MECHANISMS OF ISOLATED HYDROGEN-OXIDIZING BACTERIA IN PLANT GROWTH PROMOTION AND EFFECTS OF HYDROGEN METABOLISM ON RHIZOBACTERIAL COMMUNITY STRUCTURE

by

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ABSTRACT

MECHANISMS OF ISOLATED HYDROGEN-OXIDIZING BACTERIA IN PLANT GROWTH PROMOTION AND EFFECTS OF HYDROGEN METABOLISM ON RHIZOBACTERIAL COMMUNITY STRUCTURE

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Previous studies have showed that the hydrogen gas evolved from Hup^- legume nodules promotes plant growth and increases the hydrogen uptake rate of soils adjacent to Hup^- nodules. This may be resulted from hydrogen-induced variation of rhizobacterial community structure. Twenty isolates of hydrogen-oxidizing bacteria belonging to genera of *Variovorax*, *Burkhorderia* and *Flavobacterium* showed positive effect on root elongation.

This study showed that isolates belonging to *Variovorax* and *Flavobacterium* had ACC deaminase activity and isolates belonging to *Burkhorderia* had the ability to excrete rhizobitoxine or its structural analogue such as AVG, which meant that they have the ability to promote plant growth by lowering of plant ethylene levels. TRFLP studies showed that hydrogen metabolism resulted in obvious variation of bacterial community structure in hydrogen treated soils compared to the controls. TRF peaks whose intensity increased obviously in profiles from hydrogen-treated soils were possibly contributed by bacteria utilizing hydrogen gas.

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LIST OF ABBREVIATIONS

A	Ampere
ACC	1-aminocyclopropane-1-carboxylate
acyl-HSL	acyle-homoserine lactone
ARDRA	Amplified Ribosomal DNA Restriction Analysis
AVG	aminoethoxyvinylglycine
bp	base pair
BSA	Bovine Serum Albumin
BSF8/20	bacterial universal forward primer 5'-AGAGTTTGATCCTGGCTCAG-3')
BSR534/18	bacterial universal reverse primer (5'-ATTACCGCGGCTGCTGGC-3')
CE	Capillary Electrophoresis
CTAB	hexadecyl-trimethylammoniun bromide
DAPG	2,4-diacetyphloroglucinol
DC	Direct Current
DGGE	Denaturing Gradient Gel Electrophoresis
6-FAM	phosphoramidite fluorochrome 5-carboxyfluorescein
g	gram
HUP	uptake-hydrogenase
IAA	indoleacetic acid
IRS	Inhibitor Removal Solution

ISR	Induced Systemic Resistance
LIF	Laser-Induced Fluorescence
M	mol per litre
mA	milliampere
mg	milligram
mM	millimol per liter
MSA	Mineral Salt Agar
MW	Molecular Weight
SD	Standard Deviation
nt	nucleotides
nm	nanometer
O.D.	Optical Density
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promotion Rhizobacteria
ppm	parts per million
PSB	Phosphate-Soluble Bacteria
psi	pound per square inch
RDP	Ribosomal Database Project
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
SAM	S-adenosyl-L-methionine

SDS	Sodium Dodecyl Sulfate
SSCP	Single Strand Conformation Polymorphism
TGGE	Temperature Gradient Gel Electrophoresis
TRF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism
µl	microlitre
µmol	micromole
µm	micrometer
v/v	volume : volume

1. GENERAL INTRODUCTION

1.1 ROTATION WITH LEGUME AND PLANT GROWTH PROMOTION

1.1.1 Importance of Rotation in Agriculture

Crop rotation means that succeeding crops are of a different genus, species, subspecies, or variety than the previous crop. Classical rotation involves alternating a legume like alfalfa or clover with a grass crop like corn or wheat. The practice of crop rotation dates back to antiquity. Farmers have been using rotations for centuries and it is well known that crop rotations contribute to increase the growth and yield. One immediate economic benefit of crop rotations is improved yields. Cereal crop rotated with other crop result in higher yields when compared to continuous same cereal crop. Even greater benefits are usually obtained by rotating two distinctly unrelated crops, such as a grain seeded into land where the previous crop was a legume. For example, maize, in a two-year rotation with soybean, yielded about 5 to 20% more than continuous maize, and even more increased yield was achieved with rotations more than 2 years (Crookston et al., 1991; Peterson and Varvel, 1989). In addition, introduction of alfalfa soil (rhizospheric soil) to the soil supporting maize growth resulted in substantial increase of growth in maize after 5-8 weeks of growth (Fyson and Oaks, 1990). Although the abundance of chemical fertilizers and pesticides made the use of rotation-based farming systems decline during the 1950s and early 1960s, various negative influences of overusing chemical fertilizers and pesticides, such as the high cost of off-farm input, the

growing incidence of pesticide and fertilizer contamination of water and the increasing resistance of certain weeds and insects to pesticides, made the importance of rotation-based farming systems reconsidered (Crookston et al., 1991; Bullock, 1992; Mitchell et al., 1991). Long-term studies led to the current consensus that crop rotations are essential to maintain high production levels and allow for sustained production (Mitchell et al., 1991; Bowren et al., 1995).

1.1.2 Utilization of Legumes in Rotation

The Leguminosae, one of the largest families of flowering plants with 18,000 species classified into around 650 genera, mainly locates in temperate and tropical regions (Sprent, 2001). Leguminous crops, such as soybean (*Glycine max* L.) and alfalfa, are widely used in crop rotations due to their outstanding ability of biological nitrogen fixation, converting atmospheric nitrogen into nitrogenous compounds useful to plants with the help of symbiotic Rhizobia living in their root nodules. The host plant provides reducing power to rhizobia for the reaction through which produce ammonia (NH_3) from the proton (H^+) acquired from the plant's carbohydrates and nitrogen from air, while the produced ammonia provides an abundant source of nitrogen for plant growth (Sprent, 2001; Hogh-Jenson and Schjoerring, 2001; Roper, 1983). In a perennial grass and legume mixture, approximately 36% of the N needs of grass plants growing around legumes come from the support of nitrogen fixation legume nodules (Auburn, 1998). Nitrogen can also be released from the relatively high-protein legumes through bacterial decomposition

in soil (Auburn, 1998). Generally speaking, two thirds of the nitrogen fixed in nodules becomes available for later plant growth (Auburn, 1998). For perennial or biennial legumes, such as alfalfa or sweetclover, the productivity of biological nitrogen fixation is about 40 to 70 pounds per ton of forage. After the harvest of the crop, about 5 to 15 pounds nitrogen will be released from the remaining stubble and roots of each ton of removed forage into soil (Ebelhar *et al.*, 1984; Heichel and Henjum, 1991). Therefore, not only does the nitrogen fixed in legume nodules contribute to the growth of leguminous crops, it also improves soil N fertility by releasing some fixed nitrogen into soil. Thus, it has been widely believed to be the main reason that legumes have been widely used in crop rotations and inter-cropping practices for centuries.

However, it has been found that the nitrogen residue of legume plants is not a satisfactory explanation for the whole growth stimulation of rotating crops (Baldock, *et al.*, 1981, Copeland and Crookston, 1992). Recent studies showed that only about 25% of plant growth promotion induced by crop rotation is due to the nitrogen leftover by rotating legume crops (Bolton *et al.*, 1976, Fyson and Oaks, 1990). There must be some other factors responsible for the remaining 75%. This finding stimulated many researchers to look for other factors, which are responsible for the major benefit seen in crop rotation with legumes. Several factors other than nitrogen fixation have been proposed to explain the beneficial effect of legumes in rotation such as increase of soil organic matter, recycle of nutrients, diversification of soil microbial communities,

decrease of soil pH, improvement of soil water-holding capacity, breaking of insect and disease cycles and weed problems of grass-type crops, and so on (Bullock, 1992; Lugtenberg *et al.*, 1991; Doran and Smith, 1987; Tisdall and Oades, 1982; Regnier and Janke, 1990). However, most factors and mechanisms of those benefits are not completely understood, and none of those factors/or combination of the factors can satisfactorily explain the rotation benefit.

1.1.3 Byproduct of Nitrogen Fixation: Hydrogen Gas

Studies from the 70's have shown that hydrogen gas is an obligate byproduct of the biological nitrogen fixation process in legume nodules. This hydrogen evolution costs about 35% of reducing power and ATP flowing through the nitrogen-fixing enzyme, which represents an energy equivalent to about 5% of the crop's net photosynthetic C (Hunt and Layzell, 1993; Dong and Layzell, 2002). In some legume nodules, symbiotic bacteria (rhizobia) have the ability to produce uptake-hydrogenase (HUP) that re-oxidized most hydrogen within nodules. Therefore, a portion of the reducing power, used by the H_2 production during nitrogen fixation, is recovered. However, many of most productive nitrogen-fixing symbioses lack uptake-hydrogenase activity (Uratsu *et al.*, 1982). H_2 produced by those Hup$^-$ rhizobia during nitrogen fixation just diffuses out from the nodules into the soil. The existence of HUP used to be considered as a beneficial character because of its ability to recover a portion of the energy used for hydrogen production, while the loss of hydrogen from legume nodule to soil is traditionally

believed to be a disadvantage of HUP^- over HUP^+. However, it is conflicting to note that the majority (75%) of the rhizobia strain isolated from major soybean production areas in United States and all known clover and alfalfa symbioses are HUP^-. The evolutionary process, plant breeding of agricultural crops, or selection of optimal nitrogen-fixing bacteria prefer HUP^- to HUP^+, which seems that the energy loss exert beneficial influences on crop growth (Uratsu *et al.*, 1982; Welbaum *et al.*, 2004). It is supposed that hydrogen diffused from legume nodules may fertilize soils and contribute to plant growth (Dong and Layzell, 2002).

1.1.4 Hydrogen Metabolism and Plant Growth Promotion

Soil is a major sink for hydrogen. Despite high rates of H_2 evolution from legume root nodules, little or no H_2 escapes from the soil surface (Conrad and Seiler, 1979). Most H_2 was oxidized by microbes and free enzymes in the soil adjacent to Hup^- legume nodules after released (La Favre and Focht, 1983). The soil around the HUP^- legume nodules typically develops the capacity to take up H_2 within 8-10 days of exposure to H_2, which is associated with several obvious changes, such as higher H_2 oxidation kinetics (La Favre and Focht, 1983), an increase of rhizopheric microbial biomass which has a highly significant correlation with the soil H_2 uptake rate (Popelier *et al.*, 1985.), greater rates of O_2 consumption and chemoautolithotrophic CO_2 fixation (Dong and Layzell, 2001). All those changes show potential contribution of hydrogen metabolism to plant growth promotion.

Through comparing the growth of various crops in soils which were pretreated by air or by H_2 in air with the same exposure rate as the soil near legume nodules during plant growth, the growth of crops in hydrogen treated soils with high hydrogen uptake rate was found to be promoted significantly, which proved that one of the obvious benefits of rotation with legumes is that metabolism of released hydrogen in soil stimulates plant growth (Dong *et al.*, 2003). Furthermore, the promotion of plant growth due to hydrogen metabolism in soil can be eliminated by the treatment of bactericides, whereas the treatment of fungicides did not bring any significant negative effect on it. While the causative agents responsible for soil hydrogen uptake have not been conclusively identified, they appear to be bacterial in nature (McLearn and Dong, 2002; Irvine *et al.*, 2004).

1.1.5 Isolation of Soil Hydrogen-Oxidizing Bacteria

A group of bacteria is physiologically defined as aerobic hydrogen-oxidizing bacteria due to its ability to utilize gaseous hydrogen as an electron donor with oxygen as an electron acceptor, and to fix carbon dioxide to grow chemolithoautotrophically. The characteristic enzyme of this group is hydrogenases, catalyzing the reversible redox reaction with molecular hydrogen according to the following equation: $H_2 \longrightarrow 2H^+ + 2e^-$. They play a central role in energy metabolism of hydrogen-oxidizing bacteria. It was reported that aerobic hydrogen-oxidizing bacteria are not physiologically homogeneous and comprise species from diverse taxonomic units including the so called Knallgas

bacteria (Aragno and Schlegel, 1992), nitrogen fixing bacteria (Evans *et al.*, 1987) and photosynthetic microorganisms. According to the characteristics of aerobic hydrogen oxidizing bacteria, the best habitats for them should be places where both oxygen and hydrogen are available. Hydrogen gas was oxidized within a few centimeters (or less) from nodules. So, Hydrogen-oxidizing bacteria should be abundant in soils adjacent to Hup^- nodules (Bowien and Schegel, 1981).

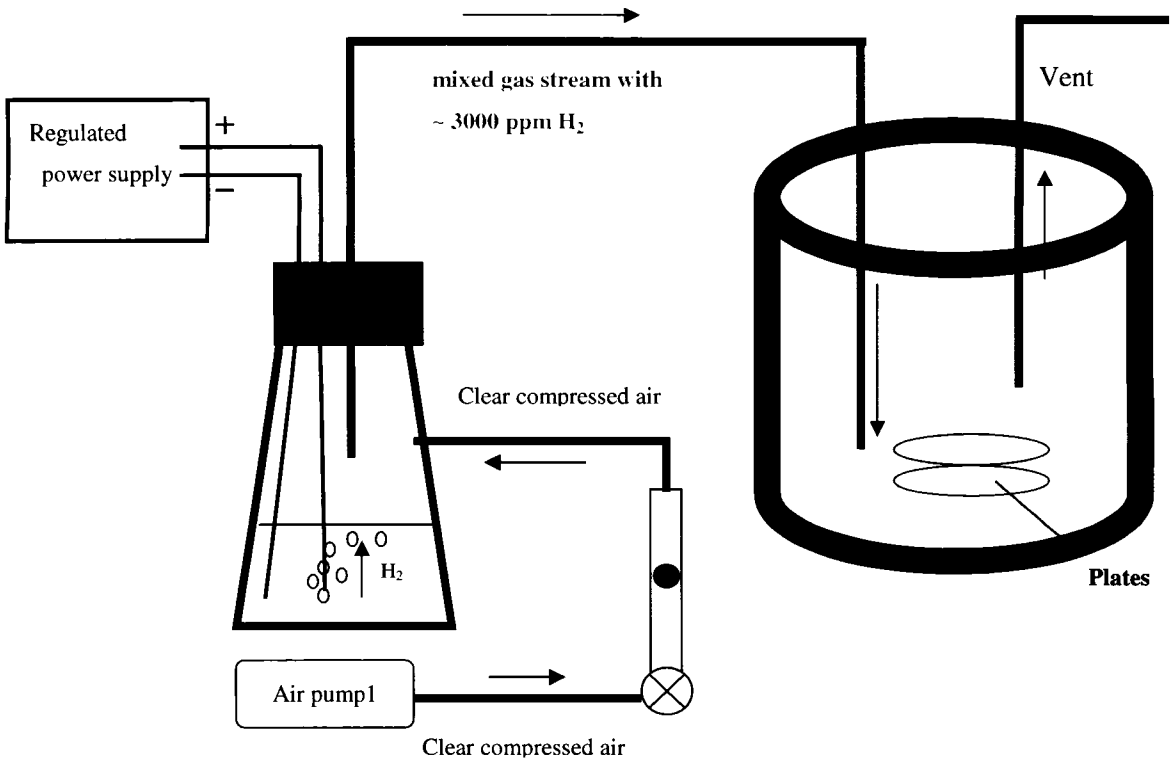
While some of the soil H_2 oxidizing bacteria and shift in microbial populations in response to soil exposure to H_2 can be detected by modern molecular techniques without prior isolation of the organisms involved (Lechner and Conrad, 1997; Stein *et al.*, 2005), ultimately it will be necessary to isolate and characterize the H_2 oxidizing bacteria before it is possible to fully understand their metabolic and physiological interactions with plant growth. Microbiologists have been investigating the isolation and characteristics of hydrogen-oxidizing bacteria over 30 years and have managed to isolate some species of hydrogen-oxidizing bacteria belonging to the gram-positive and the gram-negative genera through classical enumeration techniques, such as the direct plate technique or the liquid enrichment cultures with soil, mud, or water samples as inocula (Aragno and Schlegel, 1992). Due to the fact that most of aerobic hydrogen oxidizing bacteria studied so far are autotrophs or facultative autotrophs, the use of purely autotrophic conditions either on solid or in liquid media provides the most certain and simplest means to select bacteria representative of this group (Aragno and Schlegel, 1992). The principles of selection and

isolation of aerobic hydrogen oxidizing bacteria are simple: the aerobic hydrogen oxidizing bacteria are able to grow on mineral in the presence of a gaseous atmosphere containing hydrogen, oxygen and carbon dioxide (Veldkamp, 1970). Total 19 strains of aerobic hydrogen oxidizing bacteria were isolated by Maimaiti (2005) from soils adjacent to Hup$^-$ soybean nodules and H_2 treated soils through using an open gas flow incubation system (Figure 1) in which the H_2 generated by electrolysis in atmosphere air was kept at a stable concentration around 3000 ppm and the partial pressures of CO_2 and O_2 maintained close to the atmospheric levels, which is close to the natural growing environment of H_2 oxidizing bacteria. Most of them belong to *Variovorax*, Burkholderia, and *Flavobacterium* according to conventional identification tests and 16S rDNA sequence analysis.

1.1.6 Isolates and Plant Growth Promotion

All these isolates showed ability to significantly stimulate root elongation of spring wheat seedlings and increase dry-weight of spring wheat seeds (Maimaiti, 2005). Combining all these facts, it is obvious that soil hydrogen oxidizing bacteria stimulate plant growth by utilizing considerable energy released from the process of H_2 oxidization in soil. Several studies in our lab showed that hydrogen treatment and rotations with Hup$^-$ legume crops result in the increase of biomass (e.g. root, seed dry weight)

Figure 1: Open gas flow incubation system designed by Dong and Layzell, (2001)



or variation of morphogenesis (e.g. tiller number) in plants (Dong and Layzell, 2002). It seems that some plant growth regulators such as phytohormones might play a role in plant growth promotion. It was reported that some strains belonging to *Variovorax* and *Burkholderia* are able to lower the level of plant-produced ethylene (Glick *et al.*, 1998; Belimov *et al.*, 2001). It was hypothesized that our isolates of hydrogen-oxidizing bacteria have the ability to promote plant growth by lowing of plant ethylene levels.

1.2 RHIZOSPHERE BACTERIAL POPULATIONS AND PLANT GROWTH

1.2.1 Soil Microbial Populations

Soil is a complex and dynamic system which is an essential part of the terrestrial ecosystem. It is considered a storehouse of a wide range of microorganisms including bacteria, fungi, algae, viruses and protozoa. The microbial populations may go up to 10 billion cells of possible thousands of different species per gram soil (Pankhurst *et al.*, 1995; Bollon *et al.*, 1993). Even though the volume of soil microbes is small, the varied genetic and functional activities of extensive microbial populations are critical to plant growth through the maintenance of soil health and quality because they are involved in such key processes as soil structure formation, decomposition of organic matter, the biogeochemicial cycles of the main elements (e.g. carbon, nitrogen, sulphur, phosphorus) and trace elements (e.g. iron, nickel, mercury), the energy and nutrient exchanges, plant

growth regulator metabolism, toxin removal, suppression of soilborne plant diseases (Bloem and Breure, 1997; Wall and Virginia, 1999; Arias *et al.,* 2005; Doran and Smith, 1996; Glick *et al*, 1999). However, most soil bacteria were still unknown to us. Studies of soil microbial community structure has been focused on rhizosphere for long time. It is well known that microbial community structure is distinctly different between bulk soil and rhizosphere in which the microbial diversity is often extensive (Giri *et al.*, 2005).

1.2.2 Rhizosphere Plant-microbe Interactions

The rhizosphere is generally define as the volume of soil adjacent to and affected by the plant roots (Mantelin and Touraine, 2004). The plant roots not only absorb mineral nutrients and water from soils for plant growth but also release a wide range of organic compounds which contain sugars, amino and organic acids, fatty acids and sterols, vitamins, nucleotides and some other organic chemicals into the surrounding soil (Rovira, 1979; Curl and Truelove, 1986). Various organic compounds released from plant roots increase the concentration of nutrients and soluble carbon, which enhances the growth and populations of microbes in the rhizosphere (Norton and Firestone, 1991). In addition to soluble carbon input, plant root activity also leads to several other physical and chemical alterations of rhizosphere which influence the component and activities of bacteria in rhizosphere such as the levels of water potential, pH, oxygen content and redox potential in rhizosphere soil (Hedley *et al.*, 1982; Bolton *et al.*, 1992). Thus, the root activities provide unique microenvironments to activate and sustain microbial

communities in rhizoshpere and the composition of rhizosphere microorganisms is determined by the quantity and nature of the root exudates and physicochemical conditions of rhizosphere soil (Marschner *et al.*, 2002; Semenov *et al.*, 1999). Not surprisingly, the diversity of microbial populations on the surface of plant roots and in the rhizosphere is more complex than in soil where roots are absent (Curl and Turelove, 1986; Maloney *et al.*, 1997). Most rhizosphere microbes should have the ability to bring either detrimental or beneficial influence on the ecosystem through inducing numerous plant-microbe or microbe-microbe interactions which are applied to exert influence on the growth conditions for both the plants and the microbes in rhizosphere (Bowen and Rovira, 1999). Some rhizosphere microbes are considered microbial pathogens to plants due to their considerable damage to crops. In contrast to these plant microbial pathogens, some rhizosphere microbes were reported to have the ability to antagonize plant pathogens by competition for nutrients, stimulation of plant induced systemic resistance, and/or production of inhibitory compounds such as secondary metabolites (antimicrobial metabolites and antibiotics) and extracellular enzymes (antibiotic). Some beneficial rhizosphere microbes referred as biofertilizers can contribute to plant growth by improvement of the fertility status of the soil, including biological nitrogen fixation, decomposition of the organic matter entering the soil (e.g. plant litter), increase of mineral nutrients (e.g. phosphorus) available to plants, and so on. Similarly, some improve plant health and contribute to higher crop yield by producing certain compounds (e.g. vitamins, plant hormones,)

1.2.3 Diversity of Rhizosphere Plant Growth Promotion Bacteria

Bacteria present in the rhizosphere can be categorized into two groups. On the base of their influences on plant growth, some of these bacteria belong to plant pathogens due to their negative effects on plant growth (Lugtenberg *et al.*, 1991; Persello-Cartieaux *et al.*, 2003), while other are considered as plant growth promoting bacteria selected and enriched in rhizosphere by activities of plant roots (Barea *et al.*, 2004). The latter can be generally distinguished into two subgroups according the way they interact with plant roots. One is the bacteria belonging to the genera *Rhizobium* and *Bradyrhizobium*. They are able to form a symbiotic relationship with the roots of most legume plants, resulting in the generation of morphologically distinct structure, nodules, in which rhizobia, together with host plants, convert atmospheric nitrogen into nitrogenous compounds useful to plant growth (Broughton and Perret, 1999; Albrecht *et al.*, 1999). The other is the group of beneficial free-live soil bacteria which stimulate plant growth without developing such symbiotic associations with plant roots and is referred as plant growth promoting Rhizobacteria (Bashan and Holguin, 1998). They can survive without the supports of root exudates, but have the ability to efficiently utilize organic compounds released by roots in competition with other rhizosphere microbes (Tilak *et al.*, 2005; Kloepper *et al.*, 1991; Kloepper, 1994). Most of PGPR isolates identified in the last few decades belong to several genera such as *Acetobacter, actinoplanes, Agrobacterium, Alcaligens,Arthrobacter, Azospirillum, Azotobacter, Bacillus, Cellulomonas,*

Enterobacter, Erwinia, Flavobacteriumn, Pasteuria, Pseudomonas, Serratia, Xanthomonas (Tilak *er al.,* 2005). Among these genera, *Pseudomonas and Bacillus* are most frequently mentioned as PGPR. Several stains of PGPR are currently available as commercial products for agricultural production (Lucy *et al.*, 2004; Dobbelasre *et al.*, 2001; Vessey, 2003).

PGPR can be further divided into two classes on the base of the different ecosystem processes they are involved in. One can promote plant growth directly through nutrient cycling and regulation of Plant Growth Regulator metabolism (Bashan and Holguin, 1998). Some PGPR are described as biofertilers because they have the ability to provide plant available nutrients through mobilization of phosphoras and non-symbiotic nitrogen fixation. PGPR diazotroph isolates belonged to many genera, such as *Azomonas, Bacillus, Beijerinckia, Clostridium, Corynebacterium, Derxia, Herbaspirillum, Klebsiellas, Pseudomonas*, etc. (Kennedy *et al.* 2004; Tilak *et al.,* 2005). Many PGPR have the ability to soluble inorganic P of soil and make it available to plants, referred as Phosphate-Soluble Bacteria (PSB). The most efficient PSB belong to genera *Bacillus and Pseudomonas* (Mishra. 1985; Richardson 2001; Kucey *et al.*, 1989; Tilak *et al.*, 2005; Gaind and Gaur, 1991). In addition to non-symbiotic nitrogen fixation bacteria and PSB, this group also includes some others species belonging to *Azospirillum, Variovorax* and *Burkholderia* which improve nutrient uptake through regulating phytohormones (Glick *et*

al., 1998; Belimov *et al.*, 2001; Okon, 1994; Bashan, 1999; Lucy *et al.*, 2004; Zahir *et al.*, 2004).

The other class of PGPR promotes plant growth indirectly through biocontrol of microbial plant pathogens. Some species have the ability to constrain growth of plant pathogens and reduce root infection frequency through release of antibiotics and/or competition for nutrients or space in rhizosphere. Numerous reports showed that some *Pseudomonades* species are able to produce various antibacterial or/and antifungal metabolites (e.g. 2,4-diacetyphloroglucinol (DAPG), Oligomycin A, Oomycin A, HCN) to eliminate plant pathogens, or/and a range of iron chelating compounds or siderophores with a very high affinity for ferric iron to limit growth and activity of plant pathogens by lowering ferric iron available to them (Picard *et al.*, 2004; Nielsen *et al.*, 1998; Whipps, 1997; Loper, 1991; Loper and Henkels, 1999; O'Sullivan and O'Gara, 1992). Some non-pathogenic rhizosphere-colonizing *Bacillus* and *Pseudomonas* species control the plant pathogens through stimulating induced systemic resistance (ISR) of plants, such as forming new barriers beyond frequently infected sites (e.g. callose, lignin, phenolice), reinforcing epidermal and cortical cell wells, increasing activities of relevant enzymes (e.g. chitinase, peroxidase, polyphenol oxidase), enhancing production of phytoalexine, expressing stress-based genes (Kloepper *et al.*, 2004; Chen *et al.,* 2000; van Peer *et al.*, 1991).

1.2.4 Dynamics of Soil Microbial Communities

Different response of microbes to variation of microhabitat in soil results in the variation of microbial communities. It has long been recognized that soil microbial community structure is a dynamic concept and agriculture management regime such as crop rotation, tillage, herbicide and fertilizer application, and irrigation exerts significant influence on it (van Veen *et al.*, 1997). Even though there are many details incompletely elucidated, numerous previous studies on different plant grown in different locations showed that the key determinative factors of agricultural practice-induced variation of soil microbial populations are different carbon and energy resources provided by specific plants and physiochemical conditions of soil (e.g. distribution of different particle sizes, pH, cation exchange capacity) (Giri *et al.*, 2005; Maloney *et al.*, 1997; Semenov *et al.*, 1999; Garbeva *et al.*, 2004).

Potential microbial activities in rhizosphere were reported to vary obviously with different crops (e.g. wheat, ryegrass, bentgrass, clover). Grayston *et al* (1998) found that potential microbial activities significantly varied with plant type, while none was observed in the two types of soils. By cultivation-based and culture-independent methods (16S rRNA gene library), Germida *et al.* (1998) and Kaiser *et al.* (2001) demonstrated the important role of crop type in selection of rhizosphere bacterial communities with canola, wheat, and several species of oilseed rape grown in fields. The compounds of root exudates vary with plant growth and development, which means the stages of plant

growth and development can also exert effects on the structure of rhizosphere microbial communities. di Cello *et al.* (1997) and Seldin *et al.* (1998) observed the variation of bacterial communities in maize rhizosphere during the plant growth. It was also proved by the study of Gyamfi *et al.* (2002).

Soils are always complex and variational. They can affect structure of microbial populations either directly by providing a specific habitat for selecting microbial populations or indirectly by influencing plant root activities. By using direct PCR-DGGE, it was found that similar bacterial communities tend to present in the similar soil types through comparing DGGE patterns of 16 different soils from different geographical locations. Some other studies (e.g. Groffman *et al.*, 1996; Buyer *et al.*, 1999) also indicate that property of soil exerts marked influence on microbial populations in rhizosphere.

1.3 ASSESSMENT OF SOIL BACTERIAL DIVERSITY

The diversity of bacterial communities in soil is dynamic and exceptionally complex. One gram of soil may contain up to 10 billion cells of possibly 4,000-7,000 of different species (Bianchi and Biachi 1995). Numerous studies of microbiological ecology have long focused on assessment of soil bacterial diversity to answer two key questions: What controls the diversity of the soil bacterial communities? And how does the soil bacterial community structure change with time in response to their environment?

Traditionally, soil bacterial populations were analyzed through metabolic, morphologic and physiological traits of isolated bacteria based on cultivation-dependent approaches (e.g. plate counts, community level physiological profiling) (Gerhardt, 1981). However, studies of bacterial diversity at the genetic level showed that only about 1% of the soil bacterial populations can be cultured by a wide range of media (Kruske *et al.*, 1997). To increase the ability to obtain necessary information of hidden diversity from 99% uncultivable bacteria in soil, numerous molecular-based methods were developed to study soil bacterial populations by using total DNA extracted from the environment. Most of these methods picture the structure of bacterial populations on the basis of a phylogenetic marker, 16S rRNA gene (Woese, 1987; Torsvik and Ovreas, 2002).

1.3.1 G+C Analysis

G+C analysis first described by Holben and Harris (1995) is a method used to show the structure of bacterial populations through the separation of soil DNA with different guanine plus cytosine (G+C) content which is relate to taxonomy. The resolution of this method is relatively coarse because several taxonomic groups may appear the same G+C range (Vandamme *et al.*, 1996). This method can show the changes of bacterial communities, but nothing about other aspects of diversity such as richness, evenness, and composition. This method also requires a reasonably large amount of DNA (e.g. 50μg) and an ultracentrifuge to separate the G+C fraction (Holben et al., 1995;

Nusslein et al., 1999). All these disadvantages mentioned above made it unpopular in the assessment of soil bacterial populations.

1.3.2 Clone Libraries

Sequencing 16S rRNA genes cloned in libraries has been considered the most powerful method applied to display bacterial diversity. Even though the picture of diversity provided by this method has a fine resolution, it is too laborious, time consuming, and expensive due to the requirement of quite large cloning, especially for studying soil bacterial population dynamics (Hugenholtz *et al.*, 1998; Garbeva *et al.*, 2004). Combination of clone libraries and hybridization techniques using oligonucleotide or polynucleotide probes make it easer and simpler to display dynamics of soil bacterial populations, but it scarified part diversity of minor bacterial populations. Also, requirements of known sequence data for probe design and the specificity of probes suppress its utilization in assessing soil bacterial populations (Muyzer, 1999)

1.3.3 PCR Based Bacterial Community Fingerprinting Techniques

Fingerprinting techniques are a series of methods that are extensively applied to study bacterial community structure and dynamics through distinguishing the PCR-amplified 16S rRNA genes or other genes belonging to different taxonomic groups in different ways. Most of these approaches can be divided into three groups based on the ways used for separation of DNA with different sequences.

Firstly, Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are two similar methods because both of them differentiate PCR products with different sequences on the basis of relative helix stabilities in a denaturant or thermal gradient gel (Muyzer *et al.*, 1993). These approaches were originally developed to examine point mutations due to their high sensitivity. However, the gel system employed limits the resolution of diversity because DNA fragments with different sequences may have the similar mobility traits in gel and present in the same band. According to the report of MacNaughton *et al.* (1999), only dominant specie, about 1-2% of bacterial populations, can be detected in environmental samples. Moreover, it is impossible to establish a comparative sequence database to indicate the relationship of Tm to sequence.

Secondly, Single Strand Conformation Polymorphism (SSCP) is another method applied to separate amplified DNA with different sequences through the differentiation of electrophoretic mobility resulted from folded secondary structure of single strand DNA (Lee *et al.*, 1996). SSCP have been used to detect the changes of bacterial populations in several studies. However, it is not a popular method due to its limitations similar to those of DGGE/TGGE (Schmalenberger *et al.*, 2001; Schwieger and Tebbe, 1998).

Finally, restriction fragment length polymorphism (RFLP) based amplified ribosomal DNA restriction analysis (ARDRA) is another tool used for analyzing bacterial populations. Distinguishing of different populations is based on 16s rRNA gene fragment

length polymorphisms of the restriction digestion. This approach is frequently used to screen cloned isolates before sequencing (Pace *et al.*, 1986). Recently, RFLP based methods were frequently used to study soil bacterial population structure. It was good at detection of changes in populations, but is not effective tool to measure diversity because the exceptional complexity of RFLP profile of diverse communities lower the resolution resulting in loss of the phylogenetic information important for community analysis (Smit *et al.*, 1997).

To avoid above-mentioned disadvantages, terminal restriction fragment length polymorphism (T-RFLP) was developed from RFLP. It has the same principle as RFLP except that one end of PCR products are labeled with fluorescent dye by using one fluorescent-labeled primer. For each species, only the restriction fragments with fluorescent dye can be detected by special sensors. Thus, the profiles generated by this way are complex but interpretable (Liu *et al.*, 1997). This alteration also makes automated systems (e.g. DNA sequencer) available to obtain robust TRF data, which increases the sensitivity of signal detection. However, T-RFLP, like other PCR-based methods, can't display the whole diversity of soil bacterial populations because the universal primers used are not available to all populations, and the template DNA of numerically dominant species can suppress the PCR products from minor populations (Liu *et al.*, 1997). Even though T-RFLP still can't picture exact diversity of bacterial populations, it has been

considered as the most effective tool and commonly applied to study bacterial diversity in soil (Liu *et al.*, 1997; Osborn *et al.*, 2000; Dunbar *et al.*, 2000).

1.4 OBJECTIVES OF PRESENT STUDY

The hydrogen gas released from Hup^- legume nodules into soil plays a key role in contributing to the benefits of legumes in crop rotations. The studies from our Lab showed that hydrogen treated soil also significantly increase plant growth compared with air-treat soil. The hydrogen treatment also results in accumulation of bacteria correlative with hydrogen metabolism in soil such as hydrogen oxidizing bacteria. Three genera of hydrogen oxidizing bacteria, *Variovorax, Burkholderia,* and *Flavobacterium*, were isolated from soils with high hydrogen uptake rate such as rhizosphere soil and hydrogen-treated soil, and none from low hydrogen uptake rate soils like bulk soil and air treated soil. All of them are capable of increasing the dry-weight of roots or the tiller number of crops. It has been showed that both hydrogen uptake and plant growth promotion are bacterial in nature (Mclearn and Dong, 2002; Irvine *et al.,* 2004). But the plant growth promotion mechanisms applied by our isolates of hydrogen-oxidizing bacteria are still unknown. The variation of bacteria community structure in soils induced by hydrogen metabolism has never been studied. Therefore, the present study investigates the possible plant growth promotion mechanisms by isolated H_2 oxidizing bacteria and effects of hydrogen metabolism on variation of soil bacterial community structure, which includes:

1. To investigate the possible plant growth promotion mechanisms of our isolates of hydrogen-oxidizing bacteria;

2. To assess variation of soil bacterial community structure resulting from the metabolism of hydrogen gas through comparing the TRF profiles from hydrogen treated soils with obvious ability of hydrogen uptake (e.g. Hup^- nodule rhizosphere soil, soils treated by hydrogen gas in laboratory) with the controls (e.g. Hup^+ nodule rhizosphere soil, oils treated by air in laboratory)

3. To estimate the contributions of our isolates to hydrogen-induced variation of soil bacterial community structure by examining the characteristic TRF peaks contributed by our isolates in profiles from hydrogen-treated soils.

2. MECHANISMS OF PLANT GROWTH PROMOTION BY ISOLATES OF HYDROGEN OXIDIZING BACTERIA

2.1 INTRODUCTION

An important plant hormone, ethylene, is produced in all higher plants (Salisbury and Ross, 1992). Although predominantly associated with fruit ripening, ethylene plays a role throughout the entire life of the plant and its ubiquitous regulatory functions exert effects on almost every aspect of plant growth and development (Deikman, 1997; Frankenberger and Arshad, 1995). It is also involved in some negative effects on plant growth, such as the inhibition of root elongation and nodulation of legumes by rhizobia (Mattoo and Suttle, 1991; Ma *et al.*, 2002). Recent work has show that the inhibitory effect of ethylene on plant growth can be reduced by some soil bacteria which had the ability to lower the concentration of plant ethylene.

Ethylene is produced from methionine. During its biosynthesis, there are two important intermediates, S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylate (ACC), and three enzymes, SAM synthetase responsible for methionine to SAM, ACC synthetase in charge of the synthesis of ACC from SAM, and ACC oxidase metabolizing ACC to ethylene (Ma *et al.*, 2002).

Some strains of plant growth promoting rhizobacteria, such as *Variovorax*, are able to take up and hydrolyze some of the ACC, the immediate precursor of ethylene in

high plants, exuded from seeds or roots by the activity of ACC deaminase which catalyses the cleavage of ACC. To keep the balance of internal and external ACC level, the decrease of external ACC stimulates the exudation of internal ACC, which reduces the amount of ACC available to synthesize ethylene inside the cells (Glick *et al.,* 1998; Belimov *et al.*, 2001) (Figure 2). On the basis of more and more experimental evidences, ACC deaminase is regarded as one of the key mechanisms which rhizobacteria used to promote plant growth, mainly root elongation. Many strains isolated from different soil samples taken from geographically disparate locations have the ability to use ACC as a nitrogen source to promote seedling root elongation under gnotobiotic condition (Glick *et al.*, 1998). Moreover, Shah *et al.* (1998), Holguin and Glick (2001) also made *Escherichia coli, Pseudomonas spp.*, and *Azospirillum brasilense* strains gain the ability to stimulate the elongation of the roots of canola seedlings through introducing ACC deaminase genes.

Some strains of the legume symbionts in the genus of Bradyrhizobium and the plant pathogen *Burkholderia andropogonis* have the ability to synthesize a phytotoxin, Rhizobitoxine (2-amino-4-(2-amino-3-hydropropoxy)-trans-but-3-enoic acid), which is a structural analogue of AVG (the ethylene inhibitor) (Eaglesham and Hassouna, 1982; Mitchell and Frey, 1988; Yasuta et al., 2001). Rhizobitoxine is usually regarded as a plant toxin in that it causes foliar chlorosis symptom in soybean. However, several recent studies have shown that rhizobitoxine has the ability to lower the level of endogenous

ethylene in the host plant through strongly inhibits

1-aminocyclopropane-1-carboxylate(ACC) synthase in the ethylene biosynthesis pathway, which probably resulted in enhancing nodulation and competitiveness of the legume (Figure 3). Duodu et al., (1999) reported a positive role of rhizobitoxine in the symbiosis between B. *elknii* USDA61 and *Vignaradiate* (mung-bean). Yuhashi *et al.,* (2000) found that rhizobitoxine production in B. *elknii* USDA94 promotes nodulation and competitiveness of legume, *Macroptilium atropurpureum.* Parker and Peters, (2001) proved the positive effect of rhizobitoxine synthesized by B. *elknii* USDA61on efficient nodulation in A. *edgeworthii.*

All our isolates of soil hydrogen oxidizing bacteria, belonging to *Variovorax*, *Flavobacterium* and *Burkholderia*, obviously stimulate root elongation. It is logical to hypothesize that their beneficial effect on plant growth promotion partly results from inhibiting endogenous ethylene production in the host plant by activity of ACC deaminase or rhizobitoxine.

2.2 MATERIAL AND METHODS

2.2.1 Samples

Ten strains of hydrogen oxidizing bacteria isolated by Mainaiti (2005) were used for measurements of ACC deaminase and rhizobitoxine activities. They belonged to the genera of *Variovorax, Burkholderia, or Flavobacterium* (Figure 4).

Figure 2: Mechanism of lowering endogenous ethylene concentration in host plants through ACC-deaminase (Glick et al., 1998)
The symbol ⊥ means inhibition; Key: SAM: S-adenosyl-L-methionine, ACC: 1-aminocyclopropane-1-carboxylate

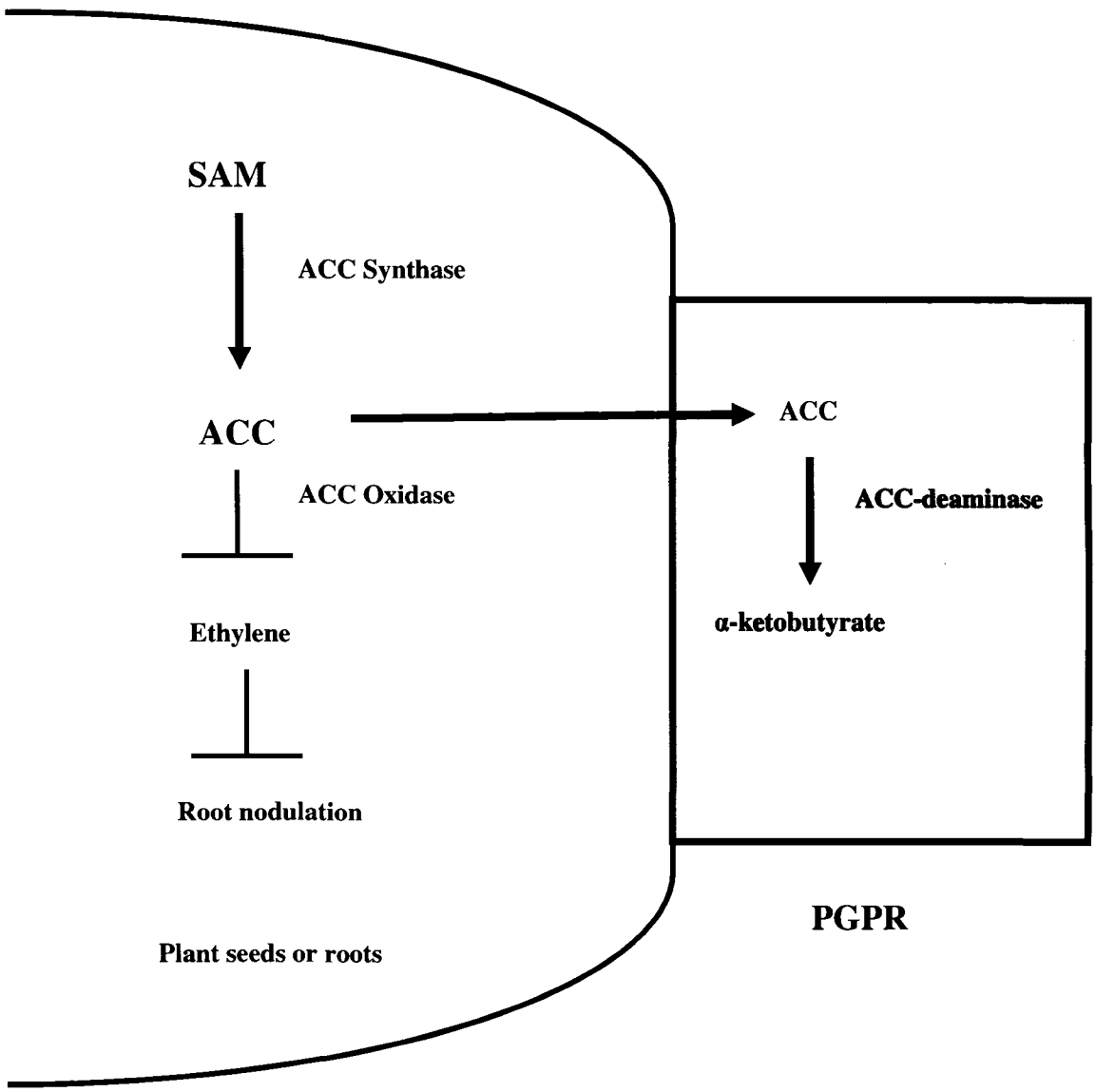


Figure 3: Mechanism of lowering endogenous ethylene concentration in host plants through rhizobitoxine (Yasuta et al., 1999)
The symbol ⊥ means inhibition; Key: SAM: S-adenosyl-L-methionine, ACC: 1-aminocyclopropane-1-carboxylate.

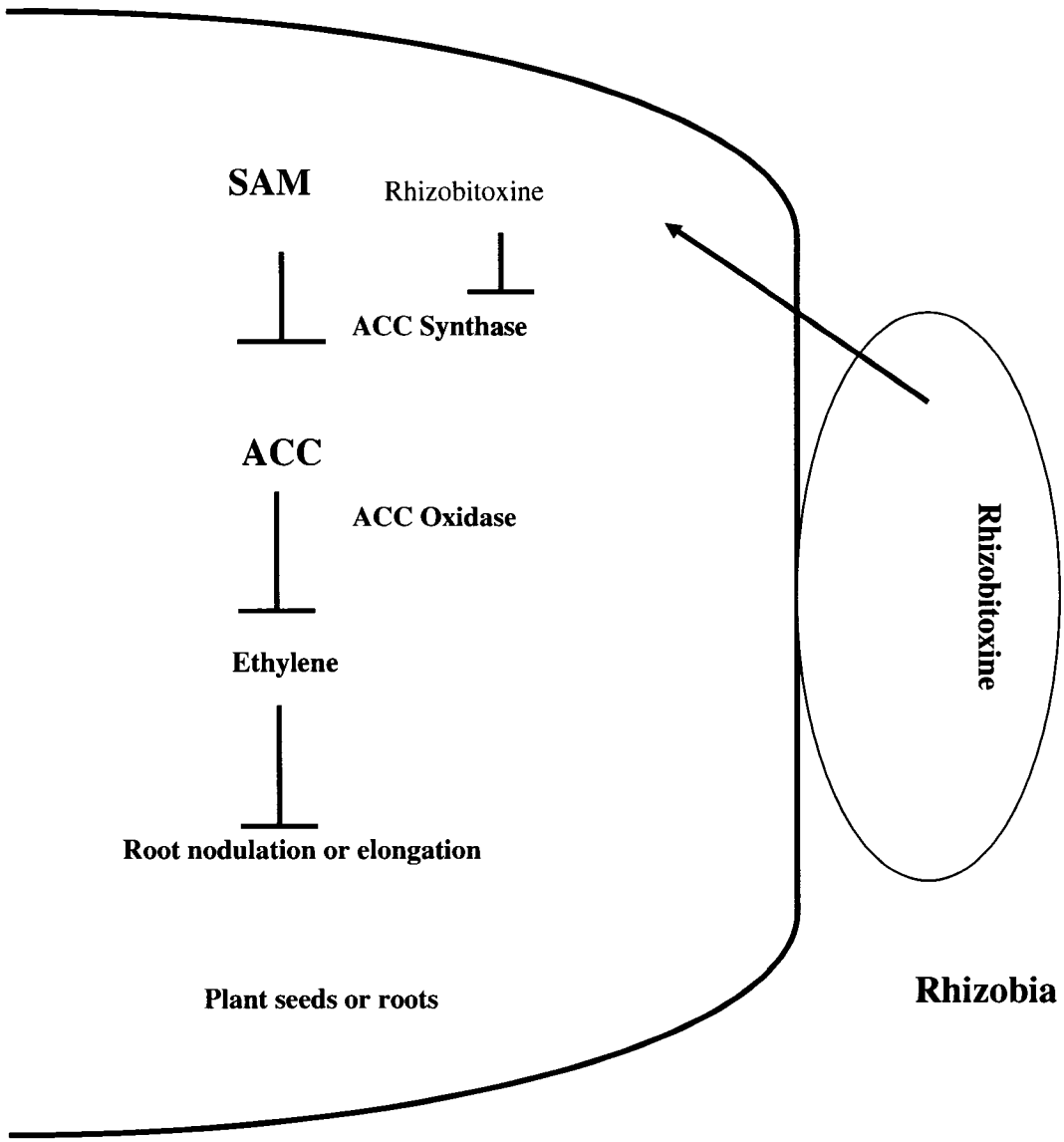
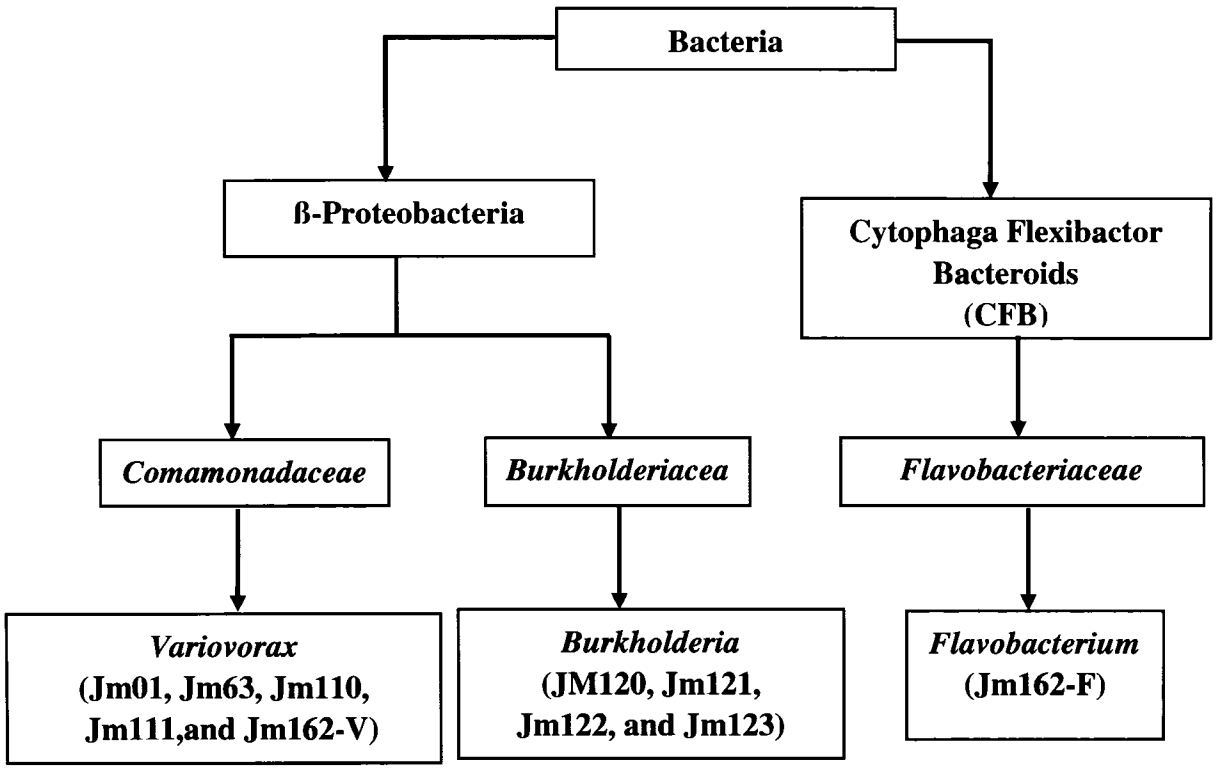


Figure 4: Classification outlines of isolates used for measurements of ACC deaminase and rhizobitoxine activities (Maimaiti, 2005)



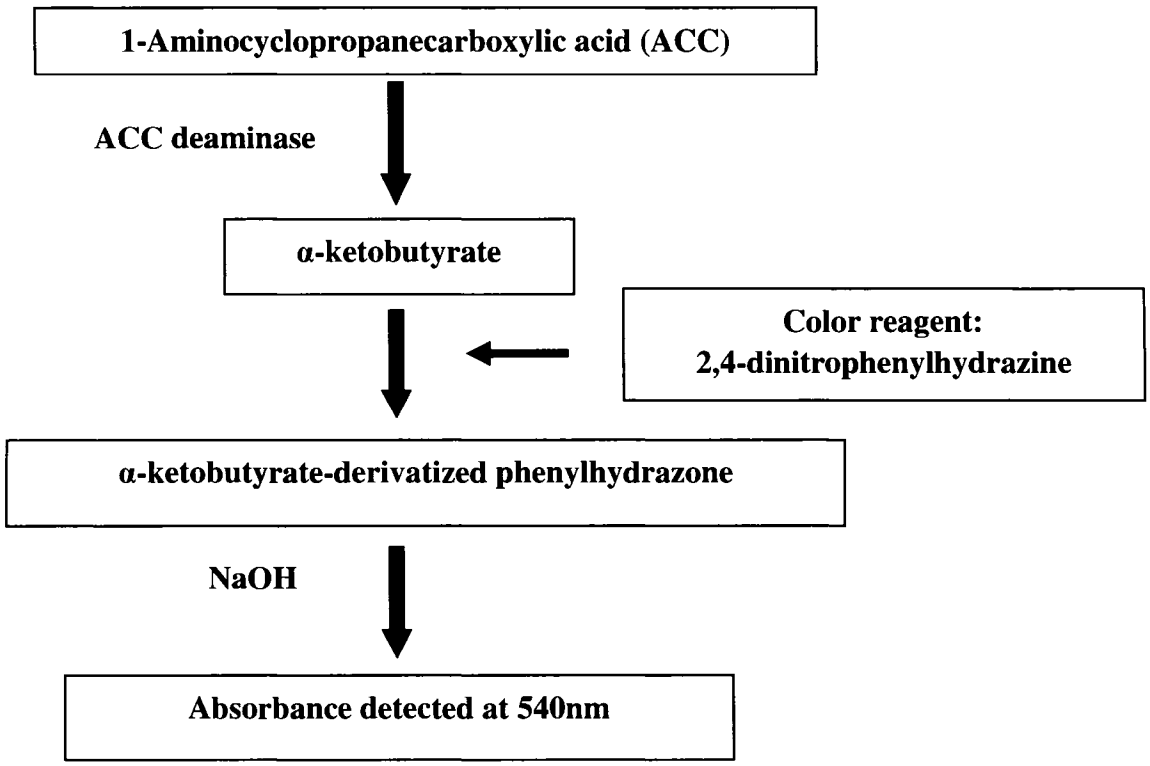
2.2.2 Measurement of ACC Deaminase Activity in Isolates

In order to assess bacterial ACC deaminase activity, the growth conditions for isolates must favor the induction of ACC deaminase. Therefore, isolates are first cultured in rich medium and then transferred to mineral medium with ACC as the sole source of nitrogen. Measurement of ACC deaminase activity is based on a modified method of Penrose and Glick (2003) which detects the amount of α- Ketobutyric Acid generated by activity of bacterial ACC deaminase (Figure 5). The number of μmol of α- ketobutyrate in solution is measured through comparing the absorbance of color reaction mixture at 540nm to a standard curve of α- ketobutyrate in the range between 0.1 to 1.0 mM.

2.2.2.1 Sample Preparation

Hydrogen oxidizing isolates (*Variovorax*: Jm01, Jm63, Jm110, Jm111, Jm162-V; *Flavobacterium*: Jm162-F; *Burkholderia*: Jm120, Jm121, Jm122, Jm123) from -80°C stock were inoculated into sterile MSA plates, and the plates were incubated for about one week at room temperature under the treatment of about 3000ppm H_2 gas generated by electrolysis in open gas flow incubation system as described by Dong and Layzell (2001) (Figure 1). Then isolates from MSA plates were inoculated in 2ml fresh LB broth and grown at 33 °C for 30-40 hr with shaking at the speed of 250 rpm. Two milliliter culture

Figure 5: Flow diagram of ACC deaminase activity measurement (Penrose and Glick, 2003)



was finally used to inoculate 30ml fresh LB broth. The culture was incubated at 33 °C with shaking at the speed of 200 rpm until the stationary phase was reached. The stationary phase cells of each isolate are harvested from broth by centrifuging at 10,000g for 10 min at 4°C. The supernatant is removed and the cells are washed twice with 0.1 M Tris-HCl (pH7.5).

2.2.2.2 Preparation of Sterile ACC Stock Solution and Mineral Medium with ACC as the Sole Source of Nitrogen

For 0.5M sterile ACC stock solution preparation: 505.5 mg ACC power (Sigma-Aldrich Co. USA, MW: 101.1) was firstly dissolved in 10ml of 0.1M Tris-HCl (pH 8.5). Then 0.5M of ACC solution was filter-sterilized through a syringe driven filter with 0.22-μm membrane (Millipore Corporation). Finally, the filtrate was collected, aliquoted (500μl/tube) and frozen at -20°C.

Just prior to inoculation, the 0.5M sterile ACC stock solution was thawed and three 500-μl aliquots were added to 500ml sterile nitrogen free MSA (mineral salt agar) broth (10g/L sucrose, 1.7g/L Na_2HPO_4, 1.2g/L K_2HPO_4, 0.5g/L $MgSO_4$, 0.5g/L KCl, 0.14g/L KH_2PO_4, 0.01g/L $Fe_2(SO4)_3$, pH 7.2±0.2).

2.2.2.3 Induction of Bacterial ACC Deaminase Activity

Following an additional centrifugation for 10min at 10,000*g at 4°C, the washed cells of each isolates were resuspended in 17ml sterile MSA broth (negative control) and 17 ml sterile nitrogen free MSA broth supplemented with 1.5 mM ACC (induction of bacterial ACC deaminase), and then incubated at 33 °C with shaking (200 rpm) for 40 hr (Ma *et al.*, 2003).

2.2.2.4 Count of Bacterial Cells in Each Culture

Each culture was divided into two potions: P-1 (15ml) for ACC deaminase activity measurement, P-2 (2ml) used to detect the O.D. value of each culture by spectrophotometer at 600nm. The number of cells per millilitre culture was calculated according to the following equation ①:

$$n\ (\text{cells/ml}) = O.D._{600} * 8.80 * 10^{8}\ \text{cells/ml} \quad \text{----------} \quad ①$$

2.2.2.5 Establishment of Alpha-ketobutyric Acid Standard Curve

A) Preparation of 1mM α- ketobutyrate stock solution from which a series of standards with known concentrations were made:

Firstly, stock solution A (100mM α- ketobutyrate) was prepared by dissolving 102 mg α- ketobutyric acid (Sigma-Aldrich Co. USA, MW: 102.09) in 10ml of 0.1 M

Tris-HCl (pH8.5) and stored at 4°C. Then, 1 ml of stock solution A (100 mM) was diluted with 9ml of 0.1 M Tris-HCl (pH8.5) to make stock solution B (10 mM α- ketobutyrate). Finally, stock solution C (1 mM α- ketobutyrate) was prepared through diluting 1 ml of stock solution B (10 mM) with 9ml of 0.1 M Tris-HCl (pH8.5).

B) Preparation of Standards (series of α- ketobutyrate solution with gradient concentrations listed in Table **1**)

C) Color reaction of α- ketobutyrate standards listed in Table **1**:

Firstly, 300μl of 0.2% 2, 4-dinitrophenyl-hydrazine in 2M HCl (Sigma-Adlrich Co. MW: 198.14) was mixed together with each α- ketobutyrate standards (200μl). Secondly, the mixture was vortexes and incubated at 30 °C for 30min to generate phenylhydrazone which has the ability to induce colored reaction after the addition of NaOH. Finally, the absorbance of the reaction mixture was determined at 540nm (Each standard has 10 replicates).

D) Generation of standard curve of α- ketobutyrate concentration VS. O.D.540nm:

All data collected from previous color reaction were divided into ten groups: A (0.1mM, O.D.540nm), B (0.2mM, O.D.540nm), C (0.3mM, O.D.540nm), D (0.4mM, O.D.540nm), E (0.5mM, O.D.540nm), F (0.6mM, O.D.540nm), G (0.7mM, O.D.540nm), H (0.8mM, O.D.540nm), I (0.9mM, O.D.540nm), J (1.0mM, O.D.540nm). Then, each

Table 1: Preparation of α- ketobutyrate standards with gradient concentrations with 1mM stock solution

Standards		Stock C (1mM)	0.1M Tris-HCl (pH8.5)	Total Volume
A series of α- ketobutyrate solutions with gradient concentrations	0.1 mM	20μl	180μl	200μl
	0.2 mM	40μl	160μl	200μl
	0.3 mM	60μl	140μl	200μl
	0.4 mM	80μl	120μl	200μl
	0.5 mM	100μl	100μl	200μl
	0.6 mM	120μl	80μl	200μl
	0.7 mM	140μl	60μl	200μl
	0.8 mM	160μl	40μl	200μl
	0.9 mM	180μl	20μl	200μl
	1.0 mM	200μl	0μl	200μl

group contributed a statistical point in 2D X-Y coordinates (X: concentration of standard, Y: Mean O.D.540nm +SD). Finally, the standard curve of α- ketobutyrate concentration VS. O.D.540nm was fitted as linear function based on all these points.

However, before the standard curve was plotted, the data validity of each group was inspected by ANOVA One-way analysis on the base of MINLAB software. Then, ANOVA one-way analysis was used to inspect the slopes of adjacent points: S1 (A, B), S2 (B, C), S3 (C, D), S4 (D, E), S5 (E, F), S6 (F, G), S7 (G, H), S8 (H, I), S9 (I, J). Due to the linearity of standard curve of α- ketobutyrate concentration against OD540nm, there was no statistically significant difference among those slopes. If there were any statistically significant difference among those slopes, the data belong to relative groups were considered inaccurate and couldn't be used to plot the standard curve.

2.2.2.6 Measurement of α-ketobutyrate Generated by Bacterial ACC Deaminase Activity

A) Condensation of induced bacterial cells:

For each strain, induced bacterial cells were harvested from 15ml culture (nitrogen free MSA broth with 1.5mM ACC) by centrifuging at 10,000*g for 10 min at 4°C after 40 hr incubation. Bacterial cells were washed twice with 5ml of 0.1M Tris-HCl (pH 7.5). Following an additional centrifugation for 5min at 16,000*g at 4°C, solution A was made by resuspending pellets of induced bacterial cells in 2ml 0.1M Tris-HCl (pH 8.5).

B) Labilization of induced bacterial cells:

One point eight milliliter of cell suspension (solution A) was transferred into a fresh 2.0-ml microcentrifuge tube. Ninety microlitre of toluene was then added, and tube was vortexed at the highest setting for 30s. The toluenized cell suspension (~1.89ml) was labeled as solution B.

C) Bacterial ACC deaminase-catalyzed ACC hydrolyzation generating α- ketobutyrate (three replicates per isolate):

Two hundred microlitre toluenized cell suspension (solution B) was transferred into a fresh 1.5-ml microcentrifuge tube. Twenty microlitre of sterile ACC stock (0.5M) was then added and mixed together with solution B, and tube was incubated at 30°C for 15 min after briefly vortexed. Finally, the reaction was completely terminated by the addition of 1ml of 0.56M HCl (briefly vortexed). Following a centrifugation for 5 min at 16,000*g at 4°C, supernatant which contained α- ketobutyrate generated by ACC deaminase activity was transferred into a fresh 1.5-ml microcentrifuge tube labelled as solution C (1.22ml).

From the previous calculation [equation ①], the following equation ② was developed to calculate the number of toluenized cells in 200 μl solution B (toluenized cell suspension).

$$N = n * 15ml * (1.80ml/2.00ml) * (0.20ml/1.89ml) \text{----------} ②$$

n (the number of cells per millilitre culture): cells/ml (equation①)

D) Measurement of α- ketobutyrat in generated by ACC deaminase activity

Firstly, solution D (1.8ml) was prepared by mixing 1000μl solution C together with 800μl of 0.56M HCl. Two hundred microlitres of solution D was mixed together with 300μl of the 2, 4-dinitrophenylhydrazine reagent (0.2% 2, 4-dinitrophenylhydrazine in 2M HCl) (Sigma Co. USA) in a fresh 10-ml glass tube. The tube was then incubated at 30°C for 30 min. Finally, two milliliter of 2N NaOH was added, and absorbance of the color reaction mixture (solution E) which was named O.D.540-S was measured at 540nm after briefly vortexed. Two references were set up to monitor background noise generated by bacterial extract (Reference A) and unhydrolyzed ACC (Reference B). Therefore, O.D.540-keto (the absorbance of α- ketobutyrate generated by the activity of ACC deaminase in solution E) is calculated by equation ③:

$O.D._{540\text{-keto}} = O.D._{540\text{-S}} - O.D._{540\text{-Ra}} - O.D._{540\text{-Rb}}$ ----------③

$O.D._{540\text{-S}}$: absorbance of reaction mixture at 540nm

$O.D._{540\text{-Ra}}$: absorbance of bacterial extract at 540

$O.D._{540\text{-Rb}}$: absorbance of unhydrolyzed ACC at 540

Finally, the concentration of α- ketobutyrate generated by bacterial ACC deaminase activity in solution D, X (mM), was calculated through comparing O.D.540-keto to the standard curve.

2.2.2.7 Calculation of ACC Deaminase Activity

Activity of bacterial ACC deaminase in 0.2ml solution B (toluenized cell suspension): B, was calculated by following equation:

$$B\ (\mu mol \cdot min^{-1}) = (X * V_D * V_C * 10^6)/15min \text{----------④}$$

V_D (volume of solution D): 1.80 ml; V_C (volume of solution C): 1.22 ml;

X (the concentration of α- ketobutyrate in solution D): mM

According to equation ①, ②, and ④ , the following equation ⑤ was developed to calculate ACC deaminase activity per 10^{11}cells: C

$$C\ [\mu mol \cdot min^{-1} \cdot (10^{-11}\ cells)] = (B * 10^{11})/N \text{ ----------⑤}$$

N (the number of toluenized cells in 0.2ml solution B): equation ②

B (activity of bacterial ACC deaminase in 0.2ml solution B): $\mu mol \cdot min^{-1}$

2.2.3 Rhizobitoxine Assay

Rhizobitoxine is an enol-ether amino acid (2-amino-4-[2-amino-3-hydroxypropoxy]-trans-3-butenoic acid) (Owens *et al.*, 1972).

As a structural analog of cystathionase, rhizobitoxine irreversibly inhibits β-cystathionase in bacteria and plants (Okazaki *et al.*, 2004). The inhibition of

β-cystathionase can be observed through measuring the variation of absorbance at 450nm generated by pyruvate after color reaction (Figure 6). Therefore, the inhibition of β-cystathionase can be used to assay activity of rhizobitoxine in samples. Rhizobitoxine inhibition of β-cystathionase isolated from *E.coli* K-12 was observed at the concentration as low as 0.1μM, and 95% inhibition occurred at about 100μM rhizobitoxine (Yasuta *et al.*, 1999; Ruan and Peters, 1991).

2.2.3.1 Preparation of β-cystathionase Extract

2.2.3.1.1 Establishment of Standard Curve for Bio-Rad Protein Assay

A) Dye reagent preparation:

One part Dye Reagent Concentrate (Bio-Rid Co. USA.) was diluted with 4 parts distilled, deionized (DDI) water, and filtered through syringe driven filters with 5-μm membrane (Millipore Corporation, USA) to remove particulates

B) Standards with gradient concentration:

BSA stock (0.9mg/ml) was prepared by dissolving 27.4mg bovine serum albumin (Bio-Rid Co. USA.) in 30ml distilled deionized water. A series of BSA standards were then prepared (Table **2**).

Figure 6: Flow diagram of rhizobitoxine assay based on the inhibition of Cysathionine activity (Penrose and Glick, 2003).
The symbol ⊥ means inhibition

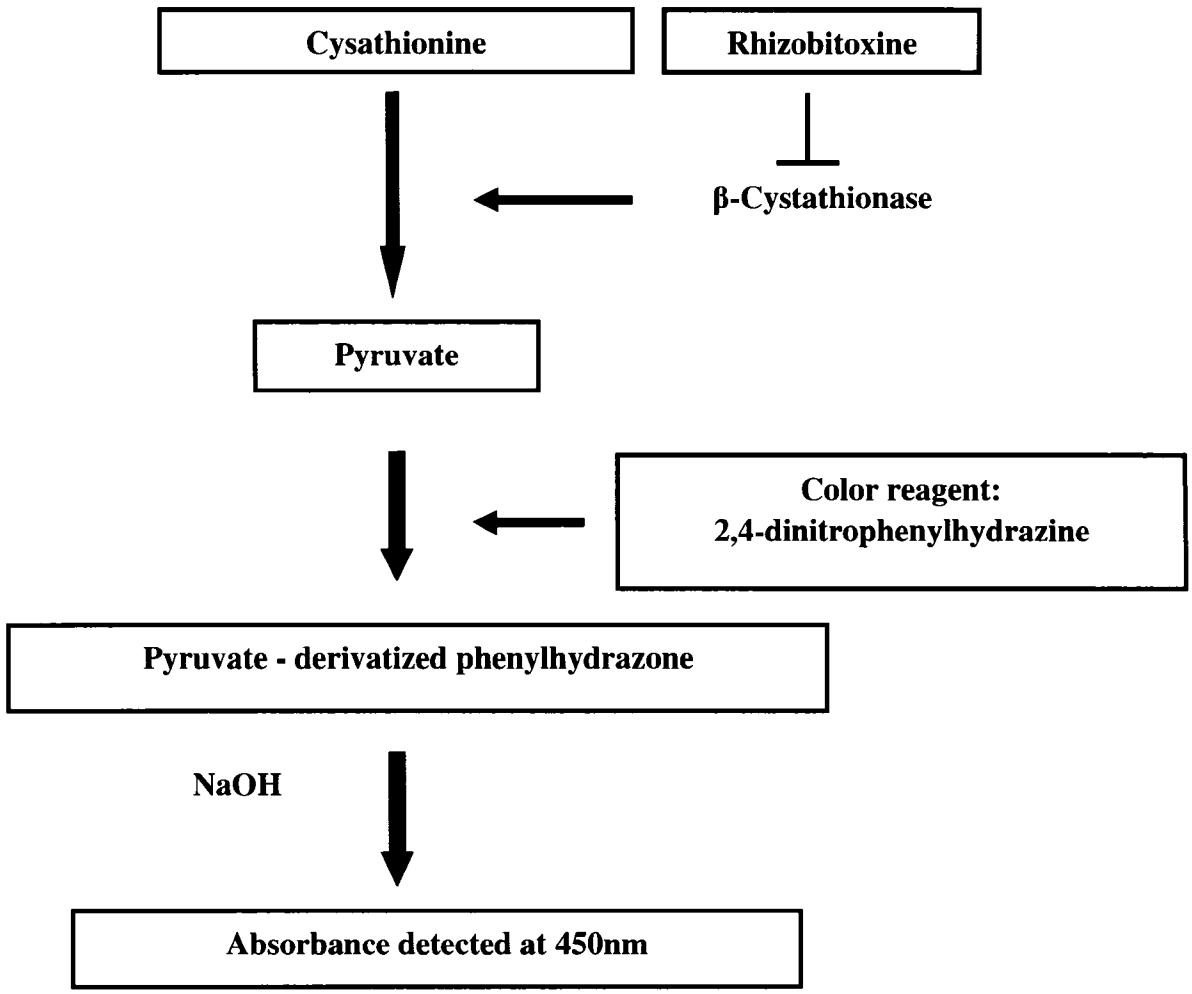


Table 2: Preparation of BSA standards by 0.9mg/ml BSA stock solution

[Extraction buffer A: potassium phosphate (K_2HPO_4): 50mM (pH 7.3), β-mercaptoethanol: 0.1mM, pyridoxal phosphate: 0.05mM]

Standards		BSA stock 0.9mg/ml	Extraction Buffer A	Total Volume (µl)
BSA standards	0.3 mg/ml	66.6µl	133.4µl	200µl
	0.4 mg/ml	88.8µl	111.2µl	200µl
	0.5 mg/ml	111.2µl	88.8µl	200µl
	0.6 mg/ml	133.4µl	66.6µl	200µl
	0.8mg/ml	177.8µl	22.2µl	200µl
	0.9mg/ml	200µl	0µl	200µl

C) Color reaction:

One hundred microlitres of each standard was firstly mixed with 5ml of diluted dye reagent in a fresh 10-ml glass tube. The tube was then incubated at room temperature for 15min. Finally, absorbance of the mixture was measured at 595nm (4 replicates per standard).

2.2.3.1.2 Preparation of *E.coli* K-12 Culture

The *E.coli* K-12 offered by Dr. Kiwamu Minamisawa (school of Agriculture, Ibaraki University, Japan) was inoculated in 8ml fresh M9 broth (12.8g/L $Na_2HPO_4.7H_2O$, 3.1g/L KH2PO4, 0.5g/L NaCl, 1.0g/L NH_4Cl, 0.5g/L $MgSO_4.7H_2O$, 0.1mM $CaCl_2$, 4g/L Glucose) and grown at 30 °C for 20 hr with shaking at the speed of 250 rpm. Eight milliliter of culture was then used to inoculate 120ml fresh M9 broth. The culture was then incubated at 30 °C with shaking at the speed of 200 rpm for 20 hr. Finally, 120ml culture was inoculated in 2L fresh M9 broth, and grown at 30 °C with shaking at the speed of 100 rpm for another 20 hr. Bacterial cells were harvested from 2L culture by a centrifugation at 15,000*g for 15 min at 4°C.

2.2.3.1.3 Extraction of β-cystathionase

A) Cell disruption:

Harvested cells were resuspended in cold extraction buffer A (potassium phosphate: 50mM [pH 7.3], β-mercaptoethanol: 0.1mM, pyridoxal phosphate: 0.05mM) at about 3ml per 1g of cells. Resuspended cells were lysed by passing through French Pressure Cell which was prechilled at 4 °C (Thermo Electron Co, Waltham, MA, USA) twice at the pressure of 16,000 psi (pound per square inch). The lysed cell suspension was centrifuged at 100,000*g (24,150 rpm in a Beckman SW41 rotor in a Beckman ultracentrifuge) for 30min at 4 °C. The protein concentration of collected supernatant was measured by the Bio-Rad protein assay.

B) Process of Cell Extract:

The concentration of protein in supernatant was adjusted to about 10mg/ml by the addition of extraction buffer A [50mM potassium phosphate (pH 7.3), 0.1mM β-mercaptoethanol, 0.05mM pyridoxal phosphate]. A final concentration of 0.2mM pyridoxal phosphate was made by diluting supernatant in 1mM pyridoxal phosphate stock in a ratio of 16:3. Finally, the pH of supernatant was adjusted to 6.5 with acetic acid.

C) Purification of β-cystathionase extract (Ruan and Peter, 1991):

The processed supernatant of cell lysate was quickly heat to 60°C. After cooled at room temperature for 3 min, the supernatant was placed in an ice-water bath until its temperature drops to 20°C. To remove the cloudy wither precipitate (denatured protein) generated by previous heat shock, solution was centrifuged at 100,000*g (24,150 rpm in a

Beckman SW41 rotor in a Beckman ultracentrifuge) for 60min at 4 ℃. After the protein concentration is measured by the Bio-Rad protein assay, the β-cystathionase extract was aliquoted (500μl/tube) and frozen at -80°C.

2.2.3.1.4 Optimal substrate (L-(+)-cystathionine) Concentration for Enzyme Assay

A) Preparation of a series of L-(+)-cystathionine standards (Table **3**)

B) Measurement of initial velocity:

During β-cystathionase-catalyzed reaction, pyruvate was generated from a series of L-(+)-cystathionine. The amount of pyruvate generated within 5 minutes was in direct proportion to the initial velocity of β-cystathionase-catalyzed reaction. Thus, the absorbance of pyruvate at 450nm in the reaction mixture (O.D.450nm) was considered as an indicator of initial velocity during this stage and later enzyme assay.

First, fifty microlitres β-cystathionase extract was mixed with 100μl LB Broth in a fresh 1.5-ml microcentrifuge tube, and incubated at room temperature for 15mins. Fifty microlitres L-(+)-cystathionine (Sigma Co. USA.) standards were added, and the reaction mixture was then incubated at 37 °C for 5min after briefly vortexed. Finally, 100μl of the

Table 3: Preparation of L-(+)-cystathionine standards of different concentrations with 30mM stock solution (L-(+)-cystathionine: Sigma Co. USA)

Standards	L-(+)-cystathionine stock (30mM)	0.1M Tris-Cl (pH 8.3)	Total Vol
1.5mM	10µl	190µl	200µl
1.7mM	10µl	170µl	180µl
1.9mM	10µl	150µl	160µl
2.1 mM	20µl	260µl	280µl
2.5 mM	20µl	220µl	240µl
3.0 mM	20µl	180µl	200µl
3.3 mM	20µl	160µl	180µl
3.8 mM	20µl	140µl	160µl
4.3 mM	30µl	180µl	210µl
5 mM	30µl	150µl	180µl
7.5 mM	50µl	150µl	200µl
15 mM	100µl	100µl	200µl
30 mM	200µl	0µl	200µl

2, 4-dinitrophenylhydrazine reagent (0.2% 2, 4-dinitrophenylhydrazine in 2M HCl) (Sigma Co. USA) was mixed together with reaction mixture, and incubated at room temperature for 15min. The absorbance of pyruvate (product of L-(+)-cystathionine) determined at 450nm showed the initial rate of reaction. The concentration of L-(+)-cystathionine which was high enough to stop the increase of initial velocity (O.D.450) was optimal for later enzyme assay.

2.2.3.2 Detection of rhizobitoxine

2.2.3.2.1 Preparation of Samples

Hydrogen-oxidizing isolates (*Variovorax*: Jm01, Jm63, Jm110, Jm111, Jm162-V; *Flavobacterium*: Jm162-F; *Burkholderia*: Jm120, Jm121, Jm122, Jm123) grown on MSA plates were inoculated in 2ml fresh LB broth with supplement of 0.1% Casamino acid at 33 ℃ for about 20 hr (Ruan and Peters, 1991).

2.2.3.2.2 Enzyme Assay

A) Sample test:

Bacterial culture was centrifuged for 10mins at 10,000∗g at 4℃, and supernatant was transferred into a fresh 2.0-ml microcentrifuge tube. L-(+)-cystathionine solution (Sigma Co. USA) with optimal concentration was catalyzed by β-cystathionase extract at the same condition mentioned above (2.2.3.1.4.B: measurement of initial velocity) except

for 100μl LB Broth which was replaced by equal volume of test samples (bacterial culture supernatant of each isolates). By using the same method mentioned above (2.2.3.1.4.B: measurement of initial velocity), the absorbance of reaction mixture was measured at 450nm.

B) Positive control (100% activity of β-cystathionase extract):

Bacterial culture of each isolate was substituted by equal volume of 0.1M Tris-Cl (pH 8.3). The absorbance of positive control was measured at 450nm

C) Calibration:

Two references were set up to monitor background noise generated by β-cystathionase extract (Reference E) and unhydrolyzed L-(+)-cystathionine (Reference AA). Therefore, the absorbance of pyruvate generated by the activity of β-cystathionase at 450nm is calculated by equation ⑥:

$$O.D._{450\text{-}Sc} = O.D._{450\text{-}S} - O.D._{450\text{-}Re} - O.D._{450\text{-}Raa} \quad \text{⑥}$$

$O.D._{450\text{-}S}$: absorbance of color reaction mixture at 450nm

$O.D._{450\text{-}Re}$: absorbance of Reference E at 450nm

$O.D._{450\text{-}Raa}$: absorbance of Reference AA at 450nm

D) Analysis:

The Minitab's ANOVA one-way analysis was applied to find out whether there existed statistically significant difference between O.D.450-Sc of tests and their positive control. If O.D.450-Sc of test were statistically lower than that of positive control, it means that the test sample (bacterial culture supernatant of each isolates) inhibited the activity of β-cystathionase. Thus, this sample shows rhizobitoxine positive. On the other side, samples are considered rhizobitoxine negative if there were no statistically significant difference between O.D.450-Sc of tests and their positive control, or the former significantly larger than the latter.

2.3 RESULTS

2.3.1 ACC Deaminase Activity

2.3.1.1 Alpha-ketobutyric Acid Standard Curve

Based on the original data (O.D.540 of 10 α- ketobutyrate standards) listed in Table **4**, numerous gradients of adjacent points (S) were generated and sorted into nine groups (Table5). Based on the Minitab's ANOVA one-way, only the group of Slop1 (A-B) showed statistically significant difference compared with the other groups (P=0.05). Thus, the data belonging to group A and B were inaccurate and improper for establishing standard curve. Finally, standard curve of concentration of α- ketobutyrate versus O.D.540 was fitted as linear function (y=0.3299x + 0.0322) based on data belonging to the other groups (C, D, E, F, G, H, I, J) (Figure 7).

2.3.1.2 Activity of Bacterial ACC Deaminase

Five isolates belonging to the genus *Variovorax* (Jm01, Jm63, Jm110, Jm111, and Jm162-V) and the genus *Flavobacterium* (Jm162-F) showed significant activity of ACC deaminase after 40-hour incubation in nitrogen free MSA broth with supplement of 1.5mM ACC, ranging from 3.28 to1.45 μmol . min^{-1} .(10^{-11} cells). While the others, belonging to the genus *Burkholderia* (Jm120, Jm121, Jm122, and Jm123) showed completely negative results of ACC deaminase activity. Among all those isolates which have the ability of ACC deaminase expression under inducement, Jm111 showed the highest ACC deaminase expression ability when induced by ACC which acts as the only nitrogen source in the medium, while *Flavobacterium* strain Jm162-F was the lowest. Minitab's ANOVA one-way teat detected statistically significant difference in ACC deaminase activity among these isolates. All six isolates can be sorted into two groups: group A (Jm01, Jm63, Jm110, Jm162-V, and Jm111); group B (Jm162-F) (Table **6**).

In order to study the effect of ACC on the induction of ACC deaminase in different isolates, a group of negative controls was set up during the stage of inducement. The stationary phase cells of each isolate, collected from LB broth, were grown in MSA (the only nitrogen source: $NaNO_3$) at 33 °C with shaking (200rpm) for 40h. There was no ACC deaminase activity measured among all strains except for the Jm162-F,

Table 4: Absorbance of α- ketobutyrate standards at 540nm

The experiment was duplicated and had ten replicates. SD: standard deviation (ten replicates for each standard).

	A	B	C	D	E
	0.1 mM	0.2mM	0.3mM	0.4mM	0.5mM
1	0.056	0.098	0.127	0.159	0.194
2	0.055	0.095	0.124	0.162	0.203
3	0.045	0.096	0.123	0.162	0.19
4	0.054	0.088	0.124	0.165	0.185
5	0.053	0.096	0.126	0.165	0.187
6	0.046	0.094	0.128	0.164	0.193
7	0.05	0.099	0.134	0.172	0.204
8	0.047	0.103	0.134	0.163	0.206
9	0.046	0.095	0.128	0.181	0.19
10	-	0.099	0.125	0.169	0.202
mean	0.05	0.096	0.127	0.166	0.195
SD	0.004	0.004	0.004	0.006	0.008
	F	G	H	I	J
	0.6mM	0.7mM	0.8mM	0.9mM	1mM
1	0.225	0.25	0.275	0.309	0.339
2	0.234	0.255	0.28	0.316	0.344
3	0.233	0.248	0.285	0.316	0.352
4	0.223	0.258	0.286	0.313	0.343
5	0.224	0.258	0.292	0.321	0.346
6	0.251	0.272	0.304	0.336	0.371
7	0.236	0.273	0.305	0.346	0.374
8	0.244	0.289	0.302	0.333	0.373
9	0.239	0.287	0.317	0.339	0.374
10	0.239	0.276	0.313	0.343	0.383
mean	0.235	0.267	0.296	0.327	0.36
SD	0.009	0.015	0.014	0.014	0.017

Table 5: The slopes of adjacent points from Table 4.

S1 = $O.D._{540}$ (B-A)/ (0.2-0.1) mM; S2 = $O.D._{540}$ (C-B)/ (0.3-0.2) mM; S3= $O.D._{540}$ (D-C)/ (0.4-0.3) mM; S4= $O.D._{540}$ (E-D)/ (0.5-0.4) mM; S5 = $O.D._{540}$ (F-E)/ (0.6-0.5) mM; S6 = $O.D._{540}$ (G-F)/ (0.7-0.6) mM; S7 = $O.D._{540}$ (H-G)/ (0.8-0.7) mM; S8 = $O.D._{540}$ (I-H)/ (0.9-0.8) mM; S9 = $O.D._{540}$ (J-I)/ (1.0-0.9) mM.

	S 1 (A-B)	S 2 (B-C)	S 3 (C-D)	S 4 (D-E)	S 5 (E-F)	S6 (F-G)	S 7 (G-H)	S 8 (H-I)	S9 (I-J)
1	0.42	0.29	0.32	0.35	0.31	0.25	0.25	0.34	0.3
2	0.4	0.29	0.38	0.41	0.31	0.21	0.25	0.36	0.28
3	0.51	0.27	0.39	0.28	0.43	0.15	0.37	0.31	0.36
4	0.34	0.36	0.41	0.2	0.38	0.35	0.28	0.27	0.3
5	0.43	0.3	0.39	0.22	0.37	0.34	0.34	0.29	0.25
6	0.48	0.34	0.36	0.29	0.58	0.21	0.32	0.32	0.35
7	0.49	0.35	0.38	0.32	0.32	0.37	0.32	0.41	0.28
8	0.56	0.31	0.29	0.43	0.38	0.45	0.13	0.31	0.4
9	0.49	0.33	0.53	0.09	0.49	0.48	0.3	0.22	0.35
10	-	0.26	0.44	0.33	0.37	0.37	0.37	0.3	0.4

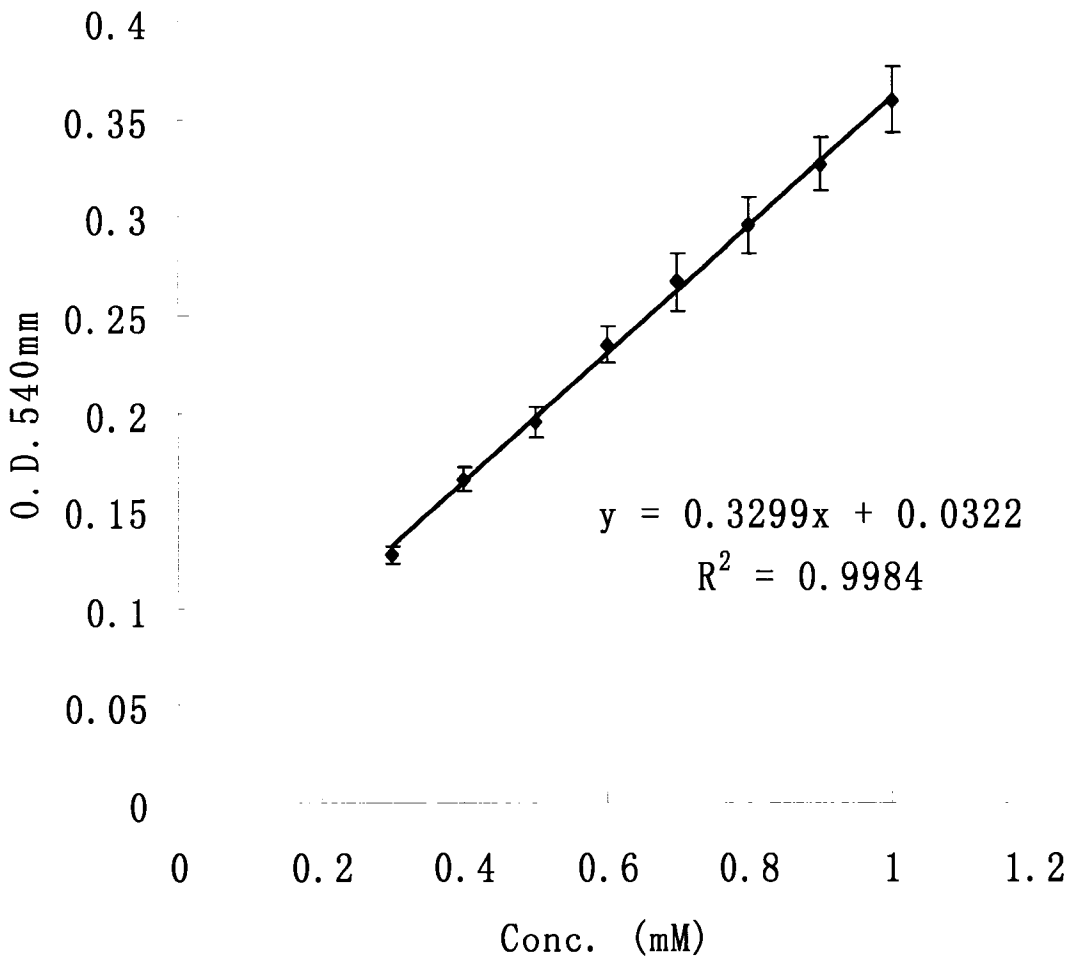
Table 6: ACC deaminase activity and Rhizobitoxine assay of isolates (±SD).
C: ACC deaminase activity [μmol·min^{-1} (10^{-11} cells)]; I: inhibition of β-Cystathionase activity; minus (-) means negative, while plus (+) represent positive; Test: bacterial cells harvested after 40-hour incubation in nitrogen free MSA broth with supplement of 1.5 mM ACC; Con-N (negative control): bacterial cells harvested after 40-hour incubation in fresh MSA broth. *: statistically significant difference (p=0.05). Each experiment was duplicated and had three replicates.

	Isolates	C		I
Variovorax	Jm01	test	2.50±0.45 a*	-
		Con-N	0	
	Jm63	test	2.60±0.43 a	-
		Con-N	0	
	Jm110	test	2.57±0.37 a	-
		Con-N	0	
	Jm111	test	3.28±0.50 a	-
		Con-N	0	
	Jm162-V	test	2.65±0.17 a	-
		Con-N	0	
Flavobacterium	Jm162-F	test	1.46±0.12 b	-
		Con-N	0.38±0.03	
Burkholderia	Jm120	Test/Con-N	0	+
	Jm121	Test/Con-N	0	+
	Jm122	Test/Con-N	0	+
	Jm123	Test/Con-N	0	+

Figure 7: Standard curve for concentration of α-ketobutyrate concentration versus $O.D._{540}$.

The experiment was duplicated and had ten replicates. The Y error bars on each point mean standard deviation of all replicates in each test. $O.D._{540}$ corrected for blank. Conc: concentration of α-ketobutyrate; O.D.540: absorbance of α-ketobutyrate at 540nm (0.3-1.0mM * 0.2 ml = 60-300 pmol α-ketobutyrate).

The curve was fitted as linear function: $y=0.3299X + 0.0322$. $R^2=0.9984$.



the only isolate belonging to *Flavobacterium* showed a little ACC deaminase activity about 0.38 μmol · min $^{-1}$· (10^{-11} cells) which is far lower than that induced by 1.5mM ACC (Figure 8).

2.3.2 Determination of Rhizobitoxine

2.3.2.1 Standard Curve for Bio-Rad Protein Assay

To detect the concentration of protein by Bio-Rad protein assay during β-cystathionase extraction, a standard curve of concentration of BSA vs. O.D.595nm (Figure 9) was fitted as linear function (y=0.5595x) based on absorbance of BSA standards at 595nm (Table7).

2.3.2.2 Optimal Concentration of L-(+)-cystathionine for Enzyme Assay

According to absorbance of pyruvate generated from a series of L-(+)-cystathionine solution with gradient concentration at 450nm (Table **8**), the initial velocity of the reaction is zero-order with the respect to the concentration of L-(+)-cystathionine over 7.5 mM, which meant that β-cystathionase extract was saturated by substrate when the concentration of L-(+)-cystathionine reaches 7.5mM. Therefore, ten mM was considered as the proper concentration of L-(+)-cystathionine for later enzyme assay (Figure 10).

Figure 8: Activity of bacterial ACC deaminase.

The experiment was duplicated and had three replicates. The Y error bars on each point mean standard deviation of all replicates in each test. Test: bacterial cells harvested after 40-hour incubation in nitrogen free MSA broth with supplement of 1.5 mM ACC; negative control: bacterial cells harvested after 40-hour incubation in fresh MSA broth; U: $\mu mol \cdot min^{-1} \cdot (10^{-11}$ cells)

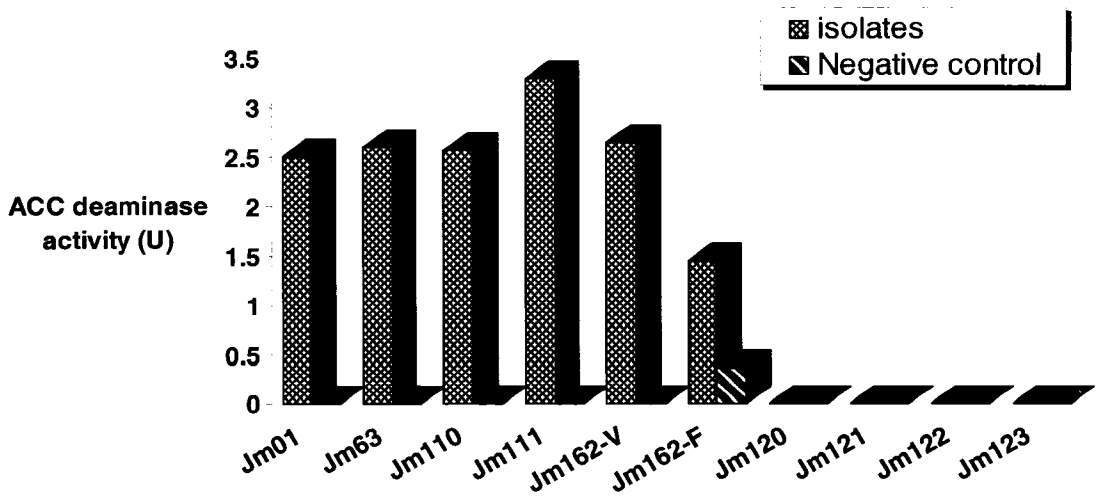


Table 7: Absorbance of BSA standard at 595nm

The experiment was duplicated and had four replicates. BSA: bovine serum albumin (Bio-Rid Co. USA.); SD (±): standard deviation.

BSA / OD_{595}	0.3mg/ml	0.4mg/ml	0.5mg/ml	0.6mg/ml	0.8mg/ml	0.9 mg/ml
replicate 1	0.187	0.216	0.282	0.330	0.463	0.512
replicate 2	0.179	0.215	0.276	0.337	0.438	0.509
replicate 3	0.182	0.241	0.275	0.345	0.453	0.494
replicate 4	0.176	0.22	0.283	0.321	0.429	0.502
Mean	0.181	0.223	0.279	0.333	0.446	0.504
SD(±)	0.005	0.010	0.004	0.010	0.015	0.008

Figure 9: Standard curve for the Bio-Rad Protein Assay.

The experiment was duplicated and had four replicates. The Y error bars on each point mean standard deviation of all replicates in each test. BSA: bovine serum albumin. The curve was fitted as linear function ($y=0.5955x$), $R^2=0.9976$.

$O.D._{595}$ corrected for blank. 300-900 μg/ml * 0.1 ml = 30-90 μg protein.

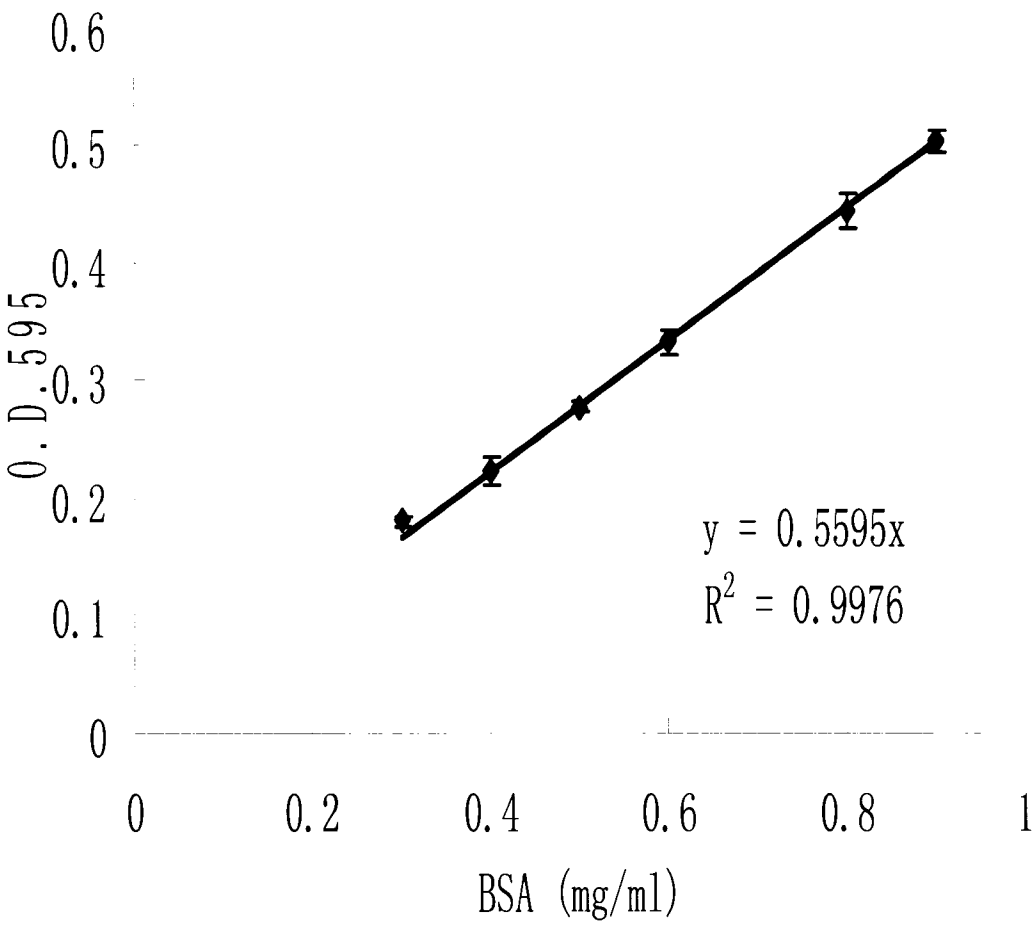


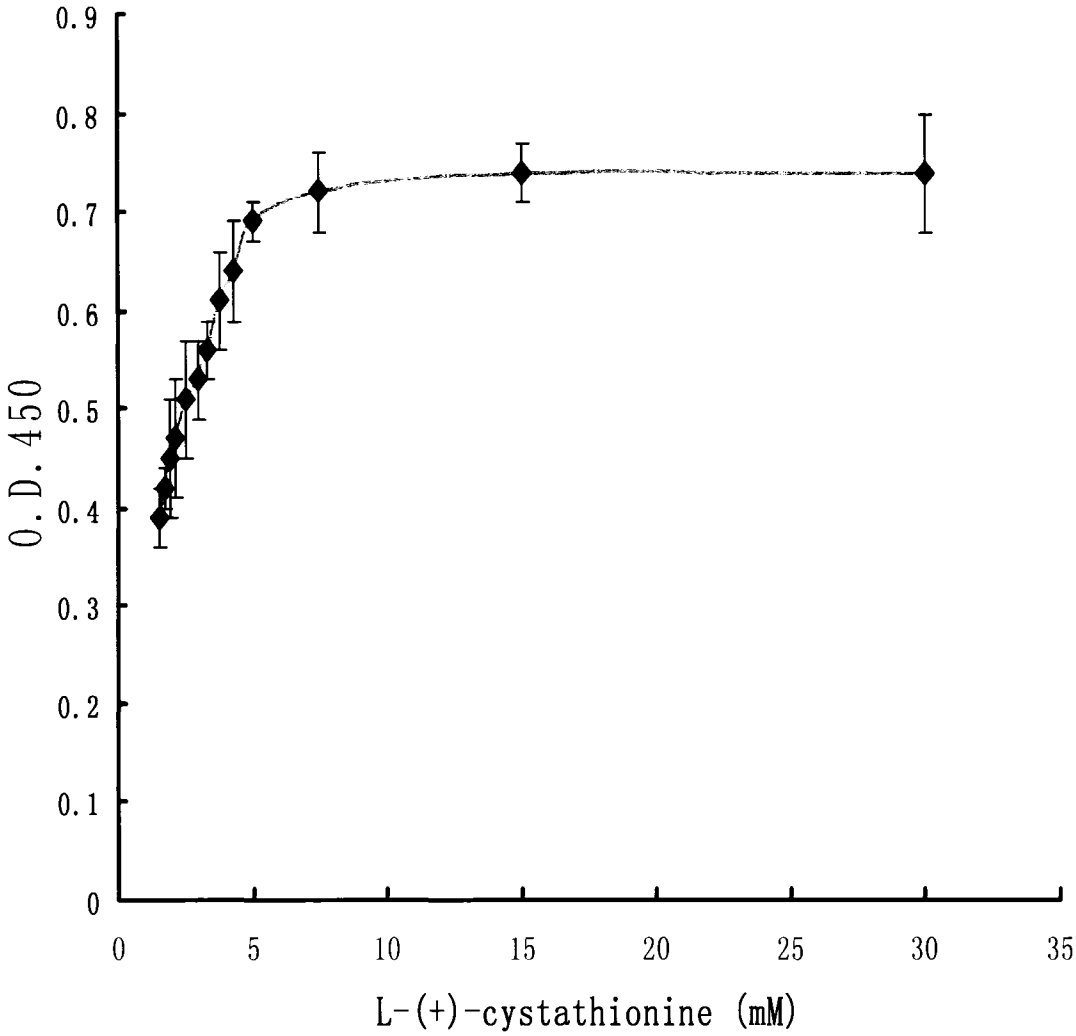
Table 8: Absorbance ($O.D._{450}$) of pyruvate generated from L-(+)-cystathionine with gradient concentrations.

The experiment was duplicated and had three replicates. S: concentration of L-(+)-cystathionine solution (mM); SD (±): standard deviation.

S(mM) / OD450	1.5	1.7	1.9	2.1	2.5	3.0	3.3	3.8	4.3	5.0	7.5	15	30
Repricate1	0.39	0.44	0.38	0.42	0.47	0.55	0.57	0.61	0.66	0.69	0.73	0.72	0.79
Repricate2	0.43	0.42	0.49	0.54	0.49	0.49	0.54	0.56	0.59	0.71	0.69	0.74	0.68
Repricate3	0.36	0.4	0.48	0.46	0.58	0.56	0.58	0.66	0.68	0.67	0.76	0.77	0.75
Mean	0.39	0.42	0.45	0.47	0.51	0.53	0.56	0.61	0.64	0.69	0.72	0.74	0.74
SD (±)	0.03	0.02	0.06	0.06	0.06	0.04	0.03	0.05	0.05	0.02	0.04	0.03	0.06

Figure 10: The initial velocity of enzyme (β-cystathionase) -catalyzed reaction versus the concentration of substrate (L-(+)-cystathionine).

The experiment was duplicated and had three replicates. The Y error bars on each point mean standard deviation of all replicates in each test. Enzyme: β-cystathionase extract; Substrate: L-(+)-cystathionine solution with gradient concentration from 1.5mM to 30mM.



2.3.2.3 Inhibition of β-cystathionase Activity

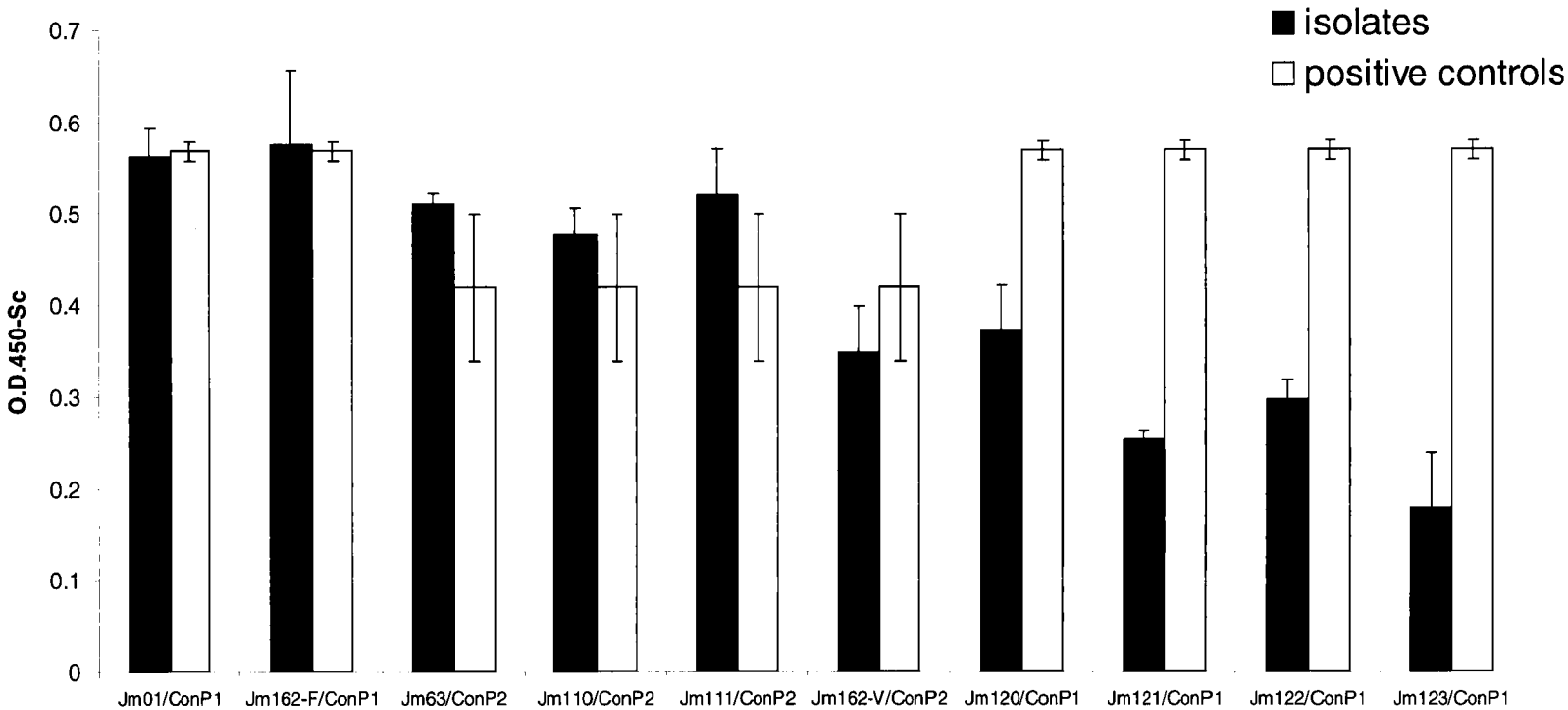
Based on the Minitab's ANOVA one-way analysis, there was no statistically significant difference between the initial rate of reaction catalyzed by β-cystathionase in bacterial culture supernatants of isolates belonging to the genera of *Variovorax* and *Flavobacterium* (Jm01, Jm63, Jm110, Jm111, Jm162-V, Jm162-V) and positive control (p=0.05) (Table **9**, Figure 11) Therefore, it was thought that none of those isolated strains belonging to *Variovorax* and *Flavobacterium* had the ability to excrete any inhibitor of β-cystathionase during growth, which meant they were rhizobitoxine-negative strains (Table **6**). However, the bacterial culture supernatant of all isolates belonging the genera of *Burkholderia* (Jm120,Jm121, Jm122 and Jm123) showed inhibition of β-cystathionase activity because the addition of bacterial culture supernatant of them in β-cystathionase extract previous reaction led to statistically significant decrease in the initial velocity of reaction compared to the initial rate of reaction detected in positive control on the basis of the Minitab's ANOVA one-way analysis(p=0.05) (Table **9**, Figure 11). Therefore, it was sure that all those isolated *Burkholderia* strains have the ability to inhibit the activity of β-cystathionase probably through expressing inhibitors such as rhizobitoxine and its structural analogue AVG.

Table 9: Initial rate of reaction catalyzed by β-cystathionase in bacterial culture supernatant of isolates and relative positive control

The experiment was duplicated and had three replicates. Con-P1: positive control of Jm01, Jm162-F, Jm120, Jm121, Jm122, Jm123; Con-P2: positive control of Jm63, Jm110, Jm111, Jm162-F; O.D.450-Sc: absorbance of pyruvate generated by the activity of β-cystathionase within the first five minutes at 450nm, calculated by equation ⑥ ($O.D._{450\text{-}S} - O.D._{450\text{-}Re} - O.D._{450\text{-}Raa}$)

O.D.450-Sc	Con-P1	Jm01	Jm162-F	Jm120	Jm121	Jm122	Jm123
Repricate1	0.557	0.532	0.66	0.378	0.24	0.316	0.24
Repricate2	0.565	0.594	0.566	0.41	0.254	0.286	0.174
Repricate3	0.583	0.562	0.502	0.328	0.264	0.288	0.124
Mean	0.568	0.563	0.576	0.372	0.253	0.297	0.179
Stdev(±)	0.013	0.031	0.079	0.041	0.012	0.017	0.058
	Con-P2	Jm63	Jm110	Jm111	Jm162-V		
Repricate1	0.324	0.526	0.506	0.464	0.298		
Repricate2	0.466	0.504	0.454	0.554	0.395		
Repricate3	0.466	0.504	0.468	0.542	0.35		
Mean	0.419	0.511	0.476	0.520	0.348		
Stdev(±)	0.082	0.013	0.027	0.049	0.049		

Figure 11: Initial rates of reaction catalyzed by β-cystathionase activity in bacterial culture supernatant (10 isolates) and relative positive control.
The experiment was duplicated and had three replicates. The Y error bars on each point mean standard deviation of all replicates in each test. $O.D._{450\text{-}Sc}$: absorbance of pyruvate generated by activity of β-cystathionase within the first five minutes at 450nm, calculated by equation ⑥ ($O.D._{450\text{-}S} - O.D._{450\text{-}Re} - O.D._{450\text{-}Raa}$); Con-P: positive control (100% activity of β-cystathionase)



2.4 DISCUSSION

Both ACC deaminase activity and Rhizobitoxine assay were based on the initial velocity of enzyme-catalyzed reaction which was monitored by color reaction. However, portion of absorbance of the complex color reaction mixture were possibly contributed by the reagents other than target reagents generated by the enzyme-catalyzed reaction. Therefore, spectrophotometric readings of color reaction mixtures were generally calibrated by using a series of references set up to measure the background noise of color reaction before calculation of the initial velocity of enzyme-catalyzed reaction.

During detection of ACC deaminase activity, the absorbance of the color reaction mixture at 540nm were possibly contributed by α-ketobutyrate generated from ACC deaminase-catalyzed reaction, together with bacterial extract, extra ACC which is unhydrolyzed. Therefore, two references were set up for calibration. The reference which was set up to measure the absorbance of bacterial extract at 540nm had the same ingredients as the reaction system designed for detecting ACC deaminase activity except the substrate of ACC deaminase, ACC, which was replaced by equal volume of reaction buffer, 0.1M Tris-HCl (pH 8.5). The other reference which was set up to monitor the absorbance contributed by unhydrolyzed ACC at 540nm had every ingredient that the reaction system designed for detecting ACC deaminase activity included except bacterial extract which was also replaced by equal volume of reaction buffer, 0.1M Tris-HCl (pH 8.5). Therefore, the amount that remains after the absorbance of color reaction mixture at

540nm is subtracted from the sum of absorbance contributed by two references at 540nm can be used to calculate the amount of α-ketobutyrate generated by the activity of bacterial ACC deaminase.

For Rhizobitoxine assay, the absorbance of β-cystathionase extract and unhydrolyzed L-(+)-cystathionine at 450nm were considered to be main sources of noise during detecting the amount of pyruvate generated by the activity of β-cystathionase. Two references were required for calibration. The reference which was set up to detect the noise resulting from the absorbance of β-cystathionase extract at 450nm had the same gradients as the reaction system designed for detecting β-cystathionase activity except the substrate of β-cystathionase, L-(+)-cystathionine, which was replaced by the equal volume of reaction buffer, 0.1M Tris-HCl (pH 8.3). The other reference which was set up to detect the noise contributed by absorbance of unhydrolyzed L-(+)-cystathionine at 450nm included every gradients that the reaction system designed for detecting β-cystathionase activity had except β-cystathionase extract, which was replaced by equal volume of reaction buffer, 0.1M Tris-HCl (pH 8.3). Therefore, calibration was carried out by subtracting the absorbance of color reaction mixture from the sum of absorbance contributed by two references at 450nm.

Rhizobitoxine assay was based on the inhibition of β-cystathionase activity reflected by the decrease in initial velocity of β-cystathionase -catalyzed reaction. When the concentration of substrate (L-(+)-cystathionine) is lower than 5 mM (Figure 10), the

initial rate of the reaction (within the first five minutes) was only resulted from portion of β-cystathionase in reaction mixture. Therefore, initial rate of the reaction doesn't slow down until the activity of extra β-cystathionase free from substrate was inhibited by added rhizobitoxine, which possibly increased false negative by lowering the sensitivity of rhizobitoxine assay. Only when the concentration of substrate becomes high enough to saturate all β-cystathionase in reaction mixture was the inhibition of β-cystathionase activity completely reflected by decrease in the initial rate of reaction. Therefore, the optimal concentration of substrate (L-(+)-cystathionine) applied for rhizobitoxine assay should be high enough to saturate all β-cystathionase in reaction mixture. However, substrate with very high concentration was still unsuitable for rhizobitoxine assay because a mass of remnant unhydrolyzed L-(+)-cystathionine possibly disturbed measurement of the amount of pyruvate generated by the activity of β-cystathionase through contributing obvious noise of spectrophotometric readings at 450nm. Thus, 10mM (Figure 10) was set as the optimal concentration of L-(+)-cystathionine in this study.

It was reported by Maimaiti (2005) that all our isolates had significant positive root elongation effect. However, it was still unsure whether these isolates stimulated plant root elongation by lowering plant ethylene levels based on the activity of ACC deaminase or rhizobitoxine or some other direct mechanisms such as the provision of bioavailable phosphorus for plant uptake, nitrogen fixation for plant use, sequestration of iron for plants by siderophores, and production of plant hormones like auxins, cytokinins and

gibberellins (Glick, 1995; Glick *et al.*, 1999). According to the studies reported by Mellado *et al.* (2004) that some species of *Burkholderia* had nitrogen fixation ability. Also some strains of *Burkholderia* (*Burkholderia vietnamiensis* TVV75) were reported to stimulated plant growth by producing a new and efficient siderophore (Tran Van *et al.*, 2000). Two strains of *Flavobacteium indologenes* (*Flavobacteium indologenes* GW2103 and LC1118), isolated by Cattelan *et al.* (1999) from the rhziosphere of soybean, had the ability to produce indoleacetic acid (IAA), one of the plant phytohormones which can increase plant growth by stimulating cell division, cell enlargements and root length (Vessey, 2003). Therefore, future work is still required to confirm whether the activity of ACC deaminase or rhizobitoxine were responsible for the obvious positive effect of these isolates on root elongation in spring wheat seedlings. For example, was the level of ethylene released from roots of spring wheat seedlings inoculated with these isolates obviously lower than the controls, or do these isolates still exert significant positive effect on root elongation after the genes responsible for activity of ACC deaminase or rhizobitoxine were inactived by insertion of bacteriophage T5 DNA?

Even though it was reported that all these isolates promoted the primary root elongation of sterilized spring wheat seedlings by 19%-254% compared to the control in two days (Maimaiti, 2005), it was still unsure whether they contributed obvious plant growth promotion in field because of the variability and inconsistency of results between laboratory, greenhouse, and field studies. Different soil types can also affect the

contribution of PGPR. The study reported by De Freitas and Germida (1990) inferred that the less fertile the soil, the greater the plant growth promoted by PGRP. Therefore, future study is required to measure the contribution of these isolates to plant growth promotion in greenhouse and field conditions.

Quorum sensing regulation involved in various activities of bacteria is a signaling mechanism that allows bacteria to control physiological functions in response to population size (von Bodman *et al.*, 2003). It was reported that the acyl-HSL (acyle-homoserine lactone) -based quorum sensing system were found to be involved in the regulation of virulence in phytopathonic bacteria such as *E. corotovora* subspecies *corotovora* (Ecc) causing soft rotting disease in a number of important crop (Andersson *et al.*, 2000; Liu *et al.*, 1998). Through quorum sensing systems, many pathogens are able to sense their surroundings and regulating the virulence based on population density, which increases the possibility of successful colonization of the infect site. A strain of *Variovorax paradoxus* (*Variovorax paradoxus* VAI-C), isolated by Leadbetter and Greenberg in 2002, was capable of degrading a number of acyl-HSLs and able to utilize acyl-HSLs as both energy and nitrogen sources. Thus, *Variovorax paradoxus* VAI-C showed the potential ability to promote plant growth because of its acyl-HSLs-degrading activity which possibly makes acyl-HSL quorum sensing -dependent pathogenic bacteria unable to sense their population density and keep expression of virulence factors blocked. It was reported that some strains of *Burkholderia* (*Burkholderia vietnamiensis* TVV75)

stimulated plant growth by inhibiting phytopathogenic fungi and producing a new and efficient siderophore (Tran Van *et al.*, 2000). Therefore, besides lowering of plant ethylene levels based on the activity of ACC deaminase or rhizobitoxine, our isolates, belonging to the genera of *Variovorax*, *Burkholderia,* and *Flavobacterium,* possibly had the ability to stimulate plant growth in greenhouse and field conditions by using indirect mechanisms, such as inhibiting the growth of pathogenic bacteria and weakening some of the deleterious effects of phytopathogenic microorganisms, which were common in many other reported PGPR (Glick, 1995; Glick *et al.*, 1999).

3. EFFECTS OF HYDROGEN METABOLISM ON RHIZOBACTERIAL COMMUNITY STRUCTURE

3.1 INTRODUCTION

Rhizosphere is a living environment supporting extremely diverse communities of bacteria which play key roles in maintaining soil quality and fertility (Lin *et al.*, 2004). Rhizosphere is also an environment in which there are complex interactions between bacteria and their plant hosts. The compositions and activities of rhizobacteria are easily influenced by a myriad of abiotic and biotic factors introduced by various agricultural practices and plant growth, which in turn influences the quality of their environment, the growth of plants, and the production of organic root exudates (Bever *et al.*, 1997). Thus, it was supposed that the process of hydrogen metabolism in soil should cause the variation of rhizobacterial community structure, and then the plant growth promotion induced by hydrogen metabolism will further amplify the effect of hydrogen metabolism on the variation of rhizobacterial community structure through activities of roots and the abundance and great diversity of organic root exudates.

It was found that most hydrogen evolved from Hup^- legume nodules was absorbed by soil (La Favre and Focht, 1983; Dong and Leyzell, 2001). Also LaFavre and Focht (1983), Popelier *et al.* (1985), and Cunningham *et al.* (1986) reported that the rhizobacterial populations were increased in H_2 rich soils around Hup^- nodules on pigeon

pea, soybean and alfalfa. McLearn and Dong (2002) proved that soil bacteria were mainly responsible for the hydrogen metabolism in soil. Thus, the increase of hydrogen uptake rate in H_2-treated soil or soil around Hup^- nodules could be an indicator of increased activities of bacteria correlated with hydrogen oxidization. Dean (2004) found that diverse white spot with a group of bacterial colonies was increased in H_2 treated soil, and the soil which had white spot had higher H_2 uptake ability compared to controls. The fact that three genera of hydrogen oxidizing bacteria had been isolated only from hydrogen-treated soils or soils adjacent to Hup^- legume nodules also showed the influence of hydrogen metabolism on abundance of bacteria related with hydrogen oxidization (Maimaiti, 2005). Thus, it has been experimentally proved that hydrogen metabolism has the ability to alter rhizobacterial community structure. However, to better understand the effect of hydrogen metabolism on rhizobacterial communities, more effective methods should be used to monitor and analyze the whole variation of rhizobacterial community structure induced by hydrogen metabolism.

Different fingerprinting techniques, such as DGGE/TGGE, RFLP/ARDRA, SSCP, and T-RFLP, have been developed over the last decade to effectively survey diversity of soil bacterial communities by using a useful prokaryotic phylogenetic marker, 16S rDNA (Muyzer *et al.*, 1993; Lee *et al.*, 1996; Liu *et al.*, 1997; Torsvik and Ovreas, 2002). Terminal restriction fragment length polymorphism (T-RFLP) is a technique following the same principle as restriction fragment length polymorphism (RFLP)/amplified

ribosome DNA restriction analysis (ARDRA) except one PCR primer is labeled with a fluorescent dye, such as TET (4,7,2′,7′-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein) (Liu *et al.*, 1997). Recently, T-RFLP analysis became increasingly popular and has been applied by many studies to investigate complex bacterial communities in the environment because the use of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection made it automated and sensitive. Moeseneder *et al.* (1999) used T-RFLP to compare complex marine bacterial community samples collected at different sites in the Mediterranean Sea and found that T-RFLP showed higher resolution than DGGE. Kaplan *et al.* (2001) reported that T-RFLP clearly has the ability to monitor the effects of probiotic dietary supplements on changes in the fecal bacterial community structure. Assessment of microbial diversity in four southwestern United States soils conducted by Dunbar *et al.* (2000) showed that T-RFLP is an effective method to elucidate similarity relationships between communities and has good detection sensitivity. Osborne *et al* (2006) made confident conclusions about the similarities of the complex bacterial communities in 17 different soil samples by developed T-RFLP. Therefore, 16S rRNA terminal restriction fragment length polymorphism is first chose as the approach towards a better understanding of effects of hydrogen metabolism on changes in rhizobacterial community structure in this study.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of Samples

The different soil samples used for TRF pattern analysis were prepared in laboratory, greenhouse and field condition.

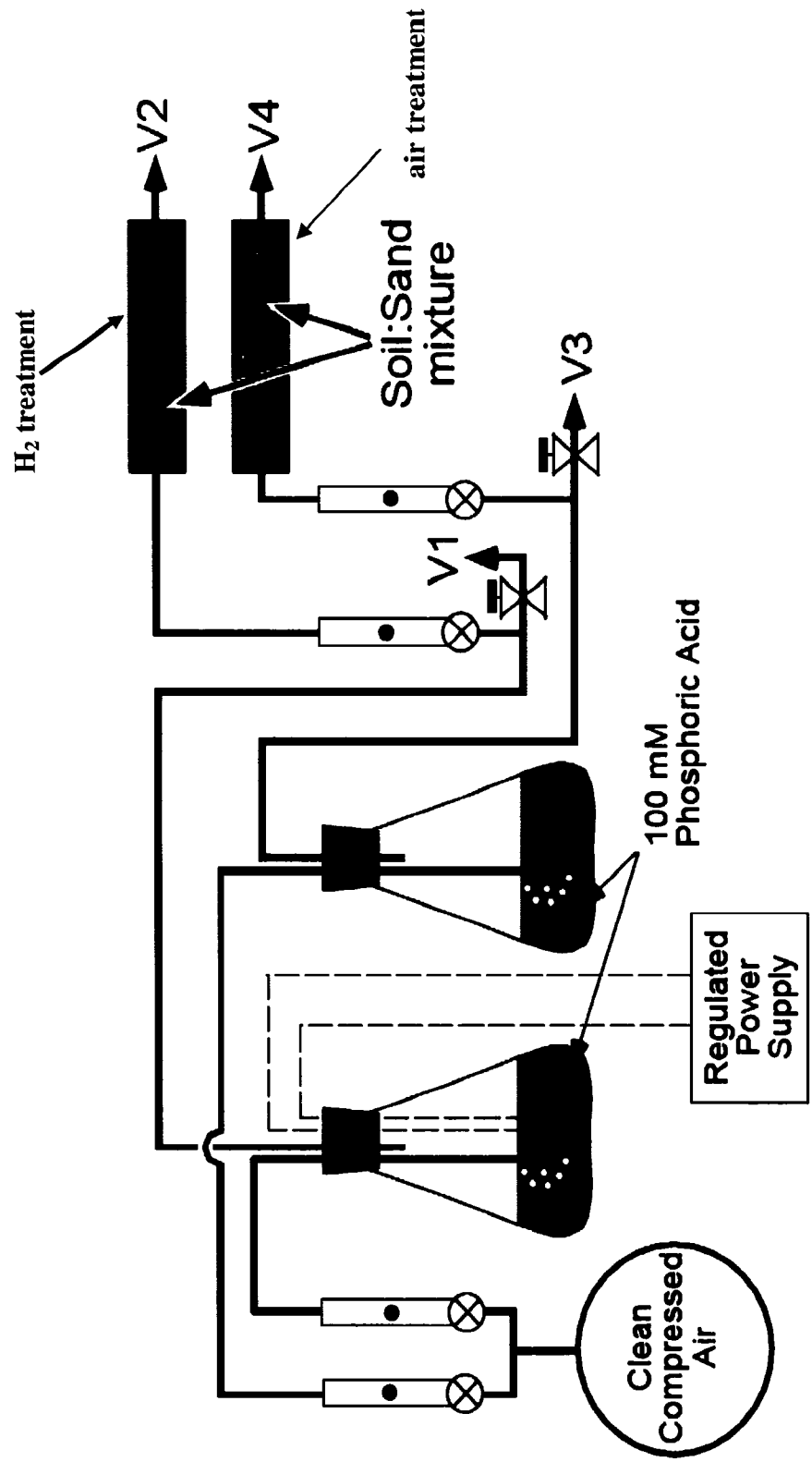
3.2.1.1 Laboratory Conditions

Soil used for later treatments was prepared by following procedures: firstly, soil (dry) collected from field in Lawrencetown, Nova Scotia two years ago was mixed with fine sand (2:1, v/v); then 500 mixture was mixed with 100 ml water. Five-gram pre H_2 treated soil with significantly high hydrogen uptake rate was mixed together with 15 ml autoclaved distilled water (dH_2O) in a small beaker. Ten milliliter supernatant was used to inoculate 500 ml prepared soil. Soil was then lightly packed into ten 60ml syringes (50ml soil per syringe). Four syringes was labeled as D1, D2, D3, and D4 and treated with the gas stream containing about 3000ppm hydrogen gas generated by electrolysis for 30 days (Figure 12). The other four syringes were labeled as E1, E2, E3, and E4 and treated by air with same flow rate for 30 days (Figure 12). Samples were frozen at -20℃ after measurement of hydrogen uptake rates (within 24 hours of sample collection).

3.2.1.2 Greenhouse Condition

Figure 12: A simplified diagram of hydrogen treatment system (Dong and Layzell, 2001).

The hydrogen gas is generated by first flask equipped with a regulated power supply to provide a direct electric current. The second flask acts as a control (air treatment). Air is provided at stable rate to both flasks. For hydrogen gas treatment, the hydrogen enriched gas stream (V1) was connected with the soil column before venting to the atmosphere at (V2). For air treatment, the air (V3) was connected with the soil column before venting to the atmosphere (V4).



A) Samples Collection

The preparation of soil samples adjacent to legume nodules grown in a green house was described in Dean (2004). Two commonly utilized commercial strains of *B. japonicum* USDA110 (Hup^+) and 532C (Hup^-), were used to inoculate soybean seeds (Cat. 32601R, First Line Seeds Ltd. Guelph, Ontario). After 10 weeks of growth in pots, the nodules and soil samples within 10mm from the nodules were collected. Soil samples adjacent to Hup^- soybean nodules (532C) were labeled as A1, A2, A3, A4, A5, A6, A7, and A8, and those adjacent to Hup^+ soybean nodules (USDA110) labeled as B1, B2, B3, and B4. Soil samples were frozen at -20 °C after their hydrogen uptake rates were measured (within 24 hours of sample collection). The collected nodules were used for later Hup status determination.

B) Nodule Hup Status Determination:

According to Lambert et al. (1985), a methylene blue reduction assay was utilized to determine Hup status of legume nodules. In order to make methylene blue reduction an indication of hydrogenase activity, inhibitors such as iodoacetic acid and malonic acid are added to prevent the respiratory electron transport processes which has the ability to reduce methylene blue (Lambert et al., 1985). After rinsed with water, fresh nodules were squashed with a small, flat surface and placed about 1 cm apart on a piece of sterile filter paper saturated with the methylene blue reduction dye solution (iodoacetic acid,

200mM; Malonic acid, 200mM; Methylene Blue , 10mM; Potassium Phosphate, 50mM; Magnesium Chloride, 2.5mM; adjust to pH 5.6 with KOH). After incubated in air for 15minutes, squashed nodules were placed in a gas chamber vacuumed and flushed with pure hydrogen gas. After 36 hours incubation of nodules in hydrogen gas, the methylene blue reduction was recorded by digital camera (Canon, Power Shot 2G) upon removal of plates from the incubation gas chamber.

For Hup^+ nodules, their symbiotic rhizobia have the ability to reduce methylene blue dye through hydrogenase-catalyzed hydrogen oxidization. Therefore, there should be white areas surrounding the nodules. For Hup^- nodules, there should be no color change around them due to the lack of hydrogenase activity which results in the inability of their symbiotic rhizobia to reduce the blue dye.

3.2.1.3 Field Condition

Soil samples were taken from no-legume field (Dr. Dong's garden) in spring of 2006. They were labeled as C1, C2, and C3, and then frozen at -20 °C after their hydrogen uptake rates were measured (within 24 hours of sample collection).

The field soil samples adjacent to the Hup^- soybean nodules, F1 and F2 were collected by Maimaiti in the same garden in 2004 and frozen at -80℃ after their hydrogen uptake rates were measured (within 24 hours of sample collection).

The soybean plants (OAC vision seeds, Nova Scotia Agriculture College, Bible Hill, Nova Scotia) were inoculated with a commercial Hup$^-$ strain of *Bradyrhizobium japonicum* (532C).

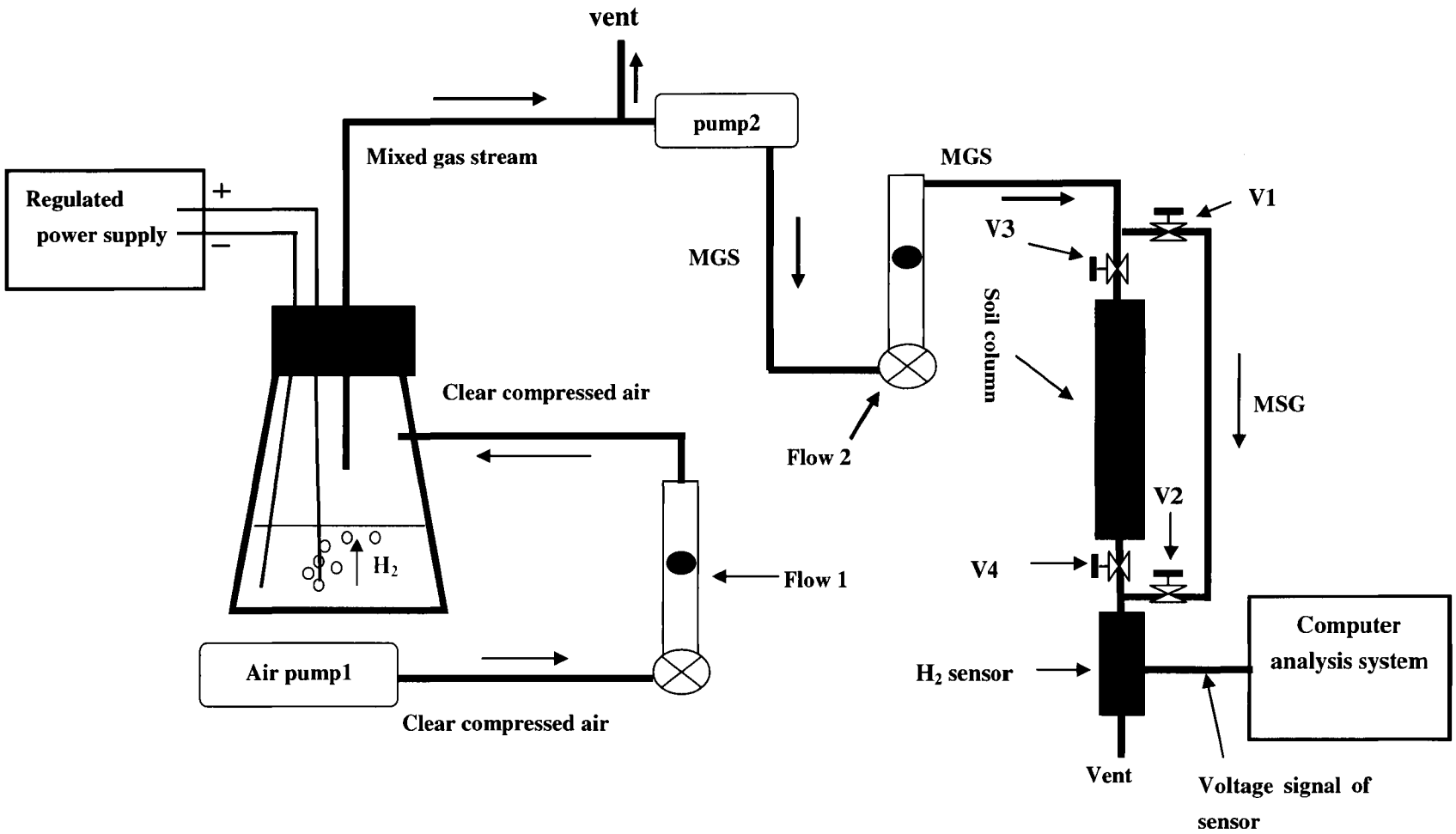
3.2.2 Measurement of Soil Hydrogen Uptake

Hydrogen uptake capability of each soil sample can be calculated by the difference between the concentrations of hydrogen before and after passing the soil sample which were measured by a hydrogen sensor (Model S211, Qubit System Inc., Kingstone, Ontario) (Figure 13).

The hydrogen senor is a semi-conductor device incorporating a heated alumina ceramic tube. A five voltage DC was applied to a circuit which contains a 10K resistor and the hydrogen sensor. The combustion of passing hydrogen gas with oxygen in the sensor caused the resistance of the semi-conductor to vary with the concentration of hydrogen in the passing gas stream, and then the voltage across the 10K resistor changed depending on the variation of the resistance of the semi-conductor and was recorded by a computer analysis system (Dong and Layzell, 2001). Thus the concentration of hydrogen on passing gas stream can be calculated by comparing the voltage of the gas stream monitored by the compute analysis system with the standard curve of voltage across 10K resistor in the hydrogen versus sensor hydrogen concentration (ppm: part per million).

Figure 13: A simplified diagram of hydrogen uptake capability measurement system.

The hydrogen gas is generated in the flask equipped with a regulated power supply to provide a direct electric current. Air is provided at stable flow rate by both pumps and combined with hydrogen gas to make a mixed gas stream before passing the soil column or hydrogen sensor. Valve 1, 2, 3, and 4 are operated to make the sensor determine the concentration of hydrogen in the mixed gas stream before and after passing the soil column. MGS: mixed gas stream.



3.2.2.1 Standard Curve of Voltage vs Hydrogen Concentration (ppm)

The amount of electrolytic hydrogen (Z: μmol/min) in the flask (Figure 13) was calculated by following equation:

$$Z\ (\mu mol/min) = (3.00 * 10^4 * C * Cu) / Av \quad \text{----------} ⑦$$

C (Coulomb Constant): $6.24 * 10^{18}\ (A^{-1})$;

Cu (current of electrolysis): mA;

Av (Avogadro Constant): $6.02 * 10^{23}\ (mol^{-1})$.

From the equation⑦, the following equation was computed to calculate the concentration of electrolytic hydrogen in mixed gas stream (H: ppm):

$$H\ (ppm) = [1.00 * 10^3 * Z * Gc * (273.15+T)]/(273.15 * FR1) \quad \text{----------} ⑧$$

Z (amount of electrolytic hydrogen per minute): μmol/min;

Gc (gas constant): 22.41 L/mol at 0 °C and 1 atmosphere pressure;

T (temperature): °C; FR1 (flow rate one): ml/min.

A series of mixed gas streams with gradient hydrogen concentration (from 0.55ppm to 147ppm) were made through regulating the current of electrolysis and flow rate one. Then V1 and V2 were turned open and V3 and V4 were closed to let the mixed gas stream passing hydrogen sensor directly. Finally, voltage across 10K resistor in

hydrogen sensor was recorded by the compute analysis system when the mixed gas stream with known concentration of hydrogen passed the hydrogen sensor (Figure 13). Based on Matlab, a standard curve of voltage vs. hydrogen concentration (ppm) was fitted as exponential function: $ppm(H_2) = a * e^{(b * v)}$ [v: voltage, e=2.718282].

3.2.2.2 Hydrogen Uptake Rate of Each Samples

Firstly, concentration of electrolytic hydrogen in the mixed gas stream before passing soil column (H_{in}: ppm) was determine by passing the mixed gas stream to hydrogen sensor directly (turning on V1 & V2 and off V3 & V4). Then, concentration of electrolytic hydrogen in the mixed gas stream after passing soil column (H_{out}: ppm) was measured when V3 & V4 were turned on and V1 & V2 were closed (Figure 13). Finally, the hydrogen uptake rate of each soil sample (Rhup: umol/hr.g) was calculated by the use of following equation

$$R_{hup}\ (umol/hr.g) = [6.00*10^{-2}*(H_{in}-H_{out})*FR2*273.15]/\ [(273.15+T)*G*W] \quad ⑨$$

H_{in} (hydrogen concentration before passing soil column): ppm;

H_{out} (hydrogen concentration after passing soil column): ppm;

FR2 (flow rate two): ml/min; T (temperature): ^{o}C;

G (gas constant): 22.41 L/mol at 0 ^{o}C and 1 atmosphere pressure;

W (weight of soil sample): g.

3.2.3 DNA Extraction

Ten soil samples were picked up for total DNA isolation: A2 & A6 (greenhouse soil adjacent to Hup^- nodules), B1 & B2 (greenhouse soil adjacent to Hup^+ legume nodules), C1, C2 & C3 (bulk field soils), D2 & D4 (30-day hydrogen treated soil), E2 & E3 (30-days air treated soils), and F1 &F2 (field soil adjacent to Hup^- nodules). For genomic DNA isolation, six strains of hydrogen oxidizing bacteria, *Variovorax* (Jm01, Jm63, Jm110, and Jm162-V), *Flavobacterium* (Jm162-F) and *Burkholderia* (Jm120), were incubated on sterile MSA plates for about one week at room temperature under air containing about 3000 ppm H_2 gas.

3.2.3.1 Soil DNA Extraction

For each sample, total DNA was extracted from 0.5g soil by using Ultraclean soil DNA isolation Kits (MO BIO Laboratory, Inc., Solana Beach,CA). For maximum yields, Alternative Protocol offered by MO BIO Laboratories, Inc. was followed. The soil (0.5g) was added to the 2ml Bead solution and vortexed to mix. Sixty microlitre of solution S1 containing SDS (sodium dodecyl sulfate), aiding cell lysis, and 200 µl IRS (inhibitor removal solution), a proprietary reagent designed to precipitate humic acids and other PCR inhibitors, were added and then vortexed at maximum speed for 10 minutes. Following a centrifugation for 30 seconds at 10,000*g, the supernatant (about 400-500µl) was transferred to a fresh 1.5 microcentrifuge tube. One hundred microlitre of IRS and

200 µl solution 2 containing a protein precipitation reagent were added and vortexed for 5 seconds and then the tube was incubated at 4 °C for 5 minutes. The tube was centrifuged at 10,000*g for 1 minute after incubation. The supernatant (about 500µl) was then mixed together with 1.3ml solution 3 (making DNA bind to silica in the presence of high salt concentration) in a fresh 2ml microcentrifuge tube. To harvest the desired DNA binding to silica, the mixture of supernatant and solution 3 was loaded onto a spin filter and centrifuged at 10,000*g for 1minute. The harvested DNA was further cleaned by loading 300µl solution 4, an ethanol based wash solution, and an additional centrifugation at 10,000*g for 1minute. After the flow through was discarded, the spin filter was centrifuged a second time for 1 minute at 10,000*g. Fifty microlitre of sterile elution buffer was added to the center of the white filter membrane and the harvested DNA was eluted from the filter membrane into the flow through (about 50µl DNA extraction) after a centrifugation at 10,000*g for 1 minute. DNA extraction was checked by running 5µl flow through in 0.8% agarose gel after transferred to a fresh 1.5ml microcentrifuge tube. Three replicate DNA extractions were pooled together to limit random bias although systematic biases always persist.

3.2.3.2 Genomic DNA Isolation (isolates)

Genomic DNA of each isolate was isolated by using modified protocol described by Lechner and Conrad (1997). For Gram-negative bacteria, such as Jm01, Jm63, Jm110, Jm162-V, and Jm162-F, genomic DNA was extracted by following procedures: plates

were washed with 1.5ml sterilized LB broth and bacterial cells were collected in a sterilized 1.5ml microcentrifugetube; the pellet was resuspended in 576μl TE buffer (100mM pH 8.0 Tris-HCl, 1mM pH 8.0 Na2EDTA); the bacterial cell suspension was mixed together with three microlitre of Protease K with concentration of 20 mg/ml and 30μl of 10% SDS and incubated at 37 °C for 30 minutes; one hundred microlitre of 5M NaCl and 80μl of 10% (wt/vl) CTAB (hexadecyl-trimethylammoniun bromid) were added to samples. Following an incubation at 60°C for 30 minutes, genomic DNA was isolated by adding an equal volume of phenol and chloroform-iosamylalcohol (24:1). After a centrifugation at 1,200*g for 5min at 4°C, supernatant containing genomic DNA was transfer into sterilized 1.5ml microcentrifugetubes.

During the genomic DNA extraction of Gram-positive bacteria, such as Jm120, French Press (Thermo Electron Co, Waltham, MA, USA) was applied to lyse cells collected by washing a plate with two ml TE buffer, and then mixed together with 100μl of 10% SDS and 10μl of 20 mg/ml Protinase K in a sterilized 15ml centrifugetube. Finally, bacterial cells were lysed by passing cell suspension through the French pressure cell prechilled at 4 °C thrice at the pressure of 16,000 psi. Following a centrifugation of lysed cell suspension at 22,000*g (14,000 rpm in a JA21 rotor in a Beckman Avanti J-E centrifuge) for 1hr at 4 °C, supernatant contain genomic DNA was mixed together with an equal volume of phenol and chloroform-iosamylalcohol (24:1) in sterilized 15ml

centrifugetubes. After a centrifugation at 1,200*g for 5min at 4°C, genomic DNA was extracted in the supernatant transferred to sterilized 15ml centrifugetubes.

For all picked isolates, isolated genomic DNA was precipitated at -20°C overnight after 0.6 volume of isopropanol was mixed together with genomic DNA-contained supernatant. The precipitated genomic DNA was collected as the pellet at the bottom of tube after a centrifugation at the highest speed for 20min at -4°C. The pellet was finally resuspended in 100-200μl sterilized TE buffer after rinsed with 70% ethanol and then dried by air. After extraction, equal volume of genomic DNA solution of Jm01, Jm63, Jm110, Jm162-V, Jm120, and Jm162-F were mixed together in a fresh microcentrifugetube and was labeled as JM.

3.2.4 PCR of 16S rRNA Genes

16S rRNA genes from all soil samples and isolates were amplified with a pair of bacterial universal primers: BSF8/20 with a fluorescent dye, 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein), labeled at the 5' terminus (6-FAM-5' - AGAGTTTGATCCTGGCTCAG - 3′) and BSR534/18 (5′ - ATTACCGCGGCTGCTGGC - 3′). The expected length of products is 527 bp (base pair).

3.2.4.1 Optimal Dilution of DNA Extract for PCR

Even though most PCR inhibitors such as humic acid were precipitated by IRS solution when total DNA was isolated from soil samples, the concentration of residual PCR inhibitors in soil DNA extract is still high enough to inhibit the activity of DNA polymerase. To make PCR more efficient, templates of each soil sample were prepared by diluting total DNA extract at the ratios of 1:10, 1:50, and 1:100 before PCR. The optimal ratio of dilution for DNA extract of each soil sample was determined by comparing gel profiles of PCRs of templates with different dilutions.

3.2.4.2 PCR Conditions

Each 50-μl reaction mixture contained: 33.6μl PCR water (molecular biology reagent, Sigma-Aldrich Canada Ltd, Oakville, On, CA), 5μl of 10x ThermoPol Reaction Buffer (New England Biolabs Ltd., Pickering, On, CA), 5μl of 2mM dNTP (dATP, dCTP, dGTP, dTTP) (New England Biolabs Ltd., Pickering, On, CA), 1μl of 20μM 6-FAM-5'-BSF8/20 and BSR534/18 (bacterial universal primers) (Applied Biosystems, Foster City, CA), and 0.4μl of 5U/μl Taq DNA polymerase (New England Biolabs Ltd., Pickering, On, CA). Amplified reactions were carried out in the Bio-rad iCycler thermal cycler (Bio-rad Laboratories, Inc., Hercules, CA) with following cycling conditions: three minutes of denaturation at 94 °C, 35cycles of 75 seconds at 94 °C, 45 seconds at 55 °C for annealing, and 45 seconds at 72 °C for extension, and a final cycle of extension at 72 °C

for 10 minutes. Multiple PCR reactions from a single sample were pooled together to minimize PCR-induced random biases. PCR products were purified with the Qiaquick PCR Purification Kit (QIAGEN Inc., Mississauga, CA).

3.2.5 Generation of TRF Profiles and Data Sets

Four restriction endonucleases were used to obtain four separate TRF profiles for each sample. TRF profiles belonging to a data set were generated by the same restriction endonuclease. Approximately 200ng purified PCR product was digested with 20 U of one of following restriction endonucleases which were applied in most previous T-RFLP analysis: *Bst*UI, *Hae*III, *Hinf*I, and *Msp*I (New England Biolabs Ltd., Pickering, On, CA) in 50-μl reaction system (Osborne *et al.*, 2006; Dunbar *et al.*, 2000; Kitts, 2001; Lui *et al.*, 1997). Each 50-μl reaction mixture was load in a sterilized 0.5ml PCR tube and incubated overnight (about 10 hours). For each restriction digestion, three replicates were set up and pooled together to minimize the artificial biases. Digested PCR products were then purified with the QIAquick Nucleotide Removal Kit (QIAGEN Inc., Mississauga, CA). Finally, 6-FAM-TRFs (6-FAM labeled terminal restriction fragments) in digested amplicons were separated and recoded by a model ABI3730 DNA sequencer (Applied Biosystems, Foster City, CA) at University core DNA services, Faculty medicine, University of Calgary, Calgary, AB, Canada.

TRF profiles consisted of TRFs were outputted by using the GeneMarker V-1.4 software (SoftGenetics LLC, USA). Each TRF was described digitally at three aspects: fragment length in nucleotides (the apex position of each peak on a base pair scale relative to a DNA size ladder, GeneScan 500 LIZ Size Standard, Applied Biosystems, Foster City, CA), the peak height at apex and the area under the peak in fluorescence units (FU). The area of any one peak calculated by integrating the fluorescence under that peak, and the total area for any profile is the amount of the areas of all peaks between 50nt and 500nt (nucleotides).

To assess the contribution of isolates (*Variovorax*: Jm01, Jm63, Jm110, and Jm162-v; *Burkholderia*: Jm120; *Flavobacterium*: Jm162-F) to the variation of bacterial community structure in soil samples exposed to hydrogen gas, it was necessary to identify the TRF peaks contributed by isolates. For each soil samples exposed to hydrogen (A2&A6, D2&D4), complex samples were generated by combining digested PCR products of total DNA extracted from soil sample and genomic DNA of isolates mentioned above with the ratio of 3:2. The complex samples were labeled as following: A2J (A2 and isolates), A6J (A6 and isolates), D2J (D2 and isolates), and D4 (D4 and isolates). TRF profiles from those complex samples were generated after running these complex samples in ABI3730 DNA sequencer. Generally speaking, peaks spiked in TRF profiles from complex samples were possible contributed by isolates.

3.2.6 Standardization of TRF Profiles

All TRF profiles within a data set were standardized by the application of the variable percentage threshold method reported by Osborne *et al.* (2006) before analysis. A unique percentage threshold value of each profile was generated by using a divisor to divide the total area of each profile belonging to the same data set (total area of a profile/divisor). For each profile, all peaks that contribute less than its unique percentage threshold value were considered as noise peaks and then discarded. For each divisor, each profile contributed one point on the plot of the number of peaks remaining after standardization vs. the total area on the original profile, and the distribution of all points generated by a divisor in co-ordinates meant the relationship between the number of peaks remaining and the total area on the original profile. A series of gradient divisors were set up and checked by using TRFLPdemo, a Matlab based program written by Luo, F. (Master student in Computer Science department, St mary's Univ.) and Zhang, Y.(Master student in Biology department, St mary's Univ.). Divisors start with 100 times the mean total area of all profiles belonging to the same data set and increased with the interval of $1.00 * 10^6$. For each divisors, the program generated a curve fitted as power function and the R square (R^2) of power curve. The relationship between the number of peaks remaining and the total area on the original profile became weakest when the minimal R^2 which normally approximated zero was resulted from the optimal divisor

applied. Thus, the divisor resulting in the most random distribution of all points contributed by profiles in the same data set was picked as the optimal divisor.

3.2.7 Comparison of TRF Profiles

Following normalization, derivative TRF profiles within a data set were aligned and TRFs which have synonymous fragment sizes were identified and binned together based on the function of Bin table report in the GeneMarker V-1.4 software. All TRFs within a bin just represented the peak which was assigned the average of the sizes of them. A single, composite list of the binned peaks (fixed within ±0.4bp) was found among all samples within a data set. For each sample, the present or absence of the binned peaks in the composite list was represented by a binary vector: present (1), and absence (0). The data set was transformed into a binary matrix whose rows represented binned peaks and columns were samples. Based on the function of pdist in Matlab7.1, the Jaccard coefficient was used to generate a matrix with upper triangular or square form to show the similarity and dissimilarity between each two samples (Jaccard, 1908). Then, Jaccard coefficient was applied to carry out the agglomerative hierarchical clustering under the rule of unweighted average distance (UPGMA) by using the function of linkage in Matlab7.1. Finally, the hierarchical, binary cluster tree created by the linkage function was plotted by using the function of dendrogram in Matlab7.1. To measure how well the cluster tree generated by the linkage function reflects the data, the cophenetic distances of the cluster tree is compared with the original distance data generated by the pdist function

by using the function of cophenet in Matlab7.1. The closer the value of the cophenetic correlation coefficient is to 1, the more accurately the clustering solution reflects the data.

3.3 RESULTS

3.3.1 The Hydrogenase (HUP) Status of Soybean Nodules

The Hup status of soybean nodules were tested using methylene blue reduction assay (Lambert *et al.*, 1985) to make sure whether the nodule were infected with applied inocula (Hup^+ and Hup^- stains of B. *japonocum*). After overnight incubation, the methylene blue dye surrounding nodules collected from 532C inoculated soybeans were not reduced, which showed no color change around nodules (blue). However, the methylene blue dye surrounding nodules collected from USDA110 inoculated soybeans were reduced, which formed a clear zone around the nodules with white. Therefore, it was proved that the nodules collected from 532C inoculated soybean were Hup^- and had no hydrogenase uptake activity, while nodules of USDA110 inoculated soybean were Hup^+ and had the hydrogenase uptake activity.

3.3.2 Standard Curve of Voltage vs. Hydrogen Concentration (ppm)

Based on the original data and the equation ⑦ & ⑧, the concentrations of electrolytic hydrogen in mixed gas stream (ppm) were calculated. For each concentration of hydrogen, a relative voltage across the hydrogen sensor was detected by computer

system (Table **10**). Using Matlab, the standard curve was generated by the voltages across the hydrogen sensor (v) against plotting the concentrations of hydrogen (ppm) (Figure 14). The standard curve was fitted as exponential function and described as following equation ⑩:

$$ppm\ [H_2] = 0.95e^{(1.158v)} \quad \text{----------} \quad ⑩$$

ppm $[H_2]$: concentration of hydrogen

v: voltage across the hydrogen sensor

e: universal constant (2.718281828)

3.3.3 Hydrogen Uptake of Different Soil Sample

As shown in Table 11, the H_2 treated soil samples (D) had significantly higher H_2 uptake rate than that of controls (air treated soil samples: E). Soil samples adjacent to Hup^- nodules (F & A) had obviously higher hydrogen uptake rate than those adjacent to Hup^+ nodules (B) and bulk soils (C). Therefore, it was sure that all the samples were qualified for studying effects of hydrogen metabolism on the soil bacterial community structure because the hydrogen uptake rate of all soil samples treated in lab, collected from greenhouse and field showed significant increase after the hydrogen exposure.

Table 10: The original data for generating standard curve of voltage across the hydrogen sensor vs hydrogen concentration (ppm)

The experiment was duplicated. The concentration of hydrogen in mixed gas stream ($ConH_2$) was calculated by using equation ⑦ & ⑧. FR1: flow rate one; FR2: flow rate two; V: voltage across hydrogen sensor.

Current (mA)	T(°C)	FR1 (ml/min)	FR2 (ml/min)	$ConH_2$(ppm)	V (v)
1.05	25.8	55	41	147.00	4.15
1.05	25.8	60	41	134.00	4.13
1.05	25.8	72	41	110.00	4.10
1.05	25.8	95	41	85.00	4.05
1.05	25.8	116	41	69.00	3.90
1.05	25.8	164	41	49.00	3.50
1.05	25.8	200	41	40.00	3.25
1.05	25.8	257	41	31.00	2.97
1.05	25.8	300	41	26.70	2.81
1.05	25.8	360	41	22.24	2.72
1.05	25.8	400	41	20.00	2.59
1.05	25.8	480	41	16.70	2.20
0.54	25.8	480	41	9.10	1.60
0.00	25.8	480	41	0.55	0.74

Figure 14: Standard curve of voltage across hydrogen sensor vs. hydrogen concentration: ppm $[H_2] = 0.95e^{(1.158v)}$ ($R^2=0.9845$)
The experiment was duplicated.

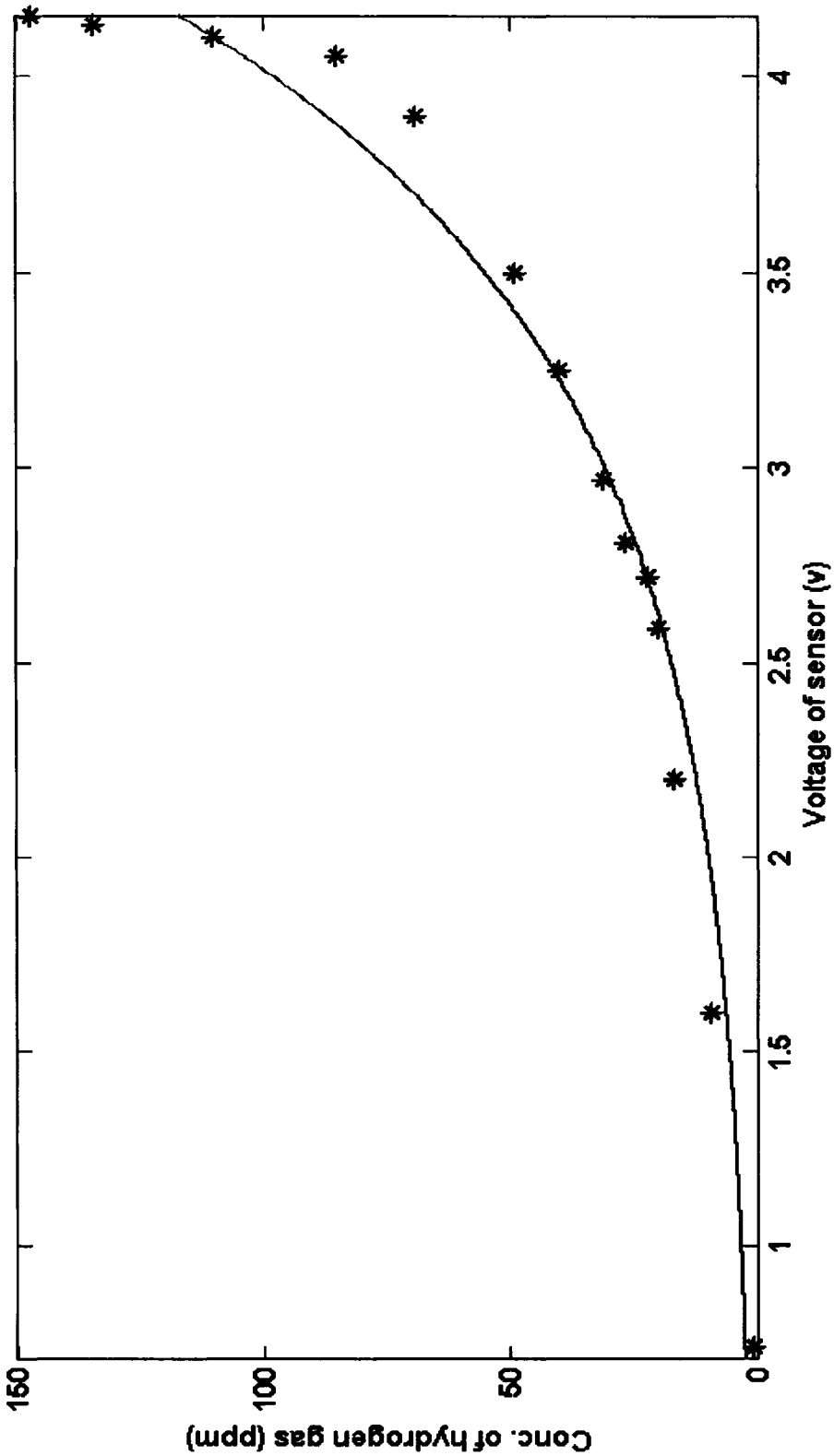


Table 11: Hydrogen uptake rates of different soil samples

This experiment was duplicated. Flow rate two (FR2) was set at 41ml/min for all samples in this experiment. Voltage-in: the voltage resulted by the concentration of hydrogen without passing the soil column; Voltage-out: the voltage corresponding to the concentration of hydrogen after passing soil column. Sample(A): greenhouse soils adjacent to Hup^- nodule (7g/sample); Sample(B): greenhouse soils adjacent to Hup^+ nodule (10g/sample); Sample(C): bulk soils in field (7g/sample); Sample(D): soils treated by hydrogen in lab (13g/sample); Sample(E): soils treated by air in Lab (20g/sample); Sample(F): field soils adjacent to Hup^- nodule offered by Maimaiti. M: mean; SD: standard deviation. The standard curve of ppm vs. voltage, were used to calculate concentrations of hydrogen corresponding to voltage-in and voltage-out. Equation ⑨ was applied to calculate the hydrogen uptake rate (R_{hup}) from the difference of the concentration of hydrogen resulted by soil samples.

Samples (A)	A1	A2	A3	A4	A5	A6	A7	A8	M±SD
Voltage-in (v)	3.58	3.58	3.58	3.59	3.6	3.59	3.59	3.6	-
Voltage-out (v)	3.45	3.38	3.43	3.48	3.46	3.38	3.42	3.41	-
R_{hup}(umol/hr.g)	0.12	0.18	0.14	0.11	0.13	0.19	0.16	0.18	0.15± 0.03
Samples (B)	B1	B2	B3	B4	-	-	-	-	M±SD
Voltage-in (v)	3.58	3.59	3.6	3.58	-	-	-	-	-
Voltage-out (v)	3.56	3.55	3.53	3.53	-	-	-	-	-
R_{hup}(umol/hr.g)	0.01	0.03	0.05	0.03	-	-	-	-	0.03± 0.01
Samples (C)	C1	C2	C3	-	-	-	-	-	M±SD
Voltage-in (v)	3.53	3.52	3.52	-	-	-	-	-	-
Voltage-out (v)	3.51	3.5	3.49	-	-	-	-	-	-
R_{hup}(umol/hr.g)	0.02	0.02	0.03	-	-	-	-	-	0.02± 0.005
Samples (D)	D1	D2	D3	D4	-	-	-	-	M±SD
Voltage-in (v)	3.53	3.51	3.52	3.51	-	-	-	-	-
Voltage-out (v)	2.94	2.62	3.03	2.74	-	-	-	-	-
R_{hup}(umol/hr.g)	0.22	0.27	0.19	0.25	-	-	-	-	0.23± 0.04
Samples (E)	E1	E2	E3	E4	-	-	-	-	M±SD
Voltage-in (v)	3.52	3.45	3.41	3.51	-	-	-	-	-
Voltage-out (v)	3.45	3.34	3.28	3.46	-	-	-	-	-
R_{hup}(umol/hr.g)	0.02	0.03	0.035	0.02	-	-	-	-	0.03± 0.01
Samples (F)	F1	F2	-	-	-	-	-	-	M±SD
R_{hup}(umol/hr.g)	-	-	-	-	-	-	-	-	0.08± 0.01

3.3.4 Generation of TRF profiles

Both total DNA extracted from soil samples (A, B, C, D, E, and F) and genomic DNA isolated from isolates (Jm01, Jm63, Jm110, Jm162-V, Jm162-F, and Jm120) showed a sharp band above 10kbp and smeared DNA bands below 10kbp in 0.8% agarose gels, which suggested that the size of most DNA fragments in soil DNA extraction and genomic DNA of isolates were bigger than 10kbp. Therefore, they were qualified as templates for amplifying 16S rRNA genes.

It was found that most PCR products against total DNA extracted from soil samples concentrated and formed a sharp band around 500bp in 1.2% agarose gels, and the rest contributed some smeared bands located between 500bp and 700bp in 1.2% agarose gels. Therefore, most PCR products were considered as copies of 16S rRNA genes because they have the same size as anticipated PCR products of 16S rRNA genes.

The sharp band around 500bp contributed by PCR products was weaken or disappeared in 2% agarose gels after PCR products were incubated together with different REs (*Bst*UI, *Hae*III, *Hin*fI, or *Msp*I) at optimal temperature for 8 to 10 hours. Furthermore, the digested PCR products contributed several weak bands below 500bp in 2% agarose gels. It suggested that PCR products were possibly completely digested by REs and those digested PCR products were qualified for generating TRF profiles.

3.3.5 Normalization of TRF Profiles

After calculation, the program found an optimal divisor for each data set (Table 12). The curves of number of peaks remaining vs. the total area on original profiles resulting from those optimal divisors became horizontal lines after fitted as power function (Figure 15). R^2 of those horizontal and linear power curves almost equaled to zero (Table 12). This meant that the optimal divisors calculated by TRFLPdemo and the unique variable percentage threshold (Table 13) for each profile derived from the optimal divisor were proper for normalizing TRFLP profiles.

3.3.6 Similarities between TRF Profiles from Different Soil Samples

The twenty-six normalized TRF profiles (13 samples with 2 replicates) generated with each RE were compiled into one data set. Then a complex data set was constructed by combining all data sets together. The distance of each pair of TRF profiles within each data set was calculated using Jaccard coefficient. Dendrograms were constructed to show the similarities between TRF profiles of different samples (Figure 16). The dendrogram of *Bst*UI data set was named Dbst. The dendrogram of *Hae*III data set was named Dhae. The dendrogram of *Hinf*I data set was named Dhin. The dendrogram of *Msp*I data set was named Dmsp. The dendrogram of combined data set (*Bst*UI, *Hae*III, *Hin*fI, and *Msp*I) was named Dcom. The cophenetic correlation coefficients of those five dendrograms

Table 12: optimal divisors for T-RFLP data sets and R squares of power curves resulting from optimal divisor generated by TRFLPdemo

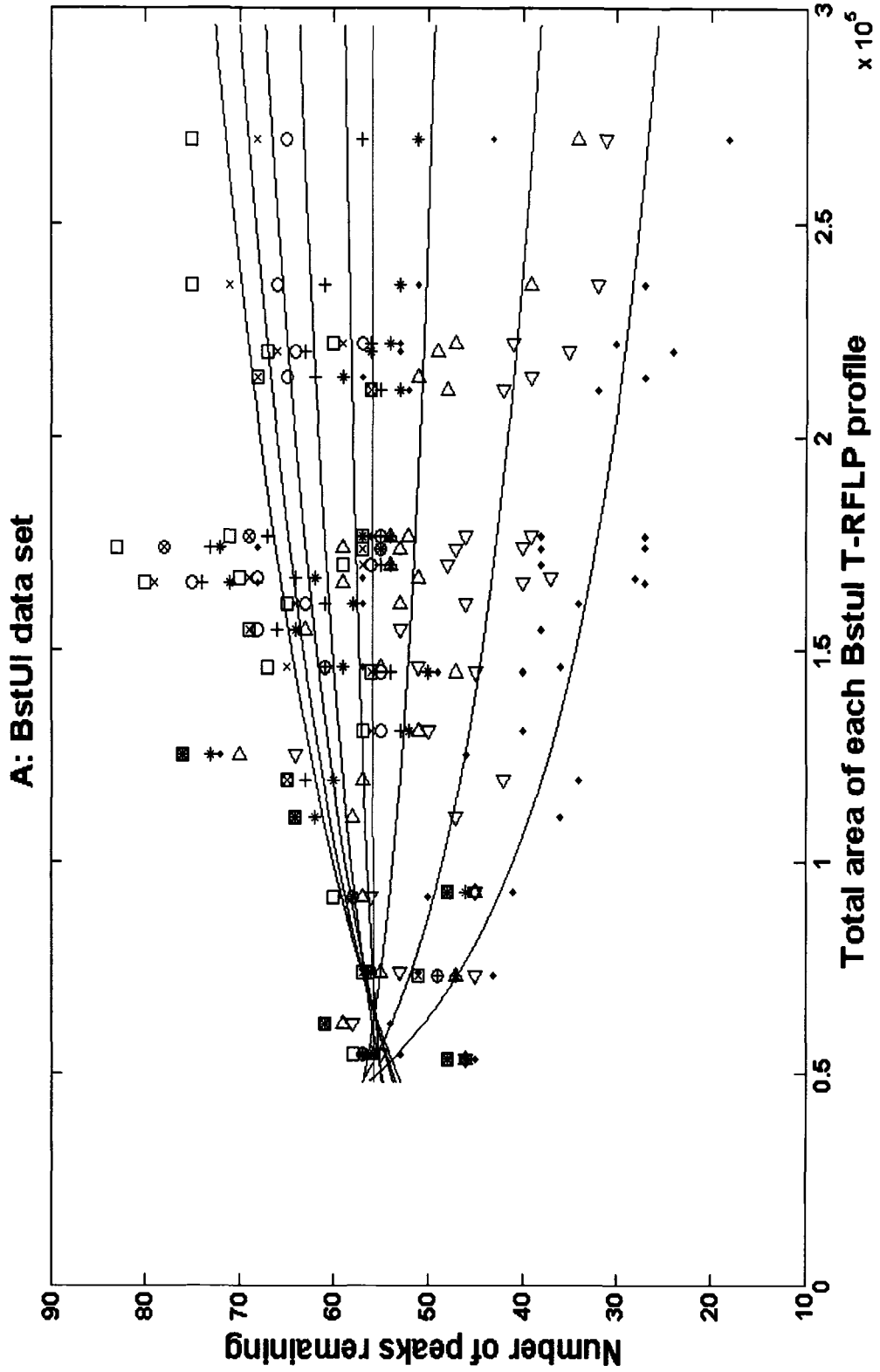
Data set	*Bst*UI	*Hae*III	*Hinf*I	*Msp*I
Optimal divisor	$4.49 * 10^{7}$	$4.70 * 10^{7}$	$4.37 * 10^{7}$	$3.75 * 10^{7}$
R square	$1.8 * 10^{-5}$	$3.6 * 10^{-6}$	$8.4 * 10^{-6}$	$1.5 * 10^{-4}$

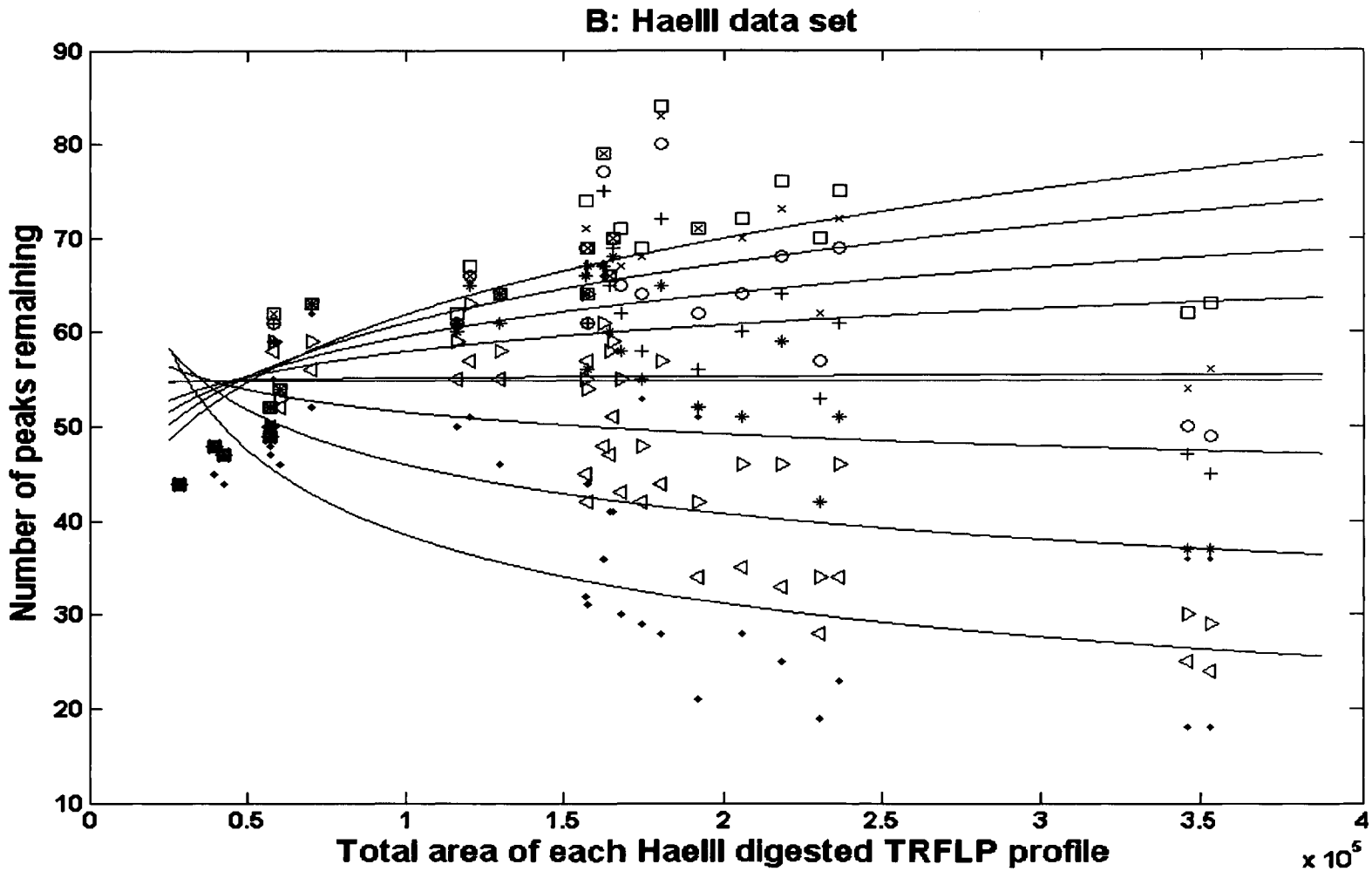
Table 13: Variable percentage thresholds for T-RFLP profiles belonging to different data sets (*Bst*UI, *Hae*III, *Hinf*I, and *Msp*I)
Threshold = (total area/optimal divisor)*100

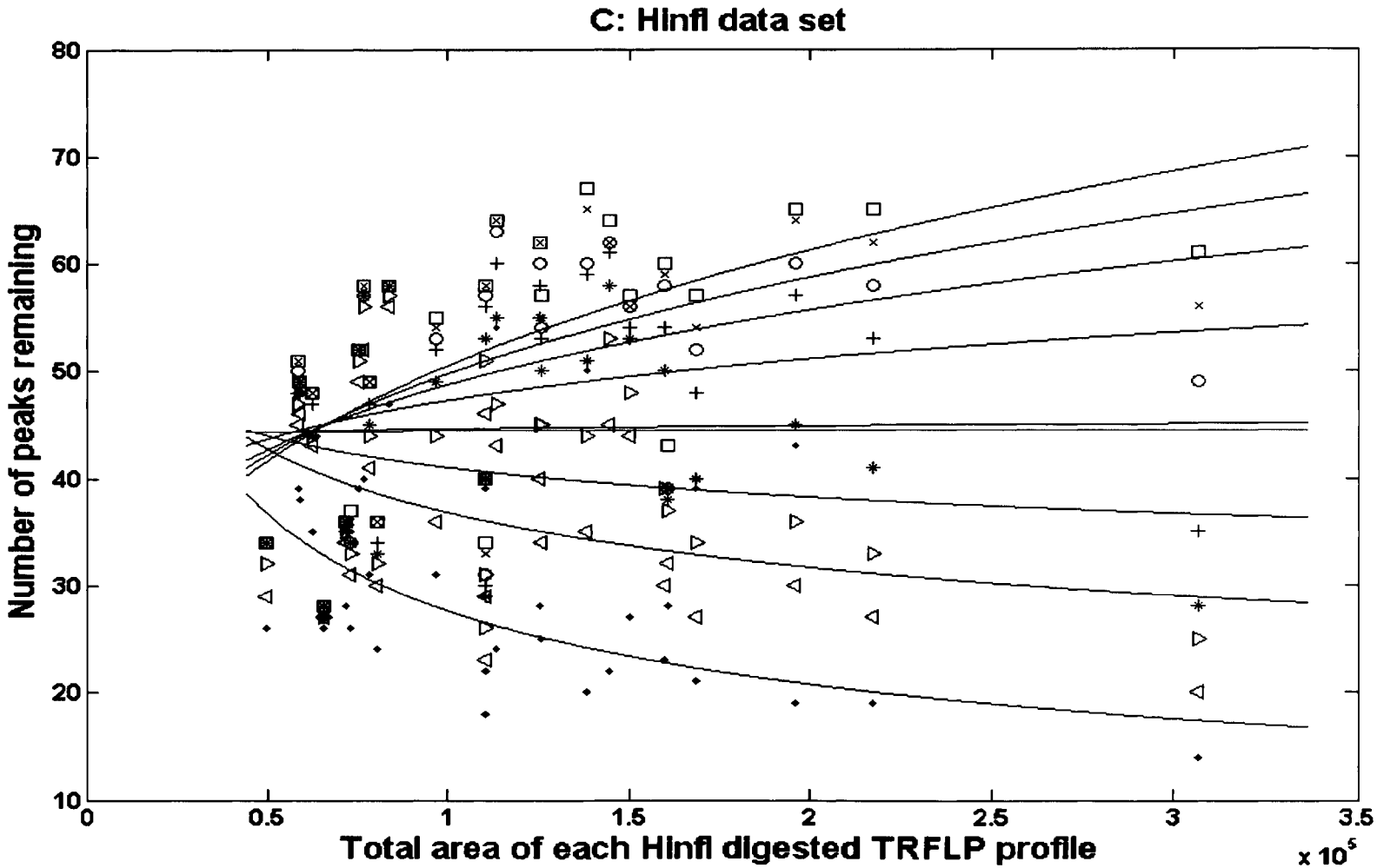
*Bst*UI data set	A2(a)	A2(b)	A6(a)	A6(b)	B1(a)	B1(b)	B2(a)	B2(b)	C1(a)	C1(b)
Total area	$1.5*10^5$	$1.5*10^5$	$2.1*10^5$	$2.2*10^5$	$0.6*10^5$	$0.5*10^5$	$0.7*10^5$	$0.9*10^5$	$1.3*10^5$	$2.2*10^5$
Threshold (%)	0.33	0.36	0.48	0.49	0.14	0.12	0.16	0.2	0.29	0.49
*Bst*UI data set	C2(a)	C2(b)	C3(a)	C3(b)	D2(a)	D2(b)	D4(a)	D4(b)	E2(a)	E2(b)
Total area	$1.8*10^5$	$1.7*10^5$	$2.1*10^5$	$1.7*10^5$	$1.7*10^5$	$1.8*10^5$	$1.1*10^5$	$1.2*10^5$	$1.5*10^5$	$1.3*10^5$
Threshold	0.39	0.38	0.47	0.39	0.37	0.39	0.25	0.27	0.34	0.28
*Bst*UI data set	E3(a)	E3(b)	F1(a)	F1(b)	F2(a)	F2(b)	-	-	-	-
Total area	$2.4*10^5$	$2.7*10^5$	$1.4*10^5$	$0.7*10^5$	$0.5*10^5$	$0.9*10^5$	-	-	-	-
Threshold	0.52	0.6	0.32	0.16	0.12	0.21	-	-	-	-
*Hae*III data set	A2(a)	-	A6(a)	A6(b)	B1(a)	B1(b)	B2(a)	B2(b)	C1(a)	C1(b)
Total area	$1.6*10^5$	-	$1.6*10^5$	$1.7*10^5$	$0.7*10^5$	$0.6*10^5$	$0.6*10^5$	$0.4*10^5$	$1.6*10^5$	$1.3*10^5$
Threshold	0.33	-	0.33	0.36	0.15	0.12	0.13	0.08	0.34	0.28
*Hae*III data set	C2(a)	C2(b)	C3(a)	C3(b)	D2(a)	D2(b)	D4(a)	D4(b)	E2(a)	E2(b)
Total area	$1.2*10^5$	$1.2*10^5$	$1.6*10^5$	$1.7*10^5$	$2.1*10^5$	$1.7*10^5$	$1.9*10^5$	$2.3*10^5$	$3.5*10^5$	$3.4*10^5$
Threshold	0.25	0.26	0.35	0.35	0.44	0.37	0.41	0.49	0.75	0.74
*Hae*III data set	E3(a)	E3(b)	F1(a)	F1(b)	F2(a)	F2(b)	-	-	-	-
Total area	$2.4*10^5$	$2.2*10^5$	$0.6*10^5$	$0.3*10^5$	$0.4*10^5$	$0.6*10^5$	-	-	-	-
Threshold	0.5	0.47	0.12	0.06	0.09	0.12	-	-	-	-
*Hinf*I data set	A2(a)	A2(b)	A6(a)	A6(b)	B1(a)	B1(b)	B2(a)	B2(b)	C1(a)	C1(b)
Total area	$1.3*10^5$	$1*10^5$	$1.4*10^5$	$1.1*10^5$	$0.8*10^5$	$0.8*10^5$	$0.6*10^5$	$0.8*10^5$	$1.1*10^5$	$0.5*10^5$
Threshold	0.29	0.22	0.33	0.25	0.18	0.19	0.13	0.17	0.25	0.11
*Hinf*I data set	C2(a)	C2(b)	C3(a)	C3(b)	D2(a)	D2(b)	D4(a)	D4(b)	E2(a)	E2(b)
Total area	$0.7*10^5$	$0.7*10^5$	$1.1*10^5$	$0.8*10^5$	$0.6*10^5$	$0.8*10^5$	$1.4*10^5$	$1.1*10^5$	$3.1*10^5$	$2*10^5$
Threshold	0.17	0.16	0.25	0.18	0.13	0.18	0.32	0.26	0.7	0.45
*Hinf*I data set	E3(a)	E3(b)	F1(a)	F1(b)	F2(a)	F2(b)	-	-	-	-
Total area	$1.7*10^5$	$2.2*10^5$	$1.6*10^5$	$0.7*10^5$	$1.5*10^5$	$0.6*10^5$	-	-	-	-
Threshold	0.39	0.5	0.37	0.15	0.34	0.14	-	-	-	-
*Msp*I data set	A2(a)	A2(b)	A6(a)	A6(b)	B1(a)	B1(b)	B2(a)	B2(b)	C1(a)	C1(b)
Total area	$1.4*10^5$	$2.7*10^5$	$1.9*10^5$	$0.9*10^5$	$0.4*10^5$	$0.5*10^5$	$0.2*10^5$	$0.3*10^5$	$1.2*10^5$	$0.7*10^5$
Threshold	0.37	0.73	0.5	0.24	0.11	0.14	0.06	0.07	0.31	0.19
*Msp*I data set	C2(a)	C2(b)	C3(a)	C3(b)	D2(a)	D2(b)	D4(a)	D4(b)	E2(a)	E2(b)
Total area	$0.6*10^5$	$0.7*10^5$	$1.1*10^5$	$1*10^5$	$2.5*10^5$	$1*10^5$	$1.3*10^5$	$1.5*10^5$	$1.4*10^5$	$1.9*10^5$
Threshold	0.16	0.18	0.28	0.27	0.68	0.25	0.33	0.4	0.36	0.52
*Msp*I data set	E3(a)	E3(b)	F1(a)	F1(b)	F2(a)	F2(b)	-	-	-	-
Total area	$1.3*10^5$	$1*10^5$	$0.4*10^5$	$0.5*10^5$	$0.7*10^5$	$0.4*10^5$	-	-	-	-
Threshold	0.35	0.27	0.1	0.13	0.2	0.11	-	-	-	-

Figure 15: Estimation of the optimal divisor for the calculation of the variable percentage threshold for four T-RFLP data sets generated by different restriction endonucleases: (A) *Bst*UI, (B) *Hae*III, (C) *Hinf*I, (D) *Msp*I.

The curves were fitted as power functions. Nine curves generated by the calculation of different divisors were showed as following: □ , $Z*10^2+7.3*10^7$; ×, $Z*10^2+6.3*10^7$; ○, $Z*10^2+5.3*10^7$; +, $Z*10^2+4.3*10^7$; ✱, $Z*10^2+3.3*10^7$; ▷, $Z*10^2+2.3*10^7$; ◁, $Z*10^2+1.3*10^7$; ◆, $Z*10^2+0.3*10^7$ (Z: the mean total area of each data set: *Bst*UI:$1.49*10^5$, *Hae*III: $1.50*10^5$,*Hinf*I: $1.17*10^5$, *Msp*I: $1.07*10^5$.). The optimum divisor for each data set was shown as '◆' (*Bst*UI: $4.49*10^7$, *Hae*III: $4.70*10^7$, *Hinf*I: $4.37*10^7$, *Msp*I: $3.75*10^7$), which resulted in the minimum R square (almost zero) of the power function which means the weakest relationship between the total area on the original T-RFLP patterns and the numbers of peaks remaining after normalized by the threshold based on that divisor.







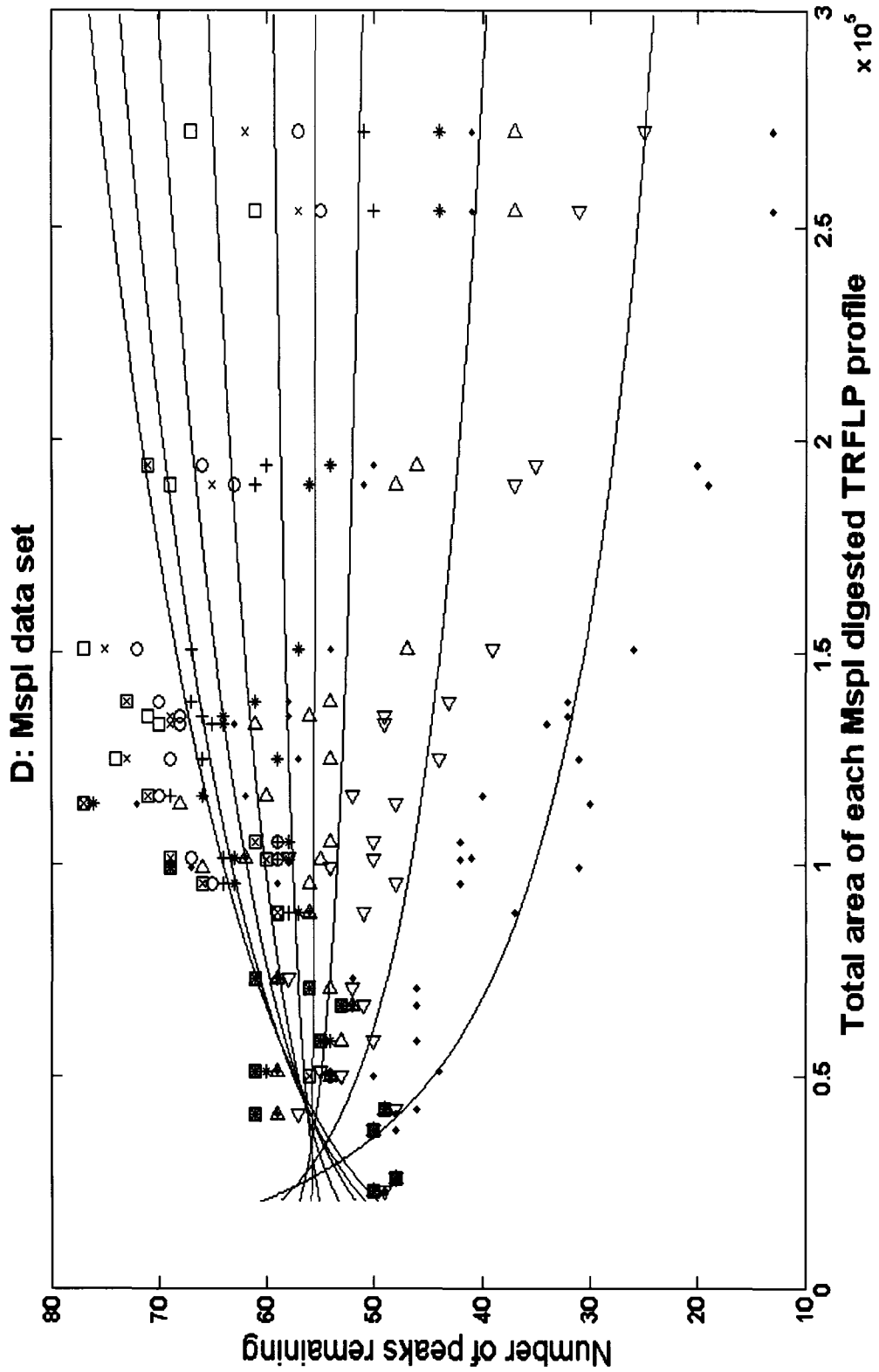
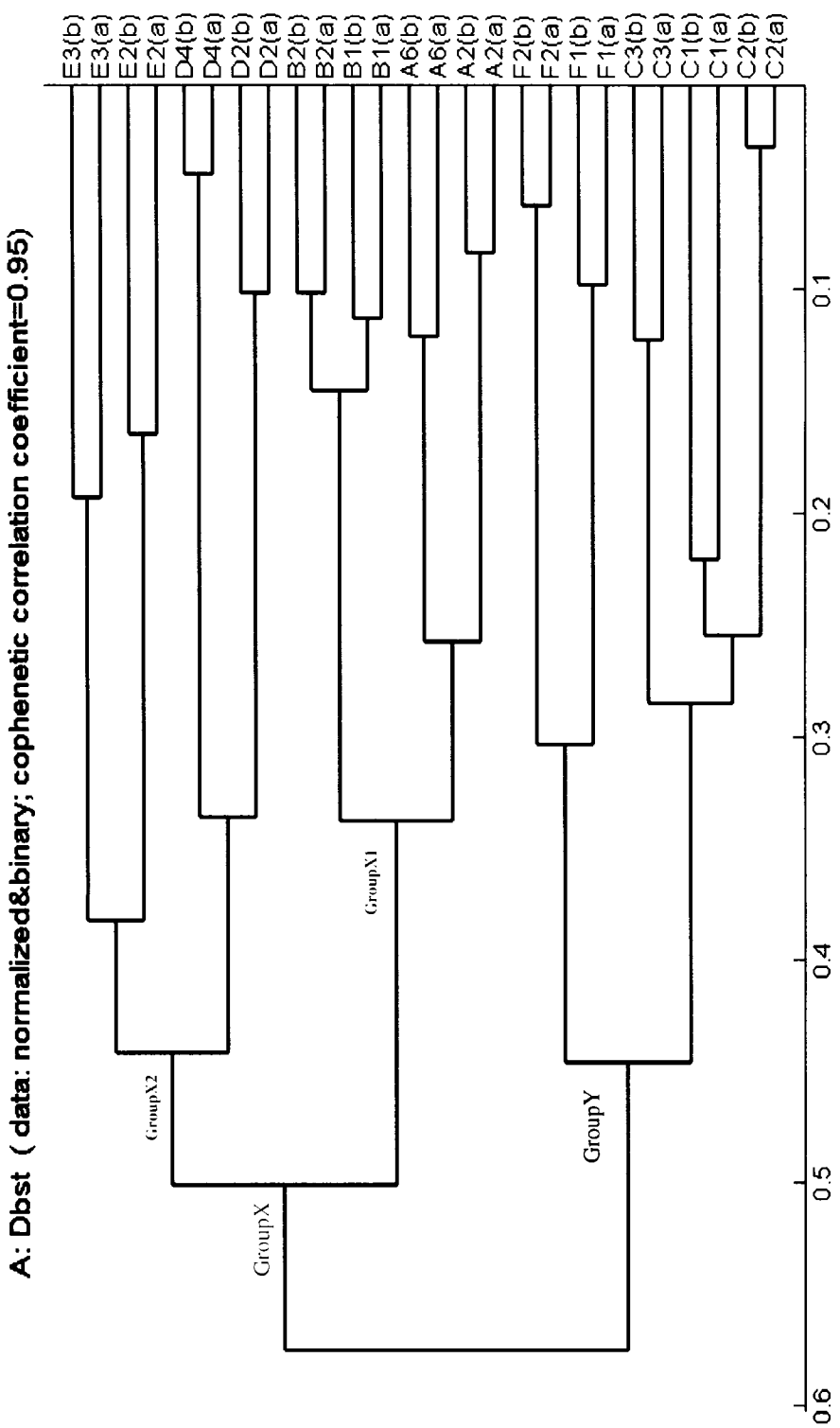
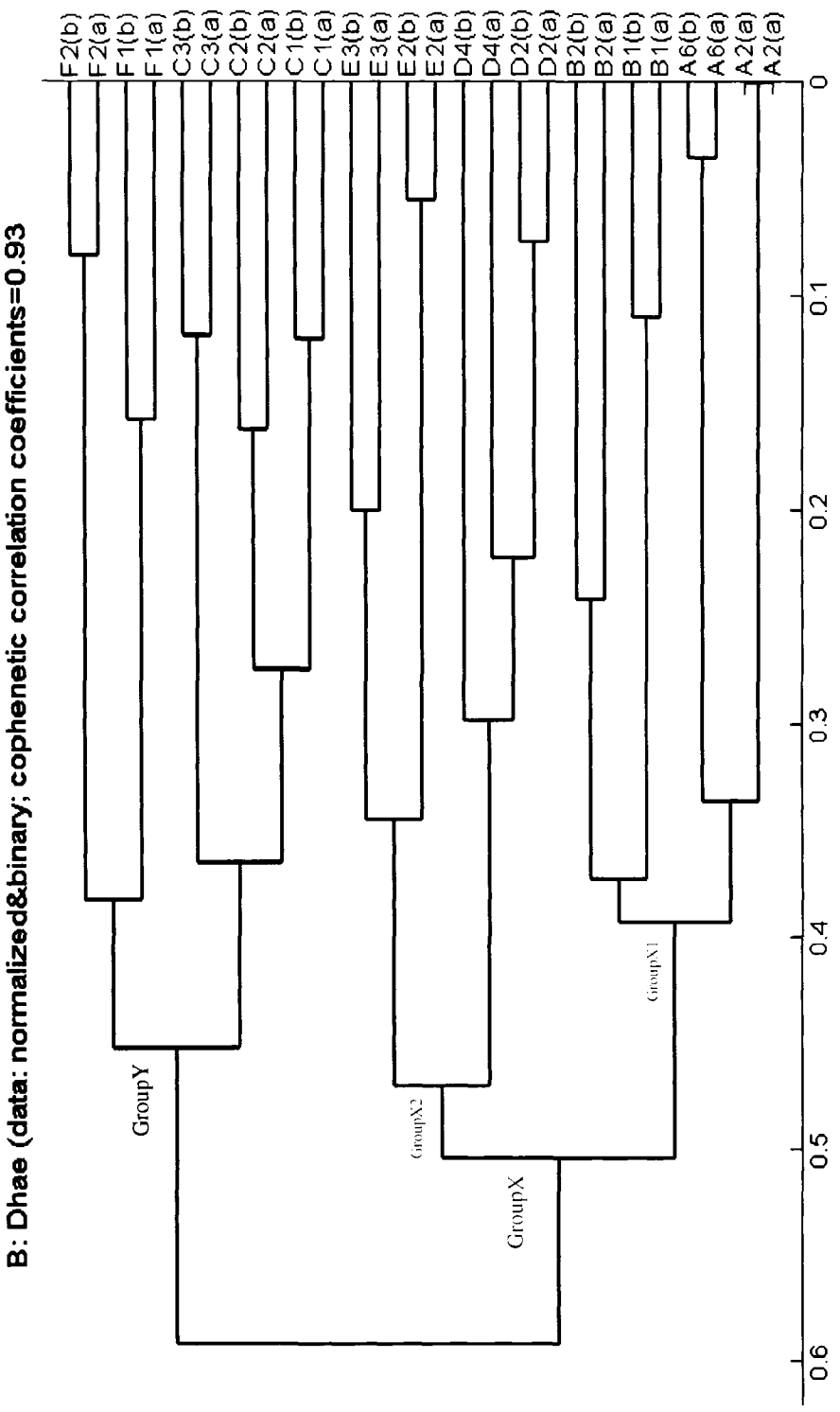
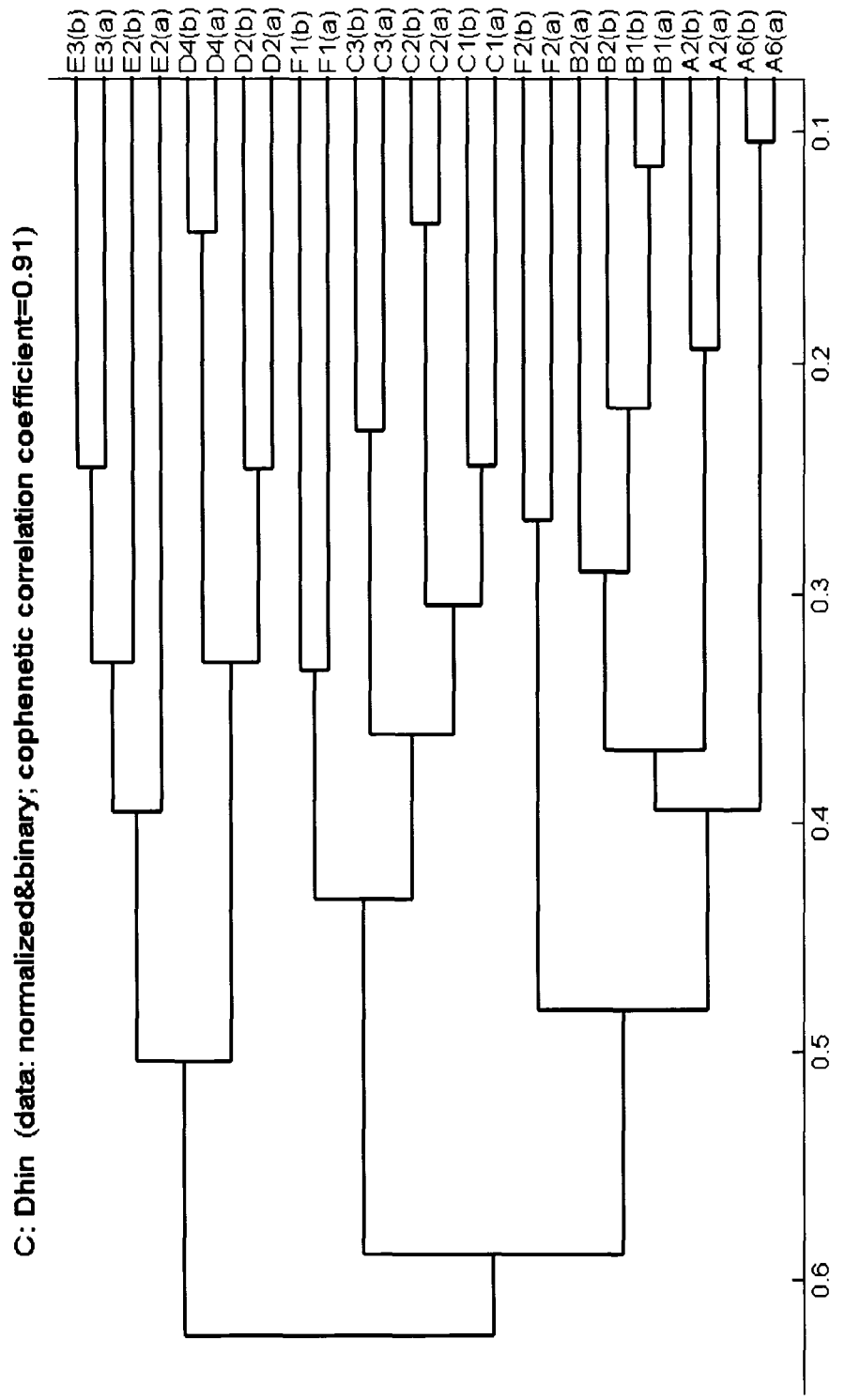


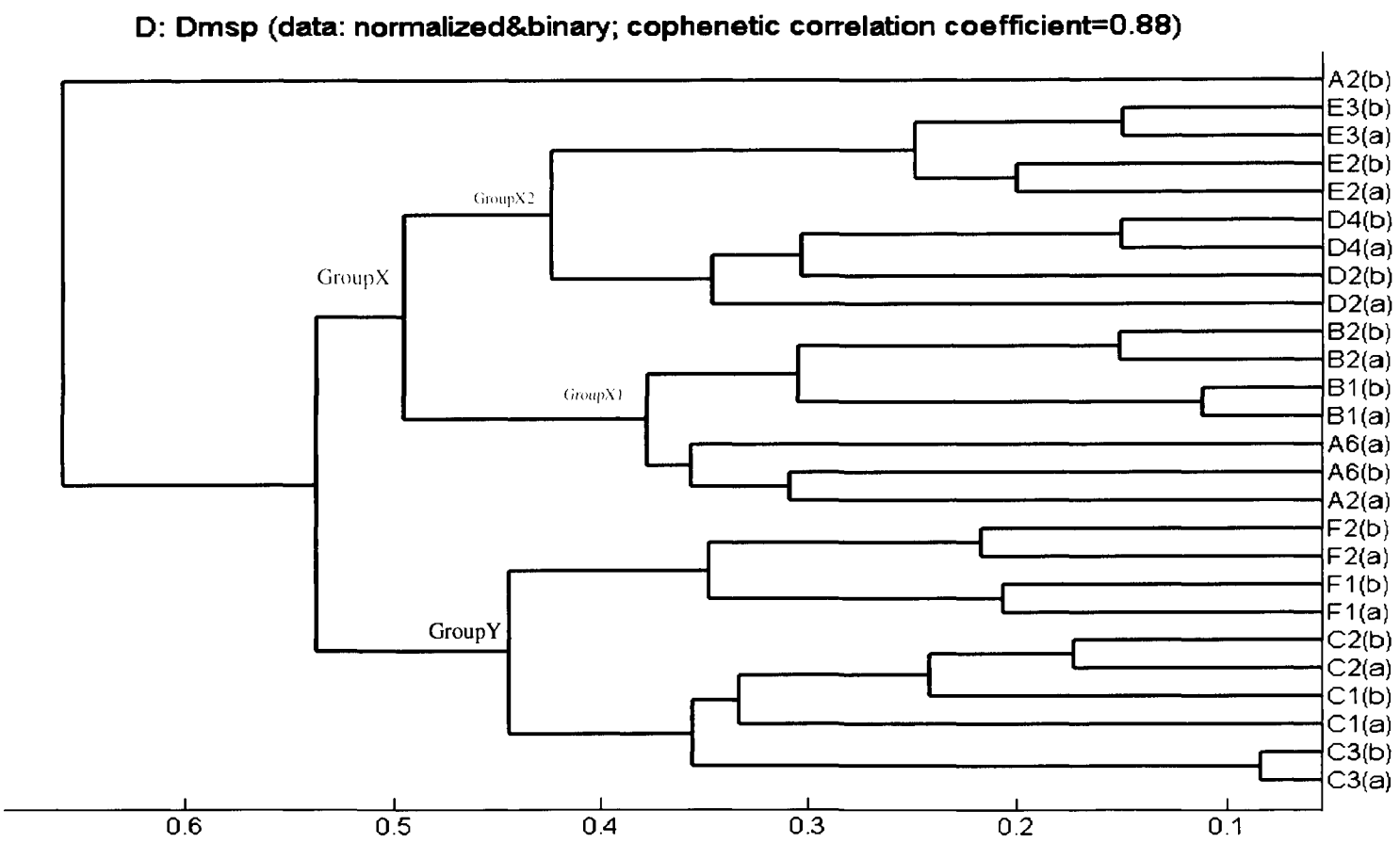
Figure 16: Dendrogram structures of TRF profile comparisons from lab-treated soil samples, greenhouse soil samples, and field soil samples.

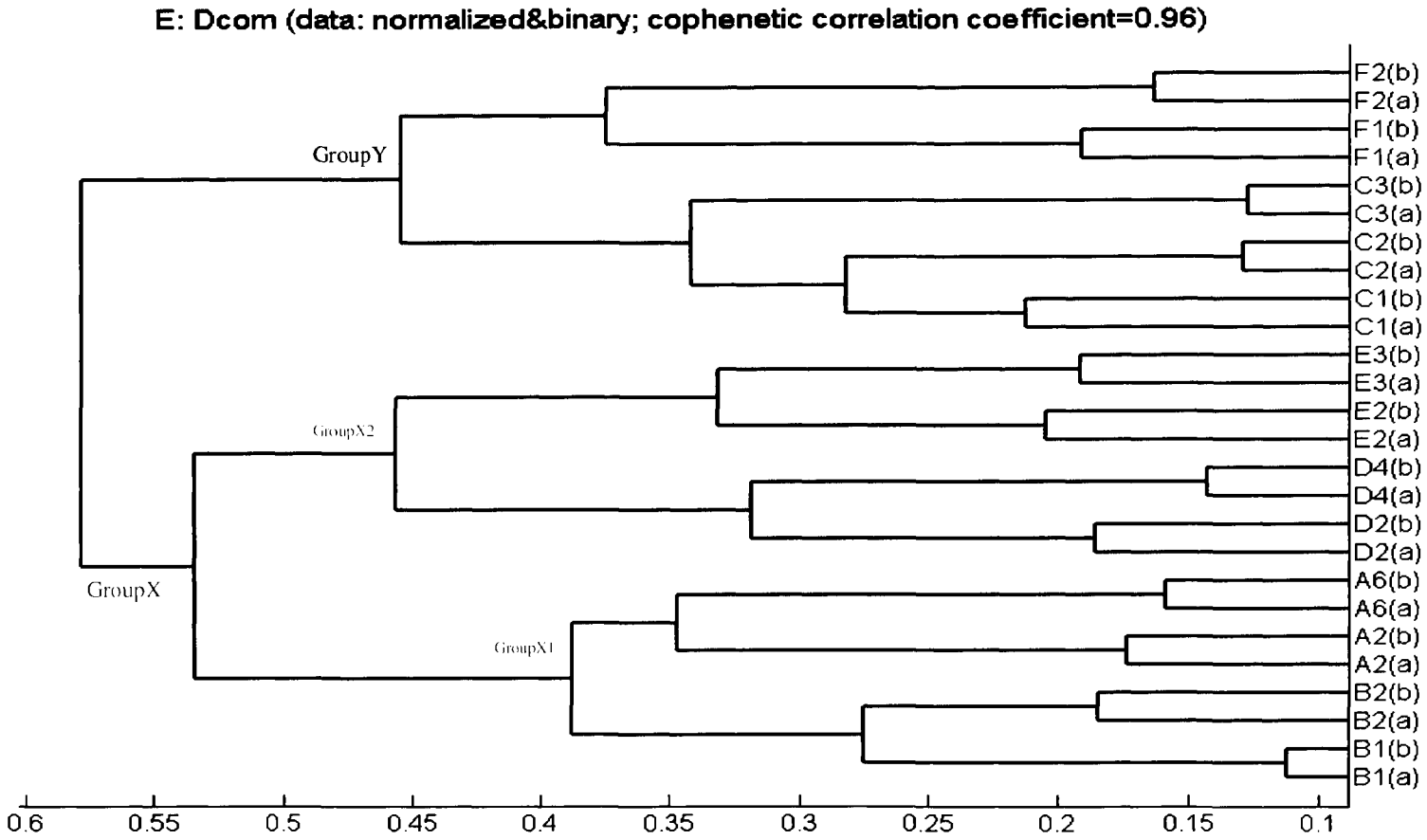
Using Matlab7.1, the similarity of binary TRF profiles was calculated by Jaccard coefficient. Unweighted average distance (UPGMA) was used for clustering. (A). Dbst: dendrogram of *Bst*UI data set (B). Dhae: dendrogram of *Hae*III data set; (C). Dhin: dendrogram of *Hinf*I data set; (D). Dmsp: dendrogram of *MspI* data set; (E). Dcom: dendrogram of combined data set (*Bst*UI, *Hae*III, *Hinf*I, and *Msp*I). The samples were indicated by letter codes at the branch termini: A (greenhouse Hup^- nodule soils); B (greenhouse Hup^+ nodule soils); C (field bulk soils); D (Lab hydrogen-treated soils); E (lab air-treated soils); F (filed Hup^- nodules soils). Replicate samples were indicated as (a)/(b).











were close to very high (Dbst: 0.95, Dhae, 0.93, Dhin, 0.91, Dmsp: 0.88, and Dcom: 0.96). Thus, those dendrograms were reliable. The more similar samples were, the more possible they were grouped together in one dendrogram. Dendrograms of different data sets showed high similarities. Relationships of different soil samples reflected by TRF profiles generated by all four REs were a nice match for anticipated results except little difference showed by the TRF profiles resulting from *Msp*I and *Hinf*I.

All dendrograms showed that four REs applied (*Bst*UI, *Hae*III, *Hinf*I, and *Msp*I) had the ability to group most replicates. One hundred percent of replicates were paired by *Bst*UI, 92% by *Hae*III, 85% by *Hinf*I, and 70% by *Msp*I. All soil samples were firstly divided into two big groups: GroupX and GroupY. All samples derived from the soil collected from field in Lawrencetown, Nova Scotia two years ago were put in GroupX (A2, A6, B1, B2, D2, D4, E2, and E3), while the others collected from the same field (Dr. Dong' garden) in the spring of 2006 samples belonged to GroupY (C1, C2, C3, F1, and F2). It was theoretically reasonable because soil bacterial community structure always varied with the places where the soil was sampled.

Then GroupX was further divided into two subgroups: GroupX1 (B1, B2, A2, & A6) and GroupX2 (D2, D4, E2, & E3). All sample belonging GroupX1 were soil adjacent to the root nodules of soybeans grown in greenhouse, while GroupX2 including all lab-treated soil samples. The activity of soybean roots should exert significant effects on

the rhizobacterial community structure. Therefore, all greenhouse samples were separated from all lab-treated soil samples which were free from the effects of the activity of soybean activity. For groups of lab-treated soil (GroupX2), greenhouse soil (GroupX1) and field soil (GroupY), they were finally divided into subgroups.

In the group of lab-treated soils (GroupX2), all one-month hydrogen-treated soil samples with high hydrogen uptake rate (D2 & D4) were separated from their controls, one-month air-treated soil samples with quite low hydrogen uptake rate (E2 & E3). The gas applied to treat soil samples was the only difference between two subgroups of lab-treated soil, which indicates that the metabolism of electrolytic hydrogen in soils should be the main reason for the obvious variation of bacterial community structure in hydrogen-treated soil samples (D2 & D4).

In the group of greenhouse soils (GroupX1), Hup^- nodule soil samples with high hydrogen uptake rate (A2 and A6) were separated from Hup^+ nodule soil samples with quite low hydrogen uptake rate (B1 & B2). Hydrogen treatment was also considered as the only difference between the subgroups of Hup^+ nodule soil samples and Hup^- nodule soil samples because Hup^- nodule have the ability to release hydrogen to rhziosphere soil while little hydrogen were released from Hup^+ nodules which had the activity of hydrogenase. Therefore, it was inferred that metabolism of hydrogen in soils was responsible for the obvious variation of bacterial community structure in greenhouse soil adjacent to the Hup^- nodules (A2 &A6).

As to the group of field soils, all bulk soils (C1, C2, &C3) and field Hup$^-$ nodule soil (F1 & F2) were completely separated into two different subgroups. In this case both of the activity of soybean roots and metabolism of hydrogen released from Hup$^-$ nodule contributed the variation of bacterial community structure in field Hup$^-$ nodule soils. Therefore, it was impossible to assess the effects of hydrogen metabolism on the variation of bacterial community structure in field soil adjacent to Hup$^-$ nodules.

3.3.7 Hydrogen Induced Variation of Bacterial Community Structure in Soil Samples

Variation in Intensity (% of total area) of TRF peaks reflected the quatitative variation of bacterial communities in soil samples. All REs digested TRF profiles from greenhouse soil samples and soil samples treated in lab showed that hydrogen metabolism resulted in both intensity increase of some TRF peaks and intensity decrease of some others in TRF profiles from hydrogen treated soils (Figure 17). The intensity increase of TFR peaks suggests that hydrogen metabolism stimulated the growth of bacteria contributing to these peaks in the soil samples, and the intensity decrease of TFR peaks indicated that the growth of bacteria responsible for those peaks was inhibited after hydrogen treatment.

Moreover, for those intensity-increased TRF peaks (Figure18 & Figure19), most of them only appeared in the TRF profiles from soil samples exposed to hydrogen gas

(hydrogen-treated lab soil: D2 & D4; greenhouse soil adjacent to Hup^- nodules: A2 & A6) compared to their controls (air-treated lab soil: E2 &E3; greenhouse soils adjacent to Hup^+ nodules: B1 & B2), which inferred that most bacteria contributing to those TRF peaks were normally dormant in the soils until they were exposed to hydrogen gas with certain concentration.

S(i), the total differences of the mean intensity (% of total area) of TRF peaks whose intensity increased obviously between profiles from soils exposed to hydrogen gas and their controls (Table 14), and S(d), the total differences of the mean intensity (% of total area) of TRF peaks whose intensity decreased obviously between profiles from soils exposed to hydrogen gas and their controls (Table 15), were calculated to study the hydrogen-induced variation of soil bacterial community structure. Table 14 showed that S(i) were 14.38 in *Bst*UI profiles, 21.09 in *Hae*III profiles, 19.27 in *Hinf*I profiles, and 17.35 in *Msp*I profiles from greenhouse soil samples (A2&A6/ B1&B2) and 39.46 in *Bst*UI profiles, 33.40 in *Hae*III profiles, 39.04 in *Hin*fI profiles, and 41.33 in *Msp*I profiles from soil samples treated in lab (D2&D4/E2&E3). Table 15 showed that S(d) were 18.90 in *Bst*UI profiles, 22.10 in *Hae*III profiles, 22.60 in *Hinf*I profiles, and 15.50 in *Msp*I profiles from greenhouse soil samples (A2&A6/ B1&B2) and 23.1 in *Bst*UI profiles, 24.1 in *Hae*III profiles, 24.5 in *Hinf*I profiles, and 21.7 in *Msp*I profiles from soil samples treated in lab (D2&D4/E2&E3). It was found that either S(i) or S(d) in TRF profiles generated by different REs (*Bst*UI, *Hae*III, *Hinf*I, and *Msp*I) from greenhouse

soil samples or soil samples treated in lab were close to each other, which meant that the profiles applied were reliable. Furthermore, the mean S(i) in TRF profiles from soil samples treated by hydrogen in lab (D2&D4) was obviously higher than that in TRF profiles from greenhouse soils adjacent to Hup$^-$ nodules (A2&A6) (D/A: 38.3±3.4/18±2.8), which matched results of hydrogen uptake rate mentioned above (D/A: 0.23±0.04/0.15±0.03). There was no obvious difference between the mean S(d) in TRF profiles from soil samples treated by hydrogen in lab (D) and greenhouse soils adjacent to Hup$^-$ nodules(A) (D/A: 23.4±1.2/ 19.8±3.3).

It was found that TRF profiles from soils exposed to hydrogen gas always included few TRFs whose intensity variation was predominant (Figure 17) and quite a few percentage of hydrogen-induced variation of bacterial community structure was contributed by them. Comparison between TRF profiles from greenhouse Hup$^-$ nodule soil samples (A2&A6) and the controls (B1&B2) showed that the ratios of Si(top5), the sum of five largest differences (intensity increases), to S(i) were 66.4% (*Bst*UI), 57.7% (*Hae*III), 48.5%(*Hinf*I), and 49% (*Msp*I) (Table 14), and the ratios of Sd(top5), the sum of five largest differences (intensity decrease), to S(d) were 61.9% (*Bst*UI), 53.7% (*Hae*III), 59.1%(*Hinf*I), and 72.8% (*Msp*I) (Table 15). Also, comparison between TRF profiles from soils treated by hydrogen gas in lab (D2&D4) and the controls (E2&E3) showed that the ratios of Si(top5) to S(i) were 70.1% (*Bst*UI), 80.5% (*Hae*III), 70%(*Hinf*I), and 61.5%

(*Msp*I) (Table 14), and the ratios of Sd(top5) to S(d) were 45.6% (*Bst*UI), 37.4% (*Hae*III), 51.7%(*Hinf*I), and 65.4% (*Msp*I) (Table 15). Most of those ratios were above 50%.

Figure 17 showed that the most of peaks whose intensity varied obviously in TRF profiles from greenhouse soils adjacent to Hup$^-$ nodules (A2&A6) didn't match those in TRF profiles from soils treated by hydrogen gas in lab (D2&D4). Thus, most of bacteria responsible for hydrogen-induced variation of bacterial community structure in greenhouse Hup$^-$ nodule soil samples (A2&A6) were different to those in soil samples treated by hydrogen gas in lab (D2&D4). Only a few of them were common both in greenhouse nodule soil samples and soil samples treated in lab. They probably contributed the intensity increase of TRF peaks: B109.5, B375.5, B391.9, Ha209.5, Hi298.8, Hi312.7, Hi313.8, Hi329, and M453.1 (Table 14) or intensity decrease of TRF peaks: B234.6, B400.5, Ha187.2, Ha188.5, Ha199.3, Ha202.6, Ha222.5, Ha225, Hi310.6, Hi316.8, Hi322.7, Hi326.6, Hi330.4, Hi337.2, M151.4, and M486 (Table 15) (Binned TRF peaks were indicated by letter and number: B, Ha, Hi, and M meant peaks generated by using *Bst*UI, *Hae*III, *Hinf*I, and *Msp*I; number was the average of the sizes of all peaks in the bin fixed within ±0.4bp).

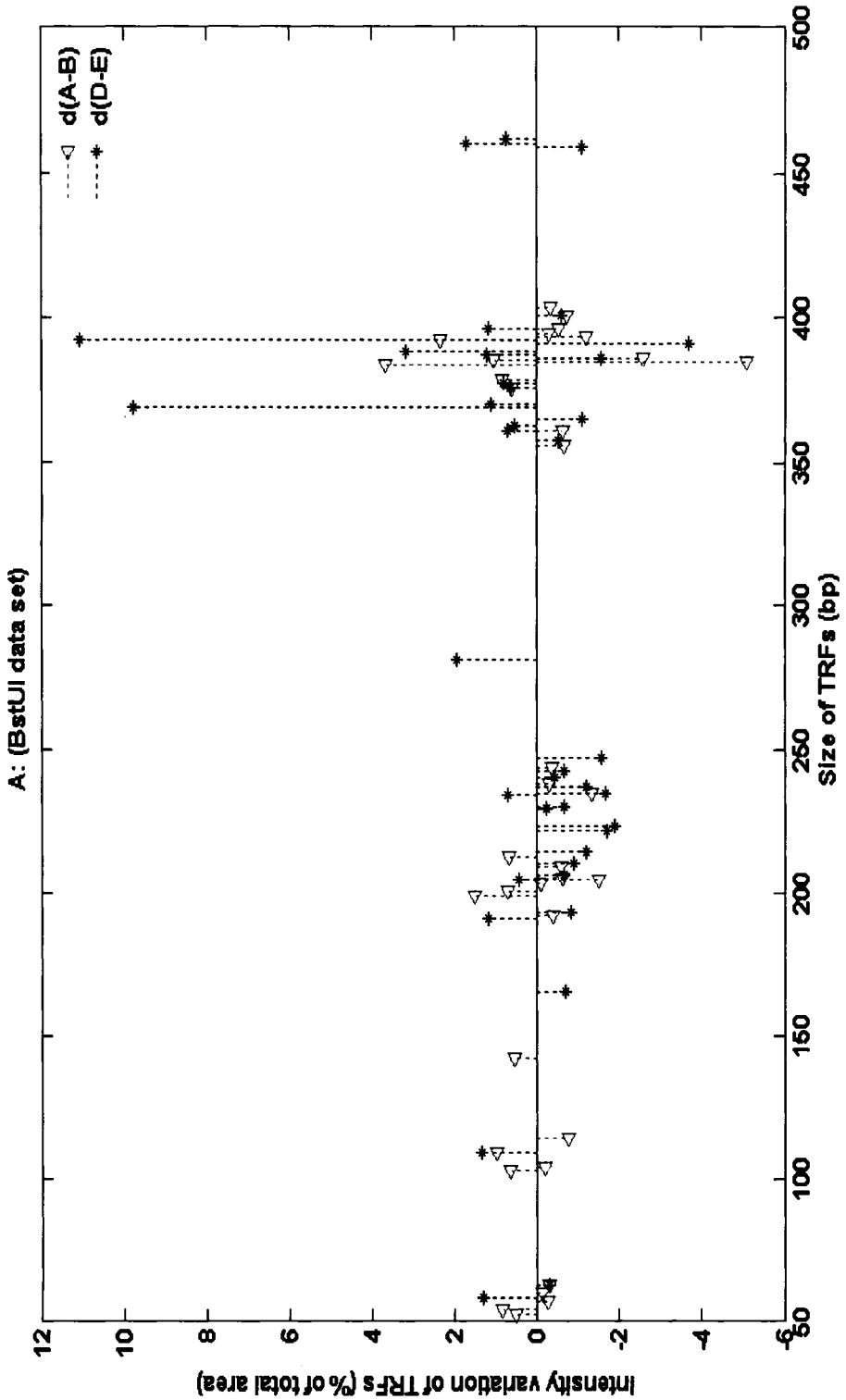
3.3.8 Contribution of Our Isolates to Hydrogen-induced Variation of Bacterial Community Structure in Soil Samples

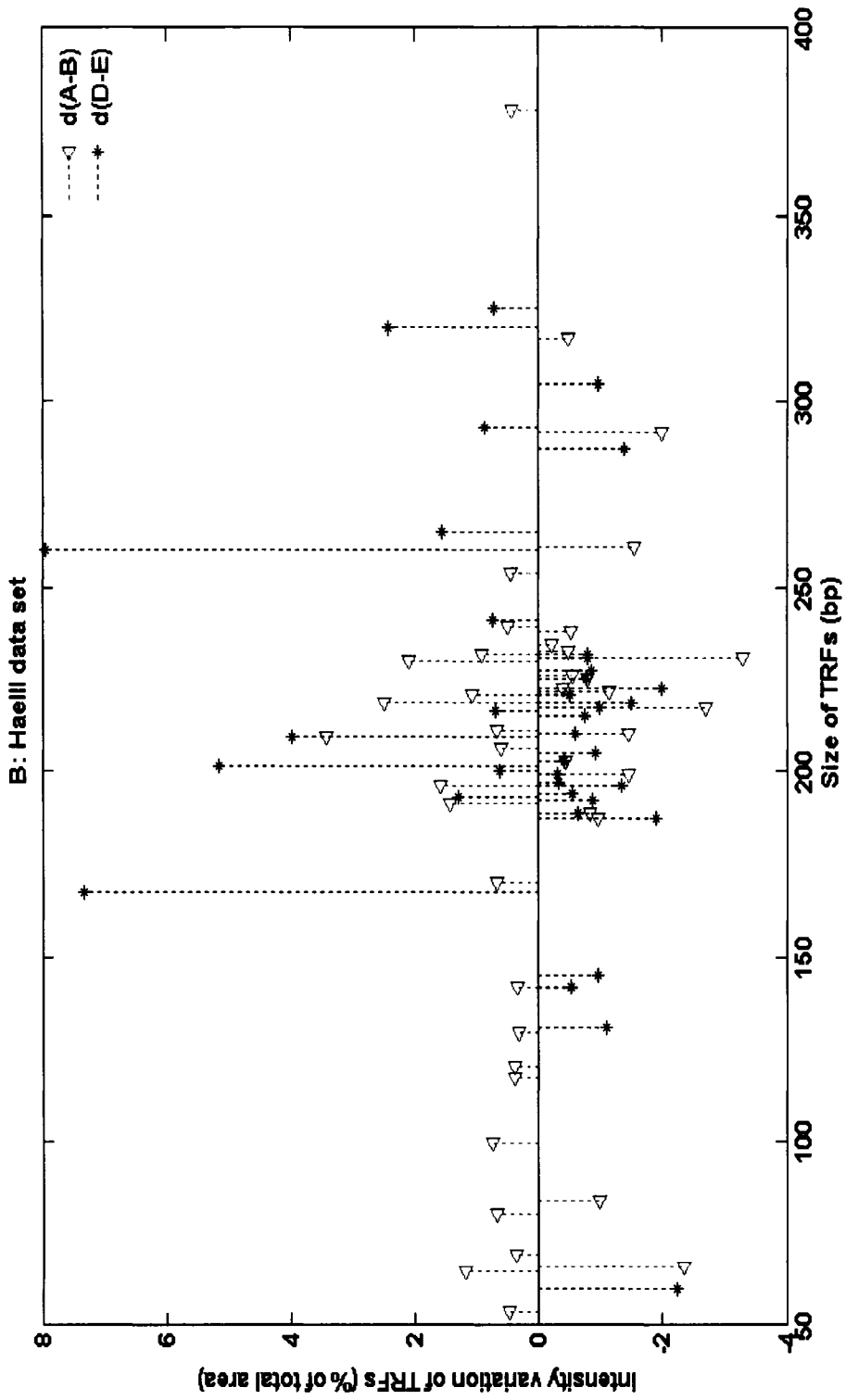
It was fond that all TRF peaks in profiles from complex samples (A2J&A6J, D2J&D4J) showed intensity decrease compared with those in profiles from hydrogen treated soil samples (A2&A6, D2&D4) except for a few peaks such as B62.5, B102, B222.4, B384.4, Ha63.2, Ha72.6, Ha217.5, Hi320.1, Hi321.6, M81.2, M275.7, and M483.6 (Table 16). However, only part of these spiked TRF peaks (B62.5, B102, Ha217.5, Hi320.1, Hi321.6, M81.2, and M483.6) were a nice match for the predicted TRF peaks (*Variovorax p*: B68, Ha220, Hi325, Hi325, and M491, *Burkholderia s.*: Ha222, Hi327, and Hi327, and *Flavobacterium j*: B106, Hi324, Hi324 and M86) generated by Restriction Enzyme analysis of DNA sequences of 16S rRNA genes from isolates published in NCBI GenBank (Table 16). Therefore, variation in intensity of these peaks (*Variovorax p*: B62.5, Ha217.5, Hi320.1, Hi321.6, and M483.6, *Burkholderia s.*: Ha217.5, Hi320.1, and Hi321.6, and *Flavobacterium j*: B102, Hi320.1, Hi321.6 and M81.2) was considered as an indicator of the status of our isolates in hydrogen-induced variation of bacterial community structure.

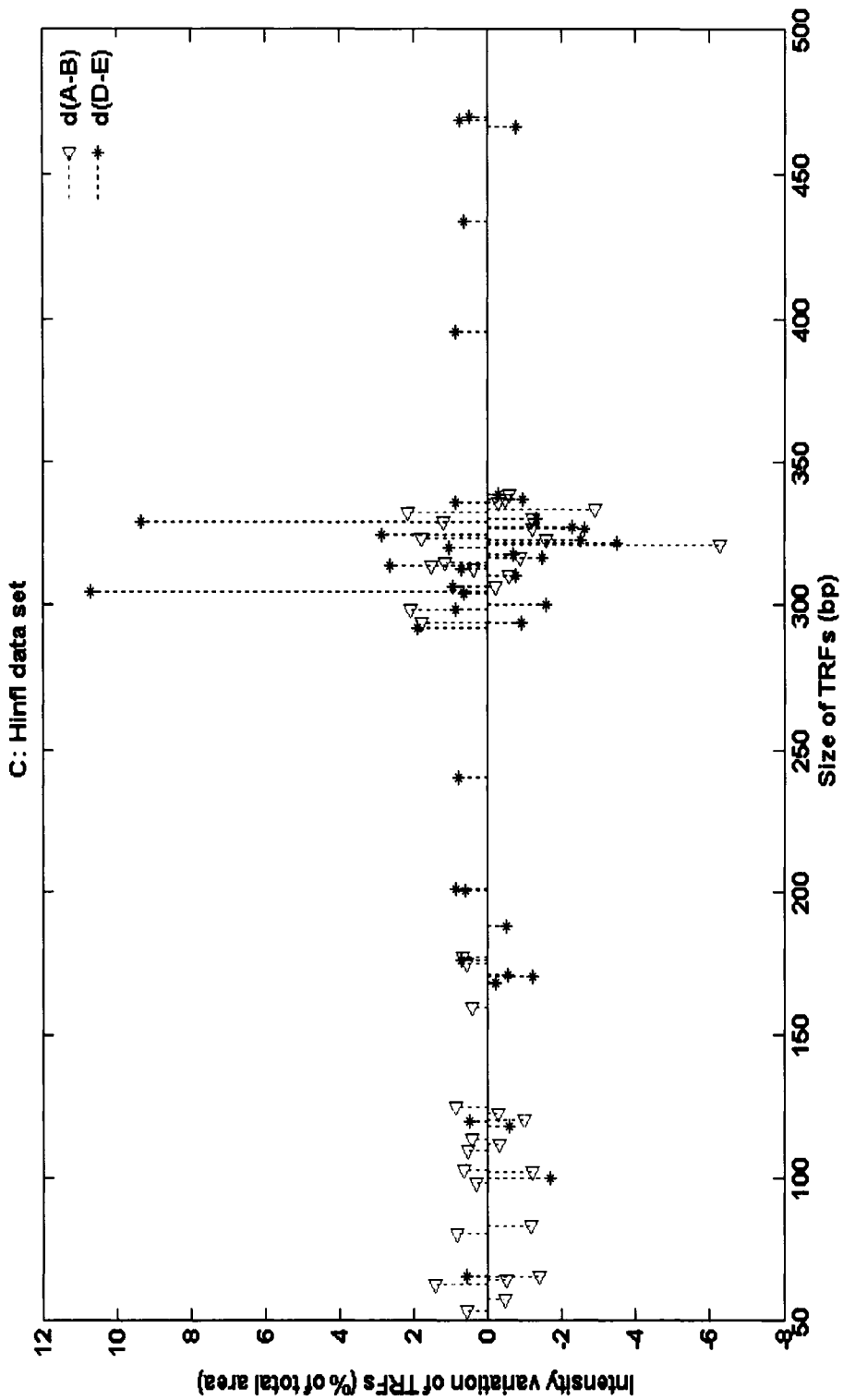
It was found that only two of them, Hi320.1 and M483.6, showed significant increase in intensity in TRF profiles from soil samples treated in lab (Figure 18 & Figure 19). Furthermore, Hi320.1 only appeared in TRF profiles (*Hinf*I) from hydrogen treated soils (D2&D4). This suggests that our isolates of hydrogen-oxidizing bacteria

Figure 17: Intensity variation of TRF peaks in profiles from hydrogen-treated soils compared to the controls

(A): Normalized data generated by *Bst*UI digestion; (B): Normalized data generated by *Hae*III digestion; (C): Normalized data generated by *Hinf*I digestion; (D): Normalized data generated by *Msp*I digestion. d (A-B): difference of the mean intensity (% of total area) of TRF peaks between greenhouse Hup^- nodules soil samples (A2&A6) and the controls (greenhouse Hup^+ nodules soil: B1&B2); d (D-E): difference of the mean intensity (% of total area) of TRF peaks between soil samples treated by hydrogen gas in lab (D2 &D4) and the controls (air-treated soil: E1&E2). Points contributed by peaks whose intensity increased obviously in TRF profiles from soil samples exposed to hydrogen gas were above X Axis; Points contributed by peaks whose intensity decreased obviously in TRF profiles from soil samples exposed to hydrogen gas were below X Axis.







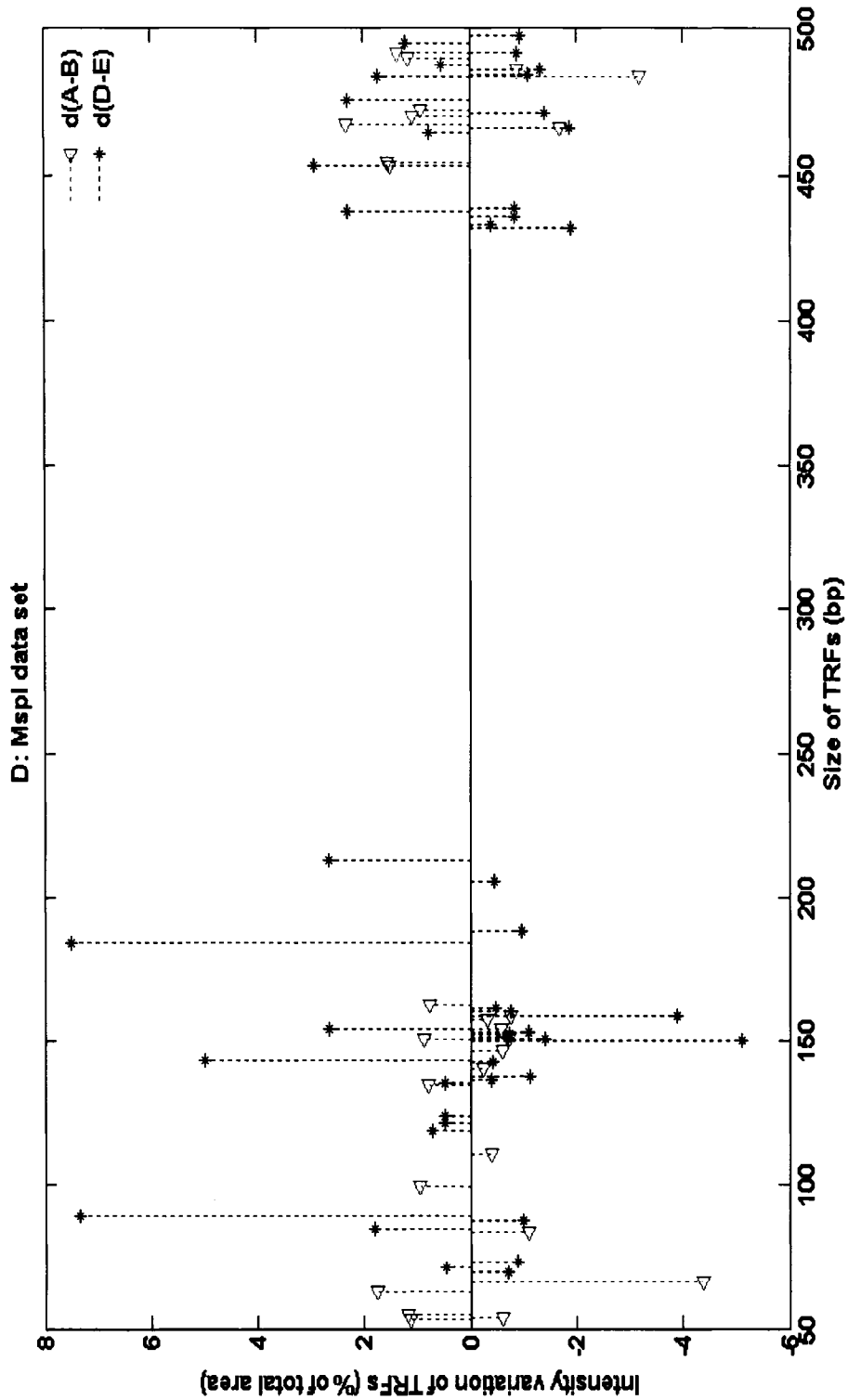
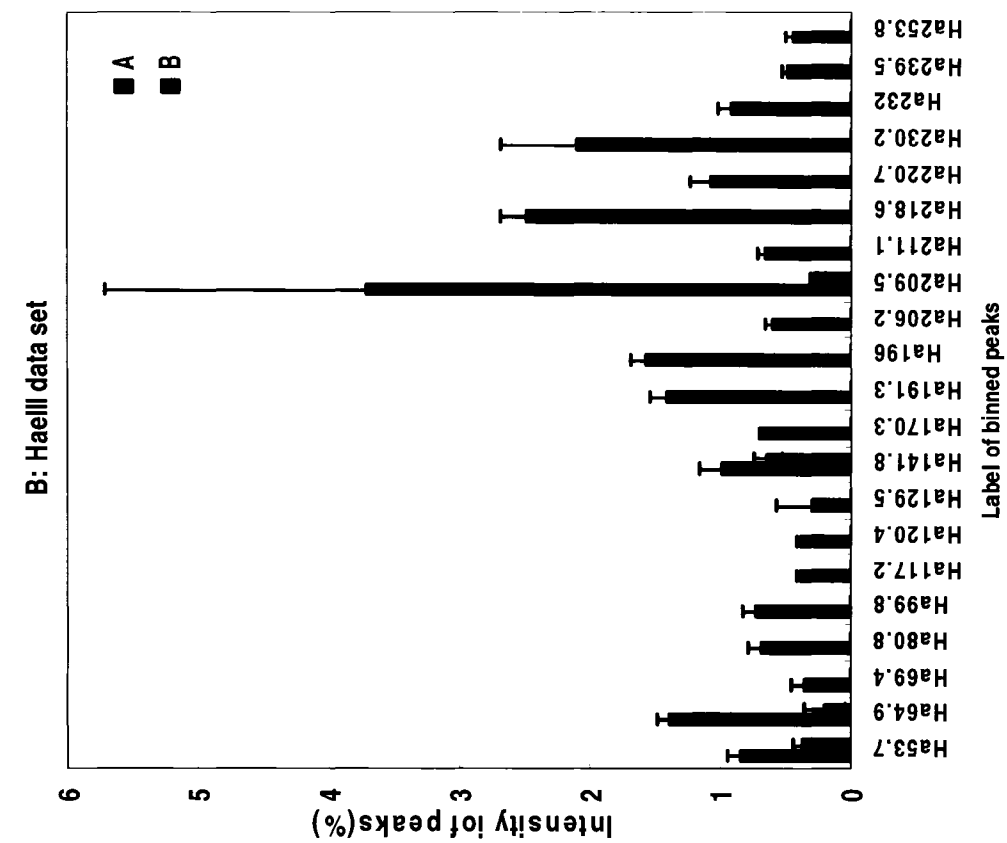
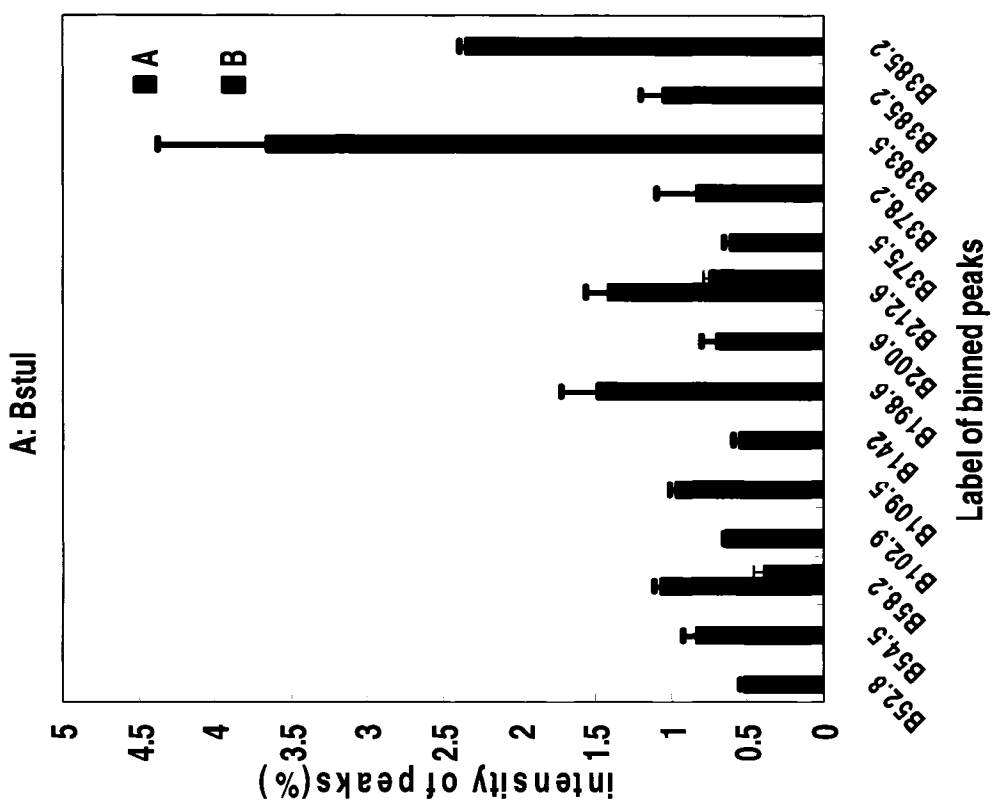


Figure 18: Average intensity with standard error bars of TRF peaks whose intensity increased obviously in profiles from greenhouse soils adjacent to Hup^- nodule compared to the controls.

(A): *Bst*UI data set; (B): *Hae*III data set, (C): *Hinf*I data set; (D): *Msp*I data set. Soil samples were indicated as: A (greenhouse Hup^- nodule soil samples: A2&A6) and B (greenhouse Hup^+ nodule soil samples: B1&B2). Binned TRF peaks were indicated by letter and number: B, Ha, Hi, and M meant peaks generated by using BstUI, HaeIII, HinfI, and MspI; number was the average of the sizes of all peaks in the bin fixed within ±0.4bp.





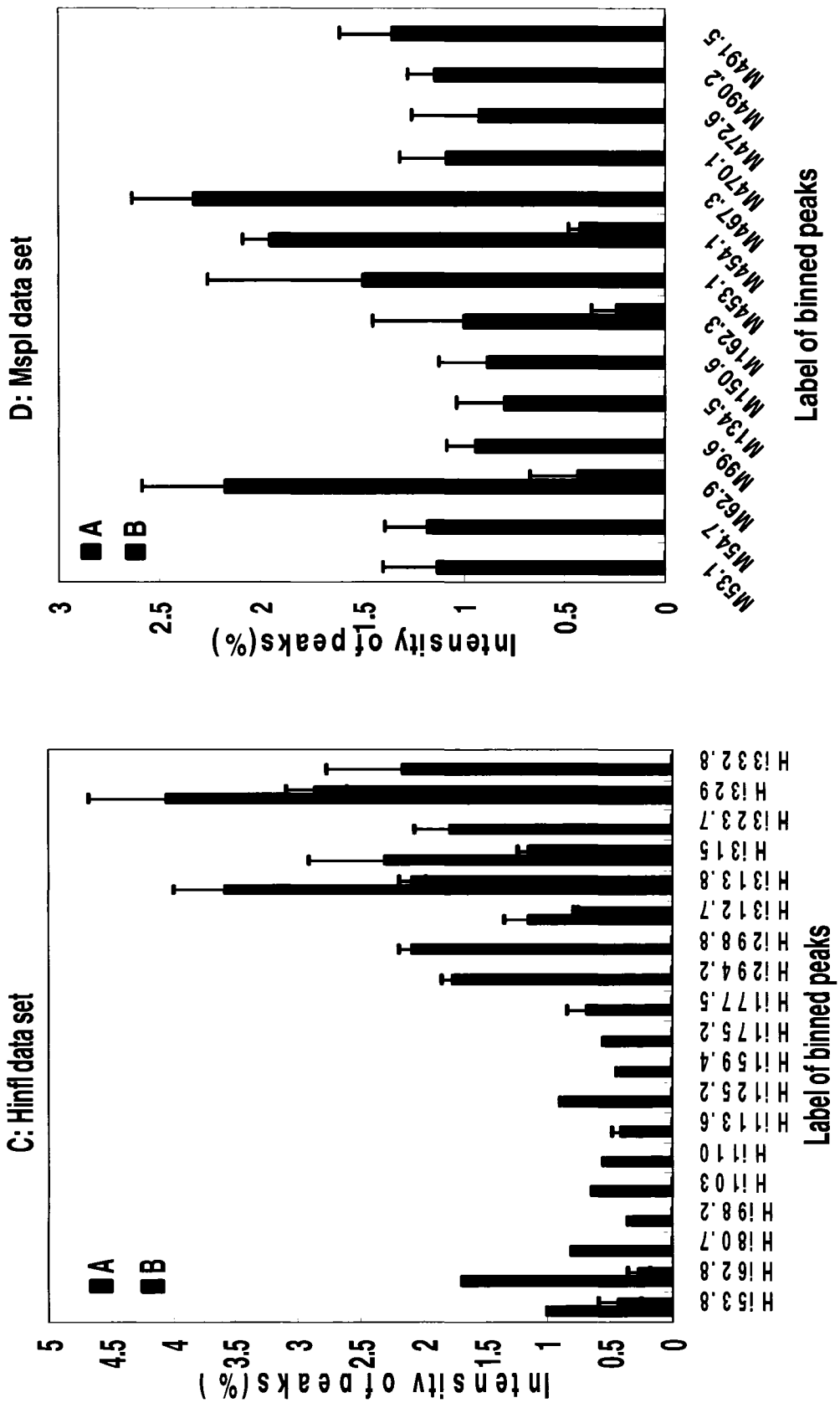
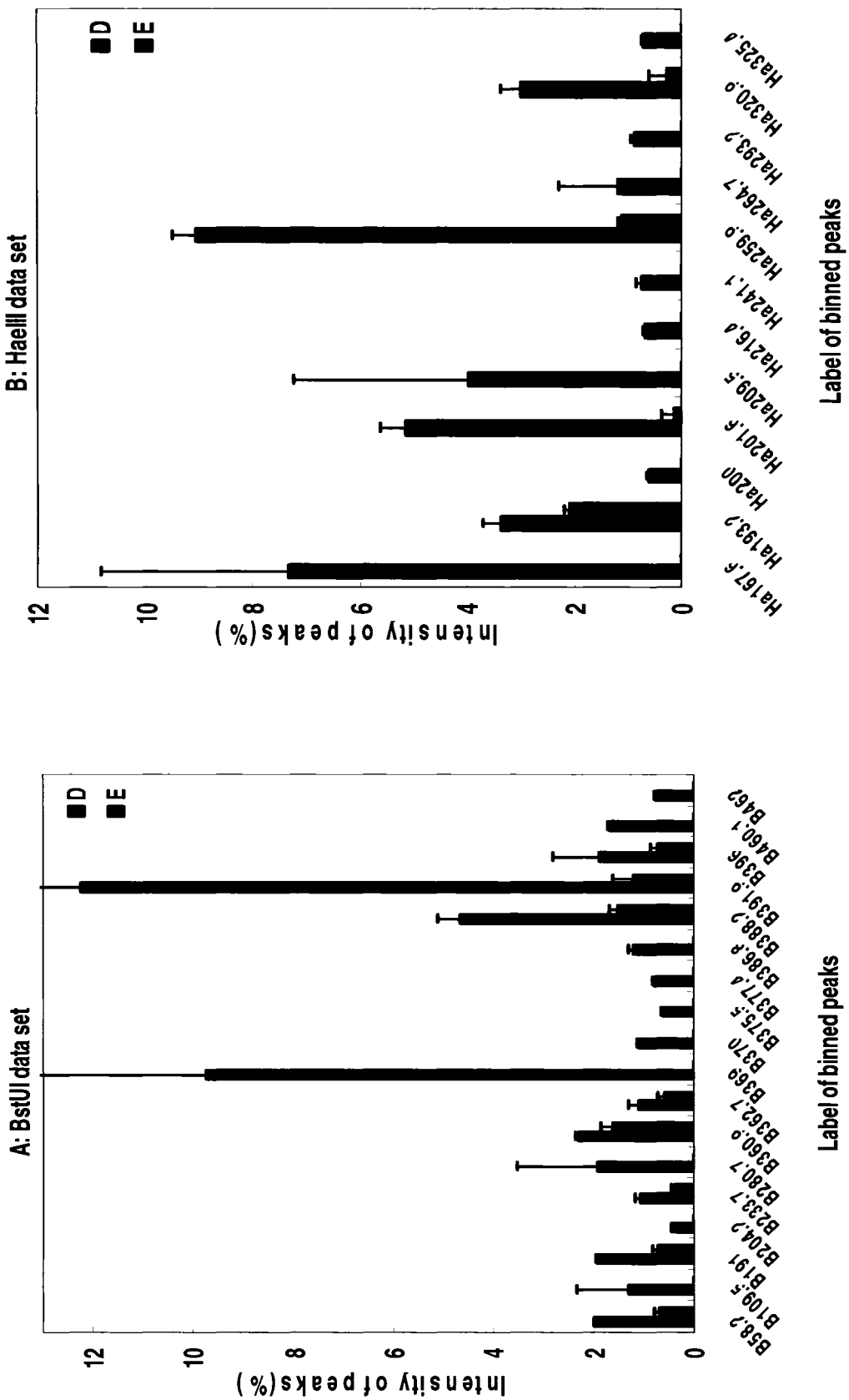
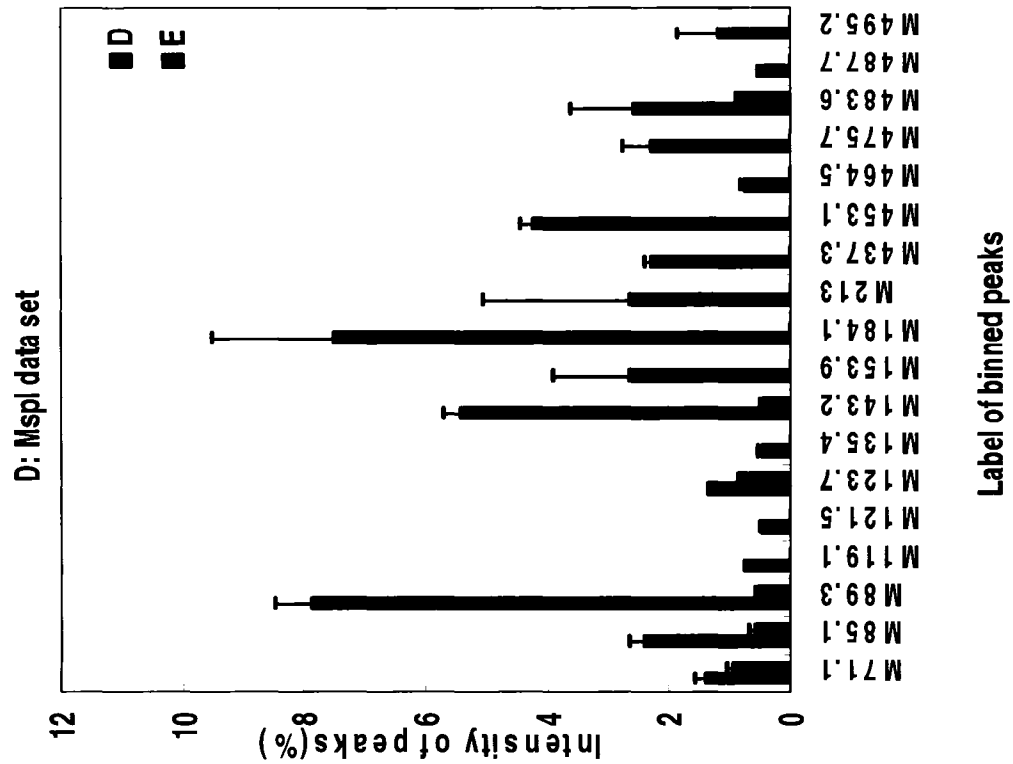


Figure 19: Average intensity with standard error bars of TRF peaks whose intensity increased obviously in profiles from soils treated by hydrogen gas in lab compared to the controls.

(A): *Bst*UI data set; (B): *Hae*III data set, (C): *Hinf*I data set; (D): *Msp*I data set. Samples were indicated as: D (soils treated by hydrogen gas in lab: D2&D4) and E (soils treated by air in lab: E2&E3). Binned TRF peaks were indicated by letter and number: B, Ha, Hi, and M meant peaks generated by using BstUI, HaeIII, HinfI, and MspI; number was the average of the sizes of all peaks in the bin fixed within ±0.4bp.





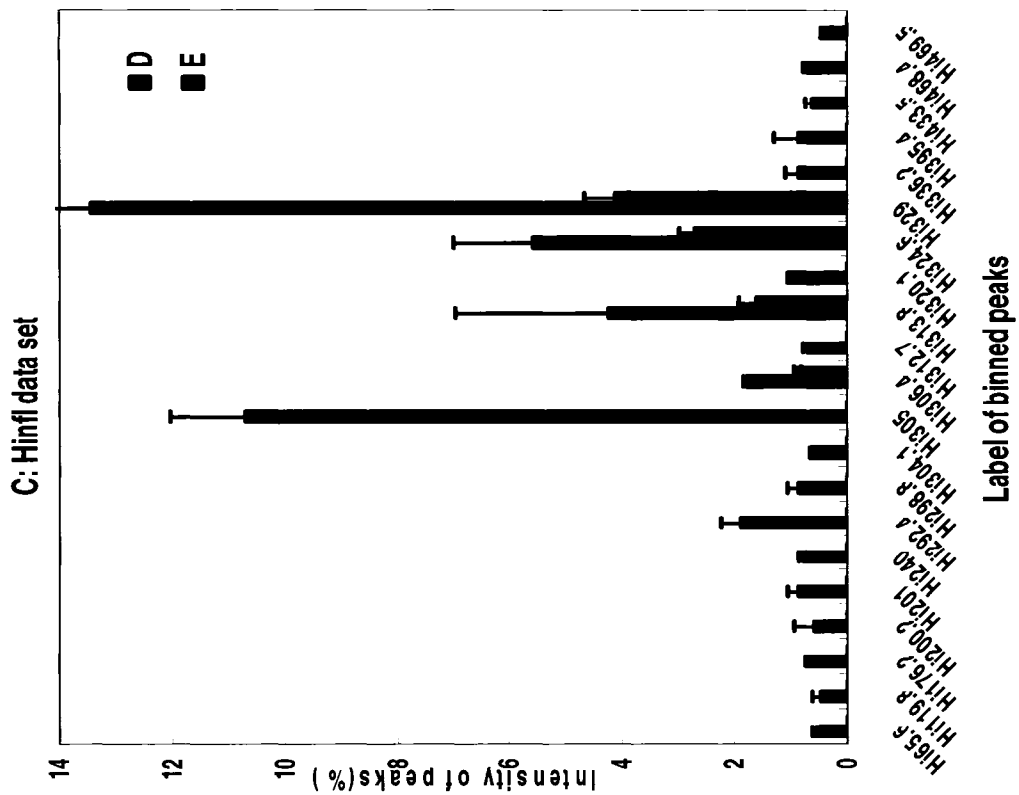


Table 14: Intensity variation of TRF peaks whose intensity increased obviously in profiles from soils exposed to hydrogen gas compared with their controls.
Bpk: Binned TRF peaks (fixed within ±0.4bp). d (A-B): difference of the mean intensity (% of total area) of TRF peaks between greenhouse Hup^- nodules soil samples (A2&A6) and their controls (greenhouse Hup^+ nodules soil: B1&B2). d (D-E): difference of the mean intensity (% of total area) of TRF peaks between soil samples treated by hydrogen gas in lab (D2 &D4) and their controls (air-treated soil: E1&E2). $S_{(i)}$: the sum of total differences [d (A-B) or d (D-E)]. $S_{i(top5)}$: the sum of five largest differences which were marked by light yellow in d (A-B) and light blue in d (D-E). N: no peak. $P_{S(i)} = x/S_{(i)} * 100$.

*Bst*UI data set										
Bpk (bp)	52.8	54.5	58.2	102.9	109.5	142	191	198.6	200.6	204.2
d(A-B)	0.52	0.84	N	0.64	0.97	0.55	N	1.5	0.71	N
$P_{S(i)}$	3.62	5.84	N	4.45	6.75	3.82	N	10.4	4.94	N
d(D-E)	N	N	1.3	N	1.32	N	1.2	N	N	0.45
$P_{S(i)}$	N	N	3.29	N	3.35	N	3.04	N	N	1.14
Bpk (bp)	212.6	233.7	280.7	360.9	362.7	369	370	375.5	377.4	378.2
d(A-B)	0.67	N	N	N	N	N	N	0.62	N	0.83
$P_{S(i)}$	4.66	N	N	N	N	N	N	4.31	N	5.77
d(D-E)	N	0.7	1.92	0.69	0.53	9.76	1.1	0.63	0.81	N
$P_{S(i)}$	N	1.77	4.87	1.75	1.34	24.7	2.79	1.6	2.05	N
Bpk (bp)	383.5	385.2	386.8	388.2	391.9	396	460.1	462	$S_{(i)}$	$S_{(i(top5)}$
d(A-B)	3.68	1.05	N	N	2.35	N	N	N	14.38	9.55
$P_{S(i)}$	25.6	7.3	N	N	16.3	N	N	N	100	66.35
d(D-E)	N	N	1.2	3.2	11.1	1.1	1.7	0.75	39.46	27.7
$P_{S(i)}$	N	N	3.04	8.11	28.1	2.79	4.31	1.9	100	70.1

*Hae*III data set										
Bpk (bp)	53.7	64.9	69.4	80.8	99.8	117.2	120.4	129.5	141.8	167.6
d(A-B)	0.46	1.18	0.36	0.68	0.73	0.38	0.39	0.3	0.34	N
$P_{S(i)}$	2.18	5.59	1.7	3.2	3.46	1.8	1.85	1.42	1.6	N
d(D-E)	N	N	N	N	N	N	N	N	N	**7.33**
$P_{S(i)}$	N	N	N	N	N	N	N	N	N	**21.9**
Bpk (bp)	170.3	191.3	193.2	196	200	201.6	206.2	209.5	211.1	216.4
d(A-B)	0.68	1.42	N	1.57	N	N	0.59	3.43	0.66	N
$P_{S(i)}$	3.22	6.73	N	7.44	N	N	2.8	16.3	3.13	N
d(D-E)	N	N	1.3	N	0.62	**5.2**	N	**3.97**	N	0.69
$P_{S(i)}$	N	N	3.9	N	1.86	**15.57**	N	**11.9**	N	2.07
Bpk (bp)	218.6	220.7	230.2	232	239.5	241.1	253.8	259.9	264.7	293.2
d(A-B)	2.48	1.07	2.1	0.92	0.49	N	0.44	N	N	N
$P_{S(i)}$	11.7	5.1	9.95	4.36	2.32	N	2.09	N	N	N
d(D-E)	N	N	N	N	N	0.74	N	**7.94**	1.6	0.87
$P_{S(i)}$	N	N	N	N	N	2.22	N	**23.8**	4.79	2.6
Bpk (bp)	320	325.4	378.2	$S_{(1)}$				$S_{1(top5)}$		
d(A-B)	N	N	0.42	21.09				12.8		
$P_{S(i)}$	N	N	1.99	100				57.7		
d(D-E)	**2.43**	0.71	N	33.4				26.9		
$P_{S(i)}$	**7.28**	2.13	N	100				80.5		

*Hinf*I data set										
Bpk (bp)	53.8	62.8	65.6	80.7	98.2	103	110	113.6	119.8	125.2
d(A-B)	0.54	1.4	N	0.8	0.32	0.65	0.52	0.42	N	0.86
$P_{S(i)}$	2.8	7.27	N	4.15	1.66	3.37	2.7	2.18	N	4.46
d(D-E)	N	N	0.54	N	N	N	N	N	0.47	N
$P_{S(i)}$	N	N	1.38	N	N	N	N	N	1.21	N
Bpk (bp)	159.4	175.2	176.2	177.5	200.2	201	240	292.4	294.2	298.8
d(A-B)	0.42	0.55	N	0.7	N	N	N	N	1.76	2.08
$P_{S(i)}$	2.18	2.85	N	3.63	N	N	N	N	9.13	10.8
d(D-E)	N	N	0.72	N	0.6	0.87	0.78	**1.88**	N	0.86
$P_{S(i)}$	N	N	1.85	N	1.53	2.23	2	**4.82**	N	2.21
Bpk (bp)	304.1	305	306.4	312.7	313.8	315	320.1	323.7	324.6	329
d(A-B)	N	N	N	0.4	1.5	1.15	N	1.8	N	1.2
$P_{S(i)}$	N	N	N	2.08	7.78	5.97	N	9.34	N	6.22
d(D-E)	0.62	**10.7**	0.94	0.7	**2.6**	N	1.05	N	**2.84**	**9.3**
$P_{S(i)}$	1.59	**27.4**	2.41	1.79	**6.67**	N	2.69	N	**7.28**	**23.8**
Bpk (bp)	332.8	336.2	395.4	433.5	468.4	469.5	$S_{(i)}$		$S_{i(top5)}$	
d(A-B)	2.2	N	N	N	N	N	19.27		9.34	
$P_{S(i)}$	11.4	N	N	N	N	N	100		48.5	
d(D-E)	N	0.85	0.86	0.64	0.75	0.47	39.04		27.3	
$P_{S(i)}$	N	2.18	2.21	1.64	1.92	1.21	100		70	

*Msp*I data set										
Bpk (bp)	53.1	54.7	62.9	71.1	85.1	89.3	99.6	119.1	121.5	123.7
d(A-B)	1.13	1.2	1.75	N	N	N	0.94	N	N	N
$P_{S(i)}$	6.51	6.91	10.1	N	N	N	5.41	N	N	N
d(D-E)	N	N	N	0.47	1.8	**7.3**	N	0.72	0.47	0.5
$P_{S(i)}$	N	N	N	1.14	4.36	**17.7**	N	1.74	1.14	1.21
Bpk (bp)	134.5	135.4	143.2	150.6	153.9	162.3	184.1	213	437.3	453.1
d(A-B)	0.8	N	N	0.88	N	0.76	N	N	N	**1.5**
$P_{S(i)}$	4.61	N	N	5.07	N	4.38	N	N	N	**8.64**
d(D-E)	N	0.48	**5**	N	**2.7**	N	**7.5**	2.65	2.3	**2.91**
$P_{S(i)}$	N	1.16	**12.1**	N	**6.53**	N	**18.1**	6.4	5.56	**7.04**
Bpk (bp)	454.1	464.5	467.3	470.1	472.6	475.7	483.6	487.7	490.2	491.5
d(A-B)	1.54	N	2.3	1.08	0.92	N	N	N	1.2	1.35
$P_{S(i)}$	8.87	N	13.2	6.22	5.3	N	N	N	6.91	7.77
d(D-E)	N	0.77	N	N	N	2.3	1.73	0.53	N	N
$P_{S(i)}$	N	1.86	N	N	N	5.56	4.19	1.28	N	N
Bpk (bp)	495.2	$S_{(i)}$					$S_{i(top5)}$			
d(A-B)	N	17.35					8.4			
$P_{S(i)}$	N	100					49			
d(D-E)	1.2	41.33					25.41			
$P_{S(i)}$	2.9	100					61.5			

Table 15: Intensity variation of TRF peaks whose intensity decreased obviously in profiles from soils exposed to hydrogen gas compared with the controls.
Bpk: Binned TRF peaks (fixed within ±0.4bp). d (B-A): difference of the mean intensity (% of total area) of TRF peaks between greenhouse Hup^- nodules soil samples (A2&A6) and their controls (greenhouse Hup^+ nodules soil: B1&B2). d (E-D): difference of the mean intensity (% of total area) of TRF peaks between soil samples treated by hydrogen gas in lab (D2 &D4) and their controls (air-treated soil: E1&E2). $S_{(d)}$: the sum of total differences [d (B-A) or d (E-D)]. $S_{d(top5)}$: the sum of five largest differences which were marked by light yellow in d (A-B) and light blue in d (D-E). N: no peak. $P_{S(d)} = x/S_{(d)} * 100$.

*Bst*UI data set										
Bpk(bp)	57.2	59.9	62.5	104.4	114.5	165.4	192.1	193.3	204.2	205.1
d(B-A)	0.26	0.13	0.31	0.2	0.75	N	0.41	N	1.5	0.61
$P_{S(d)}$	1.37	0.68	1.63	1.05	3.95	N	2.16	N	7.92	3.22
d(E-D)	N	N	0.3	N	N	0.69	N	0.82	N	N
$P_{S(d)}$	N	N	1.3	N	N	2.98	N	3.55	N	N
Bpk(bp)	206	209	209.9	214.2	221.4	223.4	229.2	230.1	234.6	236.5
d(B-A)	N	0.59	N	N	N	N	N	N	1.34	N
$P_{S(d)}$	N	3.11	N	N	N	N	N	N	7.07	N
d(E-D)	0.67	N	0.88	1.2	1.7	1.9	0.24	0.65	1.66	1.2
$P_{S(d)}$	2.9	N	3.8	5.19	7.35	8.21	1.04	2.8	7.2	5.19
Bpk(bp)	238	240.1	242.5	243.7	247.2	356	357.2	360.9	364.6	384.4
d(B-A)	0.31	N	N	0.35	N	0.67	N	0.64	N	5.1
$P_{S(d)}$	1.64	N	N	1.85	N	3.54	N	3.38	N	26.9
d(E-D)	N	0.42	0.66	N	1.57	N	0.52	N	1.1	N
$P_{S(d)}$	N	1.82	2.85	N	6.79	N	2.25	N	4.76	N
Bpk(bp)	385.9	390.7	392.9	394.5	396	400.5	403.6	458.7	$S_{(d)}$	$S_{d(top5)}$
d(B-A)	2.6	N	1.2	0.3	0.53	0.71	0.33	N	18.9	11.7
$P_{S(d)}$	13.7	N	6.3	1.6	2.8	3.75	1.74	N	100	61.9
d(E-D)	1.55	3.7	N	N	N	0.6	N	1.1	23.1	10.5
$P_{S(d)}$	6.7	16	N	N	N	2.6	N	4.76	100	45.6

*Hae*III data set										
Bpk(bp)	60.1	66.2	84	131	141.8	144.8	187.2	188.5	192.2	194
d(B-A)	N	2.34	1	N	N	N	0.97	0.85	N	N
$P_{S(d)}$	N	10.6	4.5	N	N	N	4.4	3.84	N	N
d(E-D)	**2.23**	N	N	1.1	0.52	0.98	**1.9**	0.64	0.89	0.56
$P_{S(d)}$	**9.25**	N	N	4.56	2.16	4.06	**7.88**	2.65	3.69	2.3
Bpk(bp)	196	197	199.3	202.6	205.1	210.3	215.2	217.5	218.6	220.7
d(B-A)	N	N	1.46	0.45	N	1.45	N	2.7	N	N
$P_{S(d)}$	N	N	6.6	2.02	N	6.56	N	12.2	N	N
d(E-D)	1.35	0.33	0.3	0.41	0.93	0.6	0.76	1	**1.5**	0.51
$P_{S(d)}$	5.6	1.37	1.24	1.7	3.86	2.49	3.15	4.1	**6.22**	2.1
Bpk(bp)	221.6	222.5	225	226.3	227.4	231.1	232	232.9	234.3	238
d(B-A)	1.15	0.4	0.8	0.56	N	3.3	N	0.48	0.21	0.53
$P_{S(d)}$	5.2	1.8	3.62	2.52	N	14.9	N	2.17	0.95	2.4
d(E-D)	N	**2**	0.78	N	0.86	0.8	0.8	N	N	N
$P_{S(d)}$	N	**8.29**	3.23	N	3.57	3.32	3.32	N	N	N
Bpk(bp)	261	287.2	291.6	304.8	317.4	$S_{(d)}$			$S_{d(top5)}$	
d(B-A)	1.55	N	2	N	0.48	22.1			11.9	
$P_{S(d)}$	7	N	9	N	2.17	100			53.7	
d(E-D)	N	**1.4**	N	0.97	N	24.1			9	
$P_{S(d)}$	N	**5.8**	N	4	N	100			37.4	

*Hinf*I data set										
Bpk(bp)	57.6	64.7	65.6	84	100	102.2	112.1	118.2	120.7	123
d(B-A)	0.47	0.52	1.4	1.17	N	1.2	0.32	N	0.99	0.28
$P_{S(d)}$	2.08	2.3	6.2	5.18	N	5.31	1.42	N	4.38	1.24
d(E-D)	N	N	N	N	1.71	N	N	0.57	N	N
$P_{S(d)}$	N	N	N	N	6.99	N	N	2.33	N	N
Bpk(bp)	168.4	170.2	171.1	188.2	294.2	300.2	306.4	310.6	316.8	317.8
d(B-A)	N	N	N	N	N	N	0.21	0.57	0.89	N
$P_{S(d)}$	N	N	N	N	N	N	0.93	2.52	3.94	N
d(E-D)	0.21	1.2	0.56	0.5	0.92	1.57	N	0.77	1.47	0.71
$P_{S(d)}$	0.85	4.9	2.28	2	3.76	6.4	N	3.1	6	2.9
Bpk(bp)	321	321.6	322.7	326.6	327.5	330.4	333.7	336.2	337.2	338.6
d(B-A)	6.27	N	1.58	1.23	N	1.21	2.9	0.31	0.49	0.58
$P_{S(d)}$	27.7	N	7	5.4	N	5.34	12.8	1.37	2.17	2.5
d(E-D)	N	3.52	2.5	2.62	2.3	1.32	N	N	0.97	0.3
$P_{S(d)}$	N	14.4	10.2	10.7	9.4	5.4	N	N	3.96	1.2
Bpk(bp)	466.4	$S_{(d)}$					$S_{d(top5)}$			
d(B-A)	N	22.6					13.4			
$P_{S(d)}$	N	100					59.1			
d(E-D)	0.76	24.5					12.7			
$P_{S(d)}$	3.1	100					51.7			

*Msp*I data set										
Bpk(bp)	53.9	66.1	69.5	73.5	83.8	87.5	111	136.6	137.5	140.5
d(B-A)	0.6	4.37	N	N	1.1	N	0.39	N	N	0.24
$P_{S(d)}$	3.87	28.2	N	N	7.2	N	2.5	N	N	1.55
d(E-D)	N	N	0.71	0.88	N	1	N	0.39	1.12	N
$P_{S(d)}$	N	N	3.3	4	N	4.6	N	1.79	5.16	N
Bpk(bp)	142.4	146.9	149.8	150.6	151.4	152.3	153.1	153.9	157.4	158.4
d(B-A)	N	0.61	N	N	0.74	N	N	0.59	0.32	0.76
$P_{S(d)}$	N	3.93	N	N	4.77	N	N	3.8	2.1	4.9
d(E-D)	0.42	N	**5.1**	**1.4**	0.68	0.74	1.1	N	N	**3.9**
$P_{S(d)}$	1.93	N	**23.5**	**6.45**	3.1	3.41	5.07	N	N	**18**
Bpk(bp)	160.1	161.4	188	205.5	431.8	432.8	435.8	438.3	466.4	471.1
d(B-A)	N	N	N	N	N	N	N	N	1.7	N
$P_{S(d)}$	N	N	N	N	N	N	N	N	11	N
d(E-D)	0.76	0.47	0.96	0.46	**1.91**	0.41	0.85	0.83	**1.88**	1.4
$P_{S(d)}$	3.5	2.16	4.42	2.12	**8.8**	1.89	3.92	3.82	**8.66**	6.4
Bpk(bp)	483.6	484.5	486	491.5	497.4	$S_{(d)}$			$S_{d(top5)}$	
d(B-A)	3.2	N	0.9	N	N	15.5			11.27	
$P_{S(d)}$	20.6	N	5.8	N	N	100			72.8	
d(E-D)	N	1.1	1.34	0.9	0.93	21.7			14.2	
$P_{S(d)}$	N	5.1	6.17	4.1	4.3	100			65.4	

Table 16: **Comparison between predicted TRF peaks from isolates and spiked TRF peaks from complex samples (A2J&A6J, D2J&D4J).**

The size of predicted TRF peaks was generally about 4 bp larger than that of corresponding peaks observed in real TRF profiles. The peaks matched each other were marked by yellow. Predicted TRF peaks were generated by using the function of Restriction Enzyme Analysis in Primer premier 5.0. DNA sequences applied for predicting TRF peaks were quoted from NCBI GenBank (*Variovorax p*: DQ256485, *Burkholderia s*: DQ256491, and *Flavobacterium j*: DQ256490). Binned TRF peaks were indicated by letter and number: B, Ha, Hi, and M meant peaks generated by using *Bst*UI, *Hae*III, *Hinf*I, and *Msp*I; number was the average of the sizes of all peaks in the bin fixed within ±0.4bp. N: no peak.

Variovorax p (Jm01)		*Burkholderia s* (Jm120)		*Flavobacterium j* (Jm162-f)	
Spiked peaks	Predicted peaks	Spiked peaks	Predicted peaks	Spiked peaks	Predicted peaks
B62.5	B68	B62.5	N	B62.5	N
B102	N	B102	N	B102	B106
N	N	N	B208	N	N
B222.4	N	B222.4	N	B222.4	N
B384.4	N	B384.4	N	B384.4	N
Ha63.2	N	Ha63.2	N	Ha63.2	N
Ha72.6	N	Ha72.6	N	Ha72.6	Ha77
Ha217.5	Ha220	Ha217.5	Ha222	Ha217.5	N
Hi320.1	Hi325	Hi320.1	Hi327	Hi320.1	Hi324
Hi321.6	Hi325	Hi321.6	Hi327	Hi321.6	Hi324
M81.2	N	M81.2	N	M81.2	M86
N	N	N	M142	N	N
M275.5	N	M275.5	N	M275.5	N
M483.6	M491	M483.6	N	M483.6	N

(Variovorax p: Jm01, Jm63, Jm110, and Jm162-v, Burkholderia s: Jm120, and Flavobacterium j: Jm162-f) were possibly involved in hydrogen-induced variation of bacterial community structure in soils treated by hydrogen gas in lab (D2&D4).

3.4 DISCUSSION

TRF profiles always contained numbers of small peaks resulting from either artifacts or differences in the amount of DNA loaded on a gel which cannot accurately controlled (total area of peaks in each TRF profile). They could exert negative effects on the similarity analysis of bacterial community structure in different soil samples based on the binary (presence: 1/absence: 0) TRF profiles. To limit the negative influence of fake peaks on similarity analysis, TRF data set was normalized by using an artificial threshold. Peaks below the threshold were considered as background noise and removed from the data set. Three different methods designed for normalizing data set were reported in previous TRFLP studies: the constant percentage threshold (Sait *et al.*, 2003), the constant baseline threshold (Dunbar *et al.*, 2001), and variable percentage threshold (Osborne *et al.* 2006).

The constant percentage threshold was calculated by applying a series of increasing percentage of the total area on each profile until the minimum percentage resulting in the weakest relationship between the number of peaks remaining and the total area on the original profile. Theoretically speaking, more fake peaks whose percentage

were higher than constant percentage threshold didn't detected in profiles with higher total area, while more useful peaks whose percentage were lower than constant percentage threshold were removed in profiles with lower total area.

According to the report of Dunbar *et al.* 2001, all of the peaks in each profile were reduced proportionally by the ratio of the total area of that profile to reference, the profile having the smallest total area within a data set, before a constant baseline was used to detect fake peaks. For profiles having higher total area within a data set, proportional reduction of the area of peaks not only made the constant baseline threshold powerful to remove fake peaks but also resulted in loss of more small peaks with useful information.

The method based on the variable percentage threshold reported by Osborne *et al.* 2006 was more proper for normalizing TRF profiles used in this study compared to the others mentioned above due to the widely variation in total area of each profile within a data set (Table 13). The unique percentage threshold for each profile calculated by dividing the total area of that profile by the optimal divisor made normalization reach a reasonable trade-off between removing fake peaks and keeping peaks with useful information.

One gram of soil may contain up to 10 billion cells of possibly 4,000-7,000 of different species (Bianchi and Biachi, 1995). However, the number of peaks remaining in

normalized TRF profiles from about 0.5g soil samples was no more than 100. Therefore, it was inferred that each TRF peak possibly represented more than one species.

Firstly, the TRF peaks contributed by different species could possibly have the same length in profiles from different soils. It was inconvincible to depict relationships of bacterial community structure between different soil samples based on TRF profiles generated from just one RE. Therefore, four REs were applied in our study to release more information of soil bacterial community structure by increasing the possibility of grouping these species into different peaks in profiles belonging to different data sets. The similarity of dendrograms of data sets generated by using different REs (Figure 16) meant that soil samples were grouped at a confidence level on the basis of bacterial community structure.

Secondly, different species possibly contributed the same peak in a TRF profile generated from a complex bacterial community structure in soil sample. For our isolates hydrogen-oxidizing bacteria which were isolated only in hydrogen treated soils (lab, greenhouse, and field), it seemed illogical that none of TRF peaks having the same length as those of isolates showed obvious intensity increase in hydrogen treated soils (lab: D2&D4 or greenhouse: A2&A6) except Hi320.1 and M483.6 in profiles from hydrogen-treated soils (lab: D2&D4) (Table 15 & Table 14). However, it possibly happened in TRF profiles from complex bacterial communities in soils because TRF

peaks having the same size as those of isolates were possibly contributed by some other bacteria whose growth was inhibited by hydrogen treatment.

However, it was still possible to identify species responsible for hydrogen-induced variation of bacterial community structure in soil through comparing TRF data obtained from Ribosomal Database Project (RDP) with observed TRF peaks whose intensity increased or decreased obviously in profiles from hydrogen treated soils. It was reported that the length of predicted TRF peaks was always larger than that of observed TRF peaks (Kitts, 2001), which was also revealed in this study (Table 16). Any RDP predicted TRF peak with length varying from n to n-4 were normally considered to be a probable match for the TRF peak observed at n bp. Furthermore, the sequences of 16S rDNA in hydrogen-treated soils obtained from a clone library could be used to further confirm results of RDP matching and discover some other unpublished species which were involved in hydrogen induced variation of bacterial community structure. Even though the process of cloning and sequencing is expensive and time consuming, the clone library of 16S rDNA in hydrogen-treated soils is supposed to work well for identifying bacteria responsible for interesting TRF peaks which had high intensity in profiles from hydrogen treated soils, such as B383.5, B385.2, Ha209.5, Ha218.6, Ha230.2, Hi294.2, Hi298.8, Hi313.8, Hi315, Hi323.7, Hi329, Hi332.8, M62.9, M453.1, M454.1 and M467.3 in profiles from greenhouse Hup^- nodule soils (Figure 18) and B369, B388.2, B391.9,

Ha167.6, Ha201.6, Ha259.9, Hi305, Hi324.6, Hi329, M89.3, M143.2, M184.1, and M453.1 in profiles from soils treated by hydrogen in Lab (Figure 19).

Most of intensity-increased peaks, in TRF profiles from soil exposed to hydrogen gas (greenhouse Hup^- soil and soil treated by hydrogen in lab) were considered to be contributed by hydrogen-induced PGPR responsible for hydrogen metabolism in soil because their variation were related with the treatment of hydrogen and hydrogen uptake rates of soil samples. They were in a dormant state in the soil free from hydrogen gas. After hydrogen treatment, they were activated and consumed hydrogen during their growth, which increasing hydrogen uptake rates of hydrogen-treated soil samples.

The intensity-decreased TRF peaks in profiles from soils exposed to hydrogen gas possibly meant that the growth of bacteria responsible for these TRF peaks was limited after treated by hydrogen gas. It was likely to be resulted from the increasing activity of potential hydrogen-utilizing bacteria induced by hydrogen treatment. In soil samples treated with hydrogen gas in lab (D2&D4), the growth of potential hydrogen-utilizing bacteria stimulated by the only input energy (hydrogen) resulted in competition for space, nutrients, etc. It possibly slowed down the growth of bacteria unable to utilize hydrogen gas. In soils adjacent to greenhouse Hup^- nodules (A2&A6), the hydrogen released from Hup^- nodules promoted the growth of hydrogen-utilizing bacteria which possibly had the ability to stimulate plant growth indirectly through inhibiting the growth or lowering deleterious effects of some species phytopathogenic bacteria. Therefore, the TRF peaks

which decreased obviously in intensity in profiles from greenhouse soils adjacent to Hup$^-$ nodule were possibly contributed by phytopathogenic bacteria.

McLearn and Dong, (2002) reported that the penicillin-sensitive bacterial species of *Nocadia* was possibly responsible for the main hydrogen uptake rate in hydrogen treated soils. Five strains of *Pseudonocardia* isolated from hydrogen-treated soils by Osborne *et al.* (personal communication, 2006) were known to utilize hydrogen and promote plant growth. Furthermore, it was found that several TRF peaks which showed obvious intensity increase in profiles from hydrogen treated soils (Table 14) possibly matched TRF peaks predicted from 16s rDNA sequences of *Nocadia* (AB126875)and *Pseudonocardia* (AJ252828) published in NCBI GenBank (observed peak/predicted peak: *Nocadia*. N/B222, Ha64.9/Ha68, Hi320.1/Hi323, and M153.9/M160; *Pseudonocardia*. N/B223, Ha64.9/Ha69, Hi320.1&Hi323.7/Hi324, and M135.4/M142). Therefore, both of *Nocadia* and *Pseudonocardia* were possibly responsible for increasing hydrogen uptake rate of our hydrogen treated soil samples and greenhouse Hup$^-$ nodule soils.

4. GENERAL CONCLUSION

The present study focused on the mechanisms of our hydrogen-oxidizing isolates, belonging to genera of *Variovorax*, *Burkholderia* and *Flavobacterium*, in plant growth promotion and the effect of hydrogen metabolism on the variation of rhizobacterial community structure.

Isolates belonging to *Variovorax* (Jm01, Jm63, Jm110, Jm111, and Jm162-a) and *Flavobacterium* (Jm162-F) showed ACC deaminase activity. Isolates belonging to *Burkholderia* (Jm120, Jm121, Jm122, and Jm123) had the activity of rhizobitoxine or its structural analogue such as AVG. Therefore, it was proved that our isolates of hydrogen-oxidizing bacteria have the ability to promote plant growth by lowering of plant ethylene levels. However, it was still unknown whether there were some other mechanisms used by our isolates to promote plant growth.

TRF profiles from soils treated by hydrogen gas in laboratory (D2&D4) showed significant different with those from soils treated by air in laboratory (E2&E3). TRF profiles from soils adjacent to Hup^- soybean nodules in greenhouse (A2&A6) were significant different with those from soils adjacent to Hup^+ soybean nodules in greenhouse (B1&B2). It was inferred that hydrogen metabolism in soils induced obvious variation of bacterial community structure in soils.

Some TRF peaks in profiles from hydrogen-treated soils (D2&D4, A2&A6) showed obvious increase in intensity while intensity of some other TRF peaks decreased obviously compared to TRF profiles from the controls (E2&E3, B1&B2). It meant that hydrogen treatment not only stimulated the growth of potential hydrogen-utilizing bacteria responsible for hydrogen uptake rated of soils but also inhibited the growth of some other bacteria possibly belonging to phytopathogens.

Most of TRF peaks with obvious variation in intensity in profiles from soils treated by hydrogen gas in laboratory (D2&D4) didn't match those in profiles from soils adjacent to Hup^- soybean nodules in greenhouse (A2&A6). It was suggested that hydrogen induced variation of bacterial community structure in soil soils treated by hydrogen gas in laboratory (D2&D4) were different from that in soils adjacent to Hup^- soybean nodules in greenhouse (A2&A6).

Our isolates were possibly involved in hydrogen-induced variation of bacterial community structure in soils treated by hydrogen gas in laboratory (D2&D4) because only two of TRF peaks contributed by our isolates, Hi320.1 and M483.6, showed significant increase in intensity in TRF profiles from soils treated by hydrogen gas in laboratory (D2&D4).

However, it was impossible to identify the species of bacteria contributing the TRF peaks whose intensity varied obviously in hydrogen-treated soils (D2&D4, A2&A6)

without further studies such as examining the sequences of 16s rRNA genes from database and a clone library of PCR product because most peaks in TRF profiles were possibly contributed by several species of bacteria.

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6. APPENDICES

6.1 Appendix A: Original data of 4RE (*Bst*UI, *Hae*III, *Hin*fI & *Msp*I)-Derived TRF Profiles from Greenhouse Soils Adjacent to Hup⁻ Nodules (A2&A6)

*Bst*UI digested TRF profiles											
Soil sample: A2(a)						Soil sample: A2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
52.8	787	193.3	2463	239.5	223	53.2	793	192.2	4155	247.3	855
54.3	1145	195.3	7204	247.5	802	54.7	1454	193.2	2611	293.4	199
57.1	1182	196.9	3730	293.4	168	57	1224	195.2	7455	356	281
58.8	1517	198.6	2508	356	278	58.8	1762	196.8	4043	358	1867
60.1	301	199.5	416	358	1802	60.1	277	198.5	2665	360.8	4041
61.1	141	201	1119	360.8	3901	61.1	170	199.4	493	362.3	2757
62.8	815	201.8	5218	362.3	2696	62.7	904	200.9	1033	369	646
88.9	191	202.8	2239	368.9	697	88.9	188	201.8	5505	375.7	981
90.1	9060	204.2	2415	375.7	879	90.1	9724	202.7	2537	377.1	1009
91.2	1208	205	986	378.1	1761	91.2	1185	204.2	2649	378.2	1368
93	16479	205.8	927	383.7	4667	93	18173	205.8	593	383.7	4959
94	1732	207.4	1269	388.2	1808	93.9	1925	207.3	1338	385.6	1870
95.3	8258	210.4	1560	390.6	5367	95.2	8946	210.3	1672	387.7	3999
97	823	211.8	1862	391.5	3400	96.9	900	211.8	2034	390.6	6106
101.9	460	212.7	2315	394.5	1725	101.8	571	212.6	2380	391.5	3877
103	312	214.4	2909	395.9	1608	102.9	419	214.3	3108	394.5	1707
103.9	872	219.6	275	400.4	273	103.9	912	219.6	297	396	1806
109.5	1455	220.6	2984	403.5	407	109.5	1496	220.6	3231	400.5	292
110.5	3898	222.4	1010	428.9	488	110.4	4204	222.3	1120	403.6	411
111.4	188	223.4	899	458.9	206	111.3	225	223.2	996	428.9	525
123.7	269	224.5	3075	462.1	520	112	258	224.4	3144	458.9	256
158.5	668	227.3	2871	239.5	223	123.7	353	227.2	3145	462.1	582
165.4	891	229.1	1227			158.5	771	229	1358	247.3	855
174.4	635	233.7	446			165.4	1032	233.6	492		
190.8	832	234.6	1877			174.4	717	234.5	1993		
192.2	3888	238.1	613			190.8	977	238	668		
52.8	787	193.3	2463			53.2	793	192.2	4155		

*Bst*UI digested TRF profiles												
Soil sample: A6(a)						Soil sample: A6(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
52.7	441	187	823	229.1	1239	53.3	310	191	1758	234.6	2030
57	1518	187.9	1009	233.6	773	54.9	581	192.2	5241	238	1118
58.6	874	190.8	1667	234.5	1905	57.1	1492	193.3	4083	243.6	522
60.1	280	192.1	5253	237.9	1078	58.7	863	195.3	13071	247.3	921
62.7	1685	193.2	3906	243.6	458	60.2	236	196.8	8424	293.4	206
90.1	8168	195.2	13014	247.4	913	62.8	1673	198.6	2836	356	469
91.9	1318	196.8	5019	293.4	229	90.2	8428	201.9	4067	358.1	2000
93	21648	198.5	2776	356	430	91.3	1470	202.8	4395	360.8	6661
94	2406	201.8	3932	358	2861	93.1	22374	204.3	3394	362.8	5403
95.2	15099	202.7	4449	360.7	6405	94.1	2201	205.1	1862	369	962
96.7	3640	204.2	3539	362.8	4835	95.3	15979	205.9	1277	369.9	807
98.4	419	205	1746	369	748	96.7	3725	207.5	2197	375.7	1502
100	679	205.8	1254	369.9	641	100.8	286	209	1515	378.2	1451
100.8	351	207.4	2155	375.7	1215	101.8	3085	210.5	2471	379	1077
101.7	2976	210.4	2649	378.1	1296	102.8	1381	211.5	3309	384	10257
102.6	1392	211.5	2883	384	7850	104	1616	212.7	2782	387.7	2778
103.9	1578	212.6	2807	385.6	2013	109.6	1008	214.5	3910	390.6	6093
109.5	1001	214.3	3759	387.6	2589	110.4	1066	219.8	654	391.5	5163
110.4	1078	215.4	1665	390.6	5732	123.7	1366	220.7	6342	394.5	2119
111.7	1540	216.1	2396	391.5	4923	136	993	222.4	1364	395.9	1612
123.6	1099	219.6	637	394.5	1985	142.1	1151	223.2	1397	400.4	408
142	1231	220.6	5970	395.9	1369	158.7	2042	224.3	3936	403.6	339
158.5	1987	222.3	1443	400.5	312	165.5	1751	227.1	2567	428.9	505
165.3	1733	223.2	1159	403.6	353	174.5	1202	229.2	1373	462	473
173.4	1207	224.3	4733	462.1	434	187.2	883	230.1	1339	469.9	625
174.4	1191	226.6	3523	470	613	188.1	1026	233.7	882		

*Hae*III digested TRF profiles						
Soil sample: A2(a)						Soil sample: A2(b)
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	
53.5	1323	192.2	2445	232.9	516	
55	2461	193.2	7358	234.3	915	
60.2	7635	194	2147	239.3	765	
61.2	301	194.8	600	243.6	1610	
62	343	196	2748	250.2	784	
63	4074	197	4271	253.5	602	
65.3	2165	199.4	2059	256	854	
70.7	185	201.7	1124	258.3	3906	
73.1	661	202.7	860	259.9	2015	
80.7	1084	204.9	2001	262	2438	
97.1	640	206.4	811	263.3	796	
99.5	1152	207.8	534	264.6	919	
117.1	589	208.5	736	285.4	989	Non
120.3	605	210.2	9455	287.6	333	
128.7	1569	211.1	1035	288.8	1373	
131.3	837	213.3	374	291.5	12782	
141.6	1205	215.5	633	293.2	2071	
144.6	2224	216.5	7643	301.9	726	
167.6	1169	217.7	4562	304.6	193	
168.5	406	218.9	3898	317.4	574	
176.4	616	220.8	1416	324.1	1169	
185.5	297	222.5	1107	329.4	522	
186.2	312	224.2	8489	343.6	428	
187.2	1064	227.4	6984	378.2	1417	
188.5	4136	230.5	4344			
191.5	2226	232	1450			

*Hae*III digested TRF profiles												
Soil sample: A6(a)						Soil sample: A6(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.6	350	188.4	4240	243.6	1188	53.4	414	188.3	4392	234.2	992
55.3	749	192.2	3284	250.2	603	55.2	850	192.1	3368	237.9	499
58.4	506	193.2	12570	253.4	746	58.2	459	193.1	12782	243.5	1360
60.2	11254	194.1	2702	255.6	993	60.1	11639	193.9	3157	250.1	739
61.2	252	194.9	858	258.4	2666	61.1	354	194.7	948	253.3	813
61.9	194	197	11286	259.9	1665	61.9	323	196.8	11611	255.5	1066
63.5	2420	199.4	1775	262.1	2009	63.4	2832	199.3	1780	258.3	2749
65.3	476	200.4	953	263.4	678	65.2	578	200.2	1085	259.8	1776
66.5	1113	201.8	1558	264.6	211	66.5	1377	201.6	1633	262	2098
69.6	506	205	2212	285.5	813	69.6	612	202.7	1228	263.3	755
70.7	277	206.4	964	287.6	338	70.6	358	204.9	2389	264.4	360
71.7	472	208.3	1684	288.9	999	71.6	564	206.3	1088	285.4	902
72.7	1224	209.6	3974	291.6	12579	72.6	1528	208.4	2020	287.5	392
97.1	1013	215	1541	293.6	5443	97	1103	209.5	4374	288.8	1038
99.7	206	216.6	4765	302.1	965	99.7	462	213.3	451	291.5	12575
128.7	2120	217.7	3927	304.8	178	128.6	2220	214.9	1762	293.5	5550
129.6	707	219.3	5267	324.3	879	129.6	773	216.5	4939	302	1056
131.4	1465	220.8	1895	378.3	816	131.4	1552	217.6	3991	304.7	219
141.6	1740	221.6	766			141.6	1774	219.2	5428	324.3	979
144.6	2990	222.5	797			144.6	3096	220.7	1872	378.1	822
167.6	1518	224.2	6247			167.5	1608	221.5	864		
168.5	607	225.2	2128			168.4	665	222.4	906		
170.2	1050	227.4	8554			170.1	1156	224.1	6411		
176.4	787	230.5	2769			176.3	837	225.2	2241		
186.4	474	234.3	799			186.2	571	227.3	8681		
187.3	1260	238	349			187.1	1272	230.3	2950		

*Hinf*I digested TRF profiles								
Soil sample: A2(a)					Soil sample: A2(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.2	1248	182.3	420		53.5	907	298.7	2097
54.6	1291	187.9	365		55	1876	300.3	128
55.4	1245	192.2	268		59.1	225	305	703
57.6	555	199.4	435		63	1621	306.4	758
58.9	361	293.3	4417		65.6	480	312.6	996
61.9	238	296	10390		76.3	202	313.6	3338
62.8	2109	297.6	6098		79.7	218	315	2894
65.5	721	298.8	2505		80.7	763	319.8	8350
76.2	269	300.4	148		96.9	4175	320.9	6686
79.7	320	305	789		99	555	322.3	2854
80.7	1013	306.5	879		100	9341	323.9	1587
96.8	5426	312.6	1177		100.9	345	325.1	3302
98.2	398	313.6	3884		101.6	235	327.5	5237
99	772	315	3322		102.3	247	329	3508
99.9	11927	317.9	1441		112.2	277	331.4	1668
100.8	453	319.8	9203		115.5	700	333.2	2768
101.5	287	320.9	7761		117.1	629	336.1	333
102.2	338	322.3	3316		118.2	714	337.6	653
111.2	229	323.8	1809		120.2	258	364.8	330
112.2	446	325.1	3587		125.2	804	397.1	365
115.5	1027	327.5	6398		155.2	1105	405.4	593
117.1	893	328.9	6222		168.4	314	464	1150
118.2	979	331.4	1901		169.2	356	469.4	985
120.2	414	333.2	3137		171	360		
123	1201	336.1	408		176.3	605		
125.2	1117	337.6	724		182.4	178		
155.1	1553	364.9	387		187.9	168		
168.4	567	397.2	425		192.2	120		
169.2	550	405.5	636		199.4	183		
170.9	604	464.1	1370		293.3	3736		
176.3	953	469.5	1150		296	9018		
177.5	716				297.6	4957		

HinfI digested TRF profiles								
Soil sample: A6(a)					Soil sample: A6(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.4	464	187.9	929		55.4	672	192.3	565
55.4	1029	192.2	858		57.5	261	199.3	658
57.6	485	199.3	899		58.4	883	293.3	2885
58.5	1259	293.3	3902		62.8	205	294.1	2003
62.9	480	294.1	2457		65.3	229	296.1	11297
65.3	510	296.1	13423		76.2	182	297.6	7765
76.2	413	297.6	9064		96.9	3668	300.4	118
97	4549	300.3	268		98.2	300	305.1	946
98.3	526	305	1317		99	474	306.3	873
99.1	663	306.2	1179		100	11874	311.7	870
100	14181	311.7	1176		100.9	619	312.7	1468
100.9	942	312.6	1891		101.6	520	314.3	4429
101.7	749	314.2	5469		102.8	712	315.4	1912
102.9	936	315.4	2613		110.1	544	317.9	1205
108	444	316.3	1177		111.1	438	320.2	8303
110.1	785	317.9	1743	r	112.1	603	321	7454
111.2	673	320.1	9515		113.2	405	323.9	2293
112.2	958	320.9	9489		115.4	702	325	3193
113.2	663	323.9	2815		117.2	1119	327.7	5318
115.5	931	325	3917		118.2	1256	329	4279
117.2	1508	327.6	6439		122.8	1045	331.4	1631
118.3	1673	328.9	5376		155.3	659	333.2	1763
122.9	1399	331.4	1962		159.9	436	336.1	425
155.3	986	333.2	2384		168.3	911	337.6	641
159.9	633	336.1	578		169.2	593	365	292
168.3	1384	337.6	825		171.3	702	397.3	263
169.2	847	364.9	277		175.3	592	405.6	815
171.3	1081	397.2	335		176.2	1371	464.2	893
175.3	798	405.6	947		182.4	601	469.5	2517
176.2	1834	464.1	1038		187.9	617		
177.4	1152	468.4	1374					
182.4	1014	469.4	2965					

*Msp*I digested TRF profiles											
Soil sample: A2(a)						Soil sample: A2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.2	1702	137.5	470	432.7	5761	51.6	1663	123.6	2048	197.6	440
54.7	1837	138.5	5724	435.7	6837	53.1	2569	126.6	1240	199.3	606
55.6	1587	139.4	785	437.4	2517	54.6	2778	127.8	5614	205.5	1186
59.2	405	140.4	409	438.4	2291	55.6	2860	134.6	1227	266.3	280
62.1	349	141.4	1813	453	2560	56.7	874	135.4	744	275.8	542
63	3415	144.1	1959	454.2	2592	59.2	606	136.4	2020	277.7	875
63.9	283	145.7	2287	467	2998	62.1	644	137.6	840	280.7	785
67.8	679	148	12866	470.2	1153	63	5154	138.5	9213	282.4	1623
69.5	347	149.8	8617	471.2	775	63.9	461	139.4	1275	338.7	251
71.2	877	150.7	976	472.6	1380	64.6	499	140.3	537	396.1	5650
72.3	410	151.5	670	475.6	363	65.7	1609	141.4	2905	397.1	2687
73.4	4955	152.2	716	483.8	5089	67.7	1118	142.4	1040	400	14542
78.4	394	154	659	491.4	1940	69.5	624	144.1	3035	420.6	1549
80.8	1675	156.3	1036			71.2	1401	145.7	3161	424.8	2001
83.8	527	157.4	597			72.3	692	148	21121	432.6	12913
84.9	508	160.4	2321			73.4	7088	149.7	14303	435.7	14638
87.2	2915	161.4	2106			75.6	470	151.4	947	437.4	5042
89.7	1260	162.4	2044			78.4	669	152.1	1277	438.3	4955
90.7	223	168.1	718			80.7	2617	154.1	1100	453	5512
91.7	1819	178.2	548			83.8	906	154.8	1147	454.2	5571
93.9	1722	184.1	513			84.8	893	156.3	1605	467	6885
99.5	1444	188	865			87.1	4655	157.3	836	470.1	3218
111.3	289	199.3	407			89.6	1915	158.8	1796	471.1	2058
117.1	619	205.5	729			90.7	359	160.4	3727	472.6	3305
122.5	1738	266.4	162			91.7	2823	161.4	3394	475.5	1926
123.6	1302	275.7	310			93.9	2832	162.3	3474	478.8	1033
126.6	815	277.7	469			98.6	149	165.9	789	483.6	11636
127.8	3526	280.8	270			99.5	2287	168	1189	490.2	3368
134.6	718	282.5	935			111.3	501	178.2	951	491.3	4496
135.3	560	396.2	2753			117.2	908	184.1	787	496.3	2663
136.5	1252	400	7243			122.5	2616	187.9	1533		

*Msp*I digested TRF profiles									
Soil sample: A6(a)						Soil sample: A6(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.3	465	127.8	8133	275.7	239	53	243	151.4	1719
55.6	1254	128.8	959	277.6	466	55.4	580	152.2	730
62.9	751	134.7	1823	282.4	741	62.9	365	156.3	969
65.4	480	136.5	2156	396.2	1539	65.3	270	159.9	1506
67.7	1318	138.4	4660	400.1	8526	67.7	666	161.4	1197
68.6	180	139.4	1831	432.7	6112	69.5	83	162.2	540
69.5	169	140.4	629	435.7	8444	71.1	676	168.1	575
71.2	1326	141.4	2923	438.2	3613	72.2	416	178.2	531
72	894	144.1	3110	452.9	820	73.4	3976	184.1	375
73.4	8286	145.9	3684	467	3743	78.3	194	188	705
78.3	366	147	1694	469.4	2552	81	1265	197.5	383
80	279	148	20063	471.4	1332	83.7	320	199.3	477
81	2519	149.7	16096	472.6	833	87.3	1571	205.5	573
83.7	670	150.6	1979	475.6	420	90.7	177	266.3	67
84.8	496	151.4	3524	483.6	7190	91.7	912	275.6	125
87.3	3026	152.3	1368	485.9	2409	93.9	1597	277.6	255
89.6	1003	153.2	1331	490.3	1979	111.2	337	282.3	478
90.7	294	156.3	2044	491.3	1936	122.7	1140	396.1	957
91.7	1752	158.9	2677			123.8	1061	400.1	5047
92.9	232	160	3086			126.6	867	432.6	3675
93.9	3281	161.4	2432			127.8	4025	435.7	4829
94.9	93	162.3	1158			134.7	823	438.2	2281
95.6	111	168.1	1324			136.5	1066	452.9	538
99.8	279	170.2	1270			138.4	2265	466.9	2328
101.9	552	175.4	951			139.4	938	471.3	819
111.2	876	178.2	1165			141.4	1446	472.5	491
115.7	707	184.1	896			143.2	507	475.5	240
119.1	471	188	1557			144.1	1315	483.4	4463
122.7	2524	195.1	644			145.8	1728	485.8	1539
123.8	2396	199.3	1001			147.9	9951	491.2	1187
126.7	2079	205.5	1127			149.7	8136		

6.2 Appendix B: Original data of 4RE (*Bst*UI, *Hae*III, *Hinf*I & *Msp*I)-Derived TRF Profiles from Greenhouse Soils Adjacent to Hup+ Nodules (B1&B2)

*Bst*UI digested TRF profiles							
Soil sample:B1(a)				Soil sample: B1(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
57.1	604	208.9	472	57	549	212.7	399
58.6	211	210.4	692	58.5	179	214.4	965
60.1	89	211.7	852	60.1	125	220.7	1353
62.7	637	212.7	486	62.7	574	222.3	332
90.1	2661	214.3	1143	90.3	2414	223.1	352
91.2	353	220.6	1573	91.3	381	224.2	1092
93	6574	222.3	334	93.1	5764	227	447
93.9	602	223.1	391	94.1	522	229.2	293
95.3	2814	224.2	1215	95.4	2516	234.7	928
96.5	871	226.9	445	96.6	811	237.9	391
100.7	31	229.2	344	100.9	34	247.3	339
101.6	842	234.7	1241	101.8	745	293.4	63
103.8	482	237.9	410	104	477	356	293
110.4	411	243.6	221	110.5	464	358.1	847
111.5	260	247.3	355	114.5	447	360.8	1938
114.4	425	293.3	85	123.5	194	362.7	1168
158.5	369	356	297	158.6	355	369	212
165.4	493	358.1	889	165.4	451	384	3190
174.3	196	360.8	2176	174.4	219	385.8	1653
190.8	466	362.8	1263	190.9	454	387.9	1721
192.2	1756	369	249	192.3	1662	390.7	2446
193.2	1060	384	3502	193.2	962	392.7	614
195.2	2935	385.7	1790	195.3	2810	394.5	681
197.3	970	387.8	849	196.7	946	395.9	693
200.3	208	390.7	2895	201.9	1829	400.5	325
201.8	2196	392.6	758	202.8	1463	403.5	150
202.7	1718	394.5	863	204.3	2145	462	107
204.2	2513	395.9	792	205.9	298		
205	763	400.5	350	207.4	477		
205.8	307	403.5	193	210.5	613		
207.4	560	462	113	211.8	774		

*Bst*UI digested TRF profiles								
Soil sample:B2(a)					Soil sample: B2(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
57.1	802	212.8	526		57.1	854	212.8	698
58.5	361	214.4	1114		58.5	376	214.5	1495
60	129	220.7	1565		60	104	220.8	1921
62.7	696	222.3	510		62.7	763	222.4	648
90.1	3716	223.2	633		90.2	4526	223.2	889
91.2	562	224.3	2023		91.3	712	224.3	2407
93	8635	226.9	1085		93.1	10459	226.2	709
94	851	229.2	687		94.1	1021	227	774
95.3	4310	234.7	1796		95.3	5137	229.3	858
96.6	1376	237.9	622		96.6	1729	230	767
100.8	77	243.6	171		100.8	106	234.7	3223
101.7	985	247.3	440		101.8	1270	237.9	795
103.9	632	356	642		104	840	239.4	318
110.3	344	358.1	923		110.4	455	243.6	411
123.7	356	360.9	2478		123.8	488	247.2	605
158.7	540	362.8	1691		138.6	223	356.1	767
165.4	615	369.1	298		158.7	728	358.2	1058
174.3	382	384.2	3144		165.5	790	360.9	3035
190.9	517	385.8	1770		174.4	497	362.8	2059
192.2	2224	387.9	2267		191	697	369.2	333
193.2	968	390.8	2936		192.3	2655	382.8	1019
195.3	3929	394.7	1018		193.3	1346	384.3	4383
196.7	2694	396	1123		195.4	4990	386	2051
202.7	2033	400.6	630		196.8	3371	390.9	3607
204.3	1735	403.7	276		202.8	2649	394.8	1183
205.1	849	462.2	184		204.4	2131	396.1	1485
205.9	429				206	567	400.6	738
207.4	523				207.4	739	403.7	336
209	560				209	747	462.2	211
210.5	774				210.6	968		
211.7	783				211.7	1054		

*Hae*III digested TRF profiles							
Soil sample: B1(a)				Soil sample:B1(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.8	241	210.2	1023	53.8	267	210.2	836
55.4	482	213.4	113	55.3	458	214.8	237
60.2	4530	216.6	3837	60.2	3775	216.5	3185
62	51	217.7	3121	61.9	67	217.7	2611
63.4	513	221.4	1048	63.3	440	221.4	962
65	219	222.5	709	64.9	190	222.5	559
65.8	2047	224.2	3081	65.8	1922	224.2	2551
73	275	226.3	394	70.6	47	226.3	324
83.9	689	227.4	3484	73	213	227.5	2891
97.1	393	231.2	2076	83.9	624	231.1	1694
128.7	597	232.8	309	97.1	308	232.8	254
131.3	343	234.2	531	128.7	508	234.2	409
141.6	364	237.9	388	131.3	440	238	266
144.6	1094	243.5	638	141.6	336	243.6	478
167.6	561	250.2	321	144.6	962	250.2	227
168.5	139	255.7	502	167.6	486	255.6	361
176.5	176	258.3	1456	168.4	175	258.3	1165
186.3	34	259.9	826	176.4	211	259.9	698
187.2	886	260.9	1127	186.3	64	260.9	873
188.6	2519	262.1	1135	187.2	725	262.1	835
192.2	1220	263.3	516	188.6	2161	263.3	382
193.2	3274	285.4	496	192.2	993	285.4	381
194.1	899	287.4	156	193.2	2702	287.4	135
194.9	219	288.9	597	194	840	288.9	469
197	3864	291.5	6346	194.8	222	291.5	5394
198.5	585	293.4	2170	196.9	3365	293.5	1755
199.5	2539	302	510	199.4	2133	302	353
200.4	434	304.7	121	200.3	387	304.7	37
201.9	381	317.5	589	201.7	379	317.5	502
202.8	684	324.3	666	202.7	574	325.3	119
205	892	325.3	122	205	784		
207.8	401	378.3	104	207.9	349		
209.2	191			209.2	163		

*Hae*III digested TRF profiles								
Soil sample: B2(a)					Soil sample: B2(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.9	239	214.8	337		53.8	125	217.7	1984
55.3	370	216.6	1160		55.3	219	219.4	1396
60.2	2876	217.7	4345		60.2	2461	221.4	816
62.3	133	219.4	1846		63.4	432	222.5	380
63.2	3077	221.4	976		70.7	58	224.2	2178
64.2	90	222.4	636		73.1	193	225.4	960
71.7	176	224.2	2862		128.7	512	227.5	2489
72.6	1432	225.4	1119		131.3	370	234.2	372
128.7	696	227.5	3336		141.7	288	237.9	249
131.3	326	231.1	2422		144.7	598	243.5	274
141.7	438	232.7	339		167.6	387	250.2	195
144.7	765	234.2	415		176.5	149	255.7	355
167.6	481	237.9	291		186.2	62	258.3	939
168.5	150	241.1	143		187.2	935	262.2	731
176.5	127	243.4	337		188.6	1410	264.8	199
186.3	37	250.1	247		192.2	1068	285.4	411
187.2	1205	255.7	415		193.2	2612	287.5	64
188.7	1873	258.3	1196		194	681	288.9	385
192.2	1230	262.2	977		197	2507	291.6	4394
193.2	3459	263.3	307		199.4	800	293.4	1905
194.1	744	264.7	202		202.6	531	304.8	135
195.5	790	285.5	479		205.1	468	323.6	174
197.1	3047	288.9	494		207.9	414	378.4	103
199.5	883	291.6	6017		214.8	270		
202.7	630	293.5	2593		216.6	1074		
205.1	516	304.9	214					
207.7	412	378.3	150					
209.1	189							

*Hinf*I digested TRF profiles								
Soil sample: B1(a)					Soil sample: B1(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.9	345	199.4	249		53.6	544	199.2	635
55.4	810	293.4	2705		55.3	980	293.4	2682
57.7	455	296.1	6471		57.6	615	296	6343
58.5	457	297.8	4067		58.4	589	297.7	4017
62.7	79	300.6	61		62.6	307	300.5	264
64.9	360	305	481		64.8	472	305	538
65.8	2348	306.3	669		65.7	2442	306.3	757
83.9	815	311.7	606		83.8	1070	311.7	730
97	2152	312.7	586		96.8	2313	312.7	645
99	248	314.3	1699		98.9	379	314.3	1692
100	6226	316.5	542		99.9	6124	315.4	1044
100.9	446	317.9	708		100.8	560	316.4	558
101.7	309	320.1	6568		101.6	448	320	6201
102.4	833	321	7648		102.3	1070	321	7506
108	143	322.5	3988		111	455	322.4	4082
111.1	242	325.2	2181		112.1	711	325.1	2248
112.2	489	326.6	962		115.4	559	326.4	1011
115.5	394	327.6	4373		117.1	816	327.6	4280
117.2	583	329.1	2108		118.2	849	329	2131
118.3	607	330.3	937		120.4	1062	330.3	996
119.5	209	331.5	1162		122.8	1084	331.4	1191
120.4	816	333.6	2154		155.2	580	333.5	2168
155.2	365	336.1	421		168.4	802	336	431
168.4	547	337.6	474		169.2	559	337.6	528
169.3	388	338.5	379		171.5	496	338.5	399
171.4	339	397.3	176		176.3	791	397.3	164
176.3	531	405.7	538		182.4	551	405.6	511
182.5	329	464.2	905		187.9	469	464.2	888
188	222	469.5	590		192.2	469	469.5	520
192.3	191							

HinfI digested TRF profiles							
Soil sample:B2(a)				Soil sample:B2(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.8	150	296	5984	53.7	260	192.2	302
55.3	494	297.6	3739	55.3	670	199.4	346
57.6	610	304.9	352	57.6	789	293.4	2901
58.5	491	306.2	637	58.5	668	296.1	7234
62.8	125	310.7	331	62.8	181	297.7	4596
65.7	443	311.6	429	65.7	600	304.9	417
96.9	1967	312.7	464	96.9	2503	306.2	788
99	227	314.2	1228	99	301	310.8	429
100	5747	315.4	652	100	7424	311.7	570
100.9	407	316.4	647	100.9	556	312.7	560
101.7	261	318	794	101.7	343	314.3	1485
111.1	207	320.9	9596	108	132	315.5	813
112.2	425	322.4	2179	111.1	307	316.5	800
117.2	510	323.8	1118	112.2	679	321	12131
118.3	318	325	2453	115.5	360	322.5	2756
119.4	92	327.5	2955	117.2	734	325.1	3177
120.4	434	329.2	1810	118.3	487	327.5	3696
168.4	270	331.4	1130	120.4	673	329.2	2255
169.3	207	333.7	1805	123.1	888	331.5	1386
171.5	198	336	510	168.5	581	333.7	2312
176.3	470	337.5	828	169.3	391	336.1	580
182.4	49	338.3	458	171.5	441	337.6	1266
188	39	340.4	108	176.3	842	397.4	210
192.2	57	397.3	169	177.5	646	405.8	326
199.5	55	464.2	664	182.3	332	464.3	864
293.3	2389	469.5	699	188.1	266	469.6	901

*Msp*I digested TRF profiles							
Soil sample: B1(a)				Soil sample:B1(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.7	253	146.9	217	53.4	280	148	5586
55.4	583	148	4599	55.6	528	149.9	4974
62.8	86	149.9	4079	62.8	126	151.5	1107
64.9	172	151.5	834	64.9	169	152.3	340
65.8	1830	152.3	256	65.8	2200	156.3	544
67.8	275	153.9	212	67.8	354	157.1	182
69.5	72	156.3	400	69.5	83	158.2	572
71.2	245	157.2	115	71.2	306	160.1	868
72.3	94	160	585	73.5	1836	161.4	669
73.5	1473	161.4	423	78.3	107	162.3	167
78.4	80	162.2	61	81	638	168.1	357
81	533	168.1	199	83.9	897	178.3	172
83.9	741	178.2	95	84.8	119	184.1	148
84.8	68	184.1	97	87.4	924	188	248
87.3	680	188.1	254	89.3	395	199.3	190
89.2	225	199.3	153	90.8	119	205.5	277
90.7	44	205.6	246	91.7	719	266.3	52
91.7	519	266.2	41	93.9	770	275.8	104
93.9	681	275.8	119	111.1	155	277.9	64
111.1	168	282.5	223	122.6	505	282.5	250
122.7	441	396.3	814	123.8	535	396.3	971
123.8	415	400.2	2223	126.5	431	400.2	2807
126.6	321	432.9	1668	127.8	1855	432.8	2041
127.8	1527	435.9	1931	136.6	371	435.9	2302
136.6	305	437.5	596	138.5	1849	437.5	1370
138.5	1483	454.2	187	139.5	297	454.3	198
139.5	242	471.5	289	140.4	140	471.5	317
140.4	93	475.7	135	141.4	730	475.7	105
141.4	577	483.9	3240	143.2	233	483.8	3596
144.2	620	486	1118	144.1	733	486	1261
145.9	838			145.9	1013		

*Msp*I digested TRF profiles							
Soil sample: B2(a)				Soil sample:B2(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.7	123	148	3081	53.4	168	151.3	809
55.3	272	149.8	2315	55.3	350	152.1	231
62.8	127	151.4	727	62.8	180	156.1	258
67.8	152	152.3	153	67.7	200	158.8	181
69.5	18	153.7	149	71.1	177	160.3	563
71.2	139	156.2	191	73.4	1039	161.3	443
73.5	909	158.1	105	80.9	369	168	168
81	286	160.3	449	83.7	195	178.1	56
87.4	350	161.4	333	87.3	495	184	78
89.3	143	168	101	89.2	199	188	187
91.7	316	178.2	94	91.6	440	199.3	188
93.9	253	184.1	83	93.8	314	275.6	79
111.1	90	188	184	111	113	282.4	215
122.7	296	199.4	149	122.6	389	396.3	450
123.8	273	275.7	65	123.7	293	400.3	1538
126.8	212	282.5	182	126.6	249	432.8	995
127.9	914	396.4	408	127.8	1018	435.9	1265
136.6	167	400.3	1307	136.5	177	438.4	406
138.5	496	432.9	889	138.4	550	466.5	395
139.5	164	436	1197	139.4	187	471.5	183
140.5	47	438.3	567	141.4	376	482.2	301
141.5	335	466.6	424	144	404	484	1855
144.2	379	469.7	390	145.7	519	486.1	608
146	397	484	1708	147.9	3387		
147	154	486.2	477	149.7	2613		

6.3 Appendix C: Original data of 4RE (*Bst*UI, *Hae*III, *Hinf*I & *Msp*I)-Derived TRF Profiles from Bulk Soils Sampled in Field (C1, C2&C3)

*Bst*UI digested TRF profiles											
Soil sample: C1(a)				Soil sample: C2(a)				Soil sample: C3(a)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
54.5	130	201.5	1394	54.5	336	200.7	1366	55.2	220	198.7	2002
55.3	210	203.1	3540	55.3	459	201.5	1231	56.1	6686	200.2	1217
56.2	7512	205	2404	56.2	11106	202.3	1731	58	7536	201.6	2241
58.1	6281	209.8	2005	58.1	8293	203.2	4247	59.7	242	203.2	3725
59.8	302	211.5	2175	59.8	317	205	2314	61.4	129	205.1	3692
62.4	3146	214	2201	61.4	108	206.1	3488	62.3	5506	207.6	3376
90.1	1365	222.4	1955	62.4	3180	209.8	1970	86	449	209.7	5307
92	448	223.3	1853	90.4	2935	211.7	3213	90.2	3255	211.8	3804
93	6114	224.9	2139	91.4	900	214.3	2206	91.5	2331	214.4	3528
94	816	226.3	1887	93.2	5928	221.4	887	93.2	11857	222.3	2558
95.3	3435	227.1	1192	94.1	575	222.3	4699	94.2	1075	223.3	2812
97.2	472	229.1	1456	95.4	3533	223.3	2064	95.3	9287	225	2425
99.7	809	233.7	1404	97.2	200	224.7	1753	99.7	790	227.1	2861
101.9	2644	238	2469	99.6	336	226.3	1989	102	2401	229.1	1495
102.8	1892	240.2	2184	101	194	229.1	1099	102.8	1557	233.6	1463
104.4	3197	243.6	1095	102.6	8293	233.7	2032	104.6	3590	235.8	3464
107.3	1101	247.4	2830	104.5	4995	235.7	4555	106.7	2084	237.9	2797
110.2	234	280.5	108	106.6	2084	238	3363	107.6	1345	240.1	3317
111.1	668	355.8	120	107.5	1198	240.1	2650	111.3	497	243.6	1679
112.2	773	357.8	1001	110.3	306	242.5	1080	112.4	1156	247.3	2360
123.5	278	360.9	1928	111.2	1531	243.7	1868	123.9	488	356	1008
138.6	281	362.3	811	112.3	1117	247.2	3702	148.6	1197	358.3	3856
159	742	365	83	123.4	345	357.9	1996	159.2	1079	361.1	3441
162.3	863	383.9	4425	138.8	440	361	2544	162.3	930	362.4	2621
165	656	385.9	5721	159.1	1095	362.4	1033	165	920	384.1	8696
190.9	1024	390.5	11179	162.3	1160	368.6	354	174.5	680	386.1	7951
191.7	1239	392.6	3252	165	1045	384.6	7676	191.1	1646	390.5	16856
193.3	810	394.5	5137	191	1184	386.1	5913	192.6	2572	392.8	6219
195.6	6045	396.9	2407	192.1	3750	390.5	11805	193.3	1814	394.8	8649
197.1	3502	402.9	2637	193.3	1211	392.8	3719	196.3	9675	397.1	3274
200.7	938			195.6	2402	394.7	5510	197.2	6250	402.9	2975
				196.3	2358	397	3730				
				197.2	3843	405.2	2110				

*Bst*UI digested TRF profiles											
Soil sample: C1(b)				Soil sample: C2(b)				Soil sample: C3(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
54.5	224	197.2	4219	54.5	318	202.5	1827	55.2	212	203.3	3166
55.3	419	201.6	2175	55.2	461	203.3	4094	56.2	5936	205.2	3113
56.2	11406	202.4	2581	56.2	11095	205.1	2167	58.1	6576	207	2994
58.1	9705	203.2	5153	58	8121	206.3	1460	59.1	200	209.8	4717
59.8	366	205.1	3720	59.7	363	209.9	1926	59.8	227	211.9	3377
60.6	253	207.8	1288	61.4	122	211.8	3130	60.6	142	214.5	3233
61.4	200	209.8	3134	62.3	3142	214.4	2120	61.4	135	222.4	2416
62.4	4881	211.6	5285	90.4	2838	221.4	812	62.4	4871	223.4	2781
85.9	538	214.2	3252	91.4	941	222.4	4358	90.3	2712	225	1878
90.2	2304	222.4	2789	93.2	5846	223.3	2264	91.5	1376	227.1	2606
92.1	597	223.4	3208	94.1	526	224.7	1613	93.2	10322	228	1198
93.1	9600	224.9	3089	95.4	3411	226.3	1856	94.2	949	229.2	1331
94.1	1091	226.3	2699	97.2	171	228	998	95.4	7939	233.7	1430
95.3	5436	227.1	2038	99.6	333	229.1	980	99.7	701	235.9	2245
97.4	667	229.1	2101	101	191	233.6	1735	102	2040	237.9	2704
99.7	1157	231.6	2873	102.5	7992	235.7	3998	102.9	1188	240.2	3121
101	221	233.7	2190	104.5	4829	237.9	3190	104.6	2950	243.7	1412
102	4239	235.8	5421	106.6	2032	240.1	2297	106.7	1805	247.2	2058
102.9	2727	238	3883	107.5	1283	242.5	1005	107.6	1164	356.2	827
104.5	4592	240.2	3886	110.3	297	243.6	1731	111.3	419	358.3	2224
106.6	2075	242.6	1248	111.2	1484	247.2	3435	112.4	1002	361.2	2705
107.4	1641	243.6	1721	112.2	1066	358	1586	124	423	362.5	1837
110.2	369	247.3	4340	138.8	371	361	2038	148.7	958	368.9	105
111.2	972	280.6	229	159.2	1426	362.5	1305	159.3	933	381.7	3468
112.2	1194	358.1	1697	162.4	1087	369.4	137	162.4	797	384.2	7046
117	641	361	2959	165	957	384.5	7004	165	781	386.3	6220
123.8	431	362.4	1449	192.4	4635	386.2	5508	191.2	1501	390.6	12873
138.8	402	365.1	376	193.4	1134	390.7	11050	192.7	2304	392.9	4641
159.2	1165	378.4	2407	195.2	4491	392.9	3452	196.5	8668	394.9	6751
162.4	1320	384.1	6814	196.4	2552	394.8	5354	198.8	1817	397.1	2322
165.1	931	386.1	8349	197.3	3500	397.1	3428	200.4	1090	403	2410
174.7	729	391.4	17584	200.9	1776	405.3	1873	201.7	2232		
191.1	1728	392.7	5184	201.7	1303						
191.8	1963	394.6	7878								
193.4	1211	397	4089								
195.7	8953	402.8	4238								

*Hae*III digested TRF profiles											
Soil sample: C1(a)				Soil sample: C2(a)				Soil sample: C3(a)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.6	720	218.4	6132	53.5	814	213.7	3069	53.5	799	218.3	6569
57.8	370	219.3	3377	57.7	385	215.2	1795	57.7	702	222.5	3181
59.6	2296	220.2	2905	59.5	1883	216.2	2225	58.5	271	224	1526
63.1	3215	222.5	4081	60.3	751	217.3	5130	59.5	4377	225	1599
64.7	1026	224.1	2106	61.9	169	218.5	3859	62	284	225.9	1826
66.1	688	225.1	1770	62.9	3842	219.3	3045	63	4023	227.6	3438
70.4	1006	226	1758	64.7	698	222.5	3425	64.6	1798	230.2	2193
71.6	1102	227.6	3170	65.9	301	224.1	1223	65.8	1289	232.1	4723
72.8	1141	230.2	1672	70.4	993	225.1	1009	70.4	925	234.3	2585
128.7	1526	232.1	5340	71.5	932	226.2	1967	71.6	876	236.5	3074
130.3	1329	234.4	2950	72.6	1589	227.5	1720	72.8	757	238.2	1669
131.1	1995	236.7	3201	128.6	1029	230.2	2189	127.1	911	240.9	3366
137.6	1371	238	1499	131.1	1385	232	5890	128.6	1685	242.6	2662
168.4	1555	240.9	2599	137.6	1037	234.4	2462	130.8	2948	249.3	920
176.3	779	242.7	1289	144.4	393	236.8	1760	133.3	559	250.1	1280
182.3	1078	250.2	1602	166.4	572	240.8	1908	137.7	1606	253.8	2972
185.3	635	252.9	667	168.4	1102	243.5	657	144.6	558	256.5	1375
186.2	1545	253.8	3751	176.4	465	250.2	1689	168.4	1275	257.9	6857
188	1949	255.2	1034	182.3	875	253.9	2951	182.2	874	260.3	2770
188.9	1093	258.8	2323	185.1	474	255.7	883	185	559	262.1	5260
192	2755	260.3	1622	186.3	944	258.8	2814	188.1	2279	264.5	1521
193.4	9716	262.2	4164	188.1	2093	260.3	912	189.6	1529	267.2	1108
195.5	3057	264.5	2051	193.3	7008	262.2	3814	193.2	15068	282.8	1207
196.5	5320	265.4	701	196.6	5318	264.5	1994	194.7	1612	285.6	210
199.5	2429	288.9	407	201.1	2499	288.8	766	195.5	3016	288.9	675
201.1	2929	291.7	4775	202	1399	291.7	3262	196.5	7398	291.7	6843
202	1808	293.2	719	202.8	2381	293.9	1256	199.7	2650	293.2	2262
202.9	2508	293.9	990	204.3	1476	296.6	1202	201.9	2108	296.6	744
204.3	2076	296.6	888	205.8	3014	298.9	494	202.8	1908	298.8	487
205.9	3808	298.9	603	209.5	2362	323.6	567	205.7	2641	323.4	716
209.6	3512	320.7	162	211	1548	325.8	241	209.7	3817	325.6	424
211	2359	323.8	1197					211.2	2688	329.5	499
213.6	3257	325.6	551					213.6	4483	378.3	765
215.3	2849	329.5	790					215.2	2139	405.4	750
216.2	4495	378.4	171					216.3	6019		
217.1	5202										

*Hae*III digested TRF profiles											
Soil sample: C1(b)				Soil sample: C2(b)				Soil sample: C3(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.6	598	215.3	2444	53.5	873	215.2	2105	53.5	650	218.4	7239
59.5	2023	216.3	3832	57.7	390	216.1	2638	57.7	626	222.5	3279
62	173	217.2	4486	59.5	1942	217.2	5849	58.5	238	224.1	1640
63	2925	218.4	4860	60.4	686	218.4	4186	59.5	4080	225	1740
64.6	896	219.4	2843	61.9	172	222.4	3701	62	254	226	1787
66	422	220.3	2738	62.9	3947	224.1	1410	63	3843	227.7	3455
70.4	910	222.6	3682	64.7	698	225.1	1083	64.6	1566	230.2	2063
71.5	1040	224.1	1750	65.9	335	226	2099	65.8	1107	232.2	4760
72.8	1012	225.1	1506	70.4	1005	227.5	1666	70.3	787	234.4	2543
128.7	1044	226.1	1504	71.5	956	230.1	1329	71.6	745	236.6	3304
130.4	1077	227.7	2732	72.5	1575	232	6302	72.8	673	238.3	1772
131.2	1694	230.3	1432	128.6	1179	234.4	2861	127.1	979	241	3542
137.7	895	232.1	4992	131.1	1444	236.8	1866	128.6	1768	242.7	2599
166.3	346	234.5	2569	137.6	1116	240.7	1968	130.7	3010	249.4	1030
168.5	1072	236.8	2886	144.5	387	242.6	848	133.3	622	250.2	1354
176.3	478	238.1	756	168.3	1004	243.5	721	137.7	1711	253.8	3129
182.4	750	240.9	2097	176.3	496	250.1	1416	144.7	698	256.5	1552
185.3	409	250.2	1331	182.2	922	253.8	3112	166.3	672	258	7156
186.3	1220	253.9	3131	185	498	258.7	1293	168.4	1402	260.3	2448
188.1	1589	258.9	2221	186.2	930	260.3	865	176.3	677	262.1	5347
192.1	2285	260.4	1398	188	2034	262.2	4078	182.3	838	264.5	1588
193.5	8246	262.2	3643	191.1	994	264.5	2068	188.1	2305	267.3	1201
195.6	2875	264.6	1741	193.4	6717	288.8	870	189.7	1419	282.9	1161
196.6	4541	288.9	343	196.5	5437	291.7	3557	193.3	15387	285.6	218
199.6	1960	291.7	4084	199.7	1915	293.8	876	194.8	1614	288.9	727
201.2	2527	293.2	641	201.1	2332	296.5	1142	195.6	3045	289.7	517
202.1	1656	294	810	202	1436	298.9	492	196.6	4532	291.7	6982
203	2113	294.9	316	202.8	2538	323.4	610	199.8	2657	293.1	1023
204.4	1905	296.6	725	204.3	2230	325.8	238	202	1910	293.9	1172
206	3038	298.9	507	205.8	3077	329.4	768	202.8	1885	296.6	749
209.8	2915	325.7	379	209.6	2395	361.9	306	205.7	2003	298.9	451
211.1	1794	329.6	754	211	1509	399.5	501	207.6	1823	320.6	241
213.6	4128	378.3	161	213.5	1982	410	660	209.7	3857	323.4	1020
				214.3	1273			211.3	2709	325.6	619
								213.6	3312	329.6	600
								216.3	6252	378.4	729

*Hinf*I digested TRF profiles											
Soil sample: C1(a)				Soil sample: C2(a)				Soil sample: C3(a)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.9	944	192.1	294	53.8	1019	296.3	2678	53.8	906	192.1	225
62.6	92	199.1	277	97	532	297.1	2179	96.9	524	199.3	411
96.8	573	293.4	3699	100.1	2111	297.8	2712	99	182	293.4	2793
97.7	386	294.2	940	100.9	133	306.6	719	100	4549	296.2	6131
99.1	109	296.2	5229	108	73	311.7	893	100.9	152	297.7	7026
100.1	3358	297.7	4391	110.1	432	314.1	974	111.3	898	303.8	292
100.9	128	304.4	391	111	3569	316.9	2446	113.6	1511	306.4	1853
111.3	661	306.5	2216	112	562	317.8	1562	115.9	606	311.8	2546
112.1	876	311.6	1858	113.7	1725	321.9	16050	117.1	185	314.1	3251
113.6	2375	313.8	2566	117.3	72	327	8488	118	716	317.6	7564
116	339	314.8	2124	118.2	371	329.4	2653	119.6	454	321.6	31286
117.2	135	316.7	3100	119.8	351	331.6	3313	169.2	430	327	12082
118.1	819	317.6	2927	168.6	378	334	4356	171.3	813	329.3	5614
119.7	497	321.1	33587	176.6	272	337.7	3541	176.4	219	331.5	6371
168.4	343	327.9	6827	182.2	339	340.8	2892	182.2	557	333.6	3653
169.2	657	331.4	6147	187.4	201	342.9	1279	188.3	323	337.5	3598
171.4	336	334	6436	188.3	326	367	294	189.3	321	340.7	2473
176.4	299	336.1	1843	192.2	210	469.8	70				
182.1	640	337.6	4868	293.4	3158						
187.2	351	340.8	4083								
188.2	521	342.8	1740								
189.2	284	366.8	293								

*Hinf*I digested TRF profiles											
Soil sample: C1(b)				Soil sample: C2(b)				Soil sample: C3(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
54	337	293.3	942	53.8	1060	192.1	277	53.9	623	291.5	561
65.2	95	294.2	525	97.1	618	293.4	3385	96.9	372	293.4	1860
76.5	67	296.3	2667	100.1	2258	296.3	2772	99.1	113	296.2	4209
96.9	246	297.8	2279	101	106	297.1	2022	100.1	3124	297.7	5197
100.1	1377	304.4	141	108	59	297.8	3432	101	114	306.4	1344
111.4	318	306.6	1067	110.1	438	304.5	270	111.4	953	311.8	1839
112.1	412	311.7	1054	111	3828	306.6	1087	113.7	1033	314.1	2326
113.7	1001	313.9	1361	112	584	311.7	1467	115.1	169	316.7	2764
117.2	61	314.8	1090	113.7	1802	314.1	2621	116	425	317.6	2522
118.2	369	316.7	1615	115.9	162	316.8	2460	117.2	132	321.3	22618
119.8	231	317.7	1624	117.2	54	317.8	1857	118.1	493	327.2	8807
168.5	157	321.1	17380	118.1	359	321.9	9091	119.7	323	329.3	3955
169.3	260	328	3313	119.7	329	327	9648	169.2	355	331.5	4588
171.5	144	331.5	3222	168.7	417	331.6	3661	171.3	685	333.7	3301
182.2	225	334	3436	176.5	375	334	4733	182.2	459	337.4	2468
188.2	180	340.9	2101	182.2	427	337.6	4069	188.3	271	340.7	936
192.2	93	366.7	76	187.4	285	340.8	3221	192.2	214	366.9	263
				188.3	412	342.9	1468	199.3	311	468.5	825
				189.3	257	367	234				

*Msp*I digested TRF profiles											
Soil sample: C1(a)				Soil sample: C2(a)				Soil sample: C3(a)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
52.7	252	147.9	10713	53.7	1013	144	1961	54	1165	149.8	9124
53.9	1238	149.7	6039	60.6	814	145.9	1190	62.6	1389	151.4	323
62.6	1542	152.1	895	62.4	666	147	2205	65.1	304	152.2	545
65.1	632	153.8	568	65	368	148	4953	67.7	866	153.8	309
67.6	459	155.5	1126	67.6	326	149.8	3537	69.6	226	155.5	862
69.4	190	158.1	1465	69.3	130	152.1	215	70.8	1159	158.9	1305
70.7	1424	158.9	1551	70.7	894	153.8	91	71.9	2398	160.1	1611
71.8	2087	160	2030	71.8	914	156.4	576	73.3	2378	161.4	1374
73.3	990	161.8	1674	73.5	1273	158.8	412	78.8	538	168.1	1908
74.4	1406	168.1	2927	81.2	1101	159.9	411	81.2	1854	196.3	550
78.8	841	177.9	375	84	181	161.8	570	85.3	720	197.8	1767
81.2	2663	195.2	886	85.4	372	168	1474	87.5	5740	199.3	1342
84.2	414	197.2	4434	87.4	1345	197.1	1497	89.3	1279	205.5	2111
85.2	721	199.2	1672	89.3	886	197.9	1336	90.8	1447	207.6	751
87.4	3796	205.4	1797	91.7	2238	199.2	1123	91.8	2942	213.2	383
89.3	1355	207.5	624	99.3	110	205.4	1169	111.4	479	224.1	348
91.8	2789	213	649	110	212	213.1	281	115.4	649	264.5	1059
111.1	512	224	785	110.9	3262	224	491	116.8	327	266.3	157
113.1	421	266.2	125	116.6	398	266.2	135	119.1	1037	275.8	338
115.4	551	275.8	311	117.6	67	275.7	1088	122.6	802	276.9	156
116.7	604	277.2	239	118.9	388	282.5	297	124.2	1111	280.7	1201
117.6	317	280.6	336	125.3	1096	400.3	636	127.6	2145	282.4	393
119	1089	281.3	364	128.3	2380	432.8	831	128.4	1709	288	921
122.4	855	282.6	334	134.5	727	436	1186	134.6	845	400.4	1808
123.4	1193	288	1862	135.4	1472	438.7	513	135.5	2486	431.8	937
125.2	1634	293.7	521	136.4	1333	453.5	183	136.6	3360	432.9	1694
128.3	4687	400.3	1107	138.1	2071	486	2939	138.3	4176	436.1	3025
135.4	2837	432.8	1984	140.6	982			140.8	839	437.4	2757
136.4	3511	436	1867					143.5	1734	472.9	934
138.1	4552	437.3	1316					145.7	2822	486	5273
140.5	2229	453.5	404					148.1	11168		
142.1	1033	466.7	1054								
143.6	1930	470.5	307								
145.7	2189	480.2	827								
146.9	3292	486	4907								

*Msp*I digested TRF profiles											
Soil sample: C1(b)				Soil sample: C2(b)				Soil sample: C3(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.8	843	144.6	968	53.8	1051	145.9	1436	54	1178	148.1	10222
62.4	1058	145.7	1437	62.6	667	147.1	2297	62.6	1268	149.7	7594
64.9	463	146.9	2243	65.1	373	148.1	5680	65.2	282	151.4	330
67.4	314	147.9	7681	67.7	305	149.9	3846	67.7	771	152.2	546
69.3	154	149.7	3803	69.5	146	152.2	486	69.6	194	153.8	387
70.5	1035	152.1	545	70.8	979	156.5	857	70.7	1057	155.5	837
71.7	1527	156.2	674	71.9	968	158.1	710	71.9	2242	158.9	1318
73.1	689	158.8	1006	73.6	1437	160	674	73.3	1940	160.1	1667
78.8	608	159.9	1424	78.4	281	161.9	1190	78.9	493	161.4	1441
81.2	1856	161.8	1086	81.4	1176	168.1	1889	81.3	1680	168.1	1915
84.1	306	168	1947	84.1	196	197.1	1526	85.3	672	197.8	2566
85.3	555	177.8	148	87.5	1405	197.9	1421	87.5	5262	199.2	1335
87.5	2594	184.2	54	89.5	895	199.2	1133	89.8	1158	205.4	1829
89.4	968	195.1	458	91.9	2503	205.4	1157	90.8	1353	207.6	677
91.8	1851	197.1	2936	99.6	96	213.1	383	91.9	2582	213.1	350
111.3	363	199.2	1089	110.2	273	224	519	111.3	398	264.5	991
117.6	148	205.4	1100	111.1	3490	275.8	1072	117.6	322	266.3	149
119	770	212.9	526	116.7	540	282.6	358	119.1	943	275.7	282
122.4	570	223.9	531	119.1	491	287.9	1023	122.6	737	280.6	1480
123.4	804	275.7	205	123.3	1517	293.7	606	123.5	855	282.3	605
125.2	1027	280.5	246	128.4	2726	400.3	574	124.2	983	287.9	919
127.6	3074	282.6	343	134.6	894	432.8	828	127.6	1997	400.4	1768
134.5	716	400.2	737	135.5	1671	436.1	951	128.4	1570	431.7	748
135.4	1775	432.6	1166	136.5	1726	437.5	770	134.6	832	432.8	1634
136.4	2422	435.9	1074	138.2	2243	453.5	204	135.5	2319	436.1	2871
138.1	3153	437.1	976	140.7	1334	486	3157	136.6	3200	437.3	2523
140.5	1523	453.3	219	144	2567			138.3	3945	453.5	290
142.1	678	485.9	2992					140.8	914	472.8	716
143.5	1262							143.5	1704	483.5	2445
								145.7	2749	486	5046

6.4 Appendix D: Original data of 4RE (*Bst*UI, *Hae*III, *Hinf*I & *Msp*I)-Derived TRF Profiles from Soils Treated by Hydrogen Gas in Lab (D2&D4)

*Bst*UI digested TRF profiles											
Soil sample: D2(a)						Soil sample: D2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
52	701	191.6	3206	280.6	5654	52	636	191.6	3310	282.9	376
56.6	2228	193.1	1762	283	336	56.5	2364	193.1	1697	293.5	2079
58.3	583	195.2	6237	293.5	2077	58.3	578	195.2	6486	333.7	318
59.8	718	197.1	4295	294.9	314	59.8	676	197.1	4447	355.9	1754
62.5	617	200.1	872	355.9	1691	62.5	569	200.1	917	356.9	899
70.1	381	201.1	1639	356.9	825	70.1	358	201	2865	358.1	1158
89	115	202.9	1181	358	979	89.1	129	202.9	1231	360.7	4083
90	6797	204.2	748	360.8	3727	90.1	6652	204.1	775	362.7	1673
92	640	206.1	1112	362.7	1539	92.1	594	206.1	1105	364.6	1571
93.1	14732	207.5	602	364.6	1477	93.1	14772	207.4	583	369	10644
95.3	5329	210.1	955	369	10623	94	2676	210.1	1105	375.5	1095
102.5	828	211.8	4322	375.5	1033	95.3	5551	211.7	4324	384.3	2771
104	982	212.6	762	384.3	2597	96.9	137	212.6	1412	386.4	1998
109.7	702	214.2	1006	386.4	1871	102.5	861	214.1	1203	388.1	7292
111.8	728	220.9	622	388.2	7328	104	1086	220.7	603	390.7	2754
112.7	584	222.5	5382	390.8	3036	109.7	851	222.3	5773	392.2	16889
123.7	922	223.6	3705	392.2	16212	111.9	783	223.4	3618	394.4	3250
138.9	458	224.6	3902	394.4	3203	112.7	696	224.4	4003	395.8	4573
150.4	598	226.8	1415	395.7	4544	123.8	1137	226.8	1470	400.4	1040
158.5	935	229.6	870	400.4	932	138.8	560	229.4	1069	458.7	554
159.9	458	233.8	1967	458.6	460	150.4	609	233.6	1850	460.1	533
165.3	1646	234.6	1324	460.1	524	158.6	976	234.5	1357	461.6	254
170.9	828	238.3	1426	461.6	195	159.9	541	238.2	1578		
173.7	565	240	462			165.4	1653	239.8	511		
174.6	1867	247.4	789			170.8	849	247.2	836		
						173.6	711	279.7	160		
						174.5	1998	280.7	5612		

*Bst*UI digested TRF profiles												
Soil sample: D4(a)						Soil sample: D4(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
56.5	732	204.3	300	362.9	1435	56.3	810	203.1	472	362.8	1436
58.5	2174	206.2	477	364.7	775	58.3	2359	204.2	269	364.6	796
62.4	333	207.5	285	369.1	14722	59.7	281	206	499	369.1	15857
90.1	3242	210.2	434	370.4	1216	62.3	340	207.5	261	370.4	1266
91.6	392	211.8	2154	375.6	707	90	3664	210.1	441	375.6	708
93.2	5098	213.3	1574	377.2	868	91.5	479	211.7	2342	377.2	946
95.3	2722	214.2	396	384.5	2128	93.1	5812	213.2	1697	384.5	2255
102.4	518	221.5	511	386.6	1400	95.2	2984	214.1	461	386.6	1524
104	375	222.4	3893	388.3	5570	102.4	546	220.7	231	388.2	6006
109.6	2353	223.5	2627	390.9	2750	104	404	222.3	4306	390.9	2978
110.5	137	224.5	1376	392.4	16470	108.7	83	223.4	2942	392.3	17673
111.9	370	226.9	478	394.5	2393	109.6	2620	224.3	1641	394.5	2567
123.8	326	229.5	298	396	1243	110.4	157	227	460	396.1	1251
125.2	472	233.7	1091	400.6	213	111.8	376	229.4	392	400.6	211
158.5	244	234.5	679	458.8	377	123.7	339	233.5	1238	458.9	393
165.5	584	238.2	487	460.2	1888	125.1	483	234.5	774	460.3	1954
174.6	868	247.1	422	461.7	794	165.4	561	238.1	564	461.8	918
191.9	1876	280.7	572			174.5	907	247	497		
193.3	879	293.5	2363			191.8	2024	280.8	635		
195.2	2802	294.9	295			193.2	838	293.5	2603		
197.1	1865	356.1	502			195.2	2951	294.8	399		
200.2	399	357.2	401			197.1	2147	356	460		
201.1	1319	358.2	822			200.1	414	357.1	363		
203.1	463	360.7	2599			201	729	358.1	839		
						201.7	672	360.6	2769		

*Hae*III digested TRF profiles												
Soil sample: D2(a)						Soil sample: D2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.7	1311	192.1	4033	234.3	853	50	660	186.2	296	228.1	1905
57.9	576	193.2	6285	236.6	463	53.8	1147	187.1	3345	229.9	1230
59.8	2603	194	2455	238	849	58	430	187.9	1044	232	475
63.2	7701	195.9	4058	239.7	703	59.9	2220	192.2	3471	232.9	468
65.1	1404	196.7	1859	241.3	1552	63.3	7230	193.1	5410	234.3	634
66.4	572	199.2	2113	243.5	3113	65.1	1176	193.9	2401	238	641
70.3	1366	200	1298	250.3	404	66.5	411	195.9	3661	241.3	1270
71.3	375	201.5	11312	255.8	1087	70.3	1180	199.2	1686	243.5	2630
72.2	5959	202.4	2585	257.3	306	71.4	309	199.9	1084	250.4	233
73.1	283	205.1	3965	258.6	4274	72.2	5521	201.5	9810	253.4	296
78.4	515	206.2	1329	259.9	17551	73.1	195	202.4	2184	255.9	822
128.6	873	209.4	2393	262.5	3255	78.4	310	205	3226	258.7	3759
130.9	991	211	1341	264	462	128.6	694	206.2	993	260	15633
141.5	2052	213.9	432	264.9	4346	130.9	788	209.4	1969	262.5	2772
143.4	524	216.3	1388	285.5	962	141.5	1759	211	1133	265	3697
144.5	3924	217.5	3314	287.2	757	143.4	378	213.9	262	285.5	780
145.5	442	218.6	4343	288	1695	144.5	3476	216.3	1159	287.2	625
159.8	634	219.7	1024	288.8	1556	145.5	299	217.5	2857	288	1464
166	584	220.7	4147	291.6	15924	159.8	491	218.6	4056	288.8	1359
166.7	587	221.6	475	293.1	1686	166	441	219.8	811	291.6	14235
167.6	8605	222.5	1486	300.4	300	166.7	508	220.7	3637	293.1	1415
168.4	787	224	9262	320	273	167.5	7747	221.7	308	321	5908
169.8	739	224.9	3415	320.9	6594	168.4	489	222.5	1201	322.5	144
176.6	828	226.3	766	322.5	174	169.7	642	224	8179	323.9	810
182.1	582	227.2	2753	323.9	904	176.5	678	224.9	3065	325.4	1237
186.3	407	228.1	2399	325.4	1401	182.1	425	226.3	615		
187.1	3688	229.9	1491	328.9	550	185	469	227.2	2210		
188	1219	232	625								
190.3	873	232.9	654								

*Hae*III digested TRF profiles											
Soil sample: D4(a)						Soil sample: D4(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.7	857	188	1108	227.1	2298	53.7	1054	188	1277	228.1	3202
57.8	479	191.6	2077	228	2742	57.9	556	190.4	769	229.1	696
59.7	1416	192.3	1932	229.9	1199	59.7	1712	192.2	4349	229.9	1370
62.1	152	193.2	7132	233	530	62.1	201	193.2	8327	233	553
63.1	8774	194	2022	234.2	407	63.1	10087	194	2341	234.3	477
65.1	802	196	2173	237.9	569	65.1	951	195.9	2544	238	681
70.3	1246	196.8	1530	239.6	715	70.3	1441	196.8	1732	239.7	820
72.4	1965	198.1	1119	240.9	765	72.4	2331	198.1	1278	240.9	921
120.3	535	199.2	2013	243.4	1909	120.3	582	199.2	2352	243.5	2238
128.7	546	200	1243	253.4	330	128.6	664	200	1284	253.4	394
130.9	727	201.6	9339	255.8	790	130.9	955	201.6	10761	255.8	933
141.5	2017	205	3277	257.2	173	140.5	276	205	3836	257.3	203
143.4	516	206.3	994	258.6	4970	141.5	2329	206.3	1140	258.6	5881
144.6	3579	207.2	638	259.9	18153	143.4	567	207.1	679	259.9	21510
145.6	352	208.5	555	262.4	2745	144.5	4101	207.8	556	260.7	1185
163.2	492	209.5	13325	265	839	145.6	367	208.5	674	262.4	3202
164.7	378	210.3	1073	285.5	478	150.4	687	209.5	15320	264	258
166	517	211	811	287.2	315	156.5	750	210.3	1178	265	1031
166.7	783	215.4	336	288	516	159.9	637	211	889	285.4	604
167.6	20182	216.4	1370	288.8	1217	163.2	552	211.7	572	287.2	401
168.5	878	217.4	2897	291.6	9597	166	605	213.2	302	288	645
169.2	562	218.6	3298	293.2	1782	166.7	933	215.5	336	288.8	1382
170.5	499	219.6	914	319.9	296	167.6	23591	216.3	1635	291.6	11173
176.6	705	220.7	3779	320.9	5267	168.5	993	217.4	3346	293.2	2140
182.1	573	221.6	390	323.9	834	169.2	583	218.6	3828	320	359
185.6	397	222.4	1509	325.4	1404	170.5	556	219.7	1074	320.9	5992
186.3	294	224	8742	392.2	828	174.6	782	220.7	4384	323.9	969
187.2	1745	224.9	3178			176.6	810	221.6	424	325.4	1636
						182.1	622	222.4	1767	392.2	937
						185.5	460	224	10211		
						186.3	354	224.9	3630		
						187.2	2033	227.1	2639		

*Hinf*I digested TRF profiles								
Soil sample: D2(a)					Soil sample: D2(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
54	599	304.1	376		54	556	305	6880
65.2	378	305.1	6262		65.2	393	310.5	198
76.3	326	306	1063		76.2	337	311.5	673
96.8	1785	310.6	157		96.8	1756	312.5	485
100.7	200	311.6	613		99	100	313.6	1384
111	221	312.7	403		99.9	3131	315.4	400
112.5	167	313.6	1204		111	186	316.5	927
115.4	129	315.3	347		115.4	134	317.6	442
118.2	166	316.6	901		118	116	321.6	3392
119.9	423	317.8	433		119.8	341	322.8	1818
159.9	118	321.6	2859		171.1	302	323.7	3328
171.1	415	322.8	1620		176.8	277	324.5	4984
182.2	141	323.7	2929		182.2	107	327.4	3251
187.8	71	324.5	4201		192.4	238	328.9	11192
192.4	227	327.5	3190		199.3	400	330.4	648
199.2	326	331.4	1768		201	496	331.4	2110
201	487	333.7	5821		240.2	535	333.6	6273
240.2	479	336.1	683		292.3	1271	336.1	748
292.4	1251	337	885		293.2	1829	337	919
293.2	1643	338.5	273		294	976	338.6	305
294.1	781	339.3	173		295.8	7838	395.3	353
295.9	7230	395.2	298		297.6	2970	396.8	174
297.6	2499	433.8	450		299.2	756	433.9	517
299.3	635	469.3	278		300.1	926	469.3	360
300.1	805				304.1	544		

*Hinf*I digested TRF profiles							
Soil sample: D4(a)				Soil sample: D4(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.9	1036	294.1	1377	53.9	940	299.3	803
65.2	629	295.9	10042	65.2	633	300.2	683
68.5	265	297.6	4842	76.3	799	302.9	88
76.3	836	299.3	914	96.9	3871	304.1	655
96.8	4257	300.2	761	99.1	142	305	13114
99.1	154	304.1	770	100.1	3920	305.9	2103
100	4087	305	16364	111.1	675	307.2	228
111.1	675	305.9	2450	112	366	310.7	273
112	363	307.2	174	115.5	290	311.6	725
115.5	294	310.7	276	118.1	242	312.8	883
119.5	448	311.6	796	119.5	454	313.7	7457
150.4	359	312.8	1022	155.4	221	315.3	322
155.4	238	313.7	9120	159.9	280	316.6	480
159.9	281	315.3	368	168.5	419	317.9	530
168.5	445	316.6	538	171.1	679	320.1	1201
169.6	534	317.9	563	176.8	847	321.8	4600
171.1	783	320.1	1432	182.2	413	322.9	2276
176.7	947	321.8	6037	189.7	320	323.8	3871
182.2	478	322.8	2770	192.5	674	324.6	4993
182.9	598	323.7	4256	193.5	339	326.1	541
187.9	242	324.5	5992	199.3	761	327.5	4698
188.8	433	326.1	644	200.1	705	329	14714
189.7	349	327.5	5714	201.1	1224	331.4	2420
192.4	753	329	18275	214.3	321	333.6	2262
193.5	367	331.4	2920	215.4	216	336.2	701
199.3	948	333.6	2737	216.4	281	337.1	1111
200.1	796	336.1	926	218.6	270	338.5	199
201	1300	337	1431	240.1	920	339.7	302
214.3	400	338.5	218	285.1	326	395.4	1387
215.3	292	339.7	413	293.3	2471	397	708
216.4	352	395.4	1755	294.1	1171	434	628
218.5	332	396.9	865	295.9	8192	468.5	814
240	1131	433.9	786	297.6	3949	469.4	525
285.1	440	468.5	1069				
293.2	3009	469.4	659				

*Msp*I digested TRF profiles											
Soil sample: D2(a)						Soil sample: D2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
52.9	673	137.5	754	197.8	693	52.8	229	143.2	5523	396.2	398
54	3360	138.4	3297	199.5	1043	54	1265	144.2	1178	399.9	2506
60.8	1425	140.6	692	200.2	1277	65.2	842	145.9	1399	432.7	1607
62.8	589	141.4	6414	207.7	494	67.6	276	148	6767	435	2230
63.6	430	142.3	791	212	525	69.5	162	149.8	3249	437.1	2271
65.2	2225	143.2	13818	213	10963	71	1214	152.5	1198	451.4	367
67.6	805	144.2	2856	214.4	759	72	83	153.9	1573	452.9	251
69.5	524	144.9	1210	215.3	481	73.8	958	154.9	1309	466.3	1772
71	3039	145.9	3436	216.5	297	78.5	384	156.5	448	471	719
72	300	148	16951	218.6	371	83.7	440	157.4	1044	475.6	1864
73.7	2444	149.8	8022	266.4	2744	85.1	2617	158.5	3808	483.3	1711
77.4	329	152.5	2896	275.7	2227	86.1	63	160.4	712	485.8	802
78.4	1015	153.9	3852	276.7	741	87.6	2039	161.4	670	491.4	812
81.3	309	154.9	3216	277.7	4090	89.2	7580	162.2	226	495.1	1553
83.7	1171	156.5	1188	282.5	871	90.2	246	168.1	393		
85.1	6217	157.4	2605	396.4	919	90.9	203	178.2	307		
86	203	158.5	8697	400.1	5930	91.8	1808	183.3	219		
87.6	4847	160.4	1734	420.7	709	111.1	531	184.2	5952		
89.2	17829	161.4	1648	431.5	1355	117.2	140	185	253		
90.2	622	162.3	450	432.8	4038	119.4	233	197.8	334		
90.9	547	163.4	1037	435.2	5513	122.3	1216	199.5	726		
91.8	4299	168.2	806	437.3	5621	123.1	1295	212.9	4868		
111.1	1230	173.7	760	451.5	982	127.6	1096	266.4	1174		
112.6	621	174.6	616	453	604	136.5	700	275.7	912		
115.6	395	175.6	474	464.5	1849	138.4	1327	276.6	315		
117.3	351	176.8	725	466.4	4679	141.5	2658	277.7	1743		
119.5	576	178.3	725	471.1	1841	142.3	360	282.5	360		
121.4	518	181.4	603	475.7	4718						
122.4	2699	183.3	464	483.4	4098						
123.2	3212	184.2	13456	486	1776						
126	1635	186.4	151	487.7	1260						
127.6	2739	187.9	482	491.5	2075						
136.5	1712	192.5	411	495.2	3934						

*Msp*I digested TRF profiles												
Soil sample: D4(a)						Soil sample: D4(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.9	1065	140.6	301	205.6	453	54	1061	143.2	7666	205.5	583
65.2	694	141.5	3206	213.1	741	65.2	688	144.2	1744	213	893
67.7	230	143.2	6825	214.4	331	67.6	247	145.9	1613	214.3	329
69.5	226	144.2	1575	216.4	227	69.5	247	148	6924	215.3	230
71.1	2010	145.9	1452	266.3	1681	71.1	2196	149.7	4675	216.4	227
72	159	148	6221	275.7	2158	72	159	151.5	481	266.3	2050
73.8	1291	149.8	4188	276.6	526	73.8	1395	152.5	2859	275.7	2716
78.4	441	151.6	442	277.6	1713	78.4	399	153.9	5539	276.7	660
81.4	319	152.5	2584	282.4	217	81.4	337	154.8	1445	277.6	2101
83.7	555	153.9	4792	396.4	292	83.7	589	156.4	1068	282.5	264
85.1	2879	154.8	1326	400.1	1221	85.1	3207	157.4	1670	396.4	464
86.1	106	156.4	1003	432.9	1912	86	120	158.4	3655	400.1	1652
87.6	3478	157.5	1404	435.3	2868	87.6	3903	160.1	1265	432.9	2606
89.3	10682	158.4	3006	438.3	837	89.3	12003	161.4	799	435.3	3623
90.2	354	160.2	1137	451.7	392	90.2	405	162.3	381	438.3	1188
90.9	271	161.4	712	453.1	5099	90.9	283	168.4	618	451.6	493
91.7	2041	162.3	330	466.6	1708	91.8	2188	174.6	443	453.1	6626
111.1	752	168.5	556	471.2	385	111.1	762	175.6	459	464.6	1217
117.2	194	174.6	350	475.8	3367	117.3	187	176.7	499	466.5	2191
119.5	936	178.2	532	484	4258	119.6	1045	178.2	641	471.1	457
121.4	606	183.3	601	486.1	816	121.4	682	181.3	254	475.7	4068
122.4	1804	184.1	11574	487.8	669	122.5	1861	183.2	465	483.9	5332
126.3	604	185.6	265	491.7	978	126.6	742	184.1	13886	486.1	979
127.6	1907	186.3	172			127.7	2058	184.9	658	487.7	784
135.1	646	188.1	468			135.1	667	188	540	491.6	1230
136.6	844	192.5	355			136.6	940	189.6	384	495.3	638
138.4	1197	199.4	531			138.4	1999	192.5	398		
						140.6	318	193.5	304		
						141.5	3617	197.8	439		
						142.3	495	199.3	647		

6.5 Appendix E: Original data of 4RE (*Bst*UI, *Hae*III, *Hinf*I & *Msp*I)-Derived TRF Profiles from Soils Treated by Air in Lab (E2&E3)

*Bst*UI digested TRF profiles											
Soil sample: E2(a)						Soil sample: E2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
56.3	2396	207	836	377.3	864	56.3	1615	206.1	1586	327.5	1618
58	1214	209.9	2129	384.6	2828	58.1	812	210	1541	328.9	794
59.7	423	211.7	4015	386.4	2646	59.7	319	211.7	3097	333.7	913
62.3	956	212.6	1321	388.1	2615	62.3	662	212.7	1060	356.1	1005
69.9	398	213.9	3220	390.9	11334	70	300	214	2495	357.3	1114
89	118	221.4	2262	392.3	2568	90.1	3153	221.5	1948	358.2	1198
90	4226	222.3	6070	394.4	3350	92.1	494	222.3	4495	361	1961
91.9	815	223.4	4803	395.9	1349	93.1	10470	223.4	3874	362.6	570
93	14123	224.4	3035	400.5	1539	94	1906	224.4	2342	364.7	2627
93.9	2557	225.9	731	408.2	727	95.5	2997	226	484	384.7	2086
95.3	3906	226.9	2026	458.7	2940	96.9	600	227	1624	386.5	1971
96.9	144	229.1	1176			100.1	1545	229.2	851	388.2	1803
102.1	560	229.9	1142			100.9	158	230	809	391	8420
103.9	717	233.7	654			102.3	374	233.7	415	392.4	1820
106	909	234.5	3934			104	519	234.6	2981	394.5	2384
123.7	882	237	1799			106.1	629	237	1394	396	915
138.6	330	238	898			123.8	638	238	682	400.6	1153
158.6	785	239.4	710			158.6	667	239.4	463	408.3	483
165.4	2261	242	1067			165.5	1784	242.1	771	458.9	2174
174.5	1820	243.5	447			174.5	1424	243.6	335		
191	1029	247	3670			191.1	805	247	2781		
191.9	2214	261.7	401			192	1840	281	310		
193.2	2440	293.5	2293			193.3	2016	293.5	2281		
195.3	5855	356	1490			195.4	4674	296.1	1848		
197.4	2682	357.1	1531			197.4	2244	300.2	700		
199.3	957	358.1	1700			199.3	822	316.9	652		
200.2	740	360.9	3030			200.3	642	321.6	1194		
201.2	1053	362.5	1120			201.3	903	325.3	512		
202.9	1153	364.6	3752			203	1082	326.6	811		
206	2164	375.6	755								

*Bst*UI digested TRF profiles												
Soil sample: E3(a)						Soil sample: E3(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
56.4	3249	192	4027	247.2	3925	54.7	362	174.5	3903	238	1445
58.1	1435	193.4	4174	261.7	413	56.4	2166	175.4	885	239.4	885
59.8	544	195.4	8706	293.5	2987	58.1	1409	176.6	843	242	966
62.4	1527	197.3	5701	356	1257	59.8	541	177.5	814	243.6	777
70	564	199.3	1257	357	1140	60.7	287	191	2132	247.1	4716
85.8	557	200.3	951	358.1	1458	62.4	1570	192	4469	261.6	565
88.3	133	201.2	1446	360.8	3444	70	615	193.3	5026	267.7	210
89.1	200	203.1	1678	362.6	1355	85.8	602	195.3	10130	293.4	3485
90	7688	206.2	2476	364.6	3333	88.4	144	197.3	6705	355.9	1572
92	1071	207.3	1003	369.1	285	89.1	216	199.3	1575	357	1542
93.1	17643	210.1	3367	369.9	1257	90.1	7695	200.2	1175	358.1	1691
93.9	4830	211.8	7919	375.5	1138	92	1150	201.2	1698	360.8	3939
95.4	6737	212.7	1575	377.2	934	93.1	18942	203	1915	362.6	1521
97	153	214	3371	378.2	702	94	4420	206.1	2971	364.6	3888
102.2	2847	221.6	4855	384.5	5285	95.4	7148	207.1	1170	369.9	1367
104	1137	222.5	18285	386.4	3424	97	187	210	3744	375.5	1253
106.1	1184	223.5	12299	388.2	3724	101.2	199	211.7	9036	377.2	1043
110.1	921	224.5	6497	390.8	10771	102.2	3050	212.6	1850	378.2	808
123.8	1557	226	1081	392.3	1885	104	1197	213.9	3982	384.5	5972
138.8	1733	226.9	2570	394.4	3564	106.1	1227	219.3	631	386.4	3840
150.4	836	229.3	1381	395.8	1330	110.1	862	221.4	4508	388.1	3526
158.6	1075	230	1329	400.5	1028	123.8	1628	222.3	20255	389.2	1698
163.9	926	233.7	655	408.1	702	138.8	1860	223.4	14108	390.8	11934
165.5	3304	234.6	5257	458.7	1348	150.4	959	224.4	7108	392.2	2344
169.5	1305	236.1	770			154.6	928	225.9	1506	394.4	4235
173.7	799	237.1	2198			158.6	1348	226.9	3071	395.8	1608
174.6	3373	238.1	1207			162.6	887	229.2	1637	400.5	1273
175.5	685	239.5	755			163.9	1136	229.9	1747	408.1	793
176.7	798	242	720			164.6	743	233.6	811	458.6	1530
191	1921	243.7	547			165.5	3742	234.5	6193		
						169.4	1562	236	929		
						173.6	1081	237	2619		

*Hae*III digested TRF profiles											
Soil sample: E2(a)						Soil sample: E2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
51.3	765	189.4	1832	236.7	1182	50	552	187.2	13096	235.8	1494
53.7	1660	190.3	1003	238	1880	51.4	583	188.5	4006	236.7	1245
57.8	1187	192.3	9006	241.7	1456	53.8	1394	189.4	1810	238	1688
59.8	12251	193.2	6961	243.4	2790	57.9	1101	192.3	9016	241.6	1442
62.1	380	194	5828	250.2	1918	59.9	11683	193.2	6837	243.4	2740
63.1	11533	195.1	1118	253.2	1941	62.2	295	194	5702	250.2	1681
65.1	1526	196	12028	254.7	1344	63.2	11022	195.1	1088	253.2	1868
66.5	1222	196.9	4065	255.8	1766	65.2	1253	196	11665	254.7	1315
70.4	1365	198.3	1399	257.4	624	66.7	1065	196.9	4983	255.8	1794
72.3	2702	199.2	4874	258.3	7704	70.4	1173	198.3	1394	258.3	7582
78.4	817	200.2	2006	259.8	3709	72.4	2541	199.2	4604	259.9	3628
120.3	1232	202.7	4976	260.6	1649	78.4	630	200.1	2308	260.6	1628
128.6	1597	205.1	7786	262.1	5406	120.3	1131	202.6	4809	262.1	5306
130.1	575	206.2	1627	264.1	568	128.6	1483	205.1	8025	264.1	577
131	5810	207.7	1576	269.2	639	130.1	494	206.2	2363	269.3	573
137.8	1118	208.7	1067	282.8	753	131	5766	207.7	1499	282.8	759
140.4	626	210	3627	285.2	376	137.9	1105	208.6	1014	285.2	330
141.5	4826	211.5	2309	286.4	579	140.5	567	210	3544	286.4	551
142.6	676	212.3	1561	287.2	7025	141.6	4774	211.5	2329	287.2	6969
143.5	663	213.7	1132	288.8	2475	142.7	686	212.3	1542	288.8	2417
144.5	10007	214.9	2682	291.6	22168	143.6	593	213.7	980	291.6	22023
145.6	754	216.3	1170	293.8	2153	144.6	10015	214.9	2619	293.8	2193
146.3	721	217.7	8943	295.7	1168	145.6	732	216.3	1049	295.7	1139
147.9	721	218.6	9681	302.4	1096	146.4	693	217.7	8833	302.4	1037
150.4	880	220.7	7109	304.8	3950	147.9	770	218.6	8897	304.8	3872
166.5	1665	221.6	1176	319.9	834	150.5	902	220.7	7296	319.9	815
167.4	1029	222.5	10600	320.9	2372	166.4	1047	221.6	1133	320.8	2335
168.4	1264	224	13757	322.7	1615	167.5	1060	222.5	10505	322.7	1506
169.5	1391	224.9	8391	323.8	3241	168.5	1092	224	14163	323.7	3190
173.5	1394	226.3	2644	325.4	599	169.5	1314	224.9	8212	325.3	626
176.5	2459	227.3	7989	327.8	1001	173.5	1313	226.3	2584	327.7	974
177.4	1059	229.9	3225	328.9	731	176.5	2391	227.3	7698	328.8	730
180.9	1517	231.1	2973	331.3	1282	177.4	1110	229.9	3104	331.2	1227
182.3	777	231.9	3158	375.6	506	179.1	1202	231.1	2890	399.9	421
185.4	2246	232.9	2333	399.9	424	180.9	1368	231.9	3099	406	703
187.2	13172	234.3	2433	406	754	185.4	2196	232.9	2239	466.4	578

*Hae*III digested TRF profiles											
Soil sample: E3(a)						Soil sample: E3(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
50	544	192.3	6208	232.9	1567	50	599	187.3	6279	231.1	1572
53.8	928	193.3	5171	234.4	1566	53.8	1038	188.6	2737	232	1657
57.9	1050	194	4145	236.7	809	57.9	1078	189.4	888	232.9	1491
59.9	6741	196.1	5980	238.1	1097	59.9	7063	190.3	756	234.4	1434
61.5	229	196.9	2317	241.8	1051	61.5	260	192.3	5675	236.8	659
62.2	469	198.3	916	243.6	2387	62.2	479	193.3	4689	238.1	984
63.2	16837	199.3	3006	250.3	943	63.2	17479	194	4007	241.8	1140
65.3	1342	200.2	1125	253.4	1127	65.3	1477	196.1	5834	243.5	2175
66.7	879	201.4	1160	254.9	958	66.7	1101	196.9	2207	250.3	769
70.4	1088	202.3	4419	255.9	1277	70.4	1126	198.3	863	254.8	879
72.6	3690	205.2	7422	258.5	6017	72.6	3737	199.3	2794	255.9	1073
78.5	669	206.3	2365	259.9	2781	78.5	631	201.4	1089	258.4	5389
120.4	1012	207.7	1401	260.7	1059	120.3	1045	202.3	4274	259.9	2576
128.7	1193	210.1	1500	262.2	2940	128.7	1178	205.2	6980	260.7	940
131.1	3332	211.5	3817	282.7	464	130.1	363	206.3	2204	262.2	2676
140.5	452	213.7	780	285.2	220	131.1	3158	207.7	1279	282.8	457
141.6	3953	214.9	1838	287.2	2052	140.5	435	210.1	1389	285.2	177
142.7	563	216.4	871	288.8	1644	141.6	3717	213.7	732	287.3	1854
143.6	524	217.7	6077	291.6	11568	142.7	558	214.9	1592	288.8	1471
144.6	6617	218.7	9883	293.2	857	143.6	519	216.3	686	291.6	9897
145.6	508	219.7	2821	293.9	1658	144.6	6341	217.7	5604	293.9	1490
146.3	534	220.8	6993	295.7	531	145.6	563	218.7	9381	302.4	465
150.5	682	221.7	806	302.4	506	166.5	1164	219.7	2406	304.8	1774
167.4	665	222.6	5840	304.8	1989	167.3	650	220.8	6332	320.9	1194
169.6	1109	224.1	11164	321	1332	168.6	1017	221.7	784	322.7	542
176.6	1612	225	6044	322.7	573	169.5	1058	222.5	5383	323.9	1415
182.4	544	227.4	3404	323.8	1576	176.6	1523	224.1	10325	325.4	276
185.5	1498	228.1	2556	325.4	254	182.4	450	225	5346	399.9	360
187.3	6721	230	2153	399.8	408	185.5	1384	227.3	5370		
188.6	2915	231.1	1889			186.4	681	230	2017		
189.4	948	232	1535								

*Hin*fI digested TRF profiles											
Soil sample: E2(a)						Soil sample: E2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
51.4	967	158.7	1374	299.3	1237	53.9	1715	171.2	1606	321.6	11733
53.9	2448	159.6	1710	300.2	8733	57.1	655	180.6	758	322.9	7700
57.1	938	160.6	953	306.4	2860	59.5	589	182.4	868	323.7	8133
59.5	886	167.2	1698	308.5	1291	65.2	746	183.5	723	325	5558
65.2	1138	168.6	2043	310.6	3322	68.5	373	187	669	326.6	7142
68.6	562	169.6	1478	311.6	2773	76.3	1163	187.8	957	327.5	14569
76.3	1677	170.4	5998	313.5	3903	77.3	523	192.1	989	329	7358
77.3	813	172.6	1645	314.3	3636	78.5	553	197.5	808	329.8	3463
78.5	892	175.5	1299	315.6	2729	95.2	241	198.5	832	331.5	5300
95.2	327	176.6	1714	316.9	9131	96.8	4413	199.4	1218	333.7	8398
96.9	6036	180.5	1513	317.9	4967	97.7	772	200.4	896	335.3	1172
97.7	1272	182.3	1614	321.6	16716	98.4	284	203.5	836	338.6	1612
98.4	407	183.4	1323	322.9	12969	99.1	415	240.1	438	339.7	1096
99.1	583	186.9	1198	323.7	10983	100	11983	292.3	1945	466.4	1244
100.1	17325	187.7	1820	325.2	7512	108.3	873	293.2	3591	467.4	1180
100.9	1557	192	1795	326.6	10716	109.2	298	294.1	3740	469.5	525
101.6	414	197.5	1633	327.5	21768	111.2	329	296	16134	491.7	891
102.3	581	198.4	1548	329	11048	112	505	297.7	7038		
108.4	1336	199.4	1948	331.5	7929	114.6	451	299.3	737		
109.2	597	200.3	1564	333.7	12309	115.4	765	300.2	5757		
111.2	666	201.3	1877	338.6	2473	116.3	434	301.7	259		
112.1	976	203.5	1572	339.7	1624	118.4	1246	306.4	1813		
114.6	783	231.8	971	346.2	713	120.7	433	308.5	802		
115.5	1246	232.9	993	466.5	2696	154.5	600	310.5	2091		
116.3	852	240.1	1038	467.4	1755	155.4	424	311.6	1766		
117.4	423	289.6	574	491.7	1253	159.6	856	312.7	1831		
118.4	2049	293.3	5500			167.2	942	314.2	2778		
120.7	948	294.2	5727			168.7	1050	315.5	1754		
154.5	1276	296.1	23927			169.6	830	316.9	6062		
155.5	984	297.8	10505			170.5	2109	317.8	2487		

*Hinf*I digested TRF profiles											
Soil sample: E3(a)						Soil sample: E3(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.9	1074	154.6	419	310.4	1595	53.8	1421	118.3	1333	308.4	954
57.2	538	155.5	247	311.6	1740	57.1	761	120.6	831	310.4	2242
59.6	464	159.6	327	313.4	2958	59.5	647	154.4	990	311.6	2201
62.6	248	168.5	765	315.5	1143	62.4	312	155.4	716	313.3	2066
65.2	725	169.5	496	316.8	2905	65.1	926	159.5	822	314.2	2435
68.5	362	170.3	1293	317.9	1822	68.5	509	168.4	1435	315.5	1413
76.3	1724	171.1	2123	321.8	17863	75.2	170	169.4	888	316.8	3515
77.2	318	175.5	550	322.8	9154	76.2	2134	170.3	1962	317.8	2267
78.5	555	176.5	547	323.7	9094	77.2	431	171	2836	321.8	21031
95.2	215	180.5	566	325.1	5079	78.4	743	175.5	945	322.8	11740
95.9	174	182.4	493	326.6	4668	95.1	315	176.5	1166	323.7	11571
96.9	4299	183.4	502	327.5	11134	95.8	298	180.5	1143	325.1	5681
97.7	737	187.7	634	328.9	7903	96.8	5364	182.3	1108	326.6	5352
98.4	208	192	922	329.8	3120	97.6	959	183.4	960	327.5	12848
99.1	275	199.4	928	331.7	5604	98.3	355	187.7	1118	328.9	9756
100.1	7716	201.3	797	333.6	6435	99	396	192	1642	329.8	3585
101.6	213	240.1	332	336.7	3770	100	9496	198.4	1128	331.6	6582
102.3	266	293.3	3793	338.5	941	101.5	342	199.4	1530	333.6	7830
108.4	324	294.2	3872	339.7	888	102.2	434	203.4	1244	336.7	4443
111.1	910	296.1	13510	366.8	112	108.3	582	239.9	988	338.5	1057
112	372	297.7	7224	469.4	585	111	1364	293.2	4475	339.7	1034
115.4	578	300.2	3583			111.9	575	294.1	4787	468.5	974
116.3	338	301.1	280			114.5	684	296	16782	469.4	628
118.4	752	306.4	1368			115.3	1009	297.7	7248	491.7	791
120.7	410	308.5	641			116.2	690	300.1	4438		
						117.3	411	306.3	1801		

*Msp*I digested TRF profiles												
Soil sample: E2(a)						Soil sample: E2(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.9	1437	140.4	644	213.1	226	51.4	639	136.6	1761	197.7	771
62.5	473	141.5	3790	218.5	324	53	393	138.6	4064	199.3	1094
65.2	626	142.5	548	225.6	448	53.9	2005	140.4	825	200.3	1152
67.6	2378	143.2	583	266.3	1348	62.6	610	141.5	5091	205.4	838
68.5	231	144.3	2077	275.7	1862	65.2	874	142.5	698	213.1	399
69.5	975	145.8	1641	276.6	778	67.6	3102	143.2	754	218.5	422
71.1	1176	148	10084	277.6	2204	69.5	1340	144.3	2640	225.6	558
72.1	262	149.8	13235	396.4	481	71.1	1514	145.8	2103	266.4	1905
73.8	2912	151.4	1031	400.1	4922	72.1	362	148	13318	275.8	2541
78.4	1216	152.2	3527	431.6	3341	73.8	3534	149.8	17715	276.7	1015
83.7	1030	153.2	1821	432.8	2730	75.4	242	150.7	2462	277.6	3095
85.1	961	154.9	1694	435.8	4406	78.4	1558	151.4	1364	280.3	144
87.4	5043	156.4	662	451.5	363	83.8	1310	152.3	4705	396.4	802
89.2	818	158.4	7703	452.8	424	85.1	1228	153.3	1272	400.1	7217
90.1	453	160.4	1571	466.3	6257	87.5	6579	154	994	431.7	4815
90.8	452	161.4	1574	471.1	3530	89.3	1063	154.9	2231	432.9	4027
91.7	2209	165	512	482.1	539	90.1	589	156.5	870	435.8	6796
111.3	402	168.1	923	483.4	1185	90.8	580	157.5	1865	451.6	626
117.3	155	178.2	819	484.2	1405	91.7	2905	158.4	10453	452.9	636
122.5	1772	181.4	288	485.9	2293	111.2	538	160.5	2102	466.3	8959
123.8	1135	184.7	182	491.5	1772	115.6	268	161.4	2046	471.1	5367
126.3	1273	188	1449	497.3	1081	117.3	255	165.1	697	482.1	896
127.7	1954	197.7	598			122.4	2757	165.9	587	484.2	4061
135.6	165	199.3	827			123.7	1587	168.2	1218	486	4584
136.6	1374	200.2	841			126.3	1376	178.3	1102	491.6	3010
138.6	3166	205.5	646			127.7	2644	181.5	436	497.3	2045
						130	719	184.7	223		
						135.6	192	188	1886		

*Msp*I digested TRF profiles											
Soil sample: E3(a)						Soil sample: E3(b)					
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.9	1035	138.6	1595	266.3	1739	53	173	138.6	1178	266.4	1345
62.6	497	140.4	653	275.8	5477	53.9	895	140.4	483	275.8	4261
65.2	707	141.5	3679	276.6	1513	62.6	383	141.5	2743	276.7	1103
67.7	1153	142.5	548	277.6	3688	65.2	479	142.5	447	277.7	2830
69.5	976	143.3	634	280.3	82	67.6	859	143.2	493	396.4	501
71.2	1353	144.6	2338	282.2	86	68.5	167	144.4	1808	400.1	2566
72.1	174	145.9	1686	396.4	615	69.4	721	145.8	1305	431.7	1259
73.8	2326	148	9040	400.1	3238	71.1	1014	148	6798	432.9	2045
78.4	990	149.8	9607	431.7	1790	73.8	1741	149.8	7386	435.8	2896
81.5	775	151.5	852	432.9	2731	78.4	723	150.6	1550	436.8	3096
83.8	1070	152.3	2801	435.8	3994	81.4	557	151.4	616	438.7	1606
85.2	706	153.3	1637	438.4	2074	83.7	783	152.3	2224	451.6	420
87.5	4306	154.9	2276	451.5	513	85.1	509	154.9	1787	452.7	211
89.3	635	156.5	722	452.7	283	87.4	3177	156.5	501	466.4	2343
90.1	416	158.4	10922	464.8	2257	89.2	473	157.5	1346	471.1	1555
90.8	324	160.3	2635	466.5	3317	90.8	274	158.5	8809	482.4	404
91.7	1544	161.4	1391	471.2	2182	91.7	1155	160.4	1996	486	2137
94.2	124	165.8	539	483.5	1118	94.2	44	161.5	1022	491.6	2114
111.1	983	168.1	768	484.3	1256	111.1	745	168.3	641		
117.3	246	178.2	687	486	2714	117.3	142	178.3	580		
122.5	2030	181.4	361	491.6	2552	122.5	1608	181.5	321		
123.9	1164	184.8	95			123.8	802	184.8	157		
126.3	1157	188	1159			126.3	871	188	934		
127.6	2715	199.3	712			127.6	1958	199.3	620		
135.6	293	200.2	865			136.6	1193	205.5	455		
136.6	1562	205.4	602			137.7	1083	213.1	172		
137.7	1550	213.1	216								

6.6 Appendix F: Original data of 4RE (*Bst*UI, *Hae*III, *Hinf*I & *Msp*I)-Derived TRF Profiles from Filed Soils Adjacent to Hup⁻ Nodules (F1&F2)

*Bst*UI digested TRF profiles							
Soil sample: F1(a)				Soil sample: F1(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.7	445	203.2	3683	55.4	155	209.9	1150
55.3	546	205.1	2348	57.1	4645	211.6	1525
57	7898	209.9	2229	58.7	2745	214.5	1342
58.6	4828	211.6	3010	60.3	152	221.3	1259
60.3	433	214.3	2532	61.1	79	222.3	1281
61	276	221.3	2206	62.6	1065	223.4	1498
61.7	190	222.3	2244	90.3	2716	224.9	1391
62.6	2008	223.4	2791	93.2	2999	226.2	1777
85.9	655	224.9	3097	94.1	459	227.2	1717
90.3	4739	226.2	3087	95.3	2869	229.1	932
93.1	5415	227.2	3060	99.5	99	233.6	1058
94.1	929	229.1	2016	102	1171	237.9	1515
95.3	5282	233.6	2199	102.9	583	240.2	960
97	379	235.6	4282	104.6	1820	243.6	535
99.6	368	237.9	2844	110.3	231	247.2	1020
101.1	349	240.2	2018	111.2	682	293.3	74
102	2275	243.6	1161	138.7	221	358.1	791
102.9	1165	247.2	2032	159.1	648	361	1150
104.5	3374	280.4	230	192.3	2915	362.4	1214
110.2	518	358	1446	193.3	731	368.7	108
111.2	1445	360.9	3225	196.2	4227	386.2	5066
138.8	625	362.4	2241	197.2	2315	391.5	2631
159.1	952	368.6	387	200.7	783	394.6	2155
165.3	770	386.1	8946	201.7	765	397	1453
192.2	5670	391.4	4654	203.3	1971	402.8	1426
193.3	1439	394.4	3639	205.1	1216		
196.2	8038	396.9	2584				
197.2	4210	402.8	2447				
200.7	1366						
201.7	1677						

*Bst*UI digested TRF profiles							
Soil sample:F2(a)				Soil sample: F2(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
56.9	1389	209.8	637	57	2211	211.6	2804
58.6	1071	211.5	1579	58.6	1658	214.6	1344
60.1	150	214.6	792	60.1	212	222.2	2896
62.5	668	222.2	1675	62.6	1006	223.3	1723
90.2	2377	223.3	1020	90.3	3825	224.9	2176
91.2	552	224.9	1267	91.3	791	226.2	2157
93.1	4438	226.2	1165	93.1	6946	229.1	1059
94.1	562	229.1	599	94.1	936	233.4	1049
95.2	2920	233.4	512	95.2	4628	237.8	1240
97	58	237.8	680	97	164	240.1	643
99.6	217	240	415	99.6	438	243.6	403
101	52	243.5	289	101	161	247.1	576
101.9	1300	247.1	249	101.9	2118	358.1	990
102.9	325	358	597	102.9	629	361	2735
103.7	656	361	1511	103.7	1135	362.9	1760
111.2	197	362.9	824	111.2	438	384.1	3449
159	169	384	1711	158.9	418	386.2	6814
192.3	1321	386.2	3882	192.2	2324	388.2	1984
193.2	401	388.1	953	193.2	652	389.5	3076
195.7	2853	389.4	1677	195.6	4702	391.4	6679
197	1518	391.4	3676	196.9	2529	394.6	2620
201.6	662	394.5	1636	201.6	1096	397	920
203.1	877	396.9	379	203.1	1606	402.9	578
204	776			204	1373		
207.8	250			209.8	1060		

*Hae*III digested TRF profiles							
Soil sample: F1(a)				Soil sample: F1(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.5	123	214.2	921	53.5	51	215.2	595
55.2	537	215.2	1193	55.2	288	216.3	891
60	729	216.3	1743	60	377	217.3	1217
62.4	64	218.4	2436	63.4	914	218.3	1260
63.4	1578	219.3	2015	65.2	188	222.4	643
65.1	383	220.2	1977	70.6	152	224.1	1195
66.2	183	222.4	1269	72.8	271	227.5	1309
70.6	295	224.1	2122	128.6	257	230.3	1101
71.6	412	227.5	2418	131.1	280	232.1	1385
72.7	491	229.4	1162	168.4	293	234.4	794
128.7	416	230.3	1973	182.3	162	236.8	817
131.2	459	232.1	2481	186.2	114	240.8	392
168.4	554	234.4	1442	188.1	979	243.4	241
182.3	413	236.8	1429	192.1	733	250.1	277
186.3	325	240.8	703	193.3	2544	253.8	892
188.1	1935	243.4	400	196.5	1111	258.8	473
192.2	1479	250.2	412	199.5	505	262.1	830
193.4	4316	253.8	1513	202	289	264.5	462
196.5	2059	258.8	697	202.9	564	288.8	342
199.5	911	262.2	1353	205.8	732	291.6	988
201.2	897	264.5	766	209.8	624	293.1	168
202.9	1001	288.8	609	211	426		
205.9	1415	291.7	1844	213.3	421		
207.6	859	293.2	361				
211	785	293.9	456				
213.3	1035	320.8	61				

*Hae*III digested TRF profiles								
Soil sample: F2(a)					Soil sample: F2(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.5	67	216.4	1519		53.4	151	216.3	1842
55.1	388	217.6	2602		55	525	217.6	3458
59.9	549	222.5	394		59.8	637	222.4	591
62.4	68	224.2	2514		63.3	1903	224.1	3350
63.4	1547	227.5	3288		64.8	349	227.5	4364
64.9	270	229.3	1167		65.6	1189	229.3	1426
65.8	926	230.3	977		70.3	359	230.2	1277
70.4	241	232.3	1210		72.7	456	232.3	1690
72.8	316	234.3	843		83.8	487	234.3	926
83.9	370	240.8	408		102.4	275	240.7	467
128.8	307	243.5	215		128.7	360	243.5	271
131.2	290	249.9	354		131.1	322	249.8	348
168.5	327	253.8	551		168.4	429	253.8	643
182.3	181	254.6	459		182.2	276	254.5	621
186.3	114	258.6	1260		186.2	207	256.6	556
188.5	1261	259.7	311		188.4	1670	258.6	1534
192.1	2050	262.2	1083		192.1	2785	259.6	443
193.2	5045	264.5	297		193.2	6415	262.2	1410
195.6	736	288.8	443		195.5	951	264.5	375
196.6	995	291.7	3033		196.6	1325	288.9	645
202.9	536	298.7	413		202.9	733	291.7	4067
205.2	1015	323.8	347		205.1	1359	298.7	565
211	709	325.5	85		209.4	968	323.7	464
215.2	526				210.9	973	325.4	172
					215.2	677		

*Hinf*I digested TRF profiles							
Soil sample: F1(a)				Soil sample:F1(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.7	342	293.3	5739	53.5	228	322.3	5918
55.4	1834	296.2	6410	55.3	1252	327.3	11101
62.9	177	296.9	5616	62.9	91	331.5	2804
65.3	636	297.7	7849	65.3	411	334	3682
68.6	272	304.5	548	97	1336	337.3	3115
96.9	2455	306.6	2377	100	1822	340.9	1827
99	323	311.8	2634	112	1833		
100	3538	314.8	2290	113.6	1686		
100.9	323	316.7	3280	118.1	826		
112	3593	317.8	3196	168.4	806		
113.6	3292	321.2	20366	169.3	1132		
117.3	749	322.4	11658	170.8	768		
118.1	1612	327.6	12757	182.3	684		
155.5	976	328.9	9229	188.3	770		
168.4	2014	331.5	6141	192.2	362		
169.2	2472	334	8167	293.2	2876		
170.7	2403	335.2	3617	296.1	3178		
176.4	1024	337.3	6357	297.6	4375		
182.1	1682	340.8	4145	306.4	945		
187.3	1008	342.8	1766	311.7	1163		
188.2	1919	366.9	342	316.5	1180		
189.2	937	468.6	1605	321.1	9315		
192.1	1214						

*Hinf*I digested TRF profiles							
Soil sample: F2(a)				Soil sample: F2(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.6	481	168.4	1427	53.6	72	188.2	208
55.2	1829	169.2	1538	55.3	898	192.2	143
59	386	171.2	1366	59.1	61	199.5	263
62.6	370	182.1	1314	62.7	78	293.3	2071
65.7	2105	188.2	911	65.8	1091	294.2	842
68.5	585	192.1	874	77.6	684	296.3	4420
77.6	2205	199.5	968	83.9	559	297.6	3174
83.9	1275	293.3	3838	96.9	2239	300.3	82
96.9	4449	296.2	8763	99	173	306.4	449
99	614	297.6	5993	100	4443	311.8	740
100	8278	300.4	297	100.9	254	314.7	1821
100.8	818	303.4	635	101.6	191	316.7	1130
101.6	647	306.5	1062	102.4	450	317.7	1213
102.3	1132	311.8	1599	111.2	421	320.9	5877
108.2	522	314.6	2944	112.1	2387	322.6	7218
109.1	1249	317.8	2499	113.7	779	326.8	2855
111.1	1065	320.9	11769	115.7	216	328.8	2760
112	4917	322.7	13901	117.2	576	331.4	2143
113.6	1952	324.5	6793	118.2	1742	333.9	1039
115.6	740	326.8	5902	120.3	916	336.9	1771
117.1	1492	327.8	5321	155.3	233	340.7	704
118.1	3629	328.8	5987	168.4	531	469.4	896
120.1	2109	331.5	3851	169.2	561		
122.7	1081	333.9	1850	171.3	391		
124.7	2438	336.9	3600	176.3	219		
126.8	1202	340.7	1211	182.1	323		
129	1223	468.5	1369				
136.4	1017	469.6	1767				
155.4	1032						

*Msp*I digested TRF profiles							
Soil sample: F1(a)				Soil sample: F2(b)			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
55.5	940	149.8	2952	53.4	200	146.9	2153
62.8	1054	151.4	222	55.6	964	148	4429
65.3	313	152.3	181	62.9	1169	149.7	3763
67.8	121	154	291	65.3	339	151.4	196
70.9	362	155.5	411	67.8	136	152.2	193
71.9	603	157.1	260	69.5	35	153.9	328
73.3	776	158.1	865	70.9	386	155.4	511
78.4	144	159.9	981	71.9	618	158.1	1232
81.2	583	161.9	912	73.2	834	159	549
87.4	384	168	889	78.4	165	159.9	1231
89.3	334	197.9	753	81.2	642	162.1	1139
91.8	678	199.3	626	87.5	458	168.1	1063
111.1	256	205.6	419	89.4	438	197.1	2036
119	215	213	206	90.9	208	199.2	797
122.5	421	275.7	101	91.8	771	205.6	547
123.4	641	277.3	79	93.6	89	212.9	408
127.6	754	280.7	172	119	273	266.1	35
135.5	707	396.2	221	121.4	409	275.7	132
136.5	813	400.3	365	122.5	617	280.5	250
138.3	1360	432.7	1106	123.4	804	396	231
140.5	400	436.1	1351	127.6	935	400.1	365
144.1	1531	437.3	1124	128.5	846	432.6	1549
145.9	551	453.4	160	135.5	849	436	2092
147	1785	486.1	2895	136.5	1019	437.2	1598
148.1	3445	491.7	618	138.3	1626	453.4	239
				140.6	481	472.7	322
				144.1	1194	485.9	4408
				145.8	672	491.5	1120

*Msp*I digested TRF profiles								
Soil sample: F2(a)					Soil sample: F2(b)			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.6	263	145.8	1280		53.4	113	148.1	5455
55.5	1293	148	7796		55.5	828	149.7	4065
62.8	1103	149.7	6025		62.8	708	151.4	287
65.8	1587	150.6	882		65.8	1074	152.2	256
67.8	294	151.4	508		67.7	121	154.1	433
69.5	162	152.2	443		70.8	327	155.5	305
70.8	581	154	802		71.9	523	158.1	836
71.9	814	155.5	703		73.5	605	159.9	1139
73.5	940	157.1	418		78.6	315	161.7	953
74.7	463	158.1	1293		81.3	1122	168	535
78.7	474	159.9	1713		84	728	197.8	520
81.3	1643	161.7	1523		85.5	286	199.3	242
83	399	168	848		87.4	646	205.6	266
84	1088	177.9	289		89.3	372	213	151
85.5	582	185.2	111		91.8	608	275.7	61
87.4	998	188.2	121		109.1	304	396.3	394
89.3	591	197.9	1292		111.1	255	400.3	937
91.8	877	199.3	730		119.1	205	432.7	1714
102.5	447	205.6	908		122.6	503	436	1895
109.1	497	213.1	250		123.5	730	438.3	789
111.1	420	280.5	418		126.4	514	486.1	2979
119.1	365	282.1	267		127.6	747	491.3	1742
121.4	608	396.2	600		135.5	585		
123.4	1975	400.3	1481		136.5	1000		
126.4	700	432.7	2426		138.4	1185		
127.5	1239	436	2919		140.4	489		
135.5	900	438.3	1132		144.2	1358		
136.5	1434	486.1	4521					
138.3	1760	489.2	1655					
140.3	468	491.3	2833					
144.1	2144							

6.7 Appendix G: Original data of 4RE (*Bst*UI, *Hae*III, *Hinf*I & *Msp*I)-Derived TRF Profiles from Complex Samples (A2J&A6J, D2J&D4J)

*Bst*UI digested TRF profiles								
Complex sample: A2J					Complex sample: A6J			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.5	890	214.5	4591		56.9	784	220.6	3750
55	1745	220.7	4566		62.5	13728	222.3	44370
57.1	1833	222.4	73968		90.2	4440	224.2	2796
58.9	2234	224.3	5222		91.6	1052	227.1	1425
62.7	25341	227.3	3986		93	11643	234.5	1150
90.2	11884	229	2236		95.1	8617	358.2	1091
93.1	21969	234.6	2873		96.6	1969	360.8	3877
95.3	11286	247.4	1359		102	13961	363	3001
97	1160	358.1	2291		103.9	1009	384.4	13413
102.1	23920	360.8	5723		110.4	600	387.9	1555
103.9	1632	362.4	3822		158.6	1139	390.7	3879
110.5	5355	369	836		165.5	957	392.7	1544
142.6	1195	375.7	1256		174.4	767	394.7	1428
158.6	1083	377.2	1343		190.9	978	396	1086
165.5	1480	378.3	1971		192.2	2965		
174.6	1196	384.4	23361		193.3	2259		
191	1433	387.8	5751		195.2	8037		
192.3	6348	390.7	8097		196.8	2900		
195.3	10684	394.6	2521		198.6	1449		
196.9	5708	396.1	2376		200.6	946		
198.7	3566	470	484		201.9	2123		
200.6	1772				203	1764		
201.9	6849				204.3	3638		
203	3115				207.4	1122		
204.3	6223				210.5	1539		
206	1327				212.8	1817		
207.5	1851				214.4	2165		
210.5	2284							
212.8	4688							

*Bst*UI digested TRF profiles							
Complex sample: D2J				Complex sample: D4J			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
52	410	222.5	30655	56.6	1091	293.6	3574
56.5	1464	227	993	58.5	3554	355.9	668
58.4	442	233.7	1392	62.6	13943	357.9	1257
59.8	475	238.3	942	89.9	5780	360.5	4240
62.5	7560	247.2	558	93.1	8700	362.8	2113
70	264	280.7	4913	95.3	4064	364.5	1004
90.1	5128	293.6	1624	102	14359	368.9	21589
91.8	616	355.9	1311	104	849	375.4	1047
93.1	10324	357	591	109.6	3851	376.9	1235
95.3	3858	358.1	793	111.9	699	384.2	11176
102.1	8184	360.9	3053	125.1	813	386.3	1977
104	671	362.8	1205	165.3	1096	388.1	7373
111.9	547	364.6	1086	174.6	1512	390.8	4571
158.7	614	369.1	8156	191.7	2340	392.1	22260
165.4	1205	375.5	655	193.5	1556	394.3	3836
174.5	1329	384.4	6110	195.2	4276	395.5	1672
191.6	2417	386.5	1370	197.1	2884	398.7	867
193.2	1239	388.2	5654	201	2374	460	2655
195.2	4893	390.8	2589	204.1	2345	461.6	1202
197.1	3073	392.3	12766	211.8	3220		
199	1096	394.5	2391	213.2	2681		
201.1	1171	395.9	3634	222.6	54035		
203.1	896	400.5	737	226.7	1061		
204.2	1510	458.8	428	233.8	2936		
206.1	638	460.2	442	238.4	1119		
210.1	839			280.5	849		
211.8	3051						
213.1	820						
214.3	854						

*Hae*III digested TRF profiles							
Complex sample: A2J				Complex sample: A6J			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.6	1894	218.7	4576	55.3	828	220.7	1940
55.1	3458	220.8	2217	60.2	11137	222.4	1546
58.3	994	222.5	2708	63.2	45365	224.1	6420
60.3	11379	224.2	13856	65.2	1766	227.3	9402
63.3	55171	226.2	1748	69.6	865	230.4	3033
65.3	3090	227.5	11172	72.5	21334	234.2	872
66.4	1862	230.6	6519	97	1075	243.5	1254
72.6	23998	231.9	2793	128.6	1900	250.1	642
80.8	1591	234.3	1448	131.4	1328	255.5	1185
97.2	1023	239.3	1150	141.5	1401	258.2	3226
99.6	1775	243.6	2578	144.6	2862	259.8	1527
117.2	899	250.3	1320	167.5	1406	262	2098
120.3	932	251.9	1425	170.1	982	263.3	719
128.7	2375	253.6	970	187.1	1304	285.4	924
131.4	1329	256	1468	188.4	4403	288.8	1130
141.6	1823	258.4	5991	192	3585	291.5	13390
144.7	3343	259.9	2852	193.1	12258	293.6	5705
167.7	1743	262.2	3915	196.9	5023	301.9	1129
170.1	1134	263.4	1171	199.3	1821	324.2	836
176.6	1104	264.6	1483	201.7	1674	378.1	863
187.2	1727	285.4	1618	202.8	1131		
188.6	6020	288.9	2326	204.9	2310		
193.2	11460	291.6	21311	206.4	1004		
195.9	3368	293.2	3219	208.3	1682		
197	5910	294.3	1662	209.4	1294		
199.5	3040	302	1225	213.7	795		
201.7	1780	317.5	899	214.9	833		
202.8	1788	324.4	2107	217.4	37027		
204.9	3347	329.5	953	218.6	6748		
206.5	1289	343.7	783				
210.2	8834	378.2	2529				
217.5	44872						

*Hae*III digested TRF profiles							
Complex sample: D2J				Complex sample:D4J			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
50.1	1159	213.9	896	53.9	744	220.8	3306
53.9	2226	217.4	34883	59.8	1012	222.6	1686
58	908	218.7	8161	63.1	31157	224	7238
59.9	4024	220.8	6517	65.2	1216	228.2	2256
63.1	54490	222.6	3189	70.3	883	230	976
65.2	3216	224	13772	72.6	13825	238.1	463
70.3	2190	228.2	3187	141.5	1679	239.9	509
72.6	29691	230	2279	144.5	2837	243.6	1460
78.5	789	232.9	1013	167.6	17822	255.9	610
128.8	1621	234.4	1369	176.7	457	258.8	4508
131	1812	238.1	1354	182.1	419	260	16275
138	936	241.4	2474	187.2	1482	262.5	2566
141.6	3291	243.6	4838	192	3213	264.8	762
144.6	6173	255.9	1817	193.2	6160	285.4	444
150.5	1047	258.8	6742	194.6	954	288.8	1001
159.9	1089	260	26853	196	1861	291.6	8582
167.7	13378	262.5	5250	199.2	1778	293.2	1737
176.6	1425	264.9	6748	201.7	8114	321	4691
182.2	946	285.5	1462	205.2	2765	324	813
187.2	5527	288	2654	208.4	509	325.5	1274
192.1	4066	291.6	24829	209.5	11180	391.9	748
193.2	9660	293.2	1725	211.1	858		
196	6230	321	10338	216.3	1413		
199.2	3629	324	1552	217.4	20451		
201.6	16922	325.5	2160	218.8	3380		
205.2	6022						
206.3	1756						
209.5	2242						
211	2231						

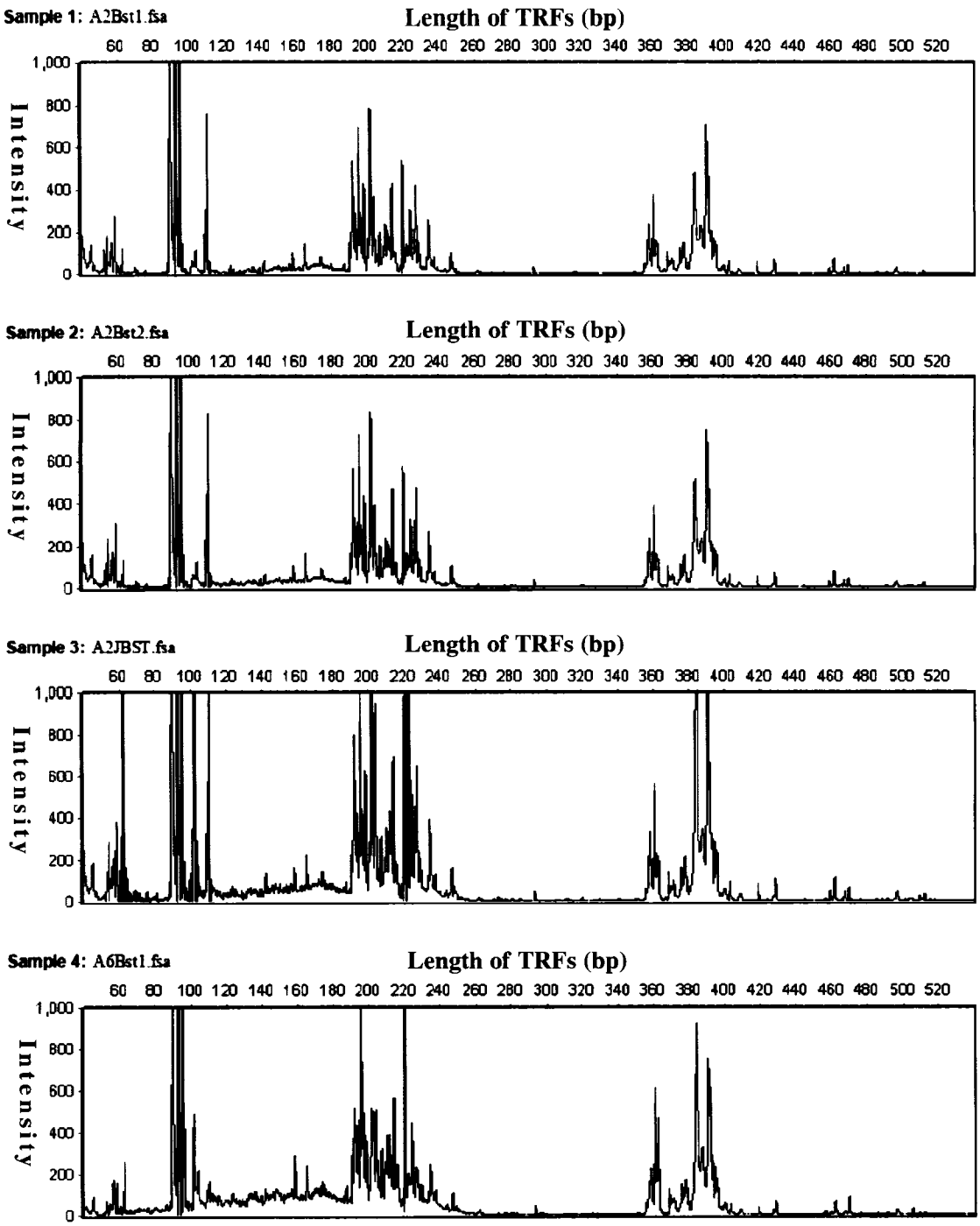
*Hinf*I digested TRF profiles								
Complex sample: A2J					Complex sample: A6J			
Size (bp)	Area	Size (bp)	Area		Size (bp)	Area	Size (bp)	Area
53.4	1410	296	14173		58.5	869	318.2	2964
54.8	3040	297.7	7338		97	3698	320.8	66220
58.9	941	298.8	3277		100	12062	321.9	56252
62.9	2342	305.1	1256		102.7	828	323.8	3064
80.8	1245	306.4	1366		115.3	1505	325.1	3229
97	6110	313.7	4565		117.1	1101	327.5	5810
100	13097	315	4169		118.2	1208	328.8	4892
112.3	938	320.9	59616		122.8	934	331.4	1954
115.5	874	322	59795		168.3	1104	333.3	2194
117.3	1253	323.9	2796		171.5	1145	337.7	919
120.2	1259	325.2	4838		176.2	1493	405.7	975
122.9	1648	327.6	8256		177.5	903	464.2	1205
125.3	1467	328.9	8353		188	898	469.5	2922
155.2	2069	331.5	2585		293.3	5802		
163.6	803	333.3	4539		296.1	12467		
169.3	1051	337.6	978		297.6	7356		
171.5	1078	405.6	958		305	1052		
176.3	1400	464.1	2001		306.2	1009		
177.5	1143	469.5	1701		311.7	1281		
188	906				312.8	2026		
293.4	5622				314.2	3209		
					315.5	2045		

*Hinf*I digested TRF profiles							
Complex sample: D2J				Complex sample: D4J			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.9	1601	313.6	2596	54.1	938	315.3	507
65.2	1088	315.4	787	65.2	516	316.6	761
68.6	557	316.5	1819	76.3	715	317.7	870
76.3	724	317.7	1287	79.1	567	320.8	27726
77.4	614	320.8	41150	96.8	3361	322	30473
79	633	322	44417	100	3141	324.6	6260
96.8	4073	324.5	9748	111.1	680	327.5	5627
100	6632	327.5	6491	166.8	588	329	17422
111.1	729	328.9	20108	168.7	478	331.5	2980
120	820	331.3	3670	176.9	799	333.7	2762
166.8	735	333.6	11327	183	674	337.1	1372
171.1	972	337	1822	192.4	707	339.7	469
176.7	899	338.5	719	199.1	1063	395.3	1686
192.4	816	395.3	583	201	1130	396.9	820
199.1	1668	433.8	975	214.3	483	433.9	742
201	1191	468.4	1276	240.2	1026	469.4	794
240.1	1191			285.1	554		
291.3	737			293.3	3083		
293.2	3557			296	9395		
295.9	15176			297.7	4421		
297.6	5798			299.3	1059		
300.1	1874			304	1003		
305	12889			305.1	15729		
311.6	1442			312.6	1128		
				313.7	8750		

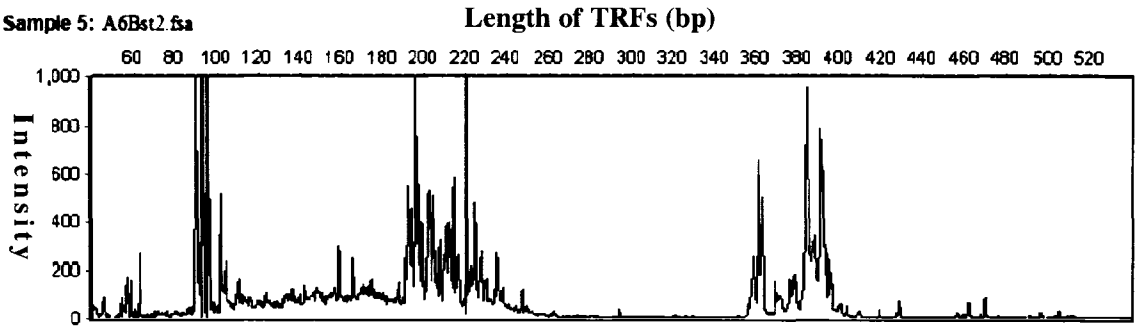
*Msp*I digested TRF profiles							
Complex sample: A2J				Complex sample: A6J			
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area
53.7	248	168.1	88	67.8	939	275.7	59349
55.3	563	178.2	125	71.3	858	282.4	666
62.9	567	184	118	73.5	5859	291.9	2301
67.9	141	188.2	201	81.4	21913	396.3	1457
71.3	163	205.4	138	87.2	2207	400.2	7357
73.6	812	223.4	274	91.7	1209	432.8	4767
78.3	101	275.7	6488	93.9	2232	435.9	7898
81.4	2384	277.5	140	122.7	1597	438.4	3050
87.1	523	280.9	190	123.9	1407	464.7	9401
89.5	252	282.5	212	126.6	1386	467	2896
91.6	364	291.9	245	127.9	5876	471.5	1531
93.8	403	396.4	519	134.8	1274	472.7	908
99.5	275	400.4	1382	136.6	1500	484.2	37453
117.2	116	432.9	1182	138.5	3011	490.3	2117
122.5	341	435.8	1231	141.5	1989	491.4	2122
126.7	109	437.4	901	144.1	1847	497.2	2309
127.9	616	454.3	512	145.8	2479		
136.5	179	472.7	238	148	14104		
138.5	923	484.3	4423	149.7	10792		
141.5	255	491.7	578	151.4	2502		
144.2	264			156.2	1711		
145.8	288			158.6	2152		
148.1	2233			160	1686		
149.8	1422			161.3	1710		
156.3	116			162.4	1065		
161.4	335			168	1042		
				188	1119		

*Msp*I digested TRF profiles								
Complex sample: D2J				Complex sample: D4J				
Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	Size (bp)	Area	
53.9	2930	157.5	2388	54	769	160.3	530	
60.8	645	158.6	8089	62.5	5452	161.4	254	
65.2	1873	161.4	1526	65.1	514	184.1	7161	
67.6	615	181.4	642	71.1	1321	213.1	549	
71	2457	184.2	12637	73.8	784	222.5	21653	
73.8	2154	212.8	10589	78.4	277	266.3	1247	
78.4	884	266.4	2508	81.4	5334	275.9	17970	
81.5	13699	275.8	46588	83.7	364	277.7	1336	
83.8	1093	277.7	4204	85.1	1789	292	366	
85.1	5594	282.5	823	87.5	2249	384.2	2993	
87.5	4554	292	828	89.2	6522	400	873	
89.2	16095	396.4	915	91.7	1248	432.9	1781	
91.8	4053	400.1	5903	102.1	5970	435.2	2542	
111.1	1392	431.6	1382	111	529	437.2	2644	
112.6	746	432.9	4298	119.5	502	438.3	761	
122.4	2760	435.2	5426	122.4	970	451.5	347	
127.5	2864	437.3	3593	127.5	956	453.1	3875	
136.5	1491	451.6	1014	136.5	335	464.7	2934	
138.4	2888	453	667	138.4	607	466.5	1546	
141.4	5999	464.7	7216	141.5	1920	471.2	318	
143.2	12513	466.4	4308	143.2	3969	475.7	2752	
145.9	3113	471.1	1620	145.9	731	484.2	9904	
148	14619	475.7	3216	148	3542	485.9	680	
149.8	7078	484.2	24897	149.8	2187	491.5	886	
152.5	2472	491.5	2070	152.6	1419			
153.9	3289	495.2	4016	153.9	2744			
155	2820			156.4	417			
				158.4	1976			

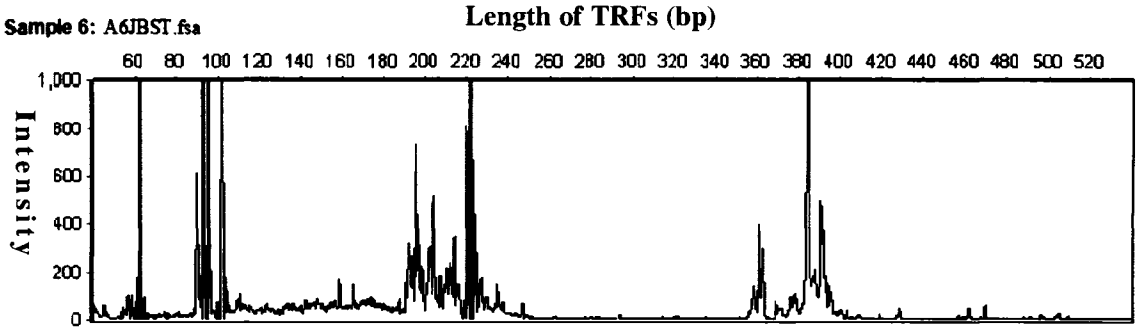
6.8 Electropherograms of *Bst*UI-Derived TRF Profiles from Soil samples (A2&A6; B1&B2; C1, C2&C3; D2&D4; E2&E3; F1&F2; A2J&A6J; D2J&D4J)



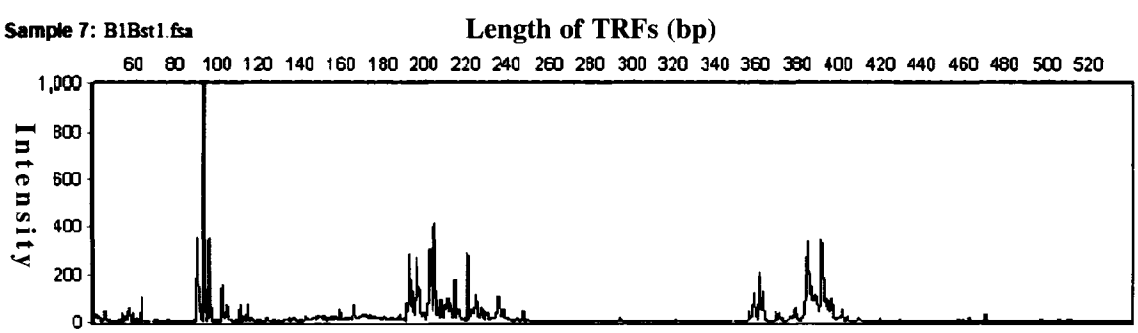
Sample 5: A6Bst2.fsa



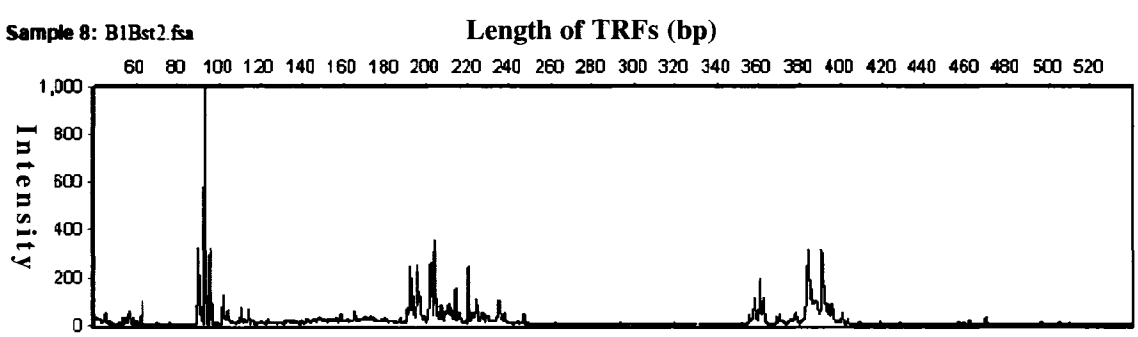
Sample 6: A6JBST.fsa



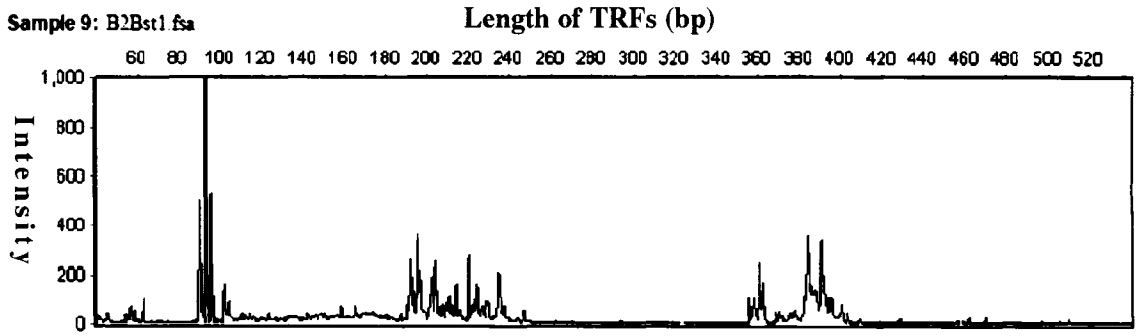
Sample 7: B1Bst1.fsa



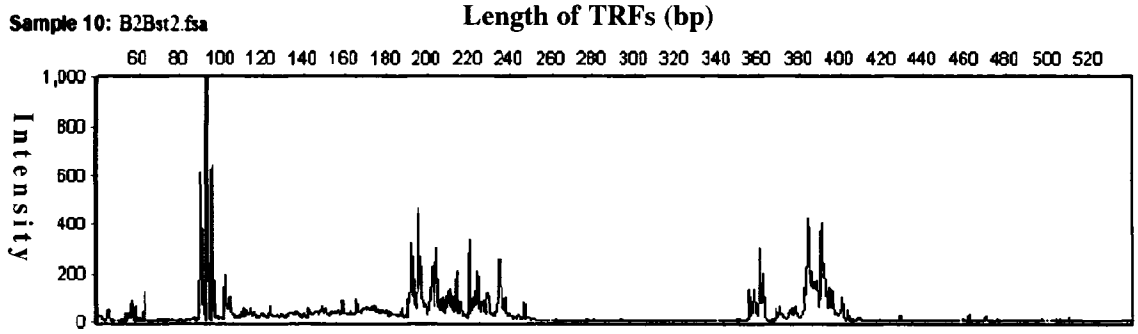
Sample 8: B1Bst2.fsa



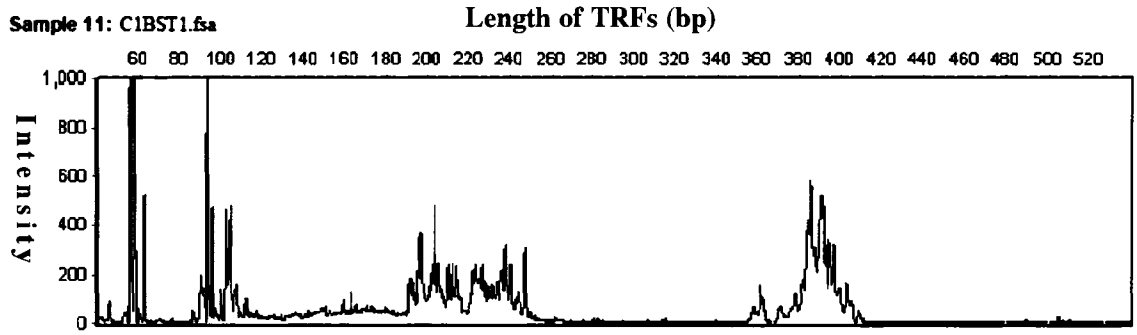
Sample 9: B2Bst1.fsa



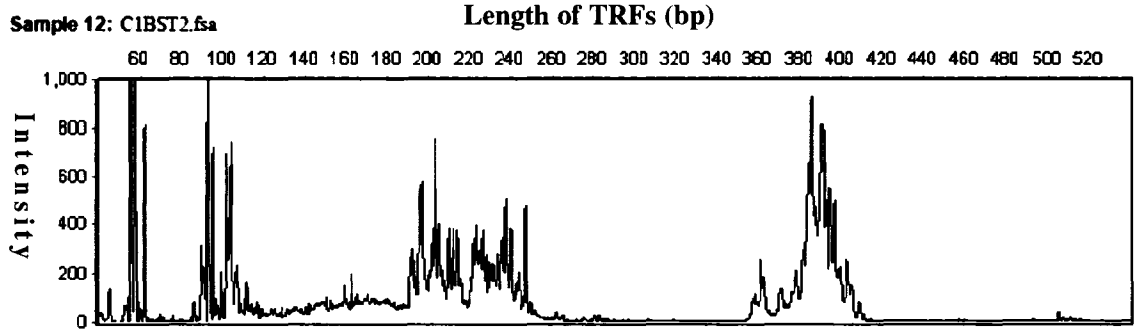
Sample 10: B2Bst2.fsa

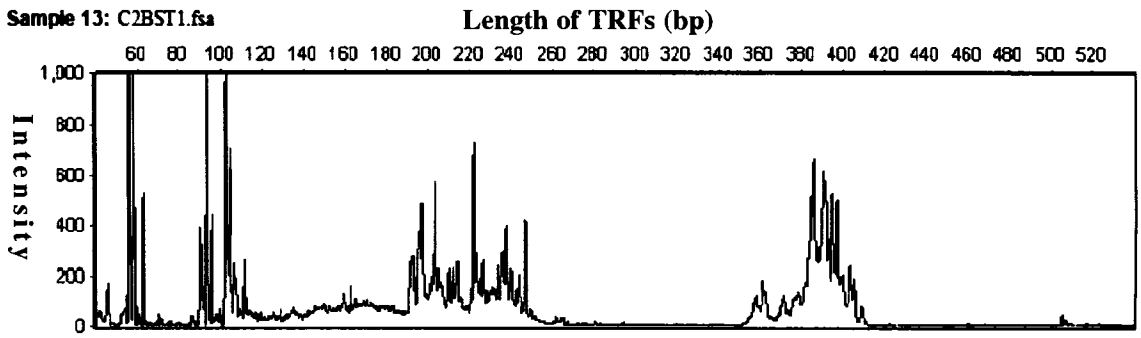


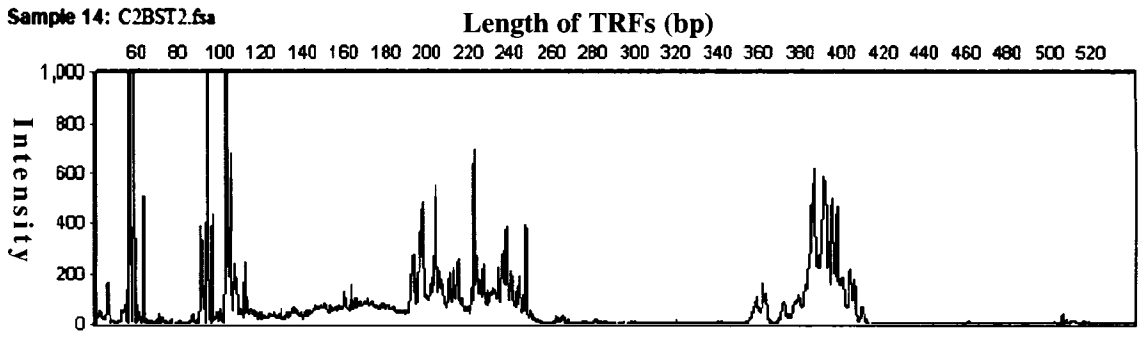
Sample 11: C1BST1.fsa

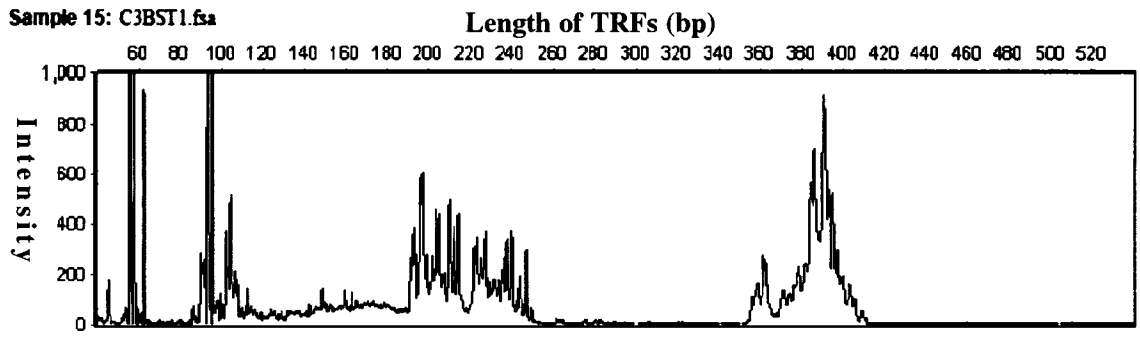


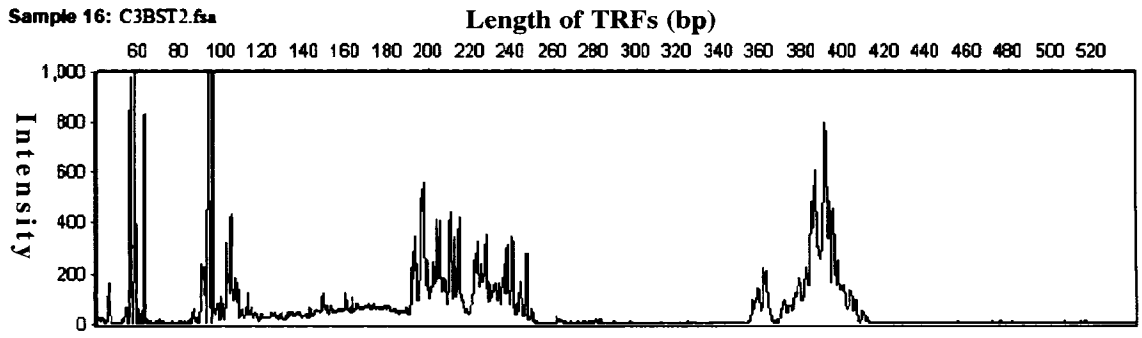
Sample 12: C1BST2.fsa



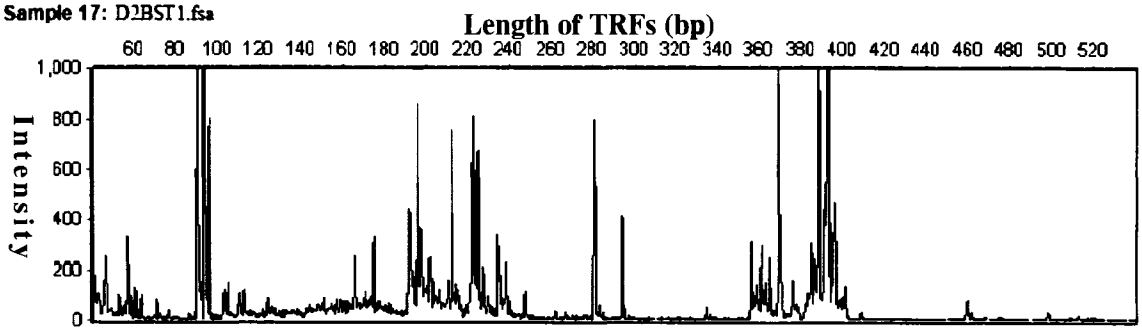




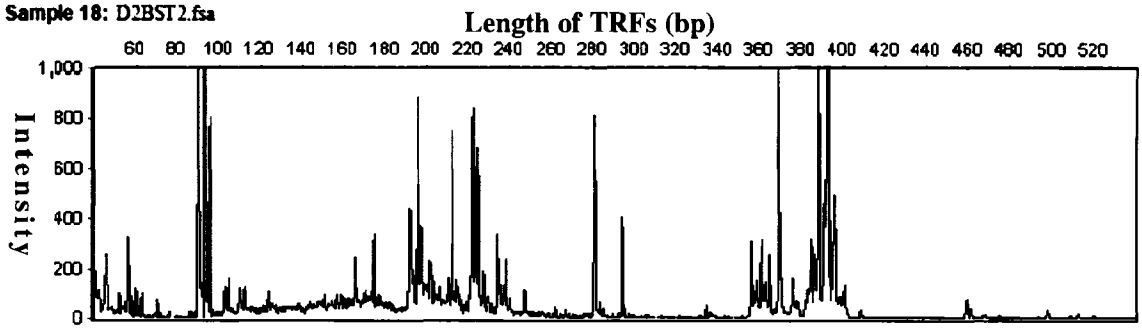




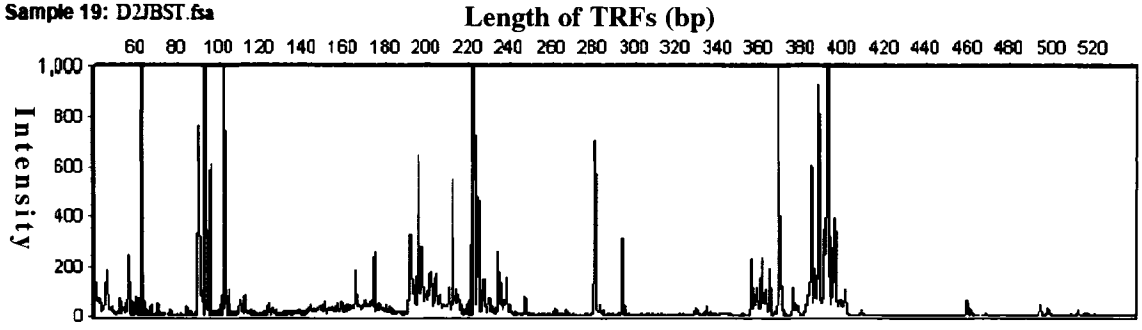
Sample 17: D2BST1.fsa



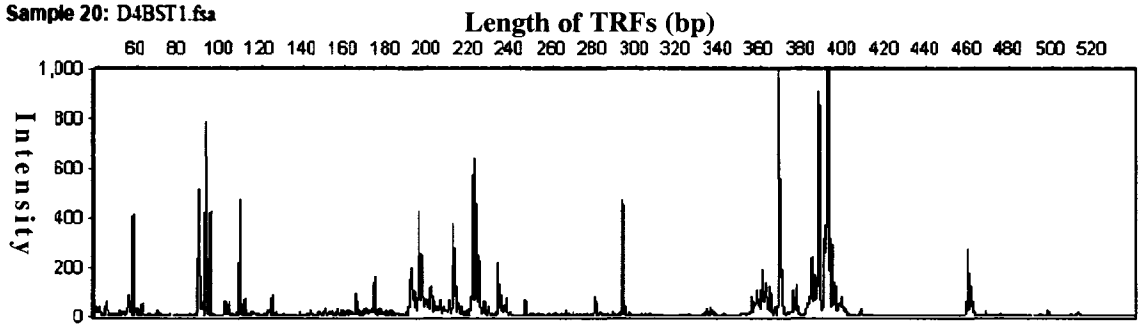
Sample 18: D2BST2.fsa



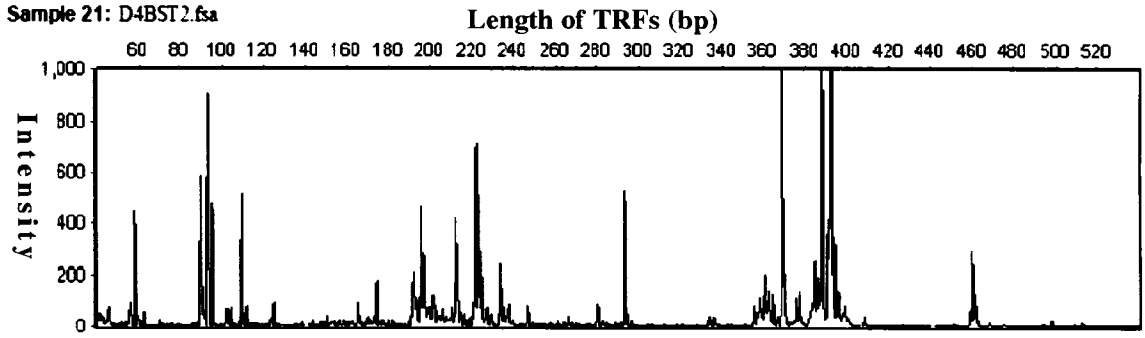
Sample 19: D2JBST.fsa



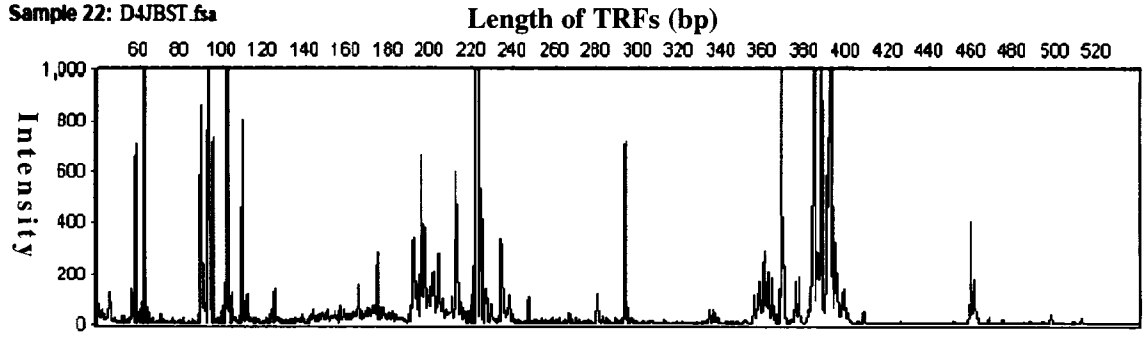
Sample 20: D4BST1.fsa



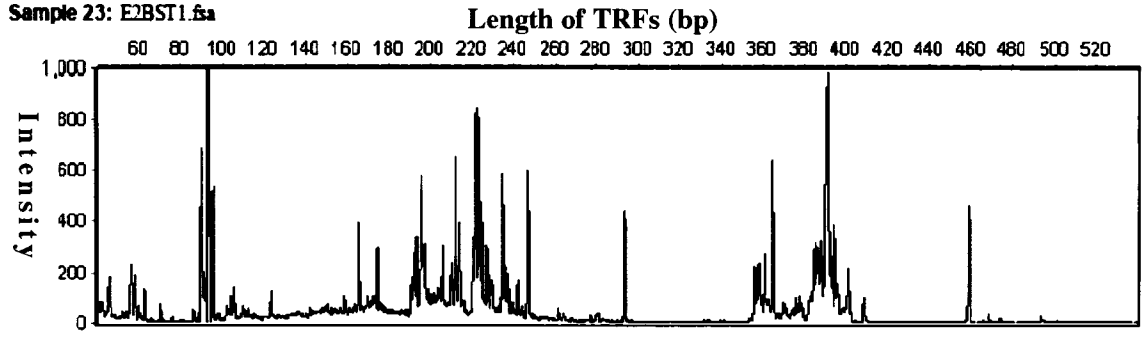
Sample 21: D4BST2.fsa



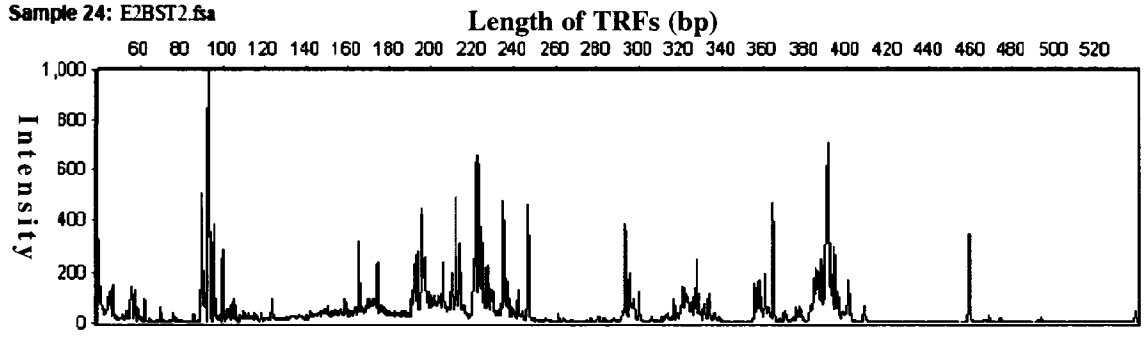
Sample 22: D4JBST.fsa



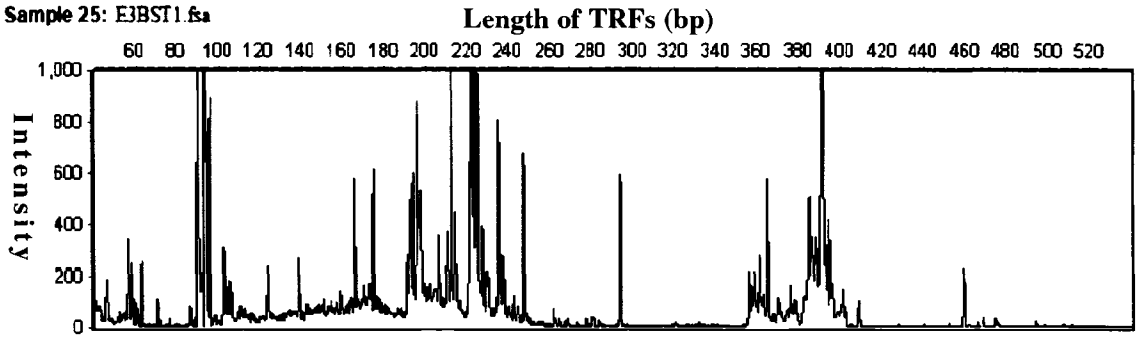
Sample 23: E2BST1.fsa



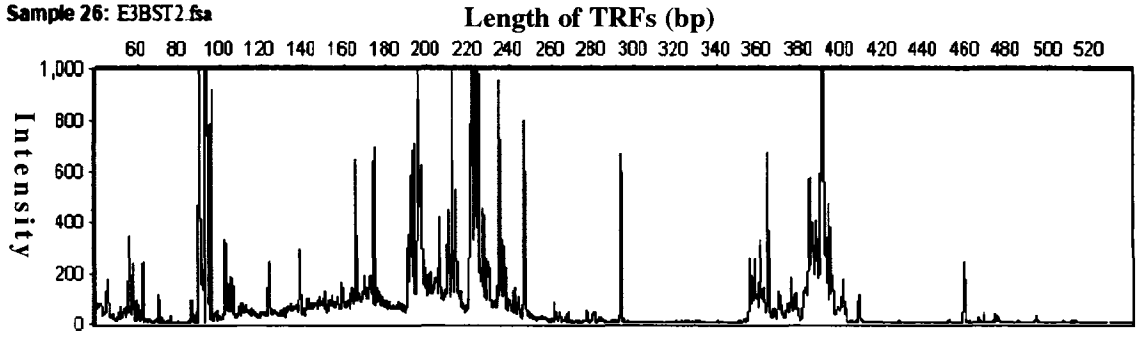
Sample 24: E2BST2.fsa



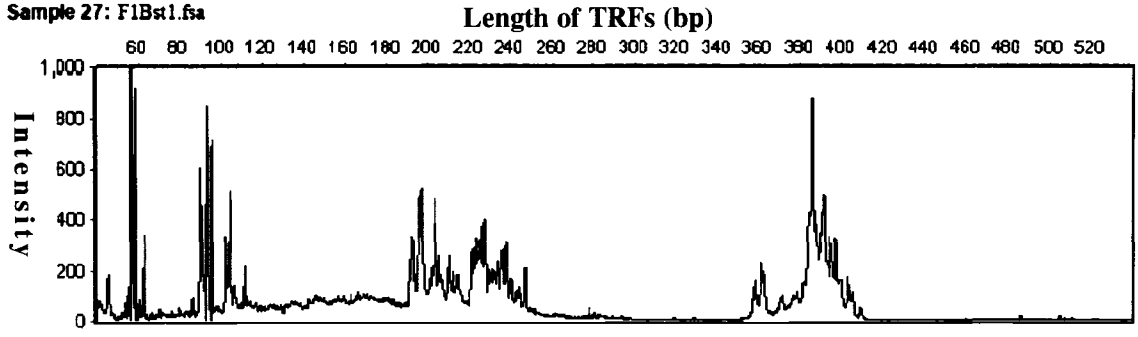
Sample 25: E3BST1.fsa



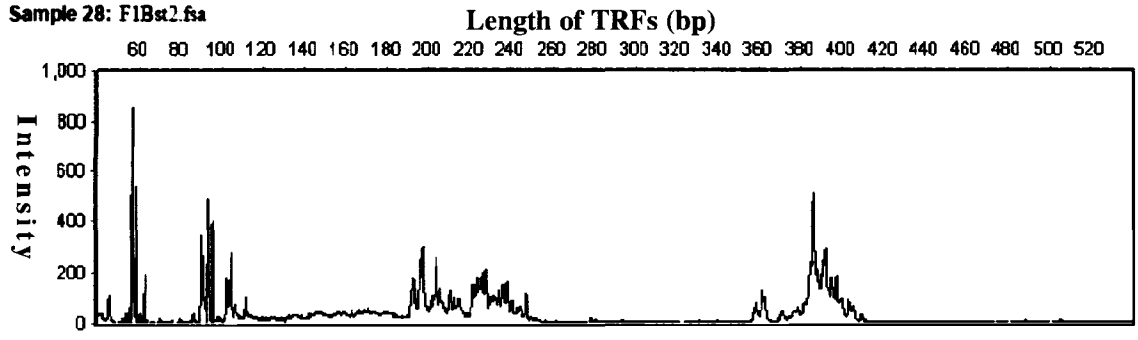
Sample 26: E3BST2.fsa



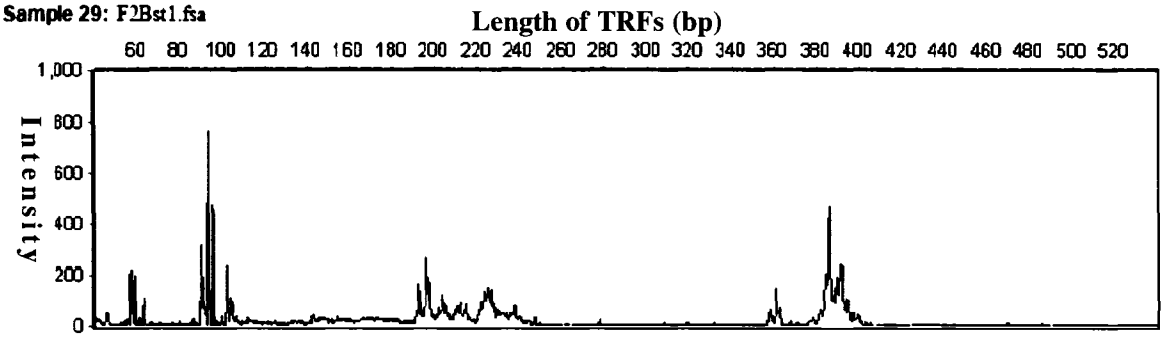
Sample 27: F1Bst1.fsa



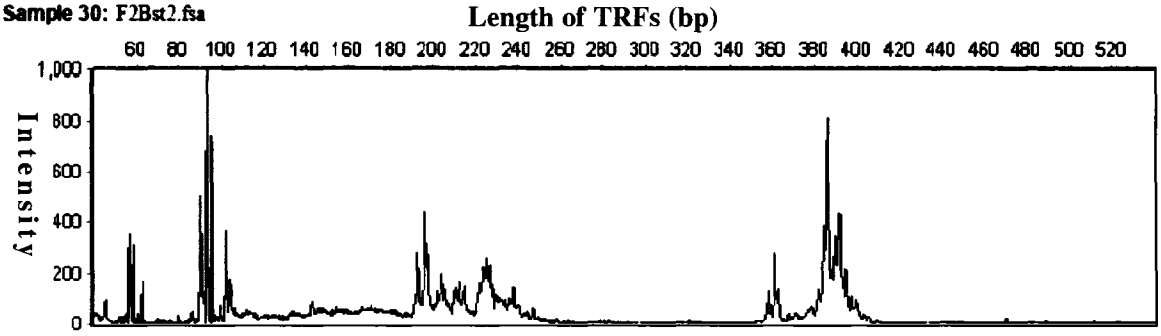
Sample 28: F1Bst2.fsa



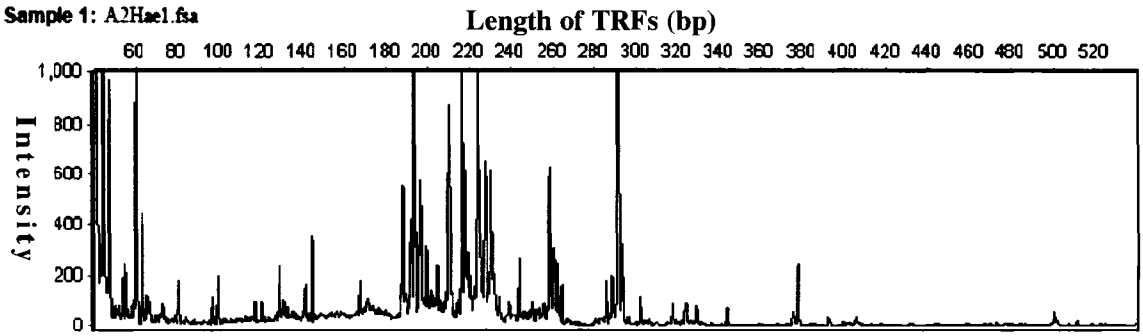
Sample 29: F2Bst1.fsa

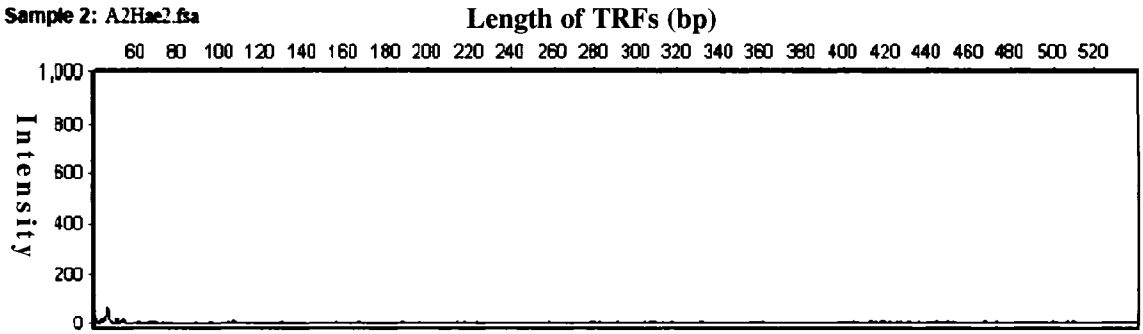


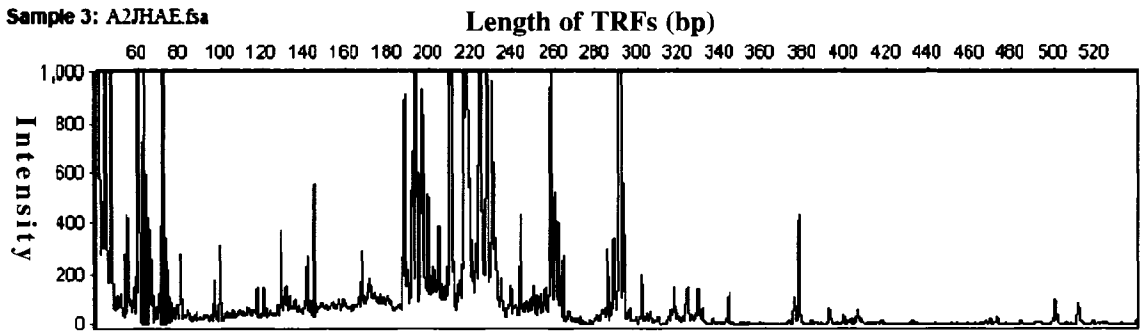
Sample 30: F2Bst2.fsa

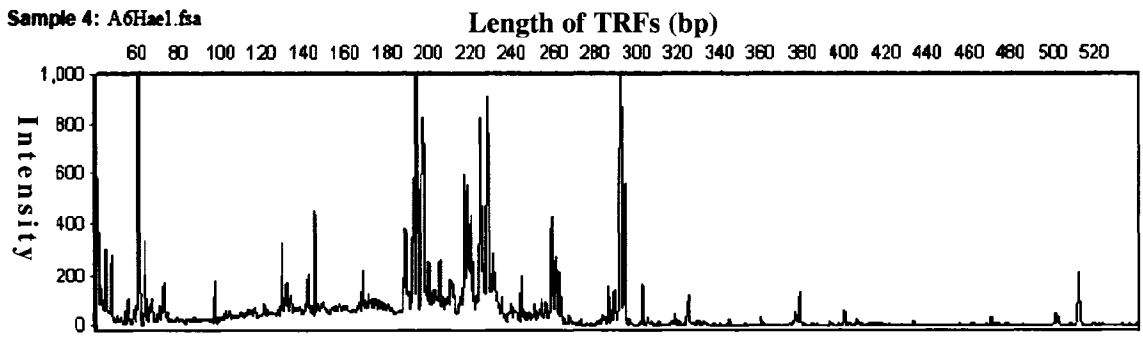


6.9 Electropherograms of *Hae*III-Derived TRF Profiles from Soil samples (A2&A6; B1&B2; C1, C2&C3; D2&D4; E2&E3; F1&F2; A2J&A6J; D2J&D4J)

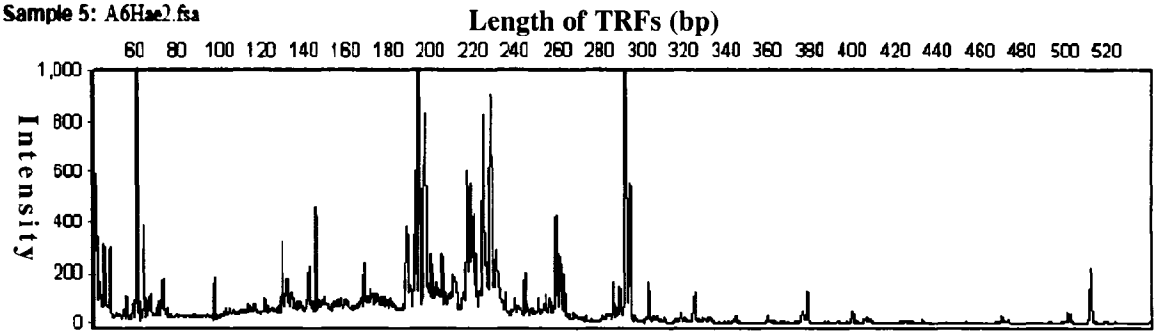




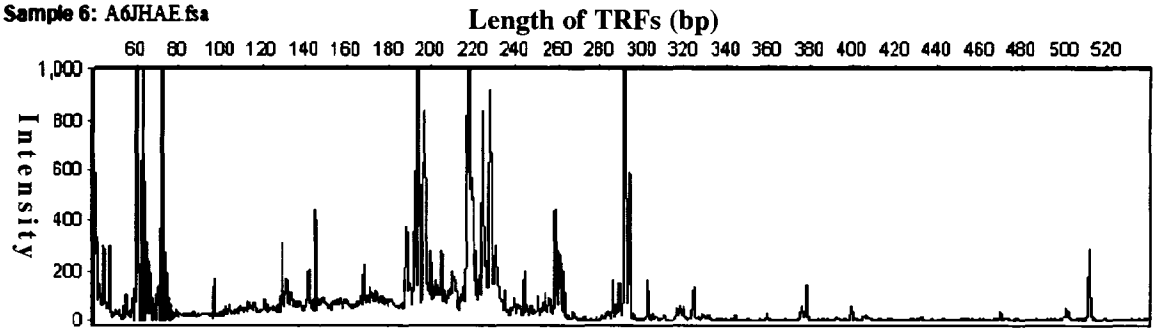




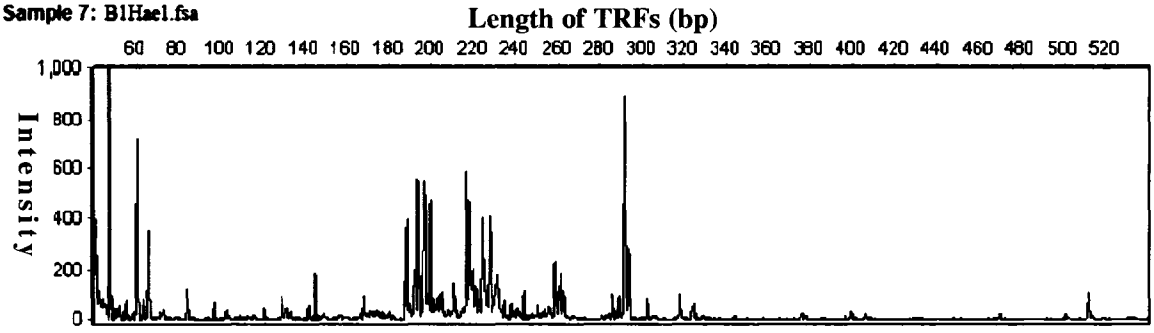
Sample 5: A6Hae2.fsa



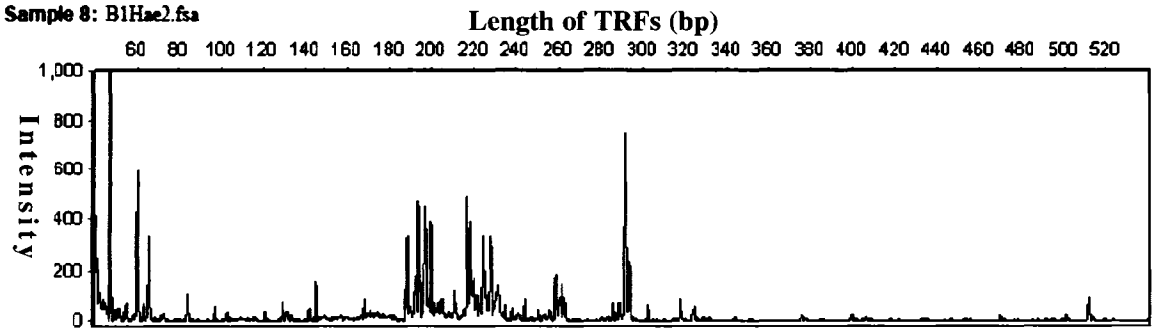
Sample 6: A6JHAE.fsa



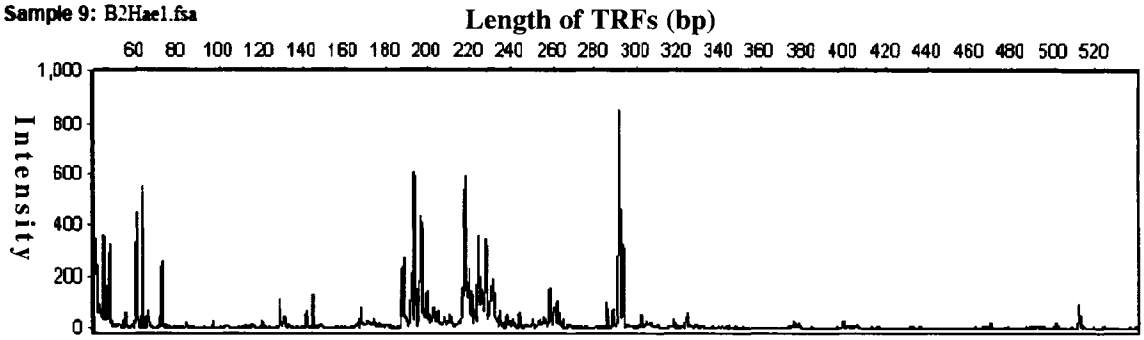
Sample 7: B1Hae1.fsa



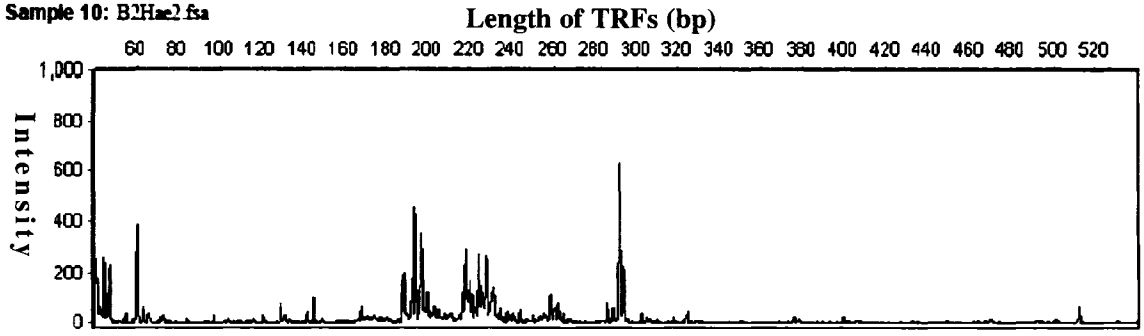
Sample 8: B1Hae2.fsa



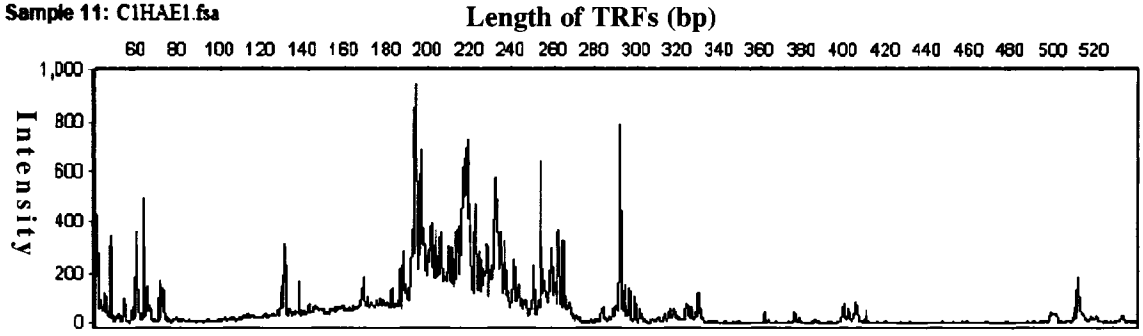
Sample 9: B2Hae1.fsa



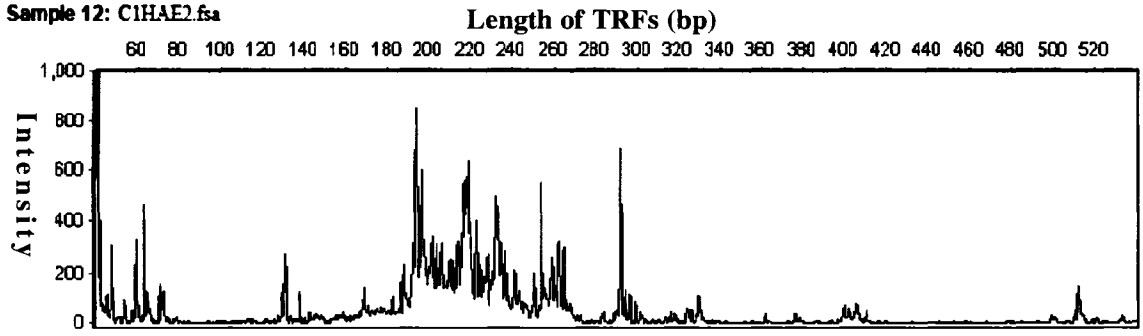
Sample 10: B2Hae2.fsa



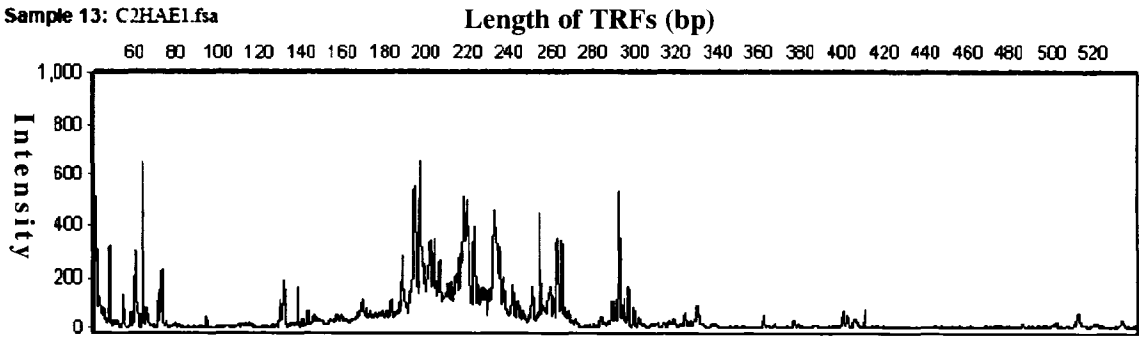
Sample 11: C1HAE1.fsa



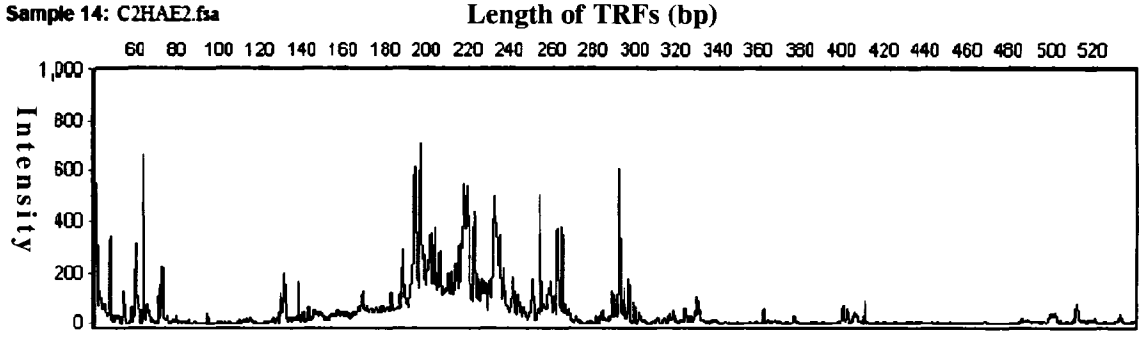
Sample 12: C1HAE2.fsa



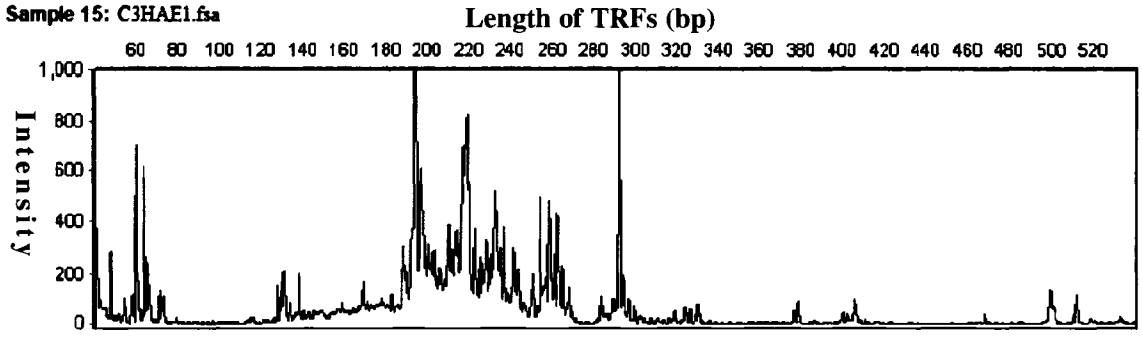
Sample 13: C2HAE1.fsa



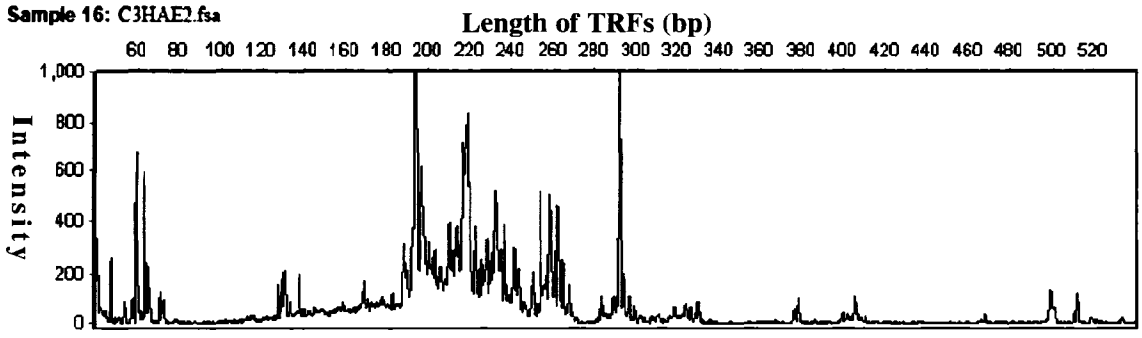
Sample 14: C2HAE2.fsa



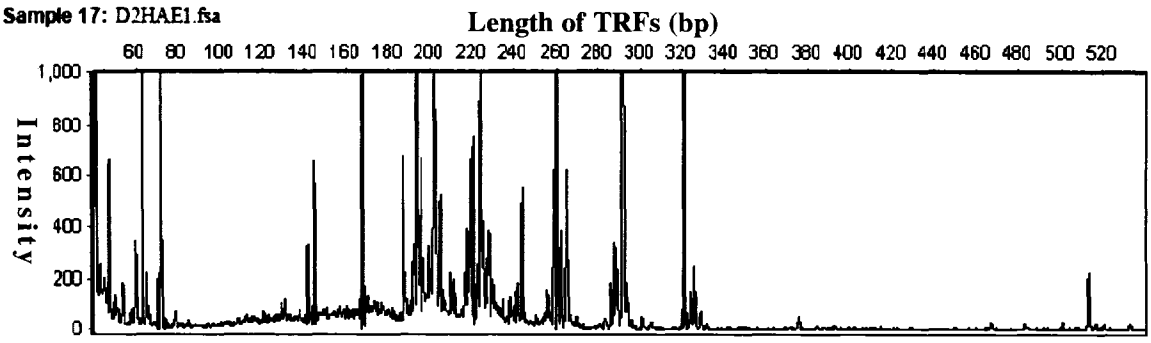
Sample 15: C3HAE1.fsa



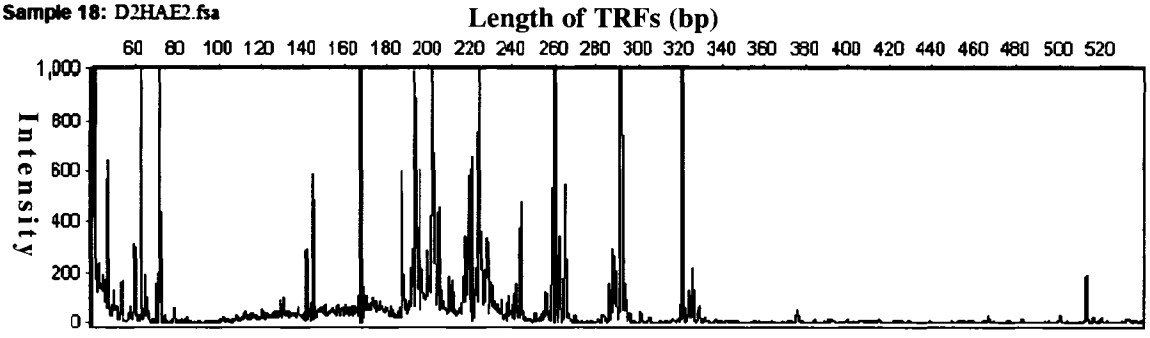
Sample 16: C3HAE2.fsa



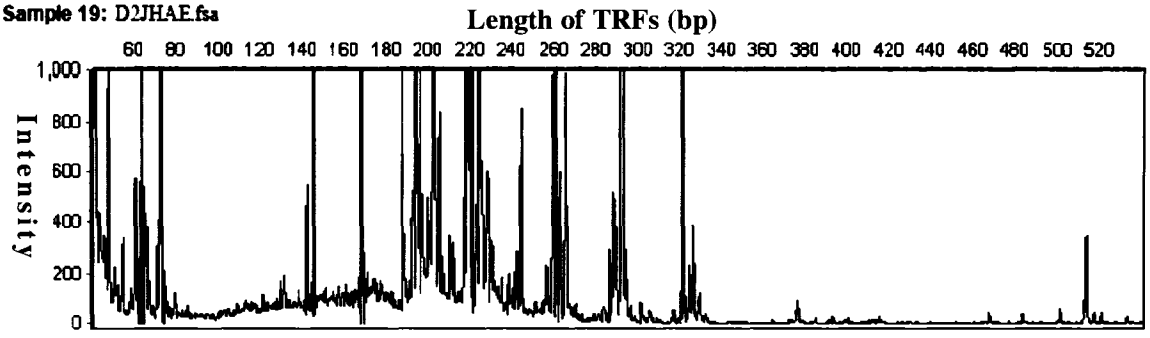
Sample 17: D2HAE1.fsa



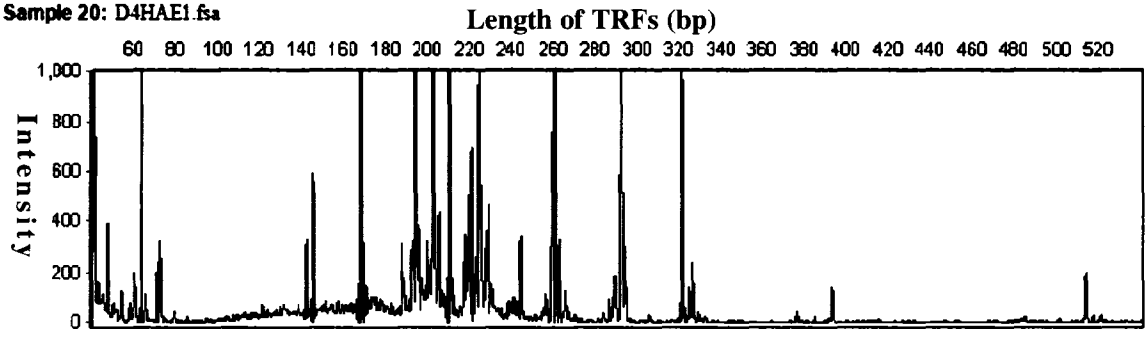
Sample 18: D2HAE2.fsa



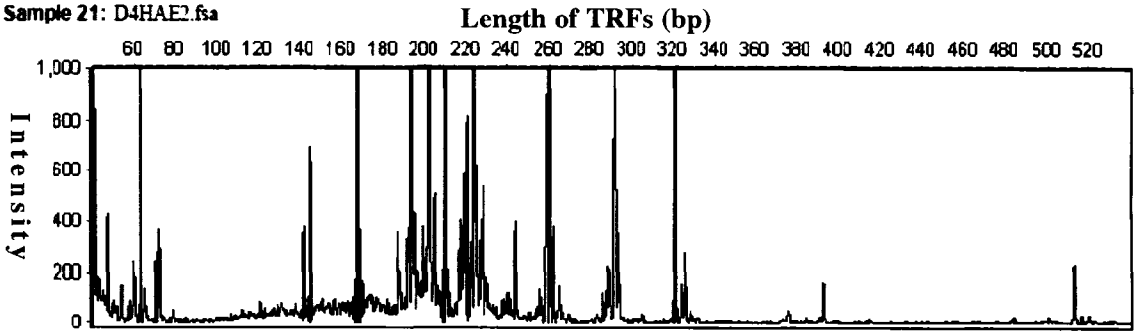
Sample 19: D2JHAE.fsa



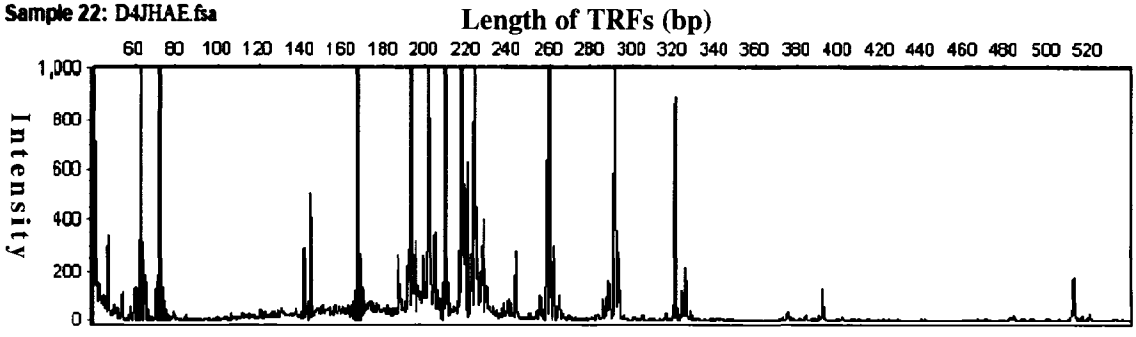
Sample 20: D4HAE1.fsa



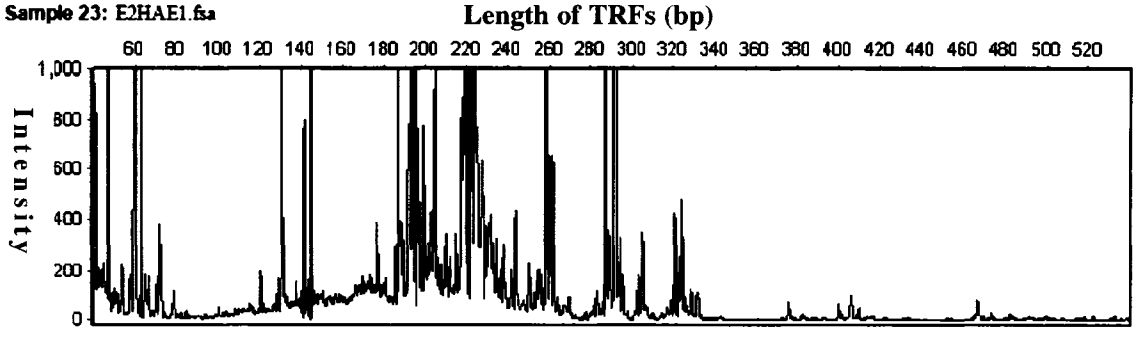
Sample 21: D4HAE2.fsa



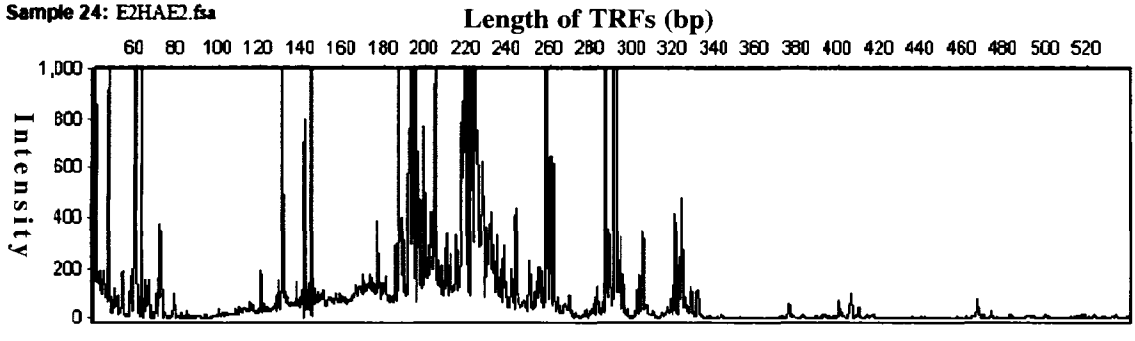
Sample 22: D4JHAE.fsa



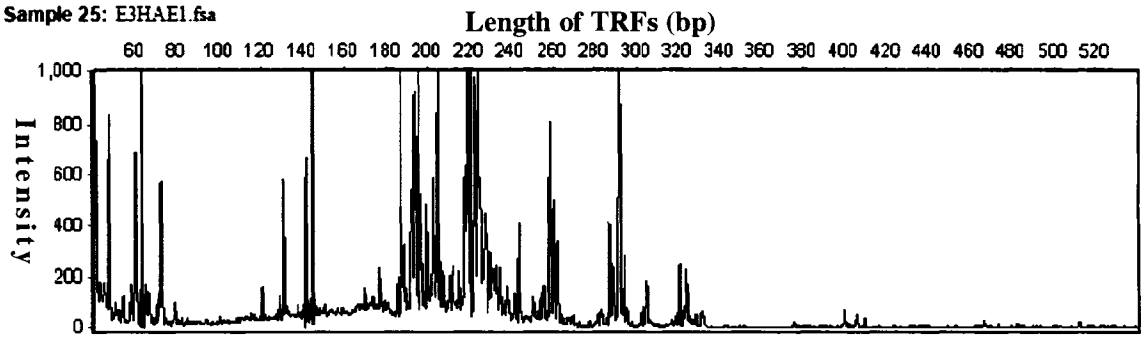
Sample 23: E2HAE1.fsa



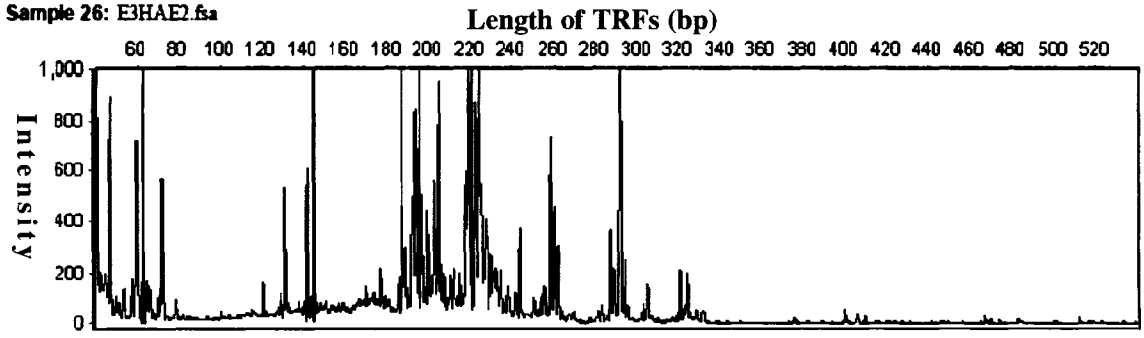
Sample 24: E2HAE2.fsa



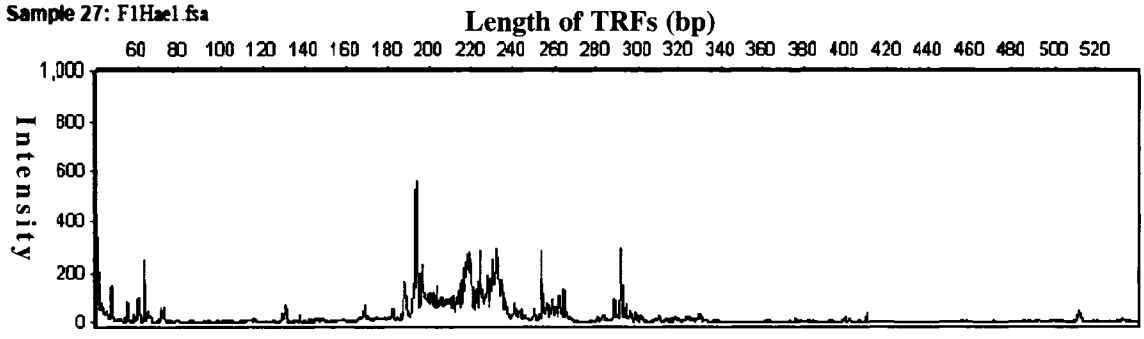
Sample 25: E3HAE1.fsa



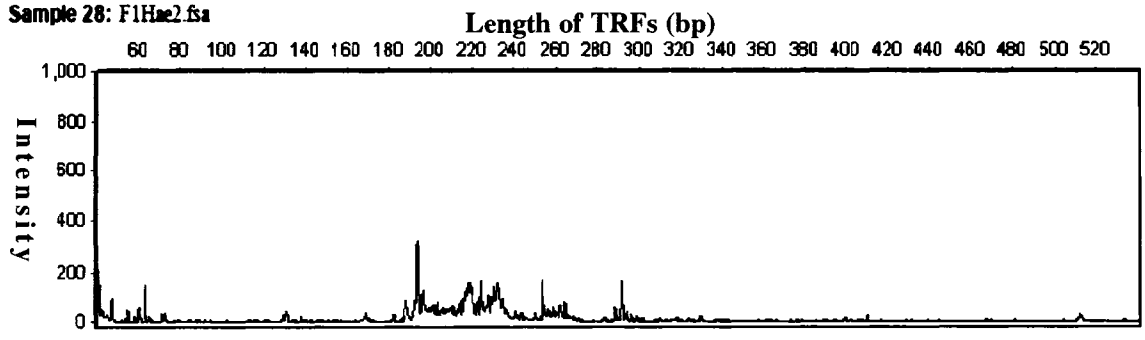
Sample 26: E3HAE2.fsa



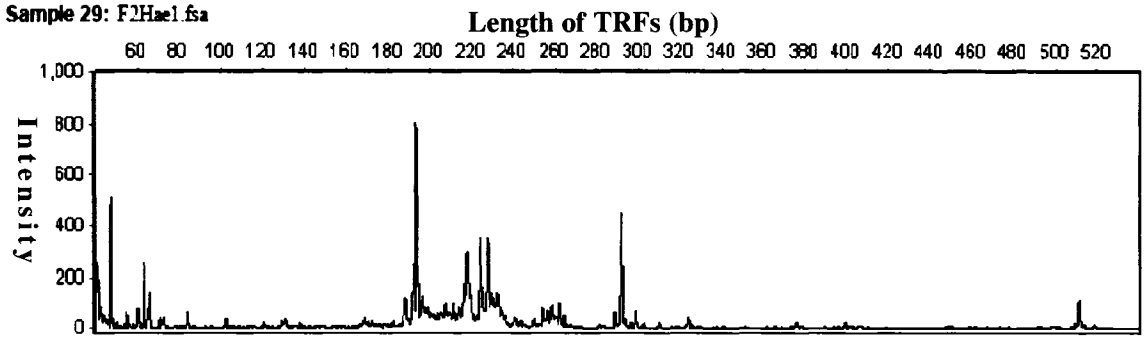
Sample 27: F1Hae1.fsa



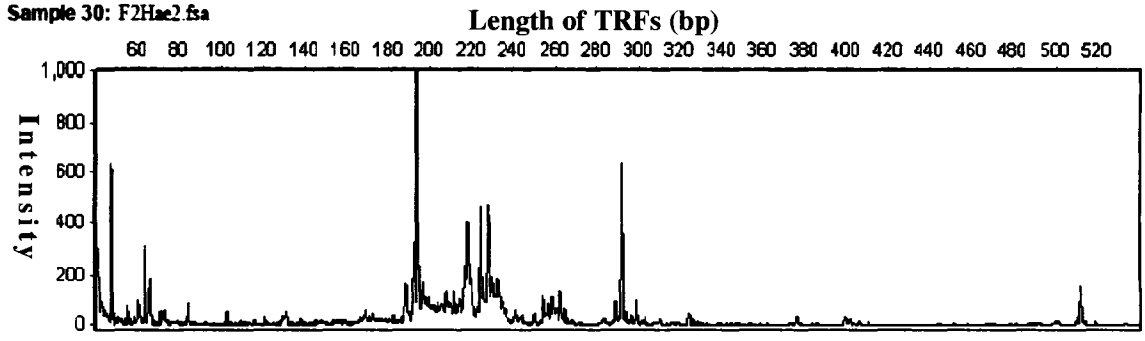
Sample 28: F1Hae2.fsa



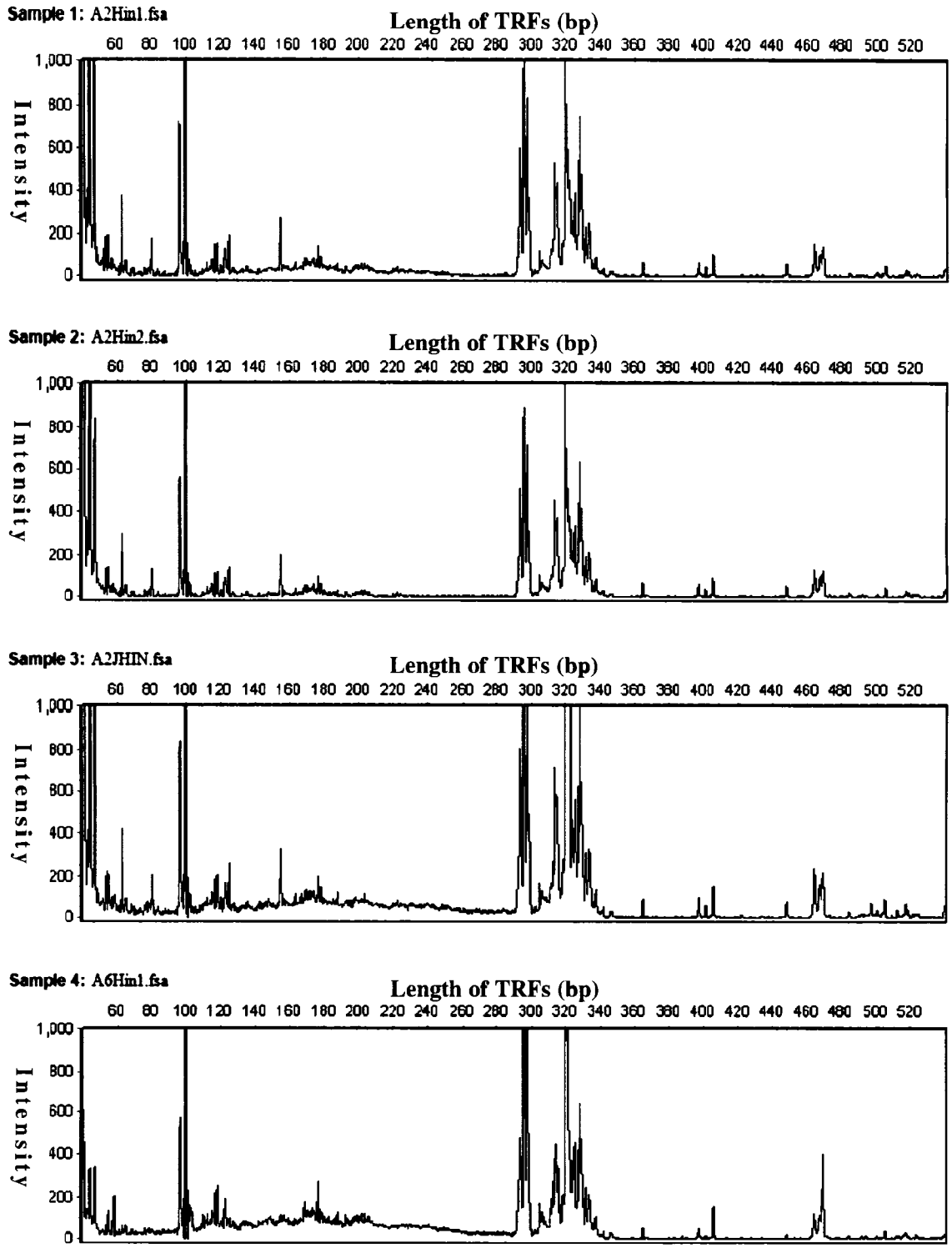
Sample 29: F2Hae1.fsa

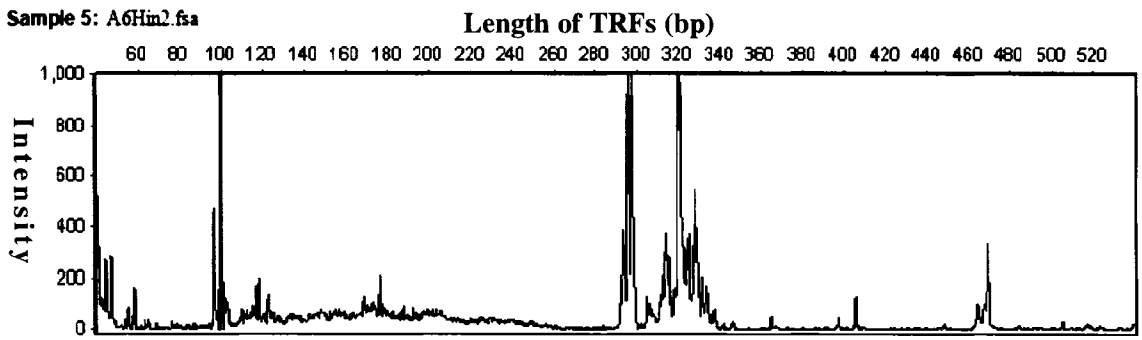


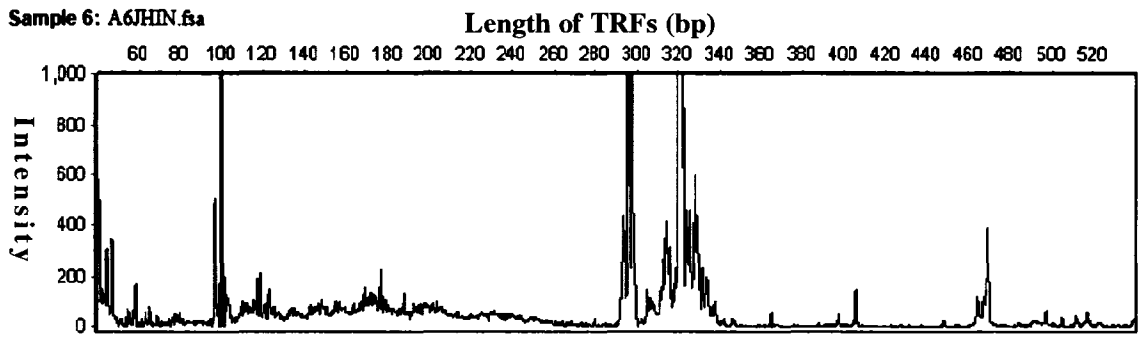
Sample 30: F2Hae2.fsa

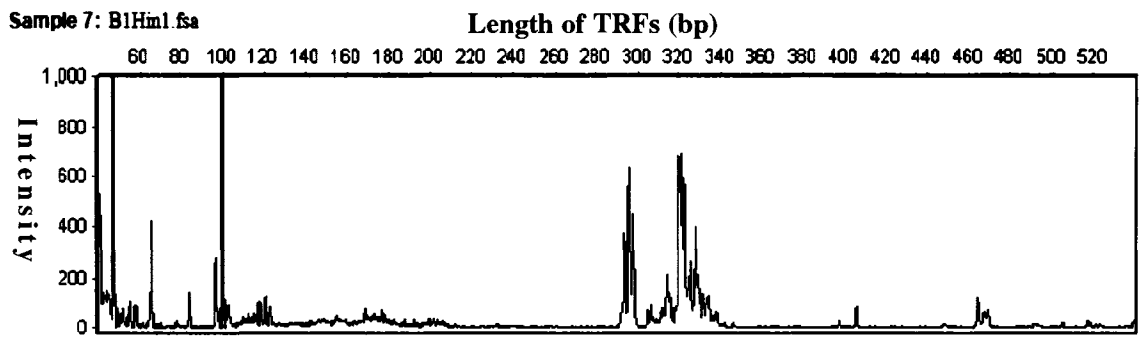


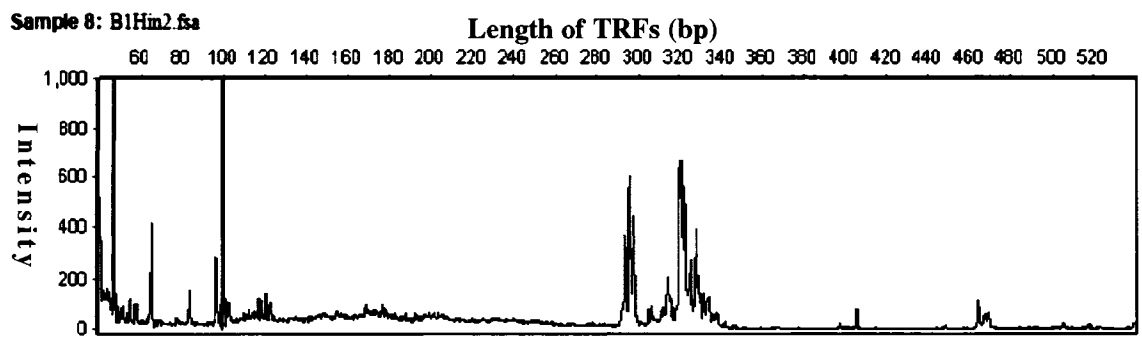
6.10 Electropherograms of *Hinf*I-Derived TRF Profiles from Soil samples (A2&A6; B1&B2; C1, C2&C3; D2&D4; E2&E3; F1&F2; A2J&A6J; D2J&D4J)



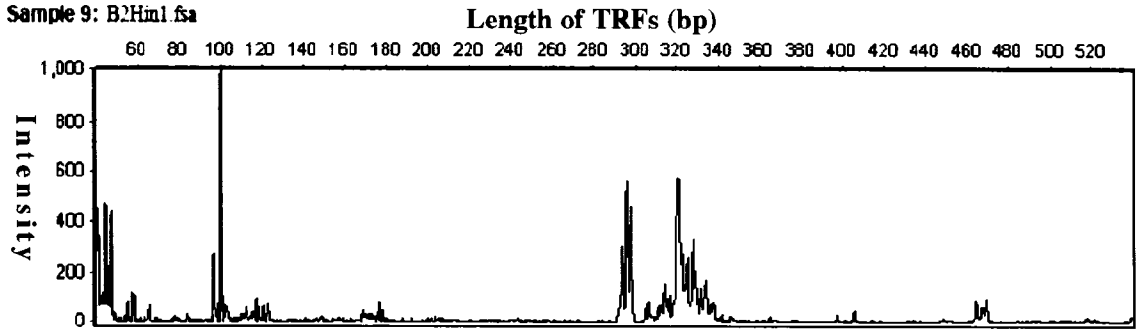




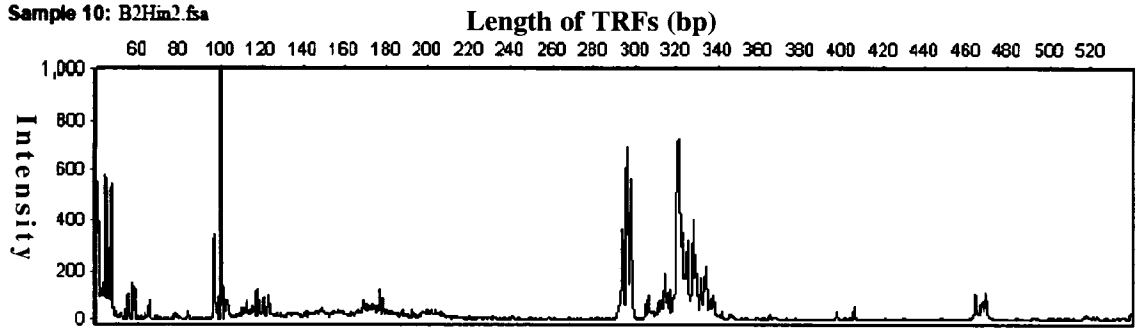




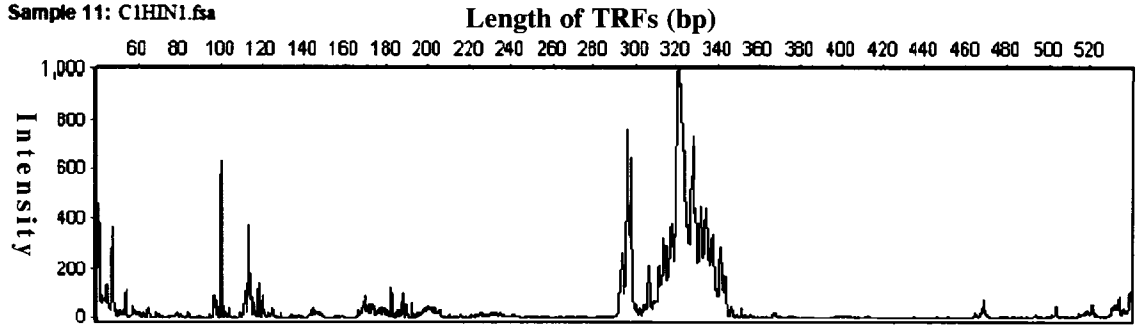
Sample 9: B2Hin1.fsa



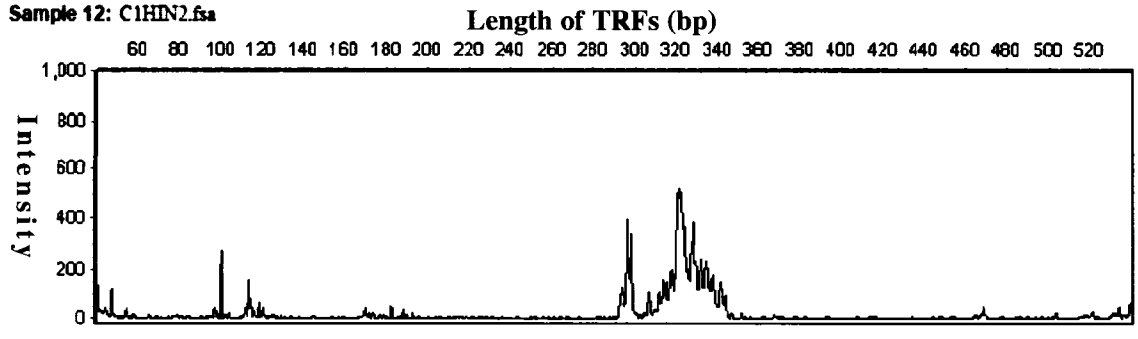
Sample 10: B2Hin2.fsa

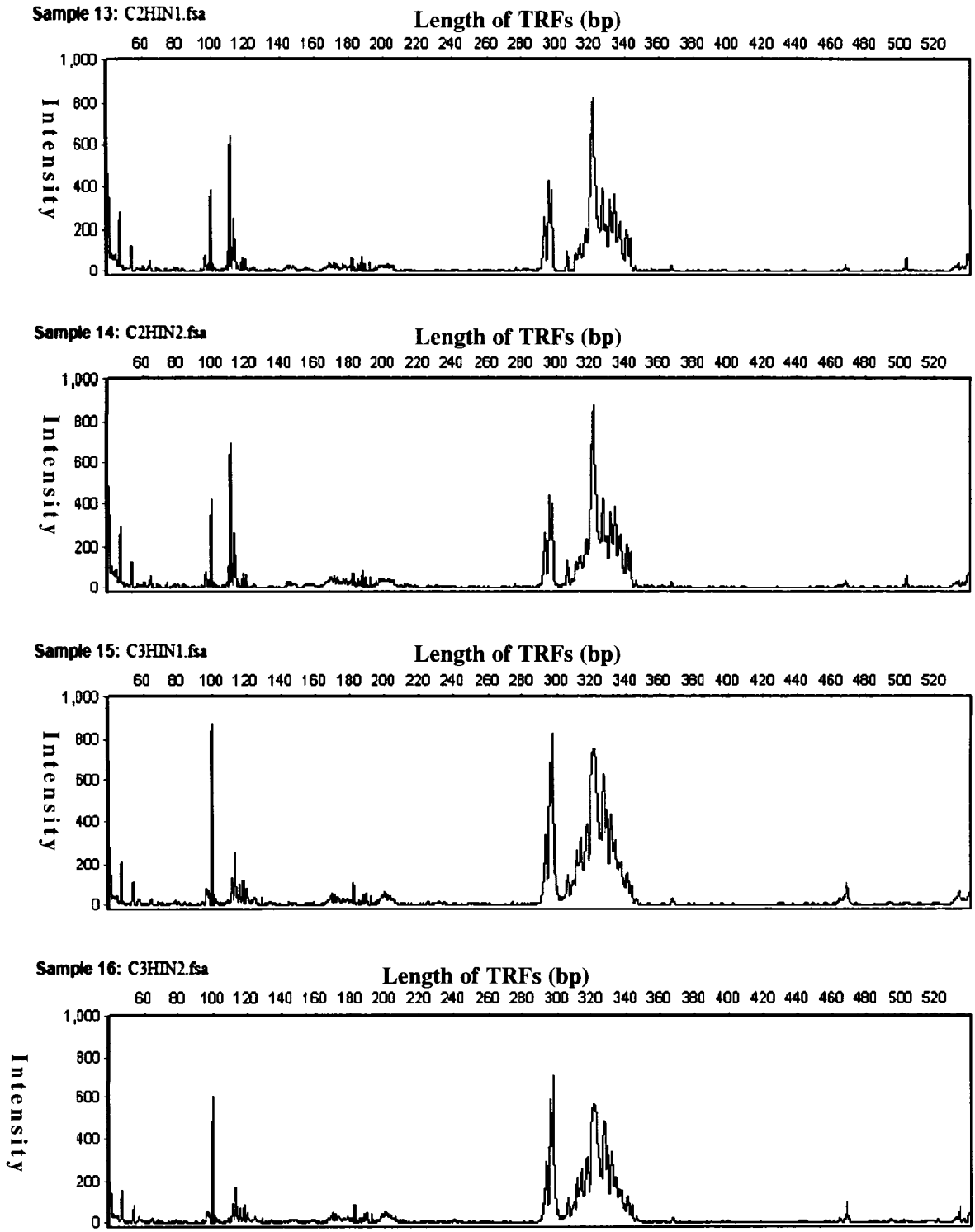


Sample 11: C1HIN1.fsa

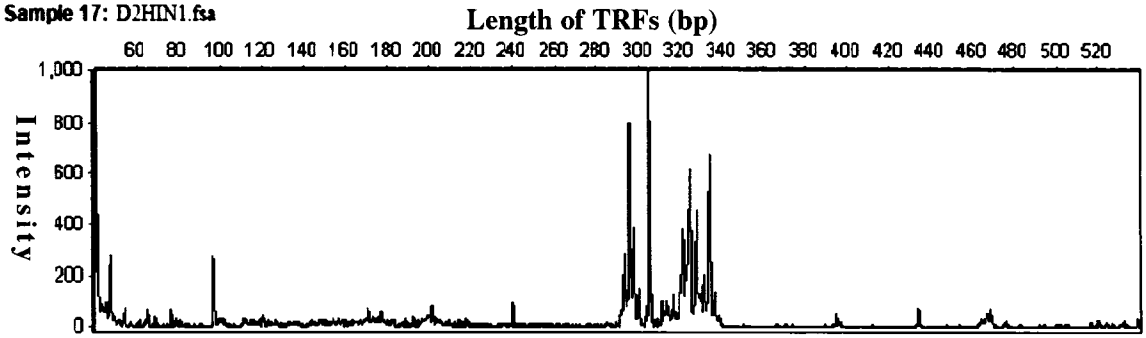


Sample 12: C1HIN2.fsa

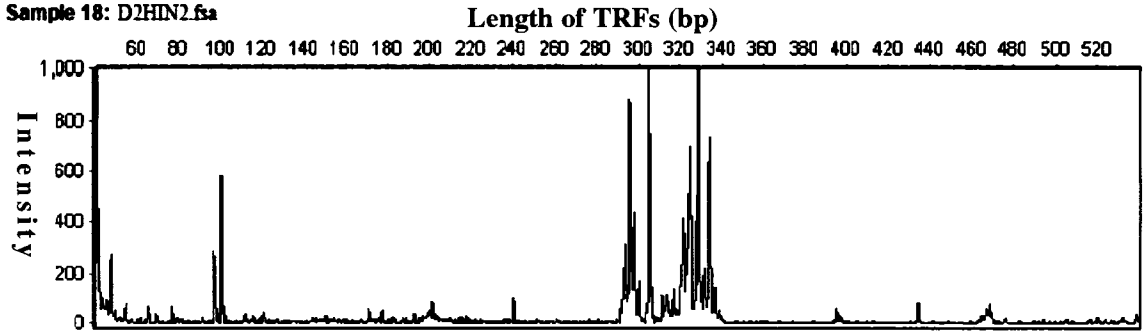




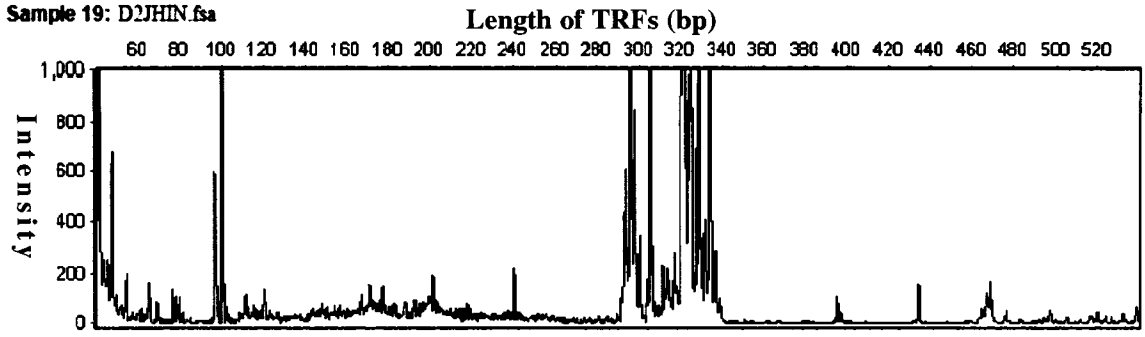
Sample 17: D2HIN1.fsa



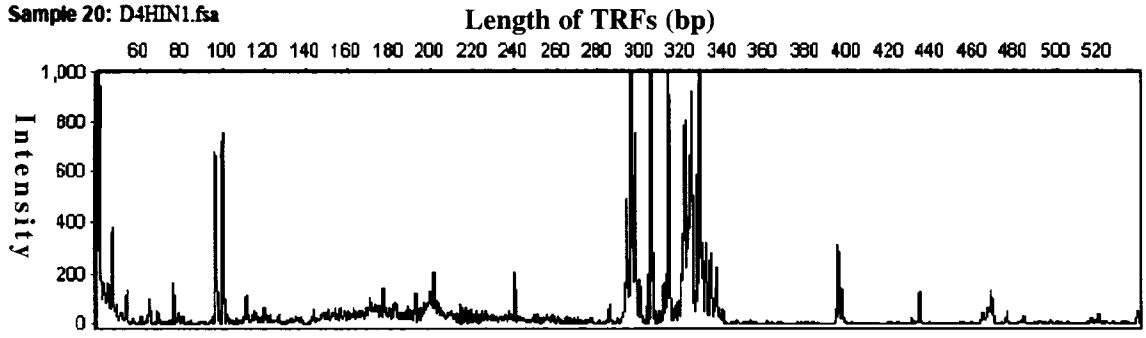
Sample 18: D2HIN2.fsa



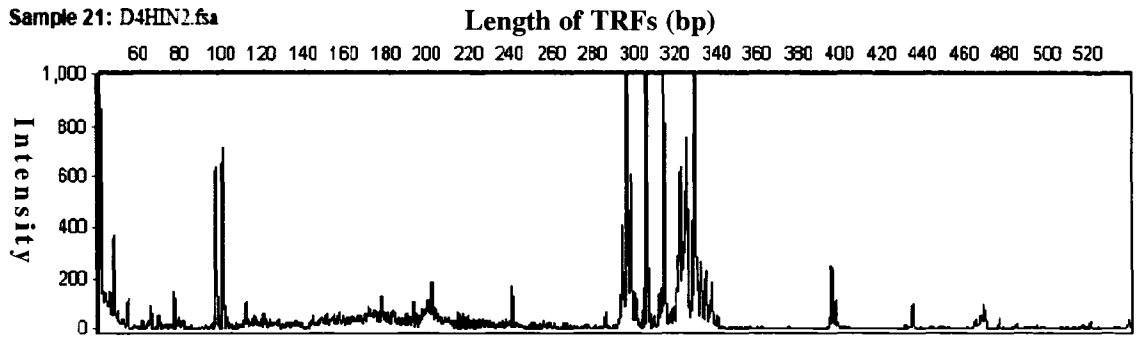
Sample 19: D2JHIN.fsa



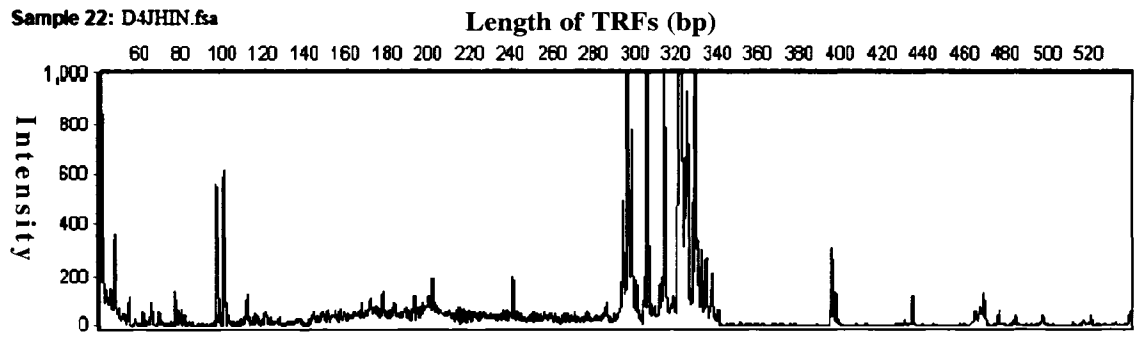
Sample 20: D4HIN1.fsa



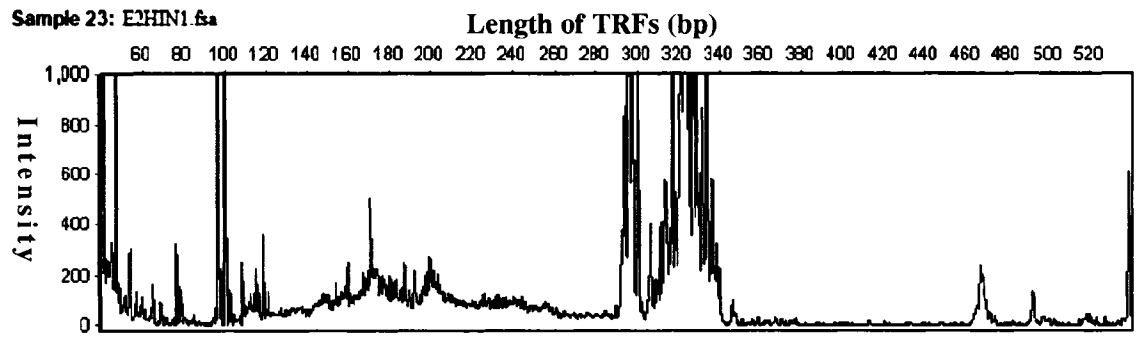
Sample 21: D4HIN2.fsa



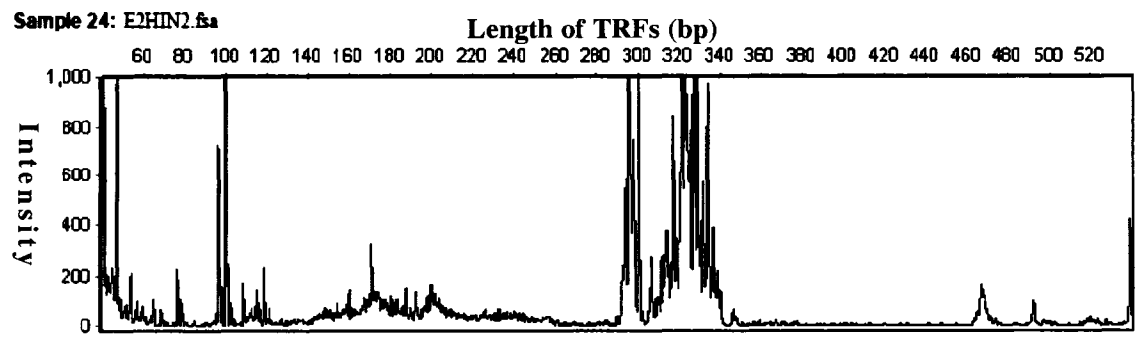
Sample 22: D4JHIN.fsa



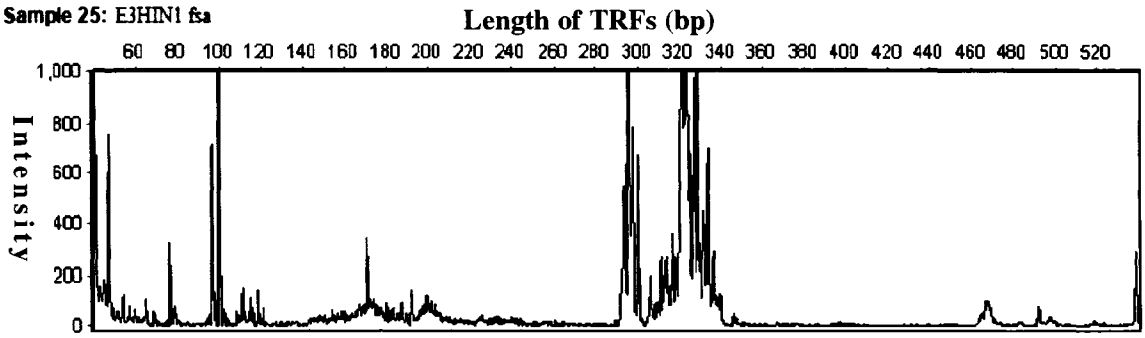
Sample 23: E2HIN1.fsa



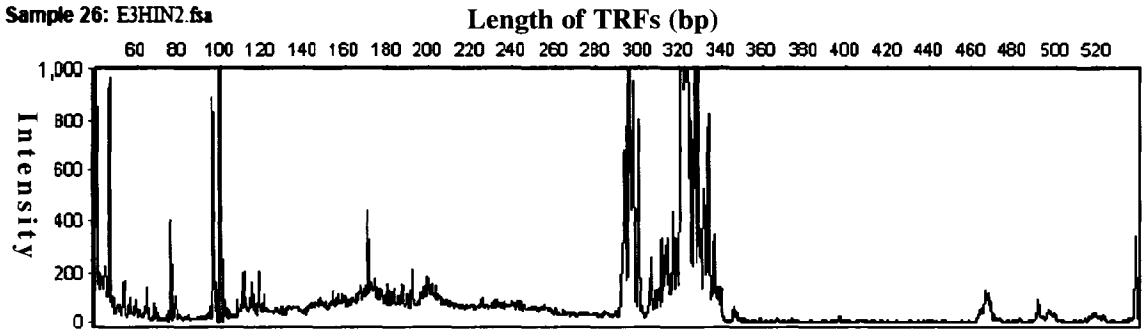
Sample 24: E2HIN2.fsa



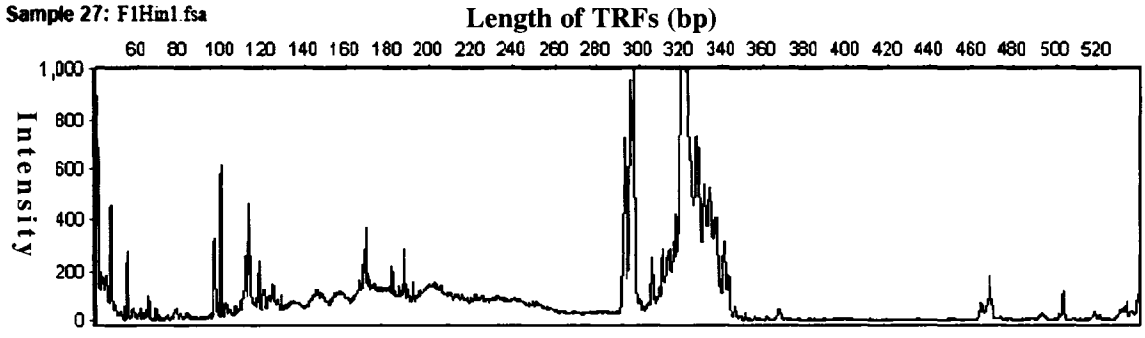
Sample 25: E3HIN1.fsa



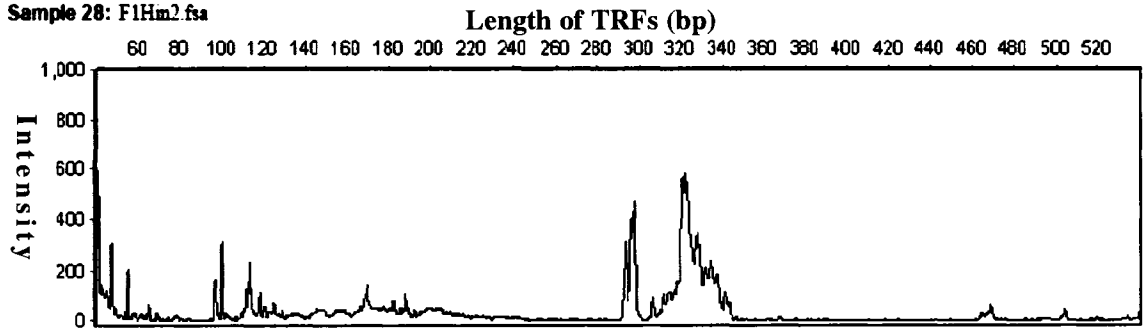
Sample 26: E3HIN2.fsa

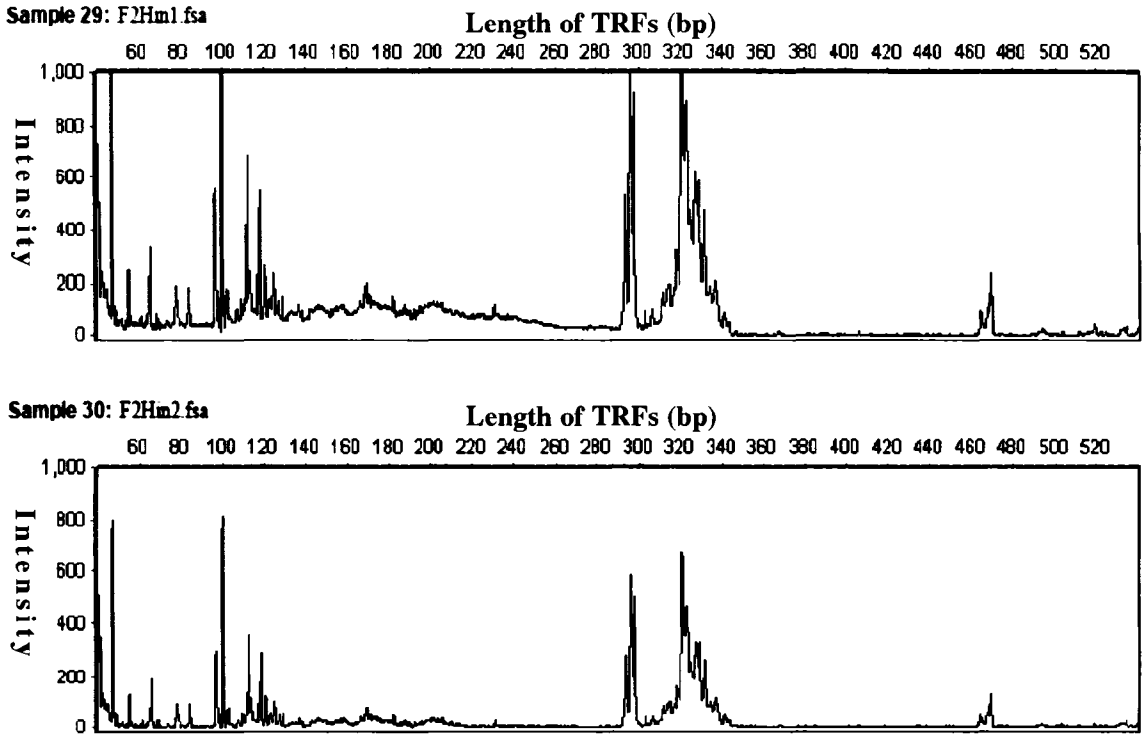


Sample 27: F1Hm1.fsa

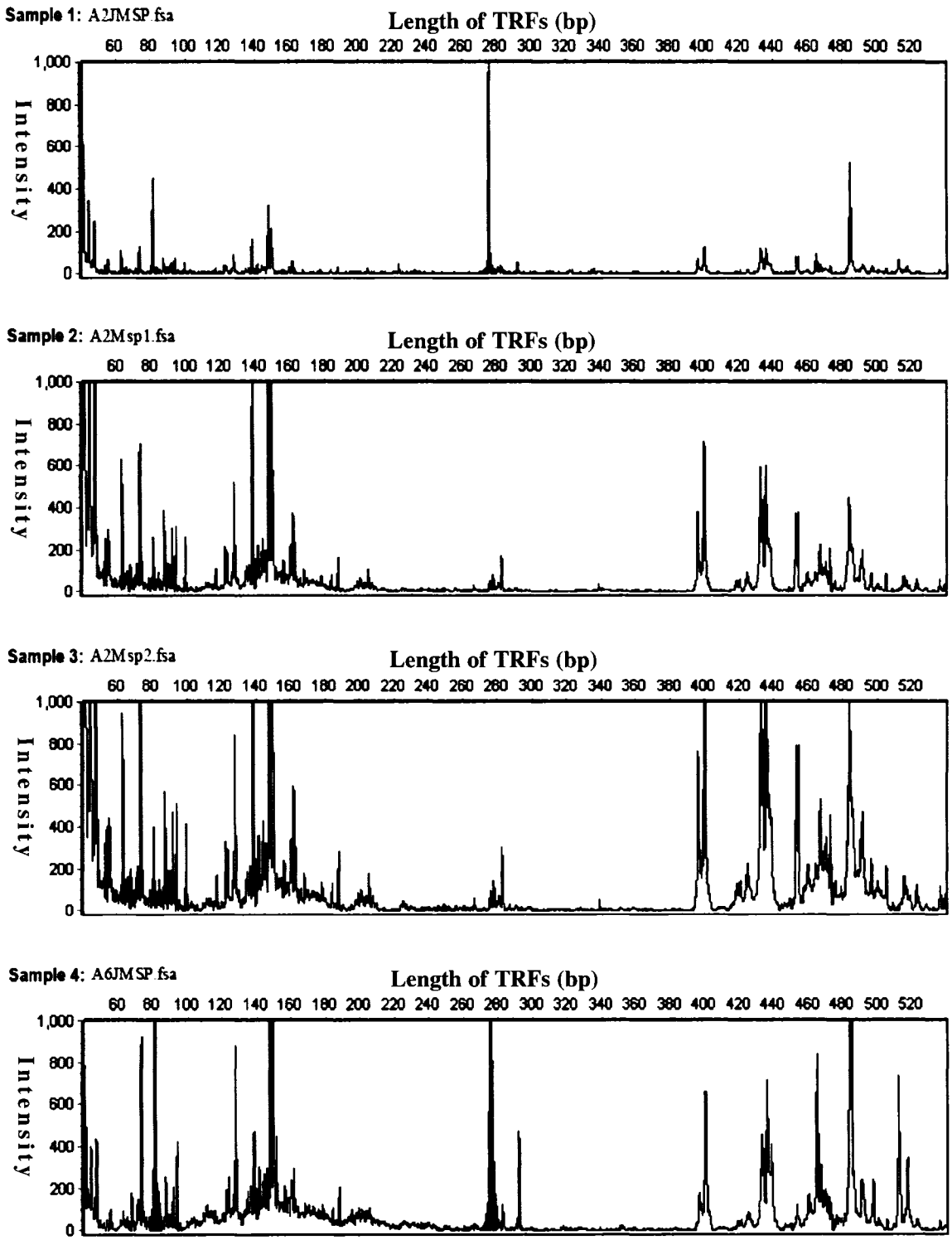


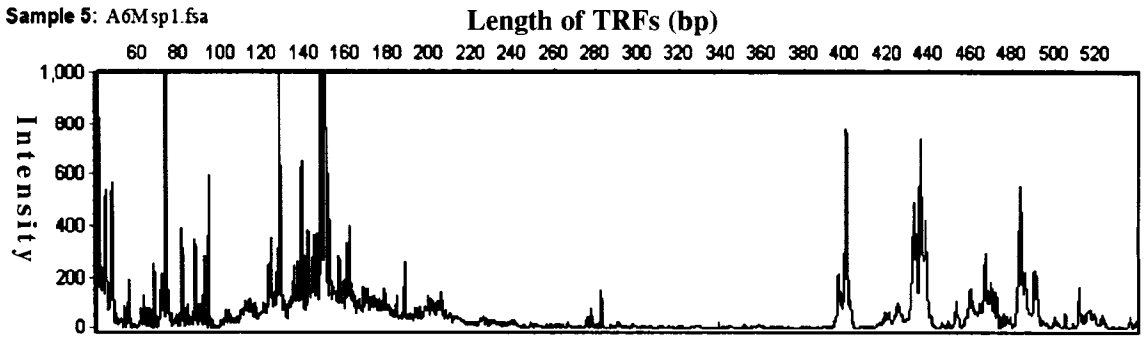
Sample 28: F1Hm2.fsa

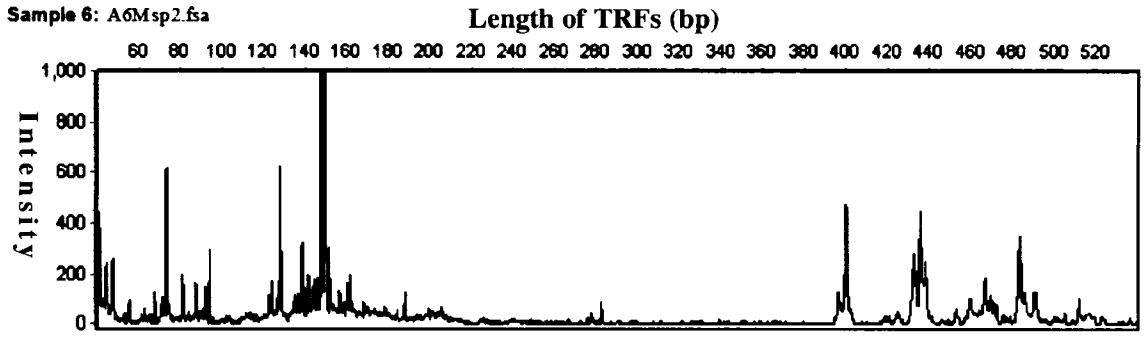


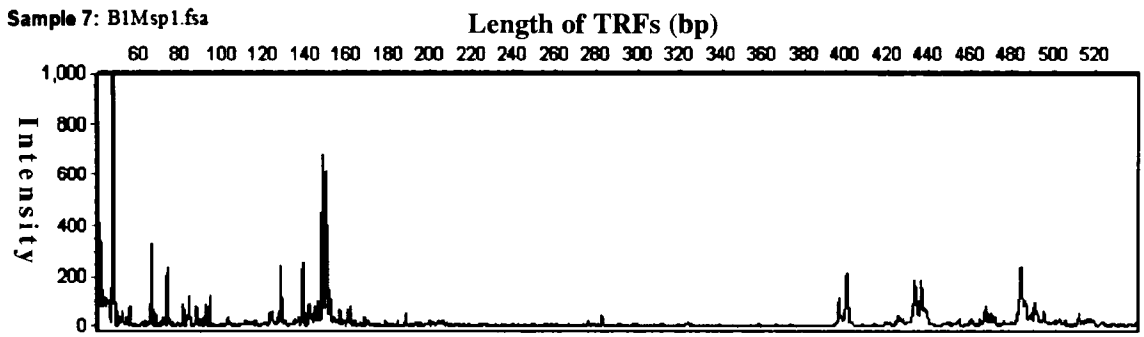


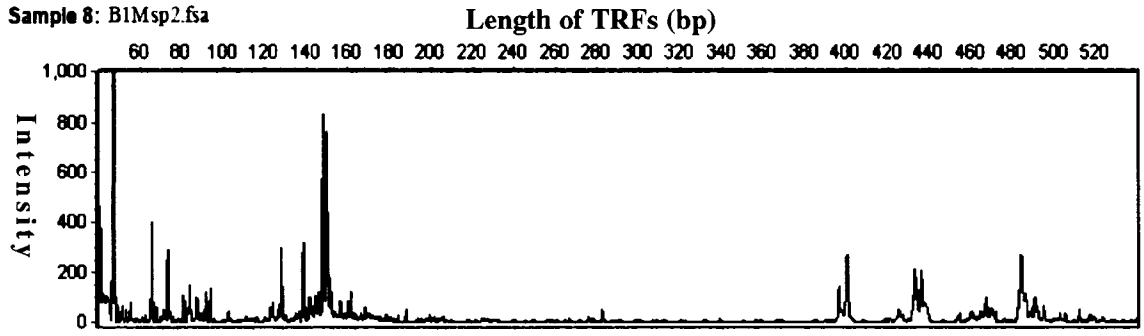
6.11 Electropherograms of *Msp*I-Derived TRF Profiles from Soil samples (A2&A6; B1&B2; C1, C2&C3; D2&D4; E2&E3; F1&F2; A2J&A6J; D2J&D4J)



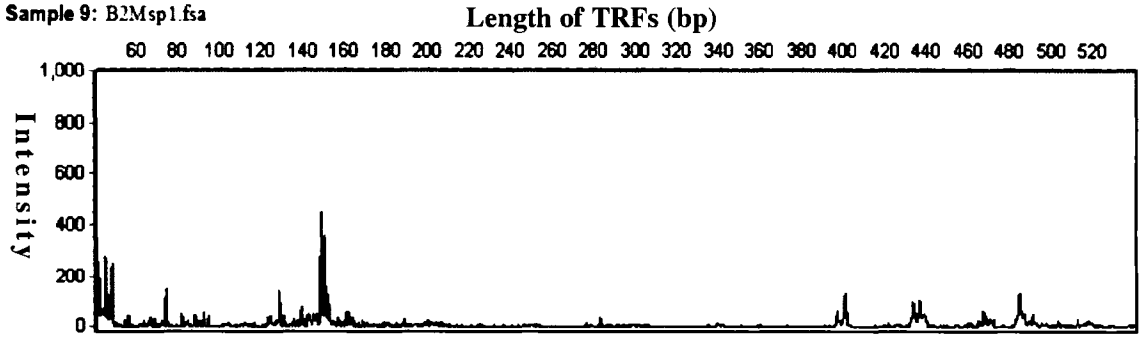




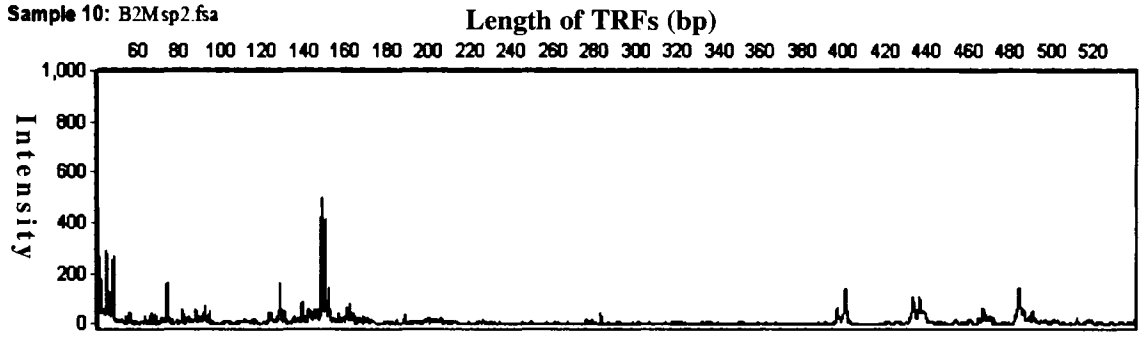




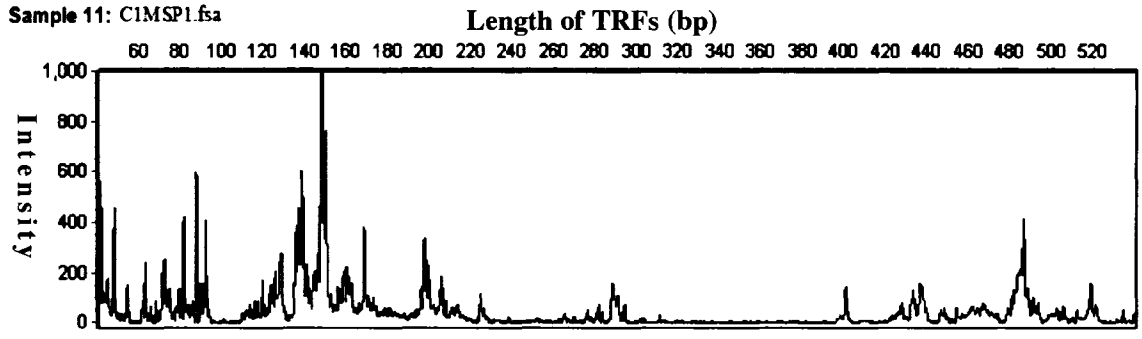
Sample 9: B2Msp1.fsa



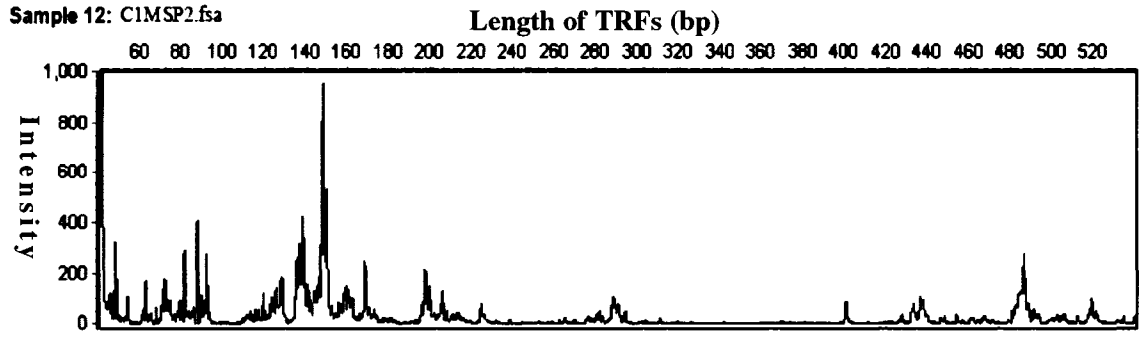
Sample 10: B2Msp2.fsa



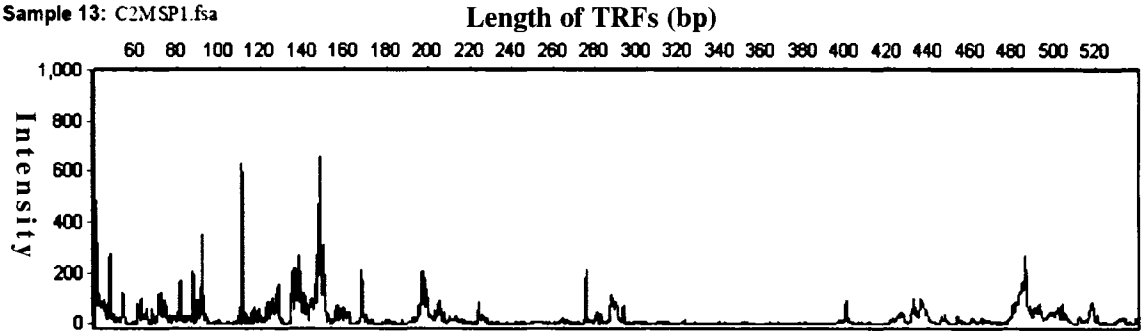
Sample 11: C1MSP1.fsa



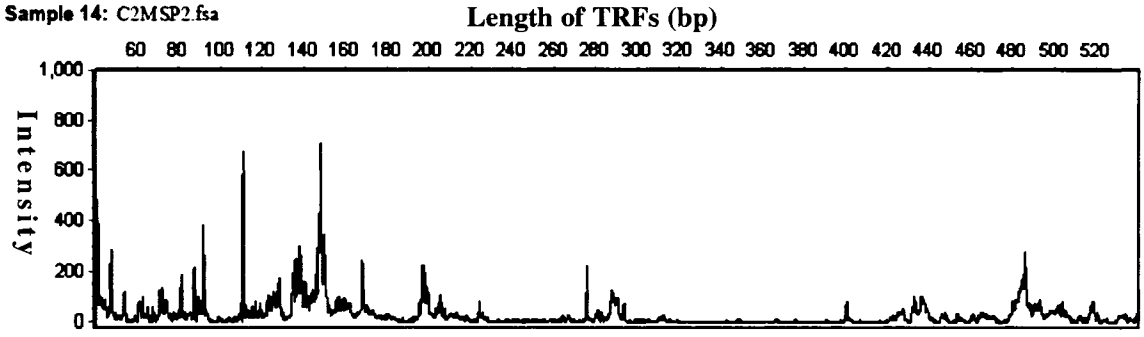
Sample 12: C1MSP2.fsa



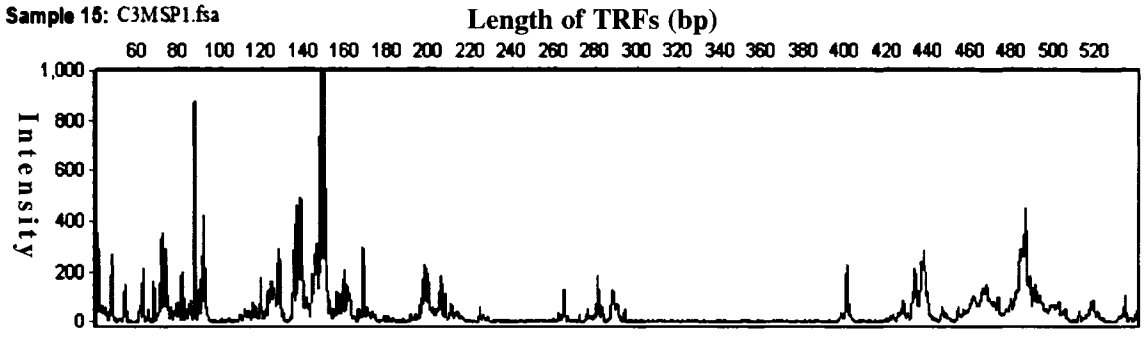
Sample 13: C2MSP1.fsa



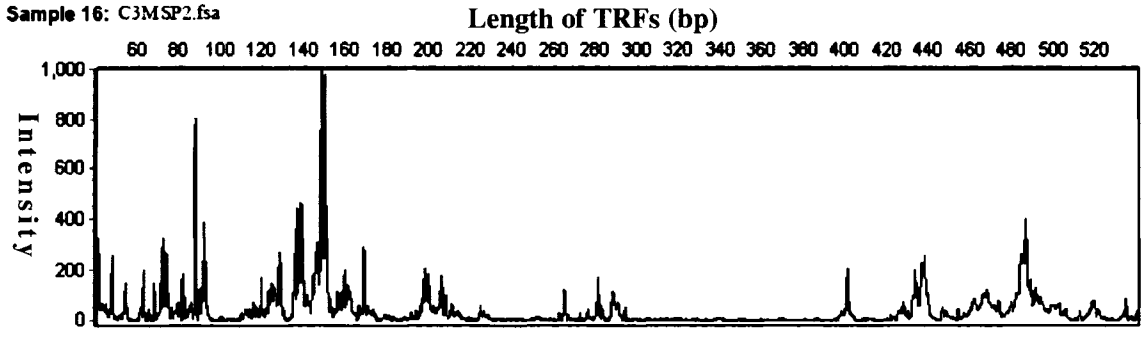
Sample 14: C2MSP2.fsa



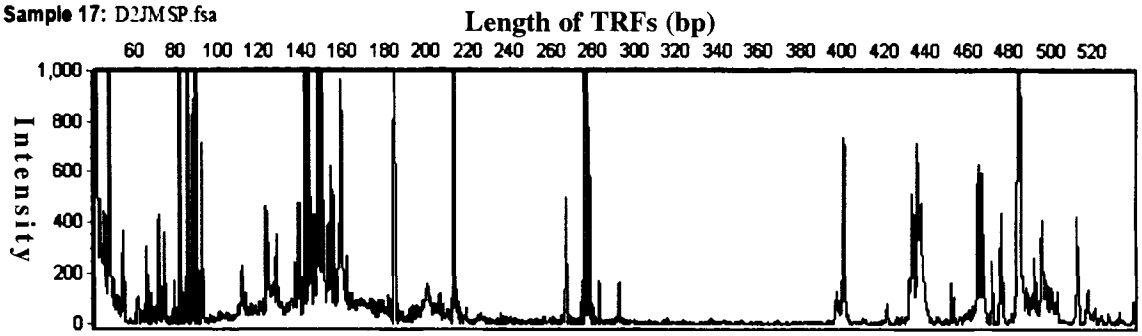
Sample 15: C3MSP1.fsa



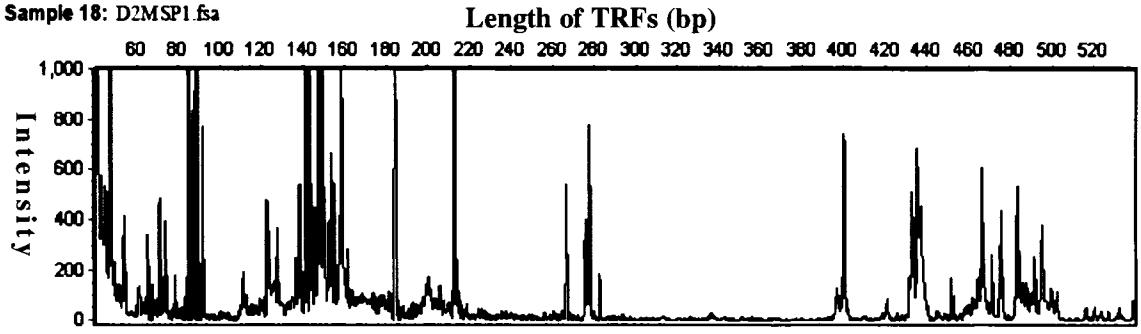
Sample 16: C3MSP2.fsa



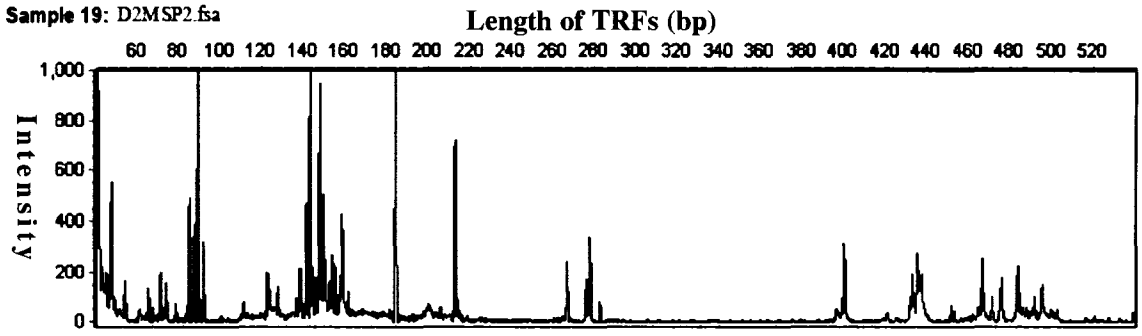
Sample 17: D2JMSP.fsa



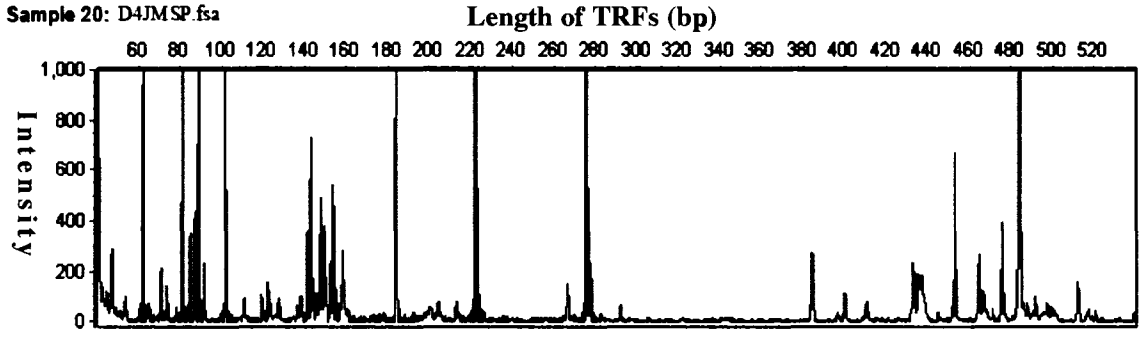
Sample 18: D2MSP1.fsa

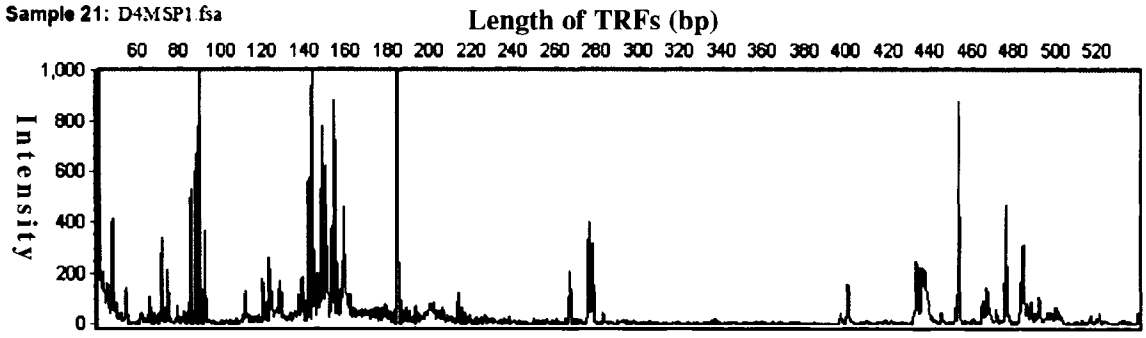


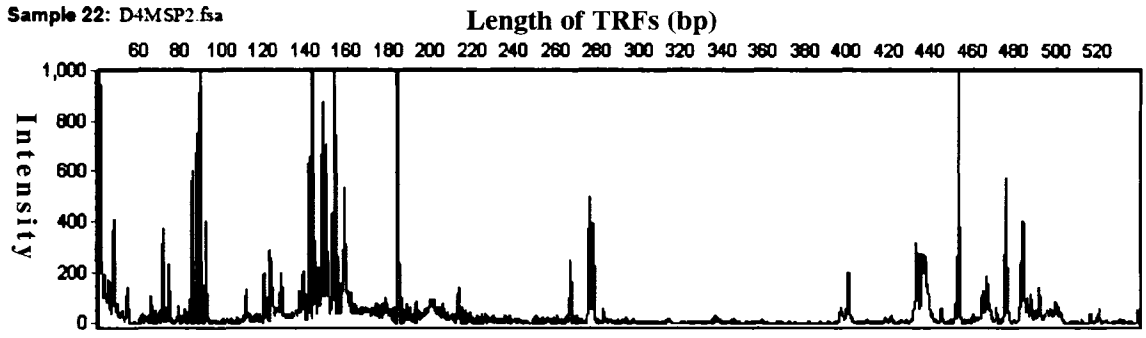
Sample 19: D2MSP2.fsa

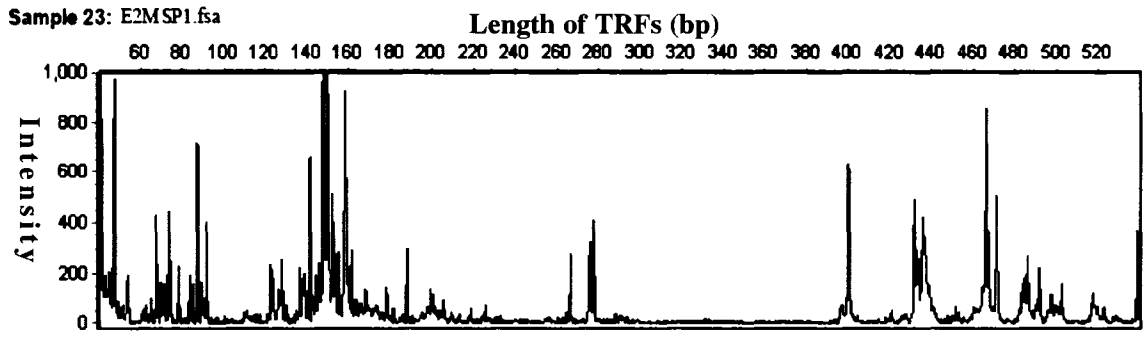


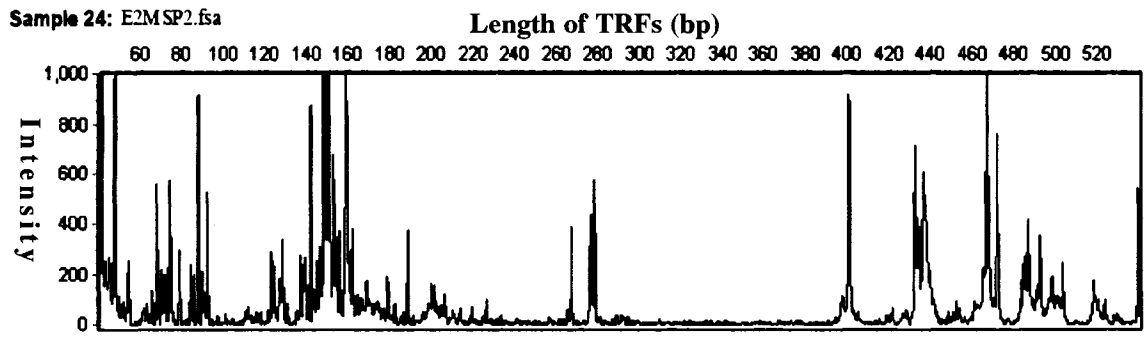
Sample 20: D4JMSP.fsa



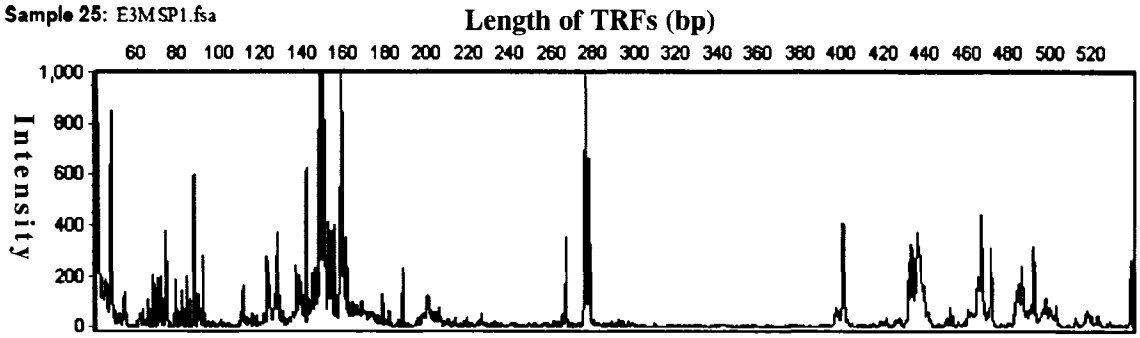




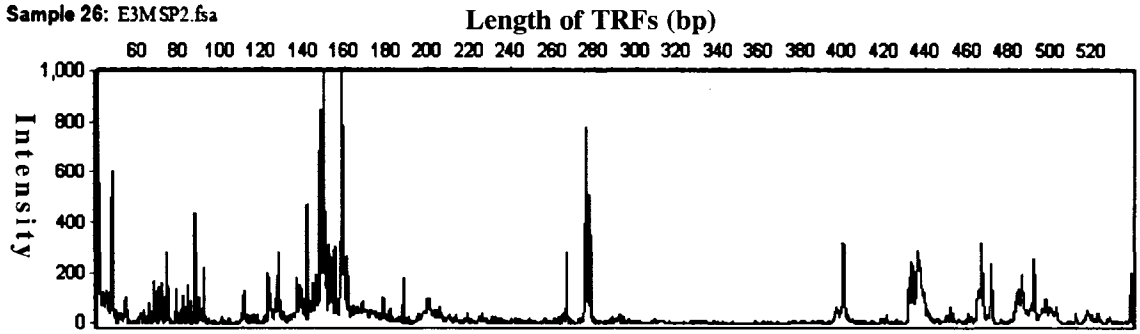




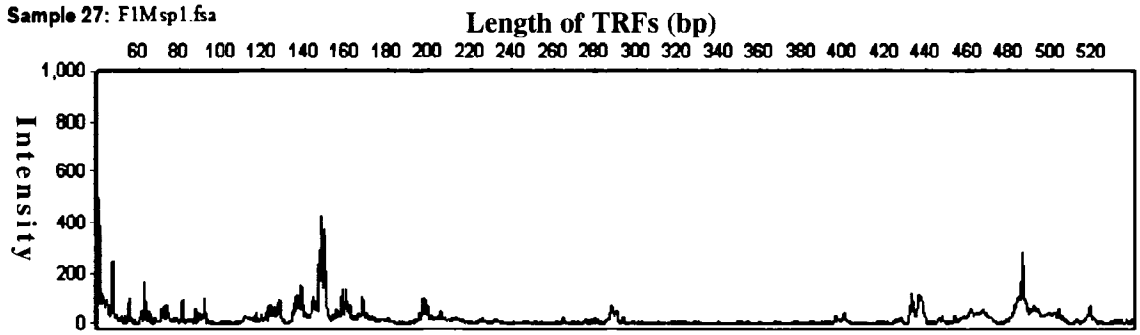
Sample 25: E3MSP1.fsa



Sample 26: E3MSP2.fsa



Sample 27: F1Msp1.fsa



Sample 28: F1Msp2.fsa