CHAPTER VI

MOLECULAR ANALYSIS OF SOIL BACTERIAL DIVERSITY

6.1 Introduction

Soil represents a complex ecosystem in which relationships and interactions between bacteria are also immensely complex. Bacteria adapt to microhabitats, live together, and interact with each other and their environment. Bacterial diversity is critical to ecosystem functioning due to diversity of processes such as decomposition, nutrient cycling, soil aggregation and pathogenicity (Dubey, *et al.* 2006). According to a current estimate, 1 g of soil may harbor up to 10 billions bacteria of possibly 4000-7000 different species and a biomass density of 300-30,000 kg ha^{-1} (Rosello-Mora and Amann, 2001). At most only a few thousand species of soil bacteria and only about 5000 non-eukaryotic organisms have been formally described in contrast to the half-million described insect species (Pace, 1997). Thus, estimates of bacterial diversity in soil should include multiple methods integrating holistic measures at the total community level and partial approaches targeting structural and functional subsets.

While analysis of bacterial diversity in soil is one of the most important aspects of microbial ecology, technically it is also one of the most challenging tasks. It is now known that the portion of bacterial diversity estimated through conventional culture techniques amounts to only 0.1–10% of the total diversity (Torsvik and Ovreas, 2002; Kirk *et al.*, 2004), indicating that techniques based on laboratory cultivation might be significantly biased. Enrichment cultures are selective for fast-growing bacterial species with high growth yields and for those adapted to grow in

media used for cultivation (Kassen and Rainey, 2004; Torsvik and Ovreas, 2002). Thus, there is no guarantee that the bacteria cultured from the soil samples might be playing significant role in their specific soil habitat. In fact, it has been observed that in many soil samples, the bacteria that are most dominant and abundant are not culturable (Torsvik and Ovreas, 2002). In recent years, the number of phylogenetic groups within Bacteria and Archaea Domains have significantly increased due to a combination of direct methods of DNA extraction from soil samples, amplification of 16S rDNA by polymerase chain reaction (PCR), and determination of its nucleotide sequence to identify the phylogenetic relatedness of 16S rDNA species (Amann and Ludwig, 2000). This has helped in discovering large number of bacteria which cannot be cultured in the laboratory but possess very significant ecological functions in their respective soil ecosystem. Together with the long time requirement for cultivation methods, the popularity of molecular approaches for studying soil bacterial populations has increased under the fast emerging discipline of molecular microbial ecology. This discipline uses research methods of molecular biology to investigate the phylogeny and ecology of soil bacteria. Bypassing the cultivation techniques, molecular microbial ecology techniques are based on extraction of nucleic acids directly from soil samples. This newly emerging discipline has opened new possibilities for the exploration of biodiversity of soil bacteria, and thus represents a step towards the exploitation of bacterial diversity in different agriculture and soil environmental problems. This chapter focuses on molecular biological tools and techniques for understanding the bacterial diversity inhabiting diverse soils from different land management in Thongphaphum district; namely the chemically-intensive farm, the organic farm, and the forest, in order to estimate the impact of land management on soil bacterial diversity.

6.2 Materials and methods

Soil Nucleic Acids Extraction

Total nucleic acids were extracted from 0.5 g (wet weight) of soil. Extractions were performed by the addition of 0.5 ml of CTAB (hexadecyltrimethylammonium bromide) extraction buffer and 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1), pH 8.0) into 2 ml screw-capped microcentrifuge tubes, each containing 0.5 g of 0.17 mm acid-washed glass beads (Sigma-Aldrich, USA.). CTAB extraction buffer, modified from the method of Griffiths *et al.* (2000), was prepared by mixing equal volumes of 10% (wt/vol) CTAB (Sigma-Aldrich, USA.) in 0.7 M NaCl with 240 mM potassium phosphate buffer, pH 8.0. Samples were lysed for 30 seconds using a vortex (Genies-2 model G560E, Scientific Industries Inc., USA.), and the aqueous phase containing nucleic acids was separated by centrifugation (16,000 x g) for 5 minutes at 4°C. The aqueous phase was then extracted, and residual phenol was removed by mixing with an equal volume of chloroform/isoamyl alcohol (24:1) followed by repeated centrifugation (16,000 x g) for 5 minutes at 4°C. Total nucleic acids were subsequently precipitated from the extracted top aqueous layer with 2 volumes of 30% (wt/vol) polyethelene glycol 6000 (Fluka BioChemika, Buchs, Switzerland)/ 1.6 M NaCl for 2 hours at room temperature, followed by centrifugation (18,000 x g) for 10 minutes at 4°C. Pelleted nucleic acids were then washed in ice cold 75% (vol/vol) ethanol and air dried prior to resuspension in 50 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8.0). Extraction of nucleic acids was confirmed and quantified by gel electrophoresis. The nucleic acids solutions were stored at 4°C for the next step.

Agarose gel electrophoresis analysis of nucleic acid extracts.

0.7% agarose gels were cast by melting the agarose in the presence of desire buffer (1X TAE Buffer: 40 mM Tris/acetate pH 7.5, 20 mM acetic acid, 1 mM Na_2EDTA) until a clear, transparent solution was achieved, and cooled down to 50 - 55°C. Then ethidium bromide was added to a final concentration of 0.5 µg/ml from a 10 mg/ml stock. The melted solution was then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field (HE99X Submarine electrophoresis unit, Hoefer Inc., USA.). The negatively charged DNA migrates toward the anode. When electrophoresis is completed, DNA bands were visualized under the UV transilluminator and photographed using GelDoc™ and ChemiDoc™ (Bio-Rad, USA.).

PCR amplification condition

Eubacterial 16S rDNA PCR products were amplified from the extracted DNA using primers conserved among all known bacteria. The universal bacteria primers used to initiate PCR were forward primer pA (5'-AGAGTTTGATCCTGGCTCAG-3'; *Escherichia coli* bases 8–28) and reverse primer pH (5'-AAGGAGGTGATCCA GCCGCA-3'; *E. coli* bases 1542–1522) (Edwards *et al.*, 1989) that amplified nearly full-length 16S rDNA products. Amplified reaction mixtures contained 1x PCR buffer (Epicenter™), 1-5 mM $MgCl_2$, 200 µM of each of dNTPs (dATP, dCTP, dGTP, dTTP), 0.2 µM of each primer, 1 unit of *Taq* polymerase (Epicenter™), approximate 25 ng of soil DNA template, in a final reaction volume of 25 µl. PCR

was performed in an automated thermal cycler (MyCycler™ thermal cycler; Bio-Rad, USA.) using an initial denaturation at 94°C for 10 minutes, followed by 30 cycles of 1 minute at 94°C (denaturation), 1 minute at 48-60°C (annealing), and 2 minutes at 72°C (extension), with a final extension step at 72°C for 10 minutes after cycling was complete. The aliquots of PCR products (1.5-Kb fragment) were examined by 0.7% agarose gel electrophoresis. The amplified products were used for cloning immediately.

Cloning of PCR products and constructing 16S rDNA libraries

The amplified PCR products were directly ligated to the pCR®2.1-TOPO® (Invitrogen). Ligation and transformation were carried out according to the manufacturer's instructions. A volume of 2 µl ligation reaction mixture was transformed by heat-shock into Mach1™ $-T1^R$ chemically competent *E. coli* (Invitrogen). The size of the PCR-amplified product was expected to be approximately 1.5 Kb if no fragment was inserted into the vector. Blue/white screening method for selected transformants was used. 40 µl of 40 mg/ml X-Gal in dimethylformamide was spread on top of the agar. After the X-Gal was let to diffuse into the agar, the selective plates were warmed in a 37°C incubator for approximately 1 hour. Only Mach1™ $-T1^R$ chemically-competent *E. coli* with the insert appeared as white colonies on LB (Luria-Bertani broth) medium containing X-Gal, whereas cells without insert will turn light to dark blue. To analyze the cloned inserts, a number of cells picked up by touching a single white colony with a sterile toothpick from an LB-ampicillin agar plate containing X-Gal was isolated to clone libraries on an LB-ampicillin agar plate.

Plasmid purification

PureLink™ Quick Plasmid Miniprep Kits (Invitrogen, USA.) was used to isolate plasmids propagated in Mach1™ $-T1^R$ chemically-competent *E. coli* (Invitrogen, USA.). The PureLink™ Quick Plasmid Miniprep Kits were designed for rapid isolation of molecular biology-grade plasmid DNA from culture. Cells were lysed using an alkaline/SDS procedure. The lysate was applied to a silica membrane column that binds plasmid DNA and the column was washed with Wash Buffers. The plasmid DNA was eluted in TE Buffer and is suitable for standard molecular biology applications including DNA sequencing.

DNA sequencing

The DNA sequences of the 16s rRNA gene were determined directly using the plasmids purified in the earlier step as the sequencing template. DNA sequences were determined by Macrogen's sequencing services (Korea) with sequencing conducted under BigDye™ terminator cycling conditions using the Automatic Sequencer 3730xl. Approximately 100 ng purified plasmid DNA was used for one sequencing reaction. The sequencing primer M13 Forward (5'-GTAAAACGACGGCCAGT-3') was used in this study.

Sequence and phylogenetic analyses

Each sequence was submitted to the CHECK_CHIMERA program of the Ribosome Database Project (RDP-II) (Cole *et al.*, 2003) to detect the presence of possible chimeric artifacts (Qui *et al.*, 2001). Sequences were generally submitted to

and can be retrieved from the Genbank via the World Wide Web. New sequences were compared with those held in the current databases by using BLAST searches (NCBI; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to determine the closest available sequences in the database (Altschul *et al.*, 1990). Sequences were then aligned using Clustal X (version 2.0.5) (Thomson *et al.*, 1997), which showed high similarity scores in the outputs of fasta in the previously aligned rDNA sequence database.

Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987), using Mega software version 4.0 (Tamura *et al.*, 2007). Bootstrap consensus analysis was performed to evaluate robustness of the inferred phenograms. Identical sequences were considered as a single phylotype. Separate phylogenic trees were constructed for the three study sites, namely the chemically-intensive farm, the organic farm, and the forest, as well as an overall tree representing bacterial sequences found in all sites.

6.3 Results and discussion

6.3.1 Soil DNA extraction

Total DNA was extracted directly from each soil sample using extraction method modified from Griffiths *et al.* (2000), which gave DNA with high molecular weight, larger than 23.1 Kb and suitable for use as PCR template. An amount of extracted DNA was about 250 ng/µl per individual sample, as estimated by comparison of its band intensity with that of λ/*Hin*dIII marker in 0.7% agarose gel electrophoresis (Figure 6.1). The extracted DNA was diluted before amplification to

decrease the effects of any remaining enzymatic inhibitors. For subsequent use in the PCR reaction, the DNA solution was adjusted to the final concentration of 25 ng/µl.

The result of this study showed that a modification of the CTAB-based method described by Griffith *et al.* (2000) produced higher yields and better quality DNA from Thongphaphum soils than the modified SDS-based method in a commercial soil DNA isolation kit. The low performance of the commercial kit could be caused by several factors, including bead composition and the composition of the extraction buffer; all of these factors might influence extraction efficacy. This result was validated through quantitative assessment of DNA by both electrophoresis and qualitative assessment by PCR analyses.

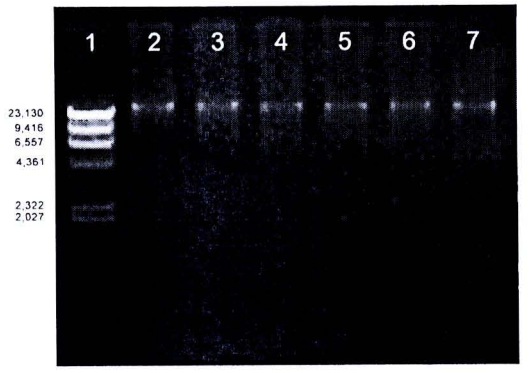

Figure 6.1 Gel electrophoresis of high molecular weight total DNA extracted from soil samples and subjected to 0.7% agarose gel electrophoresis at 100 V for 1 hour. The DNA bands on agarose gel were strained in ethidium bromide and visualized under UV light.

Lane 1 : λ/*Hin*dIII DNA standard

Lanes 2-7: Total DNA extracted from each soil sample, (Durian orchard as the chemically-intensive farm; D, Vimandin farm as the organic farm; V, and Forest; F in dry season and D, V, and F in wet season, respectively.)

CTAB can remove various types of co-contaminants such as humic acids and other polyphenolic compounds present in soil DNA extracts, thereby having positive effects on quality of soil DNA extracts (Steffan *et al.*, 1988; Berthelet *et al.*, 1996; Zhou *et al.*, 1996). PEG precipitation also improves soil DNA quality (Steffan *et al.*, 1988). Stach *et al.* (2001) also suggested that more efficient initial purification of crude environmental DNA, rather than dilution, might prevent biased amplification of the most numerically abundant taxa. Hence, to conduct a comprehensive analysis of the total bacterial diversity in chemical and organic polluted soil, a DNA extraction procedure was adopted, which provided higher yields of high molecular weight DNA

(>23.1 Kb) and PCR inhibitor-free DNA, which was suitable for molecular diversity analysis.

Quantification of soil DNA by gel electrophoresis gives good relative values for different samples (Zhou *et al.*, 2003). The low DNA yield obtained from the chemically-intensive farm soil (as compared to others) might be associated with the long term intensive agricultural management practice that has prevailed in the durian orchard. Continuous application of high rates of inorganic fertilizers and intensive tillage under conventional agriculture management practices lead to the reduction in total bacterial population in soils (Marschner *et al.*, 2003). Soils from the organic farm and the forest consistently gave high yield of DNA. This most likely reflected the fact that long-term, undisturbed or slightly disturbed, agricultural land contains high bacterial population and diversity (Ovreas and Torsvik, 1998). With regard to the DNA quality, purification steps such as polyethyleneglycol (PEG) precipitation steps increased the purity of the DNA extracted by either CTAB extraction methods.

6.3.2 Optimization of PCR conditions

A region of approximately 1.5 Kb of the 16S rRNA gene was successfully amplified using a bacterial universal primer pair, pA and pH reverse (Edward *et al.*, 1989). The 16S rRNA gene was amplified in 25 µl reaction mixture containing 1x PCR buffer, 200 µM each of dNTPs, 0.2 µM each of primers, 1 unit *Taq* DNA polymerase and 25 ng total DNA with $MgCl_2$ concentration and annealing temperature varied from 1.0-5.0 mM and 48-60 °C, respectively. The amplified products were firstly appeared at $MgCl_2$ concentration of 0.5-1.5 mM but their yields were not high enough to interfere cloning. The amplified products were consistently

increased with higher $MgCl_2$ concentration. At these conditions, non-specific products and primer dimers were observed below the specific PCR-amplified DNA. At higher $MgCl_2$ conditions (3.0-5.0 mM), the specific PCR products were concentrated, and non-specific products also appeared. Finally, the amplified products were adjusted to the appropriate $MgCl_2$ condition of 2 mM and then non specific products and primer dimer decreased or disappeared.

At low annealing temperature, the amplified products and non-specific products appeared because the primers caught non-specifically with DNA temperature. More specific catching was observed when annealing temperature was increased to the optimum annealing temperature at 53 °C. The 2 mM $MgCl_2$ concentration and annealing temperature at 53 °C were used for amplification in this study.

Amplification of 16S rDNA (~1.5 Kb) fragments directly from the undiluted total soil DNA recovered from the 6 soil samples of 3 study sites at Thongphaphum was an indicator of the high DNA purity. The undiluted of all soil DNA samples showed no amplified products but 1:10 and 1:100 dilutions resulted in better amplification. In contrast, no amplification was obtained even with 1:100 dilution of the DNA extracted using a commercial kit, which clearly suggested the presence of inhibitors in the DNA purified with the kit-protocol. Dong *et al.* (2006) made similar observations of the inefficiency of a commercial kit in removal of humic acids and PCR-inhibitors from compost soils. Braid *et al.* (2003) had used chemical flocculations prior to DNA extractions by the kit in order reduce co-precipitation of humic acids, but such flocculation treatment also resulted in loss of DNA yield.

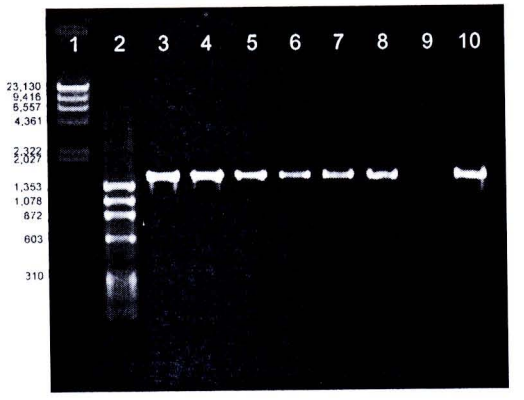

Figure 6.2 Gel electrophoresis of 16S rDNA generated during PCR amplification of total DNA extracted from soil samples. The amplified products of 16S rDNA were electrophoresed through a 1.0% agarose gel at 100 V for 1 hour.

Lane 1 λ/*Hin*dIII DNA standard

Lane 2 ϕX 174 *Hae*III DNA standard

Lane 3-8 The 1.5 Kb 16S rDNA fragments of total DNA from Durian orchard as the chemically-intensive farm; D, Vimandin farm as the organic farm; V, and Forest; F in dry season and D, V, and F in wet season, respectively.

Lane 9 and 10 Negative control and Positive control

When the PCR reaction was completed, the amplified products were electrophoresed through a 1.0% agarose gel and strained with ethidium bromide (Figure 6.2). These fragments were successfully cloned to generate six 16S rDNA libraries; each library from each study site in each season.

Results of PCR amplification, and cloning reactions clearly indicated that DNA of high purity and high molecular weight was obtained from soil samples. Further, placing quality assurance at the methodological choice will enable more meaningful within and across-study comparative analysis of soil bacterial diversity fingerprints.

6.3.3 Impacts of land management on diversity of soil bacteria

To determine phylogenetic diversity, 16 recombinant clones from each library were randomly selected for sequencing. Of the 90 partially sequenced clones analyzed, 20 clones were low-quality sequences, and were therefore disregarded; comparative analysis was performed with the remaining 70 clones. The lengths of the sequences varied between 250-865 bp with the average of 577 bp.

Comparison of sequences to public sequence databases was performed using CHECK_CHIMERA of the RDP and BLAST at NCBI server. None of sequences was detected as potential CHIMERA. Tables 6.1-6.6 list the clones along with percentage similarity with the nearest BLAST hits ranging from 71% to 100% (Tables 6.1-6.6)

Phylogenetic analyses were made on a sequence alignment of 379 bases. Phylogenetic tree constructions achieved by the neighbor-joining method with 1,000-replication bootstrap analysis revealed the presence of major and minor groups that fell into several of the established line of bacteria (Figures 6.4-6.7).

All the sequences appeared to cluster in bacterial phyla and classes generally found in soil. The majority of the clones (62.86%) had sequences that were 1-15% different from those in the current databases (Tables 6.1-6.6). About 14.29% of the clones differed by more than 15% in sequence from the database, and 22.86% of the clones differed by only 1%. The results suggested that the sequences of these clones were also highly diverse. The clones were distributed into many groups, namely *Alpha (α)*, *Beta (β)*, and *Gamma (γ) Proteobacteria*, *Actinobacteria*, *Bacteriodes*, *Firmicutes*, *Planctomycetes,* and unidentified group.

Most of clone sequences (57.14%) showed similarity to uncultured bacteria, which were present in all sites and seasons, suggesting the presence of a large numbers of uncultured bacteria which accounted for more than 99% of the bacteria in the soil. The second frequently represented group was in the *Proteobacteria* (18.57%), specifically *Alpha* (8.57%), *Beta* (2.86%), and *Gamma* (7.14%) *Proteobacteria*, and consisted of clones from all sites and seasons. The third frequently encountered group was affiliated with *Firmicutes* group (12.86%), and consisted of clones from the organic farm and the forest in both seasons. The *Firmicutes* group was not found in the chemically-intensive farm which could imply that existing substrates might inhibit or be inappropriate for *Firmicutes* growth. The forth frequently encountered group was related to *Actinobacteria* group (8.57%). This group was mostly found in the chemically-intensive farm rather than the organic farm and was not found in the forest. Other clones were affiliated with *Bacteroides* (1.43%) and *Planctomycetes* (1.43%), which are commonly found in dry soil (Zhou *et al.*, 2003). Since sampling method and DNA techniques for obtaining clones may affect the coverage and hence overall diversity in uncultured 16S rDNA library approach, the number of clones screened and sequenced in this study was not enough to cover the full bacterial diversity, as indicated by the coverage analysis.

When study sites were examined separately (Tables 6.1-6.6), molecular analysis of soil bacterial diversity showed differing composition and distribution of soil bacterial community. Based on the number of different phylotypes found, 88% of the clones represented unique phylotypes in all samples. A large proportion, approximately 61%, of the sequences from the soil of the chemically-intensive farm belonged to the unidentified uncultured bacterium, whose members are omnipresent in soil worldwide (Desmarais *et al.*, 2002; Johnsen and Nielsen, 1999; Valinsky *et al.*,

2002). Approximately 17% of the chemically-intensive farm clones belonged to the *Actinobacteria* equivalent to *Proteobacteria.* The remaining of 4% of the chemically-intensive farm clones belonged to *Firmicutes.* In the forest, 67% of clones were related to uncultured bacterium, followed by *Proteobacteria* (16.67%), *Firmicutes* (12.50%), and *Planctomycetes* (4.17%), respectively. At last, in the organic farm, there were more abundant bacterial groups than the other sites; 43% of the clones were uncultured bacteria. *Proteobacteria* and *Firmicutes* were equally represented at 22% each, followed by *Actinobacteria* (9%) and *Bacteriodes* (4%) (Figure 6.3).

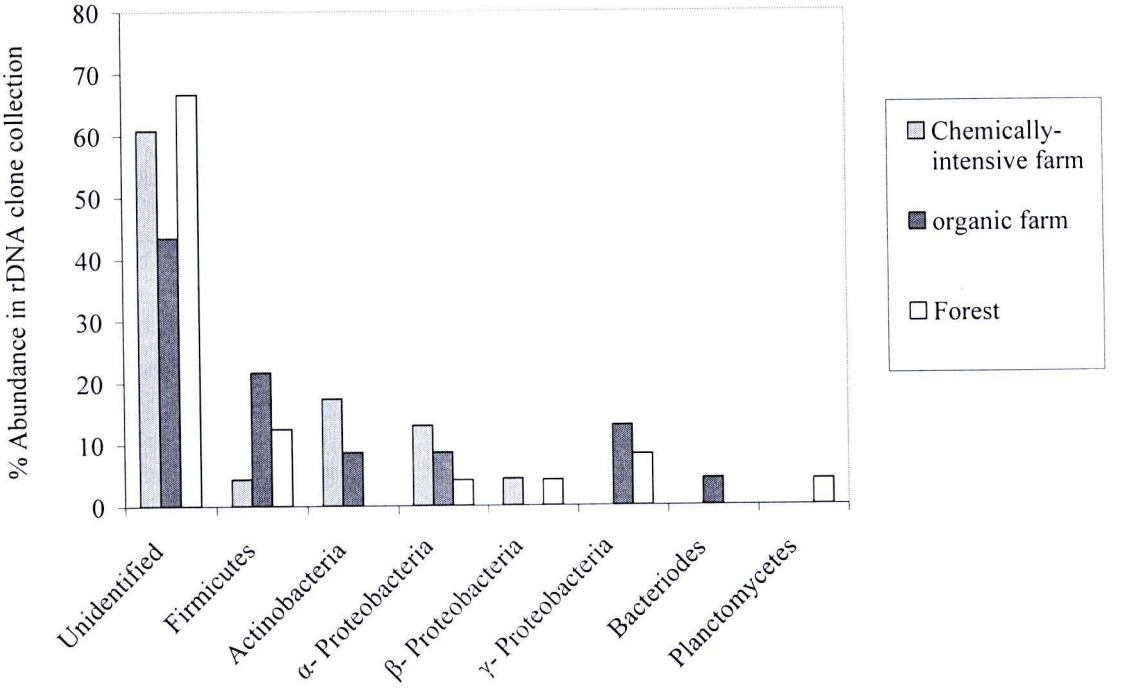

Figure 6.3 Abundance of rDNA clones of different bacteria group. Phylogenetic distribution of 16S rRNA genes amplified from DNA extracted from 3 different soils. Bars indicate the percentage of rDNA clones within each phylogenetic group. Group with a bar lacking indicates that no sequences from that particular phylogenetic group were recovered.

Similarity indices (Sørensen's, Magurran, 1988) were calculated to compare bacterial groups between study sites. Similarity index values between chemically-intensive farm and organic farm, chemically-intensive farm and forest, and organic farm and forest, were 0.67, 0.60, and 0.67, respectively. The results showed that soil bacterial communities between sites were rather similar to each other. As shown in Tables 6.1-6.6, some groups of bacteria, such as *Firmicutes* and *α- Proteobacteria*, were present in all sites, and some groups (*Actinobacteria, β-* and *γ- Proteobacteria*) were present in two sites, while some bacteria groups were unique to specific study sites. This trend was also evident in the phylogenetic analysis (Figure 6.4). However, since unidentified species, which made up to more than 50% of bacteria communities, were excluded from calculations of similarity indices, the values obtained may not represent the actual patterns of bacterial species distribution in the study sites.

Comparisons of bacterial clones showed that different bacteria were found in the wet and dry seasons. For the *Firmicutes*, more clones were viable in the wet season sample compared to those in the dry season sample. These high percentages may be linked to the physical changes during wet season, when more water content, moisture and nutrients become available and accessible for soil bacteria. This indicated that various soil bacteria could profit from these factors. In contrast, *α-Proteobacteria* and *γ- Proteobacteria* were more frequently represented in the dry season than in the wet season. The vast majority of *Proteobacteria* have not yet been cultured and their ecological functions in soils remain "to be elucidated" (Kuske *et al.*, 1997). They have been detected in a wide variety of environments worldwide (Barns *et al.*, 1999; Ibrahim *et al.*, 1997; Kuske *et al.*, 1997), suggesting *Proteobacteria* might be abundant in arid soils (Dunbar *et al.* 1999) compared to other soil types.

Smit *et al.* (2001) suggested that the ratio between the percentages of *Proteobacteria* and the *Acidobacterium* division can be indicative of the nutrient status of the soil ecosystem. However, no *Acidobacterium* was found in this study. The relatively low proportion of *Acidobacteria* indicated that the soil was of low quality which corresponded to the results of soil nutrient analysis (Chapter IV), which showed low quantities of selected nutrients. This poor soil could be due to plant roots having a preference for γ- *Proteobacteria* (Marilley and Aragno, 1999). *Bacteroides* and *Planctomycetes* were very rare, representing approximately 1% of clones.

Less than 37% of uncultured bacteria in the 16S rDNA clone library of the sample taken in the both seasons were identified. A possible explanation can be that they are diverse and most of bacteria were unculturable (Kirk *et al.*, 2004) resulting in there still has less studies for identified cover all bacteria.

Table 6.1 Diversity of the chemically-intensive farm soil 16S rDNA clones and sequences in dry season

Clone no.	Length of sequence (bp)	Accession number	Group	Bacteria with most similar sequences in the data bases	Similarlity (%)
D1_01	612	AY268251	Unidentified	Uncultured bacterium clone B5	87
D1_02	376	EU432168	Unidentified	Uncultured bacterium clone MB28	73
D1_03	374	AY217118	α-Proteobacteria	*Mesorhizobium ciceri*	96
D1_04	387	EU287095	Unidentified	Uncultured bacterium clone P13-2	99
D1_05	620	AY268248	Unidentified	Uncultured bacterium clone E3	90
D1_06	405	AY268250	Unidentified	Uncultured bacterium clone A6	79
D1_07	593	EF626595	Actinobacteria	*Streptomyces curacoi*	97
D1_08	581	AY268246	Unidentified	Uncultured bacterium clone B3	81
D1_09	705	DQ442553	Actinobacteria	*Streptomyces violaceorubidus*	87
D1_10	360	AF241608	α-Proteobacteria	Uncultured *Bradyrhizobium* sp. GU3B	92
D1_11	550	AY268246	Unidentified	Uncultured bacterium clone B3	89

Table 6.2 Diversity of the chemically-intensive farm soil 16S rDNA clones and sequences in wet season.

Clone no.	Length of sequence (bp)	Accession number	Group	Bacteria with most similar sequences in the data bases	Similarlity (%)
D2_01	456	EU137376	Unidentified	Uncultured bacterium clone Oh_3137A1A	96
D2_02	544	EU223961	Unidentified	Uncultured soil bacterium clone 149	99
D2_03	471	AB069654	α-Proteobacteria	*Bradyrhizobium* sp. PAC.24	99
D2_04	469	AJ867656	Unidentified	Uncultured bacterium clone RS 8-Bact17	98
D2_05	264	EU432158	Unidentified	Uncultured bacterium clone TB003	82
D2_06	772	AM085471	Unidentified	Uncultured bacterium clone E21	100
D2_07	343	EU432158	Unidentified	Uncultured bacterium clone TB003	92
D2_08	457	DQ442553	Actinobacteria	*Streptomyces violaceorubidus*	83
D2_09	737	EU130959	β-Proteobacteria	*Hydrogenophaga* sp. BAC120	91
D2_10	258	DQ442553	Actinobacteria	*Streptomyces violaceorubidus*	85
D2_11	446	AJ867656	Unidentified	Uncultured bacterium clone RS 8-Bact17	71

Table 6.3 Diversity of the organic farm soil 16S rDNA clones and sequences in dry season.

Clone no.	Length of sequence (bp)	Accession number	Group	Bacteria with most similar sequences in the data bases	Similarlity (%)
V1_01	700	CT573765	Unidentified	Uncultured bacterium clone 057F01	99
V1_02	457	DQ223223	α-Proteobacteria	Uncultured *Rickettsiales* bacterium clone	98
V1_03	865	AF458082	γ-Proteobacteria	*Citrobacter freundii* isolate CF-4	99
V1_04	500	EU073782	Firmicutes	Uncultured *Firmicutes* bacterium	97
V1_05	461	EU223961	Unidentified	Uncultured soil bacterium clone 149	99
V1_06	599	EU507475	Unidentified	Uncultured bacterium clone MD23_2aaa03g06	96
V1_07	471	AY649442	α-Proteobacteria	*Bradyrhizobium* sp. BR3287	99
V1_08	475	AF458081	γ-Proteobacteria	*Citrobacter freundii* isolate CF-12	94
V1_09	349	EU073782	Firmicutes	Uncultured *Firmicutes* bacterium	96
V1_10	775	AB190137	Actinobacteria	*Cellulosimicrobium cellulans* strain:S-4-25-1	89
V1_11	555	AF458082	γ-Proteobacteria	*Citrobacter freundii* isolate CF-4	93
V1_12	836	EF219748	Unidentified	Unidentified bacterium clone AI-2M_B05	97

Table 6.4 Diversity of the organic farm soil 16S rDNA clones and sequences in wet season.

Clone no.	Length of sequence (bp)	Accession number	Group	Bacteria with most similar sequences in the data bases	Similarlity (%)
V2_01	250	AY921733	Bacteroides	Uncultured *Bacteroides* bacterium	98
V2_02	823	EU048273	Firmicutes	*Bacillus pumilus* strain MASH	98
V2_03	775	AB190157	Actinobacteria	*Cellulosimicrobium cellulans* strain:S-31-25-2	89
V2_04	222	EU184876	Unidentified	Uncultured bacterium clone TC WBB100	88
V2_05	657	AJ517883	Unidentified	Uncultured bacterium clone M69.6	92
V2_06	655	AJ517883	Unidentified	Uncultured bacterium clone M69.6	86
V2_07	354	EF494280	Unidentified	Uncultured bacterium clone NR.4.42	96
V2_08	667	AJ517884	Unidentified	Uncultured bacterium clone C61.2	83
V2_09	568	AY268253	Unidentified	Uncultured bacterium clone C6	89
V2_10	520	EU240367	Firmicutes	*Bacillus samanii* strain B74	93
V2_11	492	EU240964	Firmicutes	*Bacillus subtilis* strain YC-11B	94

Table 6.5 Diversity of the forest soil 16S rDNA clones and sequences in dry season.

Clone no.	Length of sequence (bp)	Accession number	Group	Bacteria with most similar sequences in the data bases	Similarlity (%)
F1_01	475	AJ431217	γ-Proteobacteria	*Proteobacterium* BHI60-9 strain BHI60-9	94
F1_02	837	EF516786	Unidentified	Uncultured bacterium clone FCPS677	88
F1_03	741	EU289425	Unidentified	Uncultured bacterium clone 1-2D	75
F1_04	401	EF401866	Unidentified	Uncultured bacterium clone D4	98
F1_05	327	DQ404701	Unidentified	Uncultured bacterium clone 655914	85
F1_06	402	AB107544	Unidentified	Uncultured bacterium clone: 3-P03	99
F1_07	775	EF688379	Unidentified	Uncultured soil bacterium clone F6-86	90
F1_08	837	X81949	Planctomycetes	*Planctomyces brasiliensis* DSM 5305	83
F1_09	741	EF516253	Unidentified	Uncultured bacterium clone FCPP398	92
F1_10	765	EF492965	Unidentified	Uncultured bacterium clone JH-WHS68	95
F1_11	759	DQ984632	Unidentified	Uncultured bacterium clone YK_107	95
F1_12	769	AY749435	β-Proteobacteria	*Ralstonia* sp. SKJH-4	99

Table 6.6 Diversity of the forest soil 16S rDNA clones and sequences in wet season.

Clone no.	Length of sequence (bp)	Accession number	Group	Bacteria with most similar sequences in the data bases	Similarlity (%)
F2_01	403	CP000969	Firmicutes	*Thermotoga* sp. RQ2	71
F2_02	700	DQ824314	Unidentified	Uncultured bacterium clone RL304_aal74d01	98
F2_03	607	CP000076	γ-Proteobacteria	*Pseudomonas fluorescens*	99
F2_04	774	EU109461	Firmicutes	*Thermoanaerobacter subterraneus*	99
F2_05	466	CP000230	α-Proteobacteria	*Rhodospirillum rubrum*	99
F2_06	529	EU432168	Unidentified	Uncultured bacterium clone MB28	91
F2_07	470	AJ867656	Unidentified	Uncultured bacterium clone RS 8-Bact17	99
F2_08	555	AB176393	Unidentified	Uncultured bacterium clone:SSmAB09-48	97
F2_09	572	AF018052	Unidentified	Uncultured bacterium OS9C	99
F2_10	529	EU432158	Unidentified	Uncultured bacterium clone TB003	92
F2_11	535	EU240964	Firmicutes	*Bacillus subtilis* strain YC-11B	94
F2_12	555	AB176398	Unidentified	Uncultured bacterium clone:SSmAB09-53	99

The Neighbor-joining phylogenetic trees for the study sites (Figures 6.4-6.7) showed low bootstrap values, which might be due to the fact that most of the soil bacterial clones were affiliated with unculturable bacteria. Some identified bacteria were specific with locality. Accordingly, *Thermoanaerobacter* sp. was only found in the forest, *Cellulosimicrobium* sp. was only found in the organic farm, and *Streptomyces* sp. was only found in the chemically-intensive farm. *Thermoanaerobacter* sp., which belongs to thermophilic or hyperthermophilic bacteria group, could be found near a hot spring in the Thongphaphum forest. Similar findings were reported by Xue *et al.* (2001), in which *Thermoanaerobacter* sp. has been isolated from hot spring sediment. *Cellulosimicrobium* sp., a cellulolytic microbium, which was found in the organic farm that has abundant substrates essential for bacterial growth from organic input, was also reported in agricultural soil and decaying plant material (Heym *et al.*, 2005). *Streptomyces*, the largest genus of *Actinobacteria*, found predominantly in moist soil and in decaying vegetation (Postma *et al.*, 2007), was found in the chemically-intensive farm of which soil had high water content.

In other culture-independent studies for characterizing the soil microbial diversity, most clones found were also members of the described bacterial lineages but with varying percentages of participation. This might be depending on the soil, sample origin, or plant growth. For example, Felske *et al.* (1998) and Duineveld *et al.* (2001) described members of *Bacillus* as the most predominant in grassland soil in a loamy sand soil from Netherlands. Also, Smalla *et al.* (2001) studying the bulk and rhizospheric soil under three different plant species showed that *Arthrobacter* spp. prevailed in temperate loamy sand soil in Germany. The bacterial composition of soil samples from Thongphaphum were different from those previously studied, since

findings showed mostly *α- and γ- Proteobacteria*, followed by the *Firmicutes*. Representatives of the *β- Proteobacteria* were less abundant. The reasons for this might be that the tropical soil may contain other dominant bacterial populations than temperate soil or other soils, and that the populations are influenced by growth of plants. In similar soil composition such as in a forest soil of Indonesia, Krave *et al.* (2002) also found a strong dominance of *α- Proteobacteria* and *Actinobacteria,* in compliance to this study. Moreover, the most diversity of soil bacteria is in the unidentified bacteria, so the unidentified uncultured groups may relate to the dominant group as previously reported elsewhere or these clones may be suggestive of new phyla or families.

Another explanation for different dominant bacterial groups could be that extraction and/or amplification only obtained a selection of the abundant DNA from the soil which was different from those used in the other studies. The study of bacterial community in a wheat field in temperate soil in Netherlands by Smit *et al.* (2001) found no Gram-positive bacteria using molecular techniques, while these organisms dominated in the cultured isolates.

From phylogenetic analysis, it could be implied that land management had some effects on their soil bacterial diversity. Thus, the ecology of soil from various practical managements is dominated by changes in soil physical, chemical, and biological factors as shown in the results of this thesis. There are still a lot more to be elucidated since this thesis was studied only bacterial diversity. Thus, further researches in fungal diversity as other microbial diversity study would add a great credit on impact of land management on microbial diversity.

6.4 Conclusion

Total soil DNA was isolated from soils collected from the chemically-intensive farm, the organic farm, and the forest in Thongphaphum District, western Thailand. The 16S rDNA was amplified with universal bacterial primers and cloned into plasmid vectors. Phylogenetic analysis based on partial sequences (~ 379 bp) established with the neighbor-joining method with 1,000-replication bootstrap technique revealed the presence of major and minor groups that fell into several of the established lines of bacteria. Most of the clones belonged to the unidentified uncultured bacteria (57.14%), while others were classified as *Alpha* (8.57%), *Beta* (2.86%), and *Gamma* (7.14%) *Proteobacteria, Firmicutes* (12.86%), *Actinobacteria* (8.57%), *Bacteriodes* (1.43%), and *Planctomycetes* (1.43%). The results suggest that these Thongphaphum-derived clones are very diverse in phylogeny, and that many probably reflect new genera or families. Furthermore, the results of bacterial diversity indicate that different land managements affect the bacterial diversity because of large numbers of unique phylotypes specific to each site. Although there was no significant difference between bacterial community structures among sites, it does not mean the land management practices have no impact to bacterial diversity. Furthermore, impacts of land management are also related to other factors, such as chemicals remaining in the area; and long-term studies should be implemented to examine absolute changes caused by land management.

Descriptions for Figures 6.4-6.7

Figure 6.4 Neighbor-joining tree of 16S rDNA sequences cloned showed the relationship of bacteria from 3 sites in dry and wet season. Bootstrap values were shown, representing the percentage support for clusters out of 1,000 replications. D1 and D2 represented chemically-intensive farm soil (durian farm) in dry season and wet season, respectively. F1 and F2 represented forest soil in dry season and wet season, respectively. V1 and V2 represented organic farm soil (Vimandin) in dry season and wet season, respectively. The scale gives genetic distances and *Aquifex pyrophirus* was used as an outgroup.

Figure 6.5 Neighbor-joining tree of 16S rDNA sequences cloned from chemically-intensive farm soil showed the relationship of bacteria from chemically-intensive farm in dry and wet season. Bootstrap values were shown, representing the percentage support for clusters out of 1,000 replications. D1 and D2 represented chemically-intensive farm soil (durian farm) in dry season and wet season, respectively. The scale gives genetic distances and *Aquifex pyrophirus* was used as an outgroup.

Figure 6.6 Neighbor-joining tree of 16S rDNA sequences cloned from forest soil showed the relationship of bacteria from forest in dry and wet season. Bootstrap values were shown, representing the percentage support for clusters out of 1,000 replications. F1 and F2 represented forest soil in dry season and wet season, respectively. The scale gives genetic distances and *Aquifex pyrophirus* was used as an outgroup.

Figure 6.7 Neighbor-joining tree of 16S rDNA sequences cloned from organic farm soil showed the relationship of bacteria from organic farm in dry and wet season. Bootstrap values were shown, representing the percentage support for clusters out of 1,000 replications. V1 and V2 represented organic farm soil (Vimandin) in dry season and wet season, respectively. The scale gives genetic distances and *Aquifex pyrophirus* was used as an outgroup.

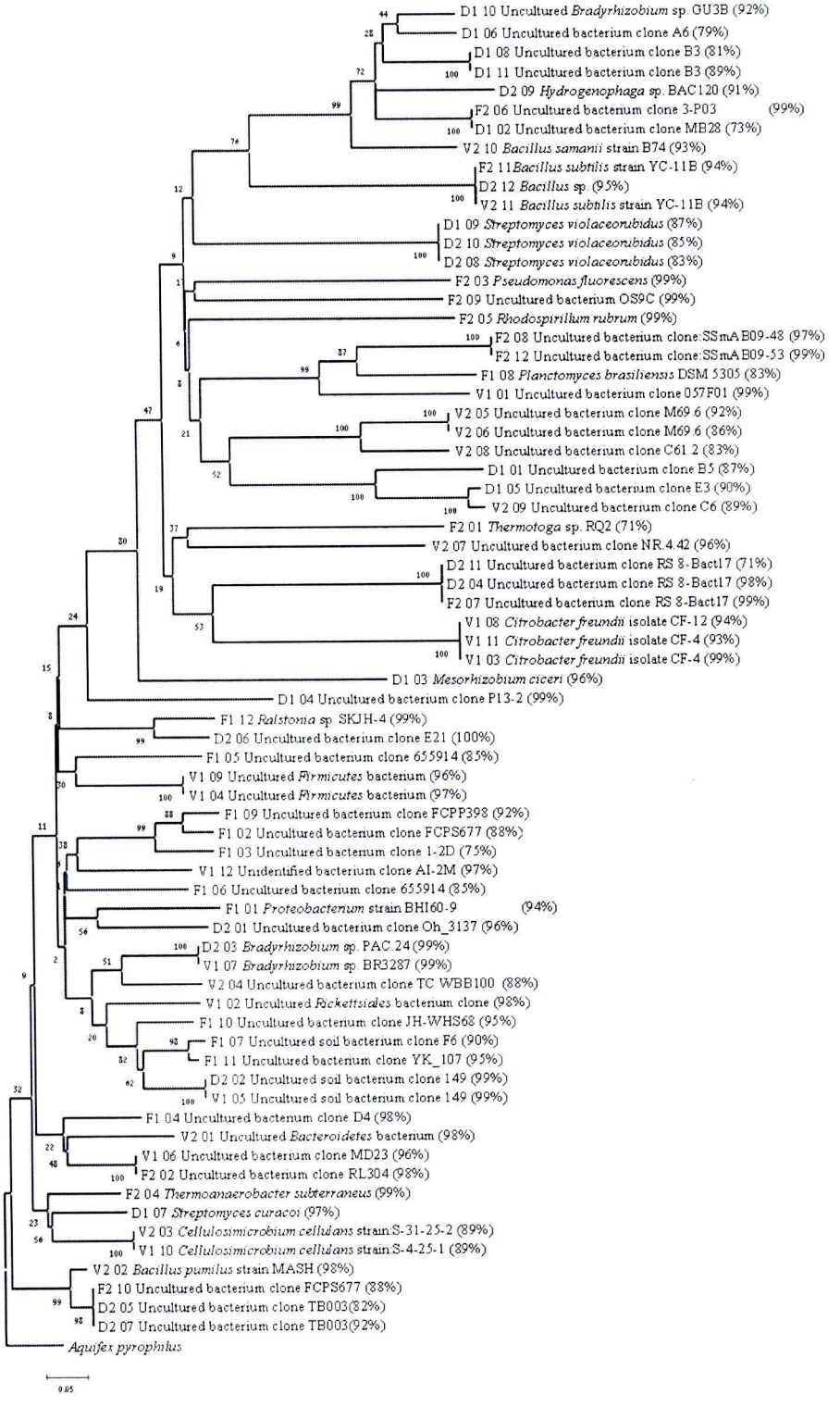
D1 10 Uncultured Bradyrhizobium sp. GU3B (92%)
D1 06 Uncultured bacterium clone A6 (79%)
D1 08 Uncultured bacterium clone B3 (81%)
D1 11 Uncultured bacterium clone B3 (89%)
D2 09 Hydrogenophaga sp. BAC120 (91%)
F2 06 Uncultured bacterium clone 3-P03 (99%)
D1 02 Uncultured bacterium clone MB28 (73%)
V2 10 Bacillus samanii strain B74 (93%)
F2 11 Bacillus subtilis strain YC-11B (94%)
D2 12 Bacillus sp. (95%)
V2 11 Bacillus subtilis strain YC-11B (94%)
D1 09 Streptomyces violaceorubidus (87%)
D2 10 Streptomyces violaceorubidus (85%)
D2 08 Streptomyces violaceorubidus (83%)
F2 03 Pseudomonas fluorescens (99%)
F2 09 Uncultured bacterium OS9C (99%)
F2 05 Rhodospirillum rubrum (99%)
F2 08 Uncultured bacterium clone:SSmAB09-48 (97%)
F2 12 Uncultured bacterium clone:SSmAB09-53 (99%)
F1 08 Planctomyces brasiliensis DSM 5305 (83%)
V1 01 Uncultured bacterium clone 057F01 (99%)
V2 05 Uncultured bacterium clone M69.6 (92%)
V2 06 Uncultured bacterium clone M69.6 (86%)
V2 08 Uncultured bacterium clone C61.2 (83%)
D1 01 Uncultured bacterium clone B5 (87%)
D1 05 Uncultured bacterium clone E3 (90%)
V2 09 Uncultured bacterium clone C6 (89%)
F2 01 Thermotoga sp. RQ2 (71%)
V2 07 Uncultured bacterium clone NR.4.42 (96%)
D2 11 Uncultured bacterium clone RS 8-Bact17 (71%)
D2 04 Uncultured bacterium clone RS 8-Bact17 (98%)
F2 07 Uncultured bacterium clone RS 8-Bact17 (99%)
V1 08 Citrobacter freundii isolate CF-12 (94%)
V1 11 Citrobacter freundii isolate CF-4 (93%)
V1 03 Citrobacter freundii isolate CF-4 (99%)
D1 03 Mesorhizobium ciceri (96%)
D1 04 Uncultured bacterium clone P13-2 (99%)
F1 12 Ralstonia sp. SKJH-4 (99%)
D2 06 Uncultured bacterium clone E21 (100%)
F1 05 Uncultured bacterium clone 655914 (85%)
V1 09 Uncultured Firmicutes bacterium (96%)
V1 04 Uncultured Firmicutes bacterium (97%)
F1 09 Uncultured bacterium clone FCPP398 (92%)
F1 02 Uncultured bacterium clone FCPS677 (88%)
F1 03 Uncultured bacterium clone 1-2D (75%)
V1 12 Unidentified bacterium clone AI-2M (97%)
F1 06 Uncultured bacterium clone 655914 (85%)
F1 01 Proteobacterium strain BHI60-9 (94%)
D2 01 Uncultured bacterium clone Oh_3137 (96%)
D2 03 Bradyrhizobium sp. PAC.24 (99%)
V1 07 Bradyrhizobium sp. BR3287 (99%)
V2 04 Uncultured bacterium clone TC WBB100 (88%)
V1 02 Uncultured Rickettsiales bacterium clone (98%)
F1 10 Uncultured bacterium clone JH-WHS68 (95%)
F1 07 Uncultured soil bacterium clone F6 (90%)
F1 11 Uncultured bacterium clone YK_107 (95%)
D2 02 Uncultured soil bacterium clone 149 (99%)
V1 05 Uncultured soil bacterium clone 149 (99%)
F1 04 Uncultured bacterium clone D4 (98%)
V2 01 Uncultured Bacteroidetes bacterium (98%)
V1 06 Uncultured bacterium clone MD23 (96%)
F2 02 Uncultured bacterium clone RL304 (98%)
F2 04 Thermoanaerobacter subterraneus (99%)
D1 07 Streptomyces curacoi (97%)
V2 03 Cellulosimicrobium cellulans strain:S-31-25-2 (89%)
V1 10 Cellulosimicrobium cellulans strain:S-4-25-1 (89%)
V2 02 Bacillus pumilus strain MASH (98%)
F2 10 Uncultured bacterium clone FCPS677 (88%)
D2 05 Uncultured bacterium clone TB003(82%)
D2 07 Uncultured bacterium clone TB003(92%)
Aquifex pyrophilus
0.05

Figure 6.4

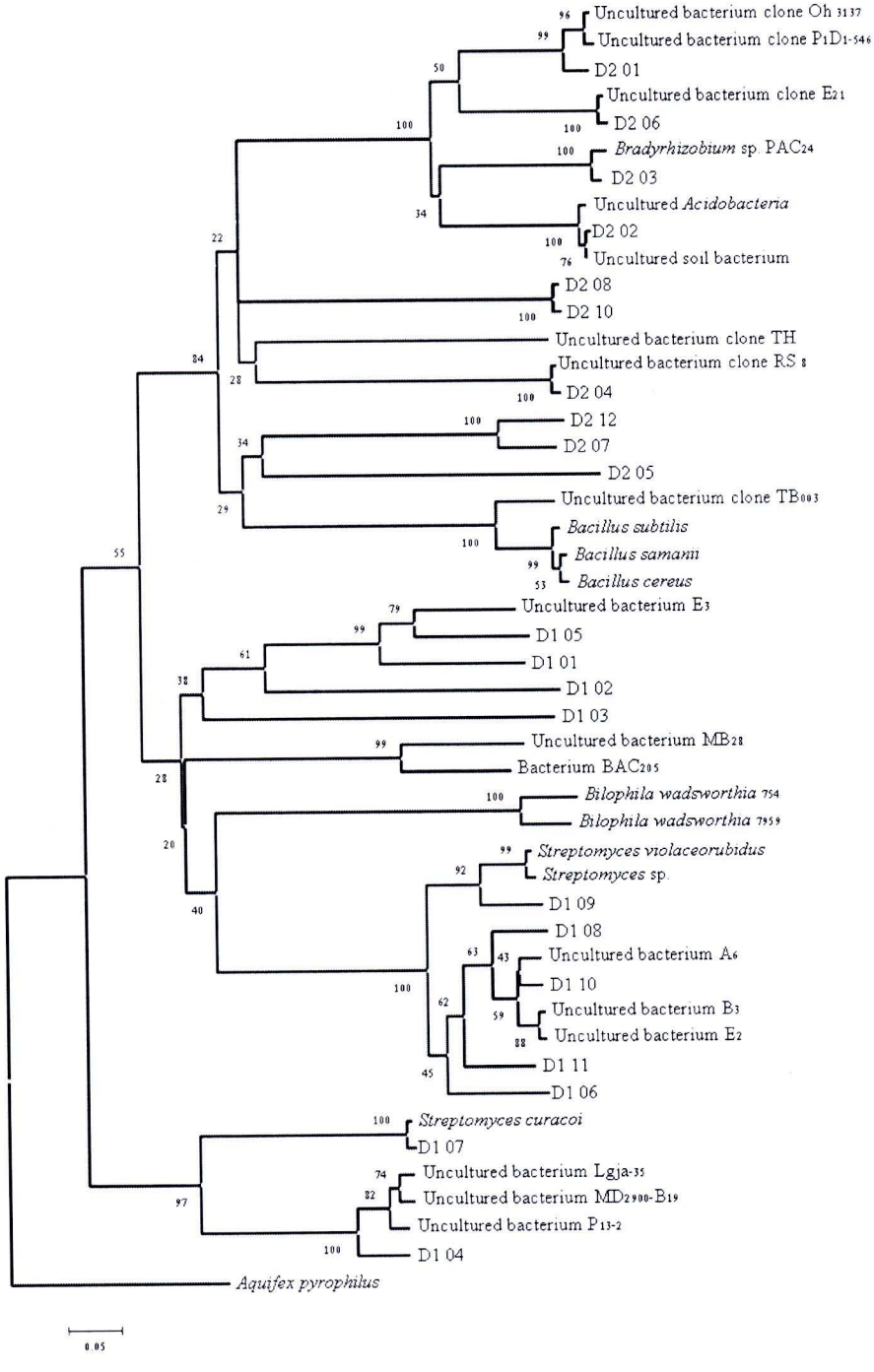
Uncultured bacterium clone Oh 3137
Uncultured bacterium clone P1D1-546
D2 01
Uncultured bacterium clone E21
D2 06
Bradyrhizobium sp. PAC24
D2 03
Uncultured Acidobacteria
D2 02
Uncultured soil bacterium
D2 08
D2 10
Uncultured bacterium clone TH
Uncultured bacterium clone RS 8
D2 04
D2 12
D2 07
D2 05
Uncultured bacterium clone TB003
Bacillus subtilis
Bacillus samanii
Bacillus cereus
Uncultured bacterium E3
D1 05
D1 01
D1 02
D1 03
Uncultured bacterium MB28
Bacterium BAC205
Bilophila wadsworthia 754
Bilophila wadsworthia 7959
Streptomyces violaceorubidus
Streptomyces sp.
D1 09
D1 08
Uncultured bacterium A6
D1 10
Uncultured bacterium B3
Uncultured bacterium E2
D1 11
D1 06
Streptomyces curacoi
D1 07
Uncultured bacterium Lgja-35
Uncultured bacterium MD2900-B19
Uncultured bacterium P13-2
D1 04
Aquifex pyrophilus
0.05

Figure 6.5

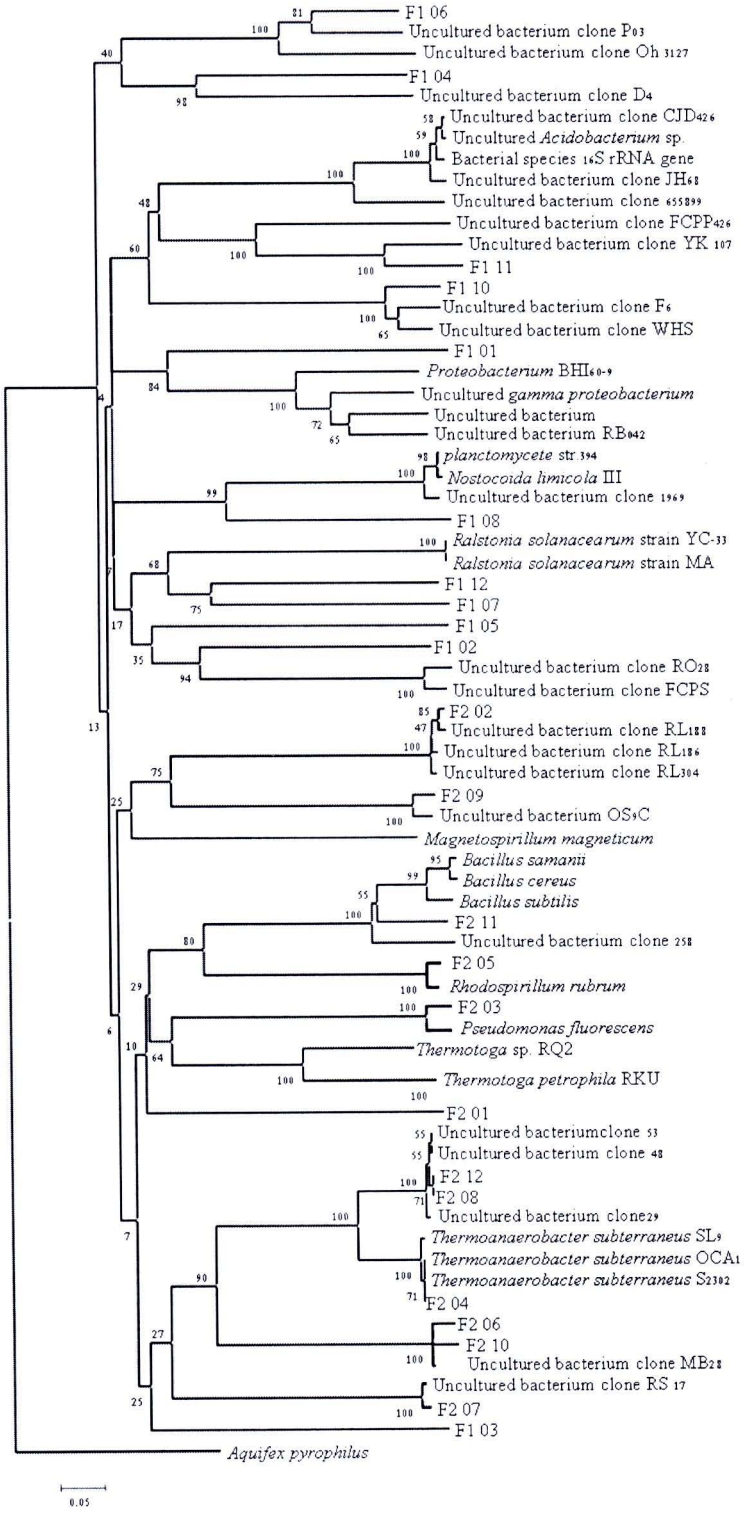
F1 06
Uncultured bacterium clone P03
Uncultured bacterium clone Oh 3127
F1 04
Uncultured bacterium clone D4
Uncultured bacterium clone CJD426
Uncultured Acidobacterium sp.
Bacterial species 16S rRNA gene
Uncultured bacterium clone JH68
Uncultured bacterium clone 655899
Uncultured bacterium clone FCPP426
Uncultured bacterium clone YK 107
F1 11
F1 10
Uncultured bacterium clone F6
Uncultured bacterium clone WHS
F1 01
Proteobacterium BHI60-9
Uncultured gamma proteobacterium
Uncultured bacterium
Uncultured bacterium RB042
planctomycete str 394
Nostocoida limicola III
Uncultured bacterium clone 1969
F1 08
Ralstonia solanacearum strain YC-33
Ralstonia solanacearum strain MA
F1 12
F1 07
F1 05
F1 02
Uncultured bacterium clone RO28
Uncultured bacterium clone FCPS
F2 02
Uncultured bacterium clone RL188
Uncultured bacterium clone RL186
Uncultured bacterium clone RL304
F2 09
Uncultured bacterium OS9C
Magnetospirillum magneticum
Bacillus samanii
Bacillus cereus
Bacillus subtilis
F2 11
Uncultured bacterium clone 258
F2 05
Rhodospirillum rubrum
F2 03
Pseudomonas fluorescens
Thermotoga sp. RQ2
Thermotoga petrophila RKU
F2 01
Uncultured bacteriumclone 53
Uncultured bacterium clone 48
F2 12
F2 08
Uncultured bacterium clone29
Thermoanaerobacter subterraneus SL9
Thermoanaerobacter subterraneus OCA1
Thermoanaerobacter subterraneus S2302
F2 04
F2 06
F2 10
Uncultured bacterium clone MB28
Uncultured bacterium clone RS 17
F2 07
F1 03
Aquifex pyrophilus
0.05

Figure 6.6

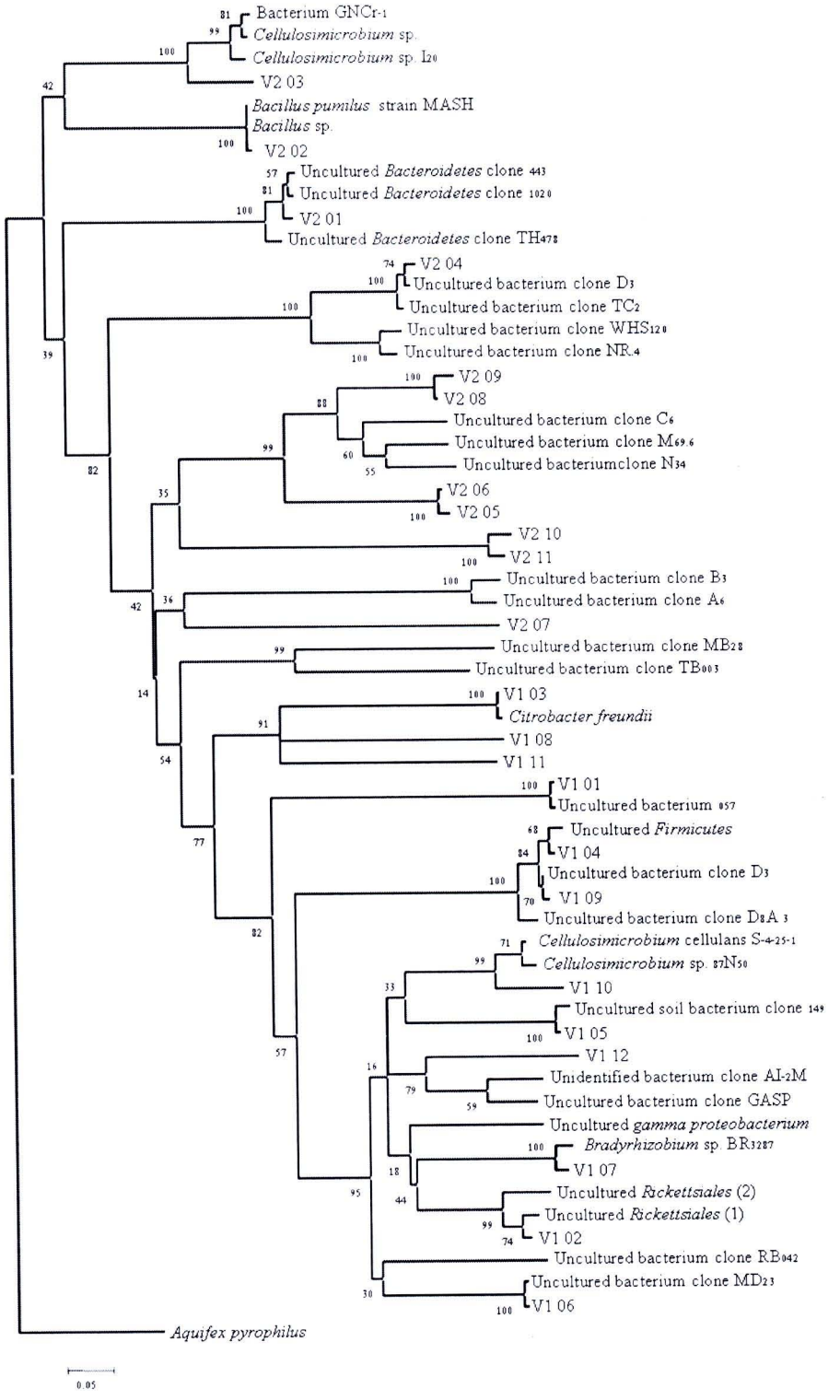
Bacterium GNCr-1
Cellulosimicrobium sp.
Cellulosimicrobium sp. L20
V2 03
Bacillus pumilus strain MASH
Bacillus sp.
V2 02
Uncultured Bacteroidetes clone 443
Uncultured Bacteroidetes clone 1020
V2 01
Uncultured Bacteroidetes clone TH478
V2 04
Uncultured bacterium clone D3
Uncultured bacterium clone TC2
Uncultured bacterium clone WHS120
Uncultured bacterium clone NR.4
V2 09
V2 08
Uncultured bacterium clone C6
Uncultured bacterium clone M69.6
Uncultured bacteriumclone N34
V2 06
V2 05
V2 10
V2 11
Uncultured bacterium clone B3
Uncultured bacterium clone A6
V2 07
Uncultured bacterium clone MB28
Uncultured bacterium clone TB003
V1 03
Citrobacter freundii
V1 08
V1 11
V1 01
Uncultured bacterium 057
Uncultured Firmicutes
V1 04
Uncultured bacterium clone D3
V1 09
Uncultured bacterium clone D8A 3
Cellulosimicrobium cellulans S-4-25-1
Cellulosimicrobium sp. 87N50
V1 10
Uncultured soil bacterium clone 149
V1 05
V1 12
Unidentified bacterium clone AI-2M
Uncultured bacterium clone GASP
Uncultured gamma proteobacterium
Bradyrhizobium sp. BR3287
V1 07
Uncultured Rickettsiales (2)
Uncultured Rickettsiales (1)
V1 02
Uncultured bacterium clone RB042
Uncultured bacterium clone MD23
V1 06
Aquifex pyrophilus
0.05

Figure 6.7