Rad	• North America
• Gloves	• Safeguard	• Malaysia
• Ice Maker	• Sastec	• Malaysia
• Laminar Flow Chamber	• Bumitech	• Malaysia
• Loading Dye	• Promega	• North America
• Magnesium chloride ($MgCl_2$) (PCR) (25 mM)	• Promega	• North America
• Magnesium chloride ($MgCl_2$) (PCR) (50 mM)	• Invitrogen	• North America
• MEGAquick-spin™ PCR & Agarose Gel DNA Extraction Kit	• iNtRON Biotechnology	• South Korea
• Medium Flasks	• Simax	• Czech Replublic
• Micropipettes	• DragonMed	• China
• Micropipette Tips	• Eppendof	• North America
• Microwave Oven	• Novia	• North America
• *Msp*I (*Hpa*II) (RFLP) (10 U/ µL)	• Thermo Scientific	• North America
• DNA-Spin™ Plasmid DNA Purification Kit	• iNtRON Biotechnology	• South Korea
• Parafilm	• Pechiney	• North America
• Petri Dishes	• Grenier Bio-One	• North America
• pH510 pH meter	• Eutech Instruments	• Singapore
• Power Pack	• Thermo EC	• North America
• Primers	• 1st Base	• Singapore
• Restriction Enzyme, *Msp*I (*Hpa*II)	• Thermo Scientific	• North America
• Shaking Incubator	• N-biotek Inc	• South Korea
• T4 DNA ligase (3 Weiss units/ µL)	• Promega	• North America
• Taq DNA Polymerase (PCR) (5 U)	• Invitrogen	• North America
• Thermal Cycler	• Bio-Rad	• North America
• Ultra Clean™ Soil DNA Isolation Kit	• MoBio Inc	• North America
• UV Transillumintor	• Gene Flash	• North America
• Vortex	• Snijders	• Netherland
• Water Bath	• Memmert	• Germany

Table 3.2: Chemicals

Chemical	Source	Country
• 1X Tris-acetic	• Merck	• North America
• 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal)	• Promega	• North America
• 6X Bromophenol Blue	• Promega	• North America
• Agar, Tryptone, Yeast Extract	• Bacto	• United Kingdom
• Agarose	• 1st Base	• Singapore
• Ampicillin	• Promega	• North America
• Bacto™ Agar	• Systerm	• Malaysia
• Calcium Chloride ($CaCl_2$)	• Systerm	• Malaysia
• Ethanol 97% (C_2H_2OH)	• Systerm	• Malaysia
• Ethyl Alcohol 99.8% (C_2H_2OH)	• Systerm	• Malaysia
• Ethylenediaminetetraacetic acid disodium salt dihyrate ($EDTA{\cdot}2H_2O{\cdot}NaOH$)	• Sigma-Aldrich	• North America
• Ethidium Bromide	• Promega	• North America
• Ficoll 400	• Sigma-Aldrich	• North America
• Glacial acetic acid (CH_3COOH)	• Sigma-Aldrich	• North America
• Glucose	• Systerm	• Malaysia
• Glycerol	• Invitrogen	• North America
• Isopropyl β-D-1-thiogalactopyranoside (IPTG)	• Promega	• North America
• Luria Bertani (LB)	• Systerm	• Malaysia
• Luria Bertani Agar (LBA)	• Systerm	• Malaysia
• Magnesium chloride ($MgCl_2$)	• Systerm	• Malaysia
• Sodium Chloride (NaCl)	• Systerm	• Malaysia
• Potassium Chloride (KCl)	• Systerm	• Malaysia
• Tris-Base	• 1st Base	• Singapore

Table 3.3: Synthetic oligonucleotide primers used

Primer	Sequence
• 27F	• 5’-AGAGTTTGATCCTGGCTCAG-3’
• 1492R	• 5’-GGTTACCTTGTTACGACTT-3’
• T7	• 5'-AATACGACTCACTATAG-3'
• SP6	• 5'-ATTTAGGTGACACTATAG-3'

3.1.1 Bacterial strain for competent cells preparation and plasmid

Escherichia coli Topo 10 cells which were purchased from Qiagen, Germany. The pGEM-T easy vector used in ligation step of cloning was purchased from Promega, U.S.A.

3.1.2 Preparation of buffer, solutions and media for agarose gel electrophoresis

In order to run the agarose gel electrophoresis, several buffers, solutions and media were prepared. These include 6X Bromophenol Blue (BPB), 1X Tris-acetic (TAE) buffer, ethidium bromide and agarose gel. Table 3.4 summarizes the preparation of 6X Bromophenol Blue (BPB) loading dye (0.15% (w/v) with BPB, 9% (w/v) Ficoll 400, 40% (v/v) glycerol), stored at 4°C

Table 3.4: Preparation of 6X BPB loading dye

Ingredient	Weight/Volume
• BPB	• 15 g
• Ficoll 400	• 0.9 g
• Glycerol, $C_3H_5(OH)_3$	• 4 mL
• Sterile distilled water top up to	• 10 mL

In order to prepare 1X TAE buffer, a total volume of one L of 50X TAE buffer, pH 8.0 (10mM Tris, 10mM glacial acetic acid, 1M EDTA) have to be prepared as follows:

Table 3.5: Preparation of 1X TAE buffer

Ingredient	Weight/Volume
• Tris-base, $C_4H_{11}NO_3$	• 242 g
• Glacial acetic acid, CH_3COOH	• 57.1 mL
• $EDTA{\cdot}2H_2O{\cdot}NaOH$	• 37.2 g
• Sterile distilled water top up to	• 1 L

Table 3.6 summarizes the preparation of the ethidium bromide solution. Firstly, the 50X TAE stock solution, stored at room temperature and used as soon as possible to avoid precipitation, was subjected to dilution of 1:50 (v/v) to give 1X TAE buffer for routine agarose gel electrophoresis. Next, the ethidium bromide solution was prepared and stored in a covered container in the dark as it is light sensitive.

Table 3.6: Preparation of ethidium bromide (EtBr) solution

Ingredient	Weight/Volume
• 1X TAE buffer	• 500 mL
• Ethidium bromide (10 mg/mL)	• 100 mL

To prepare agarose gel which will be used for electrophoresis of 16S rRNA gene fragment PCR amplification products, 2% agarose gel was prepared by pouring 40 mL of 1X TAE buffer into a conical flask containing 0.80 g of agarose powder. For electrophoresis of colony PCR, 1% agarose gel was prepared by pouring 40 mL of 1X TAE buffer into a conical flask containing 0.40 g of agarose powder (see Table 3.7).

Table 3.7: Preparation of agarose gel

Ingredient	Weight/Volume
• Agarose	• According to percentage of gel
• 1X TAE buffer	• Top up to 40 mL

3.1.3 Preparation of media for transformation

The transformation process of gene cloning requires the preparation of several media, including Luria Bertani (LB) medium, Luria Bertani agar (LBA) and super optimal broth with catabolite repression (SOC). LB and LBA can be prepared per specifications in Tables 3.8 and 3.9 respectively.

Table 3.8: Preparation of Luria Bertani (LB) in flask

Ingredient	Weight/Volume
• Bacto-typtone	• 0.5 g
• Bacto-yeast extract	• 0.25 g
• Sodium chloride (NaCl)	• 0.25 g
• Distilled waster	• 50 mL

Table 3.9: Preparation of Luria Bertani Agar (LBA) in flask

Ingredient	Weight/Volume
• Bacto-tryptone	• 2 g
• Bacto-yeast extract	• 1 g
• Sodium chloride (NaCl)	• 1 g
• Bactor agar	• 3 g
• Distilled water	• 200 mL

Following the preparation of all the ingredients in the two flasks, both flasks were autoclaved and cooled to room temperature. The flasks thus autoclaved were promptly inverted several times to ensure that the LB and LBA were mixed uniformly. Chemicals such as ampicilin, isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) need to be added into the warm LBA for plating (see Table 3.10).

Table 3.10: Preparation of LBA with ampicillin, IPTG and X-gal

Ingredient	Weight/Volume
• Ampicillin	• 210 mL
• IPTG	• 210 mL
• X-gal	• 210 mL

To increase the nutrient content in bacteria, growth medium, super optimal broth with SOC was added. SOC can be prepared in large volume as shown in Table 3.11 and transferred to 1.5 mL tubes for storage at -20°C for future use.

Table 3.11: Preparation of SOC

Ingredient	Weight/Volume
• Bacto-tryptone	• 1 g
• Bacto-yeast extract	• 0.25 g
• 1M NaCl	• 0.5 mL
• 1M KCl	• 125 µL
• Distilled water	• 49 mL
• 2M Magnesium chloride, MgCl (after autoclaved and cooled)	• 500 µL
• 2M Glucose (after autoclaved and cooled)	• 500 µL

3.2 Collection of banana rhizosphere samples

The soil samples were collected around the roots of the banana plants in the OA's settlement in Paya Mendoi, Pahang. Each soil sample was weighed 40g and was collected into the falcon tube. Two soil replicates were obtained from each tree and a total of 22 soil samples were obtained from three different banana varieties (Pisang Siam, Pisang Nangka, Pisang Nipah). Soil samples were also collected in the commercial plantation in Parit Serong, Tanjung Karang, Kuala Selangor where two soil replicates were obtained from each tree and a total of 12 soil samples were obtained from two different banana varieties (Pisang Berangan, Pisang Nipah). Subsequently soil samples were also collected from the Nipah banana rhizosphere in the OA's settlement in Kampung Pian, Pahang and the commercial plantation in Mersing, Johor. Two soil replicates from each of four trees were collected and a total of eight soil samples were obtained in Kampung Pian. In Mersing, only one soil sample was obtained from each of four trees for a total of four soil samples. All the soils were collected in sterile falcon tubes from around the roots at a depth of 20 cm. The tubes were placed in a cool box containing ice packs and brought back to the laboratory where they were stored at -20^{o}C until they were used. Details of the soil sample collections were provided in Table 3.12 below.

Table 3.12: Locations where soil samples were collected

Field Type	Location	Latitude / Longitude	Tree Number	Banana Variety
• Orang Asli	• Paya Mendoi, Pahang	N03^{o}73'206" E102^{o}34'228"	Tree 1	Siam
		N 03^{o}73'206" E 102^{o}34'230"	Tree 2	Siam
		N 03^{o}73'213" E 102^{o}34'226"	Tree 3	Siam
		N 03^{o}73'227" E 102^{o}34'215"	Tree 4	Nangka
		N 03^{o}73'224" E 102^{o}34'208"	Tree 5	Nangka
		N 03^{o}73'248" E 102^{o}34'196"	Tree 6	Nangka
		N 03^{o}73'252" E 102^{o}34'189"	Tree 7	Nangka

Continued on next page

Table 3.12, Continued,

		N 03º73’244” E 102º34’178”	Tree 8	Nipah
		N 03º73’241” E 102º34’171”	Tree 9	Nipah
		N 03º73’239” E 102º34’184”	Tree 10	Nipah
		N 03º73’231” E 102º34’185”	Tree 11	Nipah
• Commercial	• Parit Serong, Selangor	N 03º43’126” E 101º21’678”	Tree 12	Berangan
		N 03º43’106” E 101º21’670”	Tree 13	Berangan
		N 03º43’111” E 101º21’676”	Tree 14	Berangan
		N 03º43’127” E 101º21’665”	Tree 15	Nipah
		N 03º43’124” E 101º21’678”	Tree 16	Nipah
		N 03º43’148” E 101º21’666”	Tree 17	NIpah
• Orang Asli	• Kampung Pian, Pahang	N 03º47’294” E 102º14’137”	Tree 18	Nipah
		N 03º47’299” E 102º14’144”	Tree 19	Nipah
		N 03º47’312” E 102º14’144”	Tree 20	Nipah
		N 03º47’304” E 102º14’133”	Tree 21	Nipah
• Commercial	• Mersing, Johor	N 02º25’479” E 103º39’458”	Tree 22	Nipah
		N 02º25’472” E 103º39’452”	Tree 23	Nipah
		N 02º25’465” E 103º39’436”	Tree 24	Nipah
		N 02º25’461” E 103º39’426”	Tree 25	Nipah

3.3 Soil moisture analysis

Three grams of soil samples were dried in the oven at 60ºC and weighed daily until a constant weight was obtained. The soil’s weight before drying was subtracted with the final constant weight to give an overall idea about the soil’s moisture content in each soil. An average percentage of moisture content was obtained from each tree.

3.4 Soil pH analysis

Twenty grams of each soil samples were mixed with 40 mL of deionized water. Soil pH was measured using pH meter (Eutech Instrument pH510). The calibration of the instrument was performed prior to sample analyses by using pH 4 (phthalate), pH 10 (borate) and finally pH 7 (phosphate).

3.5 Extraction of soil DNA

The total DNA from the soil samples were extracted using the Soil DNA Extraction Kit (MoBio Inc., Solana, NA**)** according to the manufacture's instructions and stored at -20^{o}C for future use. The extracted DNA was quantified using a biophotometer (Eppendorf AG, NA).

3.6 PCR amplification of 16S rRNA gene region

PCR amplification of the 16S rRNA gene was carried out by using the universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') to generate amplicons of ~1,500 bp. Gradient PCR was performed to arrive at the optimum temperature needed for the best result of the primer's product. A total volume of 50 µL PCR mixture was prepared by adding 18.1 µL of master mix and 31.9 µL of diluted DNA template. The master mix for one reaction was prepared as per the following:

Table 3.13: Preparation of master mix for PCR amplification

Material	Volume
• Buffer (X5)	10.0 µL
• $MgCl_2$ (25 mM)	3.0 µL
• Forward primer, 27F (5 µM)	2.0 µL
• Reverse primer, 1492R (5 µM)	2.0 µL
• dNTP mixture (10 mM)	1.0 µL
• *Taq* polymerase (5 U)	0.1 µL
Total	18.1 µL

Dilution of DNA template:

Table 3.14: Preparation of diluted DNA template

Material	Volume
• Template DNA	1.0 µL
• Sterile distilled water, dH_2O	30.9 µL
Total	31.9 µL

The PCR program of PCR amplification was optimized and set by adjusting the temperature and time. Firstly, the template DNA was denatured at 95°C for five minutes. This was followed by 30 cycles of initial, annealing and extension processes. Initial process includes denaturing the DNA template at 94°C for 45 seconds. Annealing of the oligonucleotide primers was performed at 52.5°C for 30 seconds and extension of the new DNA at 72°C for 90 seconds. After 30 cycles of these three processes, a final extension was made at 72°C for 10 minutes before putting the PCR product in storage at 4°C. The amplification products were checked on the agarose gels.

3.7 PCR purification

Purification of pooled PCR product of 16S rRNA gene fragment amplification from the same banana variety in each respective site was made by using the MEGAquick-spin™ PCR & Agarose Gel DNA Extraction Kit (iNtRON Biotechnology, Korea). Firstly, one volume of PCR product was added with five volume of lysing buffer. The mixture was then vortexed and short spun before transferring a maximum of 800 µL mixture onto the spin column. After centrifuged at 13,000 rpm for one minute, the supernatant was discarded. Seven hundred microlitre of washing buffer was added to the spin column and centrifuged again at 13,000 rpm for one minute. The supernatant was discarded and the column was spun to dry. The spin column was then transferred to a new 1.5 mL tube. Thirty microlitre of elution buffer was added to centre of the column's membrane and incubated at room temperature for one minute, followed by centrifuged at 13,000 rpm for one minute. After centrifugation, the column was discarded. The purity of the purified product was checked by agarose gel electrophoresis and measurement of optical density (OD) at the wavelength of 260 nm was carried out by a biophotometer (Eppendorf, North America). The purified PCR product was used for ligation. The remaining purified PCR product was kept in -20°C for future usage.

3.8 Ligation

The ligation kit used was pGEM®T Easy Vectors System (Promega, USA) which contained four reagents, including 2X rapid ligation buffer, pGEM-T easy vector (50ng), a T4 DNA ligase (3 Weiss units/µL) and a control insert.

The ligation process was done by preparing a master mix, followed by the addition of a purified PCR product. The volume of each reagent used in preparing the master mix for each reaction was as follows:

Table 3.15: Preparation of master mix for ligation

Material	Volume
• 2X rapid ligation buffer	5.0 µL
• pGEM-T easy vector (50 ng)	1.0 µL
• T4 DNA ligase (3 Weiss units/ µL)	1.0 µL
Total	7.0 µL

For standard reaction, 3 µL of purified PCR product was added to 7 µL of the master mix by pipetting to give a total volume of 10 µL ligation product. For positive control, 1 µL of sterile distilled water was firstly put into the tube, followed by adding 7 µL of master mix and finally inserting 2 µL of control to give a final total volume of 10 µL ligation product. The ligation product was then incubated for 16 hours at 4°C before it was used for transformation process.

3.9 Preparation of competent cells (TOP10 *E. coli*) using $CaCl_2$

The TOP10 *E. coli* (Invitrogen, U.S.A.) was grown in 50 mL LB at 37°C, with shaking at 190 rpm for 16 hours. Then, 200 µL of the culture was transferred to 50 mL fresh LB broth and incubated at 37°C, with shaking at 190 rpm for two hours. By using the biophotometer (OD_{600}), the optimum cells' growths were checked (best at ~0.5) at the concentration of 4.0 x 10^8 cells/mL. The procedure was continued by putting 1 mL of sub-cultured *E. coli* into a sterile 1.5 mL tube and was kept in ice for 10 minutes. The tube was then centrifuged at 4° C for two minutes at 3660 rpm. The supernatant was pipetted out without disturbing the pellet. One mililitre of cold 0.1 M calcium chloride solution, ($CaCl_2$) was added into the tube, followed by re-suspending it with pellet and

kept on ice for 30 minutes before centrifuging it again at 4ºC for one minute at 3660 rpm. The supernatant was pipetted out again and resuspend in the pellet 500 µL of cold 0.1 M $CaCl_2$ solution. The resuspended pellet in the microcentrifuge tube was put on ice for five minutes, and was ready to be transformed.

3.10 Construction of clone libraries

The PCR product was ligated to a pGEM-T easy vector. The ligation process was carried out overnight in the chiller at 4ºC. Two hundred microlitre of competent *E. coli* cells was added with 8 µL of ligation reaction mixture. For positive control, 2 µL of pUC19, one type of plasmid cloning vector was added to 200 µL of competent cells. Meanwhile, for negative control, only 2 µL of distilled water was added to the competent cells. Tubes were placed in the ice for half an hour before proceeding to heat shock by putting the tube in 42ºC water bath for 90 seconds to allow the transformation process. After the transformation process, the tubes were put on ice for two minutes. Two hundred and fifty microlitre of SOC medium was added into the tube and incubated at 37°C for 1.5 hours, with shaking at 150 rpm. Once done, 100 µL of the incubated standard transformation product would be spread and plated on the media agar, LBA, contained with Ampicillin, X-Gal and IPTG. The pUC19 was transformed with competent cells earlier served as a positive control to show successful completion of the transformation of plasmid into the competent cell. Meanwhile, distilled water served as a negative control. Thirty microlitre from both positive and negative controls were taken separately and spread separately onto LBA plates containing Ampicillin, X-Gal and IPTG. Finally, the transformed cells were incubated at 37ºC, with shaking at 190rpm for 16 hours. The plates were then kept at 4°C and prepared for colony PCR.

3.11 Colony picking and colony PCR

The agar plate which has the suitable amount of colonies, about 100 to 300 colonies, with an optimal percentage of 90% of white colonies (plamids with inserts) was chosen for colony picking by using the sterile pipette tips (John, 1997). Single colonies from the incubated LBA plates were picked to grow in LB at 37°C for 16 hours, with shaking at 180 rpm, following which the LB cultures were used as template for colony PCR. A total volume of 25 µL PCR mixture was prepared by adding 15 µL of master mix and 10 µL of diluted DNA template. The master mix of PCR mixture for one reaction was prepared as follows:

Table 3.16: Preparation of master mix for colony PCR

Material	Volume
• Sterile distilled water, dH_2O	7.15 µL
• Buffer (X10)	2.50 µL
• $MgCl_2$ (50 mM)	0.75 µL
• Forward primer, T7 (5 µM)	2.00 µL
• Reverse primer, SP6 (5 µM)	2.00 µL
• dNTP mixture (10 mM)	0.30 µL
• *Taq* polymerase (5 U)	0.30 µL
Total	15.00 µL

Dilution of template:

Table 3.17: Preparation of diluted template

Material	Volume
• Template	2 µL
• Sterile distilled water. dH_2O	8 µL
Total	10 µL

The primers (T7 and SP6) were used because the DNA template was expected to be ligated between T7 and SP6 transcription site of pGEM-T easy vector (Promega, USA). As usual, gradient PCR was performed to optimize the optimum temperature needed for best result of that primer's product. The PCR program for gene amplification was set by

adjusting the temperature and time. Firstly, the template DNA was denatured at 95°C for 10 minutes, followed by 30 cycles of initial, annealing and extension processes. The initial process includes denaturing the DNA template at 94°C for 30 seconds. Annealing of the oligonucleotide primers was performed at 47.3°C for 30 seconds and the extension of new DNA was made at 72°C for two minutes. After 30 cycles of these three processes, the final extension was made at 72°C for 10 minutes before the PCR product was kept at 4°C. The amplification products (~1,700 bp) were checked on the agarose gels.

3.12 Restriction fragment length polymorphism (RFLP)

The amplicons products (~1700bp) from the T7 and SP6 PCR were subjected to RFLP and were digested using the *Msp*I (*Hpa*II) restriction enzymes (Thermo Scientific, USA). The resultant restriction fragments of each colony PCR product indicate each type of species. RFLP was prepared by adding reagents and template as follows:

Table 3.18: Preparation for restriction fragment length polymorphism

Material	Volume
• 10X buffer Tango	2 µL
• Colony PCR product	17 µL
• Restriction enzyme (10 U/ µL)	1 µL
Total	20 µL

All mixtures in tubes were then mixed gently by pipetting, followed by short spins. They were then incubated at 37° C for four hours and inactivated at 80° C for 20 minutes by using a thermal cycler. Different band patterns were obtained from each sample as the products of RFLP. Similar patterns that were observed would be grouped together, acknowledging them as a group of the same species on the assumption that each different pattern of RFLP represents one bacterial species. A representative clone from each RFLP

pattern group was selected and cultured in 20 mL of fresh LB broth at 190 rpm and 37^{o} C for 16 hours.

3.13 Plasmid extraction

This process included the use of DNA-spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Korea). Twenty microlitre of each LB culture that was previously prepared as template for colony PCR was added into a tube containing 20 mL of LB. The culture was then incubated for 16 hours, with shaking at 180 rpm. After incubation, 1 mL of culture was taken to check for the reading of OD_{600}. The reading should be between 1.0 and 1.5, which was the preferable growth condition of plasmid culture, suitable for plasmid extraction.

Five mililitre of bacterial culture was harvested by centrifugation at 13,000 rpm for 30 seconds at room temperature. The supernatant was discarded and the pellet was resuspended in 250 µL of resuspension buffer. The mixture was pipetted gently until there were no clumps to ensure high lysis efficiency. Two hundred and fifty microlitre of lysis buffer was added to the resuspended cells. The tube was closed and the mixture was gently mixed by inverting the tube. Three hundred and fifty microlitre of neutralization buffer was added and the mixture was again gently mixed by inverting the tube several times, following which the mixture was allowed to be reacted at room temperature for five minutes.

The mixture was centrifuged at 13,000 rpm for 10 minutes at 4°C and the resulting supernatant was promptly transferred into a column. The unwanted pellet formed was made up of cell debris, protein and genomic DNA. The supernatant was again centrifuged at 13,000 rpm for one minute. The column was then removed and the filtrate was discarded. Five hundred microlitre of washing buffer was added into the column and the

mixture was centrifuged in a similar way. The filtrate was then discarded. This step was necessary to remove trace nuclease activity which degrade plasmid. The column was further centrifuged at 13,000 rpm for one minute to dry the filter membrane in order to remove ethanol completely.

The dried column was then put into a clear and sterile centrifuge tube. Fifty microlitre of elution buffer was added to the upper reservoir. It was left to stand for one and a half minutes before being centrifuged at 13,000 rpm for one minute. The DNA concentration of extracted plasmids (≥80ng/μL) was measured by using a biophotometer. The end product was then kept in -20°C until sequencing and phylogenetic analysis.

3.14 Sequencing and phylogenetic analysis

Sequencing of extracted plasmids was done by sending the extracted plasmids to 1st Base Sequencing Laboratories (Selangor, Malaysia) for single pass sequencing using the primers T7 promoter. The sequences were analysed and compared to the GenBank database at the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov). The information on closest match, accession number, percentage of identity, and isolation source of each sequence analysed was recorded and tabulated. The 16S rRNA gene sequences showing 97% or higher similarity would be considered to be under the same phylotype. Sequences that had 70% or lower similarity were considered “unclassified” and listed as unknown genus and species.

3.15 Statistical and phylogenetic analysis of the 16S rDNA clone sequences

Surakasi *et al*. (2010) and Suyal *et al*. (2015) reported that the phylogenetic analysis of the clone sequences was carried out by assigning all the sequences to an operational taxonomic unit using distance-based operational taxonomic units (OTU). The diversity of OTU was further examined by using paleontological statistics (PAST) analysis (Cetecioglu *et al*., 2009). Shannon-Wiener diversity index was used to calculate Shannon index (H0), evenness and the Simpson's index (D).

3.16 Soil chemical analysis

Total nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and sulphur (S) in the soil were analysed at the Malaysian Agricultural Research and Development Institute (MARDI), Serdang. Organochlorine and organophosphorus in the soil were also analysed in MARDI as indications of pesticides. Analysis of total nitrogen was analysed by using Kjedahl method. Briefly, the Kjedahl procedure involved three major steps which were digestion, distillation and titration. In digestion step, the nitrogen bonds in the soil samples were broke down and converted all the bonded nitrogen into ammonium ions (NH_4^+). Through this step, the sample is mixed with sulfuric acid at temperature between 350^{o}C to 380^{o}C. Once complete, the samples were allowed to cool to room temperature then diluted with water and proceed to distillation step. During distillation step, NH_4^+ were converted into ammonia (NH_3) by adding sodium hydroxide (NaOH). The absorbing solution (aquous boric acid) was then added to capture dissolved ammonia gas forming solvated ammonium ions. Finally, the acid based titration was performed to determine the concentration of the captured ammonium ions.

To detect the present of N, P, K, Ca, Mg, Na and S, continuous flow analyser (SKALAR) was used. The samples were injected into a flowing carrier solution. Along the way, the samples were mixed with specified reagent which gave a colour and determine the sample concentration. CHNS/O analyser (PERKIN ELMER Series II 2400) was also used to double checked the present of N and S. The soil samples were first freeze and grind until homogeneous before loading into the ready prepared CHNS/O analyser to be analysed. These 2 analysers were programmed and analysed in a convienient method.

CHAPTER 4 : RESULTS

4.1 16S rRNA gene amplification

All samples were successfully amplified with the length of 1,500 bp on an ethidium bromide stained 2% (w/v) agarose gel (Figure 4.1).

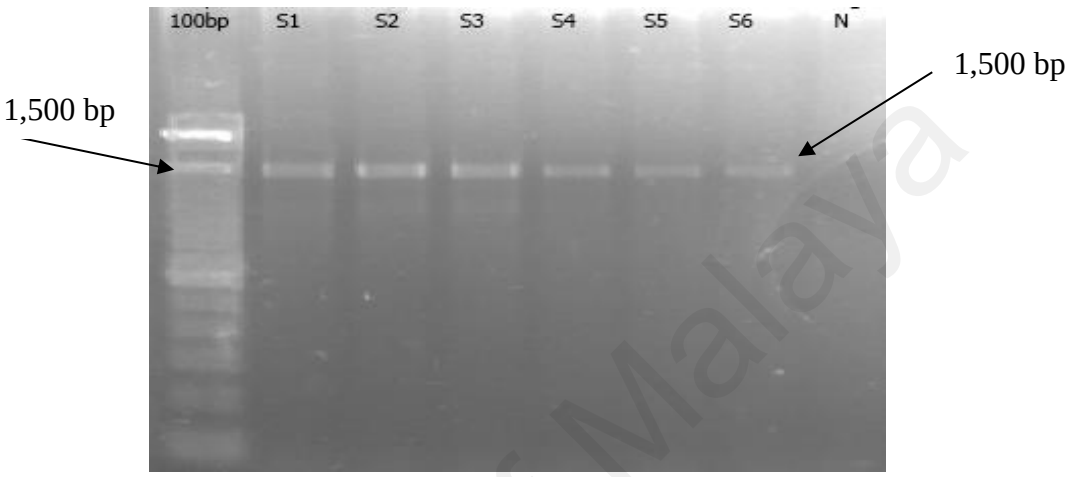

Figure 4.1: Ethidium bromide stained 2% (W/V) agarose gel of 16S rRNA gene PCR products of Pisang Siam (Paya Mendoi) rhizosphere

Lane 100bp: 100bp DNA ladder (Invitrogen, USA)

Lane S1 to S6: 16S rRNA gene PCR products from genomic DNA extracted from the Pisang Siam (Paya Mendoi) rhizosphere

Lane N: Negative control

4.2 PCR purification

The PCR products (amplicons) of 16S rRNA gene from the rhizosphere of the same banana variety and same location were pooled into one unit, followed by the PCR purification process to remove buffers, primer dimers, smearing and other contaminants which will affect the cloning process. The purified products consisted of DNA concentrations of more than 50 ng/µL. Figure 4.2 shows an example reference of a successfully purified PCR product.

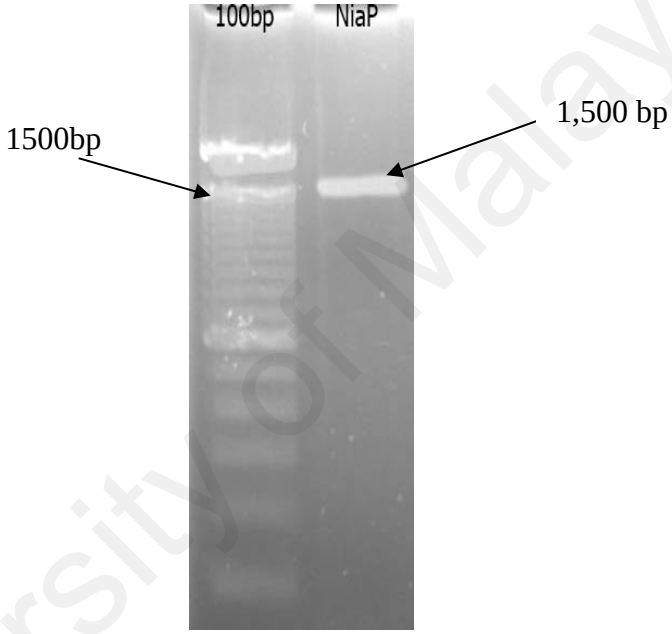

Figure 4.2: Ethidium bromide stained 2% (W/V) agarose gel of pooled purified PCR products of Pisang Nipah (Paya Mendoi) rhizosphere

Lane 100bp: 100bp DNA ladder (Invitrogen, U.S.A.)

Lane NiaP: Purified 16S rRNA gene PCR product of Pisang Nipah (Paya Mendoi) rhizosphere

4.3 Colony PCR

The white colonies obtained from cloning were picked and proceeded with colony PCR using T7 and SP6 primers. In order to achieve more than 100 positively DNA inserted colony PCR products to proceed with restriction fragment length polymorphisms (RFLP). In this study, 200 white colonies were picked at random for colony PCR. The colony PCR product with positive and correct DNA insertion was expected to have a band at molecular size of around 1,700 bp as shown in Figure 4.3.

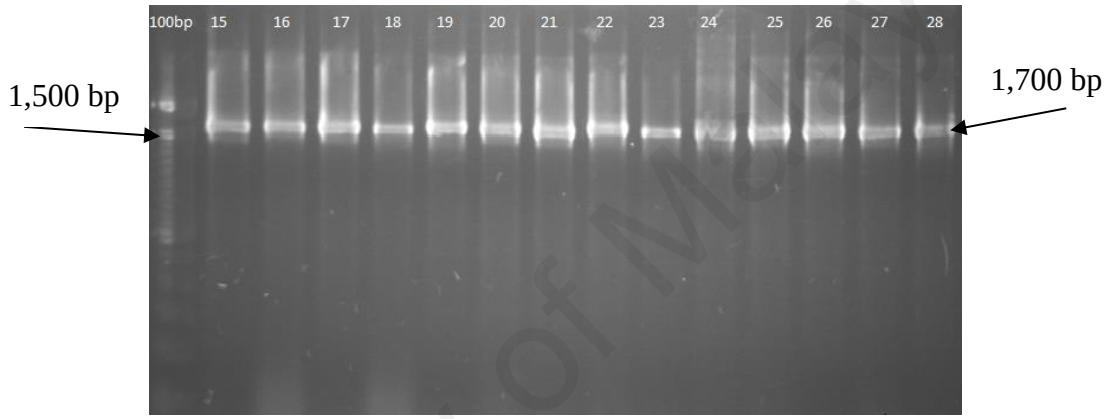

Figure 4.3: Ethidium bromide stained 2% (W/V) agarose gel of colony PCR products of Pisang Siam (Paya Mendoi) rhizosphere

Lane 100bp: 100 bp DNA ladder (Invitrogen, U.S.A.)

Lane 15 to 28: Colonies number 15 to 28

4.4 Restriction fragment length polymorphism (RFLP)

A number of colony PCR products with correct DNA insertion shown in Table 4.1 were subjected to RFLP. Each of the colony PCR products was digested with *Msp*I (*Hpa*II) (Thermo Scientific, USA) restriction enzyme. Some of them showed similar RFLP patterns while others exhibited different patterns as Figure 4.4 attached. One representative of each different RFLP pattern was chosen to proceed with plasmid extraction. The overall idea here was to identify the bacteria which had different RFLP patterns.

Table 4.1: Number of positive clones with correct DNA insert

	Orang Asli Field				Commercial Plantation		
	Paya Mendoi, Pahang			Kampung Pian	Parit Serong		Mersing
	Pisang Siam	Pisang Nangka	Pisang Nipah	Pisang Nipah	Pisang Berangan	Pisang Nipah	Pisang Nipah
Positive clones	129	123	117	165	133	167	135

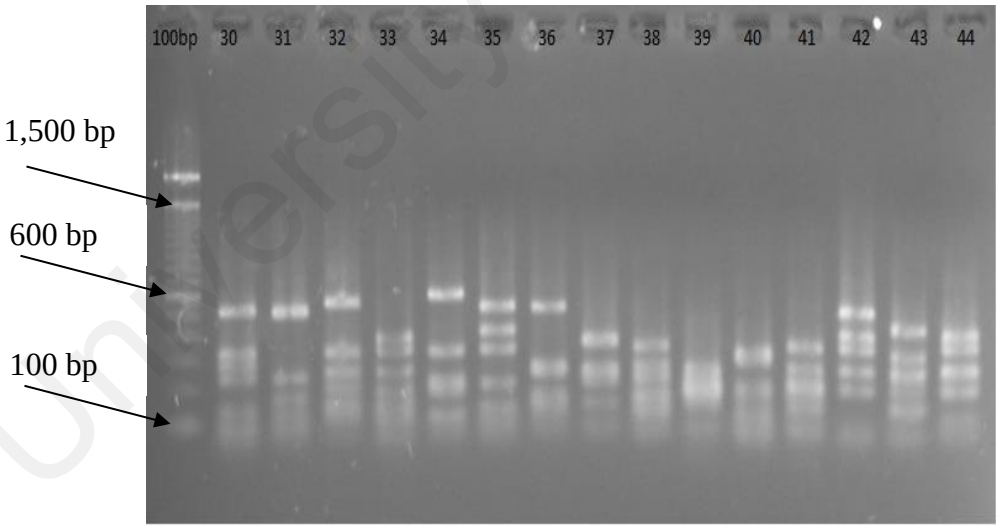

Figure 4.4: Ethidium bromide stained 2% (W/V) agarose gel of RFLP patterns of Pisang Siam (Paya Mendoi) rhizosphere

Lane 100bp: 100 bp DNA ladder (Invitrogen, U.S.A.)

Lane 30 to 44: RFLP patterns of clones number 30 to 44

4.5 Sequencing and phylogenetic analysis

The sequences obtained were compared to the GenBank database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program. The results were depicted in Figure 4.5 where it could be observed that there were both similarities and differences in bacterial composition between the banana rhizosphere in the OA Settlements and the commercial plantations. In this study, only the bacterial groups with 97% or higher homology were documented.

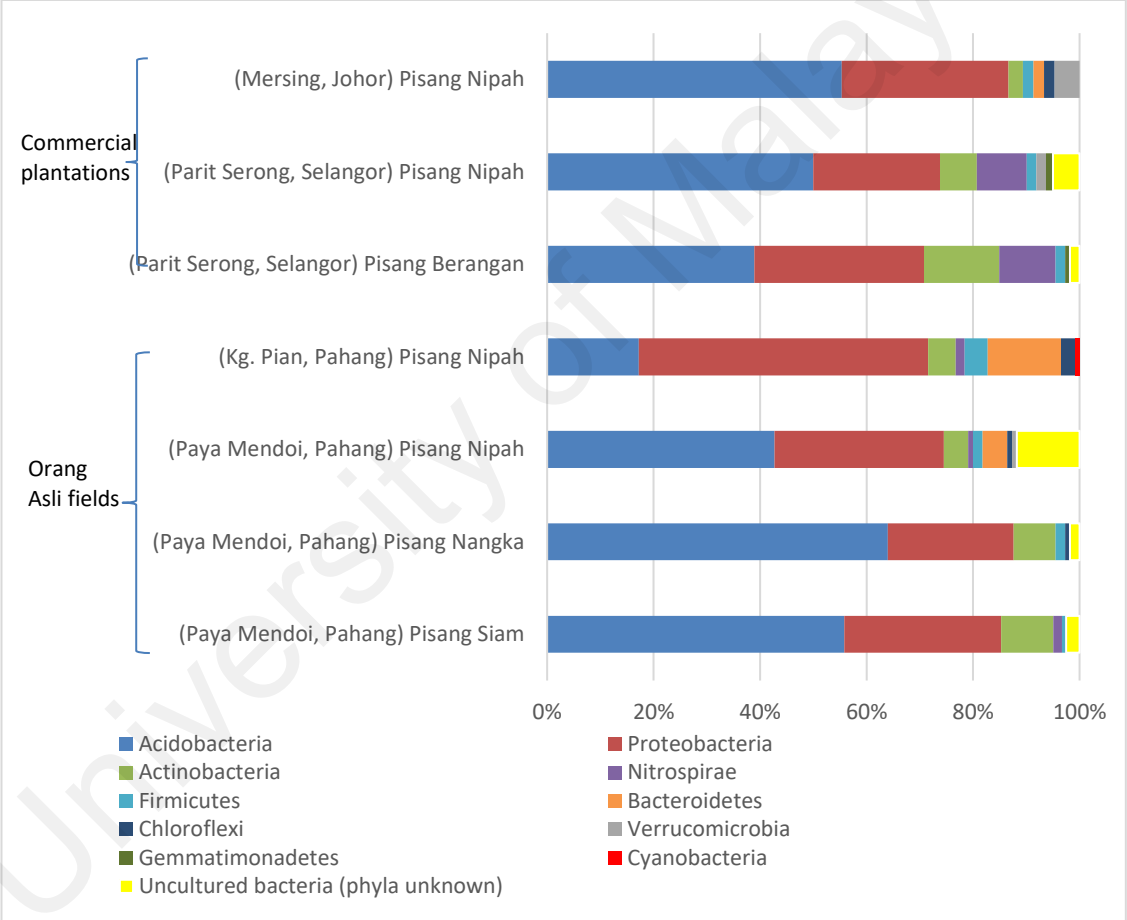

Figure 4.5: Banana rhizosphere bacterial phyla in OA fields (Kg. Pian and Paya Mendoi) and commercial plantations (Mersing and Parit Serong)

Phylogenetic analyses revealed that the rhizosphere 16S rDNA clones were classified as *Acidobacteria*, *Proteobacteria*, *Actinobacteria*, *Nitrospirae*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Gemmatimonadetes*, *Cyanobacteria* and

uncultured bacteria (phyla unknown) (see Figure 4.5). Members of *Acidobacteria* represented the largest group in the OA field in Paya Mendoi, accounting for 55.8%, 64.0% and 42.7% of the clone library for Pisang Siam, Pisang Nangka and Pisang Nipah rhizosphere respectively. *Acidobacteria* constituted 38.9% and 50% respectively of the clone library for Pisang Berangan and Pisang Nipah rhizosphere in the commerical plantation in Parit Serong, and 55.33% of the clone library in the rhizosphere of Pisang Nipah in the commercial plantation in Mersing. However, *Acidobacteria* is much less abundant in the OA field in Kampung Pian where it accounted for only 17.24% of the total clone library. Instead, members of *Proteobacteria* had a much stronger dominance, accounting for 54.31% of the clone libarary there but a smaller presence ranging from 23.6% to 31.9% in the OA field in Paya Mendoi and the commercial plantation in Parit Serong and Mersing.

The banana rhizosphere bacterial community from the result in Figure 4.5 were then further classified and categorized under different genera as shown in Table 4.2. This was to give a better understanding about certain bacteria which were found in certain soils. As well as, giving better idea about understanding whether there are similarities or differences in bacterial community and some chemical properties in the rhizosphere of banana plants in the OA fields and the commercial plantations. The Table 4.2 shows, in descending order, the three largest bacterial phyla occurring in both the OA fields and the commercial plantations: *Acidobacteria*, *Proteobacteria* and *Actinobacteria*. The phylum *Firmicutes* could be found in all of the soils but the phyla *Nitrospirae, Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Gemmatimonadetes* and *Cyanobacteria* appeared to be randomly present.

Table 4.2: Banana rhizosphere bacterial community in the Orang Asli settlements and the commercial plantations based on the 16S sequences matched with GenBank database

	Orang Asli Fields				Commercial Plantations		
Locations	Paya Mendoi			Kampung Pian	Parit Serong		Mersing
Banana variety	Pisang Siam	Pisang Nangka	Pisang Nipah	Pisang Nipah	Pisang Berangan	Pisang Nipah	Pisang Nipah
Bacterial phyla and genera							
Acidobacteria	+	+	+	+	+	+	+
Candidatus Solibacter	+	+	+		+	+	
Proteobacteria	+	+	+	+	+	+	+
Burkholderia	+	+	+			+	
Bradyrhizobium	+	+	+	+			
Dyella	+		+				
Novosphingobium	+		+				
Cupriavidus	+	+					
Variovorax		+					
Desulfoglaeba						+	
Xanthomonas			+	+			
Ralstonia			+				
Steroidobacter				+	+	+	+
Castellaniella				+			
Dokdonella				+			
Bordetella				+			
Frateuria				+			
Nevskia				+			
Pseudoxanthomonas				+			
Psudolabrys							+
Geobacter							+
Phenylobacterium							+
Actinobacteria	+	+	+	+	+	+	+
Ferrimicrobium	+	+					
Micromonosporaceae			+				
Microbacterium				+			
Nitrospirae	+		+	+	+	+	
Nitrospira			+	+	+	+	
Firmicutes			+	+	+	+	+
Bacillus	+	+	+	+			
Sporocarcina				+			
Oceanobacillus							+
Bacteroidetes			+	+			+
Chryseobacterium			+				
Flavisolibacter				+			
Chloroflexi		+	+	+			+
Verrucomicrobia			+			+	+
Gemmatimonadetes					+	+	
Cyanobacteria				+			
Uncultured bacteria (phyla unknown)	+	+	+		+	+	

* The bacterial phyla are denoted in **bold**; the indented non-bold letters denote bacterial genus.

4.6 Paleotological Statistics (PAST)

Populate with the numbers from the table below.

Table 4.3: Comparative diversity analysis using Paleontological Statistics (PAST)

	Orang Asli Field				Commercial Plantation		
Diversity Indices	Paya Mendoi, Pahang			Kampung Pian	Parit Serong		Mersing
	Pisang Siam	Pisang Nangka	Pisang Nipah	Pisang Nipah	Pisang Berangan	Pisang Nipah	Pisang Nipah
Individuals	129	123	117	165	133	167	135
Shannon_H	3.570	3.751	3.876	3.312	2.853	3.285	2.985
Menhinick	3.962	4.959	5.455	3.192	2.601	3.560	2.668
Margalef	9.054	11.220	12.180	7.834	5.930	8.793	6.116
Berger-Parker	0.124	0.149	0.103	0.206	0.113	0.102	0.096
Simpson_I_D	0.967	0.971	0.975	0.951	0.907	0.929	0.925

4.7 Abiotic analysis results

In Figures 4.6 and 4.7, the abiotic analysis (moisture content and pH) for each soil of the OA and commercial banana trees was recorded and done with four replicates each. It could be observed that there were high moisture contents in the soil of the commercial plantation in Parit Serong. From here, it was concluded that the overall pH level was slightly acidic in both the OA and commercial plantation soils.

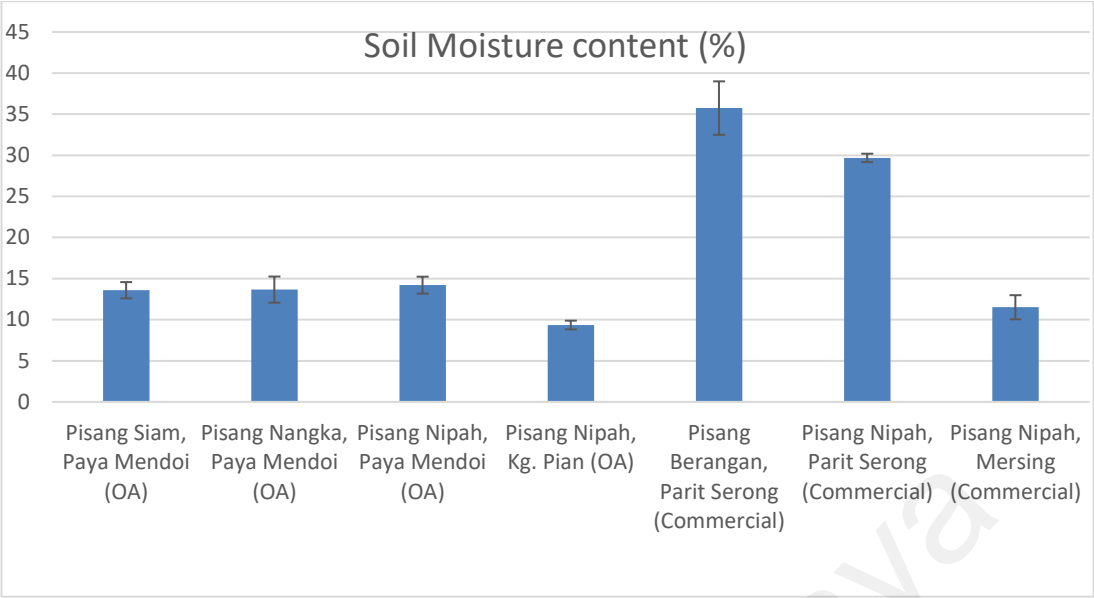

Figure 4.6: Average percentage moisture content for each soil in the Orang Asli settlements and the commercial plantations*

* All data in this work were obtained with four replicates each.

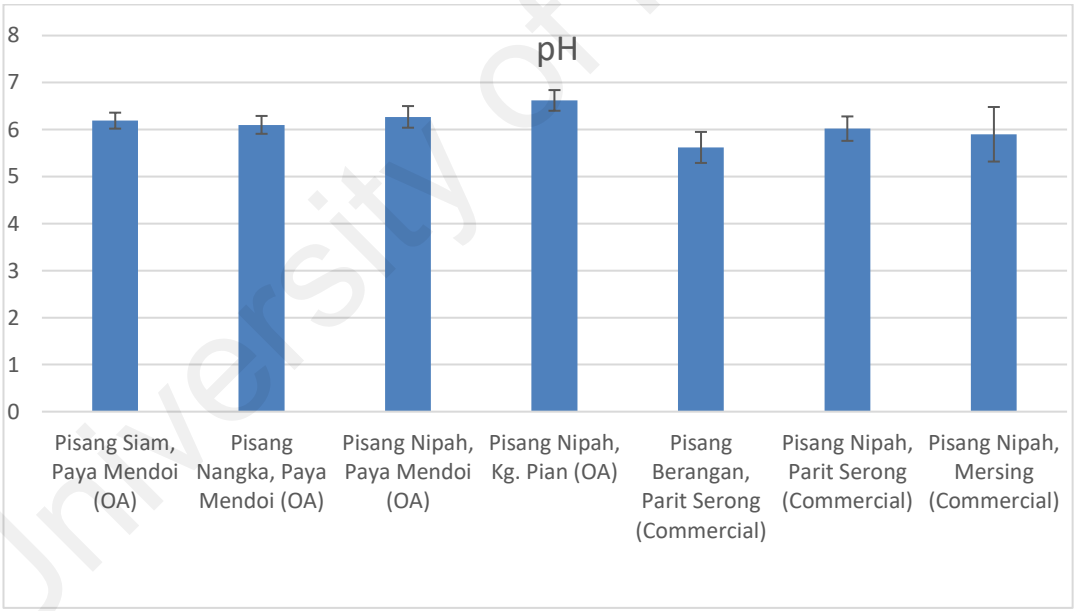

Figure 4.7: Average pH for each soil in the Orang Asli settlements and the commercial plantations*

* All data in this work were obtained with four replicates each.

In Figure 4.8, all soils samples from both the OA fields and the commercial plantations were analysed at the Malaysian Agricultural Research and Development Institute (MARDI), Serdang to test for total nitrogen (N), phosphorus (P), potassium (K),

calcium (Ca), magnesium (Mg), sodium (Na) and sulphur (S) in the soil. From the result, the rhizosphere in the commercial plantation in Parit Serong had higher N content compared to the OA banana fields, meanwhile, the rest of the elemental contents of rhizosphere samples were likely to be averagely about the same.

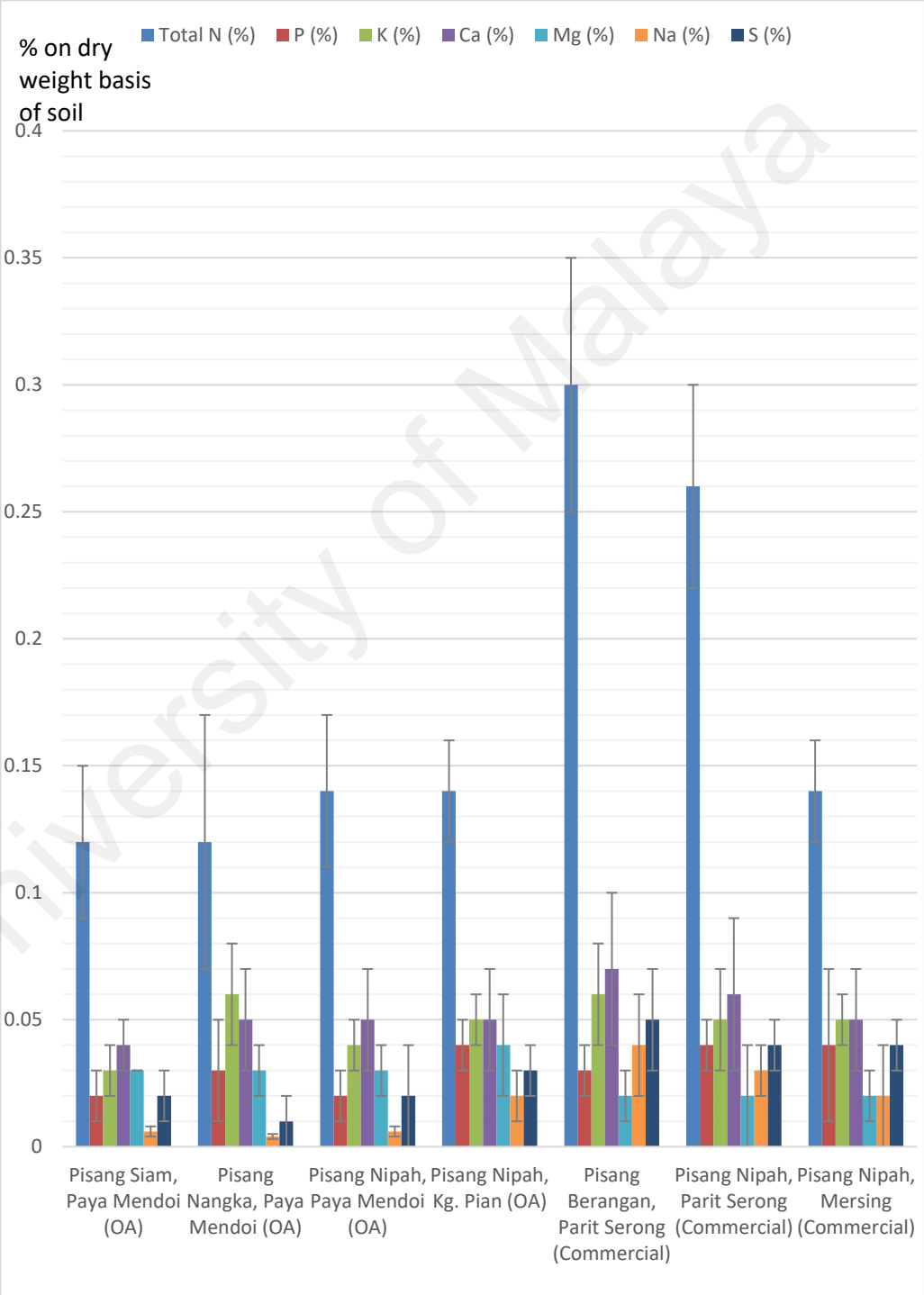

Figure 4.8: Elemental contents of rhizosphere samples from the Orang Asli settlements and the commercial plantations

CHAPTER 5 : DISCUSSION

5.1 16S cloning profiles

The 16S rRNA gene fragments were successfully amplified from the banana rhizosphere samples of both the OA settlements and the commercial plantations. The samples were also differentiated by RFLP. Clones having different RFLP patterns for the 16S fragment were sequenced and matched with the GenBank database. Similarities and differences in bacterial composition were observed between the OA's and the commercial plantations' banana rhizosphere. In this study, only bacterial groups with 97% or higher homology were documented.

These two largest phylum, *Acidobacteria* and *Proteobacteria*, had been documented in the literature as the most common and abundantly distributed bacterial groups in the environment (Faoro *et al.*, 2010). Members of *Acidobacteria* are involved in nitrate and nitrite reductions (Aislabie & Deslippe, 2013). A wide variety of *Proteobacteria* have been found to be able to degrade soluble organic molecules such as organic acids, amino acids and sugars (Eilers *et al.*, 2012). Some members of *Proteobacteria* can also aid the denitrifying process of the nitrogen cycle (Aislabie & Deslippe, 2013).

Actinobacteria was found in similar proportions in all the OA fields and commercial plantations. Like *Proteobacteria* and *Acidobacteria*, *Actinobacteria* is a phylum that is widespread and often abundant in soil (Aislabie & Deslippe, 2013). This phylum has antimicrobial compound and is capable of degrading soluble organic molecules (Aislabie & Deslippe, 2013).

The OA fields in Kampung Pian and Paya Mendoi had higher proportion of *Bacteroidetes* (between 4.6% and 13.79%) than the commercial plantations in Mersing

and Parit Serong where *Bacteroidetes* represented 0 to 2% of bacteria counts. *Bacteroidetes* are mainly involved in the degradation of complex organic molecules such as protein, starch, cellulose and chitin (Aislabie & Deslippe, 2013). This is the reason why OA fields which contained more organic matters lead to higher propotion of *Bacteroidetes* (Koyama *et al.*, 2014). Meanwhile, the proportion of *Nitrospirae* was higher in the soil of the commercial plantation in Parit Serong (9.4% in Pisang Nipah rhizosphere and 10.6% in Pisang Berangan rhizosphere) compared to the OA soils in Kampung Pian and Paya Mendoi (0% to 1.72%). *Nitrospirae* plays in an important role in the nitrogen cycle. Oxidation of ammonia to nitrite and then to nitrate was facilitated by *Nitrospirae* members (Philippot *et al*., 2007). The commercial farmers who practiced fertiliser on the soil might be the reason why there were higher *Nitrospirae* in the commercial plantations than the OA fields. This study also found a higher proportion of *Verrucomicrobia* in the commercial soil relative to the OA soil. However, the roles of *Verrucomicrobia* have not been well understood; there was very little knowledge on the role of *Verrucomicrobia*, a fermentative anaerobe, played in the fermentation of plant polysaccharides (van Passel *et al*., 2011).

The soil bacteria belonging to the phyla *Firmicutes*, *Gemmatimonadetes* and *Chloroflexi* do still occupied a certain position in soil. *Firmicutes* were found in the OA fields and the commercial plantations. *Firmicutes* were endospore forming and lactic acid bacteria capable of degrading different carbon sources including plant polysaccharides. Some genera of *Firmicutes* are fermentative and have nitrogen fixing characteristic (Aislabie & Deslippe, 2013). *Gemmatimonadetes*, classified as aerobic heterotrophs, have been found to be adapted to low soil moisture conditions (DeBruyn *et al*., 2011). Members of the genus *Gemmatimonas* were reported to be abundant in soil (Aislabie & Deslippe, 2013). In this study, *Gemmatimonadetes* were found only in the commercial plantation in Parit Serong. On the other hand, *Chloroflexi* were found in both the OA fields (Paya

Mendoi and Kg. Pian) and in the commercial plantation in Mersing. *Chloroflexi* was reported to be abundant in soils but not easily obtained from culture-dependent experiments. They are aerobic heterotrophs and had been isolated on oligotrophic media in the form of slow-growing mini colonies (Davis *et al*., 2011). To date, there is very little evidence and information on *Chloroflexi*. A small proportion of *Cyanobacteria* was found in the OA field in Kampung Pian. *Cyanobacteria* plays an important role in mutual symbiosis with fungi to form lichens which can contribute to carbon fixation in the ecosystem (Aislabie & Deslippe, 2013).

In the Paya Mendoi (OA field) and Parit Serong (commercial plantaions) clone libaries, there were uncultured bacteria clones that could not be classified into known bacterial divisions based on publicly available 16S rRNA gene sequence information and phylogenetic analyses. However, uncultured bacteria were not detected in the other OA field in Kg. Pian and the other commercial field in Mersing. Many of these clones formed bootstrap-supported clusters with each other and the GenBank member sequences were not closely related. It is interesting to note that the OA field registered a higher proportion of uncultured bacteria which could be attributed to unknown or unclassified bacteria. This might reflect a more natural bacterial community in the OA rhizosphere which had not been disturbed by commercial farming practices (e.g. addition of fertilisers and pesticides). Besides, the uncultured soil bacteria shown in Figure 4.5 were uncultured soil bacteria that do not have phyla group; the term "candidate phylum" is used to denote this group of bacteria. "Candidate phylum" refers to a 16S DNA sequence that does not appear to belong to any of the known bacterial phyla, implying that no cultures have yet existed to represent the group but they are descendents within the Bacteria domain. It is also possible that a "Candidate phylum" could be the results of a variety of artifacts (for example, Chimeric gene clones, PCR errors, sequencing errors) and methodological

errors including improper reference or outgroup taxon selection, inadequate quantity of sequence information, and improper alignment, etc (Rappe *et al*., 2003).

Results from Figure 4.5 and Table 4.2 indicate that the rhizosphere from the OA fields contained a higher diversity of bacterial phyla and genera compared to the soil from the commercial plantations, suggesting the commercial plantation soil might have exerted stronger selective pressure on the bacterial diversity.

However, the types of genera within each of these phylum varied between the soils. The presence of *Steroidobacter*, a steroid degrading bacteria (Wang *et al*., 2013), was found in the Parit Serong and Mersing commercial banana plantations but less so in the OA field in Kampung Pian. This steroid is abundant in nature through the urinary tract of mammals (Wang *et al.*, 2013) or perhaps waste dumps. This explained that the farmers in the commercial plantations which claimed to use both the synthetic fertilisers and organic fertiliser (femur) on the soils. Hence, this is why *Steroidobacter* can be found in certain side of the OA fields probably due to the present of naturally accumulated steroid from natural organic compound. Meanwhile, *Steroidobacter* is found in both the commercial plantations because of the addition of femur. It is interesting to note that the genus *Bradyrhizobium* could be found in the soils in all the OA fields but not in the commercial plantations. This nitrogen fixing bacteria is important in the nitrogen cycle (Hani *et al.,* 1998) and reflects a soil capable of generating nutrients biologically. The commercial plantations which apply fertilisers on a regular basis might have discouraged the growth of such nitrogen-fixing bacteria in the soil.

Moreover, there were some genera found in the OA's Pisang Nipah soil but not in other banana varieties in the OA fields or the commercial plantations. These include *Xanthomonas* and *Pseudoxanthomonas*, a known plant pathogen which causes severe diseases to the crop (Nadia *et al*., 2011), *Chryseobacterium,* an anti-microbial and

pathogenic soil bacteria (Kirby *et al*., 2004) and *Ralstonia*, a plant pathogenic bacteria which caused wilt disease (Meng, 2013) had all been detected in that particular soil (see Table 4.2). Variations in the rhizosphere bacterial communities might be due to spatial difference because the Pisang Nipah were planted away from the other two varieties in the OA field in Paya Mendoi or the presence of substances which inhibited the growth of pathogenic bacterial, or the presence of substances which encourage or support such bacteria. Besides, the absence of these plant pathogenic bacteria in the commercial plantation soils might be associated with the use of pesticides. The presumed selective pressure on the bacterial diversity in the commercial plantation soil (due to the use of fertiliser and pesticide) might also be a cause for the lower proportion of uncultured bacteria in those soil compared to the soils in the OA field soil. As well, a large number of uncultured bacteria clones had been detected, including uncultured *Proteobacteria*, uncultured *Acidobacteria* and uncultured *Actinobacteria*. This result was not uncommon as the amplified 16S sequences from environmental samples where the uncultured bacteria were found in abundance in nature (Brown, 2001). The uncultured bacteria were unidentified because their 16S DNA sequences did not match those of documented bacteria in the Genbank. Hence, it is difficult to speculate whether they can bring positive, negative or even no effects on the crop growth, yield and health.

Determination of OTU is one of the preferred methods currently available for comparing diversity from different clone libraries (Suyal *et al*., 2015). Based on the 16S rRNA gene clone libraries of all the sites, there were similar richness and diversity with slight differences (Table 4.3). But the PAST analysis proved that there were diversed in bacterial composition in the 16S rRNA gene clone libraries. It was also proven that the values of diversity indices indicated that there were more bacterial diversities in the OA's fields than the commercial plantations. In particular, Shannon_H value was higher in the Paya Mendoi and Kampung Pian libraries (3.570, 3.751, 3.876 and 3.312) than the Parit

Serong and Mersing commercial plantations (2.853, 3.285 and 2.985). The higher the index could be correlated by either by having more unique species, or by having greater species evenness (Shanon, 1948). These results were supported by the values of Margalef and Menhinick indices which estimate the species richness independently of the sample size (Magurran, 2004) where the index was shown to be so much higher in the OA fields than the commercial plantationa. Meanwhile, in the OA field in Kampung Pian, the values of Margalef and Menhinick indices were almost similar to the commercial plantations in Parit Serong but higher than both the commercial plantations in Parit Serong and Mersing. Besides, the value of Berger-Parker index was also higher in both the OA's fields. This index expresses the proportional importance of the most abundant species. Increase in the value of this index accompanies an increase in diversity and a reduction in dominance (Magurran, 2004). The Simpson index is used to measure diversity and homgeneity in the ecosystem. The values of the Simpson Index at or near zero indicates highly diverse or heterogeneous ecosystems. Conversely, the index of close to one indicates an almost perfectly homogeneous ecosystem (Simpson, 1949). Applying the Simpson Index to the study results, it was found that the Simpson Index values in all the sites were tending towards one (0.9668, 0.9709, 0.9752, 0.9511, 0.9068, 0.9290 and 0.9254).

Althought most of the amplicons gave bright successfully bands, however there were still faint bands appeared in the results. The intensity of amplicons reflects the quantitative abundance of species. Some amplicons were found to have relatively lower intensities, showing faint bands in ethidium bromide stained gel. This could be due to several reasons: a). the concentrations of the genomic DNAs extracted from the rhizosphere samples were relatively low, with the amplicons appearing to be less dense, indicating the absence of an abundance of species; b). the faint bands thus resulted might be due to technical errors; c). inadequate volumes of the two DNA templates pipetted out for amplification due to pipetting error, led to the usage of a lesser amount of DNA

templates, thereby resulting in less dense amplicons; d). the two DNA templates might not have been completely thawed before usage, resulting in non-uniformed distributions of genomic DNAs and faint amplicons.

5.2 Soil analysis

It could be observed that there were high moisture contents in the soil of the commercial plantation in Parit Serong. This was because the soil samples were collected there on a rainy day. Nevertheless, the high moisture did not affect the analysis nor did it show much difference in bacterial diversity compared to soils collected from the other locations. Soils from countries with a humid climate usually register a pH of between pH 6 and pH 7 (Brady *et al.*, 1999). The overall pH level was acidic in the commercial plantation soils than OA soils. The presence of a large amount of *Acidobacteria* in the commercial plantation (see Figure 4.5) was consistent with the pH result that *Acidobacteria* could contribute to a decline in the overall pH level (Lauber *et al.,* 2009). Soil pH is generally believed to be the best predictor of bacterial community composition and diversity (Rousk *et al.*, 2010).

The rhizosphere in all the commercial plantation in have higher N content compared to the OA banana fields (Figure 4.8). This finding due to the mix of both organic and inorganic fertilisers in the commercial plantations, as acknowledged by the farmers. Like most cash crops, banana plants require large amounts of N and K followed by P, Ca, Mg and Na to maintain high yields (Abdullah *et al*., 1999; Memon *et al*., 2010; Mia *et al*., 2010). Furthermore, the limitation in N uptake can be a problem for commercial cultivation of the banana plants such that the deficiency symptoms quickly develop and extra N must be frequently applied even on fertile soil (Robinson, 1996). To satisfy the plant demand for nutritional supply, it is essential to apply those elements to

the soil, most of which coming from inorganic chemical sources. This could be a reason why N content was high in the commercial plantation. This could also be a reason why bacterial diversity was higher in the OA banana fields since no fertiliser was added to the soil that might negatively affect the diversity, leaving the bacterial community undisturbed and thus having more clone library results in genus rank. The long term applications of fertilisers to the soil may affect the soil microbial diversity. Studies supporting this hypothesis reported that the number of certain numbers in the *Actinobacteria* and *Proteobacteria* groups declined due to their living on long-term fertilised soil (Li *et al*., 2014). This observation consistant with the results as shown in Figure 4.5 where there were less genera in the Proteobacteria group of the commercial plantations compared to those of the OA fields.

Eventhough the farmers claimed to add fertiliser in the commercial plantation soil in Mersing, but their nutritional values were similar to the levels in the two OA field soils. This might be due to the application of smaller amounts of fertilisers in the former over a long period of time. No organochlorine and organophosphorus were detected in all the rhizosphere even though farmers of the two commercial plantations acknowledged adding pesticides to the banana crops. It could be concluded that the pesticides used did not accumulate in the rhizosphere.

According to Beauregard *et al*. 2010, addition of pesticides and fertilisers may increase plant production and hence organic matter levels in soil may also increase. This is generally beneficial as the soil nutrient sources are increased. However, it was also reported (Aktar *et al*., 2009) that addition of pesticides and fertilisers may contribute to toxic effects on soil microorganisms. Such effects may be direct or indirect, and are dependent upon several interacting factors such as the soil environment and the nature of the microbial populations. The dynamic nature of soil biology and the effects of environment will influence the fate of pesticides and fertilisers which, in turn, will

influence the soil microbial community and function. Thus, it is difficult to draw conclusions about the impacts of various inputs in the agricultural systems.

Research studies showed that the rhizosphere microbial populations are directly or indirectly related to root exudates (Marschner *et al.*, 2001) indicating the importance of the plant. Numerous studies have also reported the importance of soil factors in influencing the composition of rhizosphere bacterial communities. (Germida *et al*., 1998; Dalmastri *et al.*, 1999; Kuske *et al*., 1999). Nucleotide extraction and PCR-based methods have proven valuable in generating independent microbial diversity data. Yet, it is recognized that PCR artifacts may cause biases (von Wintzingerode *et al.,* 1997) and the relative proportions of different bacterial groups represented in clone libraries may not reflect the relative proportions present in template DNA samples (Amann, 2000; Becker *et al.,* 2000).

CHAPTER 6 : CONCLUSION

Additions of fertiliser and pesticides would change the soil environment. This study addressed two main questions: 1). What is the bacterial community profile in the banana rhizosphere in the OA fields and the commercial plantations; 2) How do the two soil profiles differ from each other as a result of soil disturbance treatments through the use of fertilisers and pesticides.

This study was conducted to analyse the bacterial community in banana rhizosphere by constructing clone libraries of the 16S rRNA gene, and to compare the community in the OA fields and the commercial plantations. *Proteobacteria, Acidobacteria* and *Actinobacteria,* constituting the main phyla, were found in all the banana rhizosphere. It was observed that the bacterial diversity in the OA banana fields (Paya Mendoi and Kg. Pian) was different from that of the commercial plantations (Parit Serong and Mersing), the PAST analysis provided evidence of higher bacterial diversity in the OA banana fields compared to the commercial plantations. On the other hand, the bacterial communities between the rhizosphere of different banana varieties (Siam, Nangka, Nipah) in Paya Mendoi showed that they were made up of the similar main bacteria composition except for the presence of small amount of *Bacteroidetes* and Verrucomicrobia in Pisang Nipah. Meanwhile, similar results were obtained between the Pisang Nipah rhizosphere of different OA locations (Paya Mendoi and Kampong Pian) except for an extra presence of Cyanobacteria in Kampong Pian. Moving on to the bacterial communities of different banana varieties (Pisang Nipah, Pisang Berangan) in Parit Serong commercial plantation, the result strongly support the similar bacterial composition except for the presence of Verrumicrobia which only found in Pisang Nipah. However, the bacterial composition in Pisang Nipah rhizosphere in Mersing (Johor) was different to the Pisang Nipah rhizosphere in Parit Serong (Selangor) where it was

observed the presence of *Chloroflexi* and *Bacteroidetes* in Mersing only, but *Nitrospirae* and *Gemmatimonadetes* in Parit Serong only. The overall bacterial communities between Pisang Nipah rhizosphere of the OA fields and commercial plantations showed a little difference between them where certain minor bacterial phyla such as *Nitrospirae*, *Bacteroidetes*, *Chloroflexi*, *Verrumicrobia*, *Gemmatimonadetes* and *Cyanobacteria* were variable in those rhizosphere. From the results, it was concluded that the rhizosphere bacterial communities within the same location showed a similar bacterial composition but different if it was compared between the different locations. There was no difference in rhizosphere bacterial communities of different banana varieties within the same location. The bacterial diversity in the OA banana rhizosphere were a little different to the rhizosphere of the commercial plantations. As the rhizosphere samples were collected at different locations and on different dates, the bacterial composition could be influenced by geological and climatic conditions such as terrain and soil structure, rain, drought, etc. The absence of pesticide indicators (organochlorine and organophosphorus) in the commercial plantation soils might be due to degradation or washed away by rain.

This study established a library of 16S rRNA gene fragments from the banana plant rhizosphere of OA fields and commercial plantations as a means to indicate bacterial composition.

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